Investigating the Role of TLR8 in Inflammatory and Antiviral Responses in Human Polymorphonuclear Leukocytes (PMNs)

School of Biochemistry and Immunology
Trinity College Dublin

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Declaration of Authorship

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Richard Wubben
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Related Publications and Presentations

*Poster Presentations*

Oral presentations


Publications

- R. Wubben, C. Efstathiou and N.J., Stevenson *Hormones, Regulators and Viruses, The interplay between the Immune system and viruses*. August 20, 2021
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<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
</tr>
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<td>AIM2</td>
<td>Absent in Melanoma 2</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>ARDS</td>
<td>Acute Respiratory Distress syndrome</td>
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<td>C-Type Lectin Receptor</td>
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<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
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<td>Myeloid differentiation primary response 88</td>
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<td>Normal density granulocyte</td>
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<td>NET</td>
<td>Neutrophil Extracellular Traps</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B</td>
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<td>Polymorphonuclear Neutrophils</td>
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<td>PRR</td>
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<td>RIG-I-like receptors</td>
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<td>ROS</td>
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<td>Respiratory Syncytial Virus</td>
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<td>SARM</td>
<td>Sterile α-motif-containing and armadillo-motif containing protein</td>
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<td>SIP</td>
<td>Severe influenza pneumoniae</td>
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<td>SOCS</td>
<td>Suppressor of Cytokine Signalling</td>
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<tr>
<td>ssRNA</td>
<td>Single Stranded RNA</td>
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<td>Signal Transducer and Activator of Transcription</td>
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Abstract

Neutrophils are key phagocytes that migrate to the site of infection, exerting multiple anti-microbial and pro-inflammatory effects. These effector functions include the production of reactive oxygen species, alarmins, cytokines, neutrophil extracellular traps and phagocytosis. While their anti-bacterial and anti-fungal functions are well known, their anti-viral effects remain poorly understood. Single stranded (ssRNA) viruses, including respiratory syncytial virus (RSV), are associated with acute respiratory distress syndrome (ARDS), commonly correlated with a massive infiltration of neutrophils into the respiratory tract. It also remains unclear which neutrophilic intracellular signalling pathways contribute to viral control. While their anti-pathogenic mechanisms are key to the immune response, neutrophil responses are tightly regulated, thus avoiding tissue damage from their plethora of secreted inflammatory mediators. Innate immune cells utilise an array of receptors that regulate the production of inflammatory and antiviral cytokines. Of these, Toll-like Receptors (TLRs), in particular the endosomal TLR8 is important in the recognition of ssRNA viruses, such as RSV and and Human Immunodeficiency Virus (HIV); with studies been carried out in the human embryonic kidney (HEK) cell line and primary human CD4+ T-cells, respectively. While these studies have revealed novel roles for TLR8 in cell lines and adaptive immune cells, very little is known about primary human neutrophils. We hypothesised that TLR8 in primary human PMNs regulates the expression of inflammatory mediators and antiviral responses in response to ssRNA viruses, including HIV and RSV, and is therefore the focus of this project.

We found that TLR8 was a functional endosomal receptor in primary human PMNs, that participates in the induction of both inflammatory and antiviral responses, characterised by the production of IL-6, TNF, IL-1β and IL-8 and antiviral ISGs. We then sought to determine the intracellular signalling pathways responsible for these inflammatory and antiviral mediators once TLR8 was activated. Using an array of inhibitors we identified that downstream of TLR8 the NF-κB and MAPK pathways were responsible for the induction of the
inflammatory cytokines. Indeed, inhibition of TLR8 signalling in primary human PMNs inhibited their production of IL-8 in response to RSV, revealing a novel mechanism by which a ssRNA virus is detected by PMNs, leading to an inflammatory response.

In addition to the regulation of inflammatory responses, the induction of type 1 IFN from plasmacytoid Dendritic Cells (pDCs), via viral stimulation of TLR7, has been well defined. These type 1 IFNs induce autocrine/paracrine anti-viral responses through their Interferon associated receptor (IFNAR), which triggers the induction of Interferon stimulated Genes (ISG) via JAK/STAT signalling, thus creating an intracellular anti-viral state. Additionally, Pattern recognition receptors (PRRs) have been shown to induce early expression of ISGs via an IFN-independent method. While the ability of ssRNA to induce similar responses in PMNs has not been extensively studied, these antiviral pathways have been studied in cell lines and innate immune cells such as monocytes, macrophages and DCs. We hypothesised that ssRNA viruses could also induce ISG expression in PMNs. To investigate this we analysed the role of TLR8 in inducing ISG expression in response to a synthetic agonist, CLO75 and live ssRNA viruses, HIV and RSV. We found that direct activation of TLR8 in PMNs by either CLO75 or ssRNA viruses, resulted in the early upregulation of the ISG, Viperin, well characterised for its ability to restrict viral replication.

Having observed that TLR8 was important in the direct induction of antiviral ISGs in PMNs, we further investigated if it also induced type 1 IFNs from PMNs. PMNs have been demonstrated to induce mRNA transcripts of IFN-α, which participate in the pathogenesis of Lupus. Since our lab has characterised the roles of various STAT proteins in the induction of antiviral responses downstream of IFNAR, we hypothesised that TLR8 activated PMNs and peripheral blood mononuclear cells (PBMCs) could secrete type 1 IFNs, which could subsequently trigger the phosphorylation of intracellular STAT proteins, leading to ISG induction. We found that TLR8 was the principal endosomal TLR in the production of PBMC-derived type 1 IFN and that IFN-α phosphorylated STAT proteins in PMNs. Additionally, we found that primary human PMNs also respond to type 1 IFNs via IRF1 and the
TLR8 agonist CL075 induces the expression of a plethora of anti-viral effector proteins such as Viperin.

This project is the first to identify a novel role for the endosomal receptor TLR8 in the induction of inflammatory and antiviral mediators in primary human PMNs in response to ssRNA viruses. These novel findings contribute to the overall understanding of the role neutrophils play in viral infection. They provide an exciting insight into how neutrophils regulate the inflammatory response, while simultaneously promoting an anti-viral response, thus providing new information which is useful in understanding the role of these innate immune cells during disease, such as acute respiratory distress; while also providing a new angle for the identification and development of anti-viral therapeutics. Indeed, our findings highlight that the regulation of these innate immune receptors, and subsequently induced proinflammatory and antiviral mediators, may be a more efficient way of regulating the pathology of ssRNA viral infections and important when considering future therapeutics that manage the viral inflammatory response, while simultaneously enhancing the antiviral response.
Chapter 1

General Introduction
Chapter 1: Introduction

The human immune response has traditionally been divided into two divisions: innate and adaptive immunity. The innate immune system consists of “hard-wired” responses encoded by host germline pattern recognition receptors (PRRs) that recognise ‘nonself’ microbial components (1). The adaptive response consists of gene elements that are somatically rearranged to assemble antigen-binding molecules with great specificity for individual foreign structures. In contrast to the adaptive immune system which depends upon T and B lymphocytes, innate immune protection is a task performed by cells of both hematopoietic and non-hematopoietic origin. Hematopoietic cells involved in innate immune responses include macrophages (MΦ), dendritic cells (DCs), mast cell, neutrophils, eosinophils, natural killer (NK) cells and natural killer T cells. Polymorphonuclear cells (PMNs) are defined as innate cells that contain granules and are the most abundant leukocyte in humans and essential to the innate immune response against invading pathogens (2). They consist of neutrophils, basophils and eosinophils, of which the neutrophil is the most common cell type. The induction of an adaptive immune response begins when a pathogen is ingested by an antigen presenting cell (APC), such as DCs in the infected tissue. DCs bridge the gap between first line innate responses and powerful adaptive immune responses, by internalising, processing and presenting antigens on MHC and MHC-like molecules to T-cells (3). In addition to the DCs presenting antigen, macrophages, B-cells and neutrophils can also present antigens (4). Neutrophils pulsed with antigens from cytomegalovirus and influenza were able to present their antigens to autologous antigen-specific
CD4+ T cells in a major histocompatibility complex class II (MHC-II; HLA-DR)-dependent manner (5)(6). While presenting antigen is a major function of certain innate immune cells, they also function in the immunomodulation of other adaptive cells through the secretion of chemokines and cytokines (7). These inflammatory chemokines and cytokines trigger an inflammatory response, increasing the flow of lymph containing antigen and antigen-bearing cells into lymphoid tissue.

1.1 The Innate immune response

Innate immunity is a primary defence system acquired at birth. It consists of primary barrier defences, such as the skin and the respiratory mucosa, which prevent entry of pathogens into the host body and a plethora of PRRs, such as Toll like receptors (TLRs), which serve as a secondary anti-microbial defence mechanism (8). Neutrophils are phagocytic white blood cells designed to engulf and degrade microbes through a host of antimicrobial mechanisms, including the production of reactive oxygen species and degradative enzymes, such as lysozyme and matrix metalloproteases. During the aforementioned process of engulfing and degrading pathogens, phagocytic cells, including neutrophils, are involved in the production of cytokines and chemokines into the surrounding tissue which initiates inflammation, which is a vital component of the innate immune response to invading viruses and bacteria (9).

1.2 Inflammation
Inflammation is recognised by the findings of Dolor (pain), Calor (Heat), Rubor (redness) and Tumor (swelling), due to blood vessel dilation and a subsequent increase in blood flow. Inflammation is characterised by several mechanisms including vasodilation, increased permeability of capillaries, chemotaxis/phagocytosis, coagulation and subsequently tissue repair and resolution of inflammation (10). Recognition of pathogens by epithelial cells triggers a rapid host response, characterised by the production of large quantities of proinflammatory cytokines and chemokines, resulting in the influx of granulocytes including neutrophils, basophils and eosinophils (11). PMNs produce inflammatory cytokines such as IL-8, IL-6, IL-1 and TNF and anti-inflammatory cytokines such as IL-4 and IL-10 (12). These cytokines trigger intracellular signalling pathways including Nuclear factor kappa B (NF-κB), Mitogen activated protein kinase (MAPK), activator protein-1 (AP-1) and Signal transducer and activator of transcription (STAT) signalling.

1.3 PAMP associated inflammation

1.3.1 TLR signalling pathways

Recognition of pathogen associated molecular patterns (PAMPs) by TLRs leads to the transcriptional upregulation of distinct genes, dependent on the downstream signalling cascades. The difference in the signaling cascades activated by the individual TLRs can be partly explained by the TIR domain-containing adaptor molecules recruited to TLRs. These include 5 distinct TIR domain containing adaptors: 1) Myd88; 2) TIR domain-containing adaptor inducing IFN-beta (TRIF; also known as TICAM-1); 3) TIRAP/Mal; 4) TRIF-related
adaptor molecule (TRAM), and 5) Sterile-alpha and Armadillo motif-containing protein (SARM) (13). While MyD88, TRIF, Mal and TRAM adaptors have been implicated in downstream signalling of TLRs, SARM negatively regulates TRIF activity (14).

All TLRs, except TLR3, signal via MyD88 and have therefore been coined MyD88-dependent, while TLR3 is considered MyD88-independent (TRIF dependent). The MyD88-dependent pathway leads to the activation of NF-κB, MAPK and IRF transcription factor proteins, leading to the production of cytokines, such as IL-6, TNF-α and IFN-α. PAMPs bind to the receptor ectodomain and lead to the dimerisation of the TIR domains, which act as a scaffold for downstream adaptor proteins (15). The engagement of the adaptor proteins with receptor dimers occurs through TIR-TIR interaction. MyD88 is made up of an N-terminal Death Domain (DD), an interdomain and C-terminal TIR domain. Upon stimulation of the MyD88-dependent pathway, IRAK4 is recruited to MyD88 through the interaction of both DDs. This mediates the phosphorylation of IRAK1 which in turn recruits IRAK2 and IRAK4 (Myddosome) (16). This complex disassociates from MyD88 and interacts with TNFR-associated factor 6 (TRAF6), which acts as an E3 ubiquitin protein ligase. Together with an E2 ubiquitin-conjugating enzyme complex comprising Ubc13 and Uev1A, TRAF6 catalyzes the formation of a lysine 63(K63)-linked polyubiquitin chain on TRAF6 itself as well as the generation of an unconjugated free polyubiquitin chain (17). This results in the activation of two unique signalling pathways: 1) AP1 transcription through the activation of MAP kinases and 2) activation of TAK1/TAB complex, which recruits the IκB kinase (IKK) complex. This in turn phosphorylates
IκB, which leads to NF-κB translocation into the nucleus (13). TLR7, TLR8 and TLR9 signalling induces the production of type I IFNs, in addition to other NF-κB-dependent cytokines in a MyD88-dependent manner. MyD88 forms a complex with IRAK-1, TRAF6, TRAF3, IKK-α, and IRF7 and phosphorylated IRF7 translocates to the nucleus to activate the expression of genes encoding type I IFNs (18).

The MyD88-independent pathway is unique to TLR3 and TLR4/TRAM signalling and requires the recruitment of TRIF (19). TRIF is responsible for initiating signalling and associates with TRAF3 and TRAF6 through its N-terminal portion. TRAF3 activates both Tank binding kinase (TBK1) and IKKe, which phosphorylate Interferon regulatory factor 3/7 (IRF3/7) subsequently promoting the upregulation of Type 1 IFNs (20).

### 1.3.2 Endosomal TLRs

With the exception of TLR3/7/8/9, all TLRs are expressed typically on the surface of the cellular membrane. The aforementioned TLRs are expressed on the endosome and are responsible for recognising viral danger signals that include dsRNA, ssRNA and hypomethylated dsDNA (Fig 1.1). In order to get successful endosomal recognition of viral PAMPs, there first needs to be successful translocation of the receptor from the endoplasmic reticulum (ER) to the endosomal compartment through the chaperone protein UNC93B1 (21). The signalling pathways between the endosomal TLRs are homologous, with TLR3 utilising TRIF and TLR7/8/9 utilising MyDD88, resulting in the upregulation of
signalling pathways such as NF-κB, IRF and MAPK. To date, endosomal TLR research has centered on investigating the role of TLR3, TLR7 and TLR9 in the induction of Type 1 IFNs in DCs, with variations in expression across models, rendering mice either more susceptible or resilient to disease severity, dependent on the disease model (22). This has led to the hypothesis that due to selective pressure, different endosomal TLRs result in distinguishable immunological responses in a tissue specific manner. These variations in expression have been highlighted using DCs with plasmacytoid DCs (pDCs) expressing high levels of TLR7/9 and myeloid DCs expressing high levels of TLR3 (23) (24).

Figure 1.1: TLR subcellular locations and associated agonists
Diagram of TLRs and their PAMPS. Lipoproteins bind cooperative clusters of TLR1/2/6. Bacterial lipopolysaccharide binds to the complex formed by CD14 and TLR4. TLR5 binds extracellular flagellin, a common protein found on flagellated bacteria. Intracellular TLRs include TLR3, TLR9, TLR7 and TLR8 that bind double stranded RNA, CpG DNA and ssRNA, respectively. Created with BioRender.com
While less studies have investigated the role TLR8 plays in infection, early investigations have demonstrated that human TLR8 is expressed on the endosome of monocytes and macrophages (25). TLR8 is an endosomal sensor of RNA degradation products, a common feature associated with ssRNA viruses such as HIV and RSV (Fig 1.2) (26). Additionally, studies have revealed the importance of TLR8 in the differentiation of naïve T-cells towards a TH17 phenotype through the production of IL-23 from innate immune cells (27). The impact of TLR8 upon infection has remained elusive due to the lack of small animal models and effective small molecule inhibitors to date. While it is widely accepted that bacterial RNA can induce the expression of type 1 IFNs via TLR7, new evidence has implicated TLR8 in the recognition of similar RNA products in monocytes and macrophages (28). Phagocytosis of *Mycobacterium Bovis* resulted in the upregulation of TLR8 in THP-1 cells, in which splice variants of TLR8 resulted in increased susceptibility to pulmonary tuberculosis. Additionally, phagocytosis of *Helicobacter pylori* and *Borrelia burgdorferi* resulted in the upregulation of IFN-β via exclusive activation of TLR8 in THP-1 cells (29) (30). Similarly, viral RNA products have been demonstrated to be instrumental in the induction of IFN-β via TLR8 induction of IRF7 (31). While traditional beliefs suggest that TLRs signal independently of each other, some studies have highlighted a certain level of cross talk between them, including cell surface and endosomal receptors. In the context of TLR8 signalling, activation results in the upregulation of cell surface TLR2 expression, possibly through the action of autocrine IFN-β production (32). Human TLR8 is activated upon recognition of *Borrelia burgdorferi* RNA in the phagosome of human monocytes (33). Due to the
importance in the recognition of viral and bacterial products, endosomal TLRs are therefore attractive targets for vaccine adjuvants and enhancing anti-tumour immunity, due to their potential for activating a potent inflammatory and anti-viral immune response (34).

**Figure 1.2: Human TLR8**

3D structure of Human TLR8 (UniProt databases). The 3D structure is acquired using x-ray crystallography and nuclear magnetic resonance (NMR) which shows alpha helices and beta strands that form the tertiary and quaternary structure of human TLR8.
1.3.4 Cytosolic Receptors: RIG-I-Like Receptors (RLRs)

In the early 2000s, it was shown that non-pDCs could induce the type 1 IFN response, independent of TLRs (35). Soon after RIG-1, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), were identified as cytosolic nucleic acid receptors capable of inducing this intracellular dsRNA-induced IFN production (36). All three RLRs have a highly conserved structure, including the DExD/H-box helicase core and a C-terminal domain, which confers part of the ligand specificity. All, except LGP2, also contain a caspase activation and recruitment domains (CARDs), which mediate signalling to downstream adaptor proteins (37). RNAs with a triphosphate group at the 5’ end, 5-hydroxyl (5-OH) and 3- monophosphoryl short RNA molecules with double-stranded stems generated by RNase L, activate the RIG-I signalling pathway, a common feature associated with RNA viruses such as HCV (38). Human mRNAs do not have these structures and thus the structures of viral genomes have been proposed as physiological agonists for RLR activation (38). For example, it was shown that dsDNA from picornavirus acts as the ligand for the IFN response in virally infected cells via RIG-I (39).

Signaling downstream of RIG-I depends on a protein called mitochondrial antiviral signaling (MAVS), also known as CARDIF, IPS-1 or VISA. MAVS is anchored to the mitochondria and peroxisomes via its transmembrane domain (40). Recruitment of the phosphatase, PP1, dephosphorylates the CARDs of RIG-I and MDA5, leading to their activation (41). Activation of MAVS induces the formation of a “signalosome” complex, comprised of tumour necrosis factor (TNF) receptor-associated factor (TRAF) proteins, (TBK1) and IkB kinase – ε (IKKε),
which leads to the activation IRF3 and/or IRF7. As mentioned, translocation to
the nucleus of IRF3 and IRF7 induces the expression of type I IFNs (42). In RIG-
I-deficient cells, little cytokine levels are produced in response to
paramyxoviruses, Newcastle disease virus (NDV) and Sendai virus (SeV), clearly
highlighting RIG-I’s importance in viral detection (43).

1.3.5 Cytosolic Receptors: NOD-like receptors (NLRs)

In addition to RLRs, viruses are also sensed by NLRs, leading to the activation of
the inflammasome. The inflammasome is a multiprotein complex, which is
assembled following PRR detection of pathogens and danger signals in the
cytoplasm (44). Inflammasomes promote the activation of caspases, that cleave
pro-IL-1β and pro-IL-1β, thus generating their mature, active forms. Receptor
proteins that activate inflammasomes include NLR family members NLRP1,
NLRP3 and NLRC4, as well as proteins absent in melanoma 2 (AIM2) and pyrin
(45). Several viruses have been shown to activate NLRP3, including HIV-1 and
Influenza virus (46) (47). Neutrophils have been shown to express NOD2 and
NLRP3, where activation with MurNAc-l-Ala-d-isoGln (MDP) results in IL-8
secretion, CD62 down-regulation, and increased migration towards inflammatory
stimuli (48).

1.4 NF-κB signalling

Downstream in the intracellular signalling cascade of PRRs include the NF-κB
family of transcription factors. NF-κB constitutes a family of 5 transcription factors
involved in a selection of immune processes, including the upregulation of
proinflammatory and chemotactic cytokines. These 5 family members are NF-κB1
NF-κB2 (p52), RelA (p65), RelB and c-Rel, which mediate transcription of target genes by binding to a specific DNA element, κB enhancer, in a hetero and/or homodimeric manner (49). NF-κB proteins are normally sequestered in the cytoplasm through the inhibitor domain of IκB family of proteins, in which IκB-alpha is the best characterised. The two major pathways underpinning NF-κB signalling are the canonical and non-canonical signalling pathways. Canonical signalling is primarily triggered through PRR engagement and involves the degradation of IκB through site specific phosphorylation at the N terminal domain by IKK, a multi-subunit IκB kinase complex. This results in the rapid nuclear translocation of the canonical NF-κB members mentioned above. In contrast to canonical signalling, non-canonical signalling involves the recognition of TNFR family member stimuli including RANK, LTβR and BAFFR, that does not trigger IκB degradation, but instead involves the processing of the NF-κB2 precursor protein, p100 (50, 51). While the canonical signalling is involved in all aspects of innate immune inflammation, the non-canonical signalling pathway seems to have evolved as an additional signalling axis that works cooperatively in the regulation of adaptive immune responses. Inflammation is a protective response to pathogens in which NF-κB is a central mediator of inflammatory responses, characterised in innate immune cells, such as monocytes, macrophages, DCs and neutrophils, downstream of PRRs. NF-κB has been demonstrated to be instrumental in the polarisation of macrophage phenotypes particularly in the context of LPS-induced activation of TLR4. M1 (inflammatory) and M2 (anti-inflammatory) macrophages are characterised by the secretion of inflammatory cytokines, IL-1β, TNF, IL-6 and IL-12, and anti-inflammatory cytokines IL-4, IL-10 and IL-13, respectively (52). NF-κB has also been shown to play an important
role in the regulation of inflammasome activation. Canonical inflammasomes are a group of multimeric protein complexes comprised of a ligand sensing receptor, such as the NLR family members (NLRP1, NLRP3, NLRCR) and absent in melanoma (AIM), the adapter protein ASC (apoptosis-associated speck-like protein containing CARD) and pro-caspase 1. Upon activation, inflammasome receptors recruit procaspase 1 via the ASC domain and cleave it to become active caspase 1. Active caspase 1 can subsequently cleave pro-IL-1β into active IL-1β, which plays an important role in inflammatory processes in innate immune cells. In order to have successful inflammasome activation, two signals are required, a priming signal and an activation signal. PRRs provide the activation signal via NF-κB upregulation of pro-IL-1β and the activation signal is provided by NLRP3 agonists responsible for inflammasome assembly (53).

1.5 The mitogen-activated protein kinase (MAPK) signalling

In addition to the activation of NF-κB signalling, PRRs are well studied in their activation of MAPK signalling (54). Like NF-κB signalling, TAK1 downstream of PRR activation is also important in the activation of MAPK. TRAF6 can bind to TAB2/3 which results in the phosphorylation of TAK1. Subsequently, TAK1 controls activation of the ERK1/2, p38, and JNK MAPK pathways (55) (Fig 1.3). Efficient signalling requires a cascade of three kinases. MAPKs are activated by a MAPK (MAP2K), which itself is activated by an upstream MAPK (MAP3K) via phosphorylation. TAK1 acts as the MAP3K for p38 and JNK signalling, in addition to controlling ERK1/2 via IKK activation of TPL2. Under homeostatic conditions TPL2 is complexed to P105, however on activation, TPL2 is phosphorylated and is free to activate its respective MAPK, resulting in phosphorylation of
downstream targets, thereby regulating their transcription (56). MAPK signalling has been demonstrated to regulate the expression of a plethora of cytokines such as TNF, IL-8, IL-10 (57) (58) (59).

Figure 1.3: TLR activation of NF-κB and MAPK signalling pathways
Cells express membrane receptors such as toll-like receptors (TLRs). These receptors recognise pro-inflammatory stimuli such as pathogen-associated molecular patterns (PAMPs) and in turn results in the activation of adaptor proteins Myd88 and TRAF that in turn result in the activation of both the NF-κB and MAPK signalling pathways. (Created with BioRender.com)
1.6 Anti-viral cytokine signalling

Along with pro-inflammatory cytokines the activation of TLRs leads to the induction of IFNs. IFNs were first observed in 1954, when it was reported that viral growth was inhibited in pieces of chorioallantoic membrane, cut from a 10-day-old fertile hen’s egg that had previously been inoculated with heat inactivated influenza virus. It was proposed that a soluble secretory protein was responsible for viral 'interference’ (60). There are 3 classes of IFNs, type 1, type 2 (IFN-γ) and type 3 (IFN-λ). Type I IFN include IFN-α (13 subtypes), IFN-β, IFN-ε, IFN-ω and IFN-κ. The function of Type I IFNs is to induce an anti-viral state in both virus-infected and neighbouring, non-infected cells (61). Type I IFN all bind IFNAR1. IFNAR assembly involves the initial binding of type 1 IFN to IFNAR2, which in turn recruits IFNAR1 to initiate signalling (62). However, it has been demonstrated that IFN-β can bind IFNAR1 independently of IFNAR2 (63). IFN signalling through the JAK/STAT pathway has been proven to be important in controlling viral replication in acute HIV infection, and conversely been associated with a worse prognosis during chronic HIV infection (64). Additionally, RSV has been demonstrated to induce both type 1 and type 3 IFNs, which are potent activators of ISGs in both DCs and Beas-2b, lung epithelial cell line (65).

1.6.1 JAK/STAT signalling

Interestingly, the discovery of Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STAT; family = STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6), originated from the works of those investigating how IFNs functioned. Upon IFN binding the IFNAR, the cytoplasmic receptor subunits form hetero-multimers, which in turn recruit JAK tyrosine kinases (JAK1,
JAK2, JAK3 or Tyrosine kinase (Tyk2). Ligand binding is necessary for receptor activation, which stimulates the auto-phosphorylation of tyrosine residues on two JAKs in close proximity, which in turn phosphorylates docking sites on the receptor chain for latent STATs (66) (67). Phosphorylated JAK proteins catalyse the homo-dimerisation or hetero-dimerisation of STAT proteins through the SH2 domain. Tyrosine-phosphorylated STAT dimers are actively transported to the nucleus using metabolic energy and the importin α/β and RanGDP complex (68). Once inside the nucleus they bind STAT-responsive elements where they assemble with IFN-regulatory factor 9 (IRF9) and form a complex known as IFN-stimulated gene factor 3 (ISGF3) and transactivate genes (69). In addition to type 1 IFNs, more than 50 other cytokines signal via the JAK/STAT pathway to control inflammation and immune responses. Of these, the IL-6 family is an important group that consists of IL-6, IL-11, IL-27, LIF, OSM, CNTF, CT-1, and CLC. The IL-6 family binds to a unique type of receptor that expresses two protein chains: a transmembrane region and the gp130 extracellular cytokine receptor homology region (CHR). While different cytokines utilise different JAK proteins and subsequently different STAT proteins, the IL-6 family utilises JAK1 which subsequently activates STAT3 that functions in cell maturation, proliferation and cellular survival (70).

Viruses have been shown to modulate JAK/STAT signalling. The RSV protein, NS2, decreases the levels of STAT2 (71). Hepatitis C virus (HCV) downregulates the effect of type 1 IFN’s through the proteosomal degradation of STAT1 and STAT3 (72). This is in keeping with other reports, where the mumps virus
degrades STAT3 and Zika degrades STAT2 through ubiquitination and degradation (73, 74) (75).

Besides Type 1 IFN's role in anti-viral and anti-microbial responses, type 1 IFNs shape innate and adaptive immunity, influence the maintenance of homeostasis and lymphocyte development (76). Type I IFNs are needed for the control of viral infections, but they can also be protective against bacterial infection (77). Type 1 and type 2 IFNs have been shown to delay human neutrophil apoptosis through the activation of STAT3 (78). In contrast however, IFN-β has been shown to induce neutrophil apoptosis in mouse models (79).

