Investigating the Role of Fas (CD95) Signalling in the Modification of Innate Immune Induced Inflammation

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by

Caitríona Marie Lyons, BSc

Department of Pathology

National University of Ireland, Cork

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Head of Department: Prof. Michael Prentice

Supervisors: Dr. Elizabeth Brint and Dr. Aileen Houston

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DECLARATION

I certify that this thesis has not been previously submitted for a degree in this or any other University. This thesis is the result of my own investigations and any other assistance is acknowledged.

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A day you don't learn something new is a day wasted. – my Dad

Abstract

Background/Aim: The innate immune system is a complex system which maintains a fine balance between homeostatic function and the inflammatory response. It has been demonstrated that a number of pathologies occur as a result of dysregulation of the immune system. Whilst classically associated with apoptosis, the Fas (CD95) signalling pathway has also been demonstrated to play a role in inflammation. Studies have demonstrated that Fas activation augments TLR4-mediated MyD88-dependent cytokine production, whilst the Fas adapter protein FADD is required for RIG-I-induced IFNβ production. As a similar signalling pathway exists between RIG-I, TLR3 and the MyD88-independent of TLR4, we hypothesised that Fas activation may modulate both TLR3- and TLR4-induced cytokine production.

Results: The dsRNA mimetic, poly I:C, induced IFNβ, IL-8, IL-10 and TNFα which were reduced by Fas activation. However, poly I:C-, poly A:U- and Sendai virus-induced IP-10 production were augmented by Fas activation, demonstrating that this effect was not receptor specific. Overexpression studies demonstrated that the Fas adapter protein FADD inhibits TLR3-, RIG-I- and MDA5-induced IP-10 luciferase activation. Poly I:C-induced phosphorylation of p-38 and JNK MAPK was reduced by Fas activation, with overexpression studies demonstrating that FADD induces AP-1 luciferase activation. Point mutations in the AP-1 binding site also resulted in enhanced poly I:C-induced IP-10 production. LPS-induced IL-10, IL-12, IL-8 and TNFα production were enhanced by Fas activation. Fas activation reduced LPS-induced IFNβ production. Absence of FADD using $FADD^{-/-}$ MEFs resulted in impaired IFNβ production compared to wild-type MEFs whilst overexpression studies demonstrated that FADD augments TLR4-, MyD88- and TRIF-induced IFNβ

luciferase activation. Overexpression studies also suggested that enhanced TLR4-induced IFNβ production was independent of NFκB activation.

Conclusion: Viral-induced IP-10 production is augmented by Fas activation which alleviates the repressive activity of FADD on the system, with Fas activation reducing phosphorylation of p-38 and JNK MAPKs. This reduces the activation of the transcription factor AP-1, thus alleviating the negative effect of AP-1 on the IP-10 promoter, promoting IP-10 production. The Fas signalling pathway reduces LPS-induced IFNβ production, whilst augmenting all other cytokines investigated, with our data demonstrating that FADD is required for TLR4-induced IFNβ production. Studies presented here demonstrate that the Fas signalling pathway can therefore modulate the immune response. Our data demonstrates that this modulatory effect is mediated by its adapter protein FADD, which tailors the immune response by acting as a molecular switch. This ensures the appropriate immune response is mounted, thus preventing an exacerbated immune response, therefore limiting collateral damage to the host following infection.

Abbreviations

PAMP	Pathogen Associated Molecular Patterns
PRRs	Pathogen Recognition Receptors
APCs	Antigen Presenting Cells
NETs	Neutrophil Extracellular Traps
MIP-1α	Macrophage Inflammatory Protein-1α
TLRs	Toll like Receptors
NOD	Nuclear Oligodimerization Domain
NLRs	Nod like Receptors
CLRs	C-type Lectin Receptors
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-I like Receptors
LPS	Lipopolysaccharide
IL-IR	Interleukin-1 Receptor
LRRs	Leucine Rich Repeats
LBP	LPS Binding Protein
RNA	Ribonucleic acid

dsRNA	double stranded RNA
ssRNA	single stranded RNA
RSV	Respiratory Syncytial Virus
TIR	Toll/IL-1R
TIRAP	TIR domain containing adapter protein
MyD88	Myeloid differentiation primary response 88
IRAK	IL-1 receptor kinase
DD	Death Domain
TRAM	TRIF related adapter molecule
DED	Death Effector Domain
TRAF	Tumour Necrosis Factor Receptor Associated Factor 3
TAK-1	TGF-β-activated kinase
IKK	Inhibitor of kappa B kinase-β
IκB	Inhibitory κB
NFκB	Nuclear factor κB
JNK	c-Jun N-terminal Kinase
ERK	Extracellular signal related kinase

MAPK	Mitogen-activated protein kinases
AP-1	Activator-protein 1
RIP-1	Receptor interacting protein 1
RHIM	RIP homotypic interaction motif
TRADD	Tumour necrosis factor receptor type1-associated Death domain protein
NEMO	NFκB essential modulator
TANK	TRAF family member associated NFκB activator
TBK1	TANK binding kinase 1
IRF3	Interferon regulatory factor 3
BMDM	Bone Marrow-Derived Macrophage
MDA5	Melanoma Differentiation Associated Antigen 5
CARD	Caspase activation and recruitment domain
RD	Repressor domain
CTD	C terminal domain
IPS-1	Interferon-β promoter stimulator 1
MAVS	Mitochondrial anti-viral signalling protein

VISA	Virus-induced signalling adapter
TNF	Tumour Necrosis Factor
IP-10	Interferon γ-induced protein-10
IFN	Interferon
CARDIF	CARD adapter inducing IFNβ
FADD	Fas associated protein with death domain
TIM	TRAF interacting motif
PYD	Pyrin
BIR	Baculovirus inhibitor repeat
DC	Dendritic cell
SMAC	Second mitochondria derived activator of caspase
FLICE	FADD-like IL-1β-converting enzyme
cFLIP	cellular FLICE inhibitory protein
DISC	Death inducing signalling complex
dATP	2'-deoxyadenosine 5'triphosphate
IAPs	Inhibitors of apoptosis
XIAP	X chromosome-linked IAP

SLE	Systemic lupus erythematosus
ALPs	Autoimmune lymphoproliferative syndrome
TCR	T cell receptor
SH2	Src homology domain 2
MMP	Metalloproteases
FCS	Foetal calf serum
PMA	Phorbal 12-myristate 13-acetate

List of Publications

Published manuscript

Intestinal expression of Fas and Fas Ligand is upregulated by bacterial signalling through Toll-Like Receptors (TLR) 4 and TLR5, with activation of Fas modulating intestinal TLR-mediated inflammation. Fernandes P, O'Donnell C, **Lyons C**, Brint E and Houston A (2014). J Immunol 2014; 193: 6103-3113

Engagement of Fas on macrophages modulates poly I:C induced cytokine production with specific enhancement of IP-10. **Lyons C**, Fernandes P, Fanning L, Houston A and Brint E (2015), PLOS ONE

Published abstract

Double-stranded RNA (dsRNA)-induced IP-10/CXCL-10 production is augmented by Fas activation. **Lyons C**, Moran R, Houston A, Brint E (2013). Front. Immunol. Conference Abstract: 15th International Congress of Immunology (ICI).

The role of PGE_2 in Cystic Fibrosis (CF) Lung inflammation and the potential association with Ivacaftor therapy and treatment response. O Callaghan G, Ronan N, Foley N, **Lyons C**, O'Driscoll A, Ni Chroinin M, Mullane D, Murphy DM, Eustace J, Houston A, Plant BJ. 38th European Cystic Fibrosis 2015.

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Chapter 1

General Introduction

1.1.1 The Innate Immune System

The innate immune system consists of physical barriers and phagocytic immune cells. It is the first line of defence of the host, providing protection against a myriad of infections. The innate immune system relies on the recognition of evolutionary conserved molecular patterns that are common and essential for survival of microbes, but are absent in the host. These are known as pathogen associated molecular patterns (PAMPs) (1). Recognition of these PAMPs occurs through the presence of germline-encoded pattern recognition receptors (PRRs) which are a vital component of the innate immune system (2). Whilst recognition of PAMPs by PRRs can result in cytokine production, phagocytosis by antigen presenting cells (APCs) such as dendritic cells and macrophages can also occur. The pathogenic antigen is then presented on the APC, which upon recognition by antigen specific naïve T lymphocytes, results in activation of the adaptive immune response.

1.1.2 Innate immune cells

Innate immune cells are diverse in nature and represent the first line of defence of the host. These cells express and utilise the PRRs to mount an immune response against pathogenic material in the early hours of infection (3). Phagocytic leukocytes are white blood cells which are generated from haematopoietic stem cells, differentiating into the various subsets depending on the colony stimulating factor to which they are exposed (4). Neutrophils are the predominant leukocyte present in the bloodstream, are both secretory and phagocytic cells and are the first cell type to arrive at the site of infection. Recent studies have demonstrated that neutrophils target both gram-positive and gram-negative bacteria by neutrophil extracellular traps (NETs) (5). Crosstalk between both neutrophils and macrophages also aids the

innate immune response, with neutrophils producing macrophage inflammatory protein-1α (MIP-1α) and MIP-1β, resulting in migration of macrophages to the site of infection (5).

Macrophages are an essential component of the innate immune system, capable of multiple functions including phagocytosis and antigen presentation. They are formed as a result of migration of monocytes from the blood stream into various tissues, with the resultant macrophages adopting specific functions dependent on their location (6). The heterogeneous nature of macrophages can be observed when comparing macrophages from different locations. PRRs and scavenger receptors are highly expressed on alveolar macrophages present in the lungs (6), inducing the expression of inflammatory cytokines, inducing an immune response thus clearing the infection (7). As transmission of many pathogenic microorganisms occurs through exposure to infective airborne particles (8), this upregulation and production of cytokines is unsurprising, resulting in a heightened immune response towards pathogenic material.

Macrophages present in the gut are constantly exposed to commensal bacteria; therefore, intestinal macrophages lack some innate immune receptors which are present on blood monocytes, therefore reducing their capacity to react to commensal bacteria. Whilst cytokine production by these macrophages is compromised, these macrophages still retain their phagocytic and bacteriocidal capacity (9), therefore allowing these cells to function in close proximity to commensal bacteria whilst recognising and mounting an immune response against pathogens.

1.2 Pattern Recognition Receptors

A variety of PRRs exist, each designed to evoke a specific immune response targeted against specific invading pathogens. These include Toll-like Receptors (TLRs), Nuclear Oligodimerization Domain (NOD)-Like Receptors (NLRs), C-type Lectin (CLEC) receptors (CLRs) and retinoic acid-inducible gene-I (RIG-I) like Receptors (RLRs), which recognise a range of evolutionary conserved PAMPs. Secreted PRRs, such as collectins and ficolins trigger activation of the lectin pathway of the complement system, resulting in the opsonisation of micro-organisms (10). This allows phagocytosis to occur resulting in antigen expression by immune cells, activating the adaptive immune system (11). Recent studies have also demonstrated that the human caspases, caspase-4 and caspase-5, and the murine homolog caspase-11 directly bind to lipopolysaccharide (LPS) (12-14), indicating that these can be added to the list of known PRRs (14). These PRRs are poised to mount a rapid immune response culminating in the production of chemokines and cytokines.

1.2.1 Toll-like Receptors

Macrophages express a number of different PRRs, a family of which are the TLRs. The protein Toll was first identified in the fruit fly *Drosophilia melanogaster* by Nüsslein-Volhard and Wieschaus (15). It was initially demonstrated that Toll was required for the development of the dorsal-ventral axis in Drosophilia (15). Further studies demonstrated that it also played a significant role in the prevention of microbial infections in Drosophilia, whereby an absence of Toll in these flies resulted in them being highly susceptible to fungal infection (16). This subsequently led to the discovery of its mammalian homolog, Toll-like receptor 4 (TLR4) (17). Prior to the discovery of Toll in the fruit fly *Drosophilia melanogaster*, the innate

immune system was seen as a rudimentary system with its sole purpose being activation of the adaptive immune system. However, with the identification of Toll and TLR4, research in this field has led to the discovery of a multitude of other TLRs and indeed, PRRs of the innate immune system.

TLRs are a vital part of the innate immune system. To date, 13 mammalian TLRs have been identified, 10 of which are expressed in humans, each having homology to the interleukin-1 receptor (IL-1R) (18). They are defined as type 1 transmembrane glycoproteins and along with members of the IL-1R family share a conserved stretch of ~200 amino acids in their cytoplasmic region known as the Toll/IL-1R (TIR) domain (19). This TIR domain is composed of three conserved boxes, formed from a central five stranded β-sheet surrounded by five α-helices, of which BB loops connect these secondary structures, connecting β-strand B with α-helix B (20). This BB loop contains within it a conserved proline residue which is present in all TLRs except that of TLR3, which contains an alanine residue at that site (21). Conservation of this proline residue induces LPS responsiveness. Substitution of proline to histidine induces resistance to endotoxin, as observed in C3H/HeJ mice The extracellular portion of TLRs contains Leucine-Rich-Repeats (LRRs) (22, 23) appearing in a xLxxLxLxx motif (2, 19). Recognition of the ligand by its cognate receptor induces activation of intracellular signalling cascades resulting in production of appropriate cytokines and chemokines (24).

Ligands for all TLRs with the exception of TLR10 have now been elucidated (Table 1.1).

Receptor	Location	Ligand	Ligand origin
TLR1	Cell surface	multiple triacyl lipopeptides	Bacteria
TLR2	Cell surface	multiple glycolipids, multiple lipoproteins, Heat Shock Protein (HSP)70 zymosan, High Mobility Group Protein B1 (HMGB1), Lipoteichoic acid, haemaglutinin	Bacteria Bacteria Host Fungi
TLR3	Cell compartment	double-stranded RNA (dsRNA)	Viruses
TLR4	Cell surface	lipopolysaccharide (LPS), several HSPs, fibrinogen, heparin sulfate, fibronectin, hyaluronic acid, HMGB1, F-protein (RSV), lipotechoic acid	Gram-negative bacteria Bacterial and host cells Host
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	multiple diacyl lipopeptides, lipoteichoic acid, zymosan	Mycoplasma
TLR7	Cell compartment	Single stranded RNA (ssRNA)	RNA Viruses
TLR8	Cell compartment	Single stranded RNA (ssRNA)	RNA Viruses
TLR9	Cell compartment	unmethylated CpG oligodeoxynucleotide DNA	Bacteria, DNA Viruses
TLR10	Cell surface	?	?

Table 1.1 Intracellular location of each TLR with their known respective ligands and the origin of each ligand.

TLRs are present both on the plasma membrane, as well as endosomally, poising them for appropriate PAMP recognition and response (24). TLRs 1, 2, 4, 5 and 6 are located on the plasma membrane, and predominantly recognise microbial cell wall components. Whilst TLR1 and 2 are capable of recognising bacterial lipoproteins, TLR2 requires dimerization with TLR1 or TLR6 in order to mediate an appropriate immune response (18). TLR5 recognises flagellin which is present in gram-positive and gram-negative bacterial flagella (25). Recognition of LPS by LPS Binding protein (LBP) facilitates the interaction between LPS and CD14 (26, 27), resulting in shuttling of LPS to the lymphocyte antigen 96 (MD2)-TLR4 complex (28, 29). TLR4 then homodimerizes, resulting in the recruitment of its adapter proteins. This induces the activation of downstream signalling components and eventual induction of cytokines (19). While well defined as a receptor of LPS, a component of gram-negative cell walled bacteria, studies have now demonstrated that TLR4 can also recognise viral particles, as well as gram-positive bacteria (18).

Endosomally located TLRs primarily detect nucleic acids, in particular by-products of viral reproduction (24). TLR3, along with TLR7, 8 and 9 localizes to the endosome, with TLR3 recognising double-stranded RNA (dsRNA), while both TLR7 and 8 recognise single-stranded RNA (ssRNA). TLR9 recognises unmethylated CpG Oligodeoxynucleotide DNA from both DNA viruses and bacteria. TLR11 has been reported to recognize uropathogenic *E. coli* (30) and a profilin-like protein from *Toxoplasma gondii* (31). TLR10 has also been shown to be located intracellularly and whilst it has been shown to recognise the micro-organism *Listeria monocytogenes* (32), its exact ligand has yet to be elucidated.

As TLR3 and TLR4 are the main focus of this work, they will be described in more detail.

1.2.2 Toll-like Receptor 4 Signalling Pathway

Following recognition of LPS, recruitment of the adapter protein Mal to TLR4 occurs, resulting in the recruitment of Myeloid differentiation adapter protein (MyD88) (33). MyD88 can then recruit the IL-1 receptor kinase-4 (IRAK-4) through DD-DD interactions, subsequently recruiting IRAK-1. Phosphorylation and ubiquitylation of IRAK-1 then results, allowing for association between IRAK-1 and TNFR-Associated Factor (TRAF)6 to occur. This induces both oligomerization and polyubiquitination of TRAF6. The E3 ubiquitin ligase Pellino1 also induces ubiquitylation of TRAF6. Following both auto-ubiquitylation and ubiquitiylation of TRAF6, TRAF6, present in the IRAK-1 complex, becomes activated and subsequently dissociates from the receptor, allowing two TAK-binding proteins, TAB2 and TAB3 to associate with TGF-β-activated kinase (TAK1). This induces phosphorylation of inhibitor of kappa B Kinase-β (IKKβ), resulting in recruitment of TAK1. Inhibitory κB (IκB) bound in a complex with Nuclear Factor κ B (NFκB) is then degraded, resulting in translocation of NFκB to the nucleus, thus inducing production of inflammatory cytokines and chemokines (34). Similarly, TAK1 also induces activation of c-Jun N-terminal Kinase (JNK), extracellular signal related kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinases (MAPKs). This induces activation of the transcription factor activator protein-1 (AP-1), resulting in production of cytokines and chemokines. Additional proteins not detailed here are involved in this pathway as outlined in figure 1.1 (35).

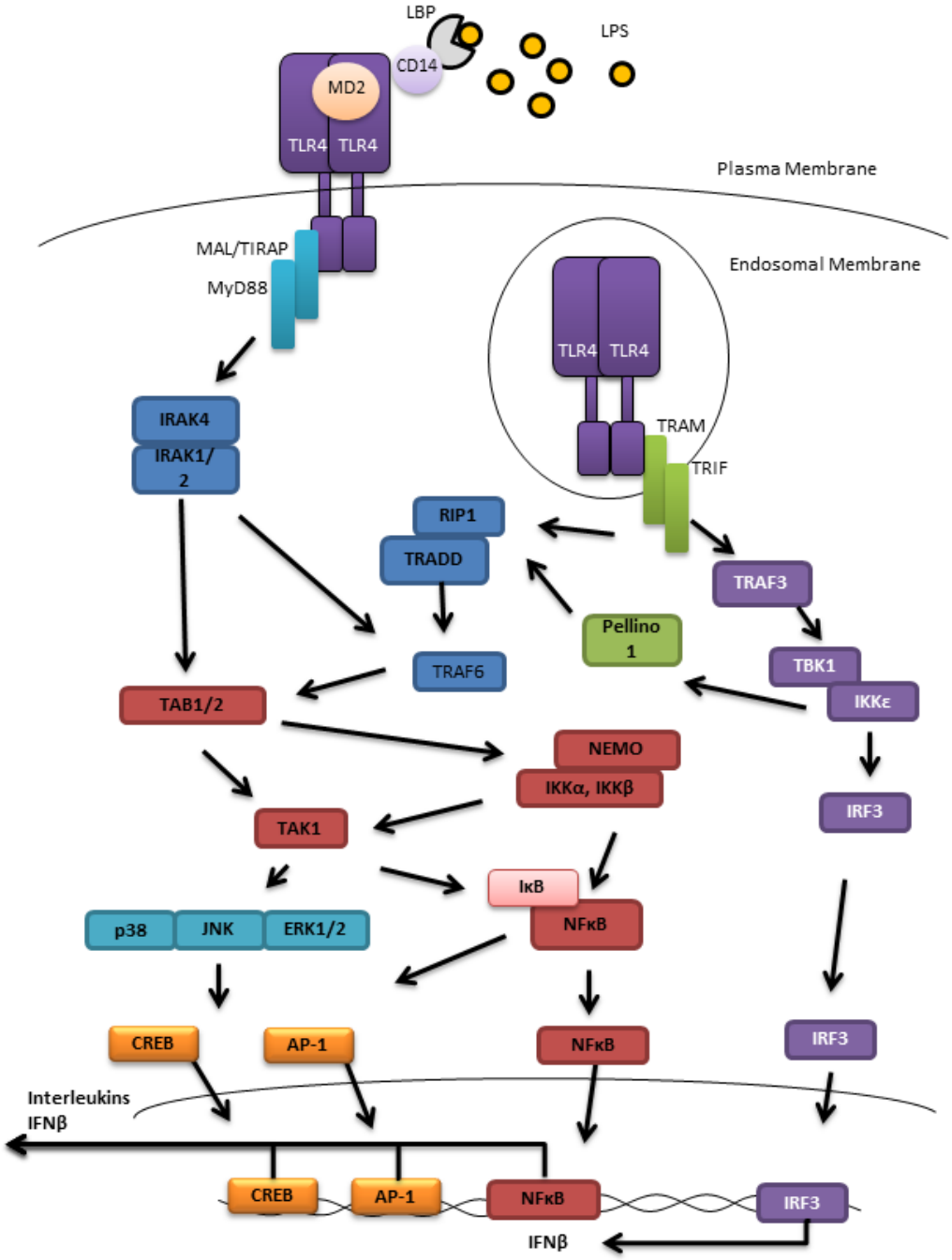

Figure 1.1: Signalling pathway of Toll like receptor 4

Following ligand recognition, TLR4 initiates a downstream signalling cascade using the adapter molecules Mal and MyD88, resulting in activation of NFκB and the MAPKs. This induces the production of inflammatory cytokines. TLR4, when trafficked to the endosome, recruits the adapter proteins TRAM and TRIF, resulting in the activation of IRF3, inducing the production of inflammatory cytokines.

Following activation of the MyD88 dependent pathway, it is thought that internalization of the receptor occurs in a clathrin and dynamin dependent manner, allowing activation of the MyD88 independent pathway to occur (36). This pathway is generally referred to as a late response pathway. It is thought that TLR4, now present in endosomes is recognised by TRIF related adapter molecule (TRAM). TRAM binds to the TIR domain in TLR4 once bound to MyD88. TRAM now binds to TRIF. TRIF then recruits Receptor Interacting Protein 1 (RIP1) through RIP homotypic interaction motif (RHIM) interactions, which can then complex with TNFR1-associated death domain protein (TRADD), which mediates downstream activation of signalling molecules, resulting in TRIF-induced NFκB activation and cytokine production (37). Whilst it is unclear whether Pellino-1 acts directly on RIP1 (38), it has been demonstrated that phosphorylation of Pellino-1 by the TBK1-IKKε complex (39) induces polyubiquitylation of RIP1, resulting in recruitment of TAK1, and TAB1/2 and NFκB essential modulator (NEMO), resulting in the activation of downstream transcription factors such as NFκB and AP-1 (Activator-protein-1), resulting in cytokine production. TRIF also recruits the signalling intermediate TRAF-3 which can interact with TRAF family member-associated NFκB activator (TANK), TANK binding kinase 1 (TBK-1) and also IKKε (IKKi). Dimerization and translocation of the Interferon regulatory factor 3 (IRF3) to the nucleus then results from interaction of TBK1 and IKKε, inducing the production of interferons (figure 1.1) (36).

The TLR4 signalling pathway is tightly regulated, with upwards of 35 regulatory proteins identified to date (40-42). IRAK-M is an inactive kinase which binds to and prevents IRAK4 and IRAK1 association with MyD88. As a result, the MyD88 signalling pathway is interrupted thus limiting NFκB activation (43). A20 is a

ubiquitin-modifying enzyme with deubiquitination of RIP1 by A20 preventing the activity of RIP1, and thus, any activation of downstream signalling intermediates again limiting NFκB activation (44). Sterile α- and armadillo-motif-containing protein (SARM) has been shown to limit TLR4-induced cytokine production (45). Whilst studies have shown that SARM and TRIF can directly interact, it has been demonstrated that SARM mediates its negative regulatory effect by inhibiting MAPK activation, thus limiting activation of NFκB, reducing the production of cytokines (46).

1.2.3 Toll-like Receptor 3 Signalling pathway

TLR3 recognises dsRNA from viruses such as respiratory syncytial virus (RSV) and rotavirus and therefore it predominantly induces production of anti-viral cytokines such as interferon-β (IFNβ). It is capable however, of inducing interleukin production through activation of NFκB. In the resting state, TLR3 is found within the endosome compartment (47, 48). This may be a mechanism preventing activation of TLR3 by self-nucleic acids (49). While TLR3 can be expressed on the cell surface, for example on human fibroblasts, it has been suggested that expression on the cell surface may be transient as viral infection of A549 lung epithelial cells with RSV led to detection of cell surface TLR3 (50). In contrast, TLR3 cell surface expression was not detected in A549 cells devoid of infection with RSV (50).

TLR3 signalling utilises the adapter molecule TRIF for signal transduction. Upon recognition of dsRNA, TLR3 becomes phosphorylated at two tyrosine residues present in its cytoplasmic tail (51). TRIF is recruited via TIR-TIR interaction to TLR3 (52), which can then associate with TRAF-3 and TRAF-6 through TRAF-binding motifs contained within its N-terminal portion (24, 53). Recruitment of

TRAF3 then results in activation of TBK-1 and the IκB kinases IKKε/i which induces phosphorylation and subsequent activation and dimerization of IRF-3 and IRF-7 which migrate to the nucleus. This results in an anti-viral response by induction of type-1 interferons (figure 1.2) (24).

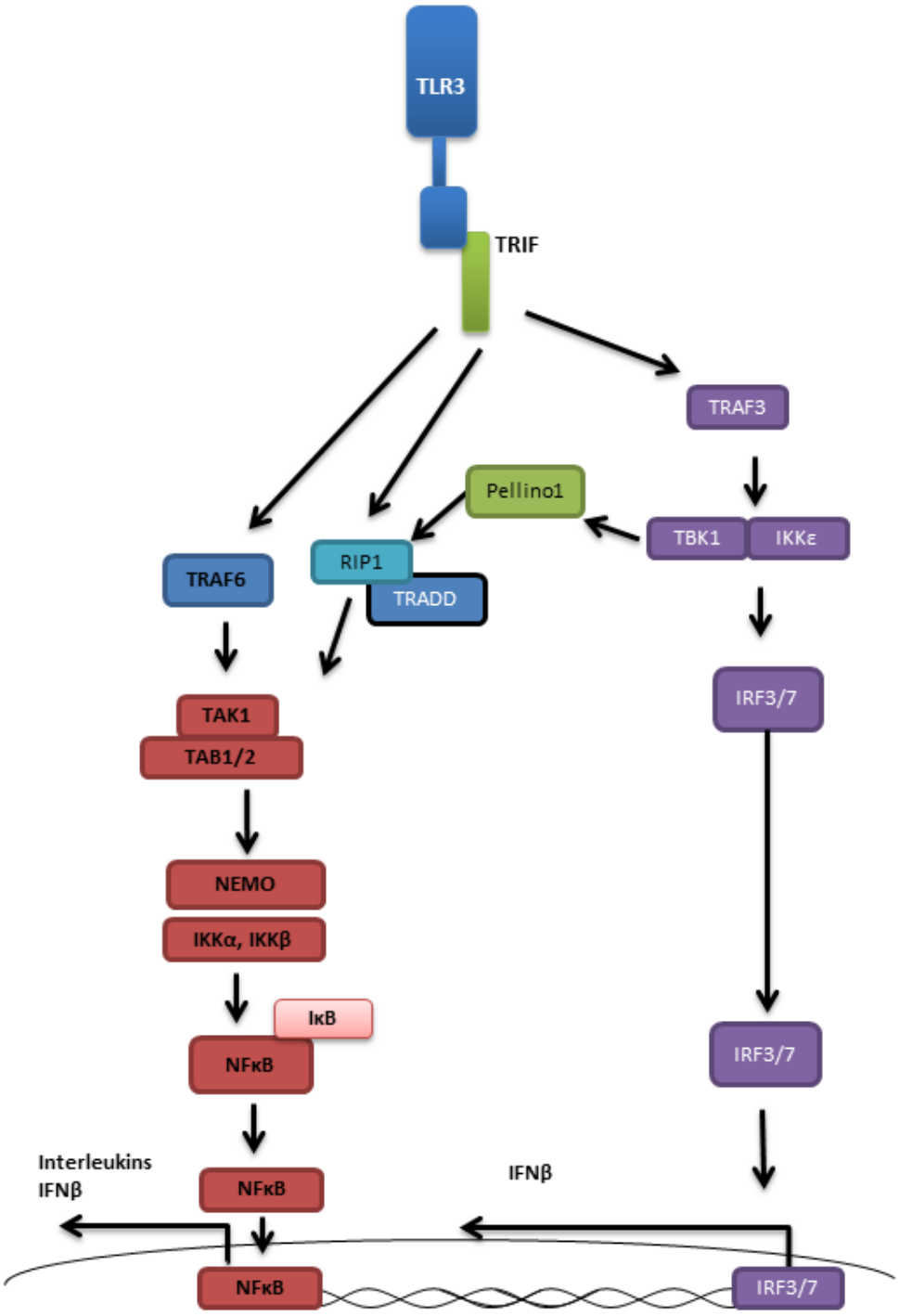

Figure 1.2: Signalling pathway of Toll like receptor 3

Following ligand recognition, TLR3 initiates the activation of a downstream signalling cascade using the adapter TRIF which initiates downstream activation of TRAF3 and subsequently TBK1 which results in activation of the transcription factor IRF3, inducing the production of anti-viral interferon genes. TRIF also recruits RIP1 through RHIM interactions, which mediates activation of TAK1, initiating the activation of the downstream signalling cascade, resulting in activation of NFκB. This induces the production of inflammatory cytokines.

Whilst the adapter proteins MyD88 and Mal are not required for TLR3-induced signalling, recent studies have demonstrated that these proteins may act as negative regulators of the TLR3 signalling pathway, thus limiting IFNβ production (54, 55). They demonstrated that MyD88$^{-/-}$ macrophages had enhanced IFNβ production compared with wild-type macrophages following poly I:C stimulation, with overexpression of MyD88 observed to inhibit IKKε activity, limiting IFNβ production (54). Similarly, poly I:C-induced production of IFNβ by Mal$^{-/-}$ Bone marrow-derived macrophages (BMDMs) was also enhanced, with Mal inhibiting the activity of Interferon Regulatory Factor (IRF)7 (55).

In addition to the TIR domain, TRIF also contains the RHIM which facilitates interaction with RIP1, required for TLR3 induced NFκB activation (37). Upon phosphorylation of Pellino-1 by the TBK1-IKKε complex, RIP1, held in a complex with TRADD, undergoes Pellino-1 mediated ubiquitylation (39). TAK1 mediates downstream TLR3-induced activation of NFκB by both TRAF6 and RIP1 (56). Both TRAF6 and RIP1 utilise the same downstream molecules, IKKα, IKKβ and NEMO, collectively known as the IKK complex which induce the phosphorylation of IκB, thus allowing the activation of the transcription factor NFκB resulting in the production of inflammatory cytokines and chemokines (figure 1.2) (57).

1.2.4 RIG-I like Receptors (RLRs)

RLRs are located intracellularly and recognise viral components present in the cytoplasm of the cell. This family comprises of RIG-I, Melanoma Differentiation-associated Antigen 5 (MDA-5) and Laboratory of Genetics and Physiology 2 (LGP2), all composed from three DExD/H box helicases which are essential for viral recognition (58, 59).

RIG-I and MDA5 have a number of similarities in their structures and are organised into three distinct domains: an N-terminal region which has sequential caspase activation and recruitment domains (CARDs), a central DExD/H box RNA helicase domain, and a C-terminal repressor domain (RD) which is enclosed within the C-terminal domain (CTD) present only in RIG-I (figure 1.3a) (59). MDA5 recognises long dsRNA, while RIG-I recognises both short dsRNA molecules and ssRNA which are phosphorylated at the 5' end (60, 61). RIG-I and MDA5 associate with dsRNA through the helicase domain (62), as unwinding of dsRNA occurs through an ATPase dependent process (63). Once the dsRNA binds to the helicase domain of the RIG-I molecule, a conformational change occurs in RIG-I (57), which releases the CARD domain enabling interaction with interferon-β promoter stimulator 1 (IPS-1, also known as mitochondrial anti-viral signalling protein (MAVS), virus-induced signalling adaptor (VISA) and CARD adaptor inducing IFNβ (CARDIF)) through CARD-CARD interactions (64).

Both RIG-I and MDA5 share common signalling intermediates as they both activate the adapter protein IPS-1 (65, 66). IPS-1 interacts with RIG-I and MDA5 through CARD-like domains present in the N-terminal, IPS-1 recruiting the downstream signalling components Fas-associated protein with death domain (FADD) and RIP1

through the C-terminal effector domain (65). TRADD has also been implicated in RLR signalling. It has been demonstrated that IPS-1 recruits TRADD which then forms a complex with both FADD and RIP1 through death domain interactions (DD-DD) (59, 67). Following ubiquitination of RIP1, NEMO is recruited, acting as a scaffold for both IKKα and IKKβ thus leading to NFκB activation (67). RLR activation also results in interaction of the RLR adapter protein IPS-1 with TRAF3 through TRAF-interacting motif (TIM) (59, 68). Recruitment of TRAF3 results in the recruitment of both TBK-1 and IKKε. This results in phosphorylation and activation of both IRF3 and IRF7, resulting in the production of type-1 IFNs (figure 1.3b) (68).

