Investigating the role of specific inflammasome components during inflammatory disease

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Supervised by
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Thesis submitted to the University of Dublin, Trinity College for the Degree of Doctor of Philosophy
I. Declaration of Authorship

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work. I agree to deposit this thesis in the University’s open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

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II Acknowledgements

First and foremost, I would like to extend my sincerest thanks to my supervisor, Emma. Emma was the most supportive mentor at all times over the last three years - both scientifically and personally. I am very fortunate to have been chosen as her PhD student and appreciate all of the time and energy she has given me.

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I would like to say a massive thank you to my family, without them this would never have been possible. To my Mam and Dad, your constant support has allowed me to grow and become whatever I wanted to be. I will always be grateful. Finally, I would like to say a huge thank you to my boyfriend Padjoe. Your love and support over the last 8 years has made this journey a lot easier.

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III. Abstract

Inflammasomes are multi-protein complexes that act as intracellular innate immune receptors. The first inflammasome was initially defined by Martinon et al in 2002 and 16 years on, this increasingly explored area has led to the identification of over 10 different inflammasomes that recognise and respond to a variety of ligands. More recent evidence has linked polymorphisms in inflammasome components to autoinflammatory syndromes and diseases. The majority of autoinflammatory syndromes are in fact linked to mutations in inflammasome components. Examples include mutations in NLRP3, Pyrin, and NLRP12 resulting in disorders such as Cryopyrin-Associated periodic Syndromes (CAPS), Familial Mediterranean Fever (FMF) and CAPS-like syndrome, respectively. This study explores novel aspects of inflammatory regulation in the context of two inflammation-associated diseases, an experimental murine model of psoriasis, and an autoinflammatory syndrome.

Psoriasis is an inflammatory-mediated disease that was previously believed to be driven by an adaptive immune response. However, more recent evidence suggests a contribution of both innate and adaptive immune cells to the pathogenesis of psoriasis. Expression of caspase-4 and 5, mediators of the non-canonical inflammasome, are reported to be increased in human psoriatic lesions. This study investigates the role of caspase-11, the murine ortholog of human caspases-4 and -5, in mediating psoriasis. Findings from this study reveal that when compared to wild-type mice, caspase-11 deficient mice are significantly protected from the symptoms arising from Imiquimod-induced psoriasis. Decreased angiogenic markers, proliferation and epidermal thickening were observed in the skin of caspase-11 deficient mice. Decreased infiltration of leukocytes to the skin and decreased pyroptosis were also observed in Casp-11/− skin, which may explain the absence of psoriasis in these mice. We propose that non-canonical inflammasome mediated inflammation is responsible for driving the pathogenesis of psoriasis. Findings from this study identify the inflammatory caspase, caspase-11 as a potential drug target for the treatment of this disease.

A clinical collaboration established with Our Lady’s Children’s Hospital, Crumlin has identified a novel mutation in NLRP6 as a causative agent of a previously undiscovered autoinflammatory syndrome. Our findings suggest that a patient presenting with Blau-like autoinflammatory symptoms has a rare loss-of-function NLRP6R653G mutation,
which is responsible for the patient’s sustained inflammatory symptoms. This study characterises the patient’s immune responses to a range of inflammatory stimuli and inflammasome activators. Findings suggest that patient NLRP6\textsuperscript{R653G} has consistently elevated basal IL-6 expression, dysregulated NFκB signalling and increased production of pro-IL-1β in response to LPS. \textit{In vitro} evidence implicates a role for NLRP6 in mediating NFκB activation in the presence of ASC, which is abolished in the presence of the mutation. Conversely, mutation NLRP6\textsuperscript{R653G} has no observable impact on inflammasome formation \textit{in vitro}. Collectively, these findings suggest that the most significant impact of the NLRP6\textsuperscript{R653G} mutation is on NFκB regulation. This study identifies the NLRP6 SNP as a causative gene for a novel monogenic autoinflammatory disease, which will have a significant impact on the autoinflammatory disease research field. Findings from this study also provide significant insight into the importance of NLRP6 signalling during innate immune responses, which is not well characterised at present.
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>AIM2</td>
<td>Absent in melanoma 2</td>
</tr>
<tr>
<td>AMP</td>
<td>Anti-microbial peptide</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoitin</td>
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<tr>
<td>AOM</td>
<td>Azoxymethane</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<tr>
<td>ASC</td>
<td>Apoptosis associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculovirus-inhibitor-of apoptosis repeats</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cryopyrin associated periodic syndrome</td>
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<tr>
<td>CARD</td>
<td>Caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CINCA</td>
<td>Chronic Infantile Neurological Cutaneous Articular syndrome</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
</tr>
<tr>
<td>CPPD</td>
<td>Calcium pyrophosphate dehydrate</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<td>dDC</td>
<td>Dermal dendritic cell</td>
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<tr>
<td>DED</td>
<td>Death effector domain</td>
</tr>
<tr>
<td>DETC</td>
<td>Dendritic epidermal T cell</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Media</td>
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<td>DMSO</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DSS</td>
<td>Dextran sodium sulphate</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix components</td>
</tr>
<tr>
<td>EDC</td>
<td>Epidermal differentiation complex</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<td>ES</td>
<td>Embryonic stem cell</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
</tr>
<tr>
<td>Term</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCAS</td>
<td>Familial Cold Autoinflammatory Syndrome</td>
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<tr>
<td>FMF</td>
<td>Familial Mediterranean fever</td>
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<tr>
<td>GBP5</td>
<td>Guanylate-binding protein 5</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage - colony stimulating factor</td>
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<td>GSMD</td>
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<td>GWAS</td>
<td>Genome wide association study</td>
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<td>H&amp;E</td>
<td>Haemotoxylin &amp; Eosin</td>
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<td>HKLR</td>
<td>Heat-killed <em>Lactobacillus rhamnosus</em></td>
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<td>Heat-killed <em>Salmonella Typhimurium</em></td>
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<td>HRP</td>
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<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<td>IFI16</td>
<td>Interferon gamma inducible protein 16</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>Interleukin</td>
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<td>IMQ</td>
<td>Imiquimod</td>
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<td>IRAK</td>
<td>IL-1R-associated kinase</td>
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<td>ISRE</td>
<td>Interferon-sensitive response element</td>
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<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
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<td>PAMP</td>
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<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TAB</td>
<td>TAK-1 binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with Tween</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming-growth forming-β</td>
</tr>
<tr>
<td>TICAM-1</td>
<td>TIR-domain-containing molecule 1</td>
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<td>TIR</td>
<td>Toll/IL-1R homology</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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<td>TRAF</td>
<td>TNFR-associated factor</td>
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<tr>
<td>TRAM</td>
<td>TRIF related adaptor molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR domain-containing adaptor inducing IFN-β</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion protein</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WGS</td>
<td>Whole genome sequencing</td>
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<td>WT</td>
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V. Presentations and Publications

Presentations

June 2018  Poster presentation  TOLL – Editing Innate Immunity, Porto, Portugal

September 2018  Poster presentation  Irish Society of Immunology, Dublin, Ireland

December 2018  Oral presentation  OLCHC Autoinflammation Clinic, Dublin, Ireland

Publications


Chapter 1: Introduction
1. Introduction

1.1 Immunity

1.1.1 Innate Immunity

The immune system is an evolutionary conserved system that has evolved to protect the host from invading pathogens and cellular damage. The human immune system is comprised of two main responses; innate immunity and adaptive immunity. The innate immune system functions as the first line of defence against invading pathogens and is crucial in maintaining homeostasis and preventing the spread of infection. The innate immune system also plays an important role in identifying “danger” signals and activating the adaptive immune system.

The first line of defence in innate immunity is the physical barrier between internal organs and the external environment. The skin and other epithelial surfaces, such as oral mucosa, respiratory epithelium and intestine, are important first lines of defence and are crucial in protecting the host from a wide onset of pathogens on a daily basis. If microorganisms succeed in surpassing these physical barriers, they enter into the body and are met with a range of complement/antimicrobial proteins and innate immune cell defence mechanisms.

The innate immune system functions as a collection of diverse cell types, including professional phagocytic cells such as macrophages and neutrophils; antigen presenting cells, such as dendritic cells; and cytotoxic cells, such as natural killer cells (NK cells) and granulocytes (mast cells, basophils and eosinophils). These cells act by engulfing and killing microorganisms/apoptotic cells or by recognising the invading pathogen and recruiting immune cells to the site of infection, through the release of chemical messengers known as cytokines. Cells of the innate immune system are believed to recognise pathogen or danger signals in an unspecific manner in comparison to the adaptive immune system. However, molecules known as “pattern recognition receptors” (PRRs), located on innate cells are capable of recognising conserved regions of invading micro-organisms (1). PRRs allow the host cells to recognise an invading microorganism in the form of “pathogen-associated molecular patterns” (PAMPs) or endogenous danger
signals released from dying cells known as “danger-associated molecular patterns” (DAMPs).

Following PAMP or DAMP recognition, the innate immune system initiates the production and release of pro-inflammatory cytokines and chemokines that mediate the recruitment of immune cells to the site of infection or damage. If the innate immune system does not succeed in the clearance of the invading pathogen or damaged cells, the adaptive immune system is initiated. While an immune response is crucial for protection of the host, dysregulation of components of either the innate or adaptive immune system can result in a plethora of autoinflammatory or autoimmune diseases (2).

*Staphylococcus aureus* is an example of a common pathogen that is responsible for initiating both an innate and adaptive immune response. *S. aureus* is the leading cause of soft tissue and skin infections and can also result in a range of life threatening illnesses such as meningitis, pneumonia, sepsis, bacteraemia and abscesses of various organs (3). The initial response to *S. aureus* is mediated by the skin which acts as a physical barrier. Keratinocytes present in the skin release antimicrobial peptides that have bactericidal activity against *S. aureus* (4). Neutrophils are an important innate cell type responsible for initial recognition of *S. aureus* infection. Neutrophils act by phagocytosing the bacteria, recognising the pathogen and releasing pro-inflammatory cytokines (5). Antigen-presenting cells (eg. dendritic cells) can prime T cells by phagocytosing bacteria and subsequently presenting antigenic peptides complexed with major histocompatibility complex-II (MHC-II) on their cell surface (6). Once activated, T-cells play important roles in the elimination of *S. aureus* infection. T-cells are responsible for driving phagocytosis via the recruitment of bone-marrow derived cells such as macrophages and neutrophils. IFN-γ production from T-helper cells helps to limit the infection of *S. aureus* to the phagosomes of macrophages (7). If the bacterium escapes the phagosome, cytotoxic T-cells then lyse the infected cells in order to release the bacteria and be re-phagocytosed. B-cell activation and effector function is determined by interactions with T cells and/or TLRs, in combination with cytokines secreted in the vicinity of the cell (6). Specific *S. aureus* antibodies then act by opsonising bacteria and mediating antibody-induced ingestion of the bacteria (3). *S. aureus* is therefore an example of a common bacterial infection that both the innate and adaptive immune response work together in order to effectively eliminate the pathogen.
1.1.2 Pattern Recognition Receptors

PRRs are a group of receptors that have a similar purpose, but differ in the various pathogens or danger signals that they recognise. All PRRs recognise either PAMPs or DAMPs, which are typically microorganism-derived molecules or mislocalised host-derived factors, both of which are essential for survival, and therefore are unlikely to be excluded from the genetic makeup of the microorganism/host. PRRs are germline encoded receptors that are capable of recognising a PAMP irrespective of the microorganism’s life cycle (8). PRRs differ structurally and can be located either on the surface of the host cell or intracellularly. Once a PRR recognises a PAMP or DAMP, it initiates the activation of downstream signalling pathways which can vary depending on the type of PRR that has been activated.