**1.6.2 Interferon stimulated genes**

Downstream of the IFN intracellular signalling pathway there are several hundred anti-viral ISGs. Even though it has been 25 years since the first ISGs were identified (80), insight into their effector functions has been limited to a select few molecules which include the classical ISGs: MxA, OAS1, APOBEC3G, TRIM5, ZAP and ISG15. Depending on the cell type, the ISG response differs with the type 1 IFN dose and time of treatment. Microarray studies have revealed >500 ISGs typical of many immune cell types. While all cells are thought to be responsive to type 1 IFNs, little is known about the neutrophil response. Interestingly, circulating neutrophils from malaria patients, albeit not a viral infection, have a strong type 1 IFN ISG signature, increased expression of surface activation markers, enhanced release of ROS, myeloperoxidase and increased recruitment of low density granulocytes (81). Well documented ISGs and their functions are shown in table 1.1
<table>
<thead>
<tr>
<th>ISG15</th>
<th>Modulate protein function by ISGylation</th>
<th>(82)</th>
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<tbody>
<tr>
<td>MxA</td>
<td>Formation of highly ordered oligomers around viral nucleocapsid structures</td>
<td>(83)</td>
</tr>
<tr>
<td>PKR</td>
<td>Inhibition of viral replication through phosphorylation of eIF2a</td>
<td>(84)</td>
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<tr>
<td>2’-5’OAS</td>
<td>Activates RNaseL to degrade the viral genome</td>
<td>(85)</td>
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<tr>
<td>SAMHD1</td>
<td>Reverse Transcription block during HIV-1 infection of myeloid cells and CD4+ T cells</td>
<td>(86)</td>
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<tr>
<td>TRIM5A</td>
<td>Interferes with the uncoating process of the HIV-1 capsid</td>
<td>(87)</td>
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<tr>
<td>Viperin (RSAD2)</td>
<td>Inhibits viral protein function Inhibits RNA replication and release</td>
<td>(88)</td>
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*Table 1.1: key anti-viral ISGs and a summary of their key functions*
1.6.3 Viral inhibition of JAK/STAT signalling

Due to persistent exposure to viruses the immune system has developed a spectrum of antiviral mechanisms. However, due to the rapid replication rate and subsequent high frequency of point mutations, viruses have also developed numerous immune evasion strategies. A common strategy is the evasion of the anti-viral type 1 IFN JAK/STAT signalling pathway. For example, Vaccinia virus has demonstrated a novel immune evasion strategy in which it has the capacity to encode a soluble IFNAR homologue named B18R, that competes with the endogenous membrane bound IFNAR for secreted type 1 IFN cytokines (89). Downstream of IFNAR it has been documented that several viruses, including HCV, HIV, RSV and human influenza virus, use evolutionarily conserved mechanisms to promote the degradation of STAT proteins via proteasomal degradation, thus effectively shutting off the type 1 IFN response (72) (90) (91). Most recently, our lab has shown that HIV accessory protein, VIF, interacts with both STAT1 and STAT3 and its expression promotes ubiquitination and subsequent degradation of both proteins, thus stunting the anti-viral IFN response (75).

1.6.4 IFN independent induction of ISGs

Viral degradation of IFN-dependent antiviral pathway may have resulted in the evolution of IFN-independent ISG induction to combat invading viruses. Studies using HCMV at 8 hours post infection upregulated the expression of the ISGs, Interferon Induced proteins with Tetratricopeptide repeats 2 (IFIT2) and IFIT3 with the introduction of IFN neutralising antibodies failing to inhibit their upregulation (92). When studying a mechanism underlying this IFN independent upregulation
of ISGs it was suggested that IRF3 might play a role (93). Constitutive upregulation of IRF3 resulted in the induction of a subset of ISGs that included IFIT1 and IFIT2. However, it was demonstrated in cells that were unable to respond to type 1 IFNs that ssRNA viruses including Sendai could induce the expression of these ISGs, independent of IRF3, downstream of TLR activation (94).

1.6.5 Viperin

Viperin is an interferon-inducible protein that inhibits the replication of a variety of viruses. It was first identified as an IFN-\(\gamma\)-inducible gene in macrophages. Human Viperin is composed of 361 amino acids with a molecular mass of approximately 42 kDa. It is composed of three domains, an N-terminal domain, a conserved central domain and a C-terminal domain. Viperin is induced in several cell types by a number of distinct IFNs, and by infection with a range of different viruses (Fig 1.4). IFN-independent induction of Viperin has also been demonstrated and is regulated via IRF1 and IRF3 (95). Viperin has been shown to inhibit a range of ssRNA viruses, including West Nile virus, Dengue and HIV-1 (96) (97) (98). The N terminal domain of Viperin is important in the localisation of Viperin to the ER, which appears to be protective in Flaviviral infections (99). Reports have suggested a role for Fe-S binding of Viperin resulting in a radical-based mechanism that underlies the anti-viral effects of Viperin. These free radicals could catalyse a diverse array of reactions that in turn modify a range of different host metabolites and proteins (100). Viperin can promote viral degradation by interfering with golgi-dependent trafficking of soluble proteins, resulting in the release of immature viral capsids. Viperin can also inhibit cholesterol synthesis by
binding to Farnesyl pyrophosphate (FPP), resulting in the disruption of lipid rafts at the plasma membrane used for viral egress (101). While most Viperin studies have focused on its anti-viral role, others have explored the possibility it is potentially pro-viral. In the context of HCMV, Viperin upregulation manipulates cellular metabolism and causes the accumulation of cytosolic lipids for use in production of the viral envelope, thus enhancing the viral lifecycle (102).

Figure 1.4: IFN dependent and IFN independent activation of Interferon response Genes
Conventional IFN induced ISG transcription is mediated through ISGF3, a complex comprised of a STAT1/STAT2 dimer complexed with IRF9. Unconventional ISG transcription has been proposed to be independent of the presence IFNs and thought to be induced through various IRF proteins including IRF1 and IRF3, downstream of various PRRs. Created with BioRender.com
1.7 Neutrophils

Neutrophils are haematopoietic cells, whose maturation is guided by the cytokine, Granulocyte Colony Stimulating Factor (GCSF), in a process known as granulopoiesis. They make up the majority of circulating immune cells and upon detection of pathogenic “alert signals” are recruited to the site of infection, classically along an IL-8 gradient. Upon detection of a pathogen, their normally short half-life is prolonged, improving their ability to tackle the infection and recruit additional immune cells. As well as cellular and intracellular receptor activation, neutrophil “alert signals” include key cytokines, such as (IFN)-γ and TNF, which go on to enhance both innate and adaptive immunity (103)(104). Neutrophils are classically portrayed as unsophisticated, first-line foot soldiers, with a role limited to the engulfment and subsequent elimination of invading extracellular pathogens, but this is unlikely to be the full picture. Upon recognition of a virus, neutrophils possess a unique arsenal of innate immune mechanisms including, phagocytosis, migration, degranulation and NETosis (105). While the antibacterial and anti-fungal role of neutrophils has been well characterised, relatively little is known about their role in viral infection (106). Besides their fundamental role in primary defence to infection, there is increasing evidence suggesting a more complex contribution to the regulation of direct immune and wider inflammatory responses, which may, in part, be controlled via their de novo production and release of cytokines and chemokines (107). As a key player in the innate immune response, neutrophils have a paradoxical role in controlling the overgrowth of bacteria and fungi, while at the other end of the spectrum can result in the overactivation of the immune response, fatal in disorders such as sepsis and Acute Respiratory Distress (ARDS) (108).
1.7.1 Neutrophil heterogeneity

Neutrophils are extremely sensitive to environmental stimuli due to the abundance of extracellular and intracellular receptors. They can integrate these signals into a changed activation status ranging from priming to full activation. It is therefore important to note the diverse pool of neutrophil phenotypes is not only dependent on the differentiation of neutrophils in the bone marrow but also on the range of stimuli they encounter. Discontinuous gradients used to isolate various immune cells has shed light on the presence of both Normal Density Granulocyte (NDGs) that reside in the high density fraction and Low Density Granulocytes (LDGs) which reside in the low density fraction. LDGs are associated with a number of disease settings, such as RA and systemic lupus erythematosus (SLE). LDGs either exhibit immature morphology with banded nuclei or mature morphology with banded nuclei and are proposed to be released from the bone marrow in response to emergency granulopoiesis. Myeloid Derived Suppressor Cells (MDSCs) named by Gabrilovich were identified as myeloid cells that suppress immune responses (109). Monocyte and granulocyte-MDSC have been identified, with G-MDSCs often found in the low density portion of peripheral blood which exhibit similar morphology to monocytes despite being bona-fide neutrophils. They express similar receptor patterns to neutrophils in which they display CD66b+, CD16+, CD15+ and CD14-. However, it’s their suppressive functions that sets them apart. Additionally, the heterogeneity of the neutrophil population was highlighted in a cancer setting with the identification of N1/N2 tumour associated neutrophils (TANs). N1 were considered to be tumour killing, and N2 immunosuppressive in a cancer setting (110). Multiple studies have
connected the expression of extracellular markers with neutrophil age. Traditionally the segmentation status of the nucleus was used as a marker for the maturation of the neutrophil, where non-segmented cells were considered immature. A clear example where a membrane marker correlates with the segmentation status of the cell is the expression of CD10. Additionally, there has been a multitude of studies examining the life span of neutrophils, in which results are conflicting. Studies showing neutrophils undergoing spontaneous apoptosis or efferocytosis (phagocytosis of dying neutrophil by a macrophage) have been limited to inflammatory states. On the contrary, there is little known about neutrophils under normal homeostatic conditions, with cells reported to survive for a reported five days (111). In addition to differences in differentiation, neutrophil counts and phenotypes differ depending on sex, age and levels of physical activity (112) (113) (114). It will therefore be paramount to determine neutrophil phenotype in viral responses, in order to tailor possible therapeutics that target the anti-viral and inflammatory responses especially where ARDS occurs during respiratory viral infections.

1.7.2 Neutrophilic processes

Migration

Previously described above, circulating levels of chemokines are important in the recruitment of neutrophils in the respiratory tract. Neutrophil trans-endothelial migration (TEM) is the process during which neutrophils cross the endothelial barrier to the site of infection. It has been demonstrated that ICAM-1 expression on neutrophils regulates TNF activated endothelial adhesion prior to crossing. While the generation of chemotactic gradients is important in the extravasation of
neutrophils, it is essential to note that histamine disrupts endothelial homeostasis, resulting in increased blood flow and vascular leakage enhancing the chemotactic ability of neutrophils. TEM has been described to have two unique stages. The first characterised by the recruitment of neutrophils through secreted products as N-Formyl-methionyl-leucyl-phenylalanine (FMLP), which is followed by the amplification step characterised by the generation of CXCL1/2/3/5/6/7/8 chemotactic gradients. Other secondary signals, such as the production of neutrophil derived IL-1β, are important for driving the production of endothelial derived CXCL1, unveiling an important chemotactic positive feedback loop. While the mechanics of neutrophil TEM in respiratory viral infection remain anecdotal they are thought to be similar to that of TEM in other inflammatory conditions, under the direct control of certain chemotactic gradients.

**Phagocytosis**

Neutrophil defence against infecting microorganisms largely depends on the phagocytic process, initiated by invagination of the plasma membrane and resulting in a membrane-enclosed phagosome. Neutrophils can phagocytose opsonised and non-opsonised pathogens. Opsonisation involves the binding of an opsonin (antibody), to the surface of a pathogen. Although several viruses have been detected intracellularly in neutrophils, such as RSV and HIV, it is not clear whether this was due to simple uptake of the virus itself or by active infection and replication of the virus within the neutrophils (115)(116)`. The primary opsonin-mediated receptors are the Fc (fragment, crystalisable) receptors (FcyRIIA [CD32], FcyRIIb [CD16] and FcyRI [CD64]) (117). However, FcR-mediated phagocytosis involves the rigorous extension of pseudopods that
physically entrap the pathogen. While the receptors that mediate phagocytosis are extracellularly similar, the underlying signalling that ensues after pathogen internalisation differs drastically.

Recent human and non-human primate HIV vaccine studies have highlighted the importance of FcR-mediated protection (118). In particular, it has been indicated that this type of Antibody-Dependent Cellular Cytotoxicity (ADCC), correlates with slower disease progression, revealing it’s importance in combating HIV (119). Additionally, it has been demonstrated that neutrophils mediate HIV-specific ADCC responses, with a peak response time of 4h, much quicker than that of NK or monocyte-mediated responses, further indicating that neutrophils have a prominent role in fighting against HIV infection via ADCC (120).

Unlike macrophages, which undertake active endocytosis, neutrophils are passive, in that they do not readily contain endosomes and lysosomes. Instead, the neutrophil phagosome acquires its anti-microbial properties through the fusion of secretory vesicles containing anti-microbial granules, which are believed to be a by-product of the trans-golgi machinery, formed during cellular development. The 4 main granules include 1) primary granules (also known as “azurophilic granules”), 2) secondary granules (also known as “specific granules”), 3) tertiary granules and 4) secretory vesicles. Primary granules are the most toxic, comprised of myeloperoxidase (MPO), elastase, cathepsins and defensins. The secondary and tertiary granules contain lactoferrin and matrix metalloproteases (121). While phagocytosis and granule secretion of neutrophils
is important in the clearance of microbes, the localisation of neutrophils to sites of infection is essential in prompting microbial clearance.

**NETosis**

NETs are a complex extracellular fibril matrix consisting of nuclear and mitochondrial DNA and proteins, such as elastase, myeloperoxidase and cathepsin G (122). In the presence of hydrogen peroxide ($H_2O_2$), myeloperoxidase (MPO) catalyses the formation of powerful reactive intermediates including hypochlorous (HOCl) and hypobromous (HOBr) which can have profound effects on cellular function by modifying proteins, lipids, and/or DNA (123). Two models of NETosis have been proposed; suicidal and vital. While suicidal NETosis is the best described, the molecular mechanisms underlying it are still poorly understood. Following 2-4h of pathogenic infection, neutrophils exhibit a gradual separation and loss of the nuclear membrane. ‘Suicidal’ NETosis is dependent on ROS for the citrullination of histones by Protein Arginase Deaminase 4 (PAD4), resulting in chromatin decondensation which facilitates the expulsion of chromosomal DNA that is coated with antimicrobial molecules into the extracellular matrix (124). ‘Vital’ NETosis involves the release of nuclear DNA without the loss of membrane structure and production of ROS (Fig 1.5). Suicidal NETosis has often been demonstrated in the context of Phorbol Myristate Acetate (PMA) stimulation whereas vital NETosis is triggered by the recognition of PAMPs through cell surface TLR4 (125). Studies have revealed that viruses can induce NETosis without the need to establish productive infection inside the cell. Viruses such as influenza and hantavirus stimulate NET production (126, 127). While the molecular mechanisms are still not fully clear, it
is believed to be through the successful ligation of cell surface TLR4, with little known about the ability of endosomal TLRs or DNA sensors to do the same (128). While the downstream signalling of PRRs triggering NETosis is still to be fully elucidated, it has been established that experimental infection of mouse neutrophils with chikungunya virus resulted in TLR7-dependent production of Netosis via a ROS dependent mechanism. Similarly, human neutrophils incubated with chikungunya virus resulted in ROS dependent production of NETS. It’s important to note that while chikungunya virus was able to induce NETosis via TLR7, other arboviruses including Dengue and Zika were not able to, revealing the highly specific nature of neutrophil NETosis (129). A recent study showing a decrease in spontaneous and LPS-induced NETosis can be achieved through the administration of tofacitinib (130). Tofacitinib being a JAK1/JAK2 inhibitor suggests an important possible role of STAT1/STAT3 in the generation of Neutrophil NETs. That viruses inducing NETosis is becoming more widely accepted, their role in anti-viral immunity is still not fully clear. It is believed that the chromatin-like structure traps virus particles, mechanically preventing their spread. While the study of NETs in SARS-CoV-2 remains in its infancy, early reports suggest that neutrophilia could be a potential source of NETS during the acute stage of infection (131).
**Figure 1.5: Neutrophilic Processes: schematic of well described antimicrobial neutrophil processes**

Phagocytosis is a process by which a microbe is internalised and degraded through the formation of the phagosome. Degranulation is a process by which neutrophils release intracellular cytotoxic antimicrobial granules into the extracellular space upon the recognition of a pathogen. Reactive oxygen species produced by NADPH oxidase and soluble secreted cytokines can be released from neutrophils where they initiate their antimicrobial effects. Finally, NETs are a network of extracellular DNA fibres complexed to antimicrobial compounds such as MPO and elastase that immobilise extracellular pathogens. Created with BioRender.com
1.8 Neutrophils and viral infection

1.8.1 Influenza

Influenza poses a concern for global public health due to the consistent emergence of novel strains with varying degrees of pathogenicity. In 2009 H1N1 was detected for the first time in North America continent, with patients experiencing a spectrum of effects, from mild to severe ARDS, when compared to seasonal influenza. In general Influenza is an ideal viral model to study pathways leading to pneumonia and ARDS. A major complication of influenza is severe influenza pneumonia (SIP) that is the single largest contributor in the development of ARDS (132). Neutrophils are present in the lower and upper respiratory tract during infection with mild seasonal strains of influenza, but also with highly pathogenic avian influenza viruses (HPAI) (133). During SIP and HPAI, neutrophil influx into the lung and accompanying release of large quantities of proinflammatory cytokines, associated with a worse prognosis and higher mortality rate. This increase in levels of IL-6, IL-8 and CXCL2 correlates with a decrease in the type 1 IFN signature measured through the expression of ISGs (134).

1.8.2 SARS-CoV

Coronaviruses are positive sense single stranded enveloped RNA viruses. To date there have been a number of endemic coronaviruses in the human population that are associated with mild respiratory symptoms. The coronaviral genome encodes of four major structural proteins: the spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and the envelope (E) protein, all of which are required to produce a structurally complete viral particle. In 2003 the
first zoonotic coronaviruses presented in the human population in Guangdong province in China in which there were a recorded 8422 infections. The virus was associated with respiratory distress and was aptly coined ‘Severe Acute Respiratory Syndrome’ (SARS). Patients presenting with virus had flu like symptoms, however there were a number presenting with dyspnea, respiratory distress, and pneumonia. Approximately 20-30% of those infected required mechanical ventilation and supplemental oxygen with a case fatality rate of 11% (135). Angiotensin converting enzyme 2 (ACE2), a metallopeptidase, was identified as the functional receptor for SARS-CoV. With respect to ACE2 the luminal surface of tracheobronchial and alveolar epithelium was found to contain a high density of the receptor (136). Interestingly while ACE2 tissue distribution mirrored that of infected organs, the lack of ACE2 expression on circulating immune cells suggested there were alternative means of infection through other possible novel receptors or phagocytosing processes. This was confirmed with the discovery that liver/lymph node-specific ICAM3-grabbing nonintegrin (L-SIGN) and dendritic-cell-specific DC-SIGN were utilised for the successful internalisation and subsequent infection in DCs by SARS-CoV (137). While the pathophysiology has been described in multiple tissue types and organs such as the intestines and kidneys, the predominant pathological finding was diffuse alveolar damage (DAD). During the acute stage, patients present with extensive edema, hyaline membrane formation, collapse of alveoli, desquamation of alveolar epithelial cells and fibrous tissue in alveolar spaces of the respiratory tract (138). It’s been hypothesised that DAD is the result of direct viral and immunopathological effects through the overactivation of the innate immune response. In many cases, cellular infiltration has been observed in which
neutrophil and macrophage influx are most common. Interestingly, a mutation introduced in the E protein of the virus (SARS-CoV-ΔE) correlates with a decrease in numbers of infiltrating neutrophils with a simultaneous decrease of NF-κB mediated TNF, IL-6, CXCL2, CXCL1 and CCL5 when compared to wild-type virus (139).

1.8.3 SARS-CoV-2

As of December 2019, novel coronavirus disease 2019 (COVID-19) (SARS-CoV2) emerged in Wuhan China and rapidly spread around the globe being officially declared as a pandemic in March 2020. As of August 2022 there are a reported 575 million cases and 6.4 million deaths worldwide. This is the third example of zoonotic β-coronavirus that has been suggested to have originated in bats, and subsequently utilised other intermediate animal hosts before jumping into the human population. Like the original SARS-CoV, this virus is a +ssRNA virus comprised of 4 structural proteins S, E, M, and N proteins. Similarly, ACE2 was identified as the primary receptor for viral entry, with an even higher binding affinity than the original SARS virus (140). The clinical spectrum of COVID-19 ranges from asymptomatic to severe clinical conditions characterised by respiratory failure and systemic multiorgan dysfunction. This is displayed through complications such as septic shock and sepsis which occurs in roughly 5% of the population. Early histopathology results have revealed edema in the lung along with proteinaceous exudates as large protein globules. In addition, histopathology revealed vascular congestion combined with inflammatory clusters of fibrinoid material and multinucleated giant cells and hyperplasia of pneumocytes. Initial studies reported that severe cases have higher neutrophil-lymphocyte ratio
(NLR), as well as having lower number of monocytes, basophils and eosinophils in the lower respiratory tract (141). Additionally it was noted that there was a significant increase in levels of circulating levels of IL-2, TNF, IL-6, IL-1 and IL-8, which is associated with increased pulmonary inflammation and lung damage (142). Age correlated with neutrophil lung infiltration and C reactive protein, with older patients reporting higher percentages of both. Concurrently, levels of circulating IL-10, known to suppress the immune response were significantly reduced in severe cases with levels returning to normal 5 days post infection as the patient recovers (143). IL-10 has been demonstrated to inhibit the production of IL-6, TNF, IL-12, IL-2 and GM-CSF all of which are upregulated during the acute stage (144). Additionally it has been reported that an increase in levels of serum amyloid A, procalcitonin and lactate dehydrogenase correlated with a decrease in numbers of lung resident lymphocytes (145). Interestingly, serum amyloid A produced by both macrophages and alveolar cells are known to prime neutrophils and increase levels neutrophil derived IL-1β via NLRP3 inflammasome activation (146). Lactate dehydrogenase, produced by LPS-activated neutrophils can promote inflammatory neutrophil mobilisation from the bone marrow via endothelial GPR81 signalling, revealing that activated neutrophils have the potential to promote this inflammatory state in an autocrine fashion (147).

1.8.4 Respiratory Syncytial Virus (RSV)

Respiratory syncytial virus (RSV) is one of the most prevalent childhood respiratory infections. While the virus is generally self-limiting, it is associated with significant morbidity and mortality. It is a negative sense RNA virus, that
encodes 10 genes and 11 proteins, both structural and non-structural. Similar to severe influenza, neutrophilic inflammation correlates with disease severity in children with RSV-induced bronchiolitis (148). Additionally, mutations in the chemoattractant IL-8 and α-defensin genes are associated with an increase in disease severity and subsequent mortality (149). While it has been hotly debated whether or not viruses undergo replication in neutrophils or are simply passively phagocytosed, studies involving RSV have demonstrated that the virus binds and undergoes transcription in neutrophils (150).

While it has generally been accepted that neutrophil influx in RSV infection is detrimental, others have suggested the potential beneficial role of neutrophils in managing viral infection (148) (151). While we are still in the early stages of investigating the anti-viral roles of neutrophils, a recent murine study has revealed that neutrophils do not change the viral load of RSV, thereby prolonging inflammatory immune responses (152).

1.8.5 Human Immunodeficiency Virus (HIV)

In addition to the dichotomy that is observed with neutrophils in viral respiratory infections, in which they have been demonstrated to be both beneficial and detrimental, studies have also explored the potential role neutrophils have in chronic tissue resident infections, such as HIV. This contradiction has been described in which neutrophils assist in the transmission of HIV, but also enhance the innate immune protection from HIV (153) (154). Gastrointestinal mucosal dysfunction is a predominant feature of HIV infection and is the result of damage
to the endothelial barrier. The damage to the endothelial barrier has been proposed to be in part caused by the HIV accessory proteins such as TAT, the apoptosis of enterocytes due the increase presence of inflammatory cytokines such as TNF and IL-1β, and finally, the loss of CD4+ T cells that produce IL-22, which maintains the integrity of the endothelial barrier (155) (156) (157). During HIV infection, neutrophil counts are increased in the GI tract. The increase in the presence of neutrophils in inflammatory bowel conditions such as irritable bowel disease (IBD) is associated with disease progression (158). However, despite not yet proven, it is postulated that the increase in the presence of neutrophils in the GI tract during HIV infection is detrimental and aids in the transmission and dissemination of the virus systemically resulting in chronic immune activation. In addition, studies have highlighted how neutrophils could modulate the adaptive immune response during HIV infection. They found that human neutrophils exposed to HIV in the presence of ROS upregulated the expression of programmed death ligand-1 (PD-L1), which upon binding PD-1 would suppress protective T-cell function (159). On the contrary, epidemiological studies have shown that high-risk female sex workers with a genetic predisposition to neutropenia (neutrophil counts <2500 cells/µl) had a 3-fold increased risk of contracting HIV compared to females with normal neutrophil counts (160). It has been proposed that neutrophils contribute to this anti-pathogenic role through the release of alpha defensins such as human neutrophil peptides (HNPs). They are thought to confer protection through the inhibition of viral replication by altering target cell signalling pathways (161). Lastly, the production of ROS and myeloperoxidase (MPO) to form hypochlorous acid has been demonstrated to be a potent viricidal agent against HIV (162).
Overall rationale underpinning thesis

Key facts:

• Endosomal TLRs (TLR7/8) are established PRRs in the detection of ssRNA viruses.
• TLR activation results in the upregulation of inflammatory and anti-viral cytokines.
• Neutrophils have been demonstrated to respond to ssRNA viruses through extracellular receptors, such as TLR4.
• TLR induced inflammatory and antiviral responses in neutrophils could have both a negative and positive impact on the host immune response.
• PRR activation has been demonstrated to upregulate the early expression of ISGs independent of IFNs in various cell lines.

Importance of investigation:

investigating the role of TLR8 in neutrophil-induced inflammatory and antiviral responses upon encountering ssRNA viruses will assist in the understanding and future treatment of hyperinflammatory immune responses, such as virally induced ARDS. Understanding the role of neutrophils in the pathogenesis of viral infections may lead to the identification of immunomodulatory therapeutic targets that could control hyperinflammation. Additionally, understanding the anti-viral signalling pathways in neutrophils could pave the way to identifying novel viral immune evasion mechanisms such as viral targeting of the TLR and/or the type 1 IFN pathways which may allow future targeted treatments to combat viral infections.
**Hypothesis:**

Given the evidence that TLR7/TLR8 is involved in the induction of inflammatory cytokines in PMNs, we propose that the endosomal TLR8 of neutrophils plays a role in the detection of viral ssRNA and subsequent regulation of various intracellular signalling pathways, that result in the upregulation of inflammatory and anti-viral cytokines, thus identifying a novel role for neutrophils in both anti-viral and proinflammatory responses during viral infection and disease. Furthermore, we hypothesise that neutrophils also respond directly to the type 1 IFN, IFN-α, thus generating an IFN-dependent response that may also enhance the overall anti-viral response.

**Overall objective:**

To investigate the function of TLR7/TLR8 in primary human neutrophils in the context of viral infections, in order to reveal the gaps in knowledge of these powerful receptors in the induction of inflammatory and anti-viral mediators.

**Specific aims:**

- Identify if endosomal TLR8 is a key receptor in the induction of inflammatory and anti-viral cytokines in PMNs.
- Examine the role of different intracellular signalling pathways involved in the induction of the aforementioned inflammatory and anti-viral responses in PMNs.
- Examine the role of PMN TLR8 upon the recognition of RSV and HIV.
- Determine the role of TLR8 in the induction of early ISG expression independent of the expression of type 1 IFNs.
• Determine the effect of IFN-α upon anti-viral signalling of PMNs.
Chapter 2

Materials and Methods
Chapter 2: Materials and Methods

2.1 Ethics

Written informed consent was obtained from each healthy individual volunteer and the study received ethical approval from the local Research and Ethics Committee in the Trinity Biomedical Sciences Institute, in accordance with the guidelines of the 1975 Declaration of Helsinki. Ethics #: BI-RW-010921

2.2 PMN isolation

PMNs were isolated from heparinized peripheral blood, collected by venepuncture from healthy volunteers. The isolation of PMN was done through the removal of red blood cells using dextran (Sigma) promoted rosette formation, followed by Ficoll-Hy-paque density gradient (GE healthcare). This involved mixing freshly isolated heparinised blood with 3% dextran in a 2:1 ratio in a 50 ml falcon tube and leaving for 30 minutes to allow red blood cells to sediment out. The top ‘straw’ coloured layer was carefully added onto 10mls of lymphoprep. Cells were spun down at 300xg for 20 minutes at room temp (20°C) with the break off resulting a cells pellet containing the granulocytes and remaining red blood cells. Red blood cells were removed by hypotonic lysis using ammonium-chloride-potassium (ACK) lysing buffer (Gibco). PMNs were removed and washed twice with PBS (300xg, 5 minutes) before resuspension in complete RPMI (cRPMI). cRPMI consisted of 10% FBS and 250U/ml penicillin, 250µg/ml streptomycin (Sigma).
2.2.1 PBMC isolation

Buffy coats derived from human blood were collected from the Irish blood transfusion service. To isolate PBMCs, the buffy coat was diluted in a 1:1 ratio with phosphate buffer saline (PBS). The resulting mixture was added to density gradient (lymphoprep) and centrifuged for 20 minutes at 300xg with the break off at room temperature. The PBMC ‘layer’ was removed and washed twice with PBS (300xg, 5 minutes) before resuspension in cRPMI.