The role of LGP2 in anti-viral signalling is not well defined. However, it is thought that RIG-I and MDA5 are regulated by LGP2 (69) potentially by sequestering dsRNA which prevents LGP2 from binding to RIG-I and/or MDA5 through the action of its repressor domain (RD) (68, 70).

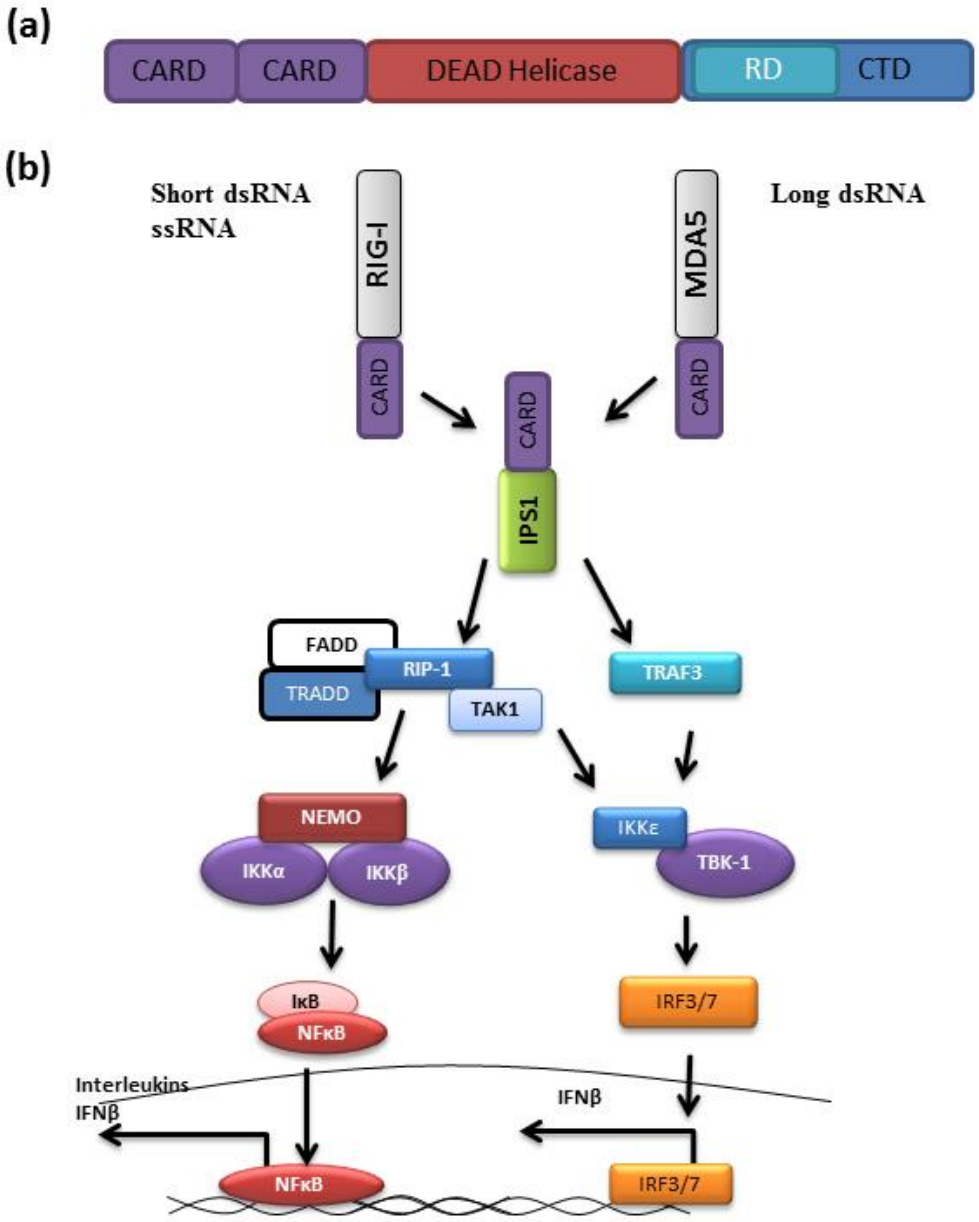

Figure 1.3: Structure representation and signalling pathway of RIG-I and MDA5

(a) RIG-I and MDA5 consists of two CARD domains; a DEAD helicase and a C-terminal domain that encodes the repressor domain. **(b)** Upon ligand recognition, RIG-I and MDA5 recruit the adapter protein IPS-1. TRAF3 mediates activation of TBK1, resulting in the activation of the transcription factors, IRF3/7. IPS-1 also interacts with RIP-1, resulting in activation of a downstream signalling cascade, culminating in activation of the transcription factor, NFκB.

1.2.5 NOD-like Receptors

NLRs are intracellular cytosolic sensors, involved primarily in bacterial recognition. They are composed of a tripartite structure, similar to that of plant R-proteins. They consist of an N-terminal effector binding region containing protein-protein interaction domains such as CARD, pyrin (PYD) and baculovirus inhibitor repeat (BIR) domains, an intermediary nucleotide binding and oligomerization domain (NOD) and an array of C-terminal LRR motifs which detect microbial PAMPs and modulate NLR activity (71). Spontaneous activation of NLR proteins is thought to be prevented by the LRRs in the C terminus folding back onto the NOD domain (72, 73). To date, 23 NLR proteins exist in humans, with 34 in mice (74). NOD1 and NOD2 are well characterised members of this family which recognise peptidoglycan components of bacterial cell walls (figure 1.4a). NOD1 recognises γ-D-glutamyl-*meso*-diaminopimlic acid, present in all Gram-negative but only some Gram-positive bacteria, while NOD2 recognises muramyl dipeptide, present in both Gram-positive and Gram-negative bacteria (75). Upon activation of these receptors, conformational changes occur causing the receptors to self-oligomerise. This in turn exposes the effector domains, recruiting and activating a downstream kinase of the NOD signalling cascade, RICK (RIP2), a serine-threonine kinase specifically required for NOD1/2 signalling. This is required for NFκB and MAPK activation (76, 77).

Several distinct members of the NLR family of receptors, including NLRP3 and NLRP4, have been described (figure 1.4b). These NLR family members can form a high molecular weight complex termed the inflammasome upon recognition of PAMPs as well as Damage Associated Molecular Patterns (DAMPs) (78, 79). Upon activation, oligomerization of these NLRs occur, resulting in the recruitment of

apoptosis-associated speck-like protein containing a CARD (ASC) through PYD-PYD interactions, resulting in the formation of multimers of ASC dimers. ASC then recruits procaspase-1 by CARD-CARD interactions, inducing self-cleavage of procaspase-1, resulting in its activation. This in turn activates both pro-IL-1β and pro-IL-18 (78-80). This pathway is termed the canonical pathway as it does not require additional co-factors for processing of IL-1β. TLR4-induced IFNβ production is however required for processing of IL-1β in the NLRP3 inflammasome, with IFNβ activating caspase-11 thereby resulting in the activation of caspase-1 and the production of IL-1β and IL-18 (figure 1.4c) (78). Inflammasome formation can also induce pyroptotic cell death as a result of caspase-1 activation which shares features with both apoptosis and necrosis, inducing an inflammatory response (78).

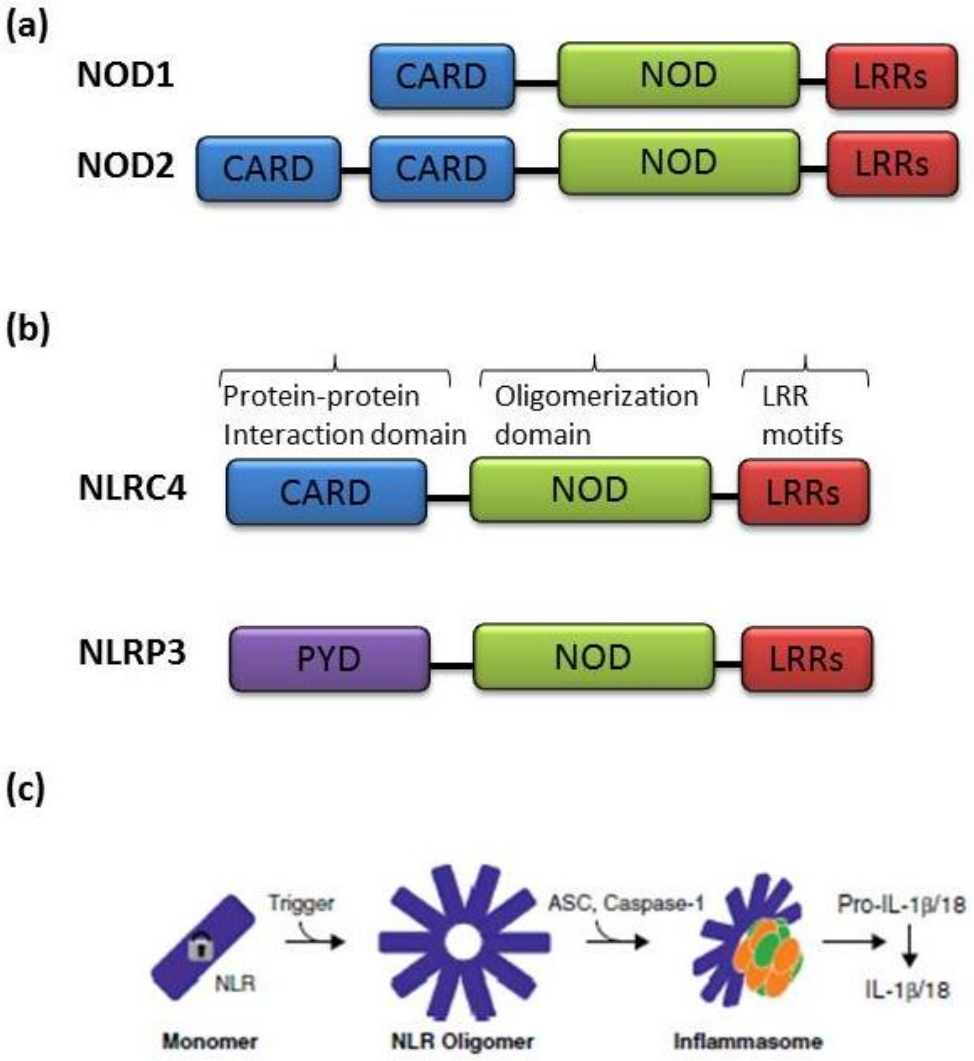

Figure 1.4 Structure of the NLRs NOD1, NOD2, NLRC4 and NLRP3 and depiction of inflammasome formation
(a) NOD1 and NOD2 are composed of three domains: a carboxy-terminal LRR domain, a central NOD domain, and an amino-terminal domain composed of a CARD. **(b)** NLCR4 and NLRP3 are composed of a carboxy-terminal LRR, a NOD domain, and either a CARD domain or PYD domain respectively. **(c)** Upon activation, oligomerization of NLRs, recruiting ASC. This results in formation of the inflammasome, resulting in the activation of caspase-1 and processing of IL-18 (Lechtenberg *et al.* (80)).

1.2.6 C-type Lectins (CLRs)

CLRs are PRRs well characterised for their role in fungal recognition, recognising carbohydrate based moieties through at least one carbohydrate recognition domain (CRD) present on the receptor. Present as both transmembrane proteins and soluble proteins, to date, 17 groups exist within this family consisting of over 1,000 members. They are distinguished from each other according to phylogeny and structure, with further subdivisions made based on the signalling potential of the CLR (81). Recent studies suggest however, that CLRs also recognise other pathogens such as bacteria and viruses (82-85). In terms of their role in bacterial infection, CLRs have been implicated in controlling *M. tuberculosis* (MTB) infection *in vitro*, however, *in vivo* studies have failed to replicate these findings (82, 83).

CLRs have also been implicated in viral infection in both a protective and detrimental manner. Activation of the CLR, DC-SIGN appears to exacerbate HIV infection by promoting viral survival, thus favouring transmission, infection and inflammation. This occurs as a result of modulating TLR-induced cytokine production and inducing accelerated dendritic cell (DC) apoptosis, dampening the maturation process of DCs in general, enhancing viral infection (84). Conversely, CLRs appear to have a protective role in the host against Herpes Simplex virus, whereby CLRs recognise dead and damaged cells, inducing activation of $CD8^+$ T cells (85).

Taken together, it is clear that PRRs in their entirety facilitate removal of invasive micro-organisms, both individually and in collaboration with each other,

demonstrating that the innate immune system is a diverse and complex entity in the host's armoury against infection.

1.3.1 The Fas Receptor

Fas (CD95/APO-1) is expressed as a homotrimer (86, 87) by numerous cells throughout the body, including lymphoid immune cells as well as in the heart, kidney and liver (88-90). Fas was first identified as a death receptor as a result of the specific apoptotic ability of anti-Fas antibodies on tumour cells (91, 92). Fas is a 45kDa type-1 transmembrane protein and is a member of the TNF receptor superfamily of death receptors, that also include TNFR1, TNFR2, DR3, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which share homology in their extracellular cysteine rich domains (93).

The Fas gene is comprised of nine exons, separated by 8 introns. It consists of three cysteine-rich domains (CRDs), the hallmark of the TNFR superfamily, present in the N terminal region encoded by exons 1 through 5, a transmembrane domain encoded by exon 6, and the intracellular domain encoded by exons 7 through 9 which contains a death domain (DD) composed of 80 amino acids (94). This facilitates the interaction with the DD of its adapter protein, FADD (figure 1.5a).

1.3.2 Fas Ligand

Activation of Fas signalling occurs following binding of the Fas receptor by its ligand, Fas Ligand (FasL/CD95L). FasL is a 40kDa type II transmembrane protein (95) expressed on activated T cells, NK cells and cytotoxic T lymphocytes (96), as well as in immune privileged sites such as the eye, testis, ovary and placenta (97, 98). The human *FasL* gene is composed of four exons. Interaction between Fas and

FasL is mediated by the specific receptor binding domain, present in the C terminal extracellular domain of FasL, which binds to the CRD of Fas. It is thought that the highly conserved proline residues contained within the N terminal intracellular domain of FasL, along with several potential tyrosine phosphorylation sites may influence FasL protein sorting to secretory lysosomes (96). Oligomerization of FasL is mediated by a self-assembly domain on FasL, whilst the function of the putative casein kinase I (CKI) motif has yet to be elucidated (figure 1.5b) (96).

1.3.3 The Fas associated death domain containing protein (FADD)

The FADD (Mort-1) gene is located on chromosome 11q13.3 in humans and chromosome 7 in mice and is organised into two exons, separated by a 2kb intron (99). Murine FADD shares 68% identity with that of human FADD (100). Within its structure, FADD contains two well conserved domains, the DD and the Death Effector Domain (DED), present in the C-terminal region in exon 2and in the N-terminal region in exon 1 respectively (figure 1.5c) (99, 101).

FADD is required for activation of the Fas signalling pathway. It has also been demonstrated that FADD is required for embryo development. Homozygous FADD mutant mice, generated by gene targeting in embryonic stem cells, did not survive *in utero* (102). Although FADD was detected in all tissues, with the expression of FADD concentrated in the brain, liver, developing vertebrae and myocardium, the requirement of FADD for embryo development is thought to be due to its role in the development of the ventricular myocardium (102).

Through the presence of the DD, FADD can also interact with other members of the TNF family of death receptors through DD-DD interactions. FADD can interact with

the TNF-R1 signalling pathway through interaction with its adapter protein TRADD, inducing NFκB activation (103). Internalisation of the TNFR1 receptor results in dissociation of TRADD, which can now interact with FADD, resulting in apoptosis (103). FADD also interacts with both DR4 and DR5 through DD-DD interactions, resulting in both apoptosis (104, 105) and in activation of NFκB (106).

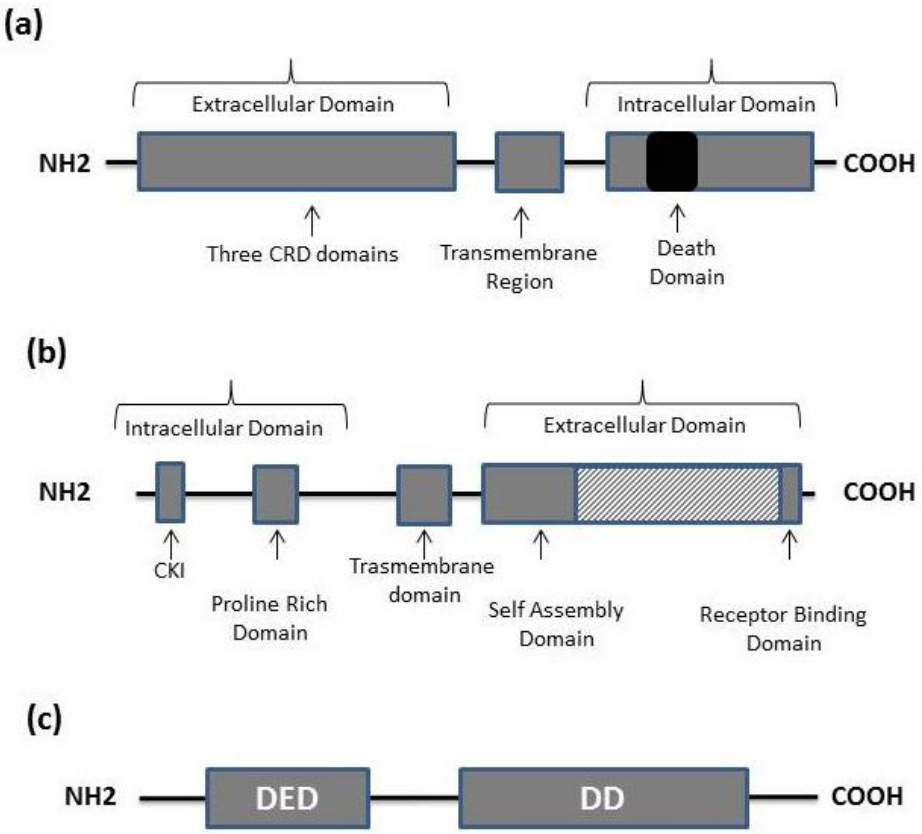

Figure 1.5: Structure of Fas, FasL and FADD protein

(a) Fas contains within its extracellular domain three cysteine-rich domains, a transmembrane domain, and a death domain contained within the intracellular domain in the N-terminus. **(b)** The C-terminal extracellular domain of FasL contains a specific receptor binding domain and self-assembly domain. FasL also contains a transmembrane domain. FasL contains a conserved proline rich domain and casein kinase I (CKI) within the N-terminal domain. **(c)** FADD contains an N-terminal DED domain and a C-terminal DD domain.

1.3.4 Apoptotic pathways of Fas

Cell death can occur by apoptosis, autophagy, necrosis (107), and the more recently identified necroptosis (108). Apoptosis is a form of programmed cell death, which results in nuclear and cytoplasmic condensation, resulting in the formation of apoptotic bodies (107, 109). These apoptotic bodies are then engulfed by neighbouring cells and degraded in a controlled manner, thus avoiding immune activation (107, 109). Fas-mediated apoptosis can be further characterised into two pathways; the extrinsic (death receptor) pathway, and the intrinsic (mitochondrial) pathway (110), with FADD, the adapter protein of the Fas signalling pathway, also contributing to programmed necrosis (necroptosis) (111).

The extrinsic pathway is induced upon binding of FasL to the Fas receptor, present on the plasma membrane, resulting in activation of the Fas signalling pathway (112). Upon activation, the intracellular domain of the receptor undergoes a conformational change (113), resulting in recruitment of the adapter protein FADD to Fas, through DD-DD interactions (114). FADD then recruits procaspase-8 (115, 116), procaspase-10 and cellular FADD-like IL-1β-converting enzyme-(FLICE)-inhibitory protein (cFLIP), a regulator of caspase-8/10 activation (117) through DED-DED interactions. This results in the formation of the Death Inducing Signalling Complex (DISC) around the cytoplasmic tail of Fas (115), with internalization of the receptor required for efficient DISC formation (118). Oligomerization of procaspase-8 occurs, resulting in auto-proteolytic cleavage of procaspase-8, which results in the activation of caspase-8. In type I cells, sufficient amounts of caspase-8 are activated at the DISC to trigger activation of additional caspases (119), including the effector

caspases, caspase-3 and caspase-7 (120). This results in enhancement in caspase activity and apoptotic death of the cell (118).

However, if insufficient levels of caspase-8 activation occur due to reduced DISC formation, the Fas signalling pathway has a compensatory mechanism, which utilises the mitochondria for activation of the effector caspases, resulting in activation of Fas-induced intrinsic apoptotic pathway (120). These are known as type II cells (121). In these cells, caspase-8 activation results in the cleavage of the protein Bid, a member of the Bcl-2 family of proteins present in the cytoplasm (122). This results in the formation of truncated Bid (tBid) which translocates to the mitochondria, causing the aggregation of both Bax and Bak, two members of the Bcl-2 family of proteins (123). This then results in the release of second mitochondria-derived activator of caspase (SMAC) and cytochrome c from the mitochondria, with the latter forming a complex with apoptotic protease-activating factor-1 (Apaf-1), 2'-deoxyadenosine 5'triphosphate (dATP) and procaspase-9, resulting in the activation of the initiator caspase in this apoptosome complex (121, 124). Caspase-9 can in turn activate the effector caspase, caspase-3, resulting in apoptosis of the cell (figure 1.6a) (123).

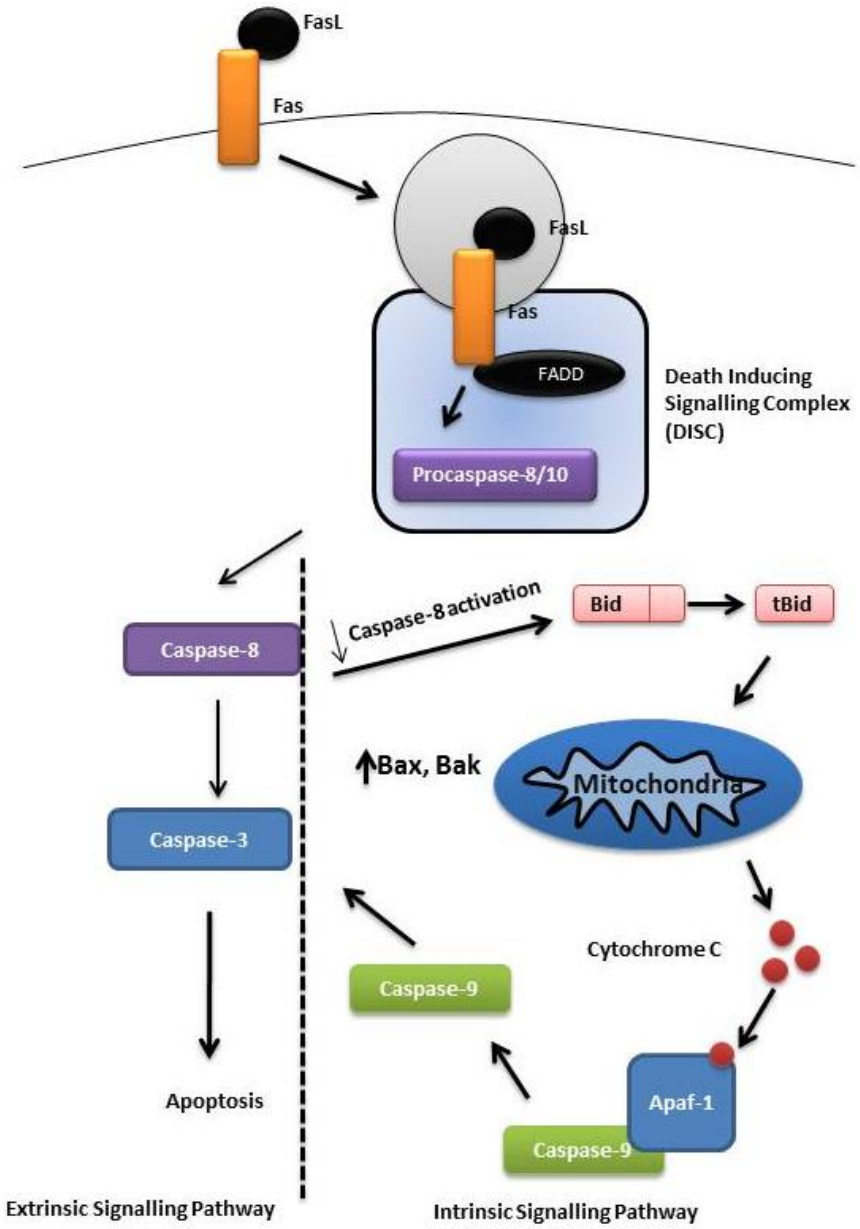

Figure 1.6a: The Fas/FasL-induced apoptotic signalling pathways

Recognition of FasL by its receptor Fas results in the oligomerisation of the receptor, initiating DISC formation. Auto-proteolytic cleavage of procaspase-8 occurs resulting in the activation of downstream effector caspases, and ultimately cell death. Reduced levels of caspase-8 can also activate the mitochondrial cell-death pathway, mediated by the activation of Bid, resulting in the release of cytochrome c from the mitochondria, resulting in the activation of Apaf-1 and caspase-9, culminating in apoptosis.

It is thought that inhibitors of apoptosis (IAPs), composed of cIAP1/2 and X chromosome-linked inhibitor of apoptosis protein (XIAP) may also regulate Fas-mediated apoptosis. XIAP, cIAP1 and cIAP2 are thought to all bind and inhibit caspase-3, -7 and -9, whilst having no effect on caspases-1, -6, -8 or -10 (125, 126).

In the absence of caspase activation, Fas activation can result in necroptosis (127, 128). The requirement for the Fas adapter protein FADD in necroptotic signalling has also been demonstrated (129, 130). Necroptotic signalling is mediated through formation of a signalling complex composed of RIP1, RIP3, FADD and caspase-8, termed the Ripoptosome, with its formation occurring independent to other death receptor signalling pathways (127, 129, 130). Ripoptosome formation is regulated by the IAPs, as well as cFLIP (129). IAPs mediate ubiquitination of RIP1, thereby limiting its availability for Ripoptosome formation (130). cFLIP is a negative regulator of Ripoptosome formation, with increased levels of cFLIP reducing Ripoptosome formation (129) by limiting the availability of FADD for complex formation. Studies have demonstrated that the kinase activity of both RIP1 and RIP3 are essential components for Ripoptosome formation, with formation of this complex inducing either apoptotic (caspase dependent) or necroptotic (caspase independent) signalling (figure 1.6b) (108, 129).

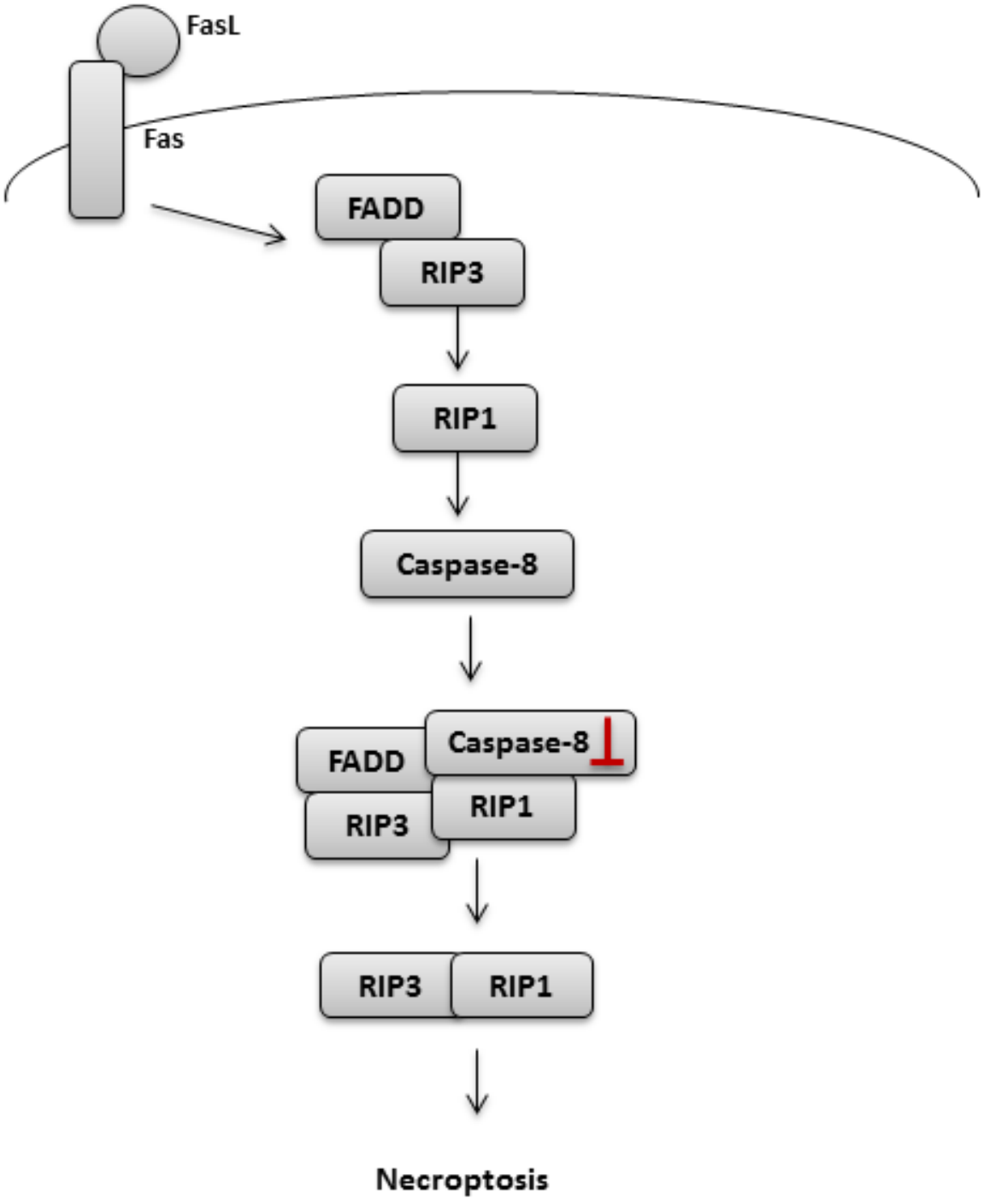

Figure 1.6b: Fas activation and necroptosis

Fas activation in the absence of caspase activity results in necroptosis of the cell. Necroptotic signalling is mediated by a complex containing RIP1, RIP3, FADD and caspase-8, with the kinase activity of RIP1 and RIP3 capable of inducing necroptosis.

1.3.5 Apoptotic functions of Fas

The Fas signalling pathway is an important means of maintaining immune privileged sites such as the eye, testis, ovary and placenta. Local expression of FasL targets invading Fas expressing cells, resulting in apoptosis. This limits inflammation, thus limiting any collateral damage from inflammatory events, maintaining anatomical integrity of these sensitive sites (97, 98, 131). Immune privilege may also be utilised by tumours to evade the immune system, with tumour cells expressing FasL inducing Fas-mediated apoptosis of infiltrating immune cells, thus allowing progression of the tumour (121, 132, 133).

The Fas signalling pathway plays a vital role in T cell homeostasis. This becomes apparent when the Fas signalling pathway is interrupted, resulting in dysregulation of Fas-induced apoptosis. It has been demonstrated in mice which contain mutations in Fas (*lpr*) or FasL (*gld*) that interruption of the Fas signalling pathway disrupts Fas-mediated apoptosis and as a result, disrupts T cell homeostasis resulting in lymphoproliferative disorders, as well as autoimmune conditions such as arthritis and systemic lupus erythromatosis (SLE) (134, 135). This is similar to the human condition, autoimmune lymphoproliferative syndrome (ALPS) which is caused by mutation in components of the Fas signalling pathway, preventing Fas signalling. This results in lymphoproliferation and a predisposition of these individuals to develop lymphomas (136-138).

1.3.6 Non-apoptotic Functions of the Fas signalling pathway

Whilst Fas is classically associated with apoptosis, recent studies have demonstrated that the Fas signalling pathway is multifaceted and is involved not only in T cell

homeostasis and the maintenance of immune privileged sites, but can also trigger migration, proliferation and inflammation (figure 1.7).