There are currently five classes of PRRs known to date, two of which will be expanded on for the purpose of this introduction. The list of PRRs include: Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), both of which are transmembrane receptors and also the cytoplasmic receptors; Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), Nod-like receptors (NLRs) and AIM2-like receptors (ALRs).

1.1.3 Toll-like Receptors

The family of TLRs are one of the best characterised of all of the PRR families. Toll was originally identified as the Drosophila gene and is an essential gene for the development of dorsoventral polarity in the embryo. It was later found to also play an important role in the anti-fungal response of a fruit fly (9). A mammalian homologue of Toll, TLR, was subsequently discovered to recognise a potent gram-negative bacterial component known as lipopolysaccharide (LPS), which was proven to be responsible for up-regulating the expression of genes involved in inflammation, to protect against bacterial infection. At present, there have been 10 TLRs identified in humans, and 12 TLRs identified in mice.

TLRs are type I integral membrane glycoproteins expressed on the surface and intracellular regions of cells (Figure 1.1). TLRs consist of N-terminal Leucine-rich repeats (LRRs), a transmembrane region, followed by a Toll/IL-1R homology (TIR) domain that extends into the cytoplasm of the cell and is responsible for interacting with cytosolic adaptor proteins. The LRR region is comprised of 19-25 tandem LRR motifs,
each approximately 24-29 amino acids long that mediate the recognition of microbial ligands (10).

TLR2 recognises numerous lipoprotein (LP) components from bacteria, viruses, fungi and mycoplasma. TLR2 recognises a ligand by forming a heterodimer with either TLR1 or TLR6, extending the ability of TLR2 to recognise diverse LP structures from different pathogens. TLR2 has also been reported to recognise many endogenous ligands such as cell damage products, inflammatory mediators, extracellular matrix and oxidised lipids. The acute phase protein, serum amyloid A has also recently been identified as a biological ligand of TLR2 that has been implicated in rheumatoid arthritis (11). TLR10 shares sequence similarity with both TLR1 and TLR6, however there is no known ligand for TLR10. TLR4 recognises LPS, a component of gram-negative bacteria, in the presence of Myeloid differentiating factor 2 (MD2) on the surface of the cell (12)(13). Two complexes of LPS-MD2-TLR4 interact together and form a homodimer to activate intracellular adaptor proteins and initiate an immune response (14). TLR5 recognises flagellin, the flagellar filament essential for bacterial motility, and is highly expressed in DCs of the small intestine (15). TLRs 3, 7, 8 and 9 recognise nucleic acids from bacteria and viruses. The recognition of ligands by these TLRs leads to the production of pro-inflammatory cytokines and type-I Interferons (IFNs). TLR3 recognises viral double stranded RNA (16). Mouse TLR7 and human TLR7/8 detect viral single stranded RNA and also small purine analog compounds such as, imidazoquinolines (17). TLR9 senses un-methylated viral and bacterial DNA with CpG motifs (18)(19). In addition, activation of TLR9 has been implicated in the autoimmune disease systemic lupus erythematosus (20).

As TLRs recognise a wide range of microbial products their activation also results in activation of a range of downstream signalling pathways, leading to the induction of pro-inflammatory genes. Following ligand recognition, TLRs dimerise and undergo a conformational change allowing the intracellular region of the TLR to form a homophillic interaction with the TIR domain of an adaptor protein. There are four main adaptor proteins that interact with TLRs: Myeloid differentiating factor 88 (MyD88), MyD88 adaptor-like (MAL)/TIR-associated protein (TIRAP), TIR-domain-containing molecule 1 (TICAM1)/TIR-domain-containing-adaptor protein-inducing IFN-β (TRIF) and TRIF-
related adaptor protein (TRAM). The differences in signalling activation are dependent on the adaptor proteins recruited to the TLR.

MyD88 is a crucial adaptor protein that is used by all TLRs except TLR3. Once a ligand binds to the TLR, MyD88 interacts via TIR domains to the cytoplasmic portion of the TLR. Activation of MyD88 then results in the recruitment and activation of IL-1R-associated kinase 4 (IRAK-4) and IRAK-1. In the case of TLR2 and TLR4 activation, an additional adaptor protein, Mal, is required to recruit MyD88 to the TLR (21). IRAK-4 phosphorylates and activates IRAK-1 and IRAK-2 which then disassociate from the MyD88/TLR complex and migrate to interact with the E3 ubiquitin protein ligase protein, TNFR-associated factor-6 (TRAF6). TRAF6, in conjunction with E2 ubiquitin-conjugating enzyme complex consisting of Ubc13 and Uev1A, catalyses the production of a K63-linked polyubiquitin chain onto TRAF6 itself and also generates a free, unconjugated polyubiquitin chain (22). The unconjugated polyubiquitin chain then activates a complex consisting of TGF-β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and TAB3. Activation of this complex mediates the phosphorylation of IκB kinase (IKK-β) and MAP kinase kinase 6. Consequently, activation of the IKK complex occurs, which is comprised of IKK-α, IKK-β and the NFκB essential modulator (NEMO). Activation of the IKK complex induces phosphorylation of IκBα, the inhibitor of NFκB. Phosphorylation of IκB promotes its degradation, resulting in the release of NFκB, and translocation into the nucleus to activate pro-inflammatory cytokine gene expression (Figure 1.1). Alternatively, activation of the TAB1/TAK1/TAB2 complex, triggers TAK1 mediated activation of the extracellular signal related kinase 1 (ERK), ERK2, Jun N-terminal kinase (JNK) and p38α mitogen-activated protein kinase (MAPK) pathways (23). Ultimately, activation of the NFκB, ERK1/2, JNK and p38α pathways result in up-regulation of expression of pro-inflammatory cytokines and chemokines that are produced to enlist further help in mounting an immune response against an invading pathogen.

Alternatively, activation of TLR3 requires the recruitment of an adaptor protein known as TRIF. Endocytosed TLR4 is also capable of recruiting TRIF to the cytoplasmic region of the receptor in a MyD88-independent manner. However, in this instance, TLR4 requires another adaptor protein to activate TRIF, known as TRAM (24). TRIF is then capable of interacting with TRAF3 and TRAF6 through TRAF binding motifs (25)(26).
TLR3 is responsible for the intracellular recognition of dsRNA. Activation of TLR3 results in the auto-ubiquitination of TRAF3 which mediates its degradation. Proteasome-mediated degradation of TRAF3 is further responsible for the activation of MAP kinases and production of pro-inflammatory cytokines.

TLRs are expressed on a variety of ‘professional’ immune cells such as DCs, macrophages, B-cells and also on ‘un-professional’ immune cells such as fibroblasts and epithelial cells. TLR expression can vary throughout the cell depending on the particular TLR. TLRs 1, 2, 4, and 6 are located on the outside surface of the cell, allowing them to sense and recognise PAMPs and DAMPs that have not yet entered into the cell. TLRs 3, 7, 8 and 9 are located in intracellular compartments in the cell, in particular on endosomes. Their ligands must therefore be internalised before signalling can occur.
Figure 1.1: An overview of the TLR signalling pathways

TLRs 1, 2, 4, 5 and 6 are located within the extracellular membrane and are activated by a variety of ligands (bacteria, viruses, fungi). TLRs 3, 7, 8 and 9 are located within the endosome membrane. All TLRs (except TLR3) result in activation of the MyD88-dependent pathway which culminates in NFκB, p38, ERK and JNK activation. TLR2 and 4 require the adaptors Mal and MyD88 to mediate signalling. TLRs 5, 7, 8 and 9 require MyD88 only to induce signalling. TLR3 requires TRIF to activate TBK1 and IKKe. Endocytosed TLR4 can also mediate MyD88-independent signalling and requires the adaptors TRAM and TRIF to mediate this. MyD88-independent signalling induces IRF3 and IRF7 activation.
1.1.4 Nod-like Receptors

The Nod-like receptors are a family of cytoplasmic pathogen sensors that are characterised by the presence of a central nucleotide-binding oligomerisation domain (NOD) which is commonly accompanied by a C-terminal Leucine-rich repeat domain, thought to be responsible for ligand recognition, and an N-terminal domain, comprised of protein interacting domains such as caspase recruitment domain (CARD), pyrin, or baculovirus inhibitor of apoptosis protein repeat (BIR), responsible for the initiation of signalling. NLRs containing a CARD domain are primarily involved in the upregulation of transcription of pro-inflammatory mediators whereas NLRs containing a pyrin or BIR domain form as a component of the inflammasome that mediates caspase-1 activation.

Several of the NLR proteins consisting of a pyrin domain (NLRPs) and the NLR protein with a CARD domain, NLRC4, have been shown to form an inflammasome complex once they become activated, following ligand recognition. Inflammasomes are large multi-protein complexes that result in the activation of the inflammatory caspase, caspase-1. The pyrin domain of NLRs such as NLRP1, NLRP3, NLRP6 or AIM2 oligomerise with the pyrin domain of ASC. ASC is an adaptor protein, consisting of a CARD and a Pyrin domain, and is responsible for the recruitment of caspase-1 via a CARD-CARD interaction, leading to the formation of the Inflammasome and the recruitment of pro-caspase-1 molecules into close proximity. Caspase-1 is subsequently auto-proteolytically cleaved via an ‘induced proximity’ model, resulting in the release of p10 and p20 subunits. The cleaved subunits form a tetramer of an active cysteine protease which mediates the cleavage and consequential activation of IL-1β and IL-18, that further act as pro-inflammatory messengers in a paracrine manner (27)(28). Caspase-1 also plays an important role in pyroptosis which is mediated by cleavage of Gasdermin D (29). In mammals, the majority of proteins are secreted through the conventional ER-golgi secretary pathway. However, caspase-1 is also believed to play a role in unconventional protein secretion, for proteins including IL-1β, IL-18 and IL-37 (30). A very recent report has outlined a clearer mechanism detailing IL-1β release (31). Monteleone et al report that IL-1β maturation results in its subsequent migration from the cytosol to the plasma membrane. IL-1β release may occur slowly, which is GSDMD-independent, or more rapidly. Rapid release of IL-1β is believed to occur through caspase-1-induced Gasdermin D pores either during or prior to pyroptosis (31).
1.2 Caspases

Caspases are a family of cysteine proteases that were originally subdivided into groups that function in either inflammation or apoptosis. The first caspases were identified over three decades ago. Ced-3 was originally discovered in the nematode Caenorhabditis elegans (32), and the mammalian homolog of Ced-3 was later identified as Interleukin-1-β-converting enzyme, (now known as caspase-1) (33). Caspases -2, -3, -7, -8, -9, and -10 were originally described as apoptotic caspases and caspases -1, -4, -5 and -12 were described as inflammatory caspases. However, it now appears the functions of caspases are most likely interchangeable between both groups. In recent years it has been shown that inflammatory caspases are responsible for initiating a programmed cell death known as pyroptosis. Conversely a typically apoptotic protein, caspase-8, has been found to play an important role in driving inflammation (34).