2.2.3 Culturing of DCs from human blood derived monocytes

Buffy coats derived from human blood were collected from the Irish blood transfusion service. CD14+ monocytes were separated from PBMCs by immunomagnetic negative selection as per the manufacturers protocol (Stemcell). PBMCs were diluted to $5 \times 10^7$ cells/ml in MACS buffer and 1 ml was added to FACS tube. 50µl of the monocyte isolation cocktail and 50µl of the platelet removal cocktail were added to the sample in the FACS tube and incubated for 5 minutes. 50µl of the magnetic particles were added to the sample and incubated for 5 minutes. The sample was topped up to a total of 3ml with MACS buffer and the FACS tube was placed into the EasySep magnet for 3 minutes. The CD14+ monocytes were poured into a fresh falcon in one continuous motion. The freshly isolated monocytes were resuspended at $1 \times 10^6$ cells/ml in RPMI. They were cultured in a 6 well non-pyrogenic cell culture plate (VWR), 3 ml per well at 37°C for 6 days. Monocytes were supplemented with GM-CSF (50 ng/ml) and IL-4 (40 ng/ml) (Miltenyi) on day 1 and again on day 3. On day 6 the immature (monocyte-derived Dendritic Cells) moDCs were harvested. DCs were seeded at a density of $1 \times 10^6$ cells/ml and 500µl was added
to each well of a 24 well plate. The cells were rested for 2h at 37°C before stimulations.

2.2.4 Culturing of CD4+ T cells

CD4+ T cells were separated from PBMCs by immunomagnetic negative selection as per the manufacturers protocol (Stemcell). The freshly isolated CD4+ T cells were resuspended at 1x10^6 cells/ml. A 96 well U bottomed plate was coated with anti-CD3 (5µg/ml) or plain PBS as a control overnight at 4°C. The plate was washed twice before adding the CD4+ T cells at 1x10^6 cells/ml.

2.2.5 Culture of HL-60 cell line

HL-60 cells are a promyelocytic cell line derived from human leukaemia. They predominantly show neutrophilic promyelocytic morphology. HL-60 cells were cultured in RPMI supplemented with 10% FBS and 250U/ml penicillin, 250ug/ml streptomycin. Cell culture density was maintained between 2x10^5 and 9x10^5 and needed to be passaged roughly every 3-4 days. Cells were maintained in a humidifier at 37°C and supplied with 5% carbon dioxide. With this cell line maturation to mature granulocytes can be induced using 1-2% DMSO for 5 days.

2.2.6 Culture of Hep2 cell line

Hep2 cells were cultured in complete DMEM (10% FBS/1% pen/strep) at 37°C.

2.2.5 Cell counting

Cells were counted using a haemocytometer, under a light microscope to ensure the cells were seeded at the same density per experiment.
2.3 Propagation of RSV

Hep2 cells were infected with full length RSV-A2-GFP stocks. Hep2 cells were cultured as previously described above before being seeded into a T175 flask at $1 \times 10^7$ cells per flask in 25ml complete DMEM and left to incubate for 24h to reach a confluency of 70-80%. To infect the Hep2 with RSV-A2-GFP, all complete DMEM was aspirated from the flasks and the cells washed with 10ml warmed PBS. The viral stock was diluted to 0.1pfu/cell in 3ml serum free (SF) media, assuming that the number of cells had doubled over night and dispersed over the cellular monolayer at 37°C for 2 hours. After 2 hours 22mL of complete DMEM was added and allowed to incubate for a further 24 hours. The following day the serum concentration was reduced to 2% and allowed to incubate until about 80% cell death. In order to harvest virus, cells were scraped from the flask and sonicated for 2 minutes in an ice bath before being aliquoted into cryovials and snap freezing in liquid nitrogen.

2.3.1 Titration of RSV

To calculate the level of virus propagated serial dilutions of the viral stock is made and used to infect permissive cells. Hep2 are used for RSV-A2-GFP. Cells are seeded into 96 well plated with $2 \times 10^4$ cells per well in 200µl complete DMEM, cells are incubated for 24h until confluent. Media is removed from the cells and gently washed with warmed PBS. Serial dilutions of viral stocks are added to the wells in triplicate, alongside stocks from the control flask. The plate is then returned to the incubator for 2h to allow the virus to infect the cells. The inoculum is then removed from the wells and replaced with 200µl complete DMEM. The
plate is returned to the incubator for 5 days. The media is discarded and the plate washed with PBS. Cells are fixed by adding 100µl of 2% hydrogen peroxide in methanol to each well, taking care not to disturb the cell monolayer. The monolayer is washed with 1% BSA in PBS and stained with biotinylated anti-RSV F diluted in 1% BSA for 1h at room temperature. The plate is washed twice with 1% BSA, followed by 100ul of a 1 in 500 dilution of Extravidin Peroxidase (Merck, USA) added to each well for 1h at room temperature. and focus forming units (FFU) are counted. To calculate the multiplicity of infection (MOI): MOI= FFU/ number of cells infected.

2.4 Propagation of HIV
HEK293T cells were transfected with HIV-1 IIIB infectious molecular clones. 48 h later virus containing supernatants were removed. Supernatants from empty vector transfected cells were used for mock infections.

2.5 HL-60 cell transfection
For transfection of HL-60 cells invitrogens neon transfection kit was chosen. 3ml of prewarmed cRPMI was plated into each well of a 6 well plate. Cells were collected when 80-90% confluent at 1million cells/ml. 18ml of cell culture was spun at 300xg for 5 minutes and washed once with PBS. The cell pellet was resuspended in a volume of 650µl of buffer R. The IRF1 and RLUC (control) siRNA was prepared and diluted to a concentration of 10nM. The resuspended cell pellet was slowly added to the siRNA in a sterile 1.5ml microcentrifuge tube and gently resuspended. The neon Transfection tube was set up and was filled with 3ml of electroporation buffer into the neon pipette station. Cells were pulsed
using different voltage(v), pulse width(pw) and pulse number(pn). The three pulse conditions chosen were (1350v, 35pw, 1pn) (1250v, 20pw, 2pn) (1650, 25pw, 1pn). The neon tip was added to the cell suspension siRNA mix and a fixed volume of 100µl was extracted. The neon pipette was placed into the neon pipette holder and the electric pulse was delivered. The pulsed cells were then immediately added to the prewarmed media in the 6 well plate and incubated for 24 hours.

2.6 Stimulations and treatments

After seeding, the PMNs were incubated at 37°C for 30 mins. Cells were stimulated for 30 minutes with a range of inhibitors before subsequently being stimulated with a range of agonists.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>CU-CPT-9a (TLR8 inhibitor)</td>
<td>5nM</td>
</tr>
<tr>
<td>MRT67307 (TBK1 inhibitor)</td>
<td>5nM</td>
</tr>
<tr>
<td>BAY11-7082 (NF-κB inhibitor)</td>
<td>5µM</td>
</tr>
<tr>
<td>SB 203580 (P38 inhibitor)</td>
<td>20µM</td>
</tr>
<tr>
<td>PD 98059 (ERK inhibitor)</td>
<td>20µM</td>
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*Table 2.1: List of intracellular signalling inhibitors*
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL075 (TLR8 agonist)</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>LPS (TLR4 agonist)</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>IFN-α</td>
<td>1000IU</td>
</tr>
<tr>
<td>PMA</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>HIV-IIIB</td>
<td>1.7ng RT/10⁶ cells</td>
</tr>
<tr>
<td>RSV-A2</td>
<td>MOI 1</td>
</tr>
<tr>
<td>DMSO</td>
<td>1%-3%</td>
</tr>
</tbody>
</table>

*Table 2.2: List of cell culture agonists and viruses*
2.7 Immunoblotting

PMNs were treated with IFN-2α (1000IU) (Sigma) over a time course of 0-60mins. All cells harvested for western blotting were lysed using radioimmunoprecipitation assay (RIPA) buffer (1M tris (hydroxymethyl) aminomethane (Tris) pH 8.0, 5M NaCl, 20% v/v Triton X-100, 20% w/v SDS made up in dH₂O, supplemented prior to use with the protease inhibitors 5µg/ml of leupeptin, 1mM of phenylmethylsulfonyl fluoride (PMSF), 1mM of sodium orthovanadate (Na₃VO₄) and 1mM of dithiothreitol (DTT). For cell lysis, cells were incubated on ice for 30mins. The cells were then centrifuged at 12,000 x g for 4°C for 15 minutes. Lysates were harvested and stored at -80°C until further use.

BIORAD’s gel kit was used to perform SDS-PAGE. This involved casting a 12% resolving gel and a 10% stacking gel. The gels were assembled using BioRad apparatus with 1X running buffer. Gels were loaded with 10µl of sample and 2µl of molecular weight marker. Gels were “run” at 80V until the protein had run through the stacking gel, before the voltage was increased to 100V and samples ran until the protein reached the end of the gel.

Proteins were transferred to a PVDF membrane. The membrane was soaked in methanol for 30 seconds and rinsed with 1X transfer buffer. Blotting paper and sponges were soaked in 1X transfer buffer. The cassettes were assembled as follows: sponge, blotting paper, membrane, gel, blotting paper, sponge. Air bubbles were removed before being run on the biorad transfer apparatus at 380 mA for 2 hours.
The membranes were then blocked for 1h at room temperature, using either 5% Milk or BSA in 1X TBST (Tris buffer saline with tween) to prevent any unspecific binding. The blots were then incubated in primary antibody at 4°C overnight.

**Resolving gel**

<table>
<thead>
<tr>
<th>Gel Percentage</th>
<th>12.5%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>6.25 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>3.55 ml</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>1.5M Tris-HCL pH 8.8</td>
<td>5.6ml</td>
<td>5.6ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150µl</td>
<td>150µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>150µl</td>
<td>150µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15µl</td>
<td>15µl</td>
</tr>
</tbody>
</table>

*Table 2.3: Composition of Resolving Gel*

**Stacking gel**

<table>
<thead>
<tr>
<th>Acrylamide</th>
<th>1ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>4.61</td>
</tr>
<tr>
<td>1M Tris-HCL PH 6.6</td>
<td>750µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>60µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>60µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>12µl</td>
</tr>
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</table>

*Table 2.4: Composition of Stacking Gel*
Running Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM Tris</td>
<td>30g</td>
</tr>
<tr>
<td>1.8 M glycine</td>
<td>144g</td>
</tr>
<tr>
<td>1% w/v SDS</td>
<td>10g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Make up to 1L</td>
</tr>
</tbody>
</table>

Table 2.5: composition of running buffer

10X Transfer Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8M glycine</td>
<td>30g</td>
</tr>
<tr>
<td>250mM Tris</td>
<td>144g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Make up to 1L</td>
</tr>
</tbody>
</table>

Table 2.6: Composition of 10X transfer buffer

1X transfer buffer is used with 20% methanol

Lysates were analysed by immunoblotting using antibodies against the following antibodies:

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Description</th>
<th>Species</th>
<th>Company</th>
<th>Probing dilution</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>p-ERK</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>p38</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
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<tr>
<td>p-p38</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>Antibody</td>
<td>Type</td>
<td>Species</td>
<td>Source</td>
<td>Dilution</td>
<td>Conjugate</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>---------</td>
<td>--------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>IκB-α</td>
<td>Mouse mAb</td>
<td>Mouse</td>
<td>University of Edinburg h gift</td>
<td>1:1000</td>
<td>Anti-mouse HRP</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Mouse mAB</td>
<td>Mouse</td>
<td>Thermo fisher</td>
<td>1:2000</td>
<td>Anti-mouse HRP</td>
</tr>
<tr>
<td>IRF1</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>PSTAT3</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>STAT1</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>pSTAT1</td>
<td>Rabbit mAb phosphorylated on sites</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>STAT2</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>pSTAT2</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>STAT3</td>
<td>Rabbit mAb</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>pIRF7</td>
<td>Rabbit mAb phosphorylated on sites Ser471 and 472</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-rabbit HRP</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Mouse mAb</td>
<td>Mouse</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Anti-mouse HRP</td>
</tr>
</tbody>
</table>

*Table 2.7: western antibodies*
2.8 RNA extraction using Norgen Biotek corp

RNA was isolated as per the manufactures protocol. Cell suspension was transferred to an RNase free tube and centrifuged for 200xg for 10 minutes to pellet the cells. 350µL of buffer RL (guanidinium salts) and 200µl of 100% ethanol was added, vortexed and added to the RNA column. The column was spun at 3500xg to allow the RNA to bind. The RNA was subsequently washed 3 times with a wash buffer before being eluted in an RNase free tube.

2.8.1 Reverse transcription (cDNA synthesis)

250ng of total RNA from each sample was reverse transcribed into cDNA using the SensiFAST reverse transcription cDNA kit (Bioline, MSC, Dublin), using the following reaction: 0.25µl of reverse transcriptase and 2.25µl of 5X Transamp buffer was added to 250ng RNA and made up to 17.5µl in RNase free DNAse free H$_2$O (Sigma). Following this, the reaction was placed on the thermocycler at 25°C for 10 minutes (annealing), 42°C for 15 minutes (reverse transcription), 85°C for 5 minutes (inactivation of the enzyme) and 4°C hold. cDNA was quantified using the NanoDrop ND-1000 spectrophotometer.

2.8.2 Quantitative RealTime-Polymerase Chain Reaction (qRT-PCR)

Each qRT-PCR reaction was carried out in duplicate in a 96-well PCR plate. Total volume per well was 10 µl:1µl of a 1 in 10 dilution of cDNA, 4µl of SYBR green PCR master mix (Biorad), 0.5 µl of forward and reverse primer and 4 µl of water. qRT-PCR was performed using the Biorad quantitative PCR system using the following cycling parameters: 95°C for 15 minutes, 92°C for 30 seconds, 65°C for
1min, 72°C for 30sec. The cycle was repeated 40 times. All gene amplifications were normalised to β-Actin.

2.8.3 qRT-PCR data analysis

Data analysis was carried out using the 2-ΔΔCt method. A ratio of gene of interest relative to housekeeping gene (β-Actin) was generated. β-Actin was selected as it was found to be the most stably expressed gene, compared to other normalisers (including glyceraldehyde 3-phosphate dehydrogenase [GAPDH] in peripheral blood cells by previous lab members). ΔCt was calculated by subtracting the housekeeping Ct value from gene of interest Ct value. To calculate the ΔΔCt values, the ΔCt value of the control was subtracted from the ΔCt value of each test sample. To calculate the expression fold change, the ΔΔCt is inputted into the formula 2-ΔΔCt.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward primer sequence (5’ – 3’)</th>
<th>Reverse primer sequence (3’ - 5’)</th>
<th>Primer supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISG15</td>
<td>TCCTGCTGGTGGTGGAACAA</td>
<td>TTGTTATTCCTCACCAGGAT</td>
<td>Sigma</td>
</tr>
<tr>
<td>MXA</td>
<td>GGTGGGTGTCACCATGTTG</td>
<td>ACCACGTCACAGCCTGTCTG</td>
<td>Sigma</td>
</tr>
<tr>
<td>OAS</td>
<td>GAAGCCCTACAAAGAATGCTAGA</td>
<td>TCGGAGTTGCTCCTTAAGACTG</td>
<td>Sigma</td>
</tr>
<tr>
<td>VIPERIN</td>
<td>CTTGGAAGGAGGAGACATGACCAGAAG</td>
<td>CCGCTCTACCAATCCAGGCTG</td>
<td>Sigma</td>
</tr>
<tr>
<td>IL-8</td>
<td>GCAGAGGGTTGTGGAGAG</td>
<td>ACCCTACAACAGACCCACCA</td>
<td>Sigma</td>
</tr>
<tr>
<td>IL-6</td>
<td>TCCACAAAGCGCCTTCCTGTCG</td>
<td>GTGGCTGTCTGTGTGGGCG</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Table 2.8: primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td><em>TLR4</em></td>
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<td>TCCCACTCCAGGTAAGTT</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>TLR7</em></td>
<td>AGTGTCTAAAGAACCTGG</td>
<td>CTTGGCCTTACAGAAATG</td>
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</tr>
<tr>
<td><em>TLR8</em></td>
<td>CAGAATAGCAGGCGTAACACATCA</td>
<td>AATGCACAGGTGCATTCAAGGG</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>ACTIN</em></td>
<td>GGACTTCGAGCAAGAGATGG</td>
<td>AGCACTGTGTAGCCGTACA</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>IRF1</em></td>
<td>ATACCTTCTCTGATGGACTC</td>
<td>GAAGTTGTACAGATCATG</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>IRF3</em></td>
<td>TCTGCCCTCAACCAGCAAGAG</td>
<td>TACTGCCTCCACCATTGGTC</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>IRF5</em></td>
<td>TagGGCTACCCAGGAGCAA</td>
<td>GCCCCACTCCAGAACACTTA</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>IRF7</em></td>
<td>CCACTCATATCTACCTACTGG</td>
<td>GCTGCTATCCAGGAAGACACA</td>
<td>Sigma</td>
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<tr>
<td><em>IRF8</em></td>
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<td>TAATCGCCACAGAAGGCTC</td>
<td>Sigma</td>
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<td>CAGCCGAGTCTTTCCAGACA</td>
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<tr>
<td><em>TNF</em></td>
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<tr>
<td><em>IL-1β</em></td>
<td>GCACGATGCACCTGTACAT</td>
<td>CACCAAGCTTTTTTGTGTAG</td>
<td>Sigma</td>
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</table>

2.9 NETosis Assay

NETs were measured according to Abcams NETosis assay protocol. Briefly PMNs were resuspended in pre warmed NETosis assay buffer (RPMI with 1% BSA and 0.1% calcium chloride). Cells were treated with 50nM of PMA and RSV at an MOI of 1 for 4 hours at 37°C. After 4 hours the supernatants were removed from the well and each well washed with prewarmed NET assay buffer. 500µl of diluted S7 nuclease (1:1000) in NET assay buffer was added to each well, and allowed to incubate for 15 mins at 37°C to disrupt the NETS. Supernatants were
added to microcentrifuge tubes in which 10µl of EDTA (500mM) was added to stop the reaction and spun at 300xg to remove any excess cellular debris. 100µl of standards and samples were added to the 96 well plate. 100µl of the 1:30 diluted NET assay neutrophil elastase substrate was added and incubated at 37°C for 2 hours. Absorbance was measured at 405nm on a 96 well plate reader.

2.10 ROS assay
PMNs (1x10^6) were stimulated with either CL075 or RSV for the indicated timepoints. Cells were treated with 5µM of cellrox (Invitrogen) green for 30 minutes at 37°C. Cells were spun at 300xg. Media discarded and cells washed twice using PBS before visualisation using flow cytometry.

2.11 Flow cytometry staining
Extracellular antibodies CD16, CD66b, CD11b, CD40, CD86, CD4, CD3, CD8, TLR8 (Biolegend) were added at a 1:100 dilution and incubated at 4°C for 20 minutes. The cells were spun at 300g for 5 minutes at room temperature and washed once with PBS. 100µl of 1X BD Cytofix/Cytoperm was added to each well, resuspended thoroughly to ensure no clumping and incubated at room temperature in the dark for 20 minutes. 100µl of BD 1x permwash (1 part permwash, 9 parts deionised water) was added. Cells were spun at 300g for 5 minutes. Cells were washed once more in 1x permwash prior to intracellular staining. Cells were resuspended in 50µl of 1x permwash that contained the intracellular antibody mastermix (1:50 dilution) and placed in the dark for 20 minutes. Cells were washed once in 1x permwash and then resuspended in PBS before acquisition.
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Company</th>
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<th>Fluorochrome</th>
</tr>
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<tr>
<td>CD8</td>
<td>Biolegend</td>
<td>563919</td>
<td>BV510</td>
</tr>
<tr>
<td>Live/Dead</td>
<td>Biolegend</td>
<td>558537</td>
<td>BV510</td>
</tr>
<tr>
<td>CD11b</td>
<td>Biolegend</td>
<td>612597</td>
<td>PE/Cyanine7</td>
</tr>
<tr>
<td>CD66b+</td>
<td>Biolegend</td>
<td>305118</td>
<td>APC</td>
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<td>CD16</td>
<td>Biolegend</td>
<td>302037</td>
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<td>48-0409-42</td>
<td>efluor405</td>
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<td>Biolegend</td>
<td>305411</td>
<td>APC</td>
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<tr>
<td>CD3</td>
<td>Biolegend</td>
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<td>APC</td>
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<tr>
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<td>AF700</td>
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</table>

Table 2.9: Fluorescently conjugated antibodies and dyes

2.12 Enzyme Linked Immunosorbent Assay (ELISA)

To measure the IL-6, IL-1β, IL-8 and TNF protein production of neutrophils primary human PMNs sandwich ELISA kits were used. 96-well high binding microplates were coated with 100μl/well of specific antibody, diluted 1:200 (according to biolegend) in 1x coating buffer and incubated at 4 degrees overnight. The following morning the plates were washed with 1% (v/v) PBS with tween by immersing the plate into wash buffer and then completely removing the
wash buffer by inverting and tapping the plate on tissue paper. This was done 3-4 times. The plate was then blocked for 2 hours with 200µl of 1x reagent diluent (FBS in PBS) in order to inhibit non-specific binding. The plates were washed as described above and in accordance with the manufacturer’s protocol the standards were added in triplicate. Samples were diluted 1/4 for IL-6 and IL-8 and 1/2 for TNF and IL-1β. Plates were allowed to incubate at 4°C overnight.

The following day the plate was aspirated and washed 4 times. 100µl of secondary biotin labelled antibody in accordance with the manufacturer’s recommended concentration were added to the plate and allowed to incubate at room temperature for 2 hours. The plate was washed 4 times before 100µl of HRP-Streptavidin conjugate (SABC) working solution was added to each well for 30 minutes at 37°C. The plate was washed 5 times. 100µl of the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and incubated for 15-30 minutes at 37°C for colour development and ELISA assays were stopped with 50µl/well of “stop solution” (Phosphoric acid). Colour development was read at 450nm on a microplate reader.

2.13 Statistical analysis
Statistical comparisons were performed using GraphPad Prism statistical analysis software (version 6). Data is represented as the mean ± SEM unless otherwise stated. Differences between two groups at a single time point were analysed using a paired t-test. Differences between multiple groups at multiple different time points were analysed using a one way ANOVA with a tukey correction post-test. A p value <0.05 was considered significant.
Chapter 3

TLR8 is an important endosomal receptor in the recognition of ssRNA virus RSV which drives an inflammatory response in primary human PMNs
Chapter 3: TLR8 is an important endosomal receptor in the recognition of the ssRNA virus, RSV, which drives an inflammatory response in primary human PMNs

3.1 Introduction

Neutrophils are bone marrow derived leukocytes with a short circulating half-life and are a fundamental part of the innate inflammatory response. It is estimated that 1 billion neutrophils are made per day for every kilogram of bodyweight, and can increase 10 fold in times of infection (163). They are classically portrayed as unsophisticated phagocytic cells with a role limited to the engulfment and subsequent elimination of invading extracellular pathogens. While their role in bacterial infections is well characterised, studies have also detected them in the lungs and bronchoalveolar lavage (BAL) of mice and humans after infection with respiratory viruses such as human metapneumovirus (HMPV), human respiratory syncytial virus (HRSV; herein referred to as RSV) and coronavirus (164) (165) (166). The infiltration of neutrophils in the respiratory tract is often associated with an increase in morbidity and mortality. It is postulated that activated neutrophils can contribute to the increase in patient morbidity through the production of neutrophil specific cytokines and NETS, which facilitate the formation of microthrombi, resulting in reduced oxygenation in the individual (167).

3.1.2 Neutrophils and the pathophysiology of RSV

The vast majority of human respiratory viral infections can cause bronchitis. RSV is the leading cause of bronchitis-induced hospitalisations in children aged 1-2 (168). RSV infects epithelial cells of both the lower and upper respiratory tract, causing extensive inflammation characterised by neutrophilia, mucus
hypersecretion and oedema in the airways (169). Due to the difficulties associated with acquiring paediatric samples it is still unclear how neutrophils respond to RSV infection. However, due to the high prevalence of these cells in the alveoli and airway walls, it’s postulated that these cells are not innocent bystanders and contribute to RSV-induced lung pathology. This has been demonstrated using a mouse RSV infection model, in which neutrophil depletion reduced the levels of MPO, MMP-9 and TNF-α (152). Additionally, it was demonstrated that neutrophil depletion in human RSV infection resulted in significantly less TNF in the BAL, highlighting that neutrophils do in part contribute to the overall inflammatory response, either directly or indirectly through the activation of surrounding immune cells (170).

3.1.3 Neutrophil Inflammatory mediators
These inflammatory and immunomodulatory properties are the result of activation of cell intrinsic pathogen sensors. Innate immune cells have evolved a unique set of molecular sensors for the detection of extracellular pathogens and intracellular pathogens, known as PRRs. There are currently 10 different TLRs found in humans which are characterised by their subcellular location. Upon TLR activation they initiate signalling pathways which allow for the activation of pro-inflammatory cytokines such as TNF-α and type 1 IFNs. In contrast to PAMP activation, neutrophils have been well documented to respond to DAMPs. These include HMGB1, which can directly activate neutrophils, as well as indirectly recruit them through the activation of epithelial cell derived IL-8. While it is important to note that PRRs are essential in the development of an antiviral protective response, over activation can result in the exacerbated pathology
associated with RSV. This double edged sword has been highlighted using mice deficient in TLR7 which develop severe pathology when infected RSV (171). In contrast, novel approaches to antagonise TLR7 have been beneficial in the treatment of immune mediated inflammatory disorders (IMID) (172). While TLR4 is well characterised by its recognition of extracellular bacteria associated LPS, it has also been implicated in the disease pathology associated with RSV. This has been highlighted in studies in which the F protein of RSV is known to interact with TLR4 via MD-2 and enhance inflammatory mediators such as IL-1β in a TLR4 expressing HEK cell line. Neutrophil-associated TLR interaction with viral respiratory infections are less well characterised. Neutrophils can detect and be activated by a range of viruses through both TLR7 and TLR8 however their role in the recognition of RSV remains poorly understood (173).

TLR8 has been shown to be required for the production of IL-6 from cardiac cells during Coxsackie B virus and the induction of IL-1β from CD4+ T-cells during HIV infection (174) (26). Additionally, it has been shown that HBV can decrease levels of TLR8 in human PBMCs, thereby potentially inhibiting the innate immune response (175). While it is known that the TLR7/8 signalling pathways are involved in recognition of virally associated ssRNA, the functional consequences of TLR7/8 activation in neutrophils remains poorly understudied. While both receptors share similarities in structure and their ability to recognise ssRNA pathogens, recent studies have demonstrated they activate different pathways. These studies have been predominantly carried out in human monocytes, therefore highlighting the need to explore the role of granulocyte endosomal TLRs in the induction of inflammatory responses during ssRNA infections (176).
Figure 3.1: RSV induced TLR8 activation in human PMNs

PMNs selectively utilise TLR8 in the recognition of ssRNA infections such as RSV intracellularly. TLR8 activation results in the activation of both the MAPK and NF-κB signalling pathways. These pathways are responsible for the production of inflammatory mediators including IL-1β, TNF-α, IL-8 and IL-6. Created using biorender.
3.2 Hypothesis

Since neutrophils are heavily implicated in the disease pathogenesis associated with viral respiratory infections, we wanted to ascertain the molecular mechanism that leads to this extreme inflammatory response. Since TLR4 has been characterised in the generation of inflammatory responses in neutrophils, we hypothesised that endosomal TLR8 which specifically recognises ssRNA infections could also exacerbate inflammatory responses observed in viral infections (Fig 3.1). As critical regulators of antiviral and inflammatory responses, we hypothesised that these endosomal receptors could regulate intracellular signalling pathways that promote the inflammatory response.

3.3 Specific aims

1. To investigate the involvement of endosomal TLR8 in the generation of inflammatory responses in PMNs.

2. Determine what intracellular signalling pathways are involved in the generation of this inflammatory response in PMNs.