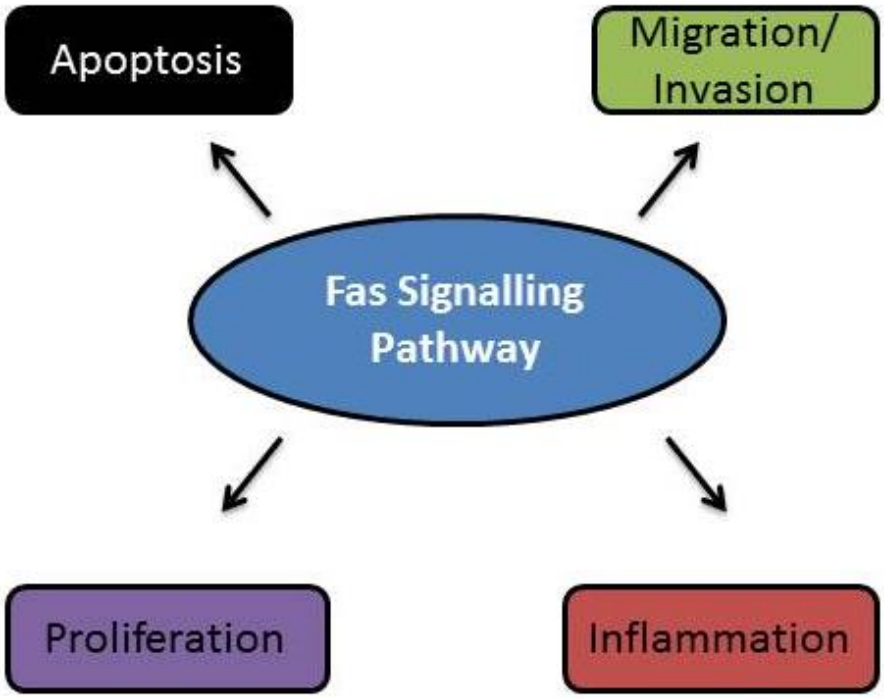

Figure 1.7: The functions of the Fas signalling pathway

In addition to apoptosis, the Fas/FasL pathway has been shown to induce migration, invasion, proliferation and inflammation.

Proliferation

Whilst studies have demonstrated that naïve T cells are resistant to Fas-mediated apoptosis, ligation of Fas induces proliferation of these cells (139, 140). Proliferation of T cells also occurred with regard to memory T cells due to their prior exposure to an antigen (141). T cells require two main signals for activation. Signal 1 results from engagement of the T cell receptor (TCR)/CD3 complex, with signal 2 provided by either IL-12, IL-4, or ligation with CD28. Activation of the TCR/CD3 complex in the absence of signal 2 would result in Fas-mediated apoptosis of naïve T cells. However, TCR/CD3-stimulated memory T cells in the absence of signal 2 do not undergo Fas-mediated apoptosis following Fas ligation. It has been demonstrated that co-stimulation of memory T cells with TCR/CD3 with Fas activation results in proliferation of memory T cells (141). Studies have also demonstrated that Fas activation can also induce proliferation of tumour cells, whereby a reduction in Fas or FasL expression resulted in reduced proliferation, possibly as a result of reduced JNK and ERK MAPK activation (142, 143). Fas and FasL are widely expressed in the nervous system (144, 145) with studies demonstrating that following sciatic nerve injury *in vivo*, Fas activation enhanced nerve regeneration (88).

Migration/Invasion

Fas ligation can also result in migration and invasion in apoptosis resistant cells, with stimulation of tyrosine kinase activity by Fas implicated in these processes (146). Fas induces tyrosine kinase activity, resulting in phosphorylation of caspase-8 (147), the scaffold protein TRIP6 (148), and the catalytic subunit of phosphatidylinositol-3-kinase (p110-PI3K) (149) however the sequence in which this occurs is unclear (146). Caspase-8 then becomes a docking site for Src

homology domain 2 (SH2) containing proteins, recruiting them to the Fas complex (146). Recruitment of Yes and p85-PI3K, members of the Src-family kinase, to the Fas complex results in PI3K activation. This results in the activation of Akt which, following a series of signalling events, can activate either NFκB through interaction with the caspase8/RIP1/TRAF2 signalling complex, or the Wnt pathway, inducing the expression of metalloproteases (MMPs) and urokinase plasminogen activator (uPA) (110, 146). Studies have demonstrated that expression of MMP9 results in increased migration of cells, with MMPs aiding the migration of cells through the extracellular matrix (110, 146).

Inflammation

Fas activation has also been implicated in inflammation (figure 1.8). Fas activation by the agonistic Fas antibody CH11 induced the production of TNFα, IL-8 and IL-10 in human monocyte-derived macrophages (150). This study demonstrated that human monocyte-derived macrophages were resistant to apoptosis, and therefore the inflammatory effect was not a by-product of cell death (150). They also demonstrated that Fas-induced cytokine production was independent of caspase activation (150).

It has also been observed that priming of macrophages with LPS induced Fas expression in macrophages which, upon ligation of Fas, resulted in the production of IL-1β and IL-18 (151). Whilst associated with the inflammasome, the maturation and secretion of IL-1β and IL-18 were determined to be independent of the inflammasome components NLRP3, ASC and caspase-1. Thus they concluded that Fas ligation induced the expression of these cytokines in a non-canonical manner (151).

Fas ligation can induce the phosphorylation of MAPKs (152, 153). The MAPKs are thought to play a key role in cytokine production, particularly ERK1/2 and JNK MAPKs. Studies have demonstrated that inhibition of the MAPKs resulted in inhibition of IL-8 production (152, 154). MAPK activation resulted in the activation of the transcription factors AP-1 and NFκB, inducing cytokine production (155).

Whilst it is well established that the MyD88 adapter protein facilitates TLR-induced cytokine production, it has now been demonstrated that MyD88 may be implicated for Fas-induced inflammation (156). In this study, they demonstrated that while Fas induced the production of inflammatory cytokines, in the absence of MyD88 cytokine production in both RAW264.7 macrophages, as well as peritoneal macrophages derived from $MyD88^{-/-}$ mice was diminished, with cytokine production occurring independent of caspase activation (156). Consistent with this, MyD88 was shown to be required for Fas-induced production of CXCL-1/KC by alveolar epithelial cells. This was also demonstrated to occur independent of caspase activation (154).

Together, these studies have demonstrated that Fas activation can indeed result in the production of inflammatory cytokines. As the Fas adapter protein FADD has been implicated in a number of innate immune receptor signalling pathways, it has now been demonstrated that Fas activation modulates their cytokine production .This occurs following recruitment of FADD to the DISC complex. This reduces the level of FADD present in the cytosol, thereby removing its effect from these immune receptors (157-159).

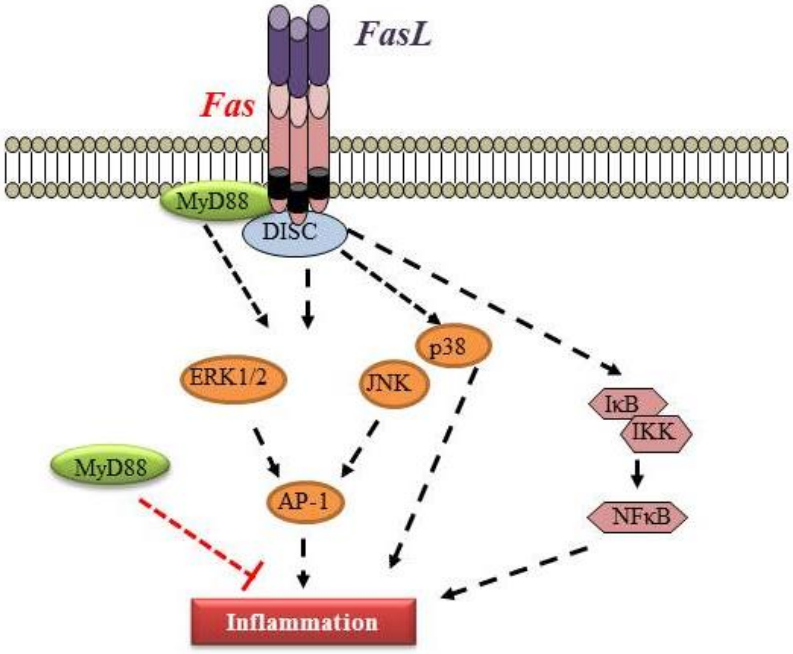

Figure 1.8: Fas-induced inflammation is induced by different signalling pathways.

Fas-mediated inflammation may be induced via activation of the NFκB transcription factor, or activation of ERK, p38 and JNK MAPK signalling pathways downstream of the DISC. MyD88 may directly interact with Fas in a FADD independent manner, resulting in Fas-mediated inflammation. Signalling pathways/intermediaries which are not fully elucidated are represented by dashed lines.

1.4 Cross-talk between Fas and Toll like receptors

Studies have demonstrated that the Fas signalling pathway can modulate TLR-induced cytokine production (158, 160-162). Interruption of the Fas signalling pathway reduced the expression of LPS-induced IL-6 production in macrophages (158), whilst Fas activation enhanced both LPS- and Flagellin-induced cytokine production in intestinal epithelial cells (160). Recruitment of inflammatory cells and therefore cytokine production was also reduced in cardiac dysfunction by blocking Fas signalling (161, 162).

The Fas adapter protein FADD has also been reported to play a negative regulatory role in TLR4 signalling (157-159). Studies have demonstrated that LPS-induced NFκB activation was reduced following overexpression of FADD (157, 158). It has also been demonstrated that LPS-induced NFκB activation was enhanced in $FADD^{-/-}$ MEFs (157), with FADD regulating NFκB activation through interaction with MyD88, one of the four adapter proteins utilised by TLR4 (158, 159) and IRAK1 (159) through DD-DD interactions, with IRAK1 proving essential for formation of this complex (159). These studies demonstrated a regulatory role for FADD in LPS-induced cytokine production using overexpression studies or removal of FADD from the cell.

Studies have demonstrated that viruses can modulate the Fas signalling pathway to their advantage (163, 164). Both equine herpesvirus type 2 (EHV-2) protein E8 and molluscum contagiosum virus (MCV) MC159 protein are DED containing proteins. It has been demonstrated that E8 interacts with the DED of caspase-8 prodomain, whilst MC159 binds to the DED of FADD, thereby preventing the activation of Fas-mediated apoptosis, resulting in the persistent infection of cells by these viruses

(163). Studies have also demonstrated that infection of cells with HSV-2 enhanced the expression of Bcl-2, thus protecting against Fas-induced apoptosis and resulting in the establishment of a persistent infection (164).

The role of FADD however is more defined in terms of viral infection. Studies have shown that FADD is required for RIG-I and MDA5 induced type 1 IFN production (165, 166). They demonstrated that RIG-I-induced IFNβ production was significantly reduced in $FADD^{-/-}$ MEFs following poly I:C stimulation as compared to wild-type cells, with reconstitution of $FADD^{-/-}$ MEFs with FADD restoring their ability to produce IFNβ (165). This group also demonstrated that FADD is associated with activation of secondary IFNα genes, with a reduction of IRF7 activation observed in $FADD^{-/-}$ MEFs following viral stimulation mediating its effect through IRF7 activation (166).

Therefore, while it is apparent that FADD is implicated in the innate immune system, its role is somewhat unclear.

1.5 Aim of this study

The Fas signalling pathway is well characterised in terms of its ability to induce apoptosis. Studies now demonstrate that this pathway also mediates inflammatory cytokine production, with an increasing body of data indicating that there is crosstalk between the TLR and Fas signalling pathways both in terms of crosstalk between the receptors themselves and crosstalk between the adaptor proteins in both pathways. Work in this field has been restricted to studies examining crosstalk between Fas and TLR4 with no studies investigating crosstalk between Fas and other TLRs. In addition there are very few studies investigating both Fas and FADD together in terms of their ability to modulate TLR responses. In this study we sought to examine the crosstalk between Fas and TLRs and the effect of Fas activation on TLR signalling, in particular, TLR3, and TLR4. Studies have alluded to the fact that crosstalk may be implicated in the modulation of TLR3- and TLR4-mediated antiviral signalling, as RIGI and TLR3, apart from their adapter proteins, share similar downstream signalling components. Equally, these signalling components are shared by the MyD88-independent signalling pathway of TLR4. To our knowledge, no study has implicated Fas activation on this pathway. Therefore, we sought to investigate more thoroughly the impact of Fas activation on TLR signalling pathways.

Specific Aims:

1. To investigate the role of Fas in the induction of anti-viral cytokine and chemokine responses, and to investigate whether there was a potential role for Fas in modulating poly I:C-induced cytokine and chemokine responses (Chapters 3, 4).

2. To further characterise the role of both Fas and its adapter protein FADD in modulating TLR4-induced inflammation using the agonistic Fas antibody, CH11, and also to identify any potential novel FADD interacting proteins (Chapter 5).

Chapter 2

Materials and Methods

2.1 Cell Culture

The leukemic monocytic cell line THP1 and Jurkat T cell line was obtained from the European Collection of Cell Cultures (ECACC). Human embryonic kidney cells stably transfected with either TLR3 or TLR4 (HEK293/TLR3, HEK293/TLR4) were obtained from Invivogen (San Diego, CA). HEK293T cell line and the murine macrophage cell line RAW264.7 were kindly donated by Dr. Ken Nally (Ailimentary Pharmabiotic Centre, UCC). The astrocytoma cell line U373 was kindly donated by Dr. Luke O'Neill (Trinity College Dublin). Both wild-type and FADD deficient murine embryonic fibroblasts (MEFs) were kindly donated by Dr. Tak Mak (University Health Networ, Ontario, Canada). Wild-type and TRIF deficient immortalised bone marrow-derived macrophages (iBMDMs) were kindly donated by Dr. Sinead Miggin (Maynooth University, Co. Kildare, Ireland).

THP1 monocytic cell line and Jurkat T cell line were maintained in Roswell Park Memorial Institute (RPMI)-1640 media (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma-Aldrich) and 1% (w/v) penicillin/streptomycin (pen/strep) (Sigma-Aldrich). HEK-293T, RAW264.7, U373, MEFs and iBMDMs were all maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% (v/v) FCS and 1% (w/v) pen/strep. HEK293/TLR3 and HEK293/TLR4 cell lines were maintained in DMEM supplemented with 10% FCS and 1% pen/strep, with selective antibiotics, with HEK293/TLR3 cells requiring 10mg/ml Blasticidin (Invivogen, San Diego, CA), with 10mg/ml Blasticidin (Invivogen) and 100mg/ml HygroGold (Invivogen) required for HEK/TLR4 cells.

Mycoplasma testing was performed quarterly using *Mycoplasma* Detection Kit (Invivogen). Cells were incubated without antibiotics for 48 hours. Cell culture supernatant was collected. A 500µl sample of cell culture supernatant was then heat inactivated and incubated with genetically engineered HEK-Blue cells for 24 hours. The presence of *Mycoplasma* was detected as follows: no colour change (pink colour) indicates a negative result and a blue/purple colour indicates the presence of *Mycoplasma*. All cell cultures used in these experiments tested negative.

2.2 Differentiation of THP1 monocytic cells into THP1 monocyte-derived macrophages

THP1 monocytes were seeded in 6 well plates at a concentration of 1 x 10^6 cells per well in full RPMI-1640 media, supplemented with 100ng of phorbal 12-myristate 13-acetate (PMA) (Sigma-Aldrich) (167). THP1 monocytic cells were allowed to differentiate for 72 hours. Media was then changed, and macrophages were allowed to rest overnight, following which stimulations occurred.

2.3 Human monocyte derived macrophages

Human monocytes were obtained from healthy volunteers. A minimum of 28ml of fresh whole blood was collected into ethylene diamine tetra-acetic acid (EDTA) vacutainers. One sample was centrifuged at 1200 x g for 20mins to retrieve the plasma which is used to supplement the media. Fresh samples were added into 50ml conical flasks, with 3mls Ficoll-Hypaque (Sigma-Aldrich) added for every 7mls of blood. Samples were centrifuged at 670 x g for 30mins with no brake applied. The buffy coat was then removed using sterile pipettes. Cells were washed by adding three times the volume of Hanks buffered salt solution, and centrifuged at 283 x gg

for 10minutes. Supernatant was removed, and cells washed twice more as described. Mononuclear cells were suspended in RPMI-1640 supplemented with 1% (w/v) pen/strep and 2% (v/v) of subject's serum and seeded in 24 well plates as required. Cells were seeded and allowed to adhere for 4 days. Non-adherent cells were removed with remaining cells gently washed three times with RPMI-1640 supplemented with 1% (w/v) pen/strep and 2% (v/v) of subject's serum. Media was replaced and cells were allowed to fully differentiate for a further 3 days.

2.4 Caspase 3/7 Assay

Cells were seeded overnight in black flat-bottomed 96-well plates at a density of 20,000 cells/well. Cells were pre-treated with human agonistic Fas antibody (clone CH11) (Merck-Millipore, Darmstedt, Germany) for 1 hr and subsequently with 20 µg/ml polyinosinic:polycytidylic acid (poly I:C) or 100ng/ml lipopolysaccharide (LPS) for 24 hr. Alternatively, cells were treated with each agonist separately. Apo-ONE caspase-3/7 reagent (Promega, Madison, USA) was added and following 1 hr incubation, fluorescence (485 excitation, 530 emission) was measured using a GENios Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland). Changes in caspase 3/7 activation were normalised relative to untreated cells.

2.5 Viability Assay

Cells were untreated or were stimulated with poly I:C (Sigma-Aldrich), poly A:U (Sigma-Aldrich), and/or CH11 (Merck-Millipore) as indicated in the figure legends. Cells were trypsinised with trypsin neutralised using double the volume of medium. Cells were centrifuged at 170 x g for 3 mins and pellets resuspended in 1ml of media. A 1:10 dilution was then performed with 10µl of cell suspension added to

90µl trypan blue (Sigma Aldrich). 10µl of this cell suspension was added to a haemocytometer. Trypan blue is excluded from living cells due to the presence of intact cell membranes (168). Cell counts were performed on both living and dead cells across all treatment groups.

2.6 Murine bone marrow-derived macrophages

Mice were sacrificed and femur and tibia bones were removed and cleaned with 1X sterile PBS and 70% ethanol. Bones were placed in sterile 1X PBS. To isolate bone marrow both ends of the bone were cut and, using a 5ml syringe, bone marrow was flushed through with sterile cold PBS. Isolated bone marrow was re-suspended to generate a single cell suspension and passed through a 45µm cell filter strainer. The cell suspension was centrifuged at 4°C at 380 x g for 5 minutes. The cell pellet was washed once in BMDM culture media (DMEM, 10% (v/v) foetal bovine serum, 1% (v/v) pen/strep, 1% L-Glutamine, 1% non-essential amino acids) and centrifuged at 4°C at 380 x g for 5 minutes. The cell pellet was re-suspended in BMDM differentiating media (BMDM culture media supplemented with 30% (v/v) L929 conditioned media) for 7 days to allow for macrophage progenitors to differentiate into mature macrophages. The cells were cultured on 100mm sterile Petri dishes. On day 3 of differentiation, fresh differentiating culturing media was added. Any non-adherent cells were removed at this stage. Adherent cell differentiate into BMDM over the 7 day period. After 7 days the cells were cultured in BMDM media without L929 conditioned media.

2.7 Stimulation of Cells

THP-1 macrophages were pre-treated with 100ng/ml CH11 (Merck-Milipore), for 1 hour prior to stimulation with either 100ng/ml LPS or 20μg/ml poly I:C (Sigma-Aldrich) for either 8 hours for RNA expression or 48 hours for protein expression unless stated otherwise.

2.8 Poly I:C-induced Fas and FasL expression of THP1 macrophages

THP1 monocytes were seeded to a final concentration of 1 x 10^6 cells/ml and allowed to differentiate into macrophages as described. Cells were then treated with poly I:C at concentrations of 5μg/ml, 10 μg/ml, 20 μg/ml and 40 μg/ml and incubated for either 8 hours for mRNA expression, or 24 hours for protein expression. An untreated control was also included.

2.9 LPS-induced Fas, FasL and FADD expression in THP1 macrophages

THP1 monocytes were seeded to a final concentration of 1 x 10^6 cells/ml and allowed to differentiate into macrophages as described. Cells were then treated with LPS at concentrations of 25ng/ml, 50ng/ml, 100ng/ml, 200ng/ml and 400ng/ml and incubated for either 8 hours for RNA expression, or 24-72 hours for protein expression. An untreated control was also included.

2.10 CH11-induced TLR3 and TLR4 expression in THP1 macrophages

THP1 monocytes were seeded to a final concentration of 1 x 10^6 cells/ml and allowed to differentiate into macrophages as described (Section 2.1.2). Cells were then treated with 25ng/ml, 50ng/ml, 100ng/ml and 200ng/ml of CH11 (Merck-

Millipore) for either 8 hours for RNA expression or 24 hours for protein expression. An untreated control was also included.

2.11 Sample Preparation - Western Blotting

Cells were rinsed once in ice cold 1x PBS and either lysed directly in 200µl Laemmli buffer supplemented with 1mM DTT (Sigma-Aldrich) and stored at -20^{o}C, or lysed on ice for 1 hour with 100µl of lysis buffer (50mM *tris*-hydroxymethyl) amino methane (Tris-Cl, 150mM sodium chloride (NaCl), 1% (w/v) Triton x-100) supplemented with 1x protease an phosphatase inhibitor cocktail (both from Merck-Millipore). Cells were then scraped and transferred to a 1.5ml eppendorf. Samples lysed in lysis buffer were centrifuged at 14000 x g at 4^{o}C for 10 minutes. The resulting cell debris was discarded and lysate stored at -20^{o}C.

2.12 Bicinchoninic Acid (BCA) Assay

Protein standards were prepared using Bovine Serum Albumin (BSA) (Thermo Scientific, IL, USA) (0, 2.5, 5, 7.5, 10, 15, 20µg/ml) and added to a 96 well plate. 2µl of each sample was added to the plate followed by 38µl of dH_20. Both standards and samples were analysed in triplicate. 160µl of BCA Protein Assay Reagent (Thermo -Scientific) was added to each well, the plate agitated and then left at 37°C for 30 minutes before reading at 595nm on a spectrophotometer. A standard curve was then generated using GraphPad Prism software and used to calculate the protein concentrations of unknown samples.

2.13 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting

50µg of cell supernatant protein was mixed with 5 x PAGE loading buffer (125mM Tris, 2% SDS, 20% Glycerol, 2.5% beta mercaptoethanol) and lysis buffer (described above) to a final volume of 20µl. Samples were boiled for 5 minutes, with 20µl loaded onto a separating and stacking SDS gel. A 19-180 kilodalton (kDa) molecular weight marker (Sigma-Aldrich) was run alongside the samples. Proteins were separated by electrophoresis at 40mA/A and then transferred for 90 minutes at room temperature onto an Immobilon –P polyvinylidene diflouride membrane (I-PVDF) (Millipore, CA, USA) at 120V/V using a wet transfer method. Protein loading and efficiency of transfer were monitored by Ponceau S staining for all experiments except those using phospho-antibodies. With the exception of phospho-antibodies which were blocked in 5% (w/v) BSA in 0.1%TBS/Tween-20, membranes were blocked for 1 hour at room temperature in BLOTTO (5% (w/v) non-fat dry milk in 0.1% PBS/Tween-20) and then incubated overnight at 4^{o}C with the appropriate primary antibody (Table 2.1). With the exception of phospho-antibody membranes which were washed in 0.1% TBS/Tween-20, membranes were washed with PBS/0.1% Tween-20 for 5 x 5 minutes, with primary antibodies detected by incubating the membrane for 1 hour at room temperature with the appropriate horseradish peroxide (HRP)-conjugated IgG secondary antibody (Dako Corp., Carpinteria, CA). Peroxidase activity was detected with the enhanced chemiluminescence system (ECL) (Millipore, Billerica, MA) using the FujiFilm LAS-3000 Lightbox. As a loading control, duplicated membranes were blocked for 1 hour in BLOTTO and incubated overnight at 4^{o}C with anti-β actin specific antibody

(Table 2.1). The primary antibody was detected using anti-mouse HRP secondary antibody (Dako Corp.).

Antibody	**Cat. Number**	**Company**
TLR4 (H-80)	sc-10741	Santa Cruz
Fas (C-20)	sc-715	Santa Cruz
FADD H-10	sc-271520	Santa Cruz
FasL	555290	Abcam
Monoclonal Anti-VSV Glycoprotein P5D4	V5507	Sigma-Aldrich
Anti-Flag	F1804	Sigma-Aldrich
βactin	AAC-74	Sigma-Aldrich
Phospho-Antibodies	**Cat. Number**	**Company**
phospho-IκBα	133462	Abcam
phospho-p42/44 MAPK	9101s	Cell Signaling
phospho-p38 MAPK	9211s	Cell Signaling
phospho-JNK	9251s	Cell Signaling
Secondary HRP antibodies	**Cat. Number**	**Company**
Polyclonal Rabbit anti-Mouse	P0260	DAKO
Polyclonal Mouse anti-Rabbit	P0448	DAKO
Fluorescent labelled Secondary antibodies	**Cat. Number**	**Company**
Donkey anti-rabbit Texas Red (TR)	sc2784	Santa Cruz
Donkey anti-mouse Fluorescein isothiocyanate (FITC)	sc2099	Santa Cruz

Table 2.1 Antibodies used for Western blotting and immunofluorescence.

2.14 Immunofluorescence

Cells were seeded at $1x10^5$ cell/ml onto EZ slides (Millipore). Cells were then fixed in 100% ice-cold methanol for 5 mins the following day. Cells were permeabilsed using 0.2% (w/v) Trition X-100 in PBS for 30mins before blocking in 10% (v/v) normal donkey serum (Sigma-Aldrich) for 1hr at room temperature. The slide was incubated with primary antibody overnight in 1.5% (v/v) normal donkey serum at 4°C. The following day, the slide was washed three times in PBS and incubated in the appropriate secondary antibody diluted in normal donkey serum for 1 hour before counterstaining with 4' 6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). DAPI preferentially stains dsDNA, binding to AT-rich regions of DNA, emitting a blue fluorescent hue (169). Anti-fade fluorescent mounting media (Dako) was utilised when applying coverslips to impede fading of fluorescence, with slides stored in the dark following application of cover-slips. Emission of primary or only secondary antibody negative controls were also included.

2.15 Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated using either the GenElute Mammalian Total RNA Mini Prep kit (RT-70-1KT, Sigma-Aldrich) or the ISOLATE kit (Bioline, UK) according to the manufacturer's instructions. 10 µl of total RNA was used as the template for cDNA synthesis (Bioline, UK). This was added to 1µl of Oligo (Dt), and 1µl of 10mM dNTP and heated to 65°C for 10 minutes. Following 2 minutes on ice, 4µl of 5x Reverse Transcriptase Buffer, 1µl of RNase inhibitor, 0.25µl of Reverse Transcriptase, and 2.75µl of DEPC treated water was added to each sample before incubation at 37^oC for 30 minutes. The reaction was terminated by a final incubation

at 70°C for 15 minutes and chilling samples on ice. cDNA samples were then diluted to 100µl using DEPC water (Sigma-Aldrich).

TaqMan MasterMix was used with TaqMan Gene Expression probes Fas, FasL, TLR3, TLR4, RIG-I, IL-8, IL-10, IL-12, TNFα, IFNβ, hIP-10/CXCL-10 and mIP-10/CXCL-10, mIFNβ (table 2.2) (Bio-Sciences, Dublin, Ireland) with qRT-PCR analysis performed using LightCycler instrument (Applied Biosystems). PCR reactions for CCL-20 were performed using 900nM of CCL-20 and 250nM of Sensi Mix II (BioLine, London, UK) in the LightCycler 480 Real-Time PCR System (Roche) software. Thermal cycling conditions were as recommended by the manufacturer (Roche). Table 2.3 below contains the primer sequences used for CCL-20. ΔΔCt analysis (170) was performed using Microsoft Excel.

Gene Name	Probe	Reaction Conditions
Human		
Fas	Hs00236330_m1	
FasL	Hs00181225_m1	
IL-8	Hs99999034_ml	
TNFα	Hs00174128_m1	
IFNβ	Hs00277188_s1	
IL-10	Hs00961622	50°C for 2 mins
IP-10	Hs00171042_m1	95°C for 10mins
IL-12	Hs01073447_m1	
TLR3	Hs01551078_m1	60 cycles@ 95°C for
TLR4	Hs00370853_m1	15secs and 60°C for 15sec
RIG-I	Hs00204833_m1	
GAPDH	GAPDH, 4352934E	
Murine		
IP-10	Mm00445235_m1	
IFN β	Mm00439552	
β-actin	β-actin, 4352341E	

Table 2.2 qRT-PCR reaction conditions for TaqMan method.

	Left Primer	Right Primer	UPL#
CCL-20	5’ gctgctttgatgtcagtgct 3’	5’ gaagaatacggtctgtgtatccaa 3’	39
β-actin	5’ attggcaatgagcggttc 3’	5’ tgaaggtagtttcgtggatgc 3’	11

Table 2.3: Sequences of primers used in qRT-PCR analysis. Primers were generated by Eurofins MWG (www.eurofinsgenomics.eu).

2.16 Migration and Attachment of T cells to THP1 monocyte-derived macrophages

Sterile coverslips were placed in each well of a 6 well plate. THP1 monocytes were seeded and allowed to differentiate as described. Stimulations were performed for 24 hours, following which Jurkat T cells were co-cultured for a further 48 hours. Coverslips were then washed in PBS and mounted on slides. Bright field microscopy was then performed.

2.17 Transwell Migration Assay

Jurkat T cells were cultured overnight in THP1 macrophage-derived supernatant to induce CXCR-3 expression. 750µl of THP-1 macrophage treated cell culture supernatant was added to each well of a 24 well plate, with 200µl of Jurkat T cells at a concentration of 1.25 x 10^6 cells/ml in serum free media added to the chamber. Plate was incubated at 37°C for 2 hrs, following which the upper chamber was removed, with the contents of the well collected. Samples were centrifuged at 12,400 x g for 5 mins, with sample incubated on ice between each centrifuge cycle. Cells were washed twice in PBS and fixed in ice cold methanol for 5 minutes. Cells were then stained in 0.1% (w/v) crystal violet in 0.1M borate pH 9.0 and 2% (v/v) ethanol for 20mins. Cells were then washed 3 x 5mins in PBS, with supernatant removed after each step. Elution of stain was then performed using 200µl of acetic acid and vortexing periodically over 15mins. Cells were centrifuged at 12,400 x g for 3 min, with supernatant collected and absorbance read at 570nm.

2.18 Flow Cytometry

THP1 macrophages were treated with 20µg poly I:C, 100ng CH-11, and pre-stimulated with 100ng CH-11 prior to poly I:C stimulation. Stimulations progressed for 48 hours. Supernatant from each treatment was then collected and centrifuged for 3 mins at 170 x g to remove any residual cells. This supernatant was then added to individual wells of a 6 well plate, with Jurkat T cells then seeded in this supernatant and incubated for 24 hours at 37°C with 5% CO_2. CXCR-3 FITC conjugated antibody (MSC) was applied to cells as per manufacturer's protocol, with appropriate isotype control (Santa Cruz BioTechnology, Dallas, TX). Flow cytometry was performed on the Facscaliber flow cytometer with analysis performed using Cell Quest Pro software.

2.19 Transformation of competent DH5α cells by heat-shock

DH5α cells were thawed on ice with 2µl of plasmid added to 25µl of competent cells (Bio-Sciences, Dublin). Suspension was left on ice for 30mins. Cells were then heated to 42°C for 2 mins, and immediately cooled on ice for 2 mins. 1ml LB broth was added, with suspension incubated at 37°C for 1hr with agitation. 100µl of this cell suspension was then plated onto agar plates containing selective antibiotic, e.g. 1mg/ml ampicillin. Plates were left overnight at 37°C, with colonies detected the following day. A single colony was then picked and added to 1ml of LB broth with selective antibiotic (starter culture). The starter culture was incubated at 37°C for 8hrs. 100µl of this starter culture was then added to 100ml of LB broth with selective antibiotic in sterile conical flasks and incubated with agitation overnight at 37°C with the bacterial culture then ready for plasmid extraction.

2.20 MaxiPrep

Plasmids were purified following the protocol for using the EndoFree Plasmid Purification Kit (Qiagen). Bacterial cultures generated above were harvested by centrifugation at 6000 x g for 60mins in a refrigerated centrifuge. The pellets were then lysed according to the manufacturer's instructions. Precipitate was removed using the QIAfilter Cartridge which removes the proteins, genomic DNA and detergent contained within the precipitate. Endotoxin was removed by addition of an endotoxin removal buffer (Buffer ER), with suspension added the QIAGEN-tip. The QIAGEN-tip is an anion-exchange tip which utilises positively charged diethylaminoethyl (DEAE) bound to the resin. The positive charge of DEAE then binds to the negatively charged phosphates of the DNA backbone forming a complex whilst removing other impurities. The QIAGEN-tip was then washed twice, with DNA eluted using elution buffer provided. Isopropanol was added to the DNA and centrifuged at 6000 x g for 60mins at 4°C. The supernatant was removed, with 5ml of endotoxin free room-temperature 70% ethanol added, with sample centrifuged at 6000 x g for 60mins at 4°C. Supernatant was carefully removed with pellet allowed to air-dry for 10mins. Pellet was then resuspended in 200µl endotoxin-free Buffer TE. The yield was then determined by measuring the plasmid DNA using the NanoDrop Spectrophotometer.