Caspases are originally synthesised as inactive, single chain, zymogen enzymes that have a primary specificity for aspartic acid residues in a protein, cleaving the protein at a P1 position before the Asp residue. Caspases are broadly classed into two main groups based on their sequence and biological functions. The first group consists of caspase-1, -4 and -5 (and murine caspase-11) and are known as the inflammatory caspases. They consist of long caspase recruitment domains (CARD) and have affinity for large hydrophobic groups at the P4 position (eg.W/Y). The second group of caspases are responsible for mediating apoptosis and can be further divided into two subgroups. The first of these are comprised of caspase-3, -6 and -7 and are known as apoptotic effector caspases that consist of short pro-domains. The second apoptotic subgroup are comprised of caspase-8, -9 and -10 and are known as initiator caspases. Initiator caspases consist of either death effector domains (DED) or CARD domains that mediate the recruitment to protein complexes and oligomerisation (35)(36). Inactive caspases consist of an N-terminal pro-domain and a protease C-terminal domain. The protease domain of a caspase consists of a large and small subunit that contains the catalytic active site.

1.2.1 Caspase-1 and the Canonical Inflammasomes

There are several inflammasome complexes identified to date. They generally include a sensor protein, an adaptor protein with a CARD domain (ASC) and caspase-1. The sensor protein is responsible for recognising PAMPs and DAMPs which then trigger the formation of an inflammasome. Inflammasome activation generally occurs via two
signals. ‘Signal 1’ involves the up-regulation of cellular levels of inflammasome components and pro-IL-1β and is mediated by NFκB signalling following TLR or NOD1/2 activation. ‘Signal 2’ involves activation of the inflammasome via ligand binding to a sensor protein. There have been eight sensor proteins identified so far with interferon-inducible protein-16 (IFI16) and absent in melanoma (AIM2) being the only non-NLR sensors (37).

The AIM2 inflammasome is one of the only inflammasome sensors that is not a member of the NLR family of PRRs. However, AIM2 is comprised of an N-terminal pyrin domain and a C-terminal HIN200 domain. Similar to other NLRs, AIM2 lacks a CARD domain, therefore requiring ASC to interact with caspase-1. Similar to other pyrin domain proteins, AIM2 is localised in the cytosol and recognises intracellular cytosolic DNA. The positively charged HIN domain forms an electrostatic attraction with the negatively charged sugar-phosphate backbone of dsDNA. Following binding of the HIN domain to dsDNA, the pyrin domain is exposed, mediating its release from the autoinhibitory PYD/HIN complex, allowing the formation of inflammasomes along the DNA helixes (38). Unintended detection of endogenous, nuclear DNA is prevented due to the localisation of AIM2 to the cytosol.

NLRP1 was the NLR that characterised the first inflammasome (39). NLRP1 is structurally different to other NLRs as it is composed of an extra domain, function to find domain (FIIND). There are variations between human and mouse NLRP1 which suggests the gene has undergone considerable diversification between species over time. There is only one human NLRP1 gene, however, three NLRP1 paralogues have been discovered in mice (Nlrp1a, Nlrp1b and Nlrp1c). Human and murine NLRP1 also appear to vary functionally. Human NLRP1 activates caspase-1 in an ASC-dependent manner, however, murine NLRP1 is capable of activating caspase-1 in the absence of ASC (40).

The most widely characterised ligand of murine NLRP1 is anthrax lethal toxin (LeTx), an important virulence factor released by the causative agent of Anthrax disease, Bacillus anthracis (41). Human NLRP1 does not recognise LeTx. In fact, the mechanism of activation of human NLRP1 is much less clear. It has, however, been shown that muramyl-dipeptide (MDP) is capable of activation of over-expressed NLRP1. In this study, a cell-free system was used to show that the peptidoglycan fragment of bacteria, MDP, induced ATP binding and oligomerisation of NLRP1, which resulted in caspase-1
activation (42). This suggests that NLRP1 contribute to host defence against pathogens by directly sensing bacterial components. However, as human and murine NLRP1 are decidedly different, this has limited the progress of NLRP1 research.

The NLRP3 inflammasome is the most widely studied of all inflammasomes and extensive research has elucidated a range of microbial and non-microbial activators of NLRP3. There are many proposed mechanisms of NLRP3 activation (Figure 1.2). One of the more consolidated mechanisms of NLRP3 activation is believed to be mediated by potassium efflux via pore-forming toxins (eg. Nigericin) and extracellular ATP. Extracellular ATP induces secretion of IL-1β via activation of the purinergic receptor (P2X7) which results in K+ efflux (43)(44). Similarly, pore forming toxins induce a K+ efflux that results in IL-1β secretion (45)(46). Interestingly, a recent study has further explored the mechanism of NLRP3 inflammasome activation (47). Green et al have reported that an efflux of Cl− results in oligomerisation of ASC and formation of an inactive inflammasome. Subsequent K+ efflux results in NEK7 binding and inflammasome activation. This report has identified that Cl− alone mediates inflammasome formation, however K+ influx is required for inflammasome activation. Interestingly Green et al also report that inflammasome formation that occurs as a result of Cl− efflux is reversible, a concept which has not been reported before (47).

NLRP3 has also been implicated in detecting a plethora of microbial pathogens such as the viral Influenza A (48), vesicular stomatitis virus (49), bacterial S. aureus, E. coli (50), fungal Candida albicans (51), Aspergillus fumigatus (52), parasitic Schistosoma mansoni (53) and Dermatophagoides pteronyssinus (54). Additionally, phagocytosis of certain endogenous and exogenous particulate matter, such as monosodium urate (MSU), amyloid-β, silica, calcium pyrophosphate dehydrate (CPPD), asbestos and alum have been reported to activate NLRP3 (55–59). These non-microbial agonists induce K+ efflux that results in subsequent NLRP3 activation. While this mechanism of activation is still unclear, it appears that phagolysosomal disruption is crucial to achieve NLPR3 activation. Endocytosis of particulate matter damages the lysosomal membrane and results in the release of Cathepsin B, a lysosomal cysteine protease into the cytosol. Destabilisation of the lysosome results in NLRP3 activation (60).

Another proposed mechanism of NLRP3 activation is the signalling of calcium. IL-1β secretion mediated by ATP requires Ca2+ influx and Ca2+ release from the endoplasmic
reticulum (ER) (61). UV radiation-induced activation of NLRP3 is also dependent on high intracellular levels of Ca$^{2+}$ (62). Additionally, cholesterol-dependent cytolysins require Ca$^{2+}$ influx to activate NLRP3 (63). Furthermore, inhibition of Ca$^{2+}$ signalling is believed to block NLRP3 inflammasome activation, suggesting Ca$^{2+}$ is likely a key mediator of NLRP3 inflammasome activation (64)(65). However, a more recent study has reported that NLRP3 activation occurs independently of Ca$^{2+}$ signalling (66). Differing results may be explained by the experimental setup used in each study. Katsnelson et al directly measured changes in both Ca$^{2+}$ and K$^+$ using minimal pharmacologic inhibitors (66). It is likely that inhibition of Ca$^{2+}$ signalling in earlier studies effects the homeostasis of other cations such as Zn$^{2+}$ or the activity of non-selective channels. Collectively, these mixed reports suggest that the proposed mechanism of NLRP3 activation via Ca$^{2+}$ signalling must be further explored.
Figure 1.2: Proposed Mechanisms of NLRP3 activation

The canonical NLRP3 inflammasome requires initial priming by activation of a TLR (eg. TLR4 recognises LPS). Priming is then is subsequently mediated by caspase-8, FADD or NFkB to induce up-regulation of expression of inflammasome components, eg. IL-1β and NLRP3. There are a variety of proposed mechanisms responsible for NLRP3 activation. K⁺ efflux, Ca²⁺ influx, pore-forming channels, mtROS, mtDNA and phagosomal destabilisation are all reported to mediate NLRP3 activation. Once activated, NLRP3 results in caspase-1 activation which mediates pyroptosis or IL-1β and IL-18 release. Image taken and modified from (67).
Activation of NLRP3 may also occur due to another proposed mechanism. Release of ROS via damaged mitochondria is believed to result in NLRP3 activation. Release of oxidised mitochondrial DNA (mtDNA) (68) may cause NLRP3 activation. Early studies suggested that mitochondrial Ca\textsuperscript{2+} overload and build-up of mtROS results in a loss of inner mitochondrial membrane potential (69). However, more recent reports have suggested that increase in mitochondria membrane potential during viral infection may be dispensable for NLRP3 activation (70).

There are also several regulators of NLRP3 activation that have been reported, such as guanylate-binding protein 5 (GBP5) (71), double-stranded RNA-dependent protein kinase (PKR) (72) and Nek7 (73). The role of both GBP5 and PKR are controversial, however the role of Nek7 has been conclusively reported in multiple studies. PKR is believed to regulate all known inflammasomes, such as AIM2, NLRP1, NLRP3 and NLRC4 and deficiency of PKR has been reported to reduce activation of caspase-1 in response to a variety of stimulants (72). However, studies using PKR\textsuperscript{-/-} mice did not confirm this hypothesis (74), suggesting the role of PKR in regulation of inflammasome activation is still unclear. Additionally, GBP5 has been reported to boost NLRP3 inflammasome activation in response to stimuli such as bacteria, ATP and Nigericin but not particulate matter (71). Similarly to PKR, GBP5\textsuperscript{-/-} mice displayed normal activation of the NLRP3 inflammasome (75).

Interestingly, recent studies have reported Nek7 as a novel NLRP3 inflammasome regulator (73). Nek7 is a member of the NIMA-related kinase family and was originally found to be responsible for regulating mitotic progression and response to DNA damage (76). Nek7 is reported to be responsible for NLRP3 activation in response to all stimuli tested, such as alum, MSU crystals, ATP and Nigericin (73)(77). Nek7 controls NLRP3 oligomerisation, formation of an ASC speck and subsequent caspase-1 activation downstream of K\textsuperscript{+} efflux (73). These findings have been reported in three independent in vivo studies and suggest Nek7 is a definite positive regulator of NLRP3 (73, 77, 78).