3. Examine the role of TLR8 recognition of the ssRNA virus, RSV, in the generation of the inflammatory response in PMNs.

4. Investigate how these PMN-derived inflammatory mediators modulate the surrounding immune response through the modulation of both DCs and T-cells.
3.4 Results

3.4.1 CL075 induces the expression of the activation marker CD11b in CD66b+/CD16+ PMNs

It has previously been reported that neutrophils express a range of receptors including markers such as CD15, CD16, CD44, CD45, CD66b and CD11b (110). CD11b is an integrin marker responsible for adhesion-related associations between granulocytes and epithelial cells. It facilitates the extravasation of granulocytes from the blood into the associated tissues (177). It has therefore been described as a marker of granulocyte activation. We found that granulocytes isolated using the dextran sedimentation protocol yielded a 95% pure population of PMNs, measured using their co-expression of both CD16 and CD66b (Fig 3.2A). In addition, to investigate the potential activation of these PMNs via endosomal TLRs, we treated with CL075 (a synthetic agonist designed to specifically bind both TLR7 and TLR8) at a concentration of 2µg/ml for 2 hours. LPS, a known activator of PMNs, was used as a positive control, at a concentration of 100ng/ml for 2 hours (178). We observed that CL075 significantly enhanced the expression of CD11b (Fig 3.2B) on the primary human PMNs, revealing a potentially novel mechanism by which pathogens could activate these cells, possibly via TLR8.
Figure 3.2: TLR8 activation of human PMNs

Human PMNs (1x10⁶) were rested in serum free media for 30min before treating with the TLR7/8 agonist CL075 (2µg/ml) or LPS (100ng/ml) for 2 hours. (A) represents the gating strategy, in which PMNs were gated on the single+ live+ CD16+ CD66+ population. (B) representative histogram of the median fluorescent intensity (MFI) fold change of CD11b relative to the untreated control, respectively. Data is represented as the mean ± SD. Paired t-test. *p<0.05 (n=4).
3.4.2 CL075 induces the mRNA expression of proinflammatory cytokines

It has previously been reported that human PMNs express and produce cytokines constitutively or upon an activation signal (179). Neutrophils express a plethora of receptors including cytokine receptors, G-protein coupled receptors and Fcy receptors and importantly, PRRs, that trigger cytokine production. Neutrophils express all TLRs, with the exception of TLR3 (173). The production of cytokines can be regulated through mRNA transcription in neutrophils. It is also important to highlight that both immature and mature neutrophils can store intracellular levels of cytokines inside vesicles (180). Human neutrophils respond well to LPS induced TLR4 activation, triggering the MyD88 dependent pathway, notably inducing the expression of proinflammatory cytokines, such as IL-6. In addition, IFN-α has been demonstrated to enhance the production of IL-6 through activation of TLR8 in human neutrophils (181). To establish whether CL075, the endosomal (TLR7/8) agonist and/or IFN-α can induce the expression of inflammatory cytokine transcripts, primary human PMNs were stimulated with CL075 (2µg/ml) and/or IFN-α (1000IU) for 12 and 24 hours. mRNA transcripts of (Fig 3.3A) IL-6, (Fig 3.3B) IL-8, (Fig 3.3C) TNF and (Fig 3.3D) IL-1β were quantified by qRT-PCR. While not statistically significant, we observed a time dependent 5-fold increase in IL-6, 50-fold increase in TNF and 15-fold increase in IL-1β, with levels for each transcript higher at the 24 hour timepoint in response to CL075. In agreement with the aforementioned study, while not significant we observed that IFN-α/CL075 co-stimulation somewhat enhanced expression of IL-6 (Fig 3.3A). We additionally observed an synergistic increase in levels of TNF (Fig 3.3C) and IL-1β (Fig 3.3D). In contrast, IL-8 (Fig 3.3B) transcripts were most highly upregulated at 12 hours and declined thereafter at 24 hours. We observed
no synergistic effect of IFN-α on CL075 induced expression of IL-8 at both 12 or 24 hours after stimulation, suggesting that IFN-α enhanced expression of cytokines varies depending on the cytokine of interest.

**Figure 3.3: The TLR7/8 agonist CL075 induces the expression of IL-6, IL-8, TNF and pro-IL-1β mRNA in primary human PMNs**

Human PMNs (1x10⁶) were rested in serum free media for 30min before treating with the TLR7/8 agonist CL075 (2µg/ml), IFN-α (1000IU) or co-stimulated with both CL075 and IFN-α for 12 and 24 hours. Using qRT-PCR (A) IL-6 (B) IL-8, (C) TNF and (D) IL-1β mRNA levels were quantified and calculated relative to the house keeping gene, β-Actin. All samples were compared with the unstimulated control (no CL075), which was normalised to 1. Data is representative of the mean ± SD (n=3).
3.4.3 TLR8/CL075 signalling of primary human PMNs induces proinflammatory protein expression of cytokines

Having observed that the TLR7/8 agonist, CL075, induces proinflammatory cytokine mRNA in primary human PMNs (Fig 3.3), we next investigated which receptor specifically induced the inflammatory response and if the increase in inflammatory transcripts correlated with an increase in secreted protein levels of the cytokines. LPS, a known ligand for TLR4, was used as a positive control for the induction of inflammatory cytokines. PMNs were pre-treated with the TLR8 inhibitor, Cu-cpt9a, for 30 minutes, prior to being stimulated with either LPS or CL075 for both 12 and 24 hours. Supernatants were collected and analysed for cytokine levels using ELISA. We observed a significant increase in levels of IL-6 (Fig 3.4A) and TNF (Fig 3.4C) at 24 hours after stimulation with CL075. Additionally we observed a significant increase in levels of IL-8 (Fig 3.4B) and IL-1β (Fig 3.4D) at both 12 and 24 hours after stimulation with CL075. Interestingly, we observed that LPS, our positive control failed to induce the expression of TNF protein from PMNs at both 12 and 24 hours, suggesting TLR8 specificity for the induction of TNF in primary human PMNs. By selectively inhibiting TLR8, using the TLR8 inhibitor, CU-CPT9a, we found a significant abrogation in levels of IL-6 (Fig 3.4A), IL-8 (Fig 3.4B) and IL-1β (Fig 3.4D) at both 12 and 24 hours PMNs treated with CL075. We also noted a significant reduction in CL075 induced TNF at the 24 hour timepoint when PMNs were pre-treated with Cu-cpt9a. It’s also important to note that pre-treating with the TLR8 inhibitor and subsequently treating with LPS there were no significant differences between the two groups, proving the high specificity of Cu-cpt9a for inhibiting TLR8.
Figure 3.4: TLR8/CL075 signalling of primary human PMNs induces protein expression of proinflammatory cytokines

Human PMNs (1x10^6) were rested in serum free media for 30 min before treating with the TLR8 inhibitor, CU-CPT9a, for 30 min prior to subsequent stimulations for 12h or 24h with either LPS or CL075. Supernatants were collected and (A) IL-6 (B) IL-8, (C) TNF and (D) IL-1β protein levels were quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 (n=6)
3.4.4 NF-κB and MAPK inhibitors do not induce cell death in primary human PMNs

In order to determine the role of intracellular signalling pathways in inducing the observed inflammatory cytokine induction in human PMNs, we next investigated the cytokine pathways involved by treating with NF-κB and MAPK inhibitors. Before our cytokine pathway analysis, we determined the effect of these inhibitors upon primary human PMN viability. PMNs were pre-treated for 30 minutes with the NF-κB (Bay-11) and MAPK (PD98059-ERK/SB303580-P38) inhibitors at a concentration of 5µM and 20µM, respectively. PMNs were also stimulated with the positive controls LPS or CL075 at both 12 and 24 hours at a concentration of 100ng/ml and 2µg/ml, respectively. Cell viability was determined using fixable viability stain 510, with positive cells indicative of dead cells using flow cytometry. The treatment of primary human PMNs with either LPS or CL075 without the pretreatment of MAPK or NF-κB inhibitors resulted in no difference in the percentage of live cells, compared to the unstimulated negative control at both 12 and 24 hours, with viability between 80-90% (Fig 3.5B/3.5C). The addition of the MAPK and NF-κB inhibitors and subsequent stimulation with either LPS or CL075 resulted in no additional cell death, with cell viability again determined to be between 80-90% after both 12 and 24 hours (Fig 3.5B/3.5C). Having determined that LPS/CL075 stimulation with or without the addition of the aforementioned inhibitors resulted in no significant cell death, we hypothesised that future variation in cytokine production was specifically due to the inhibition of specific pathways and not an artifact cell death.
Figure 3.5: Effect of MAPK and NF-κB inhibitors on the viability of primary human PMN

Human PMNs (1x10^6) were rested in serum free media for 30min before treating with the NF-κB inhibitor (BAY-11), p38 inhibitor (SB303580) and ERK inhibitor (PD98059) for 30 minutes prior to stimulations with either 100ng/ml of LPS or 2ug/ml of CL075 for 12 and 24 hours. (A) are the representative flow cytometry plots of the Live/dead Fixable viability 510 at 24 the 24 hour timepoint with inhibitors and CL075/LPS stimulation. Graph (4.B) and (4.C) are the corresponding bar charts for 12 and 24 hours, respectively. (n=1)
3.4.5 NF-κB signalling is essential for TLR8/CL075 mediated induction of inflammatory cytokines IL-6, IL-8, IL-1β and TNF in primary human PMNs.

Since our experiments highlighted the importance of TLR8 in the induction of proinflammatory cytokines from PMNs (Fig 3.4), we subsequently investigated the role of the transcription factors NF-κB and MAPK downstream of TLR8. Primary human PMNs were pre-treated with the NF-κB inhibitor, Bay-11 (5µM), for 30mins and subsequently stimulated with LPS, the positive control, or CL075, for both 12 and 24 hours before supernatants were measured for secreted proinflammatory cytokines IL-6 (Fig 3.6A), IL-8 (Fig 3.6B), TNF (Fig 3.6C) and IL-1β (Fig 3.6D) by ELISA. When pre-treating with Bay-11, the NF-κB inhibitor, TLR8-specific induction of all four aforementioned cytokines were significantly abrogated after either 12 or 24 hours CL075 treatment. These findings indicated that CL075/TLR8 signals via NF-κB to regulate the expression of IL-6, TNF, IL-1β and IL-8 production.
**Fig 3.6: NF-κB signalling is essential for TLR8/CL075 mediated induction of inflammatory cytokines IL-6, IL-8, IL-1β and TNF in primary human PMNs**

Human PMNs (1x10⁶) were rested in serum free media for 30min before treating with the NF-κB Inhibitor, bay-11, at a concentration of 5µM for 30 mins prior to subsequent stimulations. Cells were treated for 12h or 24h with either LPS (100ng/ml) or CL075 (2µg/ml). Supernatants were collected and (A) IL-6, (B) IL-8, (C) TNF, (D) IL-1β protein levels was quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. **p<0.01 ***p<0.001 (n=6)
3.4.6 TLR8 agonist CL075 signals via the canonical NF-κB pathway (degradation of IκB) in primary human PMNs

Having identified a critical role of NF-κB signalling in the induction of TLR8 induced proinflammatory cytokines (Fig 3.6), we next investigated if this was mediated via canonical NF-κB signalling. The canonical NF-κB pathway is activated through an array of different immune receptors and involves the rapid degradation of IκB and subsequent nuclear translocation of the canonical transcription factors p50, RELA and c-REL (182). The non-canonical pathway responds to signals from a subset of the tumour necrosis factor receptor (TNFR) family and involves the activation of NF-κB-inducing kinase (NIK), and subsequent nuclear translocation of non-canonical NF-κB members, including p52 and RELB (183). LPS induced TLR4 activation is well known to induce the degradation of IκB through the canonical pathway in primary human macrophages, and was therefore used as the positive control for examining the role of the canonical NF-κB pathway in primary human PMNs (184). PMNs were stimulated with either LPS or CL075 over a timecourse of 1 hour and whole cell lysates collected and blotted for IκB using western blotting. PMNs treated with CL075 (Fig 3.7A) and LPS (Fig 3.7B) over the course of 1 hour saw reductions in levels of IκB as soon as 5 mins post treatment (Fig 3.7C/D), revealing the novel rapid role canonical NF-κB signalling plays in the induction of TLR8 induced inflammatory cytokines in primary human PMNs.
Figure 3.7: The TLR8 agonist, CL075, promotes the degradation of IκB via the canonical NF-κB pathway in primary human PMNs

Human PMNs (1x10⁶) were rested in serum free media for 30 mins before being stimulated with 2µg/ml of CL075 or 100ng/ml of LPS over a timecourse ranging from 3 mins to 60 mins. Protein lysates were collected and blotted for (A+B) IκB. Densitometric analysis of 3 immunoblots was performed using the image lab software. Representative densitometric analysis for (C) CL075 degradation of IκB and (D) LPS induced degradation of IκB were analysed as a ratio of IκB: β-Actin ,relative to the untreated (PBS) control, which was normalised to 1. Statistical differences were assessed using one way ANOVA with a Tukey (n=3)
3.4.7 MAPK signalling is necessary for the induction of proinflammatory cytokines, IL-8 and IL-1β, but not IL-6 or TNF, downstream of TLR8 in primary human PMNs

Having observed that NF-κB signalling was required for proinflammatory cytokine (IL-6, IL-8, TNF and IL-1β) induction via TLR8 in primary human PMNs (Fig 3.6/3.7), we next wondered if other innate signalling pathways were also involved, including the MAPK pathway. The MAPK pathway is known to regulate a range of different functions from cell proliferation, apoptosis and innate immune defences (185). MyD88-dependent TLR signalling has been well established to regulate the AP-1 signalling pathway through MAP kinases, such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (186). These MAPKs are involved in the induction of a plethora of cytokines including IL-10, IL-4, IFN-γ, IL-8 and IL-1β (187) (188) (189). Primary human PMNs were pre-treated with SB303580 and PD98059 inhibiting both p38 and ERK, respectively, and subsequently stimulated with CL075 or LPS for 12 and 24 hours, before supernatants were measured for secreted proinflammatory cytokines IL-6 (Fig 3.8A/Fig 3.9A), IL-8 (Fig 3.8B/Fig 3.9B), TNF (Fig 3.8C/Fig 3.9C) and IL-1β (Fig 3.8D/Fig 3.9D) by ELISA. Inhibiting both ERK and p38 significantly inhibited IL-8 (Fig 3.8B/Fig 3.9B) and IL-1β (Fig 3.8D/Fig 3.9D) at both 12 and 24 hours post CL075 stimulation. In contrast, both TNF (Fig 3.8C/Fig 3.9C) and IL-6 (Fig 3.8A/Fig 3.9A) were not affected by the inhibition of ERK and p38 signalling downstream of TLR8. Additionally, the inhibition of ERK and p38 signalling in part reduced the expression of LPS/TLR4 induced IL-8 and IL-1β, however not to the same degree as TLR8, in which we observed a 50% reduction.
in secreted protein. These findings indicated that CL075/TLR8 signals via MAPK to regulate the expression of IL-1β and IL-8 production.

Figure 3.8: ERK signalling is essential for the induction of IL-8 and IL-1β, but not TNF or IL-6, downstream of TLR8 in primary human PMNs

Human PMNs (1x10^6) were rested in serum free media for 30 min before treating with the ERK Inhibitor PD98059 (20μM) for 30 mins prior to subsequent stimulations with either LPS (100ng/ml) or CL075 (2μg/ml) for 12 and 24 hrs. Supernatants were collected and (A) IL-6, (B) IL-8, (C) TNF, (D) IL-1β protein levels were quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 (n=6)
Figure 3.9: p38 signalling is essential for the induction of IL-8 and IL-1β, but not TNF or IL-6 downstream of TLR8 in primary human PMNs

Human PMNs (1x10⁶) were rested in serum free media for 30min before treating with the p38 inhibitor SB303580 (20µM) for 30mins prior to subsequent stimulations with either LPS (100ng/ml), CL075 (2µg/ml) for both 12 and 24 hrs. Supernatants were collected and (A) IL-6, (B) IL-8, (C) TNF, (D) IL-1β protein levels was quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 (n=6)
3.4.8 The TLR8 agonist, CL075, signals through the phosphorylation of ERK and p38 in primary human PMNs

Having identified a role for both ERK and p38 in PMN/CL075 signalling using their specific inhibitors (Fig 3.8/3.9), we next investigated if CL075 triggered their phosphorylation in primary human PMNs. Phosphorylation of ERK and p38 allows for the translocation of these transcription factors to the nucleus where they initiate the upregulation of various inflammatory gene transcripts. PMNs were stimulated with either LPS or CL075 over a timecourse of 1 hour and whole cell lysates collected and immunoblotted for ERK, p-ERK, p38, p-p38 and the housekeeping protein, Vinculin. Vinculin is a cytoskeletal protein and was chosen as the housekeeping protein since actin, ERK and p38 are all of similar size (~117kD). We observed phosphorylation of both p38 and ERK when stimulating with CL075 (Fig 3.10A). We observed early phosphorylation of p38 as soon as 3 minutes post stimulation with CL075 which was sustained at 30 and 60 minutes post treatment with CL075 downstream of TLR8 (Fig 3.10A). While LPS triggered early phosphorylation of p38 this was transient, reducing after 30 minutes (Fig 3.10B). While CL075 induced late pERK after 30 and 60 minutes (Fig 3.10A), LPS only induced transient pERK at 30 minutes (Fig 3.10B). Together these findings reveal that unlike LPS/TLR4 transient signalling, CL075/TLR8 ERK/p38 signalling is sustained in PMNs.
Figure 3.10: The TLR8 agonist CL075 signals through both ERK and p38 in primary human PMNs

Primary human PMNs (1x10^6) were stimulated with 2ug/ml of CL075 (A) or 100ng/ml of LPS (B) over a timecourse of 1 hour. Cells were lysed in RIPA buffer and lysates blotted for MAPK p38, ERK, phospho-p38 and Phospho-ERK and the housekeeping protein Vinculin. Densitometric analysis of 3 immunoblots was performed using the image lab software. Representative densitometric analysis for (C) CL075 phosphorylation of ERK and p38 and (D) LPS induced phosphorylation of ERK and p38 were analysed as a ratio of ERK/p38/p-ERK/p-p38: Vinculin ,relative to the untreated (PBS) control which was normalised to 1. Statistical differences were assessed using one way ANOVA with a Tukey (n=3)
3.4.9 TLR8 directly induces the expression of IL-1β independent of an activation signal, downstream of TLR8 in primary human PMNs

IL-1β has been shown to be crucial in the host defence against disease, but can also exacerbate chronic inflammatory conditions. It is produced as an inactive precursor known as pro-IL-1β in response to PRR activation, also known as the ‘priming step’ and is insufficient for the secretion of the cytokine. The primed cell must encounter an additional stimuli, such as other PAMPs or DAMPs, to induce the processing and secretion of active IL-1β, known as the ‘secondary activation signal’. This ‘secondary activation signal’ results in the cleavage of pro-IL-1β by caspase 1 through the recruitment of multiprotein complex, known as the inflammasome (NLR family pyrin domain containing 3 [NLRP3]). DAMPs that act as a ‘secondary activation signal’ include Nigericin (antibiotic derived from Streptomyces hygroscopicus), Maitotoxin, monosodium urate crystals, cholesterol crystals and aluminium salts (190). TLR4 is known to induce the expression of IL-1β without the need for a secondary ‘activation’ signal and was therefore chosen as a positive control (191). Additionally, recent studies have highlighted that in human monocytes, priming is dispensable for the NLRP3 inflammasome activation and subsequent IL-1β production (192). In contrast, cytokines like IL-6 and IL-8 have been demonstrated to not require the activation of the inflammasome complex for their secretion (193) (194). Having observed the induction of IL-1β downstream of TLR8 (Fig 3.4D), we wanted to investigate if TLR8 activation was sufficient alone for the production of active cleaved IL-1β in primary human PMNs. PMNs were stimulated with varying concentrations of CL075 or LPS for 24 hours. After the initial 4 hours PMNs were subsequently stimulated with/without Nigericin. Supernatants were measured for secreted
proinflammatory cytokines IL-6 (Fig 3.11A), IL-8 (Fig 3.11B), TNF (Fig 3.11C) and IL-1β (Fig 3.11D) by ELISA. Similarly to the TLR4 positive control, it was observed that TLR8 can induce the expression of IL-1β independent of the ‘secondary activation signal,’ Nigericin, yet the presence of the ‘secondary activation signal’ in conjunction with the priming signal provided by the TLR stimulation greatly enhanced the expression of IL-1β at both 12 hour and 24 hours post stimulation (Fig 3.11D). In contrast, we found NLRP3 independent cytokines, IL-6, IL-8 and TNF exhibited reductions in absolute quantities of secreted cytokines when stimulating with the priming signal (LPS or CL075) in conjunction with the activation signal (Nigericin) at both the 12 and 24 hour timepoint (Fig 3.11A/B/C). In summary, TLR8 activation alone, in primary human PMNs, is sufficient for NLRP3 activation and subsequent IL-1β production, without the need for any additional stimuli.
Figure 3.11: TLR8 directly induces the expression of IL-1β, independent of a secondary activation signal

Human PMNs (1x10⁶) were rested in serum free media before being stimulated with CL075 (10ng/ml-1000ng/ml) or LPS (10ng/ml-1000ng/ml) as indicated. After 4 hours PMNs were stimulated with 5μM Nigericin, an NLRP3 activator. After 24 hours cellular supernatants were collected and analysed for (A) IL-6, (B) IL-8, (C) TNF and (D) IL-1β. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 (n=6)
3.4.10 RSV upregulates the activation marker CD11b and reactive oxygen species (ROS) in primary human PMNs

Having previously established a novel way by which primary human PMNs can respond to a TLR8 agonist (Fig 3.2-3.10), we sought to explore the physiological relevance of TLR8 induced activation of primary human PMNs using the ssRNA virus, RSV. We first determined whether RSV could activate primary human PMNs in vitro. CD11b is a well-known activation marker on human neutrophils which modulates several key biological functions such as adhesion, migration, and phagocytosis (195). In addition, PMNs are characterised by their highly inducible expression of ROS, which acts as a potent anti-microbial agent (196).

To investigate the effect of RSV upon PMNs we propagated RSV using hep-2 cells, in which the virus laden supernatant was harvested for future infections. The mock infection contained no virus and was simply Hep-2 conditioned supernatant, cultured for the same length of time as the Hep-2 cells infected with RSV. Primary human PMNs were incubated with infectious RSV or a mock for 2 hours. They were subsequently stained using antibodies specific for CD11b (Fig 3.12B), a dye specific for the detection of ROS (Cellrox) (Fig 3.12C) and analysed using flow cytometry. Cellrox is a cell permeant dye weakly fluorescent in a reduced state, which exhibits bright green phospho-stable fluorescence when oxidised, due to the presence of reactive oxygen species. Primary human PMNs stimulated with RSV for 2 hours significantly upregulated the expression of CD11b 3 fold, when compared to the negative unstimulated control. Additionally, we observed no significant changes in CD11b expression in the mock infection (Fig 3.12B/D). We also detected a significant 2 fold increase in ROS expression, when compared to the negative unstimulated control or mock infection (Fig
In summary, we found that RSV activates PMNs measured by the integrin/activation marker CD11b and the production of ROS when challenged with RSV.

Figure 3.12: RSV induces the upregulation of CD11b and reactive oxygen species in primary human PMNs

Human PMNs (1x10⁶) were stimulated with RSV-A2 at an MOI of 1 for 2 hours. Cells were treated with cell rox green 30 mins, prior to the end of the 2 hour timepoint. (A) represents the gating strategy, in which PMNs were gated on the single+ live+ population. (B) representative histogram of CD11b and (C) Cell Rox green of PMNs. Graphs (D) and (E) represent the MFI of CD11b and MFI fold change of cell Rox green, relative to the untreated control, respectively. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 (n=4)
3.4.11 RSV triggers the formation of NETS and release of Matrix Metalloprotease from Primary human PMNs

Having observed the upregulation of the activation marker, CD11b, and increase in ROS expression (Fig 3.12), we next investigated whether RSV-A2 induced the production of NETs and the release of anti-microbial granules, such as MMP-9. MMP-9 regulates pathological remodeling processes that involve inflammation and fibrosis through the degradation of extracellular matrix (ECM) proteins and production of cytokines and chemokines (197). NETs have been well characterised in the context bacterial infection (198), however, recent studies have suggested a role in viral infections such as Dengue, Chikungunya and HIV (199) (200) (129). Additionally MMP-9 has been demonstrated to assist in the immune defence against bacterial infections such as *Streptococcus pneumoniae* (201). Primary human PMNs were stimulated with infectious RSV or a mock infection for 4 hours. Supernatants were measured for secreted MMP-9 (Fig 3.13A) using a standard sandwich ELISA. NET formation was quantified using a quantitative colorimetric assay that measured DNA bound elastase (a common component of NETs). PMNs were either unstimulated (negative control), treated with infectious RSV, the mock infection or PMA (positive control) (Fig 3.13B). We observed a significant 2 fold increase in the production of MMP-9 in PMNs cultured with RSV for 4 hours, when compared to the negative control and mock infection (Fig 3.13A). Additionally, we observed that PMA significantly increased NET production, proving the validity of the assay. Although not statistically significant we observed a 5 fold mean increase in NET production from primary human PMNs stimulated with RSV for 4 hours (Fig 3.13B). Together these
findings reveal that RSV causes PMNs to functionally antimicrobial through degranulation and the production of NETs.