2.21 Luciferase/Plasmids

All cells were seeded in 200µl at 2 x 10^5 cells/ml in 96 well plates, with the exception of RAW264.7 which were seeded in 1ml at 1.5 x 10^5 cells/ml in 24 well plates. RAW264.7 cells were transfected using Turbofect (Fermentas, Thermo Fisher Scientific, Waltham, MA, United States of America) with HEK cells and U373s

transfected with GeneJuice (Merck Millipore) according to the manufacturer's protocol. Cells were transfected with 40ng of full length IP-10 promoter, IP-10 containing point mutations in the ISRE, κB1, κB2 and the AP-1 binding sites (kind gifts from Prof. David Proud), full length mIP-10 promoter (a kind gift from Dr. Daniel Muruve, UC Calgary, Canada), IFNβ, ISRE, NFκB luciferase plasmid, and 5ng of Thymidine kinase Renilla luciferase (TK Renilla), along with varying amounts of expression plasmids including MyD88, TLR3, TLR4, IRAK4, RIP-1, IKKε, TRIF, TRAF3, FADD-DD (kind gifts from Prof. L. O'Neill, Trinity College Dublin, Ireland) and FADD (was a gift from Prof. A Winoto - Addgene plasmid 31814), 3xAP1pGL3 (3xAP-1 in pGL3-basic) (a kind gift from Alexander Dent) (Addgene plasmid # 40342). Stimulations involving poly I:C were performed for 6 hours prior to lysis of cells. Extracts were prepared by lysing the cells for 15 minutes in 50µl luciferase lysis buffer (10mM EDTA, 100mM DTT, 50% glycerol, 5% Triton X-100, 125mM Tris base, with pH adjusted to 7.8) and measured for Firefly luciferase and Renilla luciferase activity. Firefly luminescence readings were corrected for Renilla activity and expressed as fold stimulation over unstimulated empty vector (EV) control. Luminescent activity was then measured on Promega GloMax system (MSC).

2.22 ELISA

Supernatant from THP-1 macrophages stimulated with poly I:C +/- CH-11 for 48 hours was used for IL-8 and IL-10 ELISA (BioLegend). A dilution of 1:1000 was necessary for IL-8 ELISA due to the copious amounts of IL-8 produced by this cell type. Samples for IL-10 ELISA required no dilution. ELISA was performed as per manufacturer's instructions. IP-10/CXCL-10 protein analysis was performed using

MSD plates (Meso Scale Discovery, Washington DC, USA). A dilution of 1:20 was required due to the amount of IP-10/CXCL-10 produced by this cell type. MSD was performed as per manufacturer's instructions.

2.23 Virus Infection

Sendai virus (SeV), a kind gift from Dr. Liam Fanning (Molecular Virology and Diagnostic Research Laboratory, UCC) was supplied at 10(7.5)CEID[50]/0.2 mL (ATCC). THP-1 macrophages were washed twice in PBS. Initial dilutions of SeV were performed, with a final dilution of 1:10,000 and 1:50,000 added to THP-1 macrophages in a 1ml volume in full RPMI-1640 media. Cells were then incubated at 37^{o}C for 5 hours, after which 1ml full media was added to each well. Cells were incubated for a further 3 hours, with lysis of cells performed, with total RNA extracted (Bioline) or for a total of 24 hours, with supernatant harvested for protein analysis by ELISA.

2.24 Co-Immunoprecipitation

Initial optimisation experiments were performed by transfecting HEK293T cells either 2μg or 4μg Flag-FADD and protein expression detected by Western blotting, immunoblotting using anti-Flag. Overexpression of VSV-TRIF was also performed using 1, 2, 4 and 10μg of VSV-tagged TRIF. Cells were lysed on ice for 1 hr in 1ml RIPA buffer (50mM Tris-HCL pH8.0, 150mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Triton X-100, 1mM phenylmethylsulfonyl) supplemented with protease and phosphatase inhibitor cocktail. Cell scraping was performed, with samples collected into Eppendorf tubes. Samples were centrifuged at 14600rpm at 4^{o}C for 10 minutes. The resulting cell debris was discarded. An aliquot of the lysate

was removed for Western blotting of both VSV and Flag. Following optimisation, HEK293T cells were co-transfected with 2µg VSV-TRIF and 4µg Flag-FADD. 10µg Flag antibody or 10µg VSV antibody was added to 100µl protein A agarose beads and incubated overnight at 4^{o}C with agitation. Protein A agarose beads complexed with the relevant antibody was added to lysate and incubated at room temperature for 2hrs. Complex was washed x2 with TBS and x2 with RIPA buffer. Lysate-agarose bead suspension was then washed x1 in 500µl water, with 100µl Laemmli buffer added. Samples were then heated to 95^{o}C for 5mins. Supernatant was then transferred to an Eppendorf and stored at -20^{o}C. Samples then underwent 15% SDS-PAGE gel electrophoresis and Western blotting.

2.25 Precipitation of Endogenous Proteins

THP1 cells were seeded and differentiated into macrophages as previously described (section 2.2) in 100mm dish at 1.5 x 10^{6} cells in 10mls of media, with cells stimulated as required. Cells were lysed on ice for 1 hr in 1ml RIPA buffer supplemented with protease and phosphatase inhibitor cocktail. Cell scraping was performed, with suspension collected into eppendorf tubes. Cells were centrifuged at 14000 x g at 4^{o}C for 10mins. The resulting cell debris was discarded. An aliquot of the lysate was removed for Western blotting of FADD. 4µg FADD antibody was added to protein A agarose beads and incubated overnight at 4^{o}C with agitation after which lysate was added to this protein A agarose beads: FADD antibody complex , with immunoprecipitation performed as outlined for that of the co-immunoprecipitation (section 2.24).

2.26 siRNA transfection of THP1 macrophages

siRNA transfections were performed with Santa Cruz specific FADD siRNA (sc-35352, Santa Cruz) and control siRNA (sc-37007, Santa Cruz). THP1 siRNA transfections were performed using the Amaxa (Lonza, Basel, Switzerland) system according to the manufacturer's protocol. Cells were cultured for a further 48 hours prior to stimulation.

2.27 Statistics

Statistical Analysis was performed using GraphPad Prism software. Data are expressed as the mean ± SEM.

Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using One-Way Anova with Tukeys post-test with MEF and iBMDM experiments assessed using Two-way Anova with student t-test. Values of $p<0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*).

The results were considered to be significantly different at p values <0.05, with the significance indicated by a symbol such as asterisk as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$. $p>0.05$ was considered non-significant.

Chapter 3:

Investigating the role of Fas activation on immune responses.

3.1 Introduction

Macrophages play a key role in the innate immune response. Detection of a viral infection by macrophages results in recruitment of a variety of immune cells to the site of infection. Initial detection is dependent on recognition by host Pattern Recognition Receptors (PRRs) (171). PRRs that detect viral infections include a group of structurally related cytosolic sensors including RIG-I-like receptors (RLRs), which are sensors of RNA, and AIM2-like receptors (ALRs), which are sensors of DNA. Detection of double-stranded RNA (dsRNA) by the cytosolic sensor RIG-I results in recruitment of its adapter protein IPS-1 (MAVS/Cardif/VISA) and the downstream induction of anti-viral cytokines (172). PRRs involved in the anti-viral immune response also include endosomally located Toll-Like Receptors (TLRs) 3, 7/8 and 9, which recognise viral nucleic acids such as dsRNA, single-stranded RNA (ssRNA) and DNA, respectively (171, 173).

Macrophages also express the Fas (CD95/Apo-1) receptor, which is a well-known member of the tumour necrosis factor (TNF) family (174). Whilst activation of Fas by ligation of its ligand, Fas ligand (FasL) is best studied in terms of its role in apoptosis, Fas activation has also been implicated in inflammation (151, 152). For instance, activation of the Fas signalling pathway has been shown to induce IL-18 and IL-1β production in bone marrow derived macrophages (BMDMs) (151), and IL-8 production in THP1 macrophages(152).

Following viral infection, both Fas and FasL have been shown to be induced across a number of different cell types. Co-expression of Fas and FasL in influenza infected HeLa cells resulted in Fas-mediated apoptosis of infected cells due to cell-cell interaction (175). Although co-expression of both Fas and FasL following Herpes

Simplex Virus (HSV)-2 infection of epithelial and keratinocyte cells was shown, apoptosis of these cells was not observed (164).

As studies have demonstrated that Fas activation can result in production of pro-inflammatory cytokines, and that viral infection can induce both Fas and FasL expression, the initial aim of the work presented in this chapter was to investigate the role of Fas in the induction of anti-viral cytokine and chemokine responses. As we observed little to no induction of anti-viral immune responses, we extended this study to investigate whether there was a potential role for Fas in modulating poly I:C-induced cytokine and chemokine responses. Work presented in this chapter demonstrates that activation of Fas modifies poly I:C-induced immune responses.

3.2 Results

3.2.1 Immunofluorescence staining shows expression of CD68, TLR3, Fas and FasL in THP1 macrophages and human monocyte derived macrophages (hMDMs).

Phorbol 12-myristate 13-acetate (PMA) treated THP1 monocytes were differentiated into macrophages on glass slides over 3 days, (167) as per materials and methods. Human monocytes isolated from the peripheral blood of healthy volunteers were seeded onto glass slides and allowed to differentiate to macrophages over 7 days (167). CD68 is commonly used as a histological marker for cells of macrophage lineage. Consistent with this, positive staining of CD68 was observed following derivation of both THP1 monocytes and human peripheral blood mononuclear cells (PBMCs) into macrophages (figure 3.1a). Following this, we next determined that THP1 macrophages and hMDMs express the innate immune PRR TLR3, as well as

both Fas and FasL. Positive staining was observed for TLR3 (figure 3.1b), Fas (figure 3.1c) and FasL (figure 3.1d) in both THP1 macrophages and hMDMs.

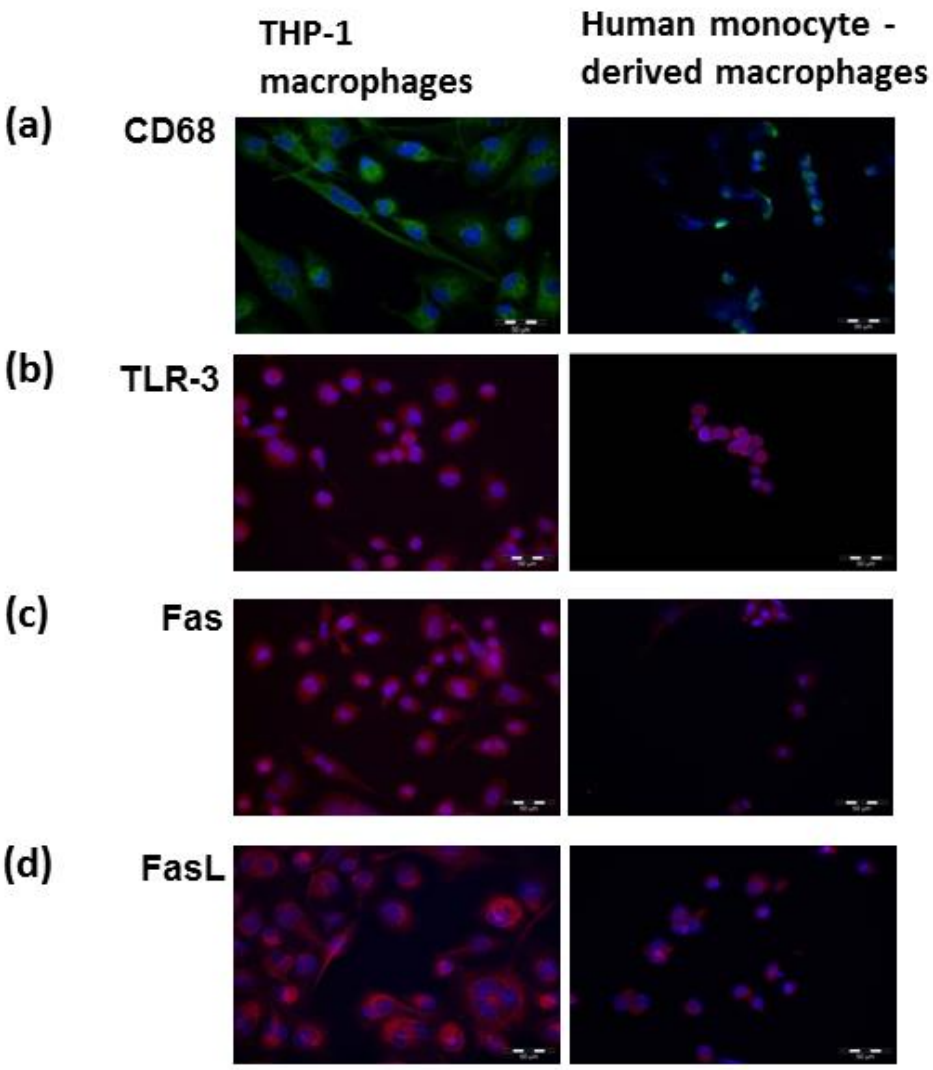

Figure 3.1: THP-1-derived macrophages and human monocyte-derived macrophages (hMDMs) express CD68, TLR-3, Fas, and Fas Ligand (FasL). THP-1 cells were differentiated with 100μg/ml PMA for 72 hrs. Human monocyte-derived macrophages were differentiated into macrophages over 7 days on glass slides. Florescent images indicate positive staining for the myeloid marker CD68 (green) **(a)**, the PRR TLR-3 (red) **(b)**, Fas (red) **(c)** and FasL (red) **(d)** superimposed with nuclear staining using DAPI (blue). Bars, 50μm. Data are representative of three individual experiments.

3.2.2 Stimulation with poly I:C increases expression of Fas and FasL by macrophages.

As previous studies have demonstrated that viral infection resulted in increased Fas and FasL expression in macrophages (175, 176), we initially confirmed that the dsRNA mimetic poly I:C could up-regulate both Fas and FasL in THP1 macrophages. Upon stimulation with increasing concentrations of poly I:C, THP1 macrophages showed increased expression of both Fas and FasL at both the mRNA (figure 3.2a, b) and protein level (figure 3.2c).

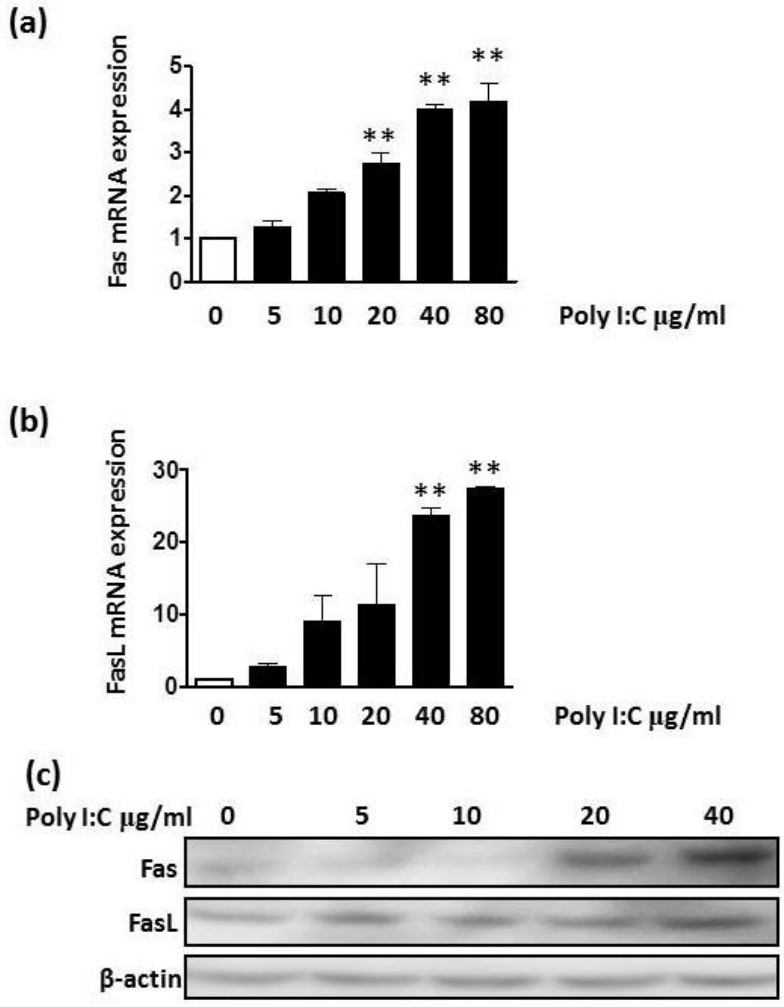

Figure 3.2: Fas and Fas Ligand expression are increased following treatment with increasing concentrations of poly I:C. THP-1 cells were differentiated with 100µg/ml PMA for 72 hrs. Cells were treated with increasing concentrations of poly I:C (5, 10, 20 and 40µg/ml). Fas and FasL expression was detected by qRT-PCR after 8 hrs (a,b) with cells normalised to GAPDH expression levels. mRNA is expressed relative to untreated cells. Western blotting was performed 48 hrs (c). Results shown representative of three separate experiments. ** $p<0.01$, values shown as Mean ± SEM, (n=3).

3.2.3 Stimulation with agonistic anti-Fas antibody (CH11) does not affect either TLR3 or RIG-I expression.

As we have demonstrated that poly I:C can induce expression of both Fas and FasL, we next investigated the effect of Fas activation on the expression levels of innate immune receptors involved in viral recognition; TLR3 and RIG-I. THP1 macrophages were stimulated with increasing concentrations of the agonistic anti-Fas antibody CH11, with expression of both TLR3 and RIG-I mRNA determined. We observed no induction of TLR3 (figure 3.3a) or RIG-I (figure 3.3b) mRNA expression following 8 hours stimulation, suggesting that crosstalk, in terms of receptor expression levels between these pathways is unidirectional.

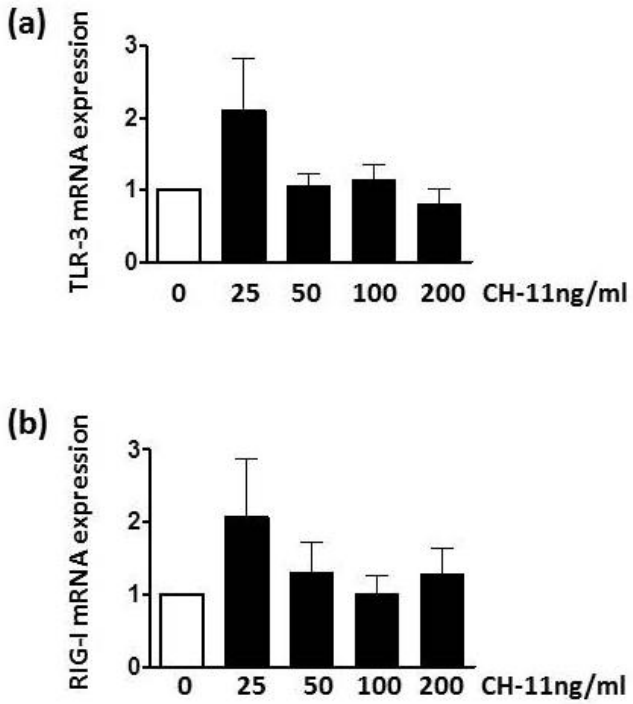

Figure 3.3: Stimulation with agonistic anti-Fas (CH11) did not alter either TLR-3 or RIG-I expression in THP-1 macrophages. THP-1 cells were differentiated with 100µg/ml PMA for 72 hrs. Cells were treated with increasing concentrations of CH11 (25, 50, 100 and 200ng/ml) for 8 hours following which qRT-PCR was performed. mRNA expression was normalised to GAPDH expresion levels. mRNA expression of TLR-3 (a) and RIG-I (b) are expressed relative to untreated cells. Values are shown as Mean ± SEM, (n=3) with results shown representative of three separate experiments.

3.2.4 Caspase 3/7 activation was not induced following Fas activation or poly I:C stimulation in THP1 macrophages.

Given the well characterised apoptotic function of Fas, we next investigated whether induction of Fas and FasL expression by poly I:C induced apoptosis in THP1 macrophages by measuring caspase 3/7 activation. Stimulation of THP1 macrophages with either poly I:C or CH11 did not result in enhanced caspase 3/7 activation (figure 3.4a). This is consistent with previous studies, whereby activation of Fas in THP1 macrophages also did not trigger apoptotic cell death (152). To confirm that CH11 was able to induce apoptosis in Fas sensitive cells, Jurkat T cells were treated with CH11 which resulted in strong caspase 3/7 activation (figure 3.4a). Cell viability of THP1 derived macrophages was also unaffected by either poly I:C or CH11 treatment (figure 3.4b). In contrast, staurosporine reduced the viability of the cells. Together, these data demonstrates that Fas activation with and without poly I:C stimulation does not induce caspase 3/7 activation, nor does it affect cell viability, despite increased Fas and FasL expression observed on poly I:C stimulated THP1 macrophages.

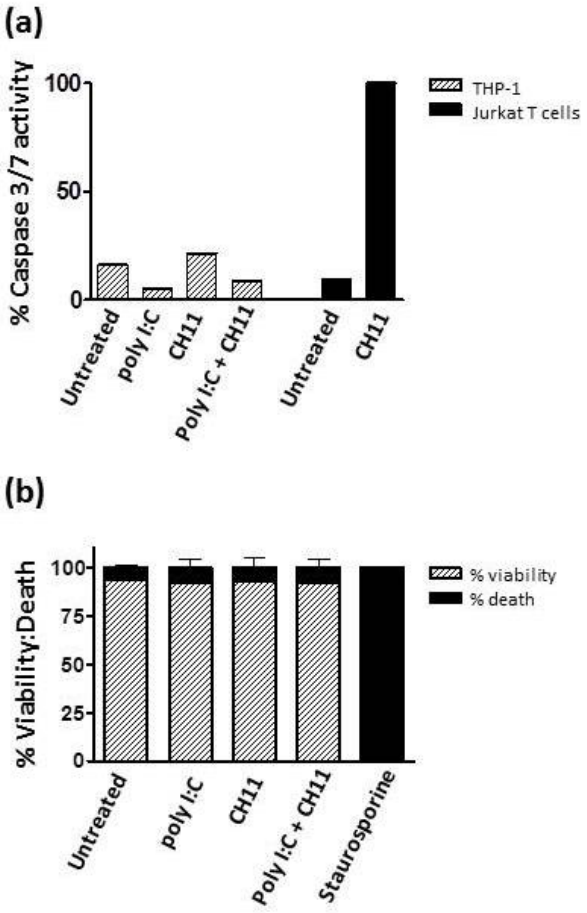

Figure 3.4: Caspases 3/7 activation and cell viability were unaffected by both poly I:C and CH11 stimulation. THP-1-derived macrophages and Jurkat T cells were treated with 200ng/ml CH11 and/or 20μg/ml poly I:C for 24 hrs, or with 5 μM staurosporine as indicated. Caspase 3/7 activity (a) was measured by fluorescence whereby the amount of caspase 3/7 activity is directly proportional to the amount of fluorescence measured. Cell viability (b) was determined by trypan blue exclusion after 24 hours. Data shown are representative of three independent experiments, with values shown as percentage Caspase 3/7 activation relative to Jurkat T cells stimulated with 200ng/ml CH11 (a) or as Mean ± SEM (b).

3.2.5 Fas activation does not induce significant inflammatory cytokine or chemokine production in THP1 macrophages.

As expression of both Fas and FasL were both augmented by poly I:C, we next investigated the ability of macrophages to produce cytokines and chemokines in response to Fas activation by the agonistic Fas antibody CH11 (figure 3.5). Initially we focused on induction of anti-viral response genes such as IFNβ and IP-10. Interestingly, we observed no up-regulation of these genes in response to Fas activation (figure 3.5a). As some previous studies have shown that Fas activation can up-regulate expression of other pro-inflammatory cytokines, we next examined expression of IL-8 and TNFα. Similar to results obtained with IP-10 and IFNβ, up-regulation of these cytokines was not observed (figure 3.5b). To confirm that the concentration of CH11 used was capable of inducing cytokine expression, SW480 intestinal epithelial cells were stimulated with CH11 and cytokine production assessed. In contrast to THP1 macrophages, Fas activation by CH11 in SW480 cells resulted in a strong induction of IP-10, IL-8 and TNFα (figure 3.5c).

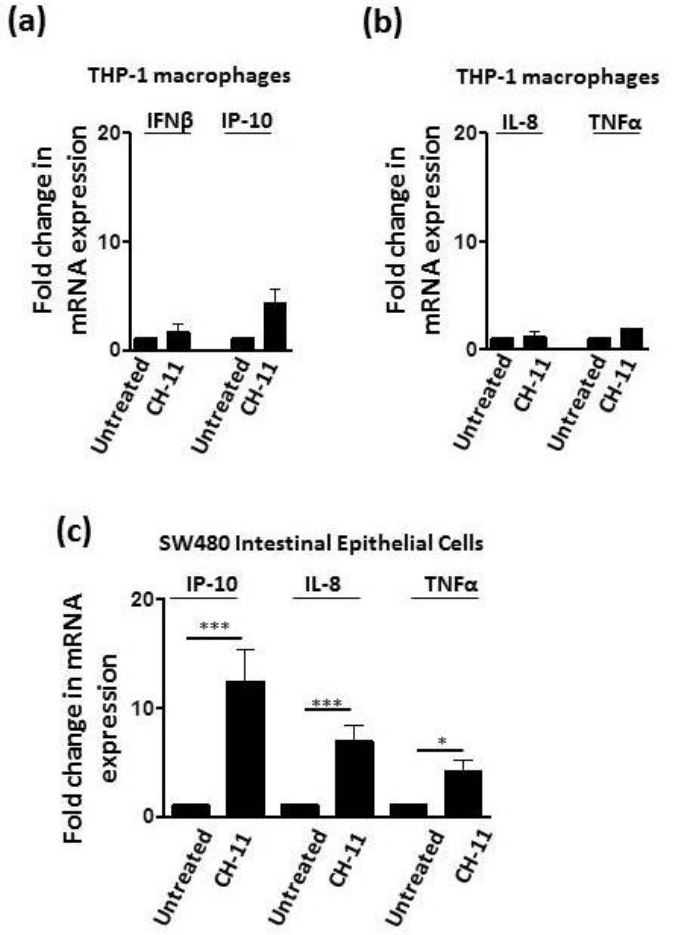

Figure 3.5: Fas activation does not induce significant inflammatory cytokine production in THP-1 macrophages. THP-1 macrophages (a, b) and SW480 cells (c) were treated with 200ng/ml CH11 for 8 hrs. Changes in cytokine expression were detected by qRT-PCR. mRNA expression was normalised to GAPDH expression levels, with changes in mRNA expressed relative to untreated cells. qRT-PCR values are shown as Mean ± SEM, (n=3), * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.2.6 Fas activation reduces poly I:C-induced TNFα, IL-10, IL-8 and IFNβ mRNA production in THP1 macrophages.

Given that poly I:C was able to increase the expression of both Fas and FasL, but that no augmentation in anti-viral response genes was observed upon Fas activation, we next investigated whether CH11 stimulation could affect poly I:C-induced cytokine production. Stimulation of THP1 macrophages with poly I:C alone induced expression of IFNβ, IL-8, IL-10 and TNFα. However, Fas activation with subsequent poly I:C stimulation resulted in a reduction in the expression of all four cytokines. Poly I:C-induced IFNβ, IL-10 and TNFα production were significantly reduced by 50% by Fas activation (figure 3.6a, c, d). A reduction in IL-8 production was also observed, albeit to a lesser extent (figure 3.6b).

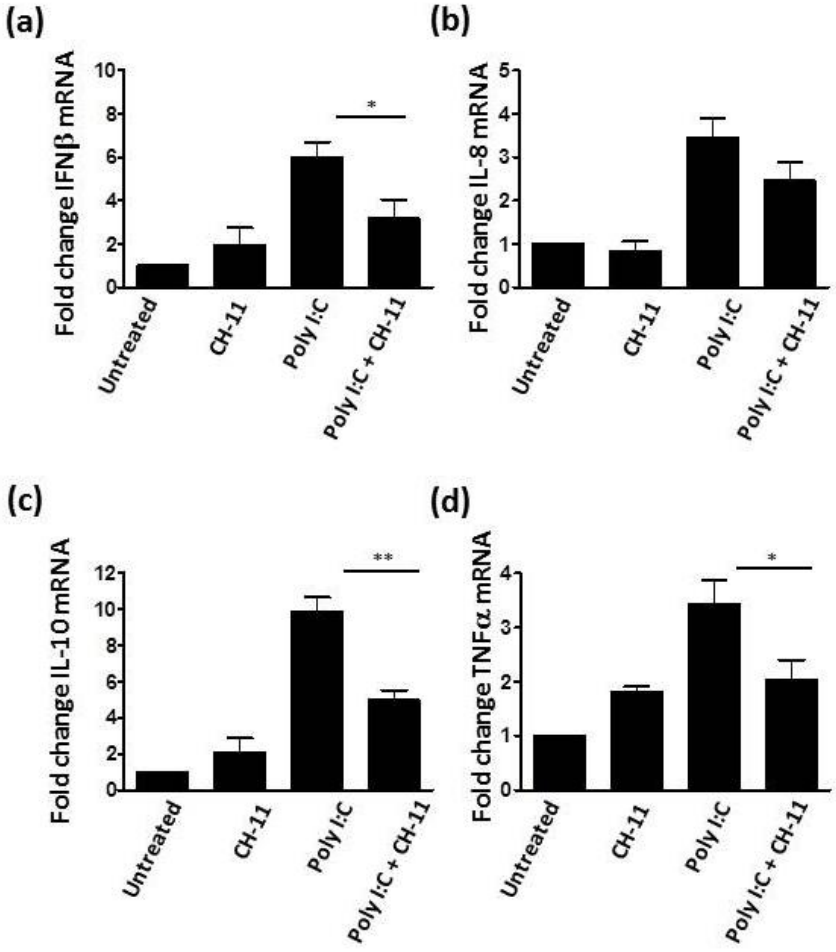

Figure 3.6: Fas activation decreases poly I:C-induced IL-8, IL-10, TNFα and IFNβ transcription. THP-1-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by stimulation with 20μg/ml poly I:C. mRNA expression was normalised to GAPDH, with changes in IFNβ (a), IL-8 (b), IL-10 (c) and TNFα (d) expressed relative to untreated cells. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM. * $p<0.05$, ** $p<0.01$.

3.2.7 Poly I:C-induced IP-10 mRNA production is augmented following Fas activation.

As our initial aim had been to investigate the role of Fas in anti-viral response, we also examined expression of IP-10 (CXCL-10), a potent T cell chemokine. In contrast to other cytokines examined, poly I:C-induced IP-10 production was significantly augmented (3 fold; $p < 0.01$) by Fas activation with CH11 (figure 3.7), suggesting that Fas activation plays a role in enhancing poly I:C-induced IP-10 production.

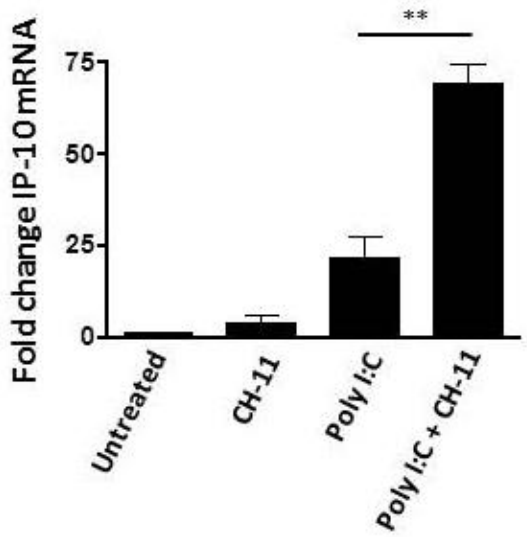

Figure 3.7: Fas activation augments poly I:C-induced IP-10 transcription. THP-1-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by stimulation with 20µg/ml poly I:C. IP-10 mRNA expression was detected by qRT-PCR at 8 hrs with mRNA normalised to GAPDH. Changes in IP-10 expression were expressed relative to untreated cells. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM. ** $p<0.01$.

3.2.8 Fas activation reduces poly I:C-induced IL-8 and IL-10 protein expression, but augments IP-10 protein expression in THP1 macrophages.

To confirm mRNA analysis, changes in IL-8, IL-10, TNFα and IP-10 protein production were detected by ELISA. THP1 macrophages were stimulated as outlined in the figure legend (figure 3.8), with supernatant harvested and ELISA analysis performed. Similar to results shown in figures 3.6 and 3.7 for mRNA expression, CH11 did not induce IL-8, IL-10, TNFα and IP-10 cytokine production (figure 3.8). However, Poly I:C-induced IL-8 production was reduced by 30% by Fas activation (figure 3.8a), while poly I:C-induced IL-10 production was reduced to that of untreated levels by Fas activation (figure 3.8b). In contrast to the mRNA data obtained, poly I:C-induced TNFα production was unchanged by pre-treatment with CH11 (figure 3.8c). Poly I:C-induced IP-10 production, however, was again significantly augmented ($p < 0.001$) by Fas activation (figure 3.8d).