The NLRC4 receptor is structurally very similar to other NLRs as it contains CARD, NACHT and LRR domains. NLRC4 has been reported to become activated in macrophages in response to a range of gram-negative bacteria such as Legionella pneumophila (79), Salmonella typhimurium (80), Pseudomonas aeruginosa (81) and Shigella Flexineri (82). The NLRC4 inflammasome recognises bacterial proteins in the
cytosolic compartments which are markers for the activity of bacteria type III (T3SS) and type IV (T4SS) secretion systems. NLRC4 is also capable of detecting flagellated bacteria, as it was shown that certain bacteria deficient in the Flagellin protein in Flagellum were inept at activating NLRC4 (83). NLRC4 is a unique inflammasome as it is also an adaptor protein in the NLR activation pathway of the NAIP family of proteins. NAIPs directly interact with one of three ligands: T3SS rod, T3SS needle or Flagellin (84–86). NAIPs are NLRs that contain a baculoviral inhibitor of apoptosis protein repeat (BIR) domain at their N-terminal as a substitute of death fold domains. There are seven NAIPs in mice but only one NAIP in humans, leading to slight uncertainty in the crossover of functions between both species (87). While NLRC4 contains a CARD domain that can interact with the CARD domain in caspase-1, it appears that ASC is still required for activation of Caspase-1 by NLRC4 (88). Conversely NLRC4 is capable of inducing cell death independently of ASC (89).

**NLRP6** is a member of the NLR family, responsible for regulating inflammation and host defence against invading pathogens. While NLRP6 is an understudied protein, there have been some interesting findings regarding the expression of NLRP6 in different tissues and cell types. NLRP6 has been found to be expressed at high levels in the small intestine and duodenum as reported by protein atlas (90). The small intestine and colon have been most widely investigated and comprise the majority of published research in relation to the role of NLRP6.

Protein atlas also reports that NLRP6 is primarily expressed in cells of myeloid or lymphoid lineage (91). However recent findings suggest that NLRP6 is found at high and specific expression in both enterocytes (92) and colonic goblet cells (92) in the intestine. Reports suggest that NLRP6 regulates the interaction of the host and microbiome in the gut. The gut is home to $10^{13}-10^{14}$ commensal microorganisms that colonise on the mucosal surface and luminal content. Commensal bacteria are crucial in maintaining homeostasis in the gut and have a variety of different roles. In the epithelium, NLRP6 has been found to be co-expressed with typical inflammasome components such as ASC and caspase-1 (93). Deficiency in NLRP6 is linked with low levels of intestinal IL-18 and caspase-1 activation (94). However, it is still unknown whether NLRP6 initiates formation of an inflammasome itself or whether it impacts other inflammasomes and subsequent IL-18 secretion.
Interestingly, the microbiome is believed to both positively and negatively regulate NLRP6. A recent metabolomics study found the bile acid-derivative, taurine is a microbiotic-produced metabolite that positively regulates NLRP6 and IL-18 production (93). Conversely, spermine and histamine have been shown to both dampen NLRP6 activity (93). Therefore, it is believed that NLRP6 may be capable of incorporating information on microbiome metabolic and colonisation status into its downstream signalling. NLRP6 may also be regulated by non-microbial means. Under water deprivation conditions, WT mice showed increased expression of stress-induced corticotrophin-releasing hormone (CRH) which, as a result, decreased expression of NLRP6 inflammasome components in the intestine, driving intestinal permeability (95). In vivo, administration of peroxisome proliferator-activated receptor-γ (PPARγ) agonist, abolished this stress-induced inflammatory response. The rationale for the use of this agonist arose when it was determined that NLRP6 harbours a PPARγ binding site at the promotor region, suggesting that NLRP6 may be regulated by this transcription factor. In in vitro studies, a PPARγ agonist was shown to induce NLRP6 expression levels in human intestinal epithelial cells. However, this has not been investigated in an in vivo situation, therefore, the actual impact of these findings have yet to be elucidated. As a whole, these findings suggest a complex network of interactions occur between both the host, microbiota present in the intestine and NLRP6.

Colonisation of microbiota in the lining of the gut epithelium is crucial for the maintenance of gut homeostasis. However, there is an important mucus layer that physically separates the bacteria from intestinal cells. Intestinal mucus is the primary defence mechanism employed by the host to protect against invading pathogens and it consists of different layers to ensure protection of the epithelium from physical and chemical damage. Goblet cells in the epithelium produce granules of mucins that are then secreted into the lumen, forming the inner mucus layer (96). Interestingly, it was discovered that NLRP6−/− mice display dysfunctional mucus granule exocytosis which may suggest that NLRP6 plays a role in the maintenance of a mucosal barrier. In addition to regulating the mucosal barrier, NLRP6 has also been implicated in regulating microbial colonisation of epithelial cells through the secretion of antimicrobial peptides. When microbiota are present, NLRP6 is considered to form an inflammasome with ASC, thereby triggering the release of IL-18. IL-18 is then believed to act on surrounding cells of the epithelium to promote the transcription of AMPs angiogenin-4, resistin-like beta
and intelectin-1 (93). Dysfunctional regulation of the microbiome – epithelial cell interaction due to NLRP6, is believed to have a direct impact on the AMP repertoire which leads to dysbiosis. This dysbiosis has been shown to be partially restored with the addition of IL-18 (93).

While there have been a multitude of studies suggesting an important role for NLRP6 in intestinal homeostasis, very recent reports present contradicting findings, highlighting the importance for littermate WT controls in murine studies. Mamantopoulos et al., have shown that separately housed NLRP6+/− and NLRP6+/+ mice do not show microbiome dysbiosis (97). They hypothesise that maternal inheritance and separate housing of NLRP6−/− and non-littermate NLRP6+/+ may in fact be the culprit for the previously observed epithelium dysfunction in NRLP6+/− mice so far. Mamantopoulos et al., showed that lifetime separation of NLRP6−/− and their littermate controls did not reveal a genetic effect of NLRP6 on the composition of the gut microbiota and intestinal function. A second study has also reported similar findings (98). These recent reports have led us to question if NLRP6 has in fact any functional role in the maintenance of intestinal homeostasis or if the lack of sufficient controls in experimental set up has led to misinterpretation of findings.

In addition to the possible role NLRP6 plays in regulating intestinal homeostasis, NRLP6 has also been found to play an important role in response to viral infection. In the epithelium, NLRP6 was found to interact with viral RNA via the helicase DHX15 which induced increased expression of IFN-stimulated genes. This increased gene expression is mediated by the mitochondrial adaptor protein known as mitochondrial antiviral signalling protein (MAVS) and is independent of caspase-1 (99).

While the focus of most research has been aimed at investigating the role NLRP6 plays in epithelium/stromal cells, there have been various studies investigating the role of NLRP6 in cells of haematopoetic lineages. A study using lethal irradiation and bone marrow transplantation of WT and NLRP6 deficient bone marrow reports that hematopoietic-derived cells are responsible for the NLRP6-mediated protection against colitis-induced tumorigenesis (100). An additional study investigated the various cells of hematopoietic lineages within the lamina propria and determined that NLRP6 expression was induced in inflammatory CD11b+ Ly6Gint Ly6Chi monocytes, during DSS-induced colitis. Interestingly, adoptive transfer of wild-type Ly6C^hi monocytes into NLRP6-
deficient mice significantly improved survival (101). *NLRP6*^−/−^ Ly6C<sup>hi</sup> monocytes were found to display decreased secretion of TNF-α which was believed to be important in preventing DSS-induced mortality and was dependent on autocrine signalling of IL-18 (101). While Seregin *et al* have reported that inflammatory Ly6C<sup>hi</sup> monocytes within the lamina propria are responsible for NLRP6-mediated protection against DSS-induced colitis, they could not identify a role for NLRP6 in intestinal epithelial cells (IECs), intraepithelial lymphocytes (IELs), or bone marrow (BM)-derived cells. These findings suggest differential roles of NLRP6 in monocyte sub-populations (101). However, collectively, these studies suggest a definitive role for NLPR6 in haematopoietic cells but warrants the need for further investigation of the role NLRP6 plays within these cell populations.

Until very recently, an agonist for the NLRP6 inflammasome had not been discovered. However, a recent publication has reported that NLRP6 recognises lipoteichoic acid (LTA), a component of gram-positive bacteria (102). LTA has now been found to bind to the LRR domain of NLRP6 which induces the recruitment of caspase-1 and caspase-11 to the NLRP6 inflammasome. NLRP6 and caspase-11 have also been determined to exacerbate *Listeria* infection *in vivo* (102). This finding has identified a novel role for NLRP6 in mediating gram-positive bacterial infection.

Various reports have also suggested that NLRP6 may regulate the innate immune system by modulating the NFκB pathway. A murine study investigating the role of NLRP6 in response to *L.monocytogenes* and *S. typhimurium* found that NLRP6 may negatively regulate NFκB and ERK signalling in response to *L.monocytogenes* infection in BMDMs (103). Further analysis suggests that NLRP6 is specifically capable of negatively regulating the canonical NFκB pathway only. Conversely, an *in vitro* study found that NLRP6 is capable of driving NFκB activation in HEK293T cells in an ASC-dependent manner (104). These contradictory findings have led to confusion as to the role of NLRP6 in relation to NFκB regulation. It is possible that NLRP6 plays various roles in different cell types and species, however it is evident that further research is crucial to determine the influence of NLRP6 on NFκB signalling pathways.

Similar to NLRP6, **NLRP12** is another NLR protein that is reported to have multiple and conflicting roles in relation to NFκB. Early reports suggested that NLRP12 co-localises with ASC and activates caspase-1 and NFκB resulting in IL-1β secretion (105).
However, as was seen with NLRP6, conflicting reports have suggested that NLRP12 negatively regulates the NFκB pathway. One study reports that NLRP12 antagonises NFκB signalling pathway by negatively regulating TNFR and TLR signalling (106). An additional study showed that silencing of NLRP12 results in increased activation of NFκB in response to TNF-α and *Mycobacterium tuberculosis* (106, 107). NLRP12 has been reported to be responsible for the activation of caspase-1, which, *in vitro* experiments have reported is mediated through ASC (108). NLRP12 has also been found to recognise the causative agent of plaque, *Yersinia pestis*. The NLRP12 inflammasome leads to caspase-1 activation and as a result, IL-1β and IL-18 secretion in response to *Y. pestis* in macrophages (109). Interestingly, the response of NLRP12 to *Y. pestis* does not interfere with NFκB signalling, suggesting differential roles in response to different stimuli. Additionally, an *in vitro* study has reported that NLRP12 controls the expression of classical and non-classical MHC class I genes (110).

![Figure 1.3: Phylogenetic tree of NLR proteins](image)

**Figure 1.3: Phylogenetic tree of NLR proteins**

Sequences of NLR1-12 were aligned using ClustalW and a phylogenetic tree was composed using Mega7. Mega7 designs the evolutionary history of sequences using the Neighbour-Joining method. The branch lengths are in the units of the number of amino acid substitutions per site.
1.2.2 Caspase-1

Caspase-1 is a fundamental mediator of innate immunity and plays an important role in a range of inflammatory diseases. Caspase-1 was originally identified in 1989 as the protease responsible for cleaving pro-IL-1β and IL-18. Interestingly, caspase-1 has very few substrates in comparison to the hundreds of substrates of apoptotic caspases. Proteomic studies have led to the belief that caspase-1 has more substrates than previously known. However, the likelihood of these events happening under physiological studies is not known. Caspase-1 has a low substrate specificity at higher concentrations, which decreases with decreasing concentrations so it is probable that in vitro experiments have led to cleavage of proteins that may not be cleaved under physiological conditions. Additionally, caspase-1 has a very low half-life, approximately 50 times less than active caspase-3, which suggests that high concentrations will never be reached in vivo situations. As mentioned previously, caspase-1 has been deemed responsible for the cleavage and activation of pro-IL-1β and IL-18. IL-1β is a potent pro-inflammatory cytokine that plays a key role in mediating inflammation. Pro-IL-1β is expressed at limited basal levels in unstimulated cells and its expression must therefore be up-regulated following TLR activation (signal 1 of inflammasome activation). IL-18 is responsible for IFN-γ production which results in the activation of T-cells, and macrophages, along with various other cell types (111).