**Figure 3.13: RSV triggers NET formation and the production of antimicrobial Matrix metalloprotease (MMP)-9**

Human PMNs (1x10⁶) were stimulated with RSV-A2 at an MOI of 1 or 100ng/ml of PMA 4 hours. (A) represents levels of matrix metalloprotease, MMP-9, measured by sandwich ELISA and (B) represents levels of DNA bound elastase measured using the quantitative colorimetric assay. Data is represented as the mean ± SD. Paired T-test. *p<0.05 (n=4)
3.4.12 RSV is detected intracellularly in primary human PMNs

Having investigated the role of TLR8 in the induction of an inflammatory response using the synthetic TLR8 agonist CL075 (Fig 3.4) and the activation of PMNs by RSV in vitro (Fig 3.12/Fig 3.13), we next investigated the role of TLR8 in the recognition of the respiratory ssRNA virus, RSV. Respiratory infections, such as RSV, are characterised by a large influx of neutrophils into the lungs and respiratory tract, which often correlate with an increase in an anti-viral and inflammatory response. The failure to regulate these inflammatory responses can lead to excessive collateral damage of tissue, progressing to significant morbidity from an RSV infection (148). RSV has been demonstrated to actively infect epithelial cells of the respiratory tract through a variety of receptors, including CX3C chemokine receptor 1 (CX3CR1), nucleolin, epidermal growth factor (EGFR), insulin-like growth factor-1 receptor (IGF1R) and heparan sulfate proteoglycans (HSPGs) (202). While there is no evidence to support active infection of RSV in human neutrophils, it has been demonstrated that neutrophils can engulf influenza virions and the apoptotic bodies containing the virus (203). To this end we determined the presence of intracellular RSV in primary human PMNs by flow cytometry. PMNs were either challenged with GFP-tagged RSV-A2 or the mock infection at an MOI of 1 for 18 hours. Levels of intracellular RSV were detected using flow cytometry to measure the GFP signature from the modified GFP-tagged virus. PMNs were gated on the single, live, CD66b+ population (Fig 3.14.A) The significant mean % of the GFP+ signal in the RSV-A2 challenged group was 7% in PMNs after 18 hours, when compared to the two negative controls (<1% GFP+), which contained either fresh media or the Mock
negative control (Fig 3.14B/3.14C). In summary we found that PMNs can enter
PMNs and thus be available inside the cell to be detected by endosomal TLR8

Figure 3.14: Intracellular RSV expression in primary human PMNs
Human PMNs (1x10⁶) were treated with the fresh media (negative control), GFP- tagged RSV-A2 or mock at an MOI of 1, for 18 hours. (A) PMNs were gated on the single, live CD66b+ Population. (B) is representative of the subsequent GFP+ signature in the negative control, RSV-A2 and mock control. (C) is the representative bar graph of the GFP+ signature of CD66b+ PMNs observed in B. Data is represented as the mean ± SD. Paired T-test. *p<0.05 (n=4)
3.4.13 Inhibition of TLR8 results in abrogated IL-8, but not IL-6, secretion from primary human PMNs challenged with RSV

Previous experiments revealed a novel role for TLR8 has in inducing IL-8 from primary human PMNs through both NF-κB and MAPK signalling (Fig 3.4/6/8/9). Additionally, we observed how RSV could activate and be successfully internalised in primary human PMNs (Fig 3.12-3.14). Previous studies have demonstrated the role of TLR8 in the recognition of viruses. This includes the role of HIV-1 in the induction of an IL-6 response in CD4+ T-cells and Coxsackie B, in the induction of IL-6/IL-1β in human cardiac cells, in a TLR8 dependent manner (26) (174). Additionally, other studies have shed light on the role of TLR4 in the induction of an inflammatory response in epithelial cells infected with RSV (204). While respiratory epithelial cells are the predominant cells that become infected with RSV, we hypothesised that rapidly migrating innate immune cells, such as neutrophils, could also contribute and exacerbate the inflammatory immune response seen during RSV infection. Therefore we next sought to investigate whether the acute viral respiratory ssRNA virus, RSV, could induce a similar response via TLR8. PMNs challenged with RSV for 18 hours, resulted in a significant increase in both levels of secreted IL-6 and IL-8 (Fig 3.15A/3.15B) when compared to the negative control or mock infection. Additionally, we observed a statistically significant reduction in levels of IL-8 in PMNs pre-treated with the TLR8 inhibitor, Cu-cpt9a (Fig 3.15A). In contrast, the addition of the TLR8 inhibitor, Cu-cpt9a, didn’t result in any significant changes in secreted IL-6 (Fig 3.15B). These findings suggest that TLR8 activation by RSV has a propensity for the production of IL-8, but not IL-6.
Figure 3.15: RSV triggers the release of IL-8 in a TLR8 dependent manner from primary human PMNs

Human PMNs (1x10⁶) were rested in serum free media for 30min before treating with the TLR8 inhibitor, CU-CPT9a, for 30 minutes, prior to subsequent stimulations. Cells were incubated for 18 hours with RSV-A2 at an MOI of 1. Supernatants were collected and (A) IL-8 (B) IL-6 protein levels were quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 **p<0.01 (n=6)
3.4.14 Inhibition of NF-κB and MAPK signalling abrogates RSV induced expression of IL-8 in primary human PMNs

Having observed that RSV induced IL-8 expression from PMNs was TLR8 dependent (Fig 3.15), we next investigated if the NF-κB or MAPK proteins ERK and p38, downstream of TLR8, were involved in transducing this inflammatory response. It has been previously reported that the NF-κB pathway is activated downstream of TLR4 in epithelial cells during RSV infection (205) (206). Additionally, recent studies have also implicated the role of MAPK proteins ERK, p38 and JNK in the regulation of inflammatory responses observed in response to Rabies and SARS-CoV-2 (207) (208). Therefore we pre-treated PMNs with the NF-κB (Bay-11), ERK (PD98059) and P38 (SB303580) inhibitors for 30 minutes and subsequently challenged with RSV-A2 or the mock for 18 hours. Supernatants were collected and analysed for IL-8 (Fig 3.16A/C/E) and IL-6 (Fig 3.16B/D/F) by ELISA. We observed significant induction of both IL-6 an IL-8 in PMNs incubated with RSV, when compared to the negative unstimulated control and the mock infection. NF-κB and ERK inhibition resulted in significant reductions in levels of IL-8 in the RSV-A2 group (Fig 3.16A/3.16C). While not significant, p38 inhibition resulted in a reduction in levels of IL-8, trending toward significance with a p value of 0.0712 (Fig 3.16E). In contrast, inhibition of NF-κB, ERK and p38 resulted in no change in levels of secrete IL-6 in the RSV group (Fig 3.16B//F).
Figure 3.16: RSV triggers the release of IL-8 but not IL-6 through both NF-κB and MAPK pathways in primary human PMNs

Human PMNs (1x10⁶) were rested in serum free media for 30min followed by pre-treating with (A-B) NF-κB inhibitor BAY-11, (C-D) ERK inhibitor PD98059 and (E-F) p38 inhibitor SB303580 for 30 mins prior to subsequent stimulations. Cells were subsequently treated with RSV-A2 at an MOI of 1 for 18 hours. Supernatants were collected and levels of (A,C,E) IL-8 and (B,D,F) IL-6 protein levels were quantified by ELISA. Data is represented as the mean ± SD. A Paired T-test was carried out. *p<0.05 **p<0.01 (n=6)
Our previous experiments indicated that the MAPK and NF-κB pathways were involved in RSV induced expression of IL-8 downstream of TLR8 in PMNs (Fig 3.16). MAPK regulation of IL-8 is well documented in current scientific literature (209). In order to confirm the role of MAPK signalling and NF-κB signalling in RSV TLR8-induced IL-8 expression of primary human PMNs, we next explored the phosphorylation of MAPK proteins, ERK and p38, and degradation of Iκb. PMNs were pre-treated with the TLR8 inhibitor, Cu-cpt9a, for 30 minutes. PMNs were subsequently incubated with either RSV or the mock infection for 60 minutes. Whole cell lysates were collected and blotted for ERK, p-ERK, p38, p-p38, Iκb and the house keeping proteins Vinculin/β-actin using western blotting. PMNs challenged with RSV resulted in an increase in phosphorylation of both ERK and p38 (Fig 3.17A/3.17B). We observed no changes in levels of total p38 or ERK. In addition, PMNs pre-treated with the TLR8 inhibitor Cu-cpt9a for 30 minutes and subsequently challenged with RSV, had a reduction in both phosphorylation levels of ERK and p38. Reduction of p-p38 with the addition of Cu-cpt9a had a near statistical significant p value of and 0.0564. In contrast we observed a smaller reduction of p-ERK with a p value of 0.233 (Fig 3.17C/3.17D). In contrast, we observed no significant degradation of Iκb (Fig 16.C/F). In agreement with Fig 3.16C we observed that TLR8 recognition of intracellular RSV triggered the phosphorylation MAPK proteins ERK and p38. In summary, we found that RSV activation of TLR8 resulted in the specific reduction of p38 phosphorylation.
Figure 3.17: RSV induces the phosphorylation of and p38 partially via a TLR8-dependent manner in primary human PMNs

Human PMNs (1x10^6) were rested in serum free media for 30 minutes before being pre-treated with 5μM of TLR8 inhibitor Cu-cpt9a for 30 mins. Cells were then stimulated with RSV at an MOI of 1 for 60 minutes. Cell pellets were lysed in RIPA buffer. Lysates were immunoblotted for p-ERK, ERK p38, p-p38, IκB and housekeeping proteins vinculin/β-actin. Representative densitometric analysis for phosphorylation of ERK/ p38 and IκB were analysed as a ratio of p-p38/p-ERK: Vinculin and IκB:β-actin ,relative to the untreated (PBS) control which was normalised to 1. Data is represented as the mean ± SD. Paired T-test. (n=4)
3.4.16 TLR8 stimulated PMN conditioned media enhances the expression of dendritic cell maturation markers CD86/CD40

Having observed a novel role of TLR8 in inducing an inflammatory response in human PMNs through the production of cytokines such as TNF, IL-1-β, IL-6 and IL-8 (Fig 3.4) we sought to explore the role of these activated PMNs in assisting in the orchestration of the global immune response. These aforementioned cytokines are secreted to induce a specific response to an invading pathogen but could interfere with DC homeostasis and induce an inflammatory response that could be responsible for the tissue pathology, often observed in viral respiratory infections (210). Conversely, DCs act as the bridge linking the innate immune response to the adaptive immune response through the presentation of antigens as well the secretion of cytokines needed for an efficient T-cell response, needed for efficient viral clearance. This requires the activation of ‘resting’ DCs, characterised by an increased ability to present antigen needed for CD4+ T-cell activation, through the upregulation of certain cell surface markers such as CD40, CD86, CD80, CD83 and CMRF-44 (211). Of the previous cytokines produced by PMNs through TLR8, TNF, IL-1β and type 1 IFNs are able to trigger DC activation in a paracrine fashion. We therefore explored how TLR8 activated PMNs ‘secretome’ could activate immature monocyte derived DCs measured by the upregulation of both CD40 and CD86. We acquired immature monocyte derived dendritic cells by magnetically isolating monocytes from peripheral blood mononuclear cells and subsequently cultured them in GMCSF and IL-4 for 5 days. Primary human PMNs were stimulated for 24 hours with either CL075 or CL075 and IFN-α. The supernatants (conditioned media) were subsequently collected and used in culturing immature DCs on day 5 after DC isolation from
the PBMCs for a further 24 hours. The necessary controls included PMNs cultured in blank media, containing no agonists, as well as DCs cultured in fresh media with no agonists (negative controls), or DCs cultured in fresh with containing LPS, a known activator of DCs (Positive control). A schematic of the experimental procedure is outlined in Fig 3.18. Levels of extracellular activation markers CD40 and CD86 were quantified using flow cytometry. We found that DCs cultured in supernatants from PMNs stimulated with CL075, or CL075 and IFN-α had a significant increase in levels of CD40 and CD86 (Fig 3.19C/D). Additionally, we observed no upregulation of CD40 or CD86 in the relevant negative controls, but observed upregulation of both CD40 and CD86 in the LPS positive control (Fig 3.19). In summary, we found that TLR8/CL075 activated neutrophil derived secreted proteins could promote DC activation.

Figure 3.18: Schematic diagram showing the experimental protocol for figures 18+19 detailing how neutrophils could modulate the adaptive responses in viral infection.
Figure 3.19: TLR8/TLR8+IFN stimulated primary human PMN supernatant enhances the expression of dendritic cell maturation markers CD86/CD40

PMNs (1 x10⁶) were cultured for 24 hours in the presence of CL075 or CL075 and IFN-α. 5 day cultured Immature DCs were then cultured in this neutrophil conditioned media for a further 24 hours and the subsequent maturation markers CD40/CD86 analysed using FACS. Mean fluorescent intensity (MFI) was measured and treated samples were compared to the unstimulated, which was normalised to 1. Graphs are the mean ± SD of 4 independent experiments and analysed using a paired T-test. *p<0.05 **p<0.01
3.4.17 DC/PMN coculture supernatant polarises CD4 T-cells towards a Th1 phenotype

Having observed an increase in DC maturation when immature DCs were cultured in supernatants from PMNs stimulated with CL075 or CL075+IFN (Fig 3.19), we next analysed whether this enhancement in DC maturation resulted in the polarisation of CD4+ T cells. CD4+ T-cells can polarise towards several different T-cell subtypes characterised by their cytokine profile. These include subtypes, such as Th1, Th2, Th9 and Th17 CD4+ T cells (212). The polarisation of CD4+ T-cells towards each individual phenotype is in part determined by the cytokine milieu they are exposed to. In order for CD4+ T cells to polarise toward a Th1 phenotype, the presence of IL-12 is required, of which PMNs and DCs are well known producers (213) (214). A Th1 phenotype has been demonstrated to be instrumental in impacting antiviral immunity at multiple stages of the immune response. These Th1 CD4+ T cells enhance early expansion of virus-specific primary cytotoxic T-lymphocytes, drive their subsequent differentiation into memory T-cells and guide their localization to sites of infection during viral re-infection (215). Th1 cells are characterised by their strong expression of intracellular and secreted IFN-γ. (216). To investigate the effect of CL075/CL075+IFN stimulated PMNs cytokines upon T-cell differentiation, CD4+ T-cells were isolated from PBMCs using magnetic sorting. CD4+ T-cells were cultured in PMN:DC supernatants (conditioned media) from the previous experiment (Fig 3.19) for 96 hours in the presence of both costimulatory molecules anti-CD3 and anti-CD28. At the 92 hour timepoint cells were stimulated with PMA and Ionomycin for 4 hours before supernatants were collected and cells stained for the presence of intracellular IL-17 and IFNγ in CD3+/CD4+ T cells. Ionomycin is a
calcium ionophore and is added because it bypasses the T-cell membrane receptor complex and will lead to the activation of intracellular signalling pathways that govern T cell activation (217). Cells were gated on the live, single, CD3+, CD4+ positive population (Fig 3.20A). Although not significant, due to the preliminary nature of the experiment (n=2), we observed that CD4+ T-cells cultured in PMN:DC conditioned media with CL075 could polarise cells towards a Th1 phenotype characterised by their expression of intracellular IFN-γ (Fig 19.B/C). In addition to intracellular levels of IFN-γ we observed a mirrored increase in levels of secreted IFN-γ in CD4+ T cell supernatants at the 96 hour timepoint in groups stimulated with CL075 (Fig 3.20D). In contrast we observed no production of intracellular IL-17 in any the groups indicative of the lack of Th17 CD4+ T cells. In summary, we hypothesise that TLR8 activated PMNs can enhance DC maturation which in turn can polarise CD4+ T-cells towards the observed Th1 phenotype.
Figure 3.20: Dendritic cell/PMN coculture supernatant polarises CD4 T-cells towards a Th1 phenotype

Dendritic cells were cultured in TLR8 conditioned PMN media for 24 hours. Isolated peripheral blood CD4+ T-cells were then cultured in this dendritic cell conditioned media for 96 OR 48 hours in the presence of anti-CD3 and CD28. live singlet CD3+/CD4+/CD8- (A)were gated on their expression of intracellular IL-17/IFN-γ (B/C). Additionally T cell culture supernatants were analysed using ELISA for IFN-γ to determine the presence of secreted CD4+ T-cell derived IFN-γ (D). Graphs are the mean ± SD of 2 independent experiments.
3.5 Discussion

Pathogen recognition is the first step in the initiation of the innate immune response and is a key determinant in the host's ability to generate a robust adaptive response. Endosomal TLR7/8 are involved in the recognition of ssRNA viruses and are important in driving an anti-viral and proinflammatory response (218). Indeed, studies have shown that ssRNA viruses are instrumental in driving a proinflammatory response through the production of proinflammatory cytokines (219). Viruses such as RSV and more recently coronaviruses including the novel SARS-CoV2 result in ARDS in patients with severe infection (220). Neutrophil infiltration in the respiratory tract is a hallmark associated with many of these respiratory viral diseases (221). Moreover, proinflammatory cytokines, including IL-6 and TNF, are key proponents in driving tissue damage (222) (223). While these individual factors are important for the disease pathology, investigating the means of viral recognition by neutrophils, the intracellular signalling and the downstream effector molecules, will be key in understanding how these innate immune cells combat infection and modulate other immune cells and responses. Therefore, the aim of this first stage of our study was to determine the role of PMN endosomal receptors in inducing inflammatory and anti-viral responses. To this end, PMNs were challenged with a range of agonists, including synthetic TLR agonists and live RSV.

We found that the TLR7/TLR8 agonist, CL075, induced the upregulation of inflammatory genes associated with acute respiratory stress in PMNs, such as TNF, IL-1β, IL-8 and IL-6. Numerous studies have revealed that neutrophils produce up to 70 different growth factors and cytokines. Cytokines, such as TGF-
IL-6 and TNF, have been demonstrated to be prestored in neutrophil granules components (224) (213) (225). This observed upregulation of inflammatory cytokine mRNA highlighted that human PMNs have the potential to respond to ssRNA viral infections and can produce de novo cytokines downstream of TLR7/TLR8. The subsequent ELISA data confirmed that the cells could produce viable secreted protein. It was of specific interest when we uncovered that human PMNs preferentially utilise TLR8 in inducing this inflammatory response. It was also interesting to note that TLR8 was observed to preferentially induce the production of TNF when compared to TLR4/LPS activation of PMNs. This was seen as an interesting comparison as LPS has been demonstrated to induce the expression of TNF in primary human monocytes (226). This was seen in stark contrast to other innate immune cells, such as pDCs, which utilise TLR7 for inducing an inflammatory and anti-viral response to viral infection (227). In addition, mouse models have been demonstrated to preferentially use TLR7 as the predominant endosomal receptor as they express low levels of TLR8 mRNA transcripts, suggesting that rodent models may not be the best candidate in understanding human neutrophil inflammation during ARDS from viral infection. Despite this limitation, scientists have developed TLR8 transgenic mice to study inflammation in collagen induced arthritis (CIA) mouse models. The importance in TLR8 driven inflammation in the development of CIA has been further postulated, with increased TLR8 detected in blood cells from patients with arthritis (228). The development of TLR8 transgenic mice could therefore be important in the study of pathogen driven inflammation and development of human therapeutics. Given that TLR8 in human PMNs induces an inflammatory response characterised by cytokines such as IL-6, IL-8, TNF and IL-1β, we hypothesised that there would be
potentially different intracellular mechanisms governing their production. To begin exploring this hypothesis we started with one of the most obvious candidates: NF-κB signalling. We observed a total abrogation of inflammatory responses when NF-κB was inhibited. Previous reports have all demonstrated that NF-κB signalling plays a role in the production of IL-8, IL-6, TNF and IL-1β (229). In order to determine if the NF-κB signalling was via canonical signalling we explored the degradation of IκB. We observed that PMNs stimulated with TLR7/TLR8 agonist CL075 utilised the canonical pathway through the degradation of IκB as early as 10 minutes post stimulation, suggesting a rapid and transient production of cytokines in contrast to the slow and persistent production of cytokines through the non-canonical pathway (230).

In addition to the NF-κB pathway we hypothesised that the MAPK pathway could also play a role in the induction of this inflammatory response downstream of TLR8. To this end, we explored the role of different MAPKs, such as ERK and p38. We observed that unlike NF-κB signalling, inhibition of ERK and p38 resulted in the reduction of IL-8 and IL-1β. While MAPK signalling has been well documented to regulate IL-8 signalling, its role in the regulation of IL-1β is poorly understood in immune cells, despite older reports showing a role of MAPK in regulating IL-1β production in keratinocytes (231). We confirmed the role of ERK and p38 in the induction of this inflammatory response in PMNs, looking at the phosphorylation of both proteins. To further explore this novel production of IL-1β via TLR8, we examined the role of the inflammasome. Classical inflammasome activation involves a two-step process: priming and activation. TLR-mediated priming results in the upregulation of pro-IL-1β. The second step requires the
proteolytic cleavage of pro-IL-1β through a range of DAMPs such as the NLRP3 inflammasome activator, Nigericin. It has been recently demonstrated that Plasmodium infected red blood cells can induce the expression of IL-1β via TLR4 and TLR8 of PBMCs, without the need for a ‘secondary activation signal’. Similarly, we observed that PMNs stimulated via TLR8 can induce the expression of IL-1β, independent of Nigericin, the secondary inflammasome activation signal. While several publications have described the concerted activation of TLR8 and NLRP3, with distinct steps for priming and activation, our study is the first to describe an alternative pathway of direct inflammasome activation by TLR8 in human PMNs, which is important for elucidating the immunopathogenesis of various diseases characterised by a large influx of neutrophils, such as viral induced ARDS (232) (233).

Having observed this inflammatory phenotype mediated by TLR8 using the synthetic agonist CL075, it was necessary to determine whether PMN TLR8 was functional in mounting an inflammatory response to a live ssRNA virus, such as RSV. PMNs are well documented to produce ROS in response to infection. This has been extensively characterised using intracellular bacterial models, such as Staphylococcus Aureus (234). In contrast, less is known about ssRNA viruses, which by their very nature are obligate intracellular organisms. We observed that PMNs stimulated with RSV upregulated the expression of CD11b, a common activation marker and induced the expression of ROS as soon as 2 hours post stimulation when treating with RSV. In addition to ROS production and the upregulation of integrins, such as CD11b. PMN degranulation is also a common mode by which neutrophils can facilitate pathogen killing (235). Degranulation
results in the expulsion of a plethora of antimicrobial proteins from the PMN, such as matrix metalloproteases (MMP), as well as neutrophil extracellular traps comprised of extracellular DNA which bind pathogens (121). We observed an increase in MMP-9, as well as an increase in DNA bound elastase, an indicator of NET formation revealing that like HIV, PMNs may facilitate the removal of RSV virions by NET bound antimicrobial proteins such as elastase, cathepsin G and MPO (199).

Due to the intracellular nature of TLR8 it we next investigated whether or not live virus could be detected intracellularly in human PMNs. The wild type RSV virus was GFP tagged and therefore it was possible to measure internalisation of the virus using cytometry. After incubating RSV with PMNs, we observed that 5%-8% of cells were GFP positive. We proposed that on internalisation the virus would bind PRRs before ultimately resulting in virus induced death, subsequently triggering an inflammatory response. Therefore, we next sought to explore the specific role of TLR8 in inducing an inflammatory response to RSV internalisation. We found the RSV triggered both a potent IL-8 and IL-6 response. Despite this, we observed that the addition of the TLR8 inhibitor only reduced the IL-8 but not IL-6 response. We therefore hypothesised that different components of RSV could be bind extracellular receptors, such as TLR2/4, resulting in the production of the observed IL-6. Previous data shows that RSV can elicit an inflammatory response via the RSV fusion (F) protein, via TLR4, in HEK cells (205). Having observed this role of TLR8 in inducing an IL-8 response, we sought to explore the role of NF-κB and MAPK signalling. Similar to the TLR8 inhibitor results, we observed a reduction in IL-8 using NF-κB and MAPK signalling.
inhibitors, with no reduction in IL-6. This failed inhibition of IL-6 led us to believe that it was feasible that IL-6 was pre-stored in neutrophil vacuoles that are released early on when encountering a pathogen with a reduced need for de novo synthesis of the cytokine. The importance of these granules may be to provide the rapid release of pattern recognition molecules to active pathways such as the lectin complement pathway and have been shown to contain cytokines during immune activation (236) (237) (238). In contrast, IL-8 was determined to be produced de novo when encountering a virus. In addition to the ELISA data we sought to explore the intracellular proteins observed in the transduction of this inflammatory response. We observed a reduction in phosphorylation of both p38 and ERK, with no change in levels of IκB degradation, suggesting an integral role of MAPK signalling downstream of TLR8 in triggering an IL-8 response in human PMNs when challenged with RSV. In agreement with our findings, that MAPK signalling is important in the production of IL-8 in primary human PMNs downstream of TLR8, studies have shown that bacterial DNA can activate the production of IL-8 through both ERK1/2 and p38 in primary neutrophils independent of CpG (239).

Finally, we sought to explore the potential of TLR8 activated PMNs to orchestrate the surrounding immune response. Previous data has demonstrated that neutrophil markers, such as CD11b, can modulate DC functions (240). We observed that PMNs cultured with TLR8 agonist can enhance the maturation of DCs. This upregulation of DCs is associated with the increase in expression of DC derived IL-12. IL-12 has been demonstrated to polarise CD4+ T-cells towards a Th1 phenotype, characterised by a strong IFN-γ response (241). We observed
that DCs cultured in media from TLR8 activated PMNs could help polarise CD4+ T-cells toward a Th1 phenotype, highlighting the potential importance of human PMNs in assisting with the global immune response to viral infection through TLR8. Neutrophil modulation of DCs has been shown to be through the expression of MAC-1 (CD11b) and the expression of neutrophil derived TNF (240). We hypothesised that our observed TLR8 induction of both CD11b and TNF enhanced DC activation and subsequently modulated CD4+ T-cell responses.

In summary, we found that TLR8 is an important receptor for the recognition of ssRNA viruses like RSV and that activation of the receptor results in an associated inflammatory response. Inflammation is characterised as a double edged sword; in that it is needed for an effective immune response, however an excessive response results in tissue damage and in the case of respiratory diseases, ARDS. Current therapeutics for treating acute respiratory stress include a plethora of monoclonal antibodies, such as IL-6 inhibitors and JAK inhibitors (242) (243). Having identified PMNs, as key inflammatory regulators we propose that targeting neutrophil processes, such as neutrophil recruitment and their associated receptors upstream of inflammatory cytokines would be beneficial in managing the immunopathology of viral respiratory diseases such as RSV.
Chapter 4

TLR8 is a key receptor involved in the recognition of ssRNA viruses in PMNs which induces an early antiviral response
Chapter 4: TLR8 is a key endosomal receptor involved in the recognition of ssRNA viruses in PMNs which induces an early antiviral response

4.1 Introduction

Viral components are detected by PRRs, including endosomal TLRs, which recognise viral genomic material (244, 245). TLR8 recognises viral ssRNA (26) and signals via the transcription factors, NF-κB as previously discussed in chapter 3 and Interferon response factors (IRFs), leading to the induction of proinflammatory and anti-viral cytokines, such as the Type 1 IFN, IFN-α and chemotactic cytokines, such as IL-8 (218, 246, 247). Secreted IFN-α acts in a paracrine/autocrine fashion, binding to its IFN receptor which activates the JAK/STAT pathway, leading to the upregulation of numerous anti-viral ISGs, such as 2’5’-oligoadenylate synthetase (OAS), MX Dynamin Like GTPase 1 (Mx)A, ISG15 and Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) (244, 248). Together these genes make up the “interferome” (249), which have broad functions in blocking viral infection and replication (245, 250). However, non-canonical induction of ISGs has been well documented over the last 20 years. This was nicely demonstrated using a human cytomegalovirus (HCMV) infection model, in which IFNAR neutralisation had no effect on the expression of certain ISG mRNAs. Indeed, viral entry alone was shown to induce the expression of selection of ISG mRNAs, demonstrated using early and late stage infection models. This IFN-independent mechanism was further highlighted using replication incompetent ultraviolet irradiated HCMV infection models, which also upregulated the expression of ISGs in early stage infection models. When looking for a mechanism that would underpin this IFN independent mechanism
early reports suggested a role of IRF3. Even in the absence of viral stimuli, constitutive expression of IRF3 resulted in the expression of a subset of ISGs, including IFIT2 and Viperin (251). However, IRF3 independent induction of these ISGs has also been noted using Interferon incompetent cells infected with ssRNA viruses, such as Newcastle disease virus (NDV) (94). In order to confirm the essential role of IRF3 in the induction of this IFN independent anti-viral response, HCMV human fibroblasts were engineered to lack IRF3. This HCMV infection model highlighted that expression of Viperin, ISG15, IFIT1, IFIT2, Mx1, and Mx2 mRNA can be induced in an IRF3-dependent, STAT1-independent manner (252).

In addition to IRF3, recent studies have revealed a possible role for IRF1 in the early anti-viral response (253) (Fig 4.1). This has been highlighted by mice deficient in IRF1 being susceptible to viral infections (254). However, IRF1’s role in the antiviral response remains unclear due to variable tissue distribution and its potential involvement varying depending on the specific virus in question. A recent study has shed light on the possible mechanism involved. It was demonstrated that IRF1 mediates IFN-independent antiviral immunity by supporting constitutive expression of antiviral effectors, by modulation of H3K4me1 at promoter enhancer regions of IRF1 dependent genes. H3K4me1 is an epigenetic modification to the DNA packaging protein histone 3 through the addition of methyl groups, which facilitate increased gene expression (255). One of the genes heavily regulated by IRF1 expression was Viperin.

Viperin inhibits the replication and egress of a selection of virus types. Viperin is also known to enhance the expression of type 1 IFNs in pDCs, by localising to lipid rafts and acting as a scaffold for the recruitment of IRAK and TRAF.
However, Viperin has also proven to be pro-viral in certain contexts. This has been demonstrated in the context of HCMV infection, where virus induced Viperin manipulated cellular metabolism and causes the accumulation of cytosolic lipids for use in production of the viral envelope (102).

4.1.1 *Functional importance of the IFN independent pathway*

Paladino *et al* has hypothesised that the IFN independent antiviral response is necessary for controlling low MOI viral infections. They demonstrated that low MOI HCMV rendered human embryonic lung fibroblasts resistant to subsequent infections, however, high MOI infections required the presence of IFNs to protect the cells from infection. They postulated that this would confer viral resistance to cells such as neurons with a low IFN threshold, that would otherwise elicit potent inflammatory responses which would prove detrimental to the host. In addition it has been postulated that this IFN independent response would confer resistance to early stage infection (<12hrs) (256). Studies using primary human monocytes highlighted that an early IFN independent anti-viral response would enhance monocyte motility/migration and would promote macrophage polarisation towards an M1 phenotype (257). While the majority of these studies have utilised epithelial cells lines, this study highlights the need for understanding these IFN independent anti-viral responses in other primary innate immune cells such as neutrophils, which provide the first line of defence to invading pathogens and form the bulk of the infiltrating immune cells to the site of epithelial cell viral infection, such as RSV and HIV.
ssRNA viruses such as RSV and HIV can induce the expression of ISGs such as Viperin in a TLR8 dependent manner independent of the presence of type 1 IFNs. This ISG induction could be dependent on the presence of intracellular proteins such as IRF1 and IRF3, that mediate early antiviral responses. Created using biorender.
4.2 Hypothesis
Since neutrophils are involved in the disease pathogenesis associated with respiratory viral infections, and that monocytes, respond to viral infections through the upregulation of IFN independent ISGs, we investigated the potential of neutrophils for also eliciting an early IFN-independent anti-viral response. Having observed that neutrophils responded to ssRNA RSV infection via TLR8, we hypothesised that TLR8 could also upregulate ISGs, via IRFs, that would have the potential for viral restriction or enhanced anti-pathogenic neutrophilic functions.