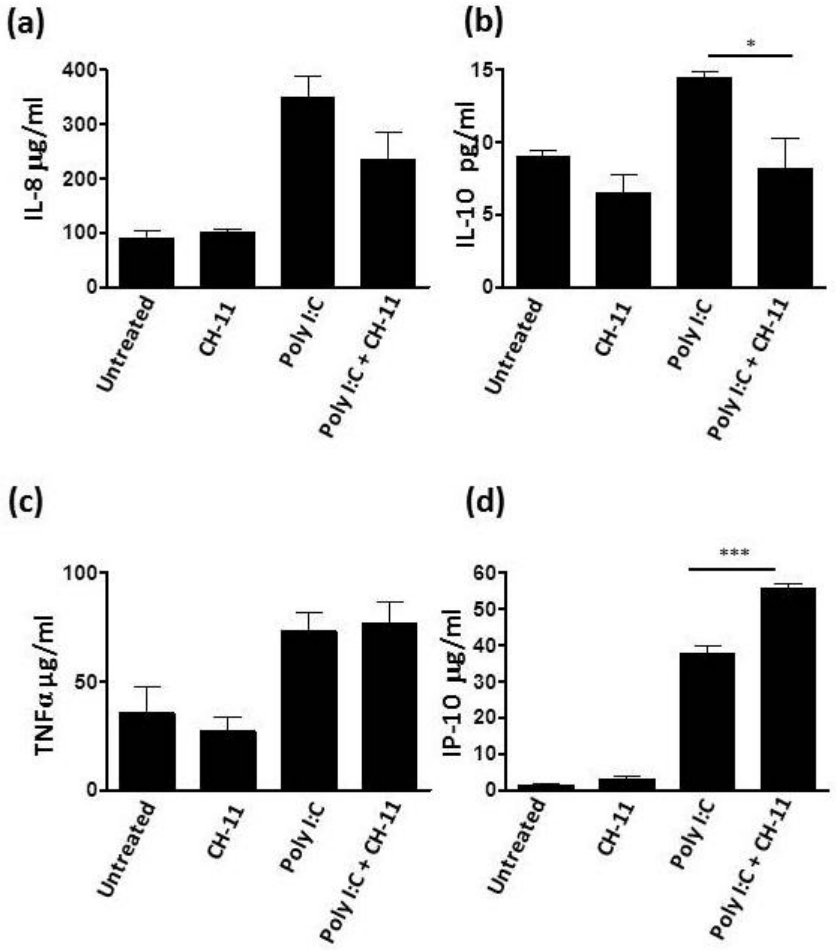

Figure 3.8: Fas activation decreases poly I:C-induced IL-8, IL-10 and TNFα protein production, whilst augmenting IP-10. THP-1-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by stimulation with 20µg/ml poly I:C. Changes in IL-8 **(a)**, IL-10 **(b)**, TNFα **(c)** and IP-10 **(d)** protein production were detected by ELISA after 48 hrs. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM. * $p<0.05$, *** $p<0.001$.

3.2.9 Fas activation reduces poly I:C-induced TNFα, IL-10 and IFNβ mRNA production, while augmenting poly I:C-induced IP-10 mRNA production in human monocyte-derived macrophages.

As we had observed that Fas activation could modulate poly I:C-induced cytokine production in THP1 cells, we wished to confirm this result in a primary cell line. Fresh whole blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were then isolated, with monocytes derived into macrophages for 7 days. Similar to THP1 macrophages, Fas activation did not significantly induce IFNβ, IL-10, TNFα or IP-10 cytokine expression (figure 3.9). Both poly I:C-induced expression of IFNβ and TNFα were reduced by 50% upon co-stimulation with CH11(figure 3.9a, c), while poly I:C-induced IL-10 production was reduced by 40% by Fas activation (figure 3.9b). However, poly I:C-induced P10 production was again augmented upon Fas activation (figure 3.9d).

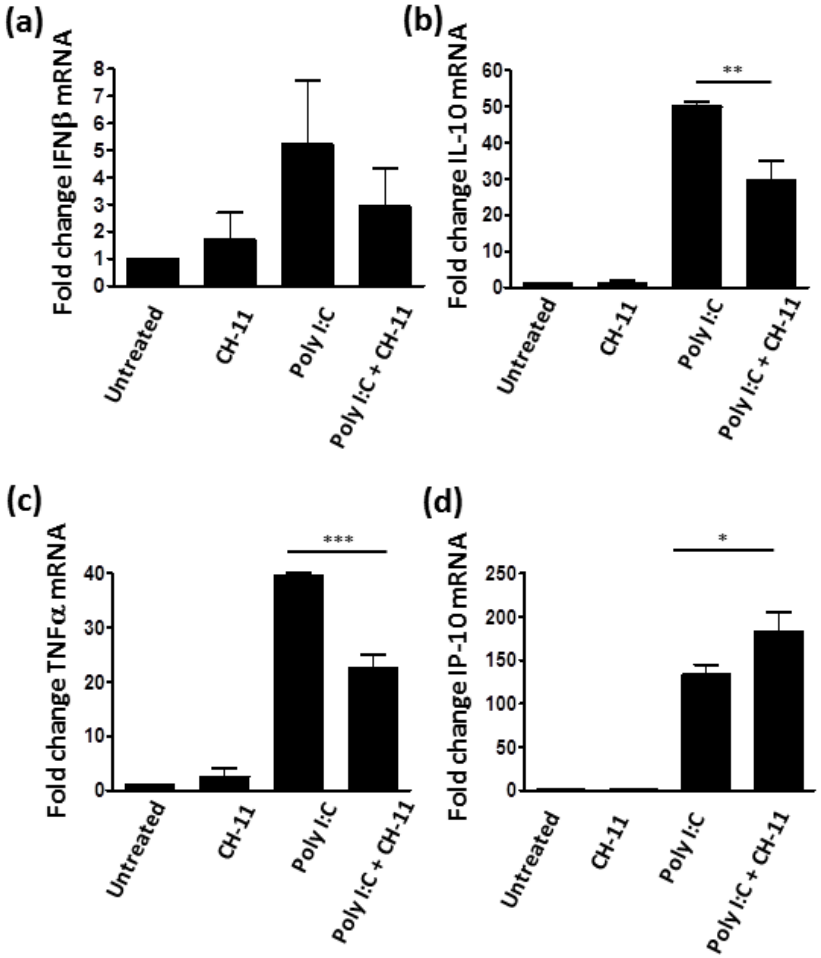

Figure 3.9: Fas activation decreases poly I:C-induced IFNβ, IL-10 and TNFα whilst augmenting production of IP-10 cytokine production in hMDMs. Human monocyte-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by stimulation with 20μg/ml poly I:C. mRNA expression was detected by qRT-PCR after 8 hrs and normalised to GAPDH. Changes in IFNβ (a), IL-10 (b), TNFα (c) and IP-10 (d) are expressed relative to untreated cells. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.2.10 Poly I:C-induced IP-10 production in THP1 macrophages was unaffected when pre-stimulated with the IgM isotype control.

To ensure that modulation of cytokine expression observed following stimulation of Fas with CH11 is specific to Fas activation, we next stimulated cells with a non-specific isotype IgM control antibody. IgM stimulation alone had no effect on IP-10 production. Poly I:C stimulation strongly induced IP-10 mRNA expression, which was unaffected by stimulation with the IgM isotype control (figure 3.10).

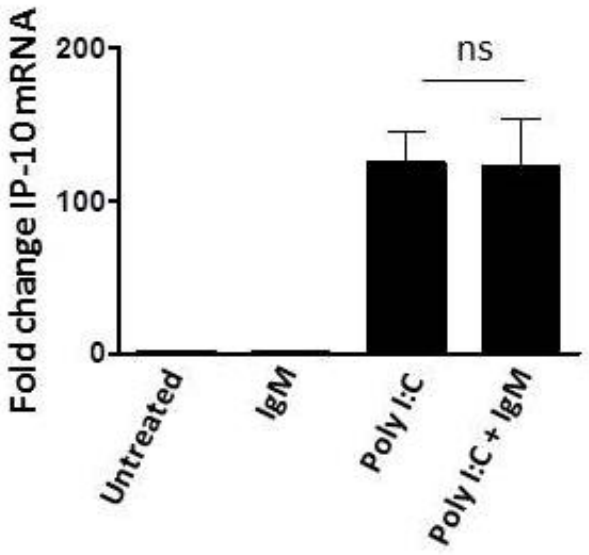

Figure 3.10: Poly I:C-induced IP-10 production was unchanged by stimulation with IgM isotype control. THP-1 macrophages were treated with 200ng/ml IgM isotype control for 1 hr followed by stimulation with 20μg/ml poly I:C. mRNA expression were detected by qRT-PCR after 8 hrs and normalised to GAPDH. Changes in IP-10 mRNA expression are expressed relative to untreated cells. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM, ns – no significant change observed.

3.2.11 Pre-treatment of macrophages with CH11 modulates both Sendai Virus (SeV) and poly A:U-induced cytokine production.

As poly I:C is recognised by both TLR3 and RIG-I, we next investigated which of these receptors was involved in the altered cytokine production seen upon Fas activation by CH11 and subsequent poly I:C stimulation. We initially investigated the effect of CH11 stimulation on SeV-induced cytokine production. SeV is a negative sense single-stranded RNA virus which signals specifically through the receptor RIG-I. SeV induced robust expression of IP-10 (figure 3.11a and b). Similar to data observed following co-stimulation with poly I:C and CH11, SeV-induced IP-10 mRNA expression was significantly augmented by Fas ligation (2 fold; $p < 0.05$) (figure 3.11a). Similarly, SeV-induced IP-10 protein production was also significantly increased by Fas ligation (2 fold; $p < 0.01$), compared with SeV-induced IP-10 protein production alone (figure 3.11b).

SeV-induced IL-8 expression was also examined, with SeV-induced IL-8 mRNA expression increased (2 fold). Unlike previous results obtained whereby CH11 reduced poly I:C-induced cytokine production, the induction of IL-8 mRNA or protein by SeV was unchanged when pre-stimulated with CH11 (figure 3.11c and d).

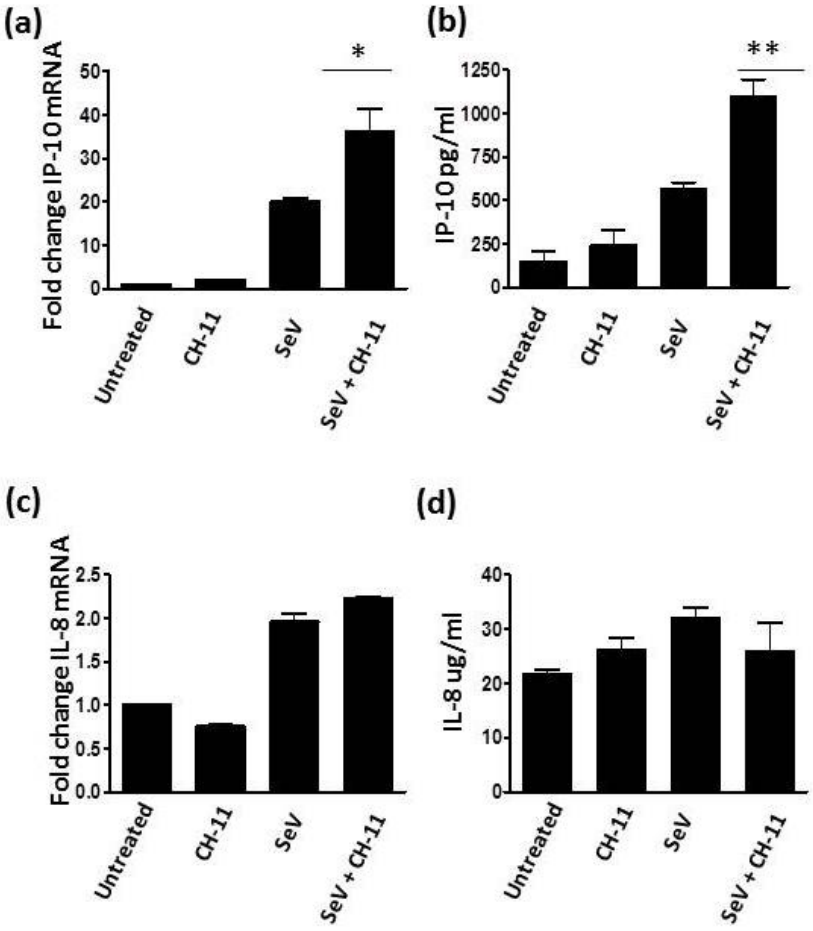

Figure 3.11: Ligation of macrophages with CH11 increases SeV-induced IP-10 production.THP-1-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by infection with Sendai virus. mRNA expression was determined by qRT-PCR after 8 hrs and normalised to GAPDH with changes in IP-10 and IL-8 production expressed relative to untreated cells **(a, c).** Changes in expression of IP-10 and IL-8 were also detected by ELISA after 48 hrs **(b, d)**. Data shown are representative of three independent experiments, with values shown as Mean ± SEM. * $p<0.05$, ** $p<0.01$

To further investigate through which receptor modulation of poly I:C-induced IP-10 production by CH11 is mediated, we next used the TLR3 specific agonist, poly A:U. While CH11 did not induce significant levels of IP-10 cytokine production, poly A:U stimulation of THP1 macrophages resulted in an increase of IP-10 mRNA production. Fas activation by CH11 with subsequent poly A:U stimulation resulted in augmentation of IP-10 production (2.5 fold; $p < 0.01$) compared to poly A:U alone (figure 3.12a), suggesting that Fas activation is required for enhanced TLR3 mediated IP-10 production. The effect of Fas ligation on poly A:U-induced IFNβ expression was also examined. Poly A:U stimulation resulted in a 2 fold increase of IFNβ production in THP1 macrophages. Although co-stimulation with CH11 did not significantly affect IFNβ production, a downward trend was observed (figure3.12b).

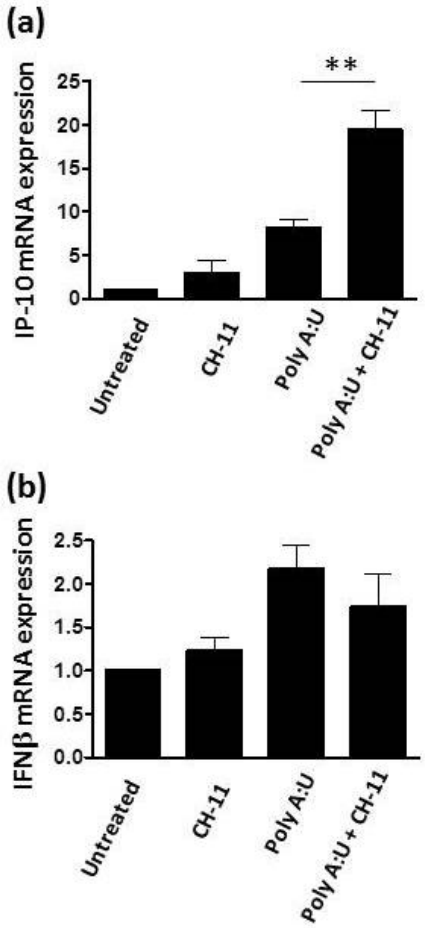

Figure 3.12: The augmentation in poly I:C-induced IP-10 production following ligation of Fas is also mediated by TLR3. THP-1-derived macrophages were treated with 200ng/ml CH11 for 1 hr followed by stimulation with poly A:U. mRNA expression was determined by qRT-PCR after 8 hrs and normalised to GAPDH with changes in IP-10 **(a)** and IFNβ **(b)** expressed relative to untreated cells. Data shown are a combination of three independent experiments, with values shown as Mean ± SEM. ** $p<0.01$

Taken together, this data indicate that the ability of CH11 to modulate poly I:C-induced cytokine production may occur through an interaction with the RIG-I and TLR3 signalling pathways.

3.2.12 Poly I:C-induced IP-10 production was augmented in immortalised bone marrow derived macrophages (iBMDMs), with no augmentation observed in TRIF$^{-/-}$ iBMDMs.

No report has previously implicated cross-talk between the TLR3 pathway and the Fas signalling pathway. As we have demonstrated that poly I:C enhances both Fas and FasL expression, we wished to further investigate potential cross-talk between the TLR3 and Fas signalling pathways. Therefore, we examined the effect of Fas activation on poly I:C-induced IP-10 production in immortalised wild type bone marrow-derived macrophages (iBMDMs), and iBMDMs lacking the TLR3 adapter protein TRIF (TRIF$^{-/-}$ iBMDMs). Consistent with our data using the human agonistic Fas antibody CH11, stimulation of Fas with the murine agonistic Fas antibody, Jo2, significantly augmented poly I:C-induced IP-10 production by wild type iBMDMs ($p < 0.001$) (figure 3.13) . This is noteworthy as we have now observed augmented poly I:C-induced IP-10 production by Fas activation in three different cell lines, indicating this is not a cell line specific effect. In contrast, no augmentation of poly I:C-induced IP-10 production was observed in the TRIF$^{-/-}$ iBMDMs by Fas activation (figure 3.13). This data provides further evidence that the ability of Fas to augment poly I:C induced IP-10 production involves the TLR3 signalling pathway.

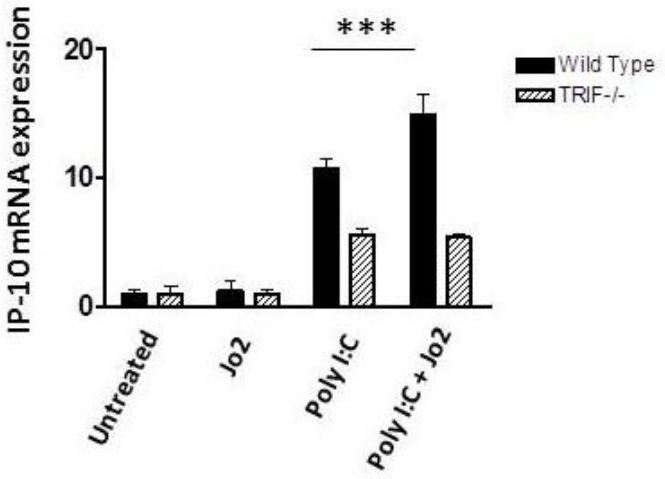

Figure 3.13: Poly I:C-induced IP-10 production is augmented following Fas activation in wild-type immortalised bone marrow-derived macrophages (iBMDMs), which was absent from TRIF-/- iBMDMs. Immortalised wild type and TRIF-/- BMDMs were stimulated with 1ng/ml murine agonistic Fas antibody (Jo2) for 1 hr followed by stimulation with 20μg/ml poly I:C for a further 8 hrs. mRNA expression was detected by qRT-PCR and normalised to GAPDH. Changes in IP-10 were expressed relative to untreated cells. Data shown are representative of three independent experiments, with values shown as Mean ± SEM. ***$p<0.001$

3.2.13 Optimal poly I:C-induced IP-10 production by Fas activation occurs between 4 and 8 hours.

IP-10 is an early phase potent T cell chemokine. As a result, we next wished to determine the time-frame by which poly I:C-induced IP-10 expression is augmented by Fas ligation. IP-10 production was examined over a 24 hour time-course in the presence or absence of CH11. In contrast to all other time points, CH11 was seen to induce IP-10 mRNA at 4 hours of stimulation to a level comparable to that of poly I:C. However, the level of poly I:C-induced IP-10 following Fas ligation was dramatically increased compared to poly I:C alone (75 fold) (figure 3.14a). Poly I:C-induced IP-10 production following Fas ligation was also increased at 8 hours (3 fold) and at 12 hours (1.5 fold), compared to poly I:C alone (figure 3.14b and c respectively). In contrast to 4, 8 and 12 hours, poly I:C-induced IP-10 production following Fas ligation was reduced at 24 hours (50% reduction) (figure 3.14d). This data suggests that the ability of CH11 to modulate poly I:C-induced IP-10 production is an early event with maximum augmentation observed at 4 hours, an effect which is reversed by 24 hours.

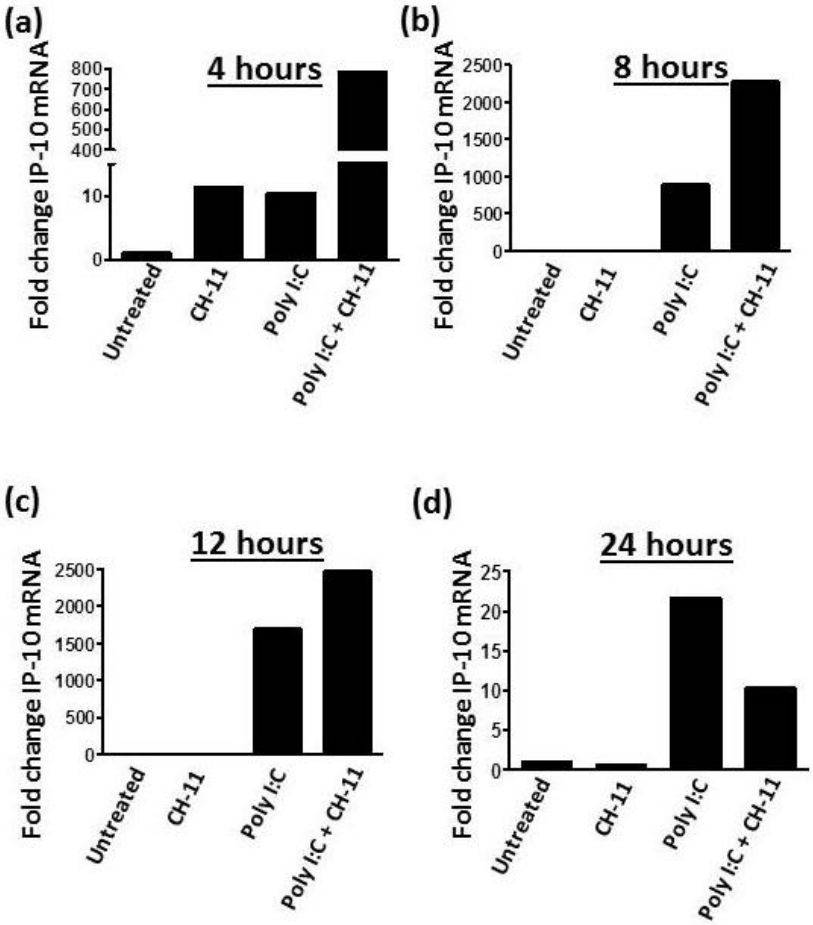

Figure 3.14: Poly I:C-induced IP-10 production is augmented following Fas activation at 4, 8 and 12 hrs with reduction in IP-10 production at 24 hrs. THP-1 macrophages were stimulated with 200ng/ml CH-11 for 1 hr followed by poly I:C stimulation with 20μg/ml for 4 hrs (a), 8 hrs (b), 12 hrs (c) and 24 hrs (d). Cytokine expression was detected by qRT-PCR and normalised to GAPDH. Changes in cytokine expression were expressed relative to untreated cells.

3.2.14 Stimulation of THP1 macrophages with CH11 and poly I:C results in enhanced Jurkat T cell recruitment and increased CXCR-3 expression.

We have demonstrated that IP-10 production is enhanced in THP1 macrophages stimulated with both CH11 and poly I:C. As IP-10 is a potent T cell chemokine, we next investigated what effect this would have on T cell recruitment. Optimisation experiments were initially performed. Increased migration of Jurkat T cells towards RPMI-1640 media supplemented with 10% serum and IP-10 was observed compared to media supplemented with 10% serum alone (figure 3.15a). Next, supernatant from THP1 macrophages treated with poly I:C in the presence and absence of CH11 for 24 hours was harvested and migration of Jurkat T cells towards these supernatants assessed using a transwell migration assay. We were unable to obtain consistent results for this assay due to a high variation between experiments, resulting in large error bars (figure 3.15 b). Therefore, we next performed a cell recruitment experiment, examining the recruitment of Jurkat T cells to THP1 macrophages which were stimulated with poly I:C with and without CH11 stimulation for 24 hours prior to addition of Jurkat T cells (figure 3.15 c-f). Recruitment and attachment of Jurkat T cells to these macrophages was then examined following 24 hours of co-culture. We observed that both untreated and CH11 stimulated THP1 macrophages had minimal T cell recruitment and attachment, as determined by the number of T cells surrounding macrophages (figure 3.15 c, d), while enhanced recruitment of T cells was observed following stimulation of THP1 macrophages with poly I:C (figure 3.15 e). Co-stimulation with both Fas and poly I:C increased T cell recruitment (figure 3.15 f). This data is also represented graphically, whereby we calculated the ratio of T cells attached to macrophages to T cells not attached to macrophages (figure 3.15 g) for all stimulations.

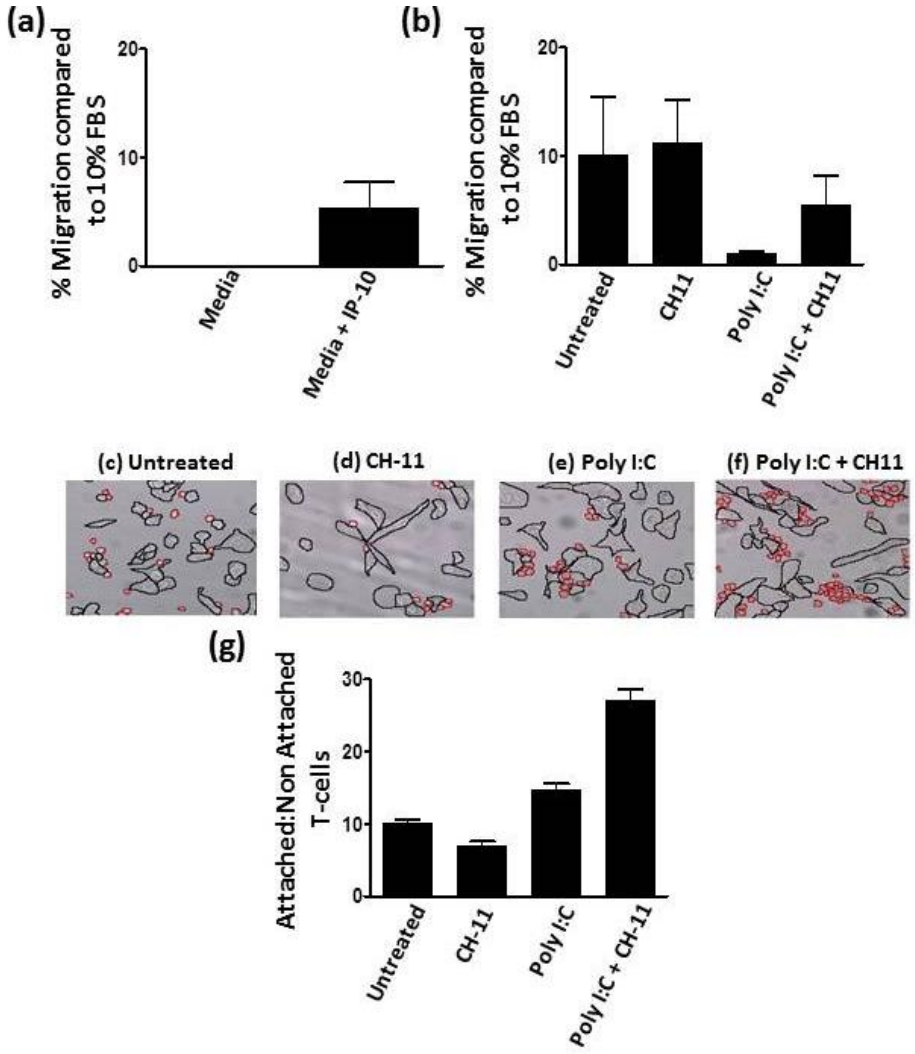

Figure 3.15: THP-1 macrophages stimulated with both poly I:C and CH11 had enhanced Jurkat T cell recruitment compared to other treatment groups. Jurkat T cells were added to the upper well of a transwell and migration towards complete media and complete media with 50ng IP-10 determined **(a)**. Migration of Jurkat T cells towards supernatant harvested from THP-1 macrophages treated with poly I:C with and without CH11 was also determined **(b)**. After 2 hrs, migrated cells were collected, stained with crystal violet and absorbance measured **(a, b)**. THP-1 macrophages were treated with 200ng/ml CH11 for 1 hr, followed by 20μg/ml poly I:C for 24hrs. Jurkat T cells were added to treated macrophages and cultured for 24 hrs, with cell aggregation observed microscopically. Macrophages are outlined in black, with Jurkat T cells outlined in red **(c, d, e, f)**. The ratio of T cells attached to macrophages : non-attached T cells was determined with a minimum of 18 visual fields counted **(g)**. Data shown are representative of three independent experiments.

As we had observed increased poly I:C-induced IP-10 production following Fas activation, and also increased recruitment of T cells to macrophages following co-stimulation of cells with poly I:C and CH11, we next wished to determine if enhanced expression of the IP-10 receptor, CXCR-3, on Jurkat T cells may account for the increased recruitment of T cells to treated THP1 macrophages. Resting Jurkat T cells expressed low levels of CXCR-3 (4.1%) (figure 3.16a). THP1 macrophages were either untreated or stimulated with poly I:C, CH11, or co-stimulated with both poly I:C and CH11 for 24 hours. Supernatant was then harvested and used for sub-culturing of Jurkat T cells. CXCR-3 expression increased 10-fold upon culturing of T cells in untreated THP1 macrophage supernatant (figure 3.16b). Whilst culturing of T cells in macrophage supernatant with CH11 (figure 3.16c) resulted in only a modest augmentation of CXCR-3 expression on T cells above unstimulated, most augmentation in CXCR-3 expression was observed following stimulation with poly I:C and CH11 (figure 3.16e). Taken together, these results suggest optimal T cell recruitment of T cells to IP-10 requires induction of CXCR-3 expression on T cells by an as of yet unknown macrophage-derived factor. These studies also show that augmented IP-10 production seen upon co-stimulation of macrophages with poly I:C and CH11 may have a functional consequence regarding increased recruitment of T cells to macrophages.

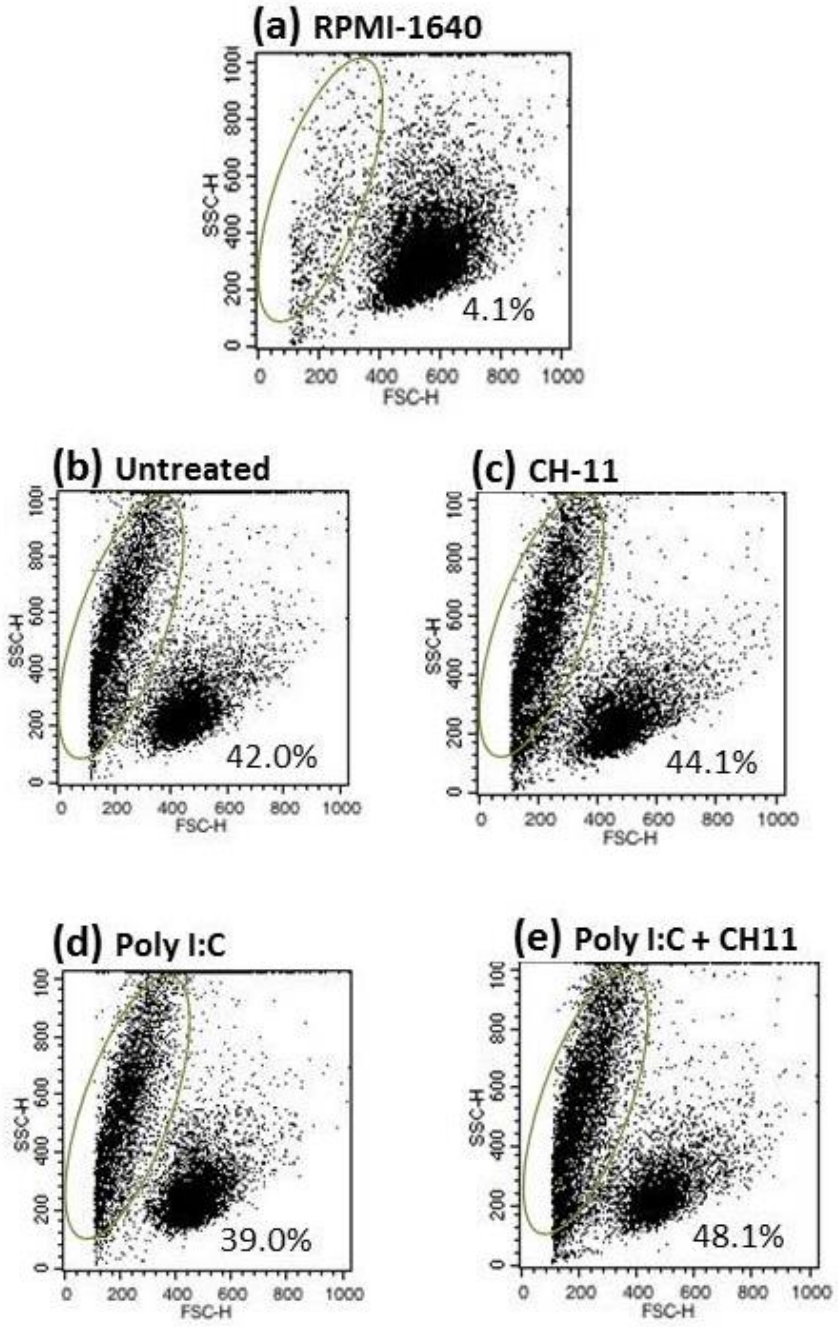

Figure 3.16: The IP-10 receptor, CXCR-3, is induced by untreated macrophage supernatant and increased by a combination of poly I:C and CH-11. Jurkat T cells were cultured in RPMI-1640 media (a), or in macrophage-derived supernatant which was either untreated (b) or treated with (c) 200ng/ml CH11, (d) 20ug/ml poly I:C or (e) poly I:C and CH11 for 24 hrs. Expression of CXCR-3 was determined by flow cytometry. Data are representative of three individual experiments.