Caspase-1 mediates pro-inflammatory signalling via NFκB activation through a range of pathways. Caspase-1 mediated IL-1β secretion results in activation of the IL-1 receptor and further NFκB activation (112). Caspase-1 has also been found to proteolytically cleave caspase-7 which mediates caspase-7 induced cleavage of poly(ADP-ribose) polymerase-1 (PARP1). Release of PARP1 from chromatin results in increased expression of NFκB genes (113). Furthermore, caspase-1 activity has been implicated in the non-enzymatic activation of NFκB, via CARD-CARD associations of caspase-1 and receptor-interacting protein kinase 2 (RIP2)(114).

Caspase-1 also plays an important role in mediating cell death. Originally, reports suggested that caspase-1 was capable of inducing apoptosis, however the classification of apoptosis in these cases were based on features such as DNA fragmentation, morphological changes, and chromatin condensation. On closer examination of these forms of microbial-induced cell death, clear differences were determined in comparison
to classical apoptosis. Now, it is characterised as an entirely different form of cell death and is known as pyroptosis (115). Pyroptosis does not require the presence of apoptotic caspases, rather, it requires inflammatory caspases alone. Additionally, pyroptosis is characterised by an increase in cell size as a result of osmotic swelling, followed by rupturing of the cytoplasmic membrane. There is also no damage to the mitochondrial membrane observed during pyroptosis. A by-product of pyroptosis is the release of pro-inflammatory mediators such as pro-IL-1α, ATP and HMGB1 (116). Activated caspase-1 during pyroptosis also results in cleavage of Gasdermin D (GSDMD), a newly discovered executioner of pyroptosis (117). The pore forming, N-terminal fragment of GSDMD has been shown to mediate the release of IL-1β and IL-18 from the pyroptotic cell and is responsible for driving pyroptosis (29). However, caspase-1 mediated GSDMD activation has recently been implicated in mediating IL-1β release independently of pyroptosis (31). When released, these mediators subsequently recruit neutrophils and other immune cells, enhancing inflammation at the site of infection. In contrast, apoptosis is believed to be an immunologically silent form of cell death.

1.2.3 Caspase 11 and its role in the Non-Canonical Inflammasome

Murine caspase-11, previously known as ich-3, was originally discovered due to its sequence similarity with caspase-1. The caspase-11 non-canonical inflammasome is believed to be a critical responder during bacterial infections. In vitro and in vivo experiments have shown that caspase-11 can restrict the replication of gram-negative intracellular bacteria. Caspase-11 has been found to play a significant role in responding to a range of gram-negative bacterial infections including Citrobacter rodentium (118), Escherichia coli (119), Shigella Flexneri (50), Burkholderia thailandensis (120), Legionella pneumophila (121), and Salmonella typhimurium (122). While it was known that caspase-11 mediated a response against gram-negative bacteria, the exact PAMP responsible for initiating this response was still unknown. It was not until 2013 that two independent groups published findings that unravelled this mystery. Studies from the laboratories of Dixit and Miao revealed that caspase-11 is capable of interacting directly with LPS, a major component of the outer wall of gram-negative bacteria (123)(124). Kayagaki et al. showed that only electroporation or transfection of LPS into the cytosol of cells activated caspase-11 (124). This group also reported that LPS was necessary for caspase-11 to recognise a gram-negative bacterial infection, as E. coli mutants lacking structurally intact LPS were incapable of instigating activation of caspase-11 (124).
Caspase-11 responds to bacterial infection in two steps: caspase-1 independent activation of pyroptosis and caspase-1 dependent secretion of IL-1β and IL-18. Caspase-11 responds to intracellular LPS and initiates pyroptosis, similarly to caspase-1 (125). Release of these cytokines can be observed following caspase-11-mediated NLRP3 inflammasome formation (126).

As previously mentioned, upon recognition of a pathogen by multiple PRRs, intracellular signalling cascades induce the up-regulation of inflammasome components such as pro-IL-1β, NLRP3 and caspase-11. Expression of caspase-11 is highly inducible with an upstream regulatory promoter region with a STAT1 binding site and multiple NFkB binding sites (127). Caspase-11 upregulation in BMDMs is largely dependent on type I IFN signalling, as LPS-induced caspase-11 upregulation is severely impaired in IFNAR⁻/⁻ BMDMs (50, 128, 129). Therefore, multiple TLR and IFN activators are capable of up-regulating caspase-11 expression. At a physiological level, bacteria consist of multiple PAMPs, most of which are detected by a TLR, causing up-regulation of caspase-11 expression before the bacteria even enters into the host cell.
Figure 1.4: Caspase-11 mediates non-canonical inflammasome activation and drives pyroptosis.

Intracellular recognition of LPS, which is mediated by Guanylate-binding proteins (GBPs) results in activation of caspase-11/4/5 (75, 125). Once activated, caspase-11 mediates cleavage of GSDMD to allow the release of an N-terminal fragment, which drives pyroptosis (130)(29). Activation of caspase-11 also drives the formation of an NLRP3 inflammasome, which mediates the activation of caspase-1 and subsequent activation of IL-1β and IL-18 (126). Image taken from (131)
1.2.4 Caspase-4 and -5 – the Human Orthologs of Murine Caspase-11

Caspase-4 and -5 are two inflammatory caspases known to form a non-canonical inflammasome but very little was known of their function until recent years. Caspase-4 and -5 are the human orthologs of murine caspase-11. However, whether the precise signalling mechanisms of caspase-4 and -5 are analogous to those of caspase-11 has yet to be determined. Substrate profiling studies have shown they have different preferred substrate cleavage sites. Caspase-4 and -5 cleave at (W/L)EHD sites whereas caspase-11 prefers (I/L/V/P)EHD sites (132)(133).

Expression of caspase-4 is relatively constitutive in comparison to caspase-5 and caspase-11, whose expression is only up-regulated following stimulation with LPS. Caspase-4 has been shown to induce pyroptosis following transfection of LPS in monocyte cell lines such as THP-1s and U937s. Additionally, expression of human caspase-4 in mice has complemented the activation of caspase-1 and enhanced the release of IL-1β and IL-18 following stimulation with LPS (134). In a recent publication, it was shown that in response to gram-negative bacterial infection in macrophages, caspase-4 mediates the release of IL-1α and inflammasome activation but not IL-1β release (135). A publication in 2014 brought us closer to discovering if caspase-4 and -5 are functional orthologs of caspase-11. Shi et al. revealed that caspase-4, -5 and -11 were all capable of interacting with intracellular LPS (136). This study demonstrated that the lipid A moiety of LPS binds directly with high affinity to the CARD domain of caspase-4, -5 and -11, leading to oligomerisation and subsequent activation of the caspases (136). Additionally, recent studies have identified GSDMD as a biological substrate of caspase-4, 5 and 11, responsible for mediating pyroptosis. (29). These discoveries unearthed a new paradigm for inflammatory caspase activation and function. These finding also lead researchers away from the belief that PRRs are responsible for recognition alone and leads us to believe that some sensors do in fact have enzymatic capabilities.

1.2.5 Inflammasome-mediated diseases

While inflammasome activation is a key mechanism responsible for mediating the host innate response to protect against infection, dysregulation of inflammasome activation can incur in autoinflammatory diseases. Autoinflammatory syndromes are generally classed as those with chronically recurring fevers with various forms of systemic and localised inflammation. Now, we know that some of the most well-characterised
autoinflammatory syndromes occur as result of mutations in inflammasome-associated genes.

Mutations in *NLRP3* have been linked to a group of disorders collectively known as CAPS. Familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory disorder (NOMID) (also known as chronic infantile neurologic, cutaneous and articular (CINCA)) are collectively known as CAPS (137–139). All three sub-phenotypes occur as a result of a dominantly inherited gain-of-function mutation that results in systemic inflammation with blood neutrophilia and fever (138). Localised neutrophilic inflammation is also observed in various tissues, such as skin, muscles, joints and cerebrospinal fluid. Both *in vitro* and *in vivo* studies have suggested that CAPS mutations result in increased activation of NLRP3. Transfection of mutant constructs with CAPS-associated mutations resulted in increased activation of caspase-1, IL-1β secretion and cell death mediators (140, 141). Additionally, mononuclear cells from CAPS patients display consistently activated inflammasomes when compared to healthy controls after mild stimulation with stimuli such as minimal hypothermia or low doses of LPS (142, 143). Downstream markers of inflammation, such as IL-6 are also consistently elevated in patients with CAPS after mild cold infliction, however, IL-1β and IL-18 levels do not appear to be significantly increased (144). Interestingly, treatment with anti-IL-1 therapies results in significant improvement in CAPS symptoms, suggesting a causative role for IL-1β in mediating these disorders (144).

Another common autoinflammatory syndrome occurs as a result of mutations in Pyrin (*MEFV*) and results in a disorder known as Familial Mediterranean Fever (FMF). Patients with FMF display symptoms such as periods of fever, synovitis, skin rash or serositis. Original reports led to the belief that FMF-associated mutations in *MEFV* resulted in loss-of-function mutations, suggesting pyrin functions in an inhibitory manner under normal conditions. However, genetic ablation of FMF in mice did not recapitulate the typical FMF phenotype. Additionally, overexpression of pyrin in *vitro* led to the discovery that pyrin interacts with ASC resulting in subsequent caspase-1 activation and further release of IL-1β (145)(146). To identify the exact role of pyrin, a group genetically engineered mice to harbour a knock-in FMF-causing mutation (147). The genetically altered mice displayed FMF-like symptoms but also secreted high levels of IL-1β in an ASC-
dependent manner (147). As a result, it is now believed that FMF-associated mutations are in fact gain-of-function.

Mutations in NLRP12 have also been linked to an autoinflammatory disease known as familial cold inflammatory syndrome 2. Also known as CAPS-like, familial cold inflammatory syndrome is a rare autosomal dominant disease that is generally characterised by a recurring fever and urticaria. Studies of patients with mutations in NLRP12 have reported that mutations effect the ability of NLRP12 to negatively regulate NFκB (148). Additionally, mutations in NLRP1 have been linked to disorders such as Vitiligo, Addison’s disease, Type 1 diabetes and Autoimmune thyroid disease (149–151). However, in the case of NLRP1, it is believed that these effects are exerted at a protein level. Similar to patients with mutations in NLRP3, heterozygous NLRP1 polymorphisms display increased secretion of IL-1β from monocytes (151). NLRC4 is an additional NLR protein associated with rare inflammatory syndromes such as enterocolitis and macrophage-activation syndrome-like illness (152, 153).