4.3 Specific aims
1. To explore the role of TLR8 in the induction of anti-viral ISGs
2. Determine the potential of ssRNA viruses RSV and HIV in eliciting an early antiviral response, independent of type 1 IFNs
3. To investigate the TLR8 downstream effector proteins involved in this early anti-viral response
4. To elucidate the role of IRF1 in the induction of this early antiviral ISG Viperin expression
4.4.1 The TLR8 agonist, CL075, induces the expression of the ISG, Viperin, in primary human PMNs

Previous studies have explored the roles of IFN-independent mechanisms by which ISGs are induced (251). Having previously explored the role of TLR8 in the induction of the inflammatory cytokines IL-8, IL-6, TNF and IL-1β, when challenged with CL075 and live RSV (chapter 3), we next explored if anti-viral ISGs were induced via TLR8 in primary human PMNs. It has been shown that PMNs isolated from patients presenting with acute respiratory distress exhibit higher levels of ISG induction and were associated with worse clinical outcomes (258). Therefore to explore the role of TLR8 in the induction of antiviral responses in PMNs, we stimulated cells with 2µg/ml of CL075 for a time course of 1-4 hours. mRNA transcripts of (Fig 4.2A) Viperin, (Fig 4.2B) 2’5-OAS, (Fig 4.2C) MxA and (Fig 4.2D) ISG15 were quantified by qRT-PCR. While 2’5-OAS, MxA and ISG15 mRNA were not induced by CL075, Viperin mRNA was significantly upregulated with an 20 fold increase after 4 hours.
Figure 4.2: The TLR7/8 agonist, CL075, induces mRNA expression of the anti-viral ISG, Viperin, in primary human PMNs

Primary human PMNs were treated with CL075 (2ug/ml) for 0, 1, 2 and 4h, before mRNA levels of A) Viperin, B) 2’5’-OAS, C) MxA and D) ISG15 were measured by qRT-PCR. ISG mRNA was calculated relative to the β-actin house-keeping gene and CL075 stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Graphs represent the mean ± SD of 5 independent blood donors. *p<0.05 paired T-Test.
4.4.2 TLR8 induces Viperin mRNA in primary human PMNs

Having observed that CL075 triggers Viperin induction via TLR7/8, we next investigated if TLR8 specifically induced Viperin in response to CL075. PMNs were isolated and pretreated with the TLR8 inhibitor, CU-CPTa for 30 minutes, before being subsequently stimulated with CL075 for 4 hours. RNA was isolated and analysed for the expression of Viperin (Fig 4.3A), OAS (Fig 4.3B), MxA (Fig 4.3C) and ISG15 (Fig 4.3D) by qRT-PCR. By selectively inhibiting TLR8 we found a nearly significant (p=0.0625) reduction in levels of Viperin. While the other ISGs were slightly reduced by TLR8 inhibition, the reduction was not near statistical significance.
Figure 4.3: CLO75 induces anti-viral ISG Viperin specifically via TLR8, in primary human PMNs

Primary human PMNs were stimulated with CL075 (2µg/ml) for 4h, either in the presence or absence of the TLR8 inhibitor CU-CPT9a (5nM). Using qRT-PCR (A) Viperin (B) 2’5’-OAS, (C) MxA and (D) ISG15 mRNA levels were quantified and calculated relative to the housekeeping gene, β-Actin. All samples were compared with the unstimulated control which was normalised to 1. Graphs represent the mean ± SD of 5 independent blood donors. Statistical analysis was carried out using a paired T-test.
4.4.3 HIVIIIB induces the expression of Viperin in primary human PMNs through TLR8

since our experiments indicated that TLR8 induced Viperin in primary PMNs (Fig 4.2/4.3), we next investigated whether live ssRNA virus challenge could also induce this anti-viral response. Upon recognition of its associated ssRNA, TLR8 forms a dimer that suffers a conformational change and allows for the recruitment of subsequent downstream proteins, such as MyD88 (259). HIV-IIIB was chosen to due to its ability to trigger early and chronic ISG induction (260). Previous studies have explored ISG activation in macrophages and DCs (261) (260), yet very little has been explored in PMNs, despite them, also being recruited to the site of infection. Therefore we challenged neutrophils with the HIV-IIIB molecular clone for 30, 60, 120 and 240 minutes or mock control, before analysing the expression of Viperin (Fig 4.4A), 2’5’OAS (Fig 4.4C) and MxA (Fig 4.4B) mRNA by qRT-PCR. We observed a continual increase in Viperin mRNA, with statistical significance observed at both 2 hours and 4 hours after treatment (Fig 4.4A). Additionally, while not significant, we observed a transient increase of 2’5’-OAS (Fig 4.4C) and MxA (Fig 4.4B), both peaking after 30 minutes and declining thereafter. Having observed this early increase in Viperin ISG expression in PMNs 2 hours after exposure to HIV-IIIB, we hypothesised that this induction was direct and not the product of paracrine or autocrine IFN effect. We therefore next explored the role of TLR8 in the induction of this early antiviral response. We treated PMNs with HIV-IIIB for 2 hours, with or without the TLR8 inhibitor (which was added to cells 30 minutes before HIVIIIB treatment), CU-CPT9a, before measuring Viperin (Fig 4.5A), 2’5’OAS (Fig 4.5C) and MxA (Fig 4.5B) mRNA by qRT-PCR. We found that HIV-mediated induction of Viperin was significantly
reduced when TLR8 was inhibited, indicating that PMNs use TLR8 to detect HIV, which leads to the induction of Viperin (Fig 4.4A/4.5A). In contrast we observed no significant reduction in levels of MxA and 2’5 OAS when TLR8 was inhibited (Fig 4.5B/4.5C). Overall these results reveal a new mechanism of viral detection in neutrophils via TLR8, which promotes the induction of specific antiviral ISGs such as Viperin.
Figure 4.4: The HIV-III B molecular clone induces the expression of Viperin in primary human PMNs

Primary human PMNs were exposed to HIV-III B for 0, 30, 60, 120 and 240 minutes. PMNs were harvested for total RNA, before (A) Viperin (B) MxA and (C) OAS mRNA was quantified by qRT-PCR. mRNA was calculated relative to the β-Actin house-keeping gene and HIV-stimulated samples were compared to unstimulated (0h), which was normalised to 1. Graphs represent the mean ± SD of 5 independent blood donors. Graphs are the mean ± SD (n=5) **p<0.01. statistical analysis was carried out using a one way anova with a turkey.
Figure 4.5: The HIV-IIIB molecular clone induces the expression of Viperin mRNA in primary human PMNs via TLR8

Primary human PMNs were exposed to HIVIIIB for 2h, with or without 30min pre-treatment with the TLR8 inhibitor CU-CPT9a (5nM). PMNs were harvested for total RNA, before (A) Viperin (B) MxA and (C) OAS mRNA was quantified by qRT-PCR. mRNA was calculated relative to the β-Actin house-keeping gene and HIV-stimulated samples were compared to unstimulated (0h), which was normalised to 1. Graphs represent the mean ± SD of 5 independent blood donors. *p<0.05. statistical analysis was carried out using paired T test.
4.4.4 RSV-A2 induces the expression of Viperin in primary human PMNs through TLR8

Having observed early induction of TLR8 mediated Viperin in primary human PMNs using both synthetic agonists (Fig 4.3A) and HIV-IIIB (Fig 4.5A), we wondered whether an acute respiratory virus such as RSV could also induce a similar response. Similar to HIV, RSV has been demonstrated to induce the expression of a subset of ISGs in the absence of type 1 IFNs in alveolar macrophages (262). To investigate this PMNs were next challenged with RSV-A2 for 30, 60, 120 and 240 mins or the mock control, before analysing the expression of Viperin, (Fig 4.6A) 2′5′OAS (Fig 4.6C) and MxA (Fig 4.6B) mRNA by qRT-PCR. We observed a continual increase in levels of Viperin mRNA with induction observed as early as 30 mins post-stimulation and significant maximal levels observed at 4h after stimulation (Fig 4.6A). In contrast, we observed no statistically significant variation in levels of MxA (Fig 4.6B) and 2′5 OAS (Fig 4.6C) levels in primary human PMNs stimulated with RSV. We also sought to investigate the role of TLR8 in RSV induced ISG expression. PMNs were pre-treated with CU-CPT9a for 30 mins before being stimulated with RSV-A2 or the mock control for a further 2h. Total RNA was harvested and levels of Viperin (Fig 4.7A), MxA (Fig 4.7B) and OAS (Fig 4.7C) were measured by qRT-PCR. We found that RSV-mediated induction of Viperin was reduced when TLR8 was inhibited (Fig 4.7.A). In contrast levels of MxA and OAS were not reduced through the inhibition of TLR8. Together these results indicate that PMNs use TLR8 to detect RSV, which leads to the induction of Viperin. Overall these results reveal a new mechanism of respiratory viral RSV detection by PMNs via TLR8, which promotes the induction of the antiviral ISG Viperin.
Figure 4.6: RSV-A2 induces the expression of Viperin mRNA in primary human PMNs

Primary human PMNs were exposed to RSV for 0, 30, 60, 120 and 240min. PMNs were harvested for mRNA, before (A) Viperin (B) MxA and (C) OAS mRNA was quantified by qRT-PCR. mRNA was calculated relative to the β-Actin house-keeping gene and RSV-A2-stimulated samples were compared to unstimulated (0h), which was normalised to 1. Graphs represent the mean ± SD of 4 independent blood donors. *p<0.05. statistical analysis was carried out using a one way anova with turkey.
Figure 4.7: RSV induces the expression of Viperin in primary human PMNs partly via TLR8
Primary human PMNs were exposed RSV-A2 for 2h, with or without the TLR8 inhibitor CU-CPT9a (5nM). PMNs were harvested for mRNA, before (A) Viperin (B) MxA and (C) OAS mRNA was quantified by qRT-PCR. mRNA was calculated relative to the β-Actin house-keeping gene and RSV-stimulated samples were compared to unstimulated (0h), which was normalised to 1. Graphs represent the mean ± SD of 4 independent blood donors. statistical analysis was carried out using paired T test.
4.4.5 Activation of TLR8 induces the expression of IRF1 mRNA

Since IRFs are crucial components of TLR8 signalling (263), we initially investigated if IRF genes were induced via TLR8 in PMNs. Previous reports have highlighted a role for both IRF3 and IRF1 in the induction of ISGs independent of IFNs (253), and IRF7 is known to induce ISGs through the IFNAR (264). Since our previous results (Fig 4.4/Fig 4.6) demonstrated that ssRNA viruses could enhance the early expression of Viperin in PMNs, we hypothesised that IRFs would be responsible for this induction. Therefore, to investigate this, PMNs were pre-treated with the TLR8 inhibitor Cu-cpt9a for 30 minutes before being stimulated with CL075 for 4 hours. Total RNA was then collected before measuring levels of IRF1 (Fig 4.8A), IRF3 (Fig 4.8B), IRF5 (Fig 4.8C), IRF7 (Fig 4.8D), IRF8 (Fig 4.8E) and IRF9 (Fig 4.8F) mRNA by qRT-PCR. We found IRF1 was the only significantly upregulated IRF in PMNs stimulated with the TLR8 agonist, CL075 (Fig 4.8A). Additionally, while not significant we observed a small rise in levels of IRF3 (Fig 4.8B) and IRF7 (Fig 4.8D) mRNA with a mean increase of 5 and 10 fold, respectively. In contrast we observed no upregulation of IRF5 (Fig 4.8C), IRF8 (Fig 4.8E) or IRF9 (Fig 4.8F) mRNA. We observed a near significant reduction (p=0.0601) in levels of IRF1 mRNA when TLR8 was inhibited with Cu-cpt9a prior to stimulating with CL075. These findings indicate that TLR8 can induce the expression of IRF1 and suggest that IRF1 may be important in PMN TLR8-induced Viperin.
Figure 4.8: TLR8 activation results in the upregulation of IRF1 mRNA in primary human PMNs

Primary human PMNs were stimulated with CL075 (2µg/ml) for 4h, either in the presence or absence of the TLR8 inhibitor CU-CPT9a (5nM) which was used as a 30 min pre-treatment. Using qRT-PCR (A) IRF1, (B) IRF3, (C) IRF5, (D) IRF7, (E) IRF9 and (F) IRF9 mRNA levels were quantified and calculated relative to the house keeping gene, β-Actin. All samples were compared with the unstimulated control which was normalised to 1. Graphs represent the mean ± SD of 5 independent blood donors. *p<0.05 paired T-Test.
4.4.6 Activation of TLR8 induces Viperin via TBK1

TANK-binding kinase 1 (TBK1) is an essential intracellular protein that is involved in the signal transduction of TLRs eliciting both a proinflammatory and anti-viral response (265). Once viral nucleic acids are sensed by TLR8, host cells recruit various adaptor proteins, such as TIR domain-containing adapter-inducing interferon-β (TRIF) or mitochondria antiviral-signalling protein (MAVS) activate TBK1. TBK1 triggers the phosphorylation of IRF proteins, that subsequently results in their oligomerisation and translocation to the nucleus, where they upregulate the expression of a plethora of genes (266). Having observed an increase of IRF1 mRNA levels in primary human PMNs stimulated with the TLR8 agonist CL075 (Fig 4.8A), we hypothesised that TBK1 might also be required for the early upregulation of ISGs such as Viperin. In order to confirm the role of TBK1 in the TLR8-induced early ISG response in primary human PMNs, primary human PMNs were pre-treated with TBK1 inhibitor, MRT67307, for 30 minutes and subsequently stimulated with 2µg/ml of CL075 for 4 hours. Total RNA was collected and levels of Viperin (Fig 4.9A), OAS (Fig 4.9B), MxA (Fig 4.9C) and ISG15 (Fig 4.9D) mRNA were analysed by qRT-PCR. We found that TBK1 inhibition significant reduced CL075-induced Viperin expression in PMNs (Fig 9.A). In contrast we observed no effect of TBK1 inhibition on OAS, MxA and ISG15 expression. In summary, we found that the TBK1 signalling pathway was needed for the TLR8 induction of Viperin in primary human PMNs.
Figure 4.9: CLO75 induces Viperin via TBK1 in primary human PMNs
Primary human PMNs were stimulated with CL075 (2µg/ml) for 4h, either in the presence or absence of 5nM of the TBK1 Inhibitor (MRT67307) which was used as a pre-treatment for 30 minutes. Using qRT-PCR (A) Viperin (B) 2'5'-OAS, (C) MxA and (D) ISG15 mRNA levels were quantified and calculated relative to the house keeping gene, β-Actin. All samples were compared with the unstimulated control which was normalised to 1. Graphs represent the mean ± SD of 4 independent blood donors. *p<0.05 paired T-Test
4.4.7 Differentiation of HL-60 cells with DMSO (towards a neutrophil phenotype) results in the reduced expression of TLR8

Since we observed IRF1 was upregulated in response to TLR8 stimulation in PMNs (Fig 4.9), we hypothesised that IRF1 was involved in TLR8-mediated Viperin induction. Since HL-60 cells can be differentiated into neutrophil-like cells, we next wanted to use HL-60 cells to specifically determine the role of IRF1 in TLR8-mediated ISG induction. HL-60 cells are promyeoloblasts isolated from the peripheral blood by leukophoresis from a 36-year-old, Caucasian female with acute promyelocytic leukaemia (120, 267). They can be differentiated into mature granulocytes using retinoic acid or DMSO (268). It is important to note that while they exhibit similar characteristics to primary PMNs, they are not identical. In order to characterise the role of TLR8 in HL-60 cells, we first investigated the basal levels of the receptor in both the DMSO differentiated and undifferentiated cells. HL-60 cells were treated with DMSO over the course of 5 days, at a concentration of 0.5% to 1.5% in order to determine the optimal concentration for differentiation. Levels of the neutrophil activation marker CD11b (Fig 4.10A) were used as a marker of differentiation. We observed a dose dependent increase in levels of CD11b, with the highest levels of CD11b observed at 1.5% DMSO (Fig 4.10A). Next, we compared basal levels of TLR4 and TLR8 mRNA between undifferentiated and differentiated HL-60 cells (1.5% DMSO over 5 days) using qRT-PCR (Fig 4.10B). Previous studies have highlighted the differences in TLR4 mRNA expression between undifferentiated and differentiated HL-60 cells, with higher levels observed in differentiated (269). We therefore hypothesised that TLR8 levels may vary depending on the HL-60 cells differentiation state. In agreement with the previous study, we observed an increase in levels of TLR4 in
differentiated cells. However, we observed a decrease in levels of TLR8 on differentiation of the HL-60 by qRT-PCR (Fig 4.10B). In order to investigate if TLR8 protein levels mirrored the mRNA expression we used intracellular flow cytometry to assess levels of TLR8 (Fig 4.10C/4.10D). Mirroring our TLR8 mRNA findings, we found undifferentiated HL-60s express higher levels of TLR8 protein, and that when differentiated (DMSO+) they downregulated the expression of the endosomal receptor (4.10B/C/D). In summary, we found that HL-60 cells express lower levels of TLR8 when undifferentiated using DMSO.
Figure 4.10: Undifferentiated HL-60s express higher levels of TLR8 compared to DMSO differentiated granulocytic HL-60s cells

(A) HL-60s were differentiated over the course of 5 days using a gradient concentration of DMSO. MFI of CD11b was measured using flow cytometry. Fold change was measured by normalising to the unstimulated negative control (0% DMSO). HL-60s were harvested and total RNA collected from undifferentiated cells (DMSO-) and differentiated cells (DMSO+) before levels of (B) TLR4 and TLR8 mRNA were quantified by qRT-PCR. mRNA was calculated relative to the β-Ac house-keeping gene and differentiated samples (DMSO+) were compared to undifferentiated (DMSO-), which was normalised to 1. (C) MFI of TLR8 was measured using flow cytometry and (D) MFI fold change of TLR8 was measured by normalising the differentiated (DMSO+) to the undifferentiated HL-60 cells (0% DMSO). Graphs represent the mean ± SD of 4 independent experiments. *p<0.05. **p<0.01 paired T test.
4.4.8 CL075 induces ISG expression in undifferentiated HL-60 cells

Having identified that undifferentiated HL-60 cells express higher levels TLR8 than differentiated we decided to use undifferentiated HL-60 cells to further investigate the role of TLR8/IRF1 in ISG induction (Fig 4.10). To this end we initially analysed CL075 mediated induction of ISGs by stimulating HL-60s with 2µg/ml of CL075 for 4 hours. Total RNA was collected and the ISGs, Viperin (Fig 4.11A), MxA (Fig 4.11B), and ISG15 (Fig 4.11C), were measured by qRT-PCR. We observed that HL-60s stimulated with endosomal TLR7/8 agonist for 4 hours induced early expression of Viperin mRNA, (4 fold increase and p=0.0625) (Fig 4.11A). Indeed, this was a similar observation to that seen in primary human PMN (Fig 4.2A). Additionally we saw some induction in levels of MxA mRNA, with a mean 3 fold increase (Fig 4.11B), but no induction in levels of ISG15 mRNA (Fig 4.11C).

**Figure 4.11: The TLR7/8 agonist, CL075, induces mRNA expression of the anti-viral ISG, Viperin, in HL-60 cells**

HL-60s were treated with CL075 (2ug/ml) for 4 hours, before mRNA levels of A) Viperin  B) MxA and C) ISG15 were measured by qRT-PCR. ISG mRNA was calculated relative to the β-actin house-keeping gene and CL075 stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Graphs represent the mean ± SD of 4 independent blood donors. paired T test.
4.4.9 CL075 induces the upregulation of IRF1 in HL-60s

Having identified that CL075 could induce the expression of Viperin in HL-60s and PMNs (Fig 4.11A) and (Fig 4.2A), and having observed a significant increase in IRF1 mRNA in PMNs upon stimulation with CL075 (Fig 4.8A), we hypothesised that IRF1 might be an important component of TLR8/CL075 signalling induction of Viperin. In order to examine the potential of IRF1 in the induction of Viperin prior to knocking down IRF1 we first analysed the expression of IRF1 in HL-60 cells treated with CL075. HL-60 cells were stimulated with CL075 over a timecourse spanning 4h. Cells were lysed in RIPA buffer and immunoblotted for IRF1 and IRF7 (Fig 4.12A). Preliminary results demonstrated a time dependent increase in levels of IRF1 protein, with levels rising to a maximum at 2h and reducing again at 3h and 4h. In contrast, we saw early phosphorylation of IRF7, our control at 1 hour. Having established that levels of TLR8 varied between HL-60s in the undifferentiated and differentiated state (Fig 4.10), we sought to explore whether varying levels of the TLR8 receptor would also affect downstream inducible IRF1 levels, following CL075 stimulation (Fig 4.12B). HL-60 cells were differentiated using 1.3% DMSO over 5 days and were subsequently stimulated with the TLR8 agonist CL075, alongside undifferentiated cells. Cells were lysed in RIPA buffer and immunoblotted for IRF1 (Fig 4.12B). We observed that both differentiated and undifferentiated HL-60s upregulated the expression of IRF1 following CL075 stimulation (Fig 4.12B), although undifferentiated cells (DMSO-) induced the expression of IRF1 to a higher degree, possibly due to the higher expression of TLR8 observed undifferentiated HL-60s (Fig 4.10).
Figure 4.12: CL075 induces IRF1 protein expression in HL-60s

(A) undifferentiated HL-60s were treated with CL075 (2ug/ml) over a timecourse of 4 hours. Cells were lysed in RIPA buffer and lysates blotted for IRF1 and our control IRF7. (B) undifferentiated (DMSO-) and differentiated (DMSO+) cells were stimulated with CL075 (2µg/ml) for 2 hours. Cells were lysed in RIPA buffer and lysates blotted for IRF1 and β-Actin
4.4.10 siRNA Knockdown of IRF1 in HL-60s

In order to determine if IRF1 is required for the induction of ISGs via TLR8, we next knocked down its expression before treating with the TLR8 agonist CL075. Due to the difficulty in transfecting suspension cells, such as HL-60s, with lipid based transfection reagents, we chose to use electroporation to improve our transfection efficiency. To choose the optimal conditions for transfection, we first investigated the knockdown efficiency using 3 different conditions, based on their pulse voltage (v), pulse width (pw) and the actual number of pulses (pn). The three pulse conditions chosen were; condition 1 (1350v, 35pw, 1pn) (Fig 4.13A/D/G), condition 2 (1250v, 20pw 2pn) (Fig 4.13B/E/H) and condition 3 (1650 25pw 1pn) (Fig 4.13C/F/I). HL-60 cells were transfected with IRF1 siRNA and RLUC siRNA (control siRNA) for 24h. Cells were collected and total RNA and protein lysates harvested. Using qRT-PCR we observed a statistically significant reduction in IRF1 when using condition 1 relative to the control siRNA and normalised to the housekeeping gene (Fig 4.13A). In addition to checking the knockdown of IRF1 at mRNA level we also analysed whether this was reflected at protein level. Using the same 3 conditions as above and immunoblotting for IRF1 protein we found that condition 1 was statistically significant in generating an optimal knockdown for IRF1 protein relative to the control siRNA and normalised to the housekeeping (Fig 4.13G). In contrast, IRF1 in condition 2 (Fig 4.13E/H) and condition 3 (Fig 4.13F/I) were not significantly reduced when compared to the control siRNA RLUC.
Figure 4.13: siRNA knockdown of IRF1 in HL-60s

HL-60s were transfected for 24 hours with either a specific IRF1 siRNA or RLUC, the control siRNA using 3 different transfection conditions. (A-C) mRNA levels of IRF1 were measured by qRT-PCR. IRF1 mRNA was calculated relative to the $\beta$-actin house-keeping gene and IRF1 knockdowns were compared to the control siRNA, which was normalised to 1. (D-I) Cells were lysed in RIPA buffer and lysates blotted for IRF1 by western blot. Graphs (D), (E) and (F) are the representative blots for condition 1, condition 2 and condition 3, respectively. Graphs (G), (H) and (I) is the associated densitometry for condition 1, condition 2 and condition 3, respectively. IRF1 expression was calculated relative to the $\beta$-actin house-keeping gene and IRF1 knockdowns were compared to the control siRNA, which was normalised to 1. Graphs represent the mean $\pm$ SD of 4 independent experiments. *p<0.05. paired T test
4.4.11 IRF1 knockdown results in a reduction in levels of TLR8-induced Viperin expression

Having established a successful knockdown of IRF1 using transfection condition 1 (Fig 4.13A/D/G), we next analysed the role IRF1 in TLR8 signalling of HL-60 cells and its specific role in ISG induction. HL-60s were transfected for 24h with either IRF1 siRNA or RLUC siRNA (control siRNA). After the 24 hour transfection, the IRF1 and RLUC siRNA transfected HL-60s were stimulated with CL075 for 4 hours and total RNA collected before analysing the expression of Viperin (Fig 4.14A), ISG15 (Fig 4.14C) and MxA (Fig 4.14B) mRNA by qRT-PCR. Upon IRF1 knockdown, we observed a twofold decrease in levels of Viperin with it trending towards significance (p=0.0625). Interestingly, while not significant, we observed a reduction in levels of MxA and ISG15 mRNA upon IRF1 knockdown of HL-60 cells suggesting a role for IRF1 in the induction of ISGs in TLR8/CL075 signalling in these.

Figure 4.14: TLR8 mediated induction of Viperin mRNA is IRF1 dependent

The HL-60 cell line was transfected with 10nM of either control (RLUC) or IRF1 siRNA for 24 hours before being treated for 4 hours with 2ug/ml of CL075. mRNA levels of A) Viperin  B) MxA and C) ISG15 were measured by qRT-PCR. ISG mRNA was calculated relative to the β-actin house-keeping gene and CL075 stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Graphs represent the mean ± SD of 4 different experiments. *p<0.05, paired T test
4.5 Discussion

The IFN dependent induction of ISGs is a system found in all nucleated cells and is required for the restriction of various stages of the viral life cycle, from viral entry to viral replication. Type 1 IFN expression is induced following the detection of viral PAMPs by PRRs. Depending on the virus, the innate immune arsenal is equipped with an array of PRRs that can detect the pathogen, such as cytosolic DNA receptors, but also the endosomal TLRs, TLR7/8/9. TLR7/8 are specifically designed for the recognition of ssRNA viruses, such as HIV-1 and RSV (270) (271). Several viral species hijack or disrupt this biological mechanism in order to evade the innate antiviral response. These immune evasion strategies are demonstrated using viral models such as HCV and RSV, which target various proteins in the Type 1 IFN JAK/STAT pathway (90)(72). In order to combat this cells have developed mechanisms to induce anti-viral of ISGs independent of type 1 IFNs being present (272). Viperin is an ISG whose expression inhibits a host of DNA and RNA viruses. Viperin acts by inhibiting the release of new virions from infected cells. Viperin previously has also been demonstrated to be induced through an IFN independent mechanism (273). While neutrophils are traditionally thought to play roles in only bacterial and fungal infections, there is an ever growing body of evidence highlighting their role in viral infection (274). Therefore, one of the aims of this study was to determine the role of TLR8 in inducing an early IFN independent anti-viral response in primary human PMNs. To this end we challenged human PMNs with a synthetic TLR7/TLR8 agonist and live viruses including HIV and RSV, with or without the presence of a number of pathway inhibitors.
While few studies have explored the role of ISG induction independent of IFN induction of ISGs, recent studies have begun to shed light on the phenomenon. Such a study includes the upregulation of ISGs such as MxA and ISG15 in response to cytomegalovirus via IRF3 activation in cells engineered to be unresponsive to IFNs (275). The upregulation of ISGs is generally associated with an improved clinical outcome. ISGs have been recorded to inhibit various stages of the viral lifecycle from replication to viral budding. However, ISG expression in PMNs can be associated with neutrophil dysfunction in ARDS, a condition often associated with respiratory diseases such as RSV. Understanding the nature of this double edged ISG induction in primary human PMNs could be pivotal in recognising and treating viral infections, especially those associated with ARDS.

We found that PMNs upregulate the expression of Viperin when stimulated with the TLR7/8 agonist CL075. In addition, we observed that this induction was in part mediated by TLR8. Having observed this induction in ISGs we sought to explore the role live viruses would have in the induction of Viperin in primary human PMNs. Using both HIV-IIIB and RSV-A2 we observed a time dependent increase in levels of Viperin. In contrast, MxA and OAS were not upregulated, nor inhibited through TLR8 antagonism, suggesting that Viperin is particularly important in the early anti-viral response and that its expression is regulated by TLR8 detection of viral components. While we hypothesised that the induction of Viperin might be virus specific we found that two different ssRNA viruses induce its expression. RSV predominantly infects epithelial cells of the respiratory tract whereas HIV infects CD4+ T cells and macrophages (276) (277) (278).
Therefore, this ISG induction was demonstrated not to be virus specific but was likely dependent on a component both viruses share, such as their ssRNA genomes. By selectively inhibiting TLR8 in the PMNs, we were able to significantly reduce the expression of Viperin at 2 hours after viral challenge. We proposed that this ISG induction in PMNs was independent of receptor mediated viral entry due to lack of studies evidence that supports the idea that HIV/RSV can actively replicate intracellularly. Instead we proposed that this viral internalisation and subsequent activation of TLR8 was mediated by some form of passive phagocytosis of viral components. Having determine this novel induction of ISGs in human PMNs in response to ssRNA viruses we sought to explore the potential associated intracellular mechanisms. To start we explored the EMBL-EBI human genome atlas to explore variations in levels of intracellular proteins, such as IRFs. We found that levels of IRF proteins varied massively across human and mouse species. IRF1 was the predominant IRF expressed in human immune organs and cells, whereas IRF3 was the predominant IRF expressed in mice, indicating intraspecies variations in anti-viral pathways (Appendix Fig 8.1).