3.3 Discussion

Our findings in this study demonstrate that activation of the Fas signalling pathway by ligation of Fas to its ligand, FasL, modulates poly I:C-induced inflammatory cytokine production. We have demonstrated augmentation of Fas and FasL expression in response to poly I:C stimulation of THP1 macrophages. We have also showed that poly I:C-induced production of IFNβ, IL-8, IL-10 and TNFα were reduced upon activation of Fas. Conversely, we have shown that poly I:C-induced IP-10 production is augmented by Fas activation, with caspase 3/7 activation unaffected. We also observed enhanced CXCR-3 expression on Jurkat T cells when cultured in THP1 macrophage supernatant. Finally, we observed increased T cell recruitment towards THP1 macrophages treated with poly I:C and CH11, compared to poly I:C alone.

We have demonstrated increased Fas and FasL expression following poly I:C stimulation in THP1 macrophages. Previous studies have also demonstrated increased Fas expression following exposure to other pathogen-associated membrane proteins (PAMPs). Lipopolysaccharide (LPS) has been shown to enhance Fas expression in bone marrow-derived macrophages (BMDMs) (151). Similarly, the double-strand RNA mimetic, poly I:C, has been shown to enhance Fas expression in the murine macrophage cell line, RAW 264.7 (176). Consistent with this, viral proteins have also been shown to enhance both Fas and FasL expression. Basal FasL expression on human monocyte-derived macrophages was enhanced following HIV (human immunodeficiency virus) infection (177), while influenza was also shown to induce expression of both Fas and FasL in HeLa cells (175). Given that we too have observed enhanced Fas and FasL expression following stimulation with poly I:C, our

findings add further evidence that Fas and FasL can be increased by TLR and viral stimuli.

Fas and FasL are best known for inducing apoptosis. Whilst we have demonstrated increased Fas and FasL expression following poly I:C stimulation, we did not detect any change in cell viability, nor did we see activation of the executioner caspases, caspases-3/7. Measurement of caspase-3/7 activation was used as a marker of apoptosis in this study, as activation of caspase-3 results in irreversible commitment to apoptosis of the cell (178). Indeed, some viruses target the Fas apoptotic pathway, thereby inducing apoptosis (179, 180). Similar to our findings however, studies have also demonstrated that viral infection does not consistently induce Fas mediated-apoptosis. The DNA virus, equine herpesvirus type 2 (EHV-2) can bind to the pro-domain of caspase-8 (163), while the DNA virus molluscum contagiosum virus (MCV) binds to FADD, thus preventing Fas mediated apoptosis (163). Moreover, although Herpes Simplex Virus 2 (HSV-2) Fas and FasL expression in keratinocytes and epithelial cells, increased levels of the anti-apoptotic protein Bcl-2 was also observed in HSV-2 infected cells, protecting the infected cells, and thus the virus, from Fas-mediated apoptosis (164). We too observed increased Fas and FasL expression following poly I:C stimulation of THP1 macrophages with no caspase 3/7 activation, and this may be due to enhanced levels of the anti-apoptotic proteins cFLIP or Bcl-2. However, the mechanisms responsible for resistance to Fas-mediated apoptosis were not investigated as part of this study. Taken together, these studies suggests that the up-regulation of Fas and FasL observed may play an alternative function, possibly in host defense against some viral infections.

As we observed increased Fas and FasL expression following poly I:C stimulation in THP1 macrophages, and several studies have now described a variety of non-apoptotic functions for Fas including migration, proliferation and inflammation, we next investigated the effect of Fas activation on inflammatory cytokine production by THP1 macrophages. Previous studies have demonstrated that Fas activation induced IL-18 and IL-1β production in macrophages (151). However, we did not see any induction of cytokine production upon Fas ligation. This may have been due to the concentration of CH11 used as in another study which used a similar concentration of CH11, cytokine production in THP1 cells was also not observed. Increasing the concentration of CH11 however, resulted in the induction of IL-8 to a level similar to that seen upon LPS stimulation (152). As poly I:C-induced both Fas and FasL expression, with no induction of cytokines or chemokines following Fas activation, we hypothesised that Fas may be modulating poly I:C-induced cytokine and chemokine production.

We have demonstrated that Fas ligation is capable of modulating poly I:C-induced cytokine production through both the RIG-I and TLR3 pathways, with specific enhancement of IP-10. Whilst no study to date has examined the effect of Fas ligation on poly I:C signalling, studies have demonstrated that the Fas signalling pathway plays a role in modulating TLR4 and LPS responses (162, 176, 181). LPS-induced IL-6 production was reduced upon interruption of Fas ligation, with reduced LPS-induced IL-6 production seen in both Fas (*lpr*) and FasL (*gld*) deficient mice, compared to wild-type mice (181). Consistent with this, blocking of Fas-FasL interaction in two models of cardiac dysfunction was shown to attenuate inflammatory cell infiltration and cytokine secretion in the myocardium without affecting apoptosis *in vivo* (161, 162), significantly preserving cardiac function.

Additionally Fas ligation on macrophages promoted TLR4-mediated chronic inflammation in a model of collagen-induced arthritis (181). These studies illustrate the importance of the Fas signalling pathway in TLR4 mediated signalling. Given our findings that the Fas signalling pathway also plays a role in signalling through both RIG-I and TLR3 pathways, our data indicates that the cross-talk between Fas and pathogen recognition receptors is more extensive than previously thought.

IP-10 is an early response gene; therefore we investigated the time-frame for the augmentation in IP-10 production. The augmentation in IP-10 following activation with both poly I:C and agonistic anti-Fas was most dramatic at the earlier time points of 4 and 8 hours, with reduced augmentation observed at 12 hours. A reduction in poly I:C-induced IP-10 occurred at 24 hours was seen following co-activation of both receptors. Changes in the expression levels of the receptors may account for this divergent effect at 24 hours as it is well-known that Fas is internalised following activation (181). Alternatively it is possible that Fas ligation is primarily required for up-regulation of IP-10 production in the early phase of the anti-viral response, consistent with the high level of IP-10 observed at 4 hours compared to 24 hours.

IP-10 is a potent T cell chemokine which affects T cell generation and trafficking *in vivo* (174), recruiting T cells expressing its receptor CXCR-3 (182, 183). While we have shown a specific enhancement of IP-10 by Fas activation and poly I:C stimulation, we have also shown that expression of the IP-10 receptor, CXCR-3 in Jurkat T cells is increased upon culturing in macrophage-derived supernatant, suggesting that Fas activation may play a significant role in IP-10 production following viral infection. The importance of IP-10 in clearance of viral infection is well established (174, 184, 185). IP-10 has been shown to protect mice infected with

neurotropic coronavirus mouse hepatitis (MHV), as blocking of IP-10 production increased mouse death and reduced the ability of infected mice to clear the infection (184). Moreover, mice lacking Fas or FasL showed reduced IP-10 production following infection with herpes simplex virus (HSV)-2, with a reduction in immune cell recruitment resulting in delayed clearance of the virus (186). We have also demonstrated increased T cell recruitment and attachment towards macrophages treated with poly I:C and CH11 compared to other treatment groups. Taken together, these studies demonstrate the importance of both IP-10 production and the Fas signalling pathway in protecting the host following viral infection.

Excessive IP-10 production however, has implications for a variety of human diseases including infectious diseases, chronic inflammation, immune dysfunction, tumour development, metastasis and dissemination (182). Increased IP-10 production may exacerbate inflammation and induce significant tissue damage as a result of increased immune cell trafficking (187). It is important, therefore, that following viral infection, IP-10 levels are regulated to ensure an adequate immune response whilst avoiding an exacerbated inflammatory response. It has been demonstrated that RNA derived from necrotic synovial fluid cells induces IP-10 expression by rheumatoid arthritis synovial fibroblasts, potentially by activation of TLR3 (188). The majority of T cells present in the synovial fluid of joints affected by rheumatoid arthritis (RA) are positive for the IP-10 receptor CXCR-3 (189, 190), with an increase in IP-10 chemotactic gradient favouring migration of CXCR-3 positive T cells from the serum to synovium of RA affected joints (189). As activated T cells express FasL, our results suggest that in situations of excessive T cell recruitment as characterised by many chronic inflammatory conditions, binding of FasL expressed by T cells to macrophage-expressed Fas may result in activation

of Fas, which may further exacerbate the inflammatory response. Therefore, blocking Fas may represent a potential therapeutic target for pathologies requiring T cell involvement, such as rheumatoid arthritis (189-191) and more recently systemic lupus erythematosus (SLE) (192).

In conclusion, we have shown that the chemokine IP-10 is specifically enhanced following Fas activation and subsequent stimulation with poly I:C, poly A:U and SeV, and that the ability of Fas to modulate poly I:C-induced cytokine production is not specific to either TLR3 or RIG-I, but is rather common to both pathways. This indicates that Fas ligation will affect cytokine production in response to a myriad of viral infections.

Chapter 4

Identification of a mechanism by which Fas activation augments poly I:C-induced IP-10 production.

4.1 Introduction

Fas is a well characterised death receptor, with ligation of Fas by its ligand, FasL, resulting in the recruitment of the adaptor protein Fas-associated death domain (FADD). FADD contains two functional domains, the Death Effector Domain (DED) and the Death Domain (DD), with binding of FADD to Fas by DD-DD interaction. This in turn recruits the cysteine protease pro-caspase-8 via a DED-DED interaction, resulting in the formation of the death-inducing signalling complex or DISC. Following activation in the DISC, caspase-8 in turn activates the effector caspases, culminating in the apoptotic cell death of sensitive cells (193). As mentioned in the previous chapter, the Fas signalling pathway, together with its adaptor protein FADD, can also mediate other biological processes. FADD has been shown to be essential for embryonic development *in utero* (102). FADD has also been shown to have a functional role in processes such as autophagy and apoptosis, tumour growth, necrosis and innate immunity (194).

In the previous chapter, we demonstrated that Fas activation augmented both TLR3 and RIG-I induced IP-10 production. While a role for FADD in dsRNA-induced type I interferon (IFN) production through the RIG-I signalling pathway has been demonstrated (165), the role of FADD in TLR3-induced cytokine production is unclear. IP-10/CXCL-10 is a potent chemokine, recruiting immune cells such as Th1 cells and Natural Killer (NK) cells expressing its receptor, CXCR-3 (182). IP-10 plays an important role in viral clearance (174, 184, 185). However, dysregulation of IP-10 production has been implicated in a variety of pathologies (182, 187). It is important, therefore, that following viral infection, IP-10 levels are regulated to ensure an adequate immune response whilst avoiding an exacerbated inflammatory

response. Thus, identifying mechanisms involved in regulating IP-10 production in response to viral infection is important. The aim of the current chapter was to investigate the mechanism involved in the augmentation of poly I:C-induced IP-10 production by Fas and to characterise the role of the Fas adapter FADD in this pathway.

4.2 Results

4.2.1 Attempt at optimising siRNA knockdown of FADD expression in THP1 macrophages.

In the first chapter we identified a role for Fas in modifying poly I:C-induced cytokine production with specific upregulation of IP-10. Given the important role of FADD in the Fas signalling pathway, we next wished to analyse the role of FADD in poly I:C-induced IP-10. We initially aimed to investigate the role of FADD in poly I:C-induced IP-10 production by reducing endogenous levels of FADD by siRNA transfection. THP1 cells were transfected with FADD-specific siRNA or scrambled control siRNA. Changes in FADD expression were detected by Western blotting. Compared to untreated cells, endogenous levels of FADD were reduced following transfection with 100nM, 150nM or 300nM FADD siRNA (figure 4.1a). As we wanted to determine what effect a reduction in FADD would have on poly I:C-induced IP-10 production, THP1 monocytes were transfected with either 100nM of FADD siRNA or100nM scrambled control siRNA and differentiated into macrophages, and then stimulated with and without 20μg/ml poly I:C (figure 4.1b). However, transfection of THP1 macrophages with both scrambled control siRNA and FADD-specific siRNA completely abrogated the capacity of the cells to respond to poly I:C (figure 4.1b). To ensure that THP1 macrophages were still responsive to

poly I:C, non-transfected cells were also stimulated with poly I:C, which induced a 3.5 fold increase in IP-10 protein production (figure 4.1c). This indicates that the effect observed following siRNA knockdown was most likely due to the transfection of the cells with siRNA and as a result we were unable to utilise this technique.

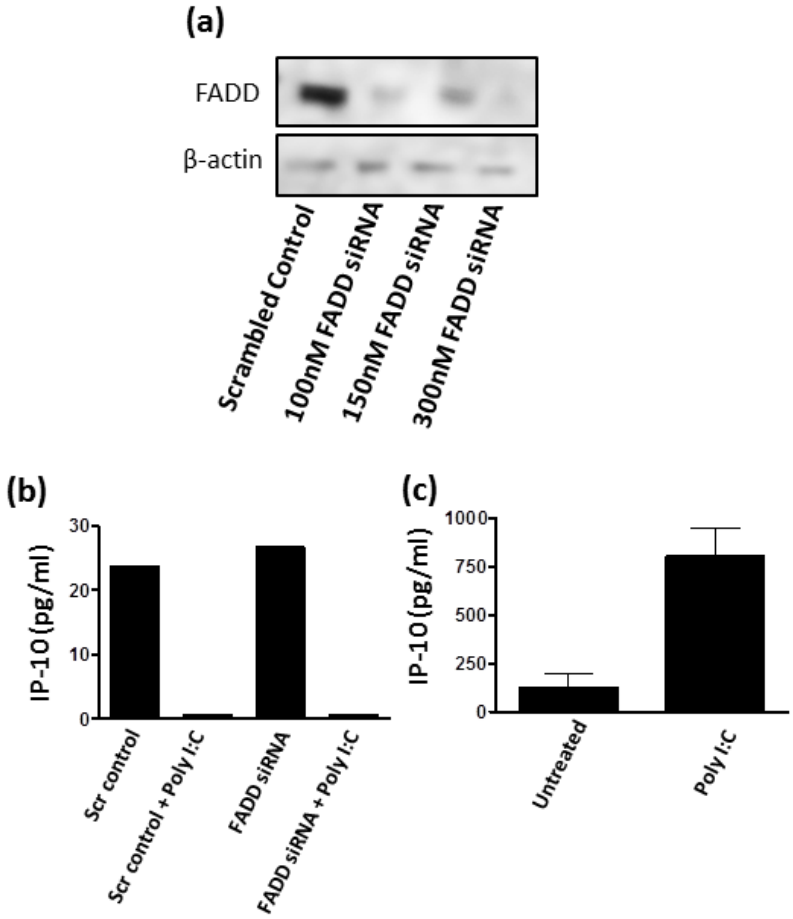

Figure 4.1: Transfection of THP-1 macrophages with either scrambled control siRNA or FADD-specific siRNA suppressed the ability of the cells to produce IP-10 in response to poly I:C. THP-1-derived macrophages were transfected with 100nM, 150nM and 300nM FADD siRNA. 24 hrs later, FADD expression was detected by Western Blotting **(a)**. THP-1 monocytes were transfected with either scrambled siRNA control or FADD specific siRNA and derived into macrophages over 72 hrs. Cells were then stimulated with 20μg/ml poly I:C for 48 hrs, and IP-10 production detected by ELISA. **(b)**. Non-transfected THP-1-derived macrophages were stimulated with 20μg/ml poly I:C for 48 hrs, and IP-10 production detected by ELISA **(c)**. Results shown are representative of three separate experiments.

4.2.2 Absence of FADD augments poly I:C-induced IP-10 transcription and inhibits poly I:C-induced IFNβ production.

As we encountered significant difficulties with the siRNA method, we next investigated the role of FADD in poly I:C-induced IP-10 production using murine embryonic fibroblasts (MEFs) which lack the Fas adaptor protein FADD ($FADD^{-/-}$ MEFs). Wild-type and $FADD^{-/-}$ MEFs were stimulated with increasing concentrations of poly I:C and expression of IP-10 analysed by qRT-PCR. Induction of IP-10 by poly I:C was significantly increased in $FADD^{-/-}$ MEFs as compared to wild-type MEFs at all doses examined ($p < 0.05$) (figure 4.2a). As previous studies have demonstrated an essential role for FADD in the type-1 interferon production via RIG-I (166), we also investigated the ability of poly I:C to induce IFNβ production in these cells (figure 4.2b). In contrast to IP-10 production, poly I:C-induced IFNβ production was decreased in $FADD^{-/-}$ MEFs when compared to wild-type MEFs (figure 4.2b). While these results were not statistically significant, they are consistent with the previously published findings that FADD is required for poly I:C-induced IFNβ production (165, 166). We also investigated the role of FADD on poly I:C-induced CCL20 production. Both wild-type and $FADD^{-/-}$ MEFs showed similar levels of CCL-20 expression following poly I:C stimulation, suggesting that FADD may not be required for poly I:C-induced CCL-20 production.

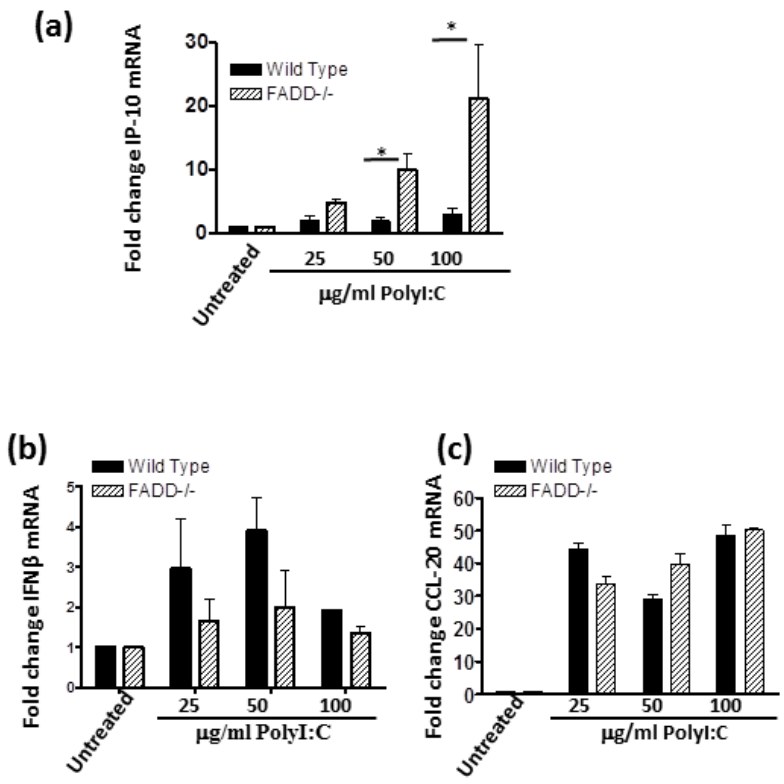

Figure 4.2: Absence of FADD augments poly I:C-induced IP-10 transcription and inhibits poly I:C-induced IFNβ production. Wild type and FADD $^{-/-}$ MEFs were stimulated with increasing concentrations of poly I:C (25, 50 and 100ng/ml) for 8 hrs, with mRNA expression measured using qRT-PCR and normalised to GAPDH. Changes in IP-10 (a), IFNβ (b) and CCL-20 (c) mRNA were expressed relative to untreated cells. Values shown as Mean ± SEM, (n=3). * $p<0.05$

4.2.3 IP-10 production is reduced following overexpression of FADD in three cell lines.

Given that deletion of FADD resulted in enhanced poly I:C-induced IP-10 production, we sought to further confirm a role for FADD in this pathway using overexpression studies. The murine macrophage cell line RAW264.7 was transfected with plasmids encoding an IP-10 luciferase reporter construct and increasing doses of FADD (figure 4.3a). Stimulation of these cells with poly I:C induced IP-10 luciferase activation, with IP-10 luciferase production reduced by FADD in a dose dependent manner (figure 4.3a). Based on these results, a concentration of 40ng FADD was selected for further luciferase experiments. The ability of 40ng of FADD to suppress poly I:C-induced luciferase activation was first confirmed in RAW 264.7 cells (figure 4.3b). This finding was subsequently confirmed using the human astrocytoma cell line U373 (figure 4.3c) and human embryonic kidney cells HEK-293/TLR3 (figure 4.3d), with poly I:C-induced IP-10 luciferase activation reduced upon overexpression of FADD in both cell lines (figure 4.3c, d).

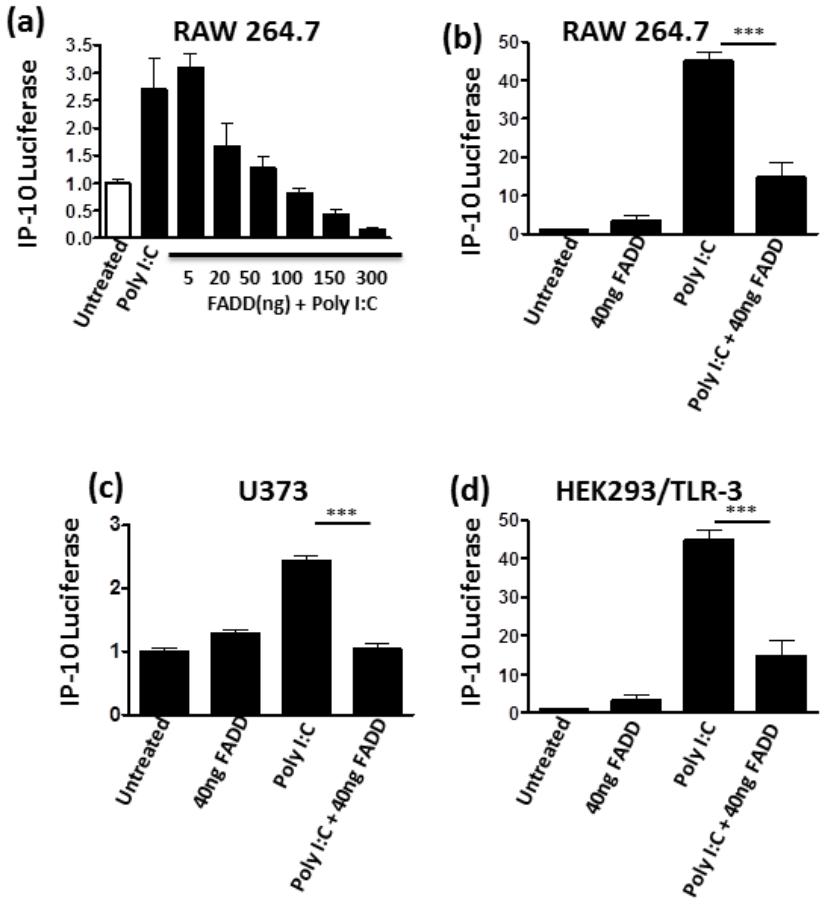

Figure 4.3: FADD reduces poly I:C-induced IP-10 production. Cells were transfected with IP-10 luciferase and TK-renilla reporter plasmid. RAW264.7 cells were also transfected with increasing concentrations of FADD plasmid (4, 20, 50 and 100ng) and stimulated 24 hrs after transfection with 25µg/ml poly I:C for 6hrs (a). RAW 264.7 cells were transfected with 40ng IP-10 luciferase construct and/or stimulated 24 hrs after transfection with 25µg/ml poly I:C (b). U373 and HEK-293/TLR-3 cells were also transfected with 40ng FADD and/or stimulated 24 hrs after transfection with 25ng/ml poly I:C and 20ng/ml respectively for 6 hrs (c, d). IP-10 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. *** $p<0.001$

4.2.4 Overexpression of cells with FADD-DD plasmid enhances poly I:C-induced IP-10 luciferase activation in U373 cells.

The role of FADD was also confirmed using a plasmid encoding the death-domain of FADD (FADD-DD), a known dominant negative of FADD, which binds to FADD and inhibits its activity. In contrast to the findings obtained following transfection of FADD plasmid (figure 4.3c), transfection of U373 cells with the FADD-DD construct augmented poly I:C-induced IP-10 luciferase activation (figure 4.4), adding further evidence that FADD inhibits poly I:C-induced IP-10 production.

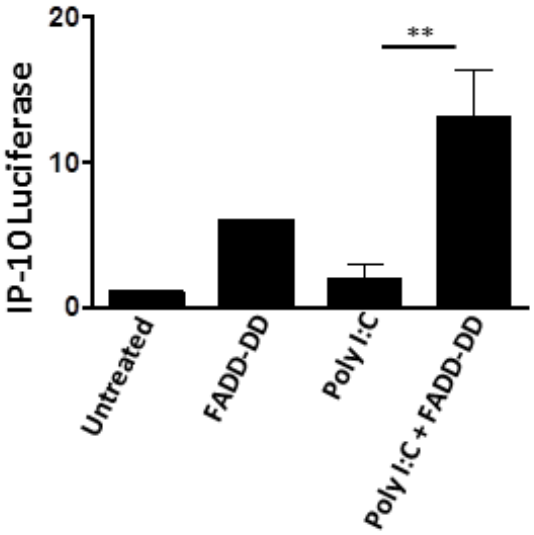

Figure 4.4: Poly I:C-induced IP-10 luciferase production is augmented by overexpression of the death domain of FADD (FADD-DD). U373-CD14 were transfected with the IP-10 luciferase construct and TK-renilla construct with/without 40ng FADD-DD. Cells were stimulated 24 hrs after transfection with 25μg/ml poly I:C for 6 hrs. IP-10 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. ** $p<0.01$

4.2.5 FADD is required for poly I:C-induced ISRE luciferase activation.

Our data thus far has demonstrated that poly I:C-induced IP-10 luciferase activity is reduced following overexpression of FADD, implying that FADD may inhibit poly I:C-induced IP-10 production. Given the findings of previously published studies demonstrating that FADD is required for poly I:C-induced type-1 IFN production we next investigated the role of FADD in poly I:C-induced interferon-stimulated response element (ISRE) luciferase activation. U373 cells were transfected with plasmids encoding an ISRE luciferase reporter construct and FADD. Stimulation of these cells with poly I:C resulted in a 6 fold increase in ISRE luciferase activation. In contrast to the inhibitory effect of FADD overexpression seen on the IP-10 luciferase construct (figure 4.3), poly I:C-induced ISRE activation was slightly enhanced by FADD (figure 4.5a). This requirement for FADD in poly I:C-induced ISRE luciferase production was confirmed using a plasmid encoding the FADD-DD. In contrast to transfection of full-length FADD construct, transfection of U373 cells with the FADD-DD construct reduced poly I:C-induced ISRE luciferase activation (figure 4.5b). Thus, in contrast to the inhibitory effect seen with FADD on poly I:C-induced IP-10 production, our results demonstrate that FADD is required for poly I:C-induced ISRE luciferase activity. This is consistent with our results which showed that poly I:C-induced IFNβ production is reduced following Fas activation (chapter 3, figure 3.6a), and augmented in $FADD^{-/-}$ MEFs (figure 4.2b).

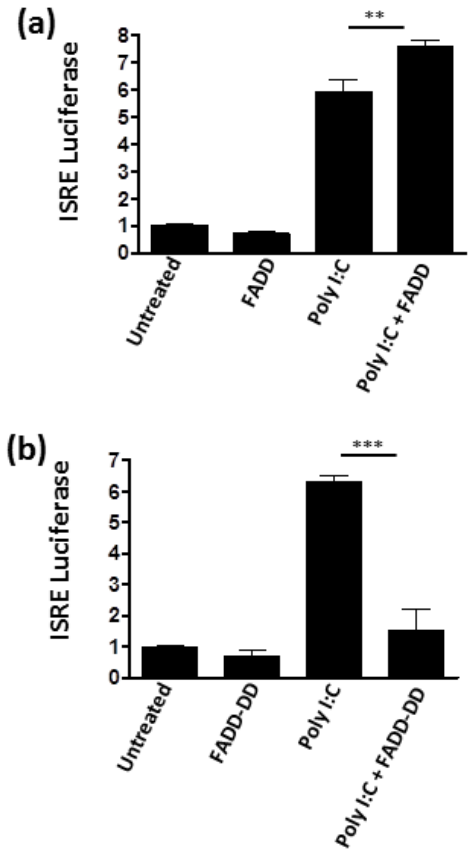

Figure 4.5: FADD is required for poly I:C-induced ISRE luciferase activation. U373 cells were transfected with ISRE luciferase and TK-renilla reporter plasmid, with 40ng FADD (a) or 40ng FADD-DD (b). After 24 hrs, cells were stimulated with 20μg/ml poly I:C. ISRE luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. ** $p<0.01$, *** $p<0.001$

4.2.6 Overexpressing FADD reduces RIG-I-, MDA5-, TLR3- and TRIF-induced IP-10 luciferase activity.

Our data thus far demonstrates that FADD modulates poly I:C-induced cytokine and chemokine production, with FADD specifically inhibiting poly I:C-induced IP-10 production. Whilst FADD has been previously implicated in modulating RIG-I-induced cytokine production (165, 166), poly I:C is known to be detected by the viral receptors RIG-I, MDA5 and TLR3, and the role for FADD in MDA5 and TLR3 signalling is not fully characterised. Therefore, we first wished to investigate if FADD was having a specific effect on the ability of any of these receptors to induce IP-10 production. To assess this, cells were initially transfected with plasmids encoding an IP-10 luciferase reporter construct, FADD and/or RIG-I (figure 4.6a). Transfection of cells with RIG-I plasmid alone induced IP-10 production of luciferase activity regulated by full length IP-10 promoter, which was reduced upon co-transfection with FADD (figure 4.6a). Cells were also transfected with an IP-10 luciferase construct, FADD and/or MDA5 (figure 4.6b). Transfection of cells with MDA5 also induced IP-10 luciferase activation, with overexpressing FADD reducing MDA5-induced IP-10 luciferase activation (figure 4.6b).

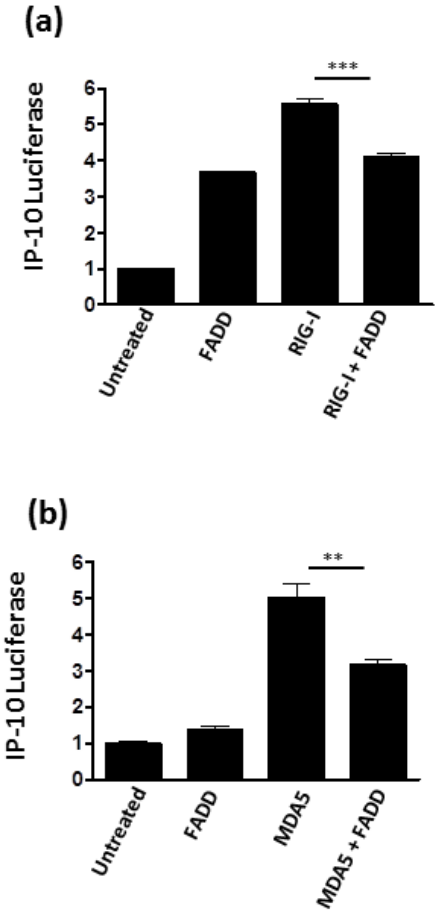

Figure 4.6: FADD reduces RIG-I and MDA5-induced IP-10 production. HEK-293T cells were transfected with IP-10 luciferase and TK-renilla reporter plasmid. Cells were also transfected with 40ng/ml FADD and 80ng/ml RIG-I **(a)** or 40ng/ml FADD and 80ng MDA5 **(b)**. IP-10 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. ** $p<0.01$, *** $p<0.001$

As poly I:C is also recognised by TLR3, we next investigated the role of FADD in TLR3-induced IP-10 luciferase production. Cells were transfected with plasmids encoding an IP-10 luciferase reporter construct, FADD and/or TLR3 (figure 4.7a). Transfection of cells with TLR3 plasmid alone induced IP-10 production, which was reduced upon co-transfection with FADD (figure 4.7a). TLR3-induced IP-10 luciferase activation was not inhibited when cells were co-transfected with FADD-DD plasmid, indeed levels were slightly augmented (figure 4.7b).

Cells were also transfected with plasmids encoding an IP-10 luciferase reporter construct, FADD and/or the TLR3 adaptor protein TRIF (figure 4.7c). Similar to results shown with TLR3, transfection of cells with TRIF induced IP-10 luciferase activation, which was reduced upon co-transfection with FADD (figure 4.7c). TRIF induced IP-10 luciferase activation was enhanced however, following co-transfection with FADD-DD (figure 4.7d). Taken together, these results demonstrate that TLR3- and TRIF-induced IP-10 luciferase activity was reduced by FADD, demonstrating a role for FADD in the TLR3/TRIF mediated pathway of IP-10 production.