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Table 1.1: List of Inflammasome-related disorders
1.3 Inflammation in Skin

1.3.1 The Skin

The skin is a multifunctional organ that plays a major role in defence as it forms a barrier against the external environment. The skin is subjected to physical, microbial and chemical assaults, such as sun, stress, smoking and physical trauma on an on-going basis. The skin has a surface area of approximately 1.8 m² and is therefore one of the largest organs in the body. The skin has both cellular and immunological functions. Besides acting as the main chemical and physical barrier, the skin has a variety of other cellular functions such as sensory and reception, maintaining hydration and allowing the synthesis of hormones and vitamins. The skin also actively defends internal organs by acting as an immuno-protective organ. In a similar manner to lung and gut immunity, the skin exploits an immune surveillance technique combining epithelial and immune cells together thus enabling the skin to mount a very effective immune response against invading PAMPs and DAMPs.

1.3.2 The Multifaceted Layers of the Skin Epithelium

The ability of the skin to carry out such a wide range of functions is due to its cleverly engineered structure. The skin is comprised of an outer epidermis, an inner dermis which are separated by a thin basement membrane and a subcutaneous layer comprised of adipose tissue. The stratum basale is the first layer of the epidermis located just above the basement membrane and is followed consecutively by the stratum spinosum, stratum granulosum and finally the stratum corneum (Figure 1.5). In healthy skin, the keratinocytes in the epidermis undergo proliferation and differentiation. The stratum corneum consists of dead keratinocytes, known as corneocytes. The skin is capable of forming a physical barrier as a result of a combination of the terminally-differentiated epidermal keratinocytes and also the acidic nature of the skin (due to the release of sweat, lipids, sebum, and a range of antimicrobial peptides). Any changes in the composition of lipids results in a disturbed skin barrier and is the cause of many immune-mediated skin diseases. The epidermis also consists of melanocytes and various immune cells such as T-lymphocytes and Langerhan cells (LCs). Additionally, it is host to Merkel cells (nerve-ending cells) that allows for light touch and the skin’s ability to discriminate between shapes and textures.
The dermis is comprised of an upper papillary dermis (stratum papillare) containing thin collagen fibres and a lower reticular dermis (stratum reticulare) which contains thick collagen fibres. The toughness of the collagen fibres forms the mechanical barrier and also provides a structural framework to hold the many blood vessels and immune cells present in the dermis. In comparison to the epidermis, the dermis plays host to a wider range of immune cells, such as dermal dendritic cells, macrophages, mast cells, natural killer (NK) cells, αβ T cells and γδ T cells. The dermis is also comprised of several appendages such as sweat and sebaceous glands and hair follicles.

Figure 1.5: Structure of Skin

The skin is comprised of three main layers, the innermost being the hypodermis that is comprised of adipose tissue. The dermis contains two main regions; the reticular and papillary regions. The dermis is host to veins, arteries, sweat glands, hair follicles, sebaceous glands and is largely composed of collagen fibres. The epidermis is the outermost layer and it is comprised of the stratum basale, stratum spinosum, stratum granulosum and stratum corneum.
For the purpose of research, scientists rely on the use of mice to gain a greater understanding of skin immunology and to tease out the mechanisms of many skin pathologies. There are, however, a few limitations worth noting, in the use of murine models in the study of skin diseases. The major difference between murine and human skin is the thick layer of fur that covers the mouse skin as opposed to the thinly dispersed hair in the human skin. Hair is an important aspect of skin immunity as it plays a role in forming a waterproof barrier, prevents the skin from drying out, and also protects the host from some fungal infections. Another structural difference is the thinner skin in mice and faster epidermal cell turnover rate, resulting in a faster recovery of wounds. Murine skin also consists of a thin layer of muscle, known as the panniculus carnosus, which allows the skin to contract and aid in a quicker healing process. In spite of these limitations, murine skin is widely used as a model to study skin inflammation, once these factors are taken into account.

1.3.3 Immunological Functions of the Skin

The skin was not believed to have immunological functions until 1978, when Streilein described the skin as “skin-associated lymphoid tissue” (SALT) as he observed the trafficking of immune cells between the lymphatic system, circulatory system and the skin (158). Now, much more is known about the crucial role that skin plays as a sentinel organ. This role is mediated by keratinocytes that recognise PAMPs and DAMPs, migratory Dendritic cells that initiate an immune response and then tissue-resident memory T (Trm) cells to recognise a re-emerging infection.

Keratinocytes are most likely the first immune cells that pathogens encounter when they invade the skin. Therefore, keratinocytes need to be fast acting in mounting an immune response to protect the host from invading pathogens. Keratinocytes express TLRs on the surface of the cell (TLR1, TLR2, TLR4, TLR5 and TLR6) or in endosomes (TLR3 and TLR9). Additionally, epidermal keratinocytes can express TLR7 following activation of TLR3 with double-stranded DNA (159). Epidermal keratinocytes recognise PAMPs via TLRs, in a similar manner to other immune cells previously described. Keratinocytes also form inflammasomes in response to PAMPs and DAMPs to induce an increased upregulation and activation of potent pro-inflammatory cytokines such as IL-1β, and IL-18 (160). Additionally, keratinocytes are known for their production of antimicrobial peptides (AMPs) in response to invading pathogens. Cathelicidin (LL-37), defensins and
S100 family proteins are examples of crucial AMPs produced by keratinocytes following invasion of pathogens. AMPs are responsible for forming holes in the cell walls of bacteria. Inflammatory cytokines such as IFN-γ, TNF-α, IL-1α and IL-1β cause the increased expression of genes that encode for AMPs. IL-22, IL-17A and IL-17F are also crucial inducers of AMPs (161). Overall, with the activation of keratinocytes, an increase in the production of pro-inflammatory cytokines and chemokines occurs, which results in the recruitment of neutrophils, macrophages and T-cells to the site of infection. This would suggest that keratinocytes are a critical component of the initial immune response to skin infection.

Plasmacytoid DCs (pDCs) are another example of a key cell type that play a role in maintenance of skin homeostasis. pDCs are not normally present in peripheral organs, but they migrate to the skin following tissue damage and viral infection (162). Once activated, pDCs produce extremely high levels of type I IFNs to promote wound healing or to limit viral damage and spread within the skin (163). The release of type I IFNs also induces maturation and activation of DCs which promotes a cell-mediated protected immunity (163).

Another subset of DCs playing an important role in skin immunology, are the epidermal LCs. These behave similarly to dendritic cells and extend into the stratum corneum. While LCs are in an immature state, they still act as sentinel cells to sample the invading micro-organisms (164). It was originally believed that once LCs recognise a PAMP, they migrate to the skin draining lymph node, where they present antigens to naive T-cells in the lymphatic system. However, this LC paradigm is now under question, as more recent murine studies have opened up the possibility that this antigen presenting role is in fact performed by dermal dendritic cells (165).

The main adaptive cell type that play a role in skin immunity are T-cells. It is believed that a normal healthy adult contains approximately $1 \times 10^6$ T-cells/cm$^2$ of normal skin which is calculated to be approximately $2 \times 10^{10}$ T-cells in the entire skin surface (166). These 20 billion T-cells present in the skin amount to almost twice the amount of T-cells present in circulation. The skin is believed to host tissue-resident memory (Trm) cells (167) that do not circulate and are present in the epidermis and dermis to act as a first line of defence. There are however, also additional effector T-cells that are directed to the skin due to a tissue-specific homing marker imprinted by the DCs. This homing marker
sends T-cells to the site where a particular antigen was initially detected. For example it has been shown that skin DCs metabolise vitamin D3 to 1,25(OH)2D3 and in doing so, imprints the T-cells to up-regulate the skin-homing signature, CCR10 (168).

1.3.4 Psoriasis – an autoimmune inflammatory disease

Psoriasis is characterised as a chronic inflammatory skin disorder that affects approximately 2% of the world population. Originally, psoriasis was believed to occur as a result of keratinisation of the skin. However, research has now shown that both innate and adaptive immune responses play an important role in the pathology of the disease.

The histological characteristics of psoriasis are observed as the dysregulation of keratinocyte proliferation which results in a marked epidermal hyperplasia, increased infiltration of immune cells and an increase in vascularisation. Psoriasis can arise due to a broad range of trigger factors which can be either an environmental cause (stress, skin trauma, chemicals, streptococcal pharyngitis) or a genetic factor (certain gene variants cause susceptibility to psoriasis). Susceptibility to psoriasis can occur in people with mutations in genes encoding proteins in the IL-23-Th17 axis, NFκB pathway, epidermal differentiation complex (EDC) or most commonly human leukocyte antigen-C (HLA-C) (169). It has long been questioned whether psoriasis is an immune or epidermal mediated disease, as psoriasis is characterised by its prominent epidermal changes but immunosuppressive drugs have proven to be highly effective in treating psoriasis. Similar to other autoimmune-mediated diseases, psoriasis is thought to be a T-cell driven disease, however, is becoming increasingly more obvious that dysregulation in the crosstalk between the innate and adaptive system is a likely culprit for the emergence of psoriasis.

Psoriasis can be initiated following exposure to certain environmental factors previously mentioned, in individuals that may have a predisposition to psoriasis. The initiation phase of psoriasis can begin in response to trauma to the skin (Koebner effect), medications, infection or topical biologics such as Imiquimod (TLR7 agonist). To date, the majority of research in the field of psoriasis has been focused on the maintenance stage, due to the difficulty of obtaining skin samples. However, Gilliet et al, have proposed a mechanistic model of the earlier stages of the disease (Figure 1.6). Trauma to the skin causes cell death, and also causes keratinocytes to begin producing the AMP, LL37. LL37 then forms a complex with DNA released from dying cells and it binds to the intracellular receptor TLR9 in plasmacytoid DCs (pDCs) (170). Once pDCs become activated in this manner,
they produce IFN-α and IFN-β. LL37 can also form complexes with free RNA and alternatively activate pDCs through TLR7 (171). These RNA/LL37 complexes can then activate TLR8 in myeloid DCs (172). Therefore, myeloid DCs are activated by both the production of type-I IFNs released from pDCs but also by LL37/RNA complexes through activation of TLR8. The activation of myeloid DCs drives the production of pro-inflammatory cytokines such as IL-12, IL-23 that result in the activation of T-cells. Release of IL-12 and IL-23 from myeloid DCs causes the activation and differentiation of T cells such as Th1, Th17 and Th22 (173). The production of IFN-γ, IL-22 and IL-17 from these T-cell subsets are responsible for the maintenance of the psoriasis phenotype. There are however, also populations of CD8+ T cells that produce similar cytokine profiles that are known as Tc1, Tc17 and Tc22. Cytokines produced by all subsets of T-cells in the skin act on keratinocytes, causing them to up-regulate expression of a wide range of inflammatory mediators. Keratinocytes produce products that can act in a positive feedback loop, continuing the constant activation of T-cells in the skin. Keratinocytes are also responsible for the release of chemokines to recruit leukocytes such as neutrophils and DCs to the site of inflammation.
Figure 1.6 Proposed mediators responsible for the initiation and maintenance of psoriasis