In order to confirm these observations, we inhibited IRF signalling in PMNs to determine its role in the induction of Viperin expression. We found that inhibition of IRF signalling resulted in a reduction in Viperin expression. In combination we explored any possible induction of IRF proteins. We observed that CL075, specifically through TLR8 could induce the expression IRF1. In order to confirm the potential role of IRF1 in the induction of Viperin we specifically knocked down IRF1. We chose the HL-60 cell line to establish a specific knockdown. HL-60s are promyelocytes and were therefore chosen due to their close relation to mature granulocytes, albeit not fully matured cells. Promyelocytes have been used to
study neutrophil function in the past, with a protocol of differentiation using DMSO been established (279). In order to determine whether HL-60 cells were suitable for studying TLR8-mediated ISG induction, we first sought to determine the presence of TLR8. Studies have shown that on differentiation of HL-60s receptors are either upregulated or downregulated (269). Therefore we differentiated the cells over 5 days and checked for levels of intracellular TLR8 between the undifferentiated and differentiated cells. We found that differentiated HL-60s downregulate their expression of TLR8. Having observed this we next sought to determine whether TLR8 was functional in HL-60 cells. To this end we stimulated the cells with CL075 and measured ISG induction. We found that they selectively upregulate the expression of Viperin and IRF1. Next we sought to specifically knockdown the expression of IRF1 in the HL-60s. Transfecting suspension cells using Lipofectamine proved to be difficult and unsuccessful, so we decided to use electroporation of siRNA to establish a selective knockdown of IRF1. We confirmed the knockdown using western blot and qRT-PCR. Finally, we found that on IRF1 knockdown, ISG induction downstream of TLR8 was reduced. Our study indicates that neutrophils possess IFN independent anti-viral pathways which may restrict the viral life cycles. Additionally, we demonstrate the importance of TLR8 induced type 1 IFN independent induction of Viperin in neutrophils. This was demonstrated by challenging neutrophils with HIV-1 or RSV-A2 either in the presence or absence of a TLR8 inhibitor. Additionally we have demonstrated the role of IRF1 downstream of TLR8 in inducing this IFN independent induction of Viperin. In summary, we highlight a novel anti-viral role of neutrophils. We anticipate these exciting findings to have wider implications against other viruses and to be fundamental in our understanding how viruses
may be detected by the innate immune system. Viperin has been demonstrated to be instrumental in the inhibition of several structural proteins required for virus assembly. We demonstrate a potential novel anti-viral pathway independent of the presence of Type 1 Interferons through the induction of Viperin and IRF1 and may shed light on the numerous ways virus escape the immune response leading to viral persistence.
Chapter 5

TLR8 induces type 1 IFNs which themselves signal via the STATs and IRFs to upregulate Viperin
Chapter 5: Differences in IFN induced expression of STATs, IRF1 and ISGs in both PMNs and PBMCs

5.1 Introduction

ssRNA viruses are well documented to induce the expression of a plethora of IFNs in a number of different cell types through the activation of PRRs such as TLRs and NLRs. Type 1 IFNs are integral to regulating the anti-viral immune response such as regulating NK cell effector functions (280). Type 1 IFN family consists of 14 subtypes of IFN-α, along with IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-ζ, and IFN-τ (281). Immune cells known to secrete large quantities of type 1 IFNs include DCs and macrophages (282) (283). Plasmacytoid DCs are known as professional IFN-α producing cells due to their abundant expression of both TLR7 and TLR9, as well as their constitutive expression of intracellular IRF7 (284). Early studies revealed that Sendai virus could induce IFN-α in blood PBMC populations (285). The PBMC population consists of various immune cells including lymphocytes (T cells, B cells, and NK cells), monocytes, and DCs. In addition to type 1 IFNs there are both type 2 (IFN-γ) and the more recently discovered type 3 (IFN-λ). Type 2 production is associated with activated CD4+ T-cells (Th-1), CD8+ cytotoxic T cells, NK cells and γδ T cells (286). The more recently discovered IFN-λ has been shown to be predominantly produced by epithelial cells of the respiratory and gastrointestinal tract. They have been demonstrated to have overlapping functions with Type 1 IFNs, yet have recently been shown to exhibit unique functions, in particular the temporal effects, with type 1 produced early and type 3 expressed in a delayed manner. Respiratory
viruses, including influenza, also induce the expression of IFN-λ suggesting an anti-viral role (287).

IFNs exhibit their anti-viral effects by binding to their associated receptor. Type 1 IFNs bind to the IFN associated receptor (IFNAR). The type 1 IFN receptor is composed of two subunits: IFNAR1 and IFNAR2. IFNAR1 consists of only 1 isoform, whereas IFNAR2 has 3 isoforms. IFNAR2c is the only isoform that possesses the full intracellular domain capable of transducing signals once IFN binds. IFNAR2b is a truncated form and IFNAR2a is a soluble form, capable of binding IFN, but unable to efficiently transduce any signalling cascade. Binding of IFN to IFNAR2 allows for IFNAR1 to bind. It has been demonstrated that this ternary complex requires endocytosis of the receptor for efficient JAK binding (288). There are 4 members of the JAK family. They consist of JAK1, JAK2, JAK3 and TYK2. JAKs are large multidomain proteins that utilise the N terminal for binding the intracellular portion of IFNAR and a C terminal reserved for its kinase activity and associated STAT protein recruitment (289). Phosphorylation of JAKs results in the autophosphorylation of STAT proteins. The STATs that are activated by IFN-α are STAT1, STAT2, STAT3 and STAT5 (290). Phosphorylation of STATs allows dimerization and subsequent translocation to the nucleus, where they regulate the expression of a plethora of genes, such as ISGs. STATs can either form homodimers or heterodimers. An important transcriptional complex induced by type 1 IFNs if the ISG factor 3 (ISGF3). The complex consists of a heterodimer complexed with IRF9. It binds the IFN stimulated response element (ISRE) which is present at the promoter region of a number of ISGs (Fig 5.1). Other STAT complexes that are induced from type 1 IFNs include STAT1–STAT1, STAT3–
STAT3, STAT5-STAT5 homodimers and STAT1–STAT2, STAT1–STAT3, STAT1–STAT5, STAT2–STAT3 heterodimers. Such IFN-induced complexes bind another type of element — known as an IFN-γ-activated site (GAS) element— that is present in the promoter of ISG (281). Additionally, IRF1 has also been shown to be important in the upregulation ISGs in response to type IFNs (291).

ISGs have been well documented to target RNA viruses through the recognition of viral replicative intermediates and other molecular features associated with RNA viral genomes. Specifically, ISGs can elicit their anti-viral effects through the degradation of RNA, inhibition of transcription and translation which culminate in blocking viral replication. Viperin has been determined to inhibit viral replication in a number of ways and is a heavily conserved ISG across all kingdoms of life. Recently its enzymatic activity has been determined. It was shown that it converts CTP to 3′-deoxy-3′,4′-didehydro-CTP, which functions as novel chain-terminating antiviral nucleotide when mis-incorporated by viral RNA-dependent RNA polymerases. Additionally in higher order organisms it interacts with a number of host proteins. These proteins can include metabolic proteins needed for viral replication (292). MxA has been established to inhibit a host of viruses such as influenza by preventing transport of the viral genome to the nucleus (293). ISG15 overexpression in cell culture has also demonstrated to have broad antiviral effects, such as the inhibition of viral replication, and viral budding (294).

While STAT signalling and ISG induction are associated with a positive anti-viral response (295), aberrant STAT signalling is also associated with autoimmune inflammatory disorders and tumour initiation and progression (296) (297). This
double edged sword has been nicely shown, with STAT3, STAT4, and STAT5 being linked to tumour progression, whereas STAT1 and STAT2 are linked to tumour killing processes (298) (299). Type 1 IFN signalling has therefore been implicated in anti-tumour immunity. Type 1 IFNs have been shown to prime DCs through the upregulation of MHC and costimulatory molecules, which in turn aids in errant cell recognition by T cells (300). On the contrary, type 1 IFNs can upregulate the expression of STAT3 and STAT5 which are known to modulate the expression of immunosuppressive cytokines, such as IL-10 and IL-4, revealing that the impact of STAT signalling in the tumour microenvironment can be destructive as well as favourable (301) (302). Neutrophils have been shown to require STAT signalling for the recruitment and extravasation of cells from the blood into the tissues. This has been clearly demonstrated using various infection models. E.coli has been shown to induce the expression of IL-6, which in turn activates both STAT1 and STAT3, both of which are needed for neutrophil recruitment, with IL-6 deficient mice having higher bacterial burdens (303). In addition it has been shown that STAT4 is essential for neutrophil function against Staphylococcus Aureus infection. STAT4 deficient mice were unresponsive to IL-12 and therefore had reduced ROS production, chemotaxis and NET production, all of which are well established to function in microbial killing (304). While type 1 IFNs classically use the JAK/STAT pathway to induce ISGs, they have also been shown to use IRF1 in epithelial cells for the induction of anti-viral ISGs and proinflammatory cytokines. Additionally, IRF1 has been shown to be differentially expressed depending on the expression of IFNs, with type 1 IFNs highly upregulating it and type III failing to do so (305).
5.1: Type 1 IFN induced expression of ISGs
PMNs respond to Type 1 IFNs through the rapid phosphorylation of STAT homo and heterodimers (STAT1/2/3) and IRF1. These STAT/IRF proteins are responsible for the upregulation of a number of ISGs through canonical IFN signalling such as the ISRE and GAS response elements. Created using biorender.
5.2 Hypothesis

Since PMNs are key innate immune cells in the antimicrobial response utilising processes such as ROS and NET production, we sought to explore the potential STAT proteins that may be involved in those processes. Having observed in our previous chapters that primary human PMNs directly responded to the TLR7/8 agonist CL075 and to ssRNA viruses RSV and HIV resulting in the expression of a plethora of inflammatory and anti-viral ISGs, we hypothesised that TLR8 activation could also induce the expression of type 1 IFNs. Furthermore, we hypothesised that PMNs could respond to these IFNs and subsequently induce ISGs via intracellular signal transduction of STAT and IRF proteins.

5.3 Specific Aims

1. To investigate the expression pattern of Type 1 IFNs in PMNs challenged with the TLR8 agonist CL075 compared to PBMCs.

2. Determine if STAT1-3 and IRF1 proteins are involved downstream of IFNAR in response to type 1 IFNs in PMNs, compared to PBMCs.

3. Determine the effect of type 1 IFN upon ISG expression in PMNs, compared to PBMCs.

4. Explore the specific role of IRF1 in the induction of the IFN response observed in HL-60 cells.
5.4.1 TLR7/8 activation results in the upregulation of IFN-α and IFN-β in PBMCs and PMNs

Having previously explored the role of TLR8 expressing PMNs in the detection of the TLR8 agonist CL075, RSV and HIV and subsequent induction of ISGs (Chapter 4), we next sought to investigate the role of TLR8 in the induction of antiviral type 1 IFNs. PBMCs have been well documented to produce IFN-α in numerous autoimmune and pathogenic infection models (306). This production has been proposed to be mainly from DCs and monocyte/macrophages (307) (308). To confirm the potential of primary human PMNs compared to PBMCs for producing type 1 IFNs via TLR8, cells were stimulated with both CL075 and LPS for 12 and 24 hours. Supernatants were collected and analysed for IFN-α (Fig 5.2A) and IFN-β (Fig 5.2B) protein by ELISA. While PMNs did not produce large quantities of IFN-α nor IFN-β, we did observe both type 1 IFNs (IFN-β (~50pg/ml) and IFN-α (<20pg/ml)) in response to CL075 after 24 hours with p values of 0.0602 and 0.1624 respectively. In contrast, PBMCs from the same donor were shown to produce significant quantities of IFN-α and IFN-β at 24 hours post stimulation with the TLR7/8/ agonist CL075. In summary, we found that while PBMCs significantly produce type 1 IFNs, PMNs produce low levels highlighting their differential effects.
Figure 5.2: Type 1 IFNs are produced in both primary human PMNs and PBMCs in response to TLR8 agonist CL075

Human PMNs and PBMCs (1x10^6) were rested in serum free media for 30min. Cells were treated for 12h or 24h with either LPS, CL075. Supernatants were collected and (A) IFN-α (B) IFN-β protein levels was quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 (n=6)
5.4.2 TLR8 activation results in the upregulation of IFN-α/IFN-β from PBMCs

The pathways involved in the generation of a type 1 IFN response have been proposed to be due to the activation of a number of PRRs such as RIG-I, TLR7 and TLR9. In particular, TLR7 has been shown to be instrumental in the generation of and IFN-α from DCs in viral infection (309). Having previously observed that TLR8 was the predominant receptor in PMNs for inducing early antiviral and inflammatory responses to both CL075, RSV and HIV (Chapter 3/4), we next hypothesised that TLR8 may specifically induce the production of IFNs from PBMCs. Having observed that CL075 can induce the expression of type 1 IFNs in primary human PBMCs (Fig 5.2B), we next investigated if TLR8 was specifically was involved using a specific TLR8 inhibitor, Cu-cpt9a. In order to confirm the role of TLR8 in the generation of secreted IFN-α, TLR8 was inhibited and PBMCs were subsequently stimulated with the TLR7/8 agonist CL075 for 12 and 24h. Supernatants were collected and analysed for IFN-α (Fig 5.2A) and IFN-β (Fig 5.2B) by ELISA. We observed that the TLR8 inhibition resulted in the significant reduction of secreted IFN-α by about 50% in primary human PBMCs at 24h (Fig 5.2A). Similarly, the inhibition of TLR8 resulted in the significant reduction of IFN-β in PBMCs at 24 hours post CL075 stimulation (Fig 5.2B). Together these findings indicate that TLR8 is a key endosomal receptor in the generation of a type 1 IFN response in primary human PBMCs.
Figure 5.3: TLR8 is a key receptor in inducing type 1 IFNs from human PBMCs

Human PBMCs (A/B) (1x10^6) were rested in serum free media for 30min followed before treating with the TLR8 inhibitor CU-CPT9a for 30 minutes prior to subsequent stimulations. Cells were treated for 12h or 24h with either LPS or CL075. Supernatants were collected and (A) IFN-α and (B) IFN-β protein levels were quantified by ELISA. Data is represented as the mean ± SD. Paired T-test. *p<0.05 (n=6)
5.4.3 IFN-α induces IRF1 expression and phosphorylation of STAT1-3 in primary human PMNs

Having confirmed the specific role of TLR8 in the induction of IFN-α (Fig 5.3A) and IFN-β (Fig 5.3B) in PBMCs, we next explored how type 1 IFNs could modulate STAT signalling/ISG induction in primary human PMNs due to both cells coexisting in communicating when mounting an immune response. We hypothesised that type 1 IFNs secreted from PBMCs via TLR8 could activate the antiviral JAK/STAT pathway in both themselves and infiltrating PMNs in an autocrine/paracrine manner respectively. STAT1 and STAT2 proteins are key mediators of type I IFN signalling, and are essential components of the cellular antiviral response (310). STAT3 has also been demonstrated to play a role in the signal transduction of a plethora of cytokines as well as playing an essential role in embryonic development (311) (312). However, despite this, STAT3 has more recently been described to be involved in the antiviral response, proving to be both pro-viral and anti-viral, regulating HCV, HIV and influenza A virus (313) (314). These three STAT proteins are instrumental in the antiviral response, observed through the use of knockout mice which exhibit limited antiviral responses (315). STAT1 and STAT2 have been extensively studied in their signal transduction of type 1 IFNs in cell lines and in primary human cells, mainly monocytes, macrophages and DCs during viral infection (316). In contrast, since PMNs have been previously believed to play only a minor role in antiviral immunity, very little work has been done on the role of IFN-JAK/STAT signalling in PMNs. IFNs have been shown to induce transcription of the IRF1 gene, with type II IFNs shown to be the most potent inducer of IRF1 transcription in most cell types (305). This IRF1 upregulation has also been demonstrated to be
instrumental in the induction of ISGs in human myeloid cells. In order to determine the downstream effector STAT proteins involved in this IFN signal transduction, PMNs were treated with IFN-α over a time course of 2h. Protein lysates were collected and immunoblotted for phospho-STAT1/STAT2/STAT3, total STAT1/STAT2/STAT3 and IRF1. IFN-α stimulation of PMNs resulted in a significant increase in phosphorylation of STAT1 at 2 hours post treatment, with phosphorylation seen as early as 5 minutes post-stimulation (Fig 5.4A). Additionally, while not significant, we observed phosphorylation of both STAT2 and STAT3 as early as 5 minutes post stimulation (Fig 5.4B/C). In contrast, levels of total STAT proteins remained unchanged. Interestingly, we observed a later significant increase in IRF1 expression at 2 hours after IFN-α stimulation in PMNs (Fig 5.4D). Together these results reveal for the first time that PMNs respond to IFN-α which induces early and prolonged pSTAT1, but transient pSTAT2 and pSTAT3 and late IRF1 induction.
Figure 5.4: IFN-α induces the phosphorylation of STAT1-3 and expression of IRF1 in primary human PMNs

PMNs (1 x 10^6 cells/ml) were stimulated with IFN-α for the indicated times. A) Phosphorylation of STAT1, STAT2, STAT3, Total STAT1-3, total IRF1 and β-Actin were detected by immunoblotting whole cell lysates. B) Densitometric analysis of 3 immunoblots was performed using the image lab software. The bar graph illustrates a ratio of STATs/IRF1: β-Actin, relative to the untreated (PBS) control, which was normalised to 1. Statistical differences were assessed using one way ANOVA with a Tukey post-test (*p<0.05) (n=3)
5.4.4 IFN-α differentially upregulates the phosphorylation of STAT1, STAT2 and STAT3 in PBMCs

Having explored the induction of pSTATs and IRF protein in PMNs (Fig 5.4) we next analysed how the PBMC population would respond to IFN-α compared to PMNs. Previous studies have highlighted the differences between early and late ISG induction in macrophages during viral infection (260). Nasr et al. demonstrated that ISG induction occurs in two phases, the first phase occurs within 1 hour after infection and is characterised by the transient upregulation IRF1 and ISGs in response to viral extracellular vesicles, and the second phase occurs after 48 hours, which involves the persistent upregulation of ISGs in response to newly synthesised intracellular virions. These individual phases are characterised by different subsets of ISGs. Therefore, we sought to explore the whether the IFN upstream effector STAT proteins and IRF1 could underlie the temporal differences observed in viral infection between different immune cell populations (PBMCs and PMNs). PBMCs from the same donors used to isolate PMNs in Fig 5.4 were isolated and stimulated with IFN-α over a time course of 2 hours. Protein lysates were collected and immunoblotted for phospho and total STAT1/STAT2/STAT3 and IRF1. Similarly to PMNs (Fig 5.4A), we observed significant phosphorylation of STAT1 in PBMCs at 2 hours after IFN stimulation (Fig 5.5A). Additionally, while not significant, we observed phosphorylation of STAT2 and STAT3, with a mean fold increase of 50 and 5 at 2 hours post IFN-α stimulation, respectively. However, in contrast to PMNs (Fig 5.4B/C), the phosphorylation of both STAT2 and STAT3 occurred later in PBMCs than PMNs. We observed phosphorylation of STAT2 and STAT3 at 60 minutes and 120 minutes after IFN stimulation, respectively, in PBMCs, with a mean 50 and 5 fold
increase respectively (Fig 5.5B/C). In addition, IRF1, while not significantly upregulated, we observed a 1.5 fold increase in PBMCs earlier (30 minutes) than the PMN population (Fig 5.4D), in which we saw significant upregulation of IRF1 at 2 hours after stimulation with IFN-α (Fig 5.5D). In contrast to PMNs (Fig 5.4D) IRF1 levels in the PBMC population had high basal levels. Again, levels of total STAT protein remained unchanged. In summary, IFN induced later phosphorylation of STAT2 and STAT3 when compared to PMNs, as well as having higher basal levels of pSTAT1 and IRF1 in the PBMC population.

![Image of graphs showing IFN-α induced phosphorylation of STAT1 and STAT2](image-url)
Figure 5.5: IFN-α induces the phosphorylation of STAT1-3 in primary human PBMCs
PBMCs (1 x 10^6 cells/ml) were stimulated with IFN-α for the indicated times. A) Phosphorylation of STAT1, STAT2, STAT3 and IRF1 was detected by immunoblotting whole cell lysates. B) Densitometric analysis of 3 immunoblots was performed using the image lab software. The bar graph illustrates a ratio of STATs/IRF1:β-Actin, relative to the untreated (PBS) control which was normalised to 1. Statistical differences were assessed using one way ANOVA with a Tukey post-test (*p<0.05) (n=3)
5.4.5 IFN-α stimulation results in heightened levels of Viperin mRNA expression in PMNs compared to the PBMC population

Having observed temporal differences in the phosphorylation of STAT1/2/3 and expression of IRF1 between PMNs and PBMCs (Fig 5.4/Fig 5.5) from the same donor, we wondered if these changes would affect the expression patterns of the downstream ISGs in both PBMCs and PMNs. To investigate this, PBMCs and PMNs were isolated from the same donors and stimulated with IFN-α over a time course of 4 hours. RNA was extracted and used to measure levels of Viperin (Fig 5.6A), MxA (Fig 5.6B) and ISG15 (Fig 5.6C). In particular, we found increasing levels of Viperin in PMNs when compared to PBMCs from the same donor stimulated with IFN-α (Fig 5.6A). We observed a time dependent 40 fold increase in levels of Viperin in PMNs with the p value trending toward significance (p=0.0579) at 4 hours after IFN stimulation. In comparison we observed a much reduced 5 fold increase in Viperin mRNA in the PBMC fraction with the p value of 0.265 in PBMCs at 4 hours post IFN stimulation. In contrast, while there were small increases, observed at 2h, there was no significant induction of MxA or ISG15 in either PMNs and PBMCs (Fig 5.6B/5.6C). The differences observed in ISG expression between PMNs and PBMCs could be the result of differences in the observed phosphorylation of STAT1, STAT2 and STAT3, as well as a strong induction of IRF1 in PMNs when compared to PBMCs (Fig 3/4). In summary, type 1 IFNs are key regulators of Viperin expression in PMNs when compared to PBMCs. Additionally, type 1 IFNs don’t appear to regulate the expression of either MxA or ISG15 in both PMNs and PBMCs, indicating that Viperin is an important ISG downstream of the antiviral cytokine IFN-α in primary human PMNs.
Figure 5.6: PMNs but not PBMCs upregulate the early expression of Viperin in response to IFN-α

Primary human PMNs and PBMCs (1X10⁶) were treated with IFN-α (1000IU) for 0, 1, 2 and 4 hours before mRNA levels of (A) Viperin, (B) MxA and (C) ISG15 were measured by qRT-PCR. mRNA was calculated relative to the β-actin house-keeping gene and IFN-α stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Data is represented as the mean ± SD. Statistical differences were assessed using a paired T test (n=3)
5.4.6 IFN-α induces the phosphorylation of STAT1, STAT2 and upregulation of IRF1 in the HL-60 cell line

Having explored the role of type 1 IFNs in the phosphorylation of STAT proteins and IRF1 induction (Fig 5.4D), as well as the specific role IRF1 in the induction of early antiviral ISGs downstream of TLR8 in HL-60 cells (chapter 4), we hypothesised that IRF1 as well as STAT1/STAT2 may also be important for the induction of IFN-induced ISG expression. We therefore sought to explore the specific role IRF1 and STAT proteins play downstream of type 1 IFNs in PMNs using the HL-60 cell model. To investigate this, we first confirmed the role of IFN-α in the phosphorylation of STAT proteins and induction of IRF1 in HL-60s. HL-60s were stimulated over the course of 2 hours with IFN-α. Protein lysates were collected and probed for phosphorylated and total STAT1 (Fig 5.7A), STAT2 (Fig 5.7B) and expression of total IRF1 (Fig 5.7C) using western blotting. While not statistically significant we observed that IFN-α phosphorylated both STAT1 and STAT2 as early as 5 minutes post stimulation, with levels of pSTAT1 peaking at 5-10 minutes and levels of pSTAT2 peaking at 2 hours post IFN-α stimulation. We saw a 500 to 1000 fold increase at 5 minutes for both pSTAT1 and pSTAT2, respectively. We observed no significant changes in levels of total STAT1 or STAT2. In contrast to pSTAT1 and pSTAT2 levels, we observed later statistically significant 6 and 8 fold upregulation of IRF1 at 1 and 2 hours, respectively (Fig 5.7C). Like we saw in the primary human PBMCs and PMNs (Fig 5.4/5.5), we hypothesised that this early phosphorylation of STAT proteins and later expression of IRF1 could induce changes in downstream ISG expression, such as Viperin (Fig 5.6). Having previously found an important role for IRF1 in the
induction of early TLR8 induced ISG expression (Fig 4.14), we now hypothesised that it also may play a role in the induction of IFN-induced ISGs in HL-60 cells.

Figure 5.7: IFN-α induces the upregulation of IRF1 and phosphorylation of STAT1 and STAT2 in the HL-60 cell line

HL-60s (1 x 10^6 cells/ml) were stimulated with IFN-α for the indicated times. A) Phosphorylation of STAT1, STAT2 and total STAT1, STAT2 and IRF1 was detected by immunoblotting whole cell lysates. B) Densitometric analysis of 3 immunoblots was performed using the image lab software. The bar graph illustrates a ratio of STATs/IRF1: β-Actin , relative to the untreated (PBS) control which was normalised to 1. Statistical differences were assessed using one way ANOVA with a Tukey post-test (*p<0.05) (n=3)
5.4.7 IFN-α induces the expression of ISGs in HL-60 cells

Having confirmed the role of IFN-α in the phosphorylation of STAT proteins and induction of IRF1 in HL-60 cells (Fig 5.7), we next sought to examine the downstream effect of IFN-α upon ISGs in these cells. HL-60 cells were stimulated with IFN-α over a time course of 4 hours. RNA lysates were collected and measured for levels of ISGs Viperin (Fig 5.8A), MxA (Fig 5.8B) and ISG15 (Fig 5.8C) by qRT-PCR. We observed that HL-60s stimulated with IFN-α over 4 hours upregulated a significant 1000 fold increase in expression of Viperin (Fig 5.8A). Additionally, we observed a significant 20 fold increase in the expression of ISG15 at 4 hours post IFN-α stimulation. In contrast, while we didn’t observe a significant increase in the expression of MxA, we did observe a time dependent increase, with a mean 20 and 30 fold increase observed at 2 and 4 hours post IFN-α stimulation, respectively.

Figure 5.8: IFN-α stimulation upregulates the expression of antiviral genes Viperin and ISG15 in HL-60s

HL-60s (1X10⁶) were treated with IFN-α (1000IU) for 0, 1, 2 and 4h before mRNA levels of (A) Viperin, (B) MxA and (C) ISG15 were measured by qRT-PCR. ISG mRNA was calculated relative to the β-actin house-keeping gene and IFN-α stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Graphs represent the mean ± SD of 3 independent experiments. Statistical differences were assessed using a paired T test. Paired T-test. *p<0.05.
5.4.8 IRF1 is involved in antiviral ISG induction in HL-60s

Having observed the upregulation of IRF1 and ISGs in response to IFN-α in HL-60 cells (Fig 7/8), and observing the role IRF1 plays in the induction of early ISGs downstream of TLR8 in HL-60s (Chapter 4), we next investigated if IFN-α-induced ISGs were also mediated via IRF1. Previous experiments (Fig 4.13) revealed a successful knockdown of IRF1 using electroporation of IRF1 siRNA. HL-60s were transfected for 24h before being stimulated with IFN-α for both 2h and 4h. RNA lysates were collected and measured for levels of ISGs Viperin (Fig 5.9A), MxA (Fig 5.9B) and ISG15 (Fig 5.9C) by qRT-PCR. As previously observed in Fig 8.A, we again saw a time dependent increase in Viperin, MxA and ISG15 in the cells transfected with the control siRNA. In contrast, the IRF1 knockdown resulted in the reduced expression of ISGs. Albeit not significant, we observed a 3 fold reduction in Viperin (Fig 5.9A) with the p-value of 0.5374 increasing in the IRF1 knockdown when compared to the control at 4h post IFN-α stimulation (0.1928). Similarly, we observed a 5 fold reduction in MxA (Fig 5.9B) in the IRF1 knockdown when compared to the control and a 4 fold reduction in ISG15 (Fig 5.9C) at 4h post IFN-α stimulation. In summary, we found that IRF1 was involved in the induction of IFN-α induced ISG expression in HL-60 cells.
Figure 5.9: IFN-α mediated induction of ISGs is IRF1 dependent

The promyelocytic cell line HL-60 was transfected with 10nM of either control (RLUC) or IRF1 siRNA for 24h before being treated for 2 and 4h with 1000IU IFN-α. mRNA levels of A) Viperin  B) MxA and C) ISG15 were measured by qRT-PCR. ISG mRNA was calculated relative to the β-actin house-keeping gene and IFN-α stimulated samples were compared to the unstimulated (0h) control, which was normalised to 1. Graphs represent the mean ± SD of 3 different experiments. Statistical differences were assessed using a paired T test.
5.5 Discussion
Since the discovery of IFNs more than 60 years ago, researchers have unravelled their intracellular signalling and cognate receptors in various cell types, from epithelial cells to cells of the immune system. IFN signalling has been described as a double edged sword; it is required for the initiation of an effective anti-viral response, but also excessive activation can result in the recruitment of inflammatory mediators that can exacerbate inflammatory disorders, such as acute respiratory distress and autoimmune disorders (317) (318). These variable IFN responses have been determined to be dependent on context, duration and magnitude (319). In other words, a beneficial IFN response balances stimulatory signals with modulatory signals, where it promotes pathogen clearance, but also facilitates a return to homeostatic conditions. Neutrophils have long been implicated in the disease pathogenesis of inflammatory disorders, in which strong IFN signatures are often observed. Studies revealed that type 1 IFNs can trigger a chemokine signal from neutrophils, which in turn recruits further inflammatory cells such as DCs and CD4+ T cells (81) (320) (321). Until recently, neutrophils have been considered predominantly anti-fungal and anti-bacterial, yet an ever growing body of evidence suggests that are also anti-viral. Studies have highlighted that STAT proteins and ISG responses are critical for inducing these responses in neutrophils (322). Therefore, we aimed to study type 1 IFNs in inducing anti-viral responses through the upregulation of STAT proteins and ISGs in primary human PMNs.