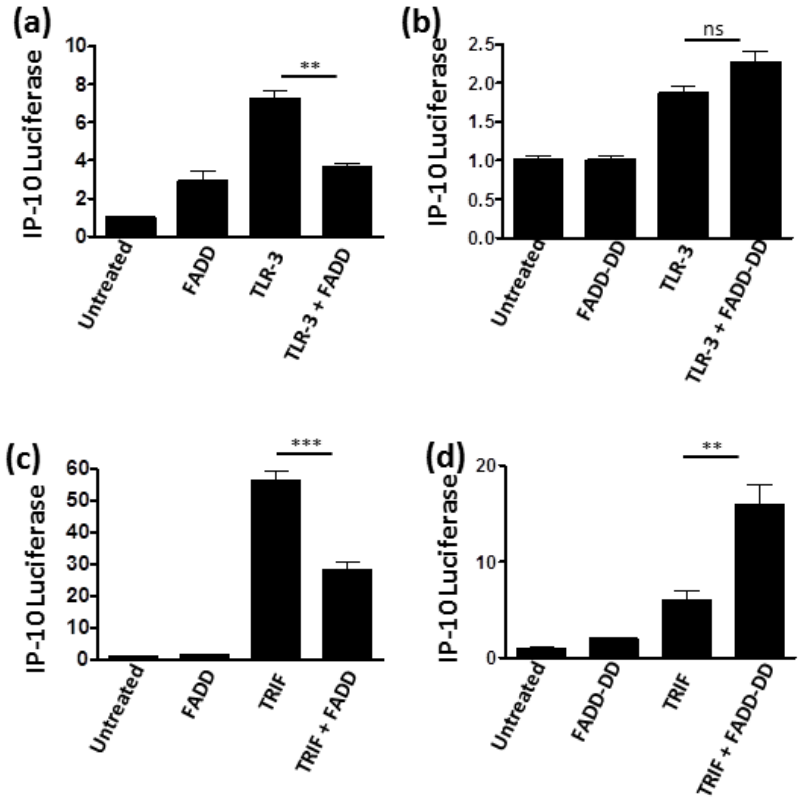

Figure 4.7: FADD reduces TLR-3- and TRIF-induced IP-10 production. HEK-293T cells were transfected with IP-10 luciferase and TK-renilla reporter plasmid. Cells were also transfected with 40ng FADD or 40ng FADD-DD, together with 40ng TLR-3 **(a, b)** or 80ng TRIF **(c, d)**. IP-10 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. ** $p<0.01$, *** $p<0.001$, ns – non significant.

Taken together, these results suggest that FADD reduces RIG-I-, MDA5-, TLR3- and TRIF-mediated IP-10 luciferase activation and also indicates that the inhibitory effect of FADD on poly I:C-induced IP-10 production is not specific to any viral RNA receptor.

4.2.7 Overexpression of FADD has no effect on RIP-1- or IKKε-induced IP-10 luciferase activation.

As it appeared that FADD was specifically inhibiting poly I:C-mediated IP-10 production, and not IFNβ and that this modulatory effect was not specific to TLR3, RIG-I or MDA5, we hypothesised that FADD may be exerting an inhibitory effect at a specific point on the pathways downstream of these receptors. In an attempt to identify where FADD may be having this effect, we next transfected cells with plasmids encoding RIP-I and IKKε. Both RIP-1 (65, 195) and IKKε (196, 197) are downstream of TLR3, RIG-I and MDA5. Transfection with either RIP-1 or IKKε alone induced IP-10 luciferase activation, which was slightly enhanced upon co-expression of FADD (figure 4.8a, c). Overexpression of the FADD-DD however, had no effect on either RIP-1 or IKKε IP-10 luciferase activation (figure 4.8b, d). As FADD did not inhibit IP-10 luciferase activation driven by signalling components downstream of poly I:C receptors or the adapter TRIF, our results suggested that FADD may reduce IP-10 luciferase activation at the level of receptors themselves or the adapter TRIF.

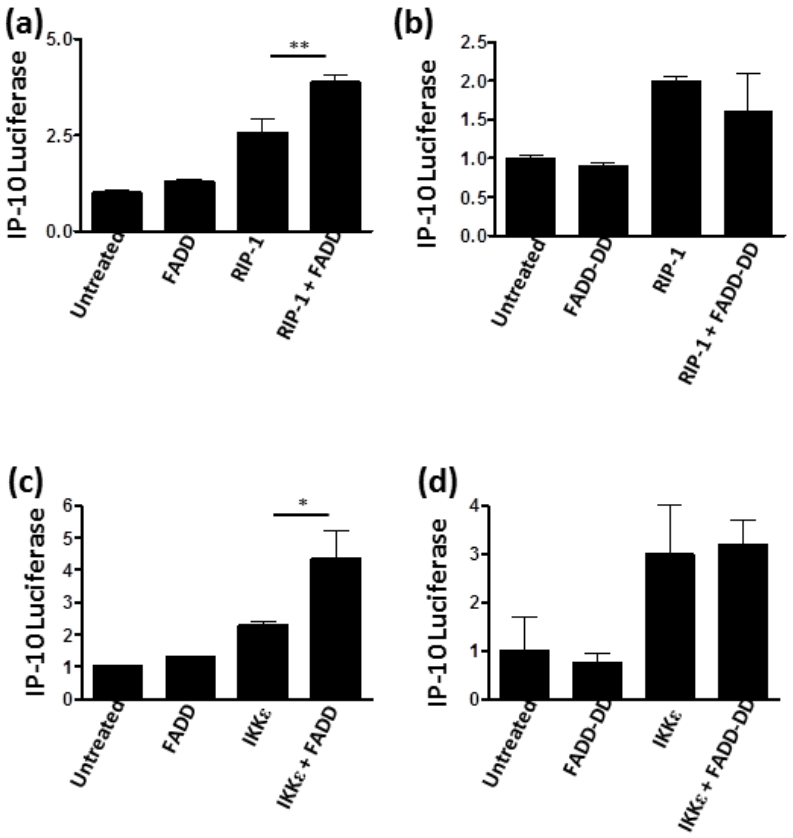

Figure 4.8: FADD does not inhibit RIP-1- and IKKε-induced IP-10 production. HEK-293T cells were transfected with IP-10 luciferase and TK-renilla reporter plasmid, with 40ng FADD or 40ng FADD-DD, together with 80ng RIP-1 (a, b) or 80ng IKKε (c, d). IP-10 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. * $p<0.05$, *** $p<0.001$

4.2.8 Co-immunoprecipitation of VSV-TRIF and FLAG-FADD was unsuccessful in detecting an interaction between TRIF and FADD.

Whilst FADD has never been shown to directly interact with TRIF, previous studies have demonstrated an indirect interaction, mediated through a complex containing TRIF/FLIP/RIP-1 (130). As our luciferase data indicated an ability of FADD to inhibit TRIF-induced IP-10 production, but not any components further downstream, we hypothesised that FADD may directly interact with TRIF. To investigate this, HEK-293T cells were initially transfected with different concentrations of a VSV-tagged TRIF construct (figure 4.9a) and a FLAG tagged FADD construct (figure 4.9b) and Western blotting performed with anti-VSV antibody and anti-Flag antibody respectively. Transfection of the FLAG-FADD construct was initially optimised. Transfection of cells with both 2μg and 4μg Flag-FADD resulted in expression of the protein (figure 4.9a). We subsequently attempted to optimise overexpression of the VSV-TRIF construct. Initial optimisation experiments were performed using 1, 2, 4 and 10μg of VSV-tagged TRIF. It was noted however, that following transfection of the higher doses of VSV-tagged TRIF, extensive cell death was observed. Therefore, we optimised the overexpression of TRIF at lower concentrations. While we did not observe a band in cells transfected with 1μg VSV-TRIF, a faint band was observed following Western blotting with anti-VSV in cells transfected with 2μg VSV-TRIF (figure 4.9b).

HEK-293T cells were next transfected with the optimal concentration of VSV-TRIF (2μg) with or without FLAG-FADD and co-immunoprecipitation was performed (figure 4.9c). Immunoprecipitation for both VSV and FLAG were performed. Following immunoprecipitation with the VSV antibody and immunoblotting for

FLAG, a band was detected in all samples at approximately 23-26kDa. This band, which is most likely Light chain of the immunoprecipitating antibody (anti-VSV), ran at the same size at FADD (26kDA) and therefore may mask any potential interactions that we may have observed (figure 4.9c). We also performed the same experiment in the reverse direction, immunoprecipitating with anti-Flag and immunoblotting with anti-VSV (figure 4.9d). In this experiment the same band at 26kDA can be observed as was seen in figure 4.9c. Moreover no band corresponding to the correct size for VSV-tagged TRIF (75kDa) was observed, perhaps reflecting the very low level of expression of VSV-TRIF as seen in figure 4.9b. Similar results were obtained three times, and therefore we were unable to identify an interaction between FADD and TRIF.

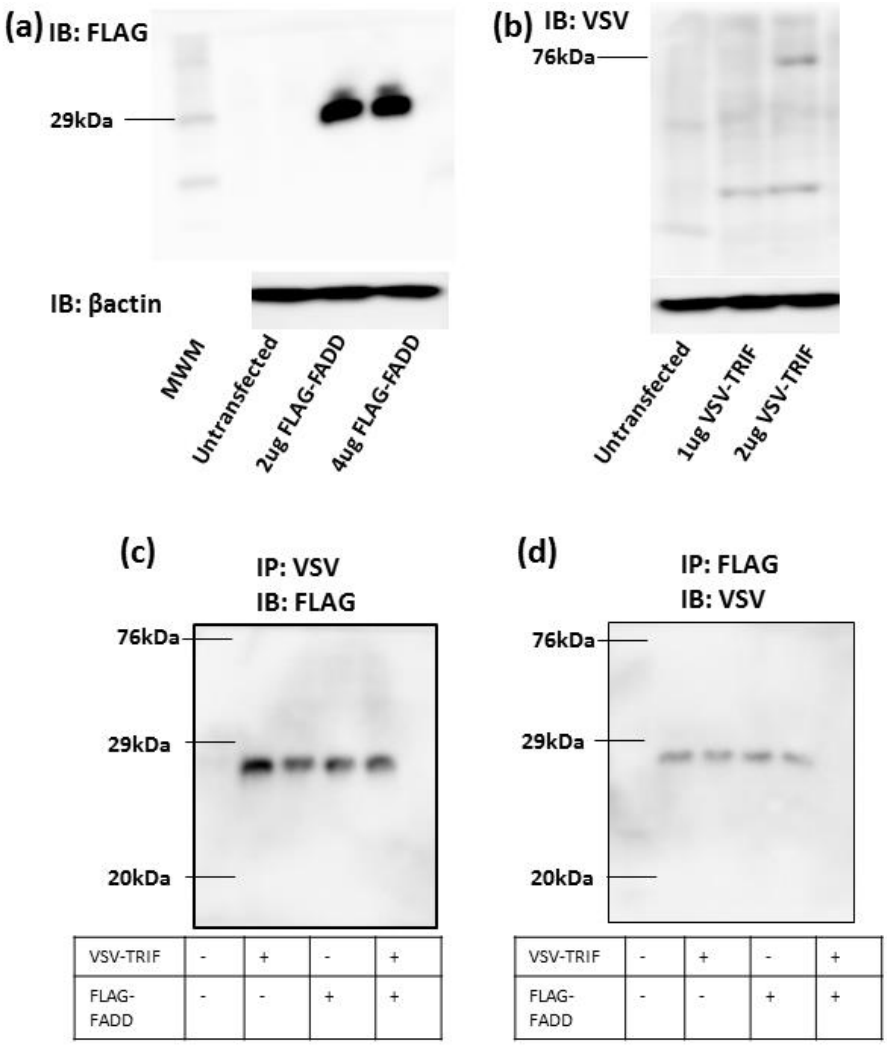

Figure 4.9: Co-immunoprecipitation of VSV-TRIF and FLAG-FADD was unsuccessful in detecting an interaction between TRIF and FADD in HEK-293T cells. HEK-293T cells were transfected with either FLAG-FADD (a) or VSV-TRIF (b) and immuno-blotting performed for anti-FLAG or anti-VSV respectively. Optimal concentrations of both FLAG-FADD and VSV-TRIF were selected, with cells either untransfected or transfected with 2μg/ml VSV-TRIF with and without 4μg/ml FLAG-FADD (c, d). Immuno-precipitation was performed. Samples which were immunoprecipitated with anti-VSV were immunoblotted for FLAG (c). Similarly, samples immunoprecipitated with anti-FLAG were immunoblotted for VSV (d).

4.2.9 Co-stimulation with poly I:C and CH11 reduces poly I:C-induced phosphorylation of JNK and p38 MAP Kinases (MAPKs).

Thus far our data demonstrates that Fas activation enhances viral-induced IP-10 production. We have also shown that over-expressing FADD inhibits TLR3-, MDA5- and RIG-I-induced IP-10 production in a non-receptor specific manner, thereby directly implicating FADD in this process. However, we had not yet defined a precise molecular mechanism by which FADD is able to modify poly I:C-induced IP-10 production. Furthermore, it was increasingly difficult to comprehend how FADD could specifically inhibit levels of IP-10, whilst either augmenting or not affecting other cytokines, such as IFNβ. To address these issues we decided to examine the ability of the Fas/FADD pathway to modify signalling components common to all three viral receptors. As these receptors can activate both NFκB and the MAP Kinase (MAPK) signalling pathways, we initially investigated whether poly I:C-induced activation of these signalling intermediates was altered in $FADD^{-/-}$ MEFs compared to wild-type MEFs. The results of these experiments however were inconclusive. In spite of loading the maximum amount of protein (i.e. lysing samples directly in sample buffer) and increasing antibody concentration, only faint bands were observed upon probing. Similarly, consistent banding patterns were not achieved over three independent experiments. This may be due to variations in protein loading. Therefore the same experimental model as utilised in the previous chapter was employed. THP1 cells were stimulated with CH11, followed by poly I:C stimulation, and phosphorylation of signalling intermediates downstream of poly I:C was measured. While poly I:C-induced phosphorylation of IκBα and p42/p44 MAPK was unaffected by CH11, Fas activation by CH11 followed by poly I:C stimulation resulted in markedly reduced phosphorylation of the MAP kinase p38 at 15, 30 and

60 minutes of stimulation, relative to poly I:C alone (figure 4.10). Similarly, phosphorylation of JNK was also reduced at 30 and 60 minutes.

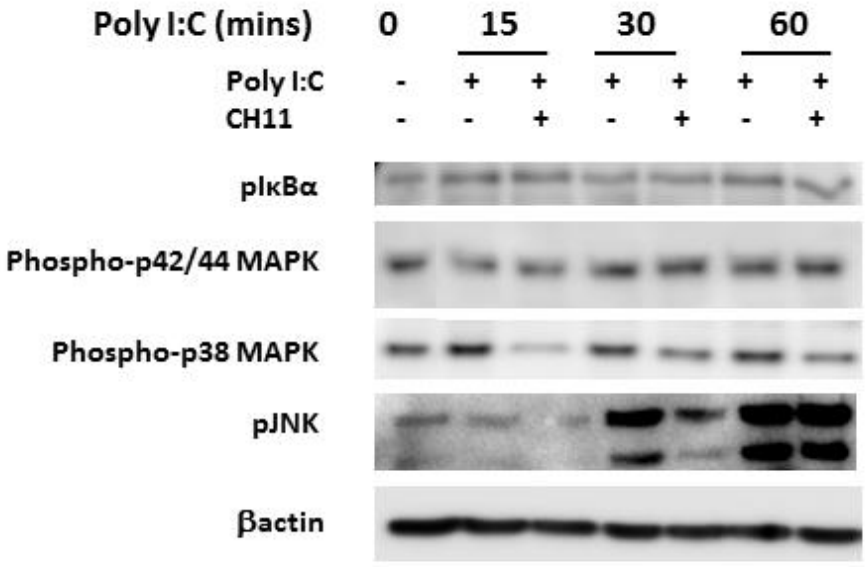

Figure 4.10: Ligation of Fas suppresses poly I:C-induced phosphorylation of JNK and p38 MAPK. THP-1 macrophages were treated with CH11 1 hr prior to poly I:C stimulation for 15, 30 and 60 mins. pIκBα, phospho-p42/44 MAPK, phospho-p38 MAPK, phospho-JNK and βactin were detected by Western blotting. Data shown are representative of three independent experiments.

4.2.10 The AP-1 transcription factor negatively regulates IP-10 production.

Phosphorylation of JNK and p38 MAPK are known to result in activation of the transcription factor AP-1. As the IP-10 promoter contains an AP-1 binding site, we next examined the IP-10 promoter using IP-10 promoter luciferase constructs mutated at the ISRE binding site, two separate NFκB binding sites (κB1, κB2) and the AP-1 binding site (figure 4.11a). Interestingly, cells transfected with the IP-10 promoter mutated at the AP-1 binding site showed augmented IP-10 luciferase activation in response to poly I:C (figure 4.11b). This augmentation parallels the increased IP-10 expression seen upon co-stimulation with CH11 and poly I:C (chapter 3, figure 3.7). In contrast, poly I:C-induced IP-10 activation was reduced following mutation of two separate NFκB binding sites (figure 4.11c, d) and also of the ISRE binding site (figure 4.11e). These results indicate that the AP-1 transcription factor represses poly I:C-induced IP-10 production.

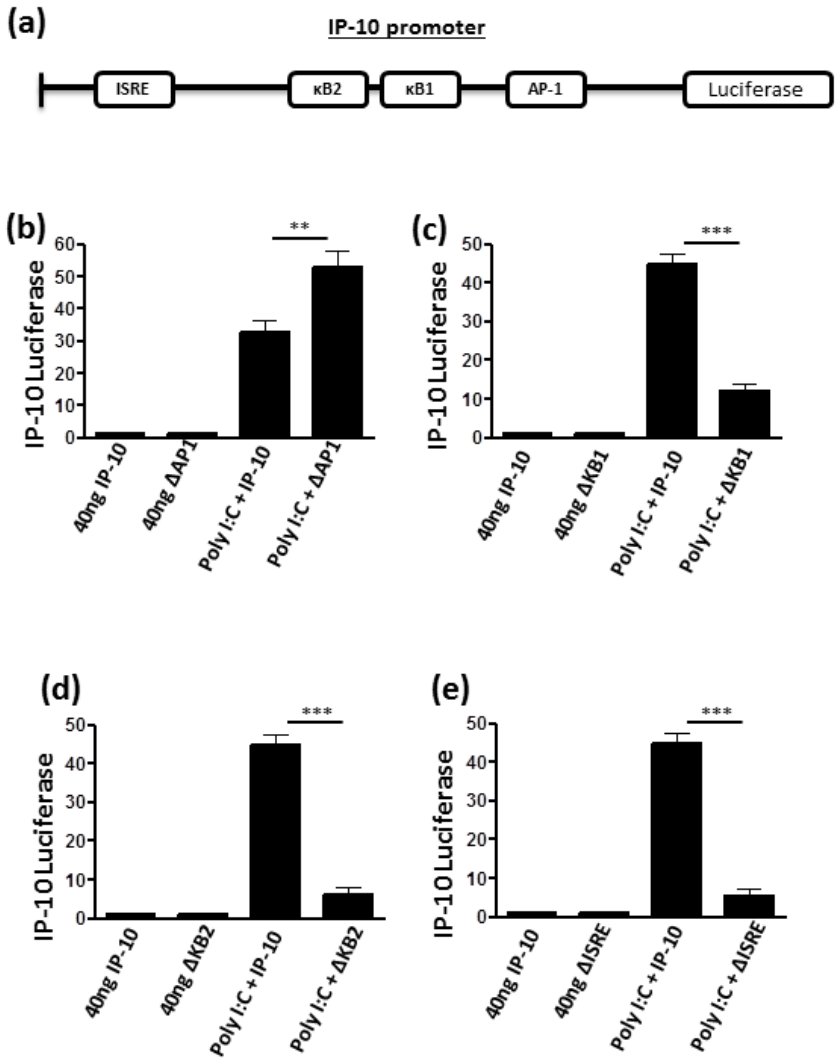

Figure 4.11: AP-1 negatively regulates the IP-10 promoter in response to poly I:C. HEK-293/TLR3 cells were transfected with full length IP-10 luciferase plasmids or IP-10 luciferase plasmids containing point deletions in the binding sites for proximal AP-1, NF-κB (κB1, κB2), or ISRE. A schematic of these putative binding sites is illustrated (a). 24 hrs post transfection, cells were stimulated with 20μg/ml poly I:C for 6 hrs with IP-10 luciferase activity expressed as fold-change over TK-renilla activity (b, c, d, e). Data shown are representative of three independent experiments with values shown as Mean ± SEM. ** $p<0.01$, *** $p<0.001$

4.2.11 Overexpression of FADD activates AP-1 luciferase activation.

Thus far we have shown that Fas ligation modifies poly I:C-induced phosphorylation of transcription factors upstream of AP-1. We have also demonstrated that AP-1 is inhibitory to poly I:C-induced IP-10 production. In order to demonstrate that Fas, through its adapter FADD, can affect AP-1 activation and thereby potentially explain the ability of Fas to upregulate poly I:C-induced IP-10, we next investigated whether transfection of cells with FADD altered activation of an AP-1 luciferase construct. Activation of AP-1 by FADD was low but was consistently enhanced in a dose-dependent manner upon transfection with FADD, with TNFα inducing a moderate induction in AP-1 activity (figure 4.12). As we have demonstrated that AP-1 negatively regulates IP-10 production, and that FADD enhances AP-1 activity, we have identified a potential mechanism by which Fas activation may modulate viral-induced IP-10 production. We hypothesise that Fas activation results in recruitment of the FADD adapter protein to the DISC complex (114), thereby limiting the levels of FADD in the cytoplasm. This would alleviate the repression of AP-1 by reducing phosphorylation of both JNK and p38 MAPK. This potential mechanism of upregulation of poly I:C-induced IP-10 by Fas is illustrated in figure 4.13.

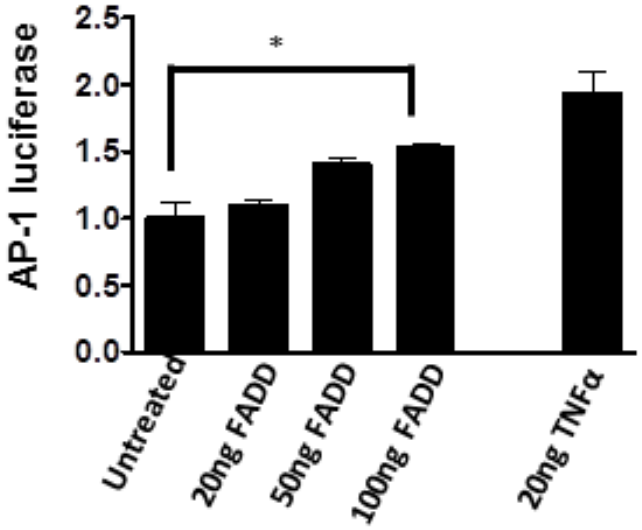

Figure 4.12: Overexpression of FADD induces AP-1 luciferase activation. HEK293/TLR3 cells were transfected with an AP-1 luciferase reporter construct. Cells were also transfected with the indicated doses of FADD. 24 hrs after transfection cells were stimulated with 20μg/ml poly I:C or 20ng/ml TNFα further 6 hrs. AP-1 luciferase activity was measured and expressed as fold-change over TK-renilla activity. Data shown are representative of three independent experiments with values shown as Mean ± SEM. * $p<0.05$

4.3 Discussion

The findings in this chapter demonstrate a role for FADD in regulating viral-induced IP-10 production. Over-expression studies revealed that FADD inhibits TLR3-, RIG-I- and MDA-5- induced IP-10 production. Investigation of signalling intermediates demonstrated that Fas activation reduces poly I:C-induced phosphorylation of both JNK and p38 MAPK, with mutation of the AP-1 transcription factor binding site on the IP-10 promoter resulting in enhanced poly I:C-induced IP-10. The Fas adapter protein FADD enhanced the luciferase activity of the AP-1 transcription factor. Therefore, we hypothesise that upon Fas activation, FADD is recruitment to the Fas receptor thus reducing the amount of FADD present in the cytoplasm. This in turn reduces activation of the AP-1 transcription factor thus alleviating AP-1 mediated repression of the IP-10 promoter and enhancing IP-10 production (figure 4.13).

In chapter 3 we observed that Fas activation reduced poly I:C-induced IFNβ production. In this chapter, we have shown that an absence of FADD ($FADD^{-/-}$ MEFs) reduced poly I:C-induced IFNβ production, indicating that FADD may be a critical signalling component in this pathway. Previous studies investigating the role of FADD in poly I:C signalling have also shown that FADD is a critical component of the RIG-I signalling pathway, whereby production of type 1 IFNs in response to intracellular dsRNA was defective in $FADD^{-/-}$ MEFs (165). This study demonstrated that $FADD^{-/-}$ MEFs had increased replication of vesicular stomatitis virus (VSV) with respect to wild-type MEFs, and while transfection of the dsRNA mimetic poly I:C still induced a low level of IFNβ in $FADD^{-/-}$ MEFs, more robust IFNβ production was observed in $FADD^{+/-}$ MEFs (165). In a separate study, this group also demonstrated that FADD was essential for RIG-I-, MDA5- and MAVS/IPS-1-

induced IFNβ production, with overexpression of these receptors inducing enhanced IFNβ luciferase activation following poly I:C stimulation in FADD$^{+/-}$ MEFs when compared to FADD$^{-/-}$ MEFs (166). They also demonstrated that FADD was also required for poly I:C-induced IRF7 expression with poly I:C inducing expression of IRF7 in FADD$^{+/-}$ MEFs, with no induction in expression observed in FADD$^{-/-}$ MEFs (166). Therefore, our data provides further evidence that FADD is required for poly I:C-induced IFNβ production.

In contrast to the data shown here concerning the essential role of FADD in IFNβ production, we have identified that overexpression of FADD inhibits IP-10 production and that a more robust induction in poly I:C-induced IP-10 production is observed in FADD$^{-/-}$ MEFs when compared to wild-type MEFs. Interestingly, this is in contrast to a study published by Zhande *et al* (159). Using a similar experimental design to our study, they exposed both wild-type and FADD$^{-/-}$ MEFs to poly I:C and analysed IP-10 production (159). IP-10 production was measured by ELISA, with wild-type MEFs showing enhanced IP-10 production when compared to FADD$^{-/-}$ MEFs (159). It seems possible that these differences could be explained by a difference in the generation of the FADD knockout MEFs. The cell line that we used was obtained from Dr. Tak W. Mak in the University of Toronto, whereas that used by Zhande *et al*. was obtained from Amgen, Inc., therefore differences in animal units, and the genetic background of the mice may account for the contrasting results observed. Also, in our study we have confirmed the specific modulation of poly I:C-induced IP-10 production by Fas stimulation using FADD$^{-/-}$ MEFs and using overexpression experiments involving both FADD and the FADD-DD. Moreover, these experiments were performed in multiple cell lines and multiple species. Our data is therefore strengthened by the variety of techniques and cell lines used as

compared to Zhande *et al*. who only examined IP-10 production in FADD$^{-/-}$ MEFs. It is worth noting that the other published studies investigating the role of FADD in poly I:C responses did not examine levels of IP-10. Furthermore, an inhibitory role for FADD in cytokine production was recently shown in another study. This study identified a novel interaction between FADD and TRIM21, and showed that overexpression of FADD and TRIM21 was able to repress both IRF7 phosphorylation and transcriptional activity. Loss of FADD and TRIM21 led to higher levels of IFNα induction in response to Sendai virus (198). These authors concluded that this complex constitutes a negative feedback loop of late IFNα pathway during viral infection (198), indicating that FADD negatively regulates IFNα (198). Our data concerning IP-10 is consistent with these findings for an inhibitory role for FADD as we have shown that, with respect to dsRNA-induced IP-10 production, FADD is able to repress IP-10 production.

The ability of FADD to inhibit poly I:C-induced IP-10 production does not appear to be receptor specific, as over-expression of FADD inhibited RIG-I-, MDA5-, TLR3 and TRIF-induced IP-10 luciferase activation. Interestingly, FADD did not either inhibit RIP-1- or IKKε-induced IP-10 production. Upon dsRNA recognition, TLR3 recruits the adapter protein TRIF for activation of downstream signalling components, while both RIG-I and MDA5 recruit the adapter protein IPS-1 (70). Both RIP-1 and IKKε are utilised by both RIG-I and TRIF, activating NFκB and IRF 3/7 respectively (70). Both NFκB and the IRFs regulate IP-10 production (199-201). Given that FADD and RIP-1 have previously been shown to be in a complex (130), it may have been expected that FADD would also inhibit RIP-1-induced IP-10 production. Instead a slight augmentation of RIP-1-induced IP-10 was observed upon co-transfection of cells with FADD and RIP-1. RIP-1 is known to be a key

component of the RIG-I signalling pathway. In this pathway RIP-1 and FADD have been shown to interact in a complex with IPS-1, and this interaction is essential for RIG-I-induced production (65). Thus, the failure of FADD to inhibit RIP-1-induced IP-10 is possibly due to the involvement of RIP-1 in the RIG-I signalling and may indicate additional complexities of FADD in RIG-I-induced IP-10 production that remain to be elucidated.

As mentioned above FADD has been shown to be in a complex with RIP-1 and this complex also includes the adapter TRIF as well as caspase-8 (130). As we had observed that overexpression of FADD could inhibit TRIF-induced IP-10 and were unable to show inhibition of other downstream signalling proteins, it seemed possible that FADD could be interacting with TRIF directly. TRIF is a 76kDa protein containing several potential binding domains for protein-protein interactions. It contains a conserved TIR domain through which it interacts with TLR3 (52). Three TRAF-binding motifs present in the amino terminal region of TRIF are necessary for association with TRAF6. Destruction of these motifs reduces the activation of NFκB (202). It also contains a RIP homotypic interaction motif (RHIM) through which it recruits RIP-1 and RIP-3 (195), essential for activation of apoptosis (172). Similarly the protein structure of FADD is well characterised. It is a 26kDa protein containing two conserved domains: the death domain (DD), and the death effector domain (DED) (194), required for downstream signalling following receptor activation. Whilst none of these domains in TRIF or FADD would be classically considered to be protein-protein interacting domains, we nonetheless performed co-immunoprecipitation experiments to try to determine whether these proteins interacted. Unfortunately, the results of the TRIF-FADD co-immunoprecipitation experiment were inconclusive. Whilst we were able to detect

overexpression of both TRIF and FADD during optimisation of the experiments, following immunoprecipitation, it is possible that the Light chain, apparent at 23-26kDa, is masking any possible interaction between FADD and TRIF. Also, as overexpression of TRIF resulted in the detection of a faint band, this suggests that the quantity of TRIF present may not have been sufficient to detect any interaction, however, a higher concentration could not be used as optimisation experiments using higher concentrations of TRIF resulted in cell death. Thus it remains to be fully clarified whether FADD and TRIF interact directly.

As we had observed that FADD over expression inhibited at the level of the receptors for poly I:C and also at the level of the adapter TRIF but were unable to pinpoint where on the pathway this inhibitory effect may be occurring we next examined the ability of the Fas/FADD pathway to modify the phosphorylation status of downstream signalling components common to all poly I:C receptors. We demonstrated that poly I:C-induced phosphorylation of p44/42, IκBα, JNK and p38 MAPK, with activation of Fas reducing the phosphorylation of JNK and p38 MAPK. IκBα is a regulatory protein which sequesters NFκB in the cytoplasm, therefore limiting the transcriptional activity of NFκB. However, upon activation, IκBα becomes phosphorylated, releasing its hold on NFκB, allowing NFκB to migrate to the nucleus, inducing its transcriptional activity. Given our earlier findings that overexpression of FADD did not alter RIP-1-induced IP-10 luciferase activity, and the important role of RIP-1 in activating NFκB, it was not surprising that Fas activation did not alter the phosphorylation status of IκBα. JNK and p38 MAPK are known to be involved in the activation of the AP-1 transcription factor. AP-1 is composed of the sub-families c-Jun and c-Fos, as well as the Maf (musculoaponeurotic fibrosarcoma) and ATF (activating transcription factor)

subfamilies, all of which are dimeric basic region-leucine zipper (bZIP) proteins (203). c-Fos and c-Jun are the main AP-1 proteins, with c-Fos proteins requiring c-Jun proteins to form stable heterodimers as they are not capable of forming homodimers (203). We have demonstrated that mutation of the AP-1 binding site augmented IP-10 transcription, indicating that AP-1 inhibits the IP-10 promoter. This finding may explain our data concerning opposing roles of FADD on the same poly I:C pathway. It would seem that it is not that FADD is exerting a direct inhibitory effect on IP-10, but rather that it is somewhat specifically modulating the AP-1 transcription factor, with the AP-1 transcription factor in turn repressing the IP-10 promoter. Thus, the ability of CH11 to reduce poly I:C-induced phosphorylation of JNK and p38 MAPK may result in a reduction in the level of AP-1 binding to the IP-10 promoter, thus alleviating the AP-1-mediated repression of IP-10. Consistent with this, a similar inhibitory role for AP-1 on the IP-10 promoter was recently shown in hepatocytes following Hepatitis C (HCV) infection (204). In this study, poly I:C-induced IP-10 production was reduced upon point mutation in the κB1, κB2 and ISRE binding sites of the IP-10 promoter, with a point mutation in the AP-1 binding site enhancing IP-10 production. Although this study did not investigate the role of Fas during HCV infection, hepatocytes are known to express Fas (205) and thus the system that we have identified here suggests that activation of the Fas signalling pathway in hepatocytes during HCV infection may play an important role in the induction of IP-10 in these cells.