Initiation of psoriasis is mediated by a variety of trauma inducing ligands in the skin, such as medications, infections or chemicals. These triggers result in activation of keratinocytes and the subsequent release of anti-microbial peptides such as LL37. LL37 can form complexes with DNA/RNA to activate TLR7/9 on pDCs or RNA to activate TLR7/8 on mDCs. pDCs produce type I IFNs to further act on mDCs, inducing the production of IL-12 and IL-23. Once activated, dDCs and mDCs produce cytokines such as IL-23, IL-12 and IFN-γ to activate T17, Th1, Tc1, Th22, Tc22 cells, which act to further amplifying a feedback loop via keratinocyte activation. Modified from (174).
1.3.5 Imiquimod-induced Psoriasis Model in Mice

1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine is an imadazoquinoline derivative and is most commonly known as R837 or Imiquimod. Imiquimod was originally designed to induce a potent type I IFN response and was anticipated to be particularly successful for antiviral immunity (175). Due to its high hydrophobicity and small molecular weight, Imiquimod was a well suited candidate for use as a topical cream. Imiquimod was formulated as a 5% cream and trademarked by 3M Pharmaceuticals under the name Aldara™. Aldara™ was first licensed in 1997 to treat perianal and genital warts commonly caused by human papilloma virus or HSV (176). Aldara™ was later licensed for the treatment of actinic keratosis (177) and basal cell carcinomas (178). While it is not fully understood how exactly Imiquimod induces an anti-tumour response, Aldara™ applications in murine melanoma transplant studies have induced a tumour regression following pDC infiltration (179). When treating actinic keratosis or basal cell carcinoma, an unpredicted side effect of the application of Aldara™ was observed. In patients with previously controlled psoriasis symptoms, an exacerbated psoriasis phenotype occurred. Interestingly, Aldara™ induced psoriasis at Aldara™ treated sites but also at previously unaffected distant sites (180). The appearance of such a phenotype suggested a systemic response was occurring, and also opened up the possibility of the use of Aldara™ for further psoriasis research.

The study of psoriasis has been hindered until recent times, due to the limited availability of patient samples and the difficulty of animal models. One of the most successful animal models used was the xenotransplant model, which involved the transplantation of healthy skin from a psoriasis patient onto an immunodeficient mouse. Psoriasis would then develop at the graft site. While this model was successful and was used to determine important mechanisms of psoriasis, it was limited in that it was difficult to obtain patient samples and the expertise required to carry out the model. Therefore the discovery of the Imiquimod model was a welcome change. The Imiquimod model is a cheap, fast and effective model that works on a large range of murine strains. The model involves the application of 50 mg of Aldara™ cream to the shaved back or ears of a mouse every 24 hours for 4-5 days.

While this is a relatively simple model for such a complex chronic disease, it has been determined that the phenotype of the skin shows direct similarities with human psoriasis.
and the cytokine production. In particular, it has been shown that both the Imiquimod-
induced model of psoriasis in mice and human psoriasis both depend on the IL-17/IL-23
axis (181). Van der Fitz et al., have characterised the Imiquimod-induced model in mice
and have identified the many similarities between human psoriasis and Imiquimod-
induced murine psoriasis (181). Imiquimod induces an increased proliferation and
differentiation of the keratinocytes in the epidermis, resulting in a thickening of the
epidermis. In conjunction with this, T-cells, neutrophils and various APCs are recruited
to the skin, in a similar manner to human psoriasis. Imiquimod also results in increased
angiogenesis, another typical characteristic of psoriasis. Spleen enlargement was also
observed, with a particular increase in Th17 cells (181). Due to evident similarities
between human psoriasis and Imiquimod-induced psoriasis, it is now a widely accepted
model used to study psoriasis.

1.3.6 Wound Healing

The skin’s primary immunological role is the maintenance of homeostasis, which is
evident in the process of wound healing following tissue damage. A wound is defined by
the breakdown of protective function in the skin, the loss of continuity of epithelium,
with or without loss of underlying connective tissue (182). The three main steps of wound
repair are: inflammation, formation of new tissue and tissue remodelling. In a very short
space of time after tissue injury, an inflammatory response is mounted. The collagen that
is exposed after injury activates a coagulation cascade that leads to an initial platelet plug.
This platelet plug is sealed and solidified by a component known as fibrin and will
eventually result in the formation of a blood clot at the site of damage, preventing any
further blood loss. Within an hour of a wound being initiated, neutrophils will arrive at
the site of damage and initiate an immune response (183). After approximately two days,
the site of damage is overcome with monocyte-derived macrophages. In mice, it is
believed there are two sub-populations of monocytes that drive the wound healing
process (184). Ly6C\textsuperscript{lo}CCR2\textsuperscript{lo}CX3CR1\textsuperscript{hi} migrate to the site of injury and begin to produce
pro-inflammatory cytokines, and phagocytose wound debris to clear the site. Following
this, Ly6C\textsuperscript{lo}CCR2\textsuperscript{lo}CX3CR1\textsuperscript{hi} monocytes leave the circulatory system and begin to
produce Transforming-growth factor-\(\beta\) (TGF-\(\beta\)) and vascular endothelial growth factor
(VEGF). The human highly expressing CX3CR1 CD16\textsuperscript{+} cells are believed to be the
ortholog of Ly6C\textsuperscript{lo}CCR2\textsuperscript{lo}CX3CR1\textsuperscript{hi}, whereas the human CD16\textsuperscript{lo}CCR2\textsuperscript{lo}CX3CR1\textsuperscript{lo}
monocytes are believed to be similar to the murine Ly6C\textsuperscript{lo}CCR2\textsuperscript{lo}CX3CR1\textsuperscript{lo} monocytes
There have been supporting evidence to confirm the theory that macrophages are the main orchestrators in wound healing. Depletion of macrophages results in less angiogenesis and delayed re-epithelialisation which also results in a decreased production of VEGF and TGF-β. It is therefore very likely that macrophages orchestrate the wound closure and repair by regulating cytokine production in the site of the wound.

pDCs are a unique subset of DCs that are also known to enter the site of injury. pDCs are not normally present in the skin and are characterised by their increased production of type I IFNs as a result of activation of TLR7 and TLR9 by nucleic acids. pDCs are believed to migrate to the wound at the same time as neutrophils and become activated by ‘self’ nucleic acids that are released from damaged, dying cells. This then initiates the production of type I IFNs. pDCs are also known to produce IL-6 during the early inflammatory response which aids in re-epithelialisation and induces the production of other inflammatory cytokines such as IL-17A and IL-22.

T-cells that reside in the epidermis, also play an important role during wound healing. In mice, Vγ5Vδ1 dendritic epidermal T cells (DETCs) act as initial responders to damage in the skin by promoting tissue repair. Following injury, DETCs recognise an unknown antigen and begin promoting the up-regulation of pro-inflammatory cytokines, chemokines, and growth factors to induce growth and proliferation of keratinocytes. DETCs also recruit macrophages and αβ T cells to the skin by increasing the deposition of extracellular matrix components (ECM) (185). DETCs are not present in humans, however, it has been shown that αβ conventional and Vγ1 unconventional T cells in the epidermis are capable of producing insulin-like growth factor-1 (IGF-1) following injury to the skin and consequently promote wound healing. Moreover, it has also been shown that a specific skin homing population of T-cells known as Vγ9Vδ2 are capable of producing a range of inflammatory mediators, similar to those produced by DETCs in mice (186).
Project Hypothesis and Aims

The overall aim of this project is to investigate the role of inflammasome components in inflammatory mediated disorders such as autoinflammation and psoriasis. We hypothesise that caspase-11, a mediator of the non-canonical inflammasome, is responsible for mediating the pathogenesis of psoriasis in mice. We also hypothesise that a mutation in NLRP6 results in an autoinflammatory disease, which highlights a functional role for NLRP6 in the regulation of NFκB during inflammatory responses.

The key objectives of this project are:

1. To determine the role of caspase-11 in mediating Imiquimod-induced psoriasis.
2. To characterise the immunological impact of a novel mutation in \textit{NLRP6} in a patient with an autoinflammatory syndrome.
Chapter 2: Materials and Methods
2. Materials and Methods

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Methods

2.2 Cell Culture

Cell lines and primary cells were cultured in level II laminar flow cabinets and dealt with in an aseptic manner. Cells were monitored daily to ensure no obvious contamination or infections were detectable in the flasks. Cells were grown in either T75 or T175 size flasks. Cells were counted using a haemocytometer under 10X magnification.

2.2.1 Hek293T Cell Line

Human embryonic kidneys 293T cells are a derivative cell line of the human embryonic kidney 293 cell line that express the SV40 large T antigen. The SV40 large T antigen can bind to SV40 enhancers to increase expression of proteins. Therefore, HEK293T cells are an adherent cell line widely used for transfecting plasmids. HEK293T cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% Foetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S). Cells were maintained at 2x10⁵ cells/mL and subcultured when cells reached 80-90% confluency and never grown beyond 1x10⁶ cells/mL. Cells were subcultured by initially washing with approximately 10 mL of Phosphate Buffer Saline (PBS) and then adding trypsin for 2-5 mins or until cells had detached sufficiently from the plate. The trypsin was deactivated by adding 3 times as much media and cells were centrifuged at 400 g for 5 mins.

2.2.2 THP-1 Cell Line

THP-1 cells are a human suspension monocyctic cell line derived from a 1 year old male infant with acute monocytic leukemia. THP-1s were cultured in Roswell Park Memorial Institution (RPMI) supplemented with 10% FBS and sub cultured every two to three days or when the cells reached 8 x 10⁵ cells/mL. Cells were sub-cultured by centrifugation at 400 g and resuspension of cells in fresh complete RPMI at 2 – 4 x10⁵ cells/mL. Cells were seeded at 1 x 10⁶ cells/mL prior to stimulation.

2.2.3 Bone Marrow Derived Macrophages

Murine bone marrow derived macrophages were extracted from the femurs and tibiae of C57BL/6J wild type and caspase-11 knockout mice. Mice were housed in a sterile pathogen free environment in Trinity College Dublin and euthanised by inhalation of CO₂, followed by cervical dislocation. Following euthanasia, mice were sprayed with
70% ethanol, dissected and leg bones were removed for further cell isolation. In a tissue culture hood, cleaned femurs were flushed to extrude bone marrow using sterile 25G needles and a syringe filled with PBS. Bone marrow cells were centrifuged at 400g for 5 min. The supernatant was discarded and cells were resuspended in 2 mL of red blood cell lysis buffer for 2 mins. 8 mL of DMEM was then added to neutralise the lysis buffer and centrifuged at 200 g for 5 mins. If red blood cells remained in the pellet, this step was repeated. Once isolated, cells were allowed to differentiate into macrophages for 8 days with the addition of Macrophage Colony Stimulating Factor (M-CSF). M-CSF was obtained from L929 cells, a cell line that secretes M-CSF into its supernatant and is then filter sterilised and added to BMDM cultures at 20% of total culture media volume. Cells were cultured for 8 days, with a change in media after 3 days.