In order to study the potential differences between PMN and PBMCs in response to IFNs, we first wanted to confirm their ability to produce type 1 IFNs
downstream of TLR8. We observed that PMNs stimulated with CL075 failed to produce large quantities of IFN-α. In contrast, PBMCs from the same donor were able to induce IFN-β and IFN-α when stimulated with CL075. Additionally, LPS also failed to trigger an IFN-α/IFN-β response in both PMNs and PBMCs, suggesting that endosomal TLR7/8 receptors have a higher propensity for the production of type 1 IFNs in immune cells, when compared to TLR4. The ssRNA receptors TLR7 and TLR8 have been shown to be involved in neutrophil responses to IAV through the production of murine IL-8 (323). Similarly, neutrophils have been shown to be implicated in the production of IFN-β in plasmid DNA transfected cells which is further increased with the addition of LPS. In contrast to our results, neutrophils have been shown to be potential sources of IFN-α, demonstrated in Lupus patients, where there was an observed increase in IFN-α transcripts (324). PBMC populations consist of a wide array of cell types. These include DCs, monocytes, macrophages, T cells and B cells. Of these, DCs have been shown to be the predominant IFN-α producing cell (325). TLR7 has been shown to be the receptor involved in the induction of this IFN-α response (227). We observed that on TLR8 inhibition of the PBMC population the secreted amount of IFN-α was reduced by half, possibly suggesting an alternative cellular source of IFN-α that utilises TLR8. We also observed that when TLR8 was inhibited in both the PBMC and PMN population, production of IFN-β was reduced by half. These results suggest that different cellular sources account for differences in type 1 IFN production and that TLR8 may be an important receptor in triggering type 1 IFNs in both PMN and PBMC populations.
Given that PBMCs can produce large quantities of IFN-α, we sought to explore how it signals in PMNs, given the close proximity of these cell types during in the immune response. Aberrant STAT signalling has been shown to be pivotal in the development of autoimmune disorders, such as Primary Sjögren's syndrome (pSS), with basal and IFN-induced levels of STAT1 shown to correlate with disease severity (326). The pathology of autoimmune disease has often been associated with the influx of neutrophils (327). We therefore thought it important to understand the differences in STAT signalling between PBMCs and PMNs. We observed that PMNs could rapidly induce the phosphorylation of STAT1, STAT2, STAT3 and IRF1. In contrast to PMNs, PBMC exhibited delayed phosphorylation of STAT1, STAT2 and STAT3. We propose that this variation could explain that neutrophils are fast responders to type 1 IFNs, accounting for an early anti-viral and inflammatory response. In addition, PMNs also significantly expressed higher levels of IRF1 in response to type 1 IFNs, when compared to PBMCs, suggesting a novel role of IRF1 downstream of IFNAR in PMNs. Although the temporal response of signalling pathways in PMNs and its implication in the global immune response has not been fully elucidated, some data has shown that specific microbial activation signals result in the temporal upregulation of distinct subsets of genes. Boutros et al demonstrated that upon microbial infection, the antimicrobial NF-κB pathway preceded the JAK/STAT pathway which exhibited a slightly delayed transient expression, suggesting that temporal pathway patterns after microbial challenge (328).

Following on from our previous observations, we set out to explore whether the variations in STAT signalling linked with differences observed in ISG induction in
response to IFN-α. While temporal differences in ISG induction have not been established between PMNs and PBMCs some work has established differences between type 1 IFN signalling and type 3 IFN signalling in hepatocytes, with IFN-α stimulating rapid ISG induction and type 3 IFN stimulating delayed transient induction of ISGs (329). We observed that PMNs and PBMCs stimulated with IFN-α displayed a stark difference in ISG induction. PMNs stimulated with IFN upregulated the early expression of Viperin. In contrast PBMCs displayed reduced levels of Viperin induction when stimulated with IFN-α. MxA levels appeared relatively consistent between PMNs and PBMCs, whereas ISG15 levels appeared to more inducible in PBMCs when compared to PMNs. We hypothesised that this increase in Viperin, specific to PMNs would be important in the restriction of a number of viruses that are detected intracellularly, thereby reducing the viral load overall.

Finally, in order to confirm the role of IRF1 in the induction of an anti-viral response in PMNs, we sought a successful knockdown. We confirmed our primary human cell studies in the HL-60 cell line prior to the knockdown. Considering the importance of IRF in inducing an IFN independent response observed in the previous chapter and that IFN-α could strongly induce its expression in PMNs and HL-60 we chose to knockdown IRF1. once knocked down we observed a stunted ISG response in HL-60s when stimulated with IFN-α indicating that IRF1 is important for the transduction of type 1 IFN anti-viral responses in HL-60 cells.
In conclusion, we found that TLR8 may be an important receptor in the induction of IFN-α in PBMCs and PMNs and that this IFN-α may be an important agonist for the induction of early and direct anti-viral responses in PMNs through the activation of STATs and IRF1. Additionally, we found that Viperin may be an important anti-viral ISG in human PMNs and HL-60s downstream of IFNAR activation for the restriction of intracellular ssRNA viruses.
Chapter 6

General Discussion
Chapter 6: General Discussion

6.1 Introduction

Inflammation is defined as a cascade of cellular events characterised by the production of inflammatory and anti-viral intermediates in response to infection. Innate immune cells employ a plethora of innate immune receptors that trigger immune responses that should culminate in the elimination of a pathogenic infection. Viruses have developed a host of innate immune escape mechanisms that allow for prolonged infection and inflammation. The TLR family of innate immune receptors consists of 10 human receptors, which are expressed on either the extracellular membrane or the endosome. They are known to induce the expression of NF-κB, MAPK and IRF signalling pathways (330). TLR7 and TLR8 are both endosomal receptors and share a large percentage of sequence homology. Unsurprisingly, they are both involved in the recognition of ssRNA sequences and are often associated with the recognition of actual ssRNA viruses (176). Research has focused on how the modulation of these intracellular signalling cascades could either manage an uncontrolled inflammatory response during something like acute respiratory distress, while also simultaneously enhancing an antiviral response, which would in turn significantly reduce patient mortality. However, recent work has revealed novel roles and uneven distribution across cell types and different species (228). While the bulk of human innate antiviral work has been characterised in cells such as macrophages and DCs, an ever growing interest has been in that of the role of neutrophils in viral infection. We therefore wondered the role of TLR8 in the induction of antiviral responses and inflammatory responses in primary human PMNs. Our results show that
TLR8 is an important receptor in PMNs responsible for the upregulation of inflammatory and anti-viral mediators in response to CL075 or the ssRNA viruses, RSV and HIV.

6.1.1 TLR8 mediated upregulation of inflammatory cytokines in primary human PMNs in response to ssRNA viruses

PMNs are the most abundant leukocytes in circulation and are generally some of the first responders to the site of infection. Although they are considered to have a short half-life when compared to their monocyte and macrophage counterparts, their sheer number and their ability to quickly home to the site of infection puts them at the forefront of immune surveillance and protection. Under homeostatic conditions, PMNs initiate inflammatory responses, but are also involved in the resolution of inflammation, subsequent to the successful clearance of a pathogen (331). However, certain pathogenic viruses are associated with dysregulated PMN responses, excessive tissue damage and inflammatory diseases such as sepsis (332) (333). PRRs are often associated with the instigation of these inflammatory responses. Human PMNs are known to express a plethora of PRRs. These include TLRs, NLRs and RLRs (334) (335). PMNs are known to express mRNA for all TLRs, with the exception of TLR3 (336). Recent studies have begun to shed light on the differences between endosomal receptors and their role in the upregulation of various inflammatory and antiviral cytokines. These differences have been nicely described using monocytes and macrophages. They showed that TLR8 was the predominant receptor involved in the induction of NF-κB pathways. Additionally, they observed that TLR8 activation resulted in elevated cytokine production in macrophages, when compared to
monocytes (337). Adding to the complexity, many studies exploring the role of endosomal receptors utilise mice (338). However, it has been shown that mice don't express TLR8, revealing potential translational issues when it comes to human disease and therapeutics (228). We hypothesised that TLR8 was the main receptor involved in the initiation of an inflammatory response in primary human PMNs. To investigate this we stimulated human PMNs with CL075, a TLR7/8 agonist and measured levels of inflammatory cytokines by qRT-PCR. We observed de novo synthesis of cytokines such as IL-6, TNF and IL-8. Additionally, we examined the unexplored differences in TLR7/8 signalling in PMNs using a specific TLR8 inhibitor. We found that TLR8 was the dominant receptor involved in the initiation of inflammatory cytokines. Furthermore, we found that TLR8 signalling regulated both MAPK and NF-κB pathways. Previous studies have shown that MyD88 dependent and independent pathways (TRIF dependent) regulate both NF-κB and MAPK pathways (339) (340). TLR8 utilises the MyD88 dependent pathways for the activation of various downstream intracellular signalling cascades (341). We observed a high degree of redundancy in the TLR8 signalling in primary human PMNs, with both NF-κB and MAPK pathways capable of inducing an IL-8 and IL-1β response. In agreement with other studies using macrophages, the cytokines IL-6 and TNF heavily relied on the NF-κB pathway (342).

Having identified a possible novel role of TLR8 in the induction of inflammatory mediators in PMNs, we next sought to examine how primary human PMNs would respond to the ssRNA virus RSV, and the role TLR8 would have in the induction of virus induced inflammatory responses. RSV is a paediatric respiratory infection
that predominantly infects cells of the lower respiratory tract. As well as epithelial cells, it has also been demonstrated to infect immune cells including B-cells, T-cells and macrophages (343) (344) (345). In contrast, there is a gap in our knowledge, with conflicting evidence of whether or not ssRNA viruses can infect human PMNs (346) (152). Due to the high prevalence and early infiltration of neutrophils into the airways during severe RSV infection of infants, we hypothesised that RSV could infect PMNs and subsequently trigger intracellular PRRs, such as the aforementioned TLR8. Previous RSV research has revealed a role for extracellular TLR4 in the induction of an inflammatory response (204). In contrast, the role of TLR8 in the recognition of RSV still remains elusive. This is not to say TLR8 has not been implicated in other respiratory viral infections. Influenza A has been shown to induce a strong IL-8 response in neutrophils through the successful activation of TLR8 (347). We first confirmed the presence of RSV intracellularly in live primary human PMNs using flow cytometry. Infection of neutrophils by viruses has proven to be pathogen specific, with Influenza RNA being detected intracellularly in neutrophils. In contrast, neutrophils can phagocytose virions, thereby promoting virus clearance and preventing viral replication within cells and blocking infection of the surrounding cells (348) (203). Even though we detected RSV within PMNs, in order for us to confirm whether or not RSV actively infected the primary human PMNs or was passively phagocytosed, further studies would be needed to clarify. We found that TLR8 was the predominant endosomal TLR involved in the upregulation of activation markers and inflammatory cytokines in response to RSV. In agreement with previous studies, PMNs challenged with RSV resulted in an enhanced activation state, with NET formation a common feature (128). Following on from this we
sought to explore the specific role of TLR8 in the induction of inflammatory responses in PMNs when challenged with RSV. We found that RSV could induce the expression of IL-6 and IL-8 in PMNs. However, it was also important to note that RSV appeared to trigger the preferential production of IL-8 via TLR8, with the inhibition of TLR8 having no effect on the production of IL-6. Additionally, inhibition of ERK and NF-κB resulted in an abrogated IL-8, but not IL-6, in PMNs stimulated with RSV, suggesting that TLR8 is an important receptor in the induction of IL-8 in ssRNA viral infections. We hypothesised that the observed IL-6 response was either due to other PRRs triggering the response or RSV triggering the release of prestored IL-6 in neutrophil granules, so as to bypass the need for de novo synthesis of cytokines. Prestored cytokines in PMN granules has been demonstrated in which toxoplasma gondi infection resulted in the recruitment of neutrophils containing prestored IL-12 indicating that PMN derived IL-6 release may also be through this mechanism (213).

6.1.2 Impact for inflammatory disorders

While it’s important to highlight that PMN influx into the lung during virally induced ARDS correlates with an increase in an inflammatory cytokine expression, it will be paramount to determine whether neutrophils are a major source. Early COVID-19 studies examined the potential role of granulocyte induced inflammation. GM-CSF is an important cytokine in the recruitment and survival of neutrophils (349). Mavrilimumab is an anti-GM-CSF receptor monoclonal antibody that improved the clinical outcome of patients with COVID-19 induced hyperinflammation, supposedly through the inhibition of neutrophil recruitment (350). Targeting certain proinflammatory cytokines in the lung using monoclonal
antibodies has proven to be successful, as seen during the 2019 SARS-CoV2 pandemic as well. The use of anti-IL-6 (Siltuximab) to treat patients presenting with ARDS was shown to reduce disease (351). IL-8 has also been demonstrated to be an important biomarker for the pathogenesis of COVID-19 and is currently under review as a possible new therapeutic target to efficiently modulate the hyperinflammatory response in ARDS (352). Targeting downstream intracellular proteins that relay inflammatory cytokine signalling, such as IL-6, have also been explored as potential treatments for ARDS. The monoclonal antibody, Baricitinib, a JAK1/JAKK2 inhibitor, showed a reduction in 30-day mortality in over 70s, with moderate-to-severe COVID-19 pneumonia, and combined with Remdesivir decreased recovery time and reduced 28-day mortality, serious events and new infections (243) (353). Therefore, targeting the JAK/STAT pathway and the cytokines that signal via it, could be useful.

We propose that targeting the pathways involved in the induction of the IL-8 and IL-6 response would be another attractive option in the treatment of severe ARDS. Therefore, understanding the molecular mechanisms, such as PRRs driving the inflammation in a viral infection, will be key to managing the numbers of acutely ill patients. Targeting PRRs in bacterial infection has been extensively characterised. Small molecule inhibitors of MyD88 have also been shown to be effective in limiting inflammation (354). While the current knowledge centers around inhibiting cell surface PRRs much more will need to be done to characterise the role of intracellular receptors responsible for the recognition of ssRNA viruses like RSV or HIV. We propose that, characterising the role of endosomal TLRs, such as TLR8, and their associated downstream signalling
effectors molecules, such as the NF-κB and MAPK signalling pathways, will be instrumental in understanding how to manage inflammation and improve anti-viral responses. Current TLR7 agonists on the market include imiquimod (Aldara) and resiquimod which are approved for the treatment of external genital warts and HSV infection respectively. Indeed these are thought to be through the upregulation of type 1 IFNs (355). Additionally, the TLR7 antagonists 2′-O-methyl-modified RNA that binds with a higher affinity to TLR7, significantly reduced IFN-α and IL-6 production in TLR7 agonist-treated murine DCs and human PBMCs (356). In theory, the regulation of these endosomal viral receptors and subsequent proinflammatory mediators may be important when considering potential therapeutics that manage the viral inflammatory response. Collectively these results highlight the importance of TLR8 in neutrophil mediated inflammatory and anti-viral responses in ssRNA infection, with an inhibitory molecule for TLR8 being an attractive target for ARDS due to its possible inflammatory effects. Collectively our findings may pave the way for the discovery of novel anti-inflammatory molecules used to manage severe inflammatory responses. We hypothesise that future studies that explore managing specific PMN derived inflammatory mediators could be beneficial in modulating acute inflammatory conditions in viral infections, but also in inflammatory autoimmune conditions, such as RA or lupus.

6.1.3 TLR8 mediated early upregulation of ISGs in primary human PMNs in response to ssRNA viruses

We also set out to uncover the antiviral roles neutrophils could play during infection. PRRs, as well as triggering inflammatory mediators via pathways such
as NF-κB and MAPK, also trigger the activation of IRF signalling pathways (357). IRF proteins regulate transcription of IFNs as well as play a critical role in immune cell development. There are 9 IRF proteins identified (1-9). IRF7 is involved in the induction of IFNs through the their self-phosphorylation and subsequent translocation to the nucleus (358). This IFN then goes onto bind its associated IFN receptor (IFNAR) and induce the expression of plethora of antiviral ISGs. Low-level IFN production in the absence of infection plays a vital role in diverse biological processes, including immune cell function and the maintenance of the hematopoietic stem cell niche. Disturbances of IFN expression and the subsequent loss of IFN homeostasis can contribute to the etiology of several inflammatory conditions (359). As well as inducing an anti-viral state in cells, ISG expression can also control chemotaxis and cell migration, two processes important for the recruitment of neutrophils (360). We previously discussed how ARDS severity maybe influenced by infiltration of neutrophils into the respiratory tract. Interestingly, studies have shown that extremes of ISG expression in neutrophils, whether they be low or high, are associated with a worse outcome in ARDS (258). In the context of an RSV infection, the transcriptome of airway neutrophils reveals a strong IFN response in individuals with life threatening ARDS (322). This increase in an ISG signature also correlated with distinct expression of neutrophil activation genes (TNFSF13B, FCER1G) (361).

Having explored the role of TLR8 in the induction inflammatory response in PMNs we next sought to explore its potential in activating an ISG signature. Studies have explored the role TLR7 has in the induction of a type 1 IFN
response, primarily in cells such as macrophages and pDCs (362). In contrast, the role of TLR7 and TLR8 remains poorly understood in primary human PMNs. We hypothesised that TLR8 activation from ssRNA viruses would result in the ISG expression commonly associated with individuals suffering from ARDS during a viral respiratory infection. We demonstrate early induction of ISGs, in particular, Viperin, in response to CL075 exclusively through TLR8. Viperin is multifunctional protein commonly associated with the inhibition of viral replication (363). Previous work has demonstrated that macrophages and neutrophils highly induce Viperin in response to chronic LCMV infection (364). Having observed that a synthetic agonist designed to activate TLR7/8 was able to induce Viperin expression in primary human PMNs, and that other studies have demonstrated that LCMV could do the same, we were interested to check the biological significance of TLR8 by using the ssRNA viruses HIV-IIIB and RSV-A2. Cells stimulated with HIV-IIIB or RSV demonstrated a time dependent increase in levels of Viperin in a TLR8 dependent manner. Since we observed early induction of ISGs, this led us to believe that the ISG expression we were observing was not due to the presence of type 1 IFNs, but instead the direct result of PRR activation. The induction of ISGs independent of IFNs remains understudied, however some investigations have begun to shed light on the topic. Human Cytomegloviruses has demonstrated the upregulation of ISGs in the HEK293t cell line via IRF3 activation (251). Others have shown that IRF1 is a potential intracellular protein involved in the induction of ISGs independent of type 1 IFNs in BEAS-2B bronchial epithelial cells (253). This IRF1 mediated IFN-independent, early, antiviral response has been proposed to function through an IRF1 interaction with IRF3. This interaction reduces the ability of the phosphatase 2A
(PP2A) to degrade phosphoryl groups, enhancing phosphorylation and subsequent nuclear translocation of IRF3, thus enhancing ISG expression. Having observed that TLR8 induced an early antiviral response, we hypothesised that either IRF1 or IRF3 could potentially be involved in the TLR8 induced activation of ISGs in primary human PMNs. In agreement with Panda, Gjinaj et al. we found that IRF1 was important in PMNs for this observed ISG expression. With limited ability to explore the role of IRF1 in more detail in PMNs, due to the short lifespan and an unstable nature we chose to use the granulocyte-like cell line, HL-60s, to next study the role of IRF1 in TLR8 signalling. We found that on knocking down IRF1 there was a steep reduction in CL075 induced Viperin levels, indicating that IRF1 downstream of TLR8 was directly involved in the induction of early Viperin expression in HL-60 cells. Viperin plays multiple roles in various cell types. It inhibits viral replication, mediates signaling pathways, and regulates cellular metabolism. While in macrophages, Viperin can inhibit IFN production, in pDCs it can enhance TLR7 mediated IFN production suggesting that viperin can play different roles in activation of the same pathway in different cell types, highlighting the importance of Viperin expression in primary human PMNs (365).

6.1.4 Differences in IFN mediated phosphorylation of STAT proteins and subsequent ISG upregulation in primary human PMNs and PBMCs

Finally, having observed proinflammatory and early antiviral signalling pathways independent of IFNs in PMNs, we next sought to explore the differences in IFN signalling between PMNs and PBMCs. Type 1 IFNs are produced by a wide array of cells. IFN-β was originally defined as the antiviral cytokine that is produced by fibroblasts in response to viral infection (366). However, IFN-β has now been
shown to be produced by nearly all cells in the body (367). Later pDCs, also known as IFN producing cells (IPCs), were described to be specialist producers of type 1 IFNs (368). Recent findings however indicate that type 1 IFNs can be produced independent of IPCs and that the cell type responsible for the type 1 IFN production depends on the spatiotemporal nature and virally infected cell type (289). Neutrophils however are not generally associated as being a traditional IFN producing cells. Decker et al. have demonstrated that neutrophils could potentially be an important source of type 1 IFNs in response to some lupus stimuli (324). Despite their ability to produce significantly less IFN when compared to their DC and macrophage counterparts, their sheer number of PMNs in circulation could prove to produce a significant cumulative amount of type 1 IFNs. In order to compare PMNs to their PBMC counterparts and explore variations in the different populations ability to produce IFNs, both cell populations were isolated from the same donors. We observed differences in type 1 IFN expression from primary human PMNs and PBMCs downstream of TLR8 activation. We found that both PMNs and PBMCs could produce small quantities of IFN-β. In contrast, we observed that only PBMCs could produce significant quantities of IFN-α in a TLR8 dependent manner. To our knowledge, TLR7 is the predominant receptor involved in the production of IFN-α from DCs in primary human PBMCs (227). However, our findings suggest another cell within the PBMC fraction, other than DCs, is responsible for the production of IFN-α in a TLR8 dependent manner.

IFNs can subsequently bind the associated IFNAR receptor and activate various JAK/STAT proteins which translocate to the nucleus and upregulate the
expression of a plethora of anti-viral ISGs (369). The tissue distribution of each
STAT is unique and it is widely accepted that STAT proteins have tissue specific
functions (370). The modulation of tissue-specific genes has been shown to be a
physiological role of STAT proteins in a variety of cell types such as adipocytes
(371). The role of STAT proteins in anti-microbial immunology is an exciting
concept. Recent studies of PMNs have shown the importance of specific STAT
proteins such as STAT4 downstream of IL-12 in the upregulation of antimicrobial
processes, including enhanced chemotaxis, ROS production and NET production
during an intracellular *Staphylococcus Aureus* infection (304). Additionally,
phosphorylation of STAT3 downstream of IL-6 has been shown to be instrumental
in the recruitment of neutrophils during *E. coli* induced pneumonia (303). In
contrast, very little research has explored the roles of STAT protein
phosphorylation and subsequent ISG activation during viral infection in primary
human PMNs. Firstly, having observed the production of type 1 IFNs, we sought
to explore the differences in IRF1 and STAT signalling between the PMNs and
PBMC populations from the same donors. Due to the fact PMNs and PBMCs are
both recruited during infections, and that they are expected to communicate in a
paracrine manner through the production of type 1 IFNs, we decided to use IFN-α
to explore the differences in phosphorylation of various STAT proteins in PMNs
and PBMCs (372). We observed differences in STAT phosphorylation patterns,
with PMNs appearing to phosphorylate STAT proteins as early as 10 minutes
post IFN stimulation and PBMCs appearing to phosphorylate STAT proteins at a
later stage (seen at 60 minutes to 2 hours after IFN stimulation). We propose that
this early phosphorylation of STATs in PMNs allows them to respond more rapidly
to invading pathogens through the upregulation of inflammatory cytokines, ROS
production and NET production and subsequently shaping the later innate and adaptive immune responses. We also propose that this early phosphorylation will facilitate the rapid activation of ISGs and thus may help restrict viral replication in the PMNs themselves and neighboring immune cells. We also observed that the expression of IRF1 downstream of type 1 IFN stimulation is required for expression of the ISG Viperin in PMNs which may be a cell specific mechanism by which PMNs can restrict intracellular viral infections. IRF1 has previously been shown to preferentially be involved in the transduction of type 1 IFNs, primarily demonstrated using epithelial cell lines (305).

6.1.5 Impact for HIV and RSV infection

Evidence shows that ssRNA viruses, such as HIV and RSV, are highly effective at suppressing different parts of the innate immune response. In confirmation of this, the majority of patients without the introduction of antiviral treatments such anti-retroviral therapy, develop a chronic infection, with a wealth of studies exploring the exact intracellular immune subversion strategies (373). While the role of PMNs in viral infection remains an understudied topic, there is an ever increasing body of evidence to support their antiviral role to which our studies now support. Since PMNs highly express TLR8, and that it is the sole endosomal receptor involved in the induction of early ISGs, indicates that this is an important pathway for eliciting an antiviral response. Our results found that Viperin was a highly inducible ISG downstream from TLR8 in response to both HIV-IIIB and RSV-A2 in PMNs. Additionally, we found that PMNs were highly responsive to type 1 IFNs through the phosphorylation of STAT proteins. Taken together, these results demonstrate two ways PMNs could confer resistance to viral infections.
This in theory, will allow for future studies to explore potential ways specific viruses inhibit anti-viral signalling in PMNs. In turn, this will allow for the development of novel therapeutics that inhibit viral proteins involved in shutting off the innate immune response in PMNs.

### 6.1.6 Conclusions

This study hypothesised that PMNs would be involved in the regulation of both inflammatory and anti-viral immune responses through endosomal PRRs. This project has demonstrated a novel role of TLR8 in primary human PMNs. We discovered that TLR8 is responsible for inducing an inflammatory response characterised by intracellular signaling activation and subsequent release of inflammatory cytokines. We highlighted PMN-TLR8 as a receptor for the detection of ssRNA viral infections, which may help in understanding the inflammatory processes often observed in viral disease. In addition we observed that PMNs can induce an antiviral response characterised by both IFN and IFN independent mechanisms, specifically through IRF1’s upregulation of Viperin. Finally we show that human PMNs can strongly respond to type 1 IFNs via a host of STAT proteins and IRF1 to induce ISGs, with the potential of generating an intracellular antiviral state. These findings highlight a new role for PMNs in combatting viral infections, and therefore may reveal targets to modulate or enhance inflammatory and anti-viral responses, thereby reducing disease severity with new therapeutics.
Chapter 7: References


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Chapter 8: Appendix

8.1 Humans express high levels of IRF1

We have previously established that TLR8 signalling might be crucial in viral infections, we set to investigate the tissue distribution of other proteins involved in transducing the TLR8 signal and eventually culminating in the induction of ISG. Of these, IRF proteins have been previously shown to be crucial in the signalling. The most prominent IRF proteins expressed in viral infections are IRF1, IRF3, IRF5 and IRF7. In order to explore the tissue distribution and species variability of these proteins we used Illumina Body Map and FANTOM5 datasets. The IRF expression levels were measured in transcripts per million (TPM) which is a normalisation method for RNA-seq. Fig 7.A and 7.B represent the immune organ distribution of IRF proteins in Human and mice respectively. Results showed that IRF1 baseline expression was higher than all other IRF proteins in leukocytes in humans at 114 TPM. In contrast levels of IRF3 and IRF7 displayed baseline expression levels at about half of what was observed for IRF1. In contrast to the high expression levels of IRF1 observed in humans, mice expressed very little IRF1. Instead levels of IRF3 were observed to be expressed to a higher degree at baseline. Our results suggest that different species utilise different IRF proteins during a viral infection and that IRF1 in humans might play a significant role in viral infections due to its high expression in leukocytes.
Figure 8.1: IRF protein expression in humans and mice
IRF1, IRF3, IRF5 and IRF7 tissue distribution was found using EMBL-EBI and Expression Atlas.
Expression levels of IRFs were generated from Illumina Body Map and FANTOM5 datasets.