As we had shown that Fas can reduce phosphorylation of poly I:C-induced p38 and JNK MAPK and that AP-1 is inhibitory to poly I:C-induced IP-10, we wished to link these findings by identifying that the Fas/FADD system could directly modify AP-1. Here we have shown that overexpression of FADD activates an AP-1 luciferase

reporter construct. Thus, our working hypothesis is that activation of Fas recruits FADD to Fas, limiting the levels of FADD available in the cytoplasm. This would reduce the inhibitory effect of FADD on p38 and JNK, reducing AP-1 activation and alleviating AP-1 mediated repression of IP-10. While our data supports this working hypothesis further work is required to make a definitive conclusion. In order to investigate this hypothesis fully, electrophoretic mobility shift assay (EMSA) could be performed on THP1 macrophages treated with poly I:C with or without CH11 stimulation, with interaction between AP-1 and its binding site on the IP-10 promoter examined. Based on our data to date, we would expect to find less AP-1 bound to IP-10 when cells were stimulated with poly I:C and CH11. Chromatin immunoprecipitation assay (ChIP) could also be performed. For ChIP analysis, a specific antibody against AP-1 could be used to immunoprecipitate the AP-1/IP-10 complex, with IP-10 expression measured by qRT-PCR. Based on our findings, we would expect to see reduced expression of IP-10 on qRT-PCR analysis in cells stimulated with both poly I:C and CH11, compared to poly I:C alone. Also whilst we and others have shown that FADD is recruited to Fas upon stimulation with CH11 (114, 206), we have not shown that this substantially decreases levels of FADD in the cytoplasm of the cell such that the ability of signalling pathways that utilise FADD would be affected. Although experiments such as these were not performed due to time constraints, they would have strengthened the working hypothesis of the potential mechanism outlined here for augmented poly I:C-induced IP-10 production by Fas activation (figure 4.13).

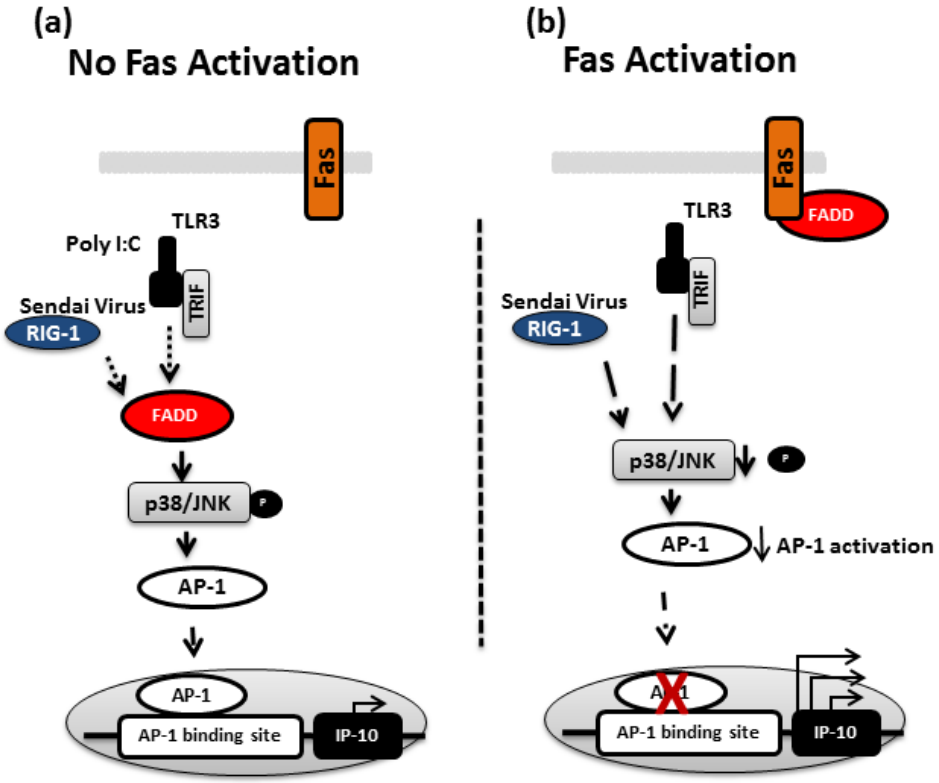

Figure 4.13: Schematic of mechanism whereby Fas activation may enhance poly I:C-induced IP-10. In the absence of Fas engagement, poly I:C, acting through both TLR3 and RIG-I, cause the phosphorylation of JNK and p38 MAPK in a FADD dependant manner. This results in AP-1 activation and translocation of AP-1 to the nucleus, where it acts to repress IP-10 production. Dashed lines indicate that the precise position of FADD in this pathway is unknown **(a)**. Upon Fas activation, FADD is recruited to Fas, reducing poly I:C-induced JNK and p38 MAPK activation. This reduces the levels of AP-1, thereby alleviating AP-1-mediated repression of the IP-10 promoter, resulting in enhanced IP-10 production **(b)**.

In conclusion, we demonstrate here a role for Fas in modulating poly I:C-induced cytokine production. These studies show that ligation of Fas specifically enhances IP-10 through modulation of p38 and JNK MAP kinases and the AP-1 transcription factor. It is not clear why AP-1 negatively regulates IP-10 and not the other cytokines examined here. This study points to a level of complexity present in the IP-10 promoter whereby levels of IP-10 are tightly regulated in the anti-viral immune response. It may be that there is a requirement for a second activating signal such as Fas ligation, ensuring that the promoter is optimally induced during active infection.

7. References

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Appendix A

Table A.1: FADD-protein interactions isolated from Mass spectrometry analysis for sample 1, untreated THP-1 macrophages.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P13796	Plastin-2 OS=Homo sapiens GN=LCP1 PE=1 SV=6 - [PLSL_HUMAN]	1689.75	51.83	3	26	26	50	627	70.2	5.43
B4DNE0	cDNA FLJ52573, highly similar to Elongation factor 1-alpha 1 OS=Homo sapiens PE=2 SV=1 - [B4DNE0_HUMAN]	1470.06	14.94	14	4	4	44	395	42.6	9.01
P02545-2	Isoform C of Prelamin-A/C n=2 Tax=Homo sapiens RepID=P02545-2	837.86	33.57	4	19	19	25	572	65.1	6.84
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	584.47	31.46	1	11	14	16	639	65.4	8.00
B4DNW7	Adenylyl cyclase-associated protein OS=Homo sapiens PE=2 SV=1 - [B4DNW7_HUMAN]	486.64	23.09	5	9	9	14	433	47.4	8.22
P14317	Hematopoietic lineage cell-specific protein OS=Homo sapiens GN=HCLS1 PE=1 SV=3 - [HCLS1_HUMAN]	442.58	17.49	1	7	7	13	486	54.0	4.81
B3KVX6	cDNA FLJ41699 fis, clone HCHON2004776, highly similar to Homo sapiens cytoskeleton-associated protein 4 (CKAP4), mRNA OS=Homo sapiens PE=2 SV=1 - [B3KVX6_HUMAN]	384.95	21.31	4	8	8	10	521	58.1	5.25
P12314	High affinity immunoglobulin gamma Fc receptor I OS=Homo sapiens GN=FCGR1A PE=1 SV=2 - [FCGR1_HUMAN]	373.79	13.37	1	6	6	13	374	42.6	7.97
P08107-2	Isoform 2 of Heat shock 70 kDa protein 1A/1B n=1 Tax=Homo sapiens RepID=P08107-2	359.84	16.04	4	6	8	9	586	63.9	5.71
Q6ZNL4	FLJ00279 protein (Fragment) OS=Homo sapiens GN=FLJ00279 PE=2 SV=1 - [Q6ZNL4_HUMAN]	275.01	15.63	3	7	7	7	563	65.7	8.85
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	266.60	14.07	1	6	8	8	590	62.3	7.74
Q5DT20	Hornerin OS=Homo sapiens GN=HRNR PE=2 SV=1 - [Q5DT20_HUMAN]	265.86	10.95	2	7	7	8	2850	282.2	10.02
E9PKE3	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=2 SV=1 - [E9PKE3_HUMAN]	263.26	12.92	3	5	7	8	627	68.8	5.52
F5H0T1	Stress-induced-phosphoprotein 1 OS=Homo sapiens GN=STIP1 PE=2 SV=1 - [F5H0T1_HUMAN]	225.30	14.45	5	6	6	7	519	59.7	6.80
B4DEK4	Sorting nexin-2 OS=Homo sapiens GN=SNX2 PE=2 SV=1 - [B4DEK4_HUMAN]	221.82	17.66	6	6	6	6	402	46.1	7.24

B4DNK4	Pyruvate kinase OS=Homo sapiens PE=2 SV=1 - [B4DNK4_HUMAN]	216.06	15.54	2	5	5	6	457	49.9	7.83
P20700	Lamin-B1 OS=Homo sapiens GN=LMNB1 PE=1 SV=2 - [LMNB1_HUMAN]	207.87	15.19	1	6	6	6	586	66.4	5.16
Q8IUK7	ALB protein OS=Homo sapiens PE=2 SV=1 - [Q8IUK7_HUMAN]	207.83	6.06	13	3	3	6	396	45.1	6.10
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	184.53	14.41	1	4	5	5	472	51.5	5.16
Q59GL1	Synaptotagmin binding, cytoplasmic RNA interacting protein variant (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q59GL1_HUMAN]	165.64	10.11	1	5	5	5	534	59.6	8.73
B4DLW8	Probable ATP-dependent RNA helicase DDX5 OS=Homo sapiens GN=DDX5 PE=2 SV=1 - [B4DLW8_HUMAN]	147.76	11.03	6	5	5	5	535	60.5	9.07
P21589-2	Isoform 2 of 5'-nucleotidase n=1 Tax=Homo sapiens RepID=P21589-2	143.75	7.82	5	3	3	3	524	57.9	6.98
Q03252	Lamin-B2 OS=Homo sapiens GN=LMNB2 PE=1 SV=3 - [LMNB2_HUMAN]	138.11	5.83	2	3	3	3	600	67.6	5.35
Q9Y3Z3-4	Isoform 4 of Deoxynucleoside triphosphate triphosphohydrolase SAMHD1 n=1 Tax=Homo sapiens RepID=Q9Y3Z3-4	133.21	8.80	3	5	5	5	591	68.1	6.90
Q8TD55	Pleckstrin homology domain-containing family O member 2 OS=Homo sapiens GN=PLEKHO2 PE=2 SV=1 - [PKHO2_HUMAN]	107.35	7.14	1	3	3	3	490	53.3	5.43
Q16555	Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1 - [DPYL2_HUMAN]	103.56	6.82	3	3	3	3	572	62.3	6.38
P23786	Carnitine O-palmitoyltransferase 2, mitochondrial OS=Homo sapiens GN=CPT2 PE=1 SV=2 - [CPT2_HUMAN]	92.94	3.34	1	2	2	2	658	73.7	8.18
B7Z2F4	T-complex protein 1 subunit delta OS=Homo sapiens GN=CCT4 PE=2 SV=1 - [B7Z2F4_HUMAN]	86.89	6.94	5	2	2	2	389	42.3	7.61
F5GZM7	Tyrosine-protein phosphatase non-receptor type 6 OS=Homo sapiens GN=PTPN6 PE=2 SV=1 - [F5GZM7_HUMAN]	84.53	24.77	12	2	2	2	109	12.4	6.93
Q8WWJ8	Integrin beta OS=Homo sapiens PE=2 SV=1 - [Q8WWJ8_HUMAN]	83.22	5.82	7	2	2	2	378	41.8	6.61
H0YMM1	Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=2 SV=1 - [H0YMM1_HUMAN]	79.67	16.11	7	2	2	2	149	16.4	5.91

B4DDC8	cDNA FLJ57252, highly similar to Protein phosphatase 2C isoform gamma (EC 3.1.3.16) OS=Homo sapiens PE=2 SV=1 - [B4DDC8_HUMAN]	79.64	4.47	4	2	2	2	515	56.1	4.34
Q70T18	BBF2H7/FUS protein (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q70T18_HUMAN]	76.12	14.65	13	2	2	2	157	16.1	9.00
Q9H3P7	Golgi resident protein GCP60 OS=Homo sapiens GN=ACBD3 PE=1 SV=4 - [GCP60_HUMAN]	74.24	7.58	1	2	2	2	528	60.6	5.06
Q5T0H9	Gelsolin OS=Homo sapiens GN=GSN PE=2 SV=1 - [Q5T0H9_HUMAN]	74.11	6.19	10	2	2	2	485	52.3	5.34
B3KTA3	Fascin OS=Homo sapiens PE=2 SV=1 - [B3KTA3_HUMAN]	70.71	4.66	4	2	2	2	472	52.2	7.65
B3KRK8	cDNA FLJ34494 fis, clone HLUNG2005030, highly similar to VIMENTIN OS=Homo sapiens PE=2 SV=1 - [B3KRK8_HUMAN]	63.11	5.90	4	2	2	2	407	46.9	5.00
Q6PK50	HSP90AB1 protein (Fragment) OS=Homo sapiens GN=HSP90AB1 PE=2 SV=1 - [Q6PK50_HUMAN]	62.97	13.11	3	2	2	2	351	40.3	4.97
Q9H834	cDNA FLJ13966 fis, clone Y79AA1001394, weakly similar to CELL DIVISION PROTEIN FTSH HOMOLOG (EC 3.4.24.-) OS=Homo sapiens PE=2 SV=1 - [Q9H834_HUMAN]	55.36	3.33	7	2	2	2	480	53.5	9.45

Table A.2: FADD-protein interactions isolated from Mass spectrometry analysis for sample 2, THP-1 macrophages treated with 200ng/ml CH11.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
Q1KLZ0	HCG15971, isoform CRA_a OS=Homo sapiens GN=PS1TP5BP1 PE=2 SV=1 - [Q1KLZ0_HUMAN]	5898.81	67.73	4	1	23	184	375	41.7	5.48
P63261	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1 PE=1 SV=1 - [ACTG_HUMAN]	5847.44	68.00	1	2	24	183	375	41.8	5.48
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	1230.07	15.96	1	1	8	37	564	60.0	8.00
Q6KB66-2	Isoform 2 of Keratin, type II cytoskeletal 80 n=1 Tax=Homo sapiens RepID=Q6KB66-2	1162.88	7.82	3	2	3	29	422	47.2	5.30
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	924.93	28.14	1	10	15	28	590	62.3	7.74
Q5DT20	Hornerin OS=Homo sapiens GN=HRNR PE=2 SV=1 - [Q5DT20_HUMAN]	671.14	17.40	2	13	13	22	2850	282.2	10.02
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	662.59	36.86	1	7	13	19	472	51.5	5.16
Q5HYL6	Putative uncharacterized protein DKFZp686E1899 OS=Homo sapiens GN=DKFZp686E1899 PE=2 SV=1 - [Q5HYL6_HUMAN]	525.29	30.40	2	9	9	13	352	39.5	5.19
Q6ZNL4	FLJ00279 protein (Fragment) OS=Homo sapiens GN=FLJ00279 PE=2 SV=1 - [Q6ZNL4_HUMAN]	486.63	19.89	3	8	8	12	563	65.7	8.85
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	467.73	6.20	1	14	14	15	2871	331.6	6.81
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	396.32	20.93	1	3	9	12	473	51.2	5.05
P04075	Fructose-bisphosphate aldolase A OS=Homo sapiens GN=ALDOA PE=1 SV=2 - [ALDOA_HUMAN]	365.10	35.71	1	9	9	11	364	39.4	8.09
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 - [DSG1_HUMAN]	356.17	7.91	1	6	6	8	1049	113.7	5.03
F2XI28	MHC class I antigen (Fragment) OS=Homo sapiens GN=HLA-A PE=3 SV=1 - [F2XI28_HUMAN]	313.14	32.23	1	3	7	8	273	31.6	6.47
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN]	287.05	9.80	1	6	6	8	745	81.7	6.14
P61160	Actin-related protein 2 OS=Homo sapiens GN=ACTR2 PE=1 SV=1 - [ARP2_HUMAN]	228.43	14.97	1	5	5	7	394	44.7	6.74
B4DU58	cDNA FLJ51488, highly similar to Macrophage capping protein OS=Homo sapiens PE=2 SV=1 - [B4DU58_HUMAN]	225.87	25.38	3	7	7	7	327	36.2	6.25
Q6YHK3-2	Isoform 2 of CD109 antigen n=1 Tax=Homo sapiens RepID=Q6YHK3-2	206.14	3.51	3	4	4	5	1368	152.6	5.64
Q08188	Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3 PE=1 SV=4 - [TGM3_HUMAN]	64.15	3.90	1	2	2	2	693	76.6	5.86

H0YKS4	Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=3 SV=1 - [H0YKS4_HUMAN]	149.14	31.82	5	5	5	5	176	19.5	5.96
H2AM05	MHC class I antigen (Fragment) OS=Homo sapiens GN=HLA-A PE=3 SV=1 - [H2AM05_HUMAN]	146.79	16.48	1	1	5	6	273	31.4	5.67
P24752	Acetyl-CoA acetyltransferase, mitochondrial OS=Homo sapiens GN=ACAT1 PE=1 SV=1 - [THIL_HUMAN]	145.77	15.69	1	4	4	4	427	45.2	8.85
D6RG15	Twinfilin-2 OS=Homo sapiens GN=TWF2 PE=2 SV=1 - [D6RG15_HUMAN]	141.51	18.11	2	3	3	4	254	28.9	6.13
E7EUT5	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens GN=GAPDH PE=2 SV=1 - [E7EUT5_HUMAN]	136.83	16.54	3	3	3	4	260	27.9	6.95
Q8N1N4	Keratin, type II cytoskeletal 78 OS=Homo sapiens GN=KRT78 PE=2 SV=2 - [K2C78_HUMAN]	135.59	8.27	1	4	4	4	520	56.8	6.02
P08567	Pleckstrin OS=Homo sapiens GN=PLEK PE=1 SV=3 - [PLEK_HUMAN]	124.02	8.29	1	2	2	3	350	40.1	8.28
H0YJC0	26S protease regulatory subunit 10B (Fragment) OS=Homo sapiens GN=PSMC6 PE=4 SV=1 - [H0YJC0_HUMAN]	112.73	15.27	2	3	3	3	262	29.6	6.40
P05089	Arginase-1 OS=Homo sapiens GN=ARG1 PE=1 SV=2 - [ARGI1_HUMAN]	112.65	9.32	2	2	2	2	322	34.7	7.21
B4DNY3	Adenylyl cyclase-associated protein OS=Homo sapiens PE=2 SV=1 - [B4DNY3_HUMAN]	107.25	10.97	7	3	3	3	401	43.7	8.54
Q9Y371	Endophilin-B1 OS=Homo sapiens GN=SH3GLB1 PE=1 SV=1 - [SHLB1_HUMAN]	104.86	7.67	2	2	2	2	365	40.8	6.04
Q93020	GTP-binding regulatory protein Gi alpha-2 chain (Fragment) OS=Homo sapiens GN=WUGSC:H_LUCA16.1 PE=2 SV=2 - [Q93020_HUMAN]	97.33	21.83	3	3	3	3	197	22.1	5.49
P04040	Catalase OS=Homo sapiens GN=CAT PE=1 SV=3 - [CATA_HUMAN]	96.47	4.55	1	2	2	3	527	59.7	7.39
B3KUZ8	Aspartate aminotransferase OS=Homo sapiens PE=2 SV=1 - [B3KUZ8_HUMAN]	95.19	7.01	4	2	2	2	371	41.3	8.84
A8K0T9	cDNA FLJ75422, highly similar to Homo sapiens capping protein (actin filament) muscle Z-line, alpha 1, mRNA OS=Homo sapiens PE=2 SV=1 - [A8K0T9_HUMAN]	93.54	9.09	2	2	2	2	286	32.9	5.69
B4DLC0	Poly(rC)-binding protein 2 OS=Homo sapiens GN=PCBP2 PE=2 SV=1 - [B4DLC0_HUMAN]	88.38	8.97	2	2	2	3	301	32.1	7.90
Q9HB00	Desmocollin 1, isoform CRA_b OS=Homo sapiens GN=DSC1 PE=2 SV=1 - [Q9HB00_HUMAN]	85.80	2.86	2	2	2	3	840	93.8	5.53
E7DVW5	Fatty acid binding protein 5 (Psoriasis-associated) OS=Homo sapiens GN=FABP5 PE=2 SV=1 - [E7DVW5_HUMAN]	79.22	13.33	1	2	2	2	135	15.2	7.01

Table A.3: FADD-protein interactions isolated from Mass spectrometry analysis for sample 3, THP-1 macrophages treated with 200ng/ml CH11.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
B2RDE1	cDNA, FLJ96568, highly similar to Homo sapiens tropomyosin 3 (TPM3), mRNA OS=Homo sapiens PE=2 SV=1 - [B2RDE1_HUMAN]	1835.02	62.10	1	13	22	65	248	29.0	4.75
P67936	Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3 - [TPM4_HUMAN]	1196.13	54.03	1	11	19	44	248	28.5	4.69
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	903.90	37.92	1	7	16	27	472	51.5	5.16
Q5DT20	Hornerin OS=Homo sapiens GN=HRNR PE=2 SV=1 - [Q5DT20_HUMAN]	847.20	16.04	2	14	14	31	2850	282.2	10.02
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	830.51	35.52	1	9	16	27	473	51.2	5.05
P04259	Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5 - [K2C6B_HUMAN]	710.30	18.44	1	1	10	24	564	60.0	8.00
B4DRR0	cDNA FLJ53910, highly similar to Keratin, type II cytoskeletal 6A OS=Homo sapiens PE=2 SV=1 - [B4DRR0_HUMAN]	514.37	22.43	3	2	11	18	535	57.8	8.00
B7Z722	Tropomyosin 1 (Alpha), isoform CRA_i OS=Homo sapiens GN=TPM1 PE=2 SV=1 - [B7Z722_HUMAN]	472.50	32.26	2	3	11	19	248	28.6	4.79
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	379.48	4.14	1	10	10	13	2871	331.6	6.81
P62258	14-3-3 protein epsilon OS=Homo sapiens GN=YWHAE PE=1 SV=1 - [1433E_HUMAN]	310.99	47.45	1	8	10	12	255	29.2	4.74
Q04695	Keratin, type I cytoskeletal 17 OS=Homo sapiens GN=KRT17 PE=1 SV=2 - [K1C17_HUMAN]	300.68	24.07	1	2	10	10	432	48.1	5.02
F5GWP8	Junction plakoglobin OS=Homo sapiens GN=JUP PE=2 SV=1 - [F5GWP8_HUMAN]	292.11	16.92	1	2	8	9	591	66.3	5.19
B4DVQ0	cDNA FLJ58286, highly similar to Actin, cytoplasmic 2 OS=Homo sapiens PE=2 SV=1 - [B4DVQ0_HUMAN]	270.45	27.33	3	7	7	10	333	37.3	5.71
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 - [DSG1_HUMAN]	234.23	6.39	1	4	4	5	1049	113.7	5.03
P00491	Purine nucleoside phosphorylase OS=Homo sapiens GN=PNP PE=1 SV=2 - [PNPH_HUMAN]	218.81	24.22	2	5	5	6	289	32.1	6.95
Q53FB0	Chloride intracellular channel 1 variant (Fragment) OS=Homo sapiens PE=2 SV=1 - [Q53FB0_HUMAN]	200.54	39.83	2	5	5	6	241	27.0	5.17
Q96C19	EF-hand domain-containing protein D2 OS=Homo sapiens GN=EFHD2 PE=1 SV=1 - [EFHD2_HUMAN]	199.71	34.58	1	7	7	8	240	26.7	5.20
Q06323	Proteasome activator complex subunit 1 OS=Homo sapiens GN=PSME1 PE=1 SV=1 - [PSME1_HUMAN]	139.03	20.88	2	5	5	5	249	28.7	6.02

D0PNI1	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide OS=Homo sapiens GN=YWHAZ PE=2 SV=1 - [D0PNI1_HUMAN]	120.89	20.00	1	2	4	4	245	27.7	4.79
P24534	Elongation factor 1-beta OS=Homo sapiens GN=EEF1B2 PE=1 SV=3 - [EF1B_HUMAN]	117.30	12.44	1	2	2	2	225	24.7	4.67
B4DQ50	cDNA FLJ56823, highly similar to Protein-glutamine gamma-glutamyltransferase E (EC 2.3.2.13) OS=Homo sapiens PE=2 SV=1 - [B4DQ50_HUMAN]	96.66	6.00	1	2	2	2	533	58.8	7.15
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	91.06	22.73	2	2	2	3	110	11.3	6.54
H0YMM1	Annexin (Fragment) OS=Homo sapiens GN=ANXA2 PE=2 SV=1 - [H0YMM1_HUMAN]	85.34	16.11	7	2	2	2	149	16.4	5.91
Q5D862	Filaggrin-2 OS=Homo sapiens GN=FLG2 PE=1 SV=1 - [FILA2_HUMAN]	83.90	2.09	1	3	3	3	2391	247.9	8.31
C9JZ20	Prohibitin (Fragment) OS=Homo sapiens GN=PHB PE=2 SV=1 - [C9JZ20_HUMAN]	79.74	10.95	8	2	2	2	201	22.3	5.96
P04040	Catalase OS=Homo sapiens GN=CAT PE=1 SV=3 - [CATA_HUMAN]	74.48	6.83	1	2	2	2	527	59.7	7.39
Q6ZNL4	FLJ00279 protein (Fragment) OS=Homo sapiens GN=FLJ00279 PE=2 SV=1 - [Q6ZNL4_HUMAN]	72.29	4.26	3	2	2	2	563	65.7	8.85
P29508-2	Isoform 2 of Serpin B3 n=1 Tax=Homo sapiens RepID=P29508-2	68.77	9.17	3	2	2	2	338	38.5	6.74
B1AHF3	NADH-cytochrome b5 reductase 3 membrane-bound form (Fragment) OS=Homo sapiens GN=CYB5R3 PE=2 SV=1 - [B1AHF3_HUMAN]	64.39	16.33	3	2	2	2	147	16.7	9.73
P00918	Carbonic anhydrase 2 OS=Homo sapiens GN=CA2 PE=1 SV=2 - [CAH2_HUMAN]	59.42	9.62	1	2	2	2	260	29.2	7.40
Q15404-2	Isoform 2 of Ras suppressor protein 1 n=2 Tax=Homo sapiens RepID=Q15404-2	52.30	7.14	4	2	2	2	224	25.5	8.72

Table A.4: FADD-protein interactions isolated from Mass spectrometry analysis for sample 4, THP-1 macrophages treated with 200ng/ml CH11.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
F8W1R7	Myosin light polypeptide 6 OS=Homo sapiens GN=MYL6 PE=2 SV=1 - [F8W1R7_HUMAN]	3092.29	57.93	9	9	9	92	145	16.3	4.65
H6VRF8	Keratin 1 OS=Homo sapiens GN=KRT1 PE=3 SV=1 - [H6VRF8_HUMAN]	3049.76	45.81	6	26	30	91	644	66.0	8.12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	1622.25	46.40	1	19	24	42	584	58.8	5.21
B4DNE0	cDNA FLJ52573, highly similar to Elongation factor 1-alpha 1 OS=Homo sapiens PE=2 SV=1 - [B4DNE0_HUMAN]	1451.44	17.22	14	4	4	43	395	42.6	9.01
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	1332.51	43.82	1	19	19	39	623	62.0	5.24
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	842.03	43.04	1	14	21	25	639	65.4	8.00
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	680.47	32.42	1	6	13	20	472	51.5	5.16
P08779	Keratin, type I cytoskeletal 16 OS=Homo sapiens GN=KRT16 PE=1 SV=4 - [K1C16_HUMAN]	610.11	32.35	1	7	14	20	473	51.2	5.05
Q6KB66-2	Isoform 2 of Keratin, type II cytoskeletal 80 n=1 Tax=Homo sapiens RepID=Q6KB66-2	546.55	7.82	3	2	3	14	422	47.2	5.30
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	482.26	20.68	1	8	12	15	590	62.3	7.74
B4DRR0	cDNA FLJ53910, highly similar to Keratin, type II cytoskeletal 6A OS=Homo sapiens PE=2 SV=1 - [B4DRR0_HUMAN]	409.54	23.18	3	6	12	13	535	57.8	8.00
A1A4E9	Keratin 13 OS=Homo sapiens GN=KRT13 PE=2 SV=1 - [A1A4E9_HUMAN]	394.16	19.21	2	4	8	13	458	49.6	4.96
P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	343.90	4.11	1	10	10	12	2871	331.6	6.81
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN]	287.68	9.93	1	6	6	7	745	81.7	6.14
P07737	Profilin-1 OS=Homo sapiens GN=PFN1 PE=1 SV=2 - [PROF1_HUMAN]	260.85	47.14	1	5	5	7	140	15.0	8.27
A4D1U3	Single-stranded DNA-binding protein OS=Homo sapiens GN=SSBP1 PE=2 SV=1 - [A4D1U3_HUMAN]	230.52	37.84	2	4	4	6	148	17.2	9.60
Q5DT20	Hornerin OS=Homo sapiens GN=HRNR PE=2 SV=1 - [Q5DT20_HUMAN]	211.01	8.49	2	5	5	6	2850	282.2	10.02
Q6ZNL4	FLJ00279 protein (Fragment) OS=Homo sapiens GN=FLJ00279 PE=2 SV=1 - [Q6ZNL4_HUMAN]	196.58	6.22	3	3	3	5	563	65.7	8.85
Q01546	Keratin, type II cytoskeletal 2 oral OS=Homo sapiens GN=KRT76 PE=1 SV=2 - [K22O_HUMAN]	194.23	7.68	1	2	5	6	638	65.8	8.12
B4DVQ0	cDNA FLJ58286, highly similar to Actin, cytoplasmic 2 OS=Homo sapiens PE=2 SV=1 - [B4DVQ0_HUMAN]	188.71	14.11	9	4	4	5	333	37.3	5.71

UPI00017BDB3D	FabOX108 Light Chain Fragment n=1 Tax=Homo sapiens RepID=UPI00017BDB3D	165.60	21.20	1	3	3	4	217	23.8	6.67
O15511	Actin-related protein 2/3 complex subunit 5 OS=Homo sapiens GN=ARPC5 PE=1 SV=3 - [ARPC5_HUMAN]	149.08	21.19	1	3	3	4	151	16.3	5.67
B8ZZQ6	Thymosin alpha-1 OS=Homo sapiens GN=PTMA PE=4 SV=1 - [B8ZZQ6_HUMAN]	142.71	26.17	7	3	3	3	107	11.8	3.81
A3KPC7	Histone H2A OS=Homo sapiens GN=HIST1H2AH PE=2 SV=1 - [A3KPC7_HUMAN]	137.16	27.34	11	3	3	8	128	13.9	10.89
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 - [DSG1_HUMAN]	131.84	4.29	1	3	3	3	1049	113.7	5.03
E7DVW5	Fatty acid binding protein 5 (Psoriasis-associated) OS=Homo sapiens GN=FABP5 PE=2 SV=1 - [E7DVW5_HUMAN]	110.32	20.00	1	3	3	4	135	15.2	7.01
O75348	V-type proton ATPase subunit G 1 OS=Homo sapiens GN=ATP6V1G1 PE=1 SV=3 - [VATG1_HUMAN]	103.17	22.03	1	2	2	2	118	13.7	8.79
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	101.70	20.00	2	2	2	3	110	11.3	6.54
Q08188	Protein-glutamine gamma-glutamyltransferase E OS=Homo sapiens GN=TGM3 PE=1 SV=4 - [TGM3_HUMAN]	101.39	3.90	1	2	2	3	693	76.6	5.86
P30049	ATP synthase subunit delta, mitochondrial OS=Homo sapiens GN=ATP5D PE=1 SV=2 - [ATPD_HUMAN]	84.62	13.69	1	2	2	2	168	17.5	5.49
B4DRV9	Glyceraldehyde-3-phosphate dehydrogenase OS=Homo sapiens PE=2 SV=1 - [B4DRV9_HUMAN]	73.21	10.23	3	2	2	2	215	23.2	8.54
P61956	Small ubiquitin-related modifier 2 OS=Homo sapiens GN=SUMO2 PE=1 SV=3 - [SUMO2_HUMAN]	72.69	18.95	4	2	2	2	95	10.9	5.50
P31025	Lipocalin-1 OS=Homo sapiens GN=LCN1 PE=1 SV=1 - [LCN1_HUMAN]	64.37	12.50	1	2	2	2	176	19.2	5.58
P13073	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial OS=Homo sapiens GN=COX4I1 PE=1 SV=1 - [COX41_HUMAN]	64.28	13.02	1	2	2	2	169	19.6	9.51
P62854	40S ribosomal protein S26 OS=Homo sapiens GN=RPS26 PE=1 SV=3 - [RS26_HUMAN]	59.10	20.87	2	2	2	2	115	13.0	11.00