2.2.4 Bone Marrow Derived Dendritic Cells

Murine bone marrow derived cells were isolated from the femurs and tibiae of C57BL/6J wild type and caspase-11 knockout mice as described in section 2.2.3. Cells were differentiated to dendritic cells by seeding in a T1 flask of 6.25 x 10^5 cells/mL in RPMI, medium supplemented with 20 ng/mL of recombinant Granulocyte-macrophage colony stimulating factor (GM-CSF) in a total of 30 mL per flask. After three days in culture, a further 30 mL of RPMI with 20 ng/mL GM-CSF was added to the flask. On day 6, the media was gently aspirated from the flask and replaced with 30 mL of fresh RPMI with 20 ng/mL GM-CSF. A further 30 mL of RPMI with 20 ng/mL GM-CSF was added to the flask of day 7. On day 10, loosely adherent cells were removed from the flask by gentle aspiration of the media and cells were subsequently centrifuged at 200 g for 5 min. Cells were then counted and seeded at 6.25 x 10^5 cells/mL. 24 hours later cells were stimulated with 1 μg/mL LPS and transfected with either 1 μg/mL Imiquimod or 1 μg/mL Aldara cream.

2.2.5 L929 Cell Line

L929 cells are a mouse subcutaneous connective tissue cell line derived from a 100 day old male C3H/An mouse. L929 cells constitutively secrete M-CSF and to a lesser extent, GM-CSF, which can augment the effects of M-CSF on monocytes and macrophages (187, 188). Supernatants from this cell line are therefore widely used to culture BMDMs. Cells were cultured in DMEM, 10% FBS and 1% Pen-Strep. L929 cells were seeded at 20 x 10^6 cells in a T175 flask containing 40 mL medium. Cells were grown in a
humidified incubator with 5% CO$_2$ at 37°C for 10 days. Then supernatants were filtered through a 0.45-µm filter and stored in 6 mL aliquots at -20°C for use as L929 conditioned media.

2.2.6 Cryopreservation of Cells

Cells were frozen down for long term storage and stored in liquid nitrogen with a cryoprotective agent, Dimethylsulfoxide (DMSO). DMSO is a widely used cryoprotectant as it applies a slower cooling rate by reducing the freezing point of the media, thereby preventing ice crystal formations which would damage the cells. Freezing medium was prepared with 40% culture medium, 50% FBS and 10% DMSO. Cell pellets were resuspended in 1 mL (1 x 10$^6$ cells) freezing medium and transferred to cryovials, before they were directly transferred to a cell freezing container (ThermoScientific) and stored at -80°C for 24 h. Cryovials, within the freezing container, achieve a rate of cooling very close to -1°C/min, the optimal rate for cell preservation. Cryovials were subsequently transferred to liquid nitrogen for long term storage.

2.2.7 Thawing of Cells

Cryovials were removed from liquid nitrogen and thawed in a water bath at 37°C until a min pellet of ice remained (approximately 2 mins). The cells were then immediately transferred into warm media and centrifuged for 5 mins at 300 g. The pelleted cells were then resuspended in 10 mL culture medium in a T25 flask and grown at 37°C until confluent.

2.3 Human Studies

2.3.1 Ethics Approval

All ethics were sought for and granted by Our Ladies Children’s Hospital Crumlin Research Ethics Committee.

2.3.2 Sequencing of human genomic DNA

PCR is an in vitro molecular technique that amplifies a particular region of DNA. The PCR technique relies on the DNA polymerase, Taq Polymerase and a set of forward and reverse primers designed to target specific regions in a DNA sequence. Taq Polymerase synthesises new DNA strands complementary to the original DNA template. Taq Polymerase can only add nucleotides to previously existing 3’-OH groups. Therefore, a
primer typically of 18-22 bps is required to bind to the template DNA in a complementary manner and allow the Taq polymerase to catalyse the addition of nucleotides.

Prior to the PCR amplification of human DNA, blood was obtained by a trained phlebotomist from consenting donors participating in the study. Genomic DNA was isolated from the blood using NucleoSpin® Blood kit (Macherey-Nagel) and the purified DNA was quantified using a nanodrop (ND-1000) spectrophotometer. Regions of the **ADA2**, **C6**, **NLRP6** and **SH3BP2** genes were amplified using primers listed in table 2.1. Amplification of the genomic DNA was performed in a thermocycler using ExTaq polymerase under the conditions listed in Table 2.2 and 2.3. Amplified DNA was then purified and quantified before sending to Eurofins Genetic Services for sequencing using the primers listed in table 2.1.

Table 2.1 Primers used to amplify genomic DNA and for Sequencing

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<td>5’ GTACCAACAGGCACATTGCG 3’</td>
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<tr>
<td>C6</td>
<td>5’ ACTTGGTTGGAGAGATTTGCTG 3’</td>
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<td>NLRP6</td>
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Table 2.2: PCR Components for amplification of human genomic genes

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Table 2.3 PCR conditions for amplification of human genes

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<td>98</td>
<td>10 seconds (35)</td>
</tr>
<tr>
<td>55</td>
<td>30 seconds (35)</td>
</tr>
<tr>
<td>72</td>
<td>1 min (35)</td>
</tr>
<tr>
<td>72</td>
<td>10 mins (1)</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

2.3.3 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Blood was collected by a phlebotomist and stored in EDTA coated 13 mL tubes. Blood was collected from the patient and three healthy age and gender matched controls at three month intervals over the course of one year. The healthy and gender matched controls varied with each phlebotomy. Multiple tubes of blood from each individual donor were then combined and centrifuged for 7 min at 300 g. 7 mL of the top plasma layer was then removed for later use and the blood was diluted with PBS 1:1. In a separate tube, approximately 15 mL of lymphoprep was added and the blood/PBS mix was gently pipetted drop by drop onto the lymphoprep layer, ensuring not to disturb it. This mix was then centrifuged at 400 g for 25 min at RT with the brake off. The thin, middle layer of white blood cells was then gently pipetted out and transferred to a new tube. Approximately 20 mL of RPMI was added to the white blood cells and the tube was then centrifuged at 650 g for 10 mins with the brake on. Supernatant was then removed, the pellet was resuspended in 15 mL RPMI, and the tube was centrifuged again at 650 g for 10 mins with the brake on. Once complete, the supernatant was removed and cells were resuspended in 10 mL RPMI and counted using a haemocytometer. Cells were seeded in 6-well, 12-well and 96-well plates for further stimulation.

2.3.4 PBMC Simulations

LPS Stimulation

PBMCs were seeded at 1 x 10⁶ cells/mL in a 6-well plate with 1 mL per well. 24 hours later, cells were treated with 1 μg/mL LPS for 0, 15, 30, 60, 120 and 240 mins. Cells were lysed in 100 μl RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 0.5%
(w/v) sodium deoxycholate and 1% (v/v) NP-40) supplemented with protease inhibitors (cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail).

**Inflammasome Stimulations**

PBMCs were seeded at 1 x 10^6 cells/mL in a 24-well plate with 0.5 mL per well. 24 hours later, cells were treated with 1 μg/mL LPS for 12 hours and stimulated with either 5 mM ATP or 3.4 μM Nigericin for the final 30 mins. Cells were then lysed as before.

PBMCs were seeded at 1 x 10^6 cells/mL in a 24-well plate with 0.5 mL per well. 24 hours later, cells were treated with 1 μg/mL LPS for 4 hours and then transfected with either 1μg/mL Poly(dA:dT) or 1 μg/mL (Poly(I:C) for a further 8 hours. 1 μL Lipofectamine reagent in 100 μL Opti-MEM was used for transfection of each well of PBMCs. The transfection technique is discussed further in section 2.10.1. Cells were then lysed as before.

**Heat-killed Bacteria Stimulations**

PBMCs were seeded at 1 x 10^6 cells/mL in a 24-well plate with 0.5 mL per well. 24 hours later, cells were treated with 1x10^6, 2x10^6 or 1x10^7 cells/mL of either heat-killed *Lactobacillus rhamnosus* or heat-killed *Salmonella typhimurium* for 24 hours. Cells were then lysed as before.

**2.4 Animal Studies**

*Casp-11*−/− mice were generated on a C57BL/6J background and obtained from the laboratory of Junying Yuan (Harvard Medical School, MA, US). The active site of caspase-11, QACRG is located within exon 5 of *CASP11* and was disrupted by a replacement type vector by homologous recombination. A neo gene encoding neomycin phosphotransferase was inserted in place of a 1.5 kb fragment of *CASP11*, resulting in a deletion of 16 amino acids from the coding region of caspase-11. This substitution deleted the active site which prevents caspase-11 from forming a functional protein. The constructed vector was subsequently transfected into and clonally expanded in J1 embryonic stem cells. C57BL/6J blastocytes were then injected with Mutant *CASP11* ES cell clones and implanted into falsely pregnant females. Chimeric male progeny were then mated with C57BL/6J female mice to obtain a germline transmission of the mutant alleles.
Mice were shipped to Trinity College Dublin and *Casp-11*−/− mice were then re-crossed with C57BL/6J mice purchased from Harlan Laboratories (UK). Both heterozygous and homozygous breeding pairs were established to maintain homozygous *Casp-11*−/− and littermate WT controls. Mice are now maintained in a specific pathogen free facility in Trinity Biomedical Sciences Institute. All animal procedures were reviewed and approved by the Trinity College Dublin Animal Ethics Committee and carried out under Health Products Regulatory Authority (HPRA).

### 2.4.1 DNA Extraction from ear punch of Caspase-11 KO and WT mice

Genomic DNA was isolated from ear punches of both caspase-11 KO and WT mice. Each ear tag was dissolved in 500 µL DNA extraction buffer consisting of 100 mM Tris, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 500 µg/mL Proteinase K and incubated at 55°C overnight. Digested ear punches were then centrifuged at 20,000 g for 10 mins. 450 µL of the supernatant were transferred to 950 µL ice cold Ethanol. Samples were then vortexed to precipitate DNA and then centrifuged at 20,000 g for 10 mins at 4°C. Supernatants were removed and pelleted DNA was left to air dry before being resuspended in 35 µL nuclease free water. DNA was then frozen at -20°C until further use.

### 2.4.2 DNA Quantification

The concentration of each DNA sample was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Nuclease free water was used as a blank. 1-2 µL of DNA sample was added to the lower pedestal of the NanoDrop and DNA the NanoDrop calculated the concentration of DNA in the sample at 260 nm. The NanoDrop calculates the ratio of sample absorbance at 260:280 nm to measure the purity of the DNA samples. A 260:280 ratio of 2.0 is an acceptable pure ratio for further analysis. A lower ratio may suggest contamination of phenols, proteins or other contaminations.

### 2.4.3 PCR Amplification of murine genomic DNA for genotyping.

Genomic DNA isolated and quantified from the caspase-11 WT and KO mice are amplified using the PCR technique. WT primers were designed to amplify a specific region of the WT caspase-11 gene and KO primers were designed to amplify a region of the truncated cassette inserted to disrupt the caspase-11 gene. The primer sequences are
listed below in Table 2.4. The reaction was set up with GoTaq Hot Start Green Master mix which is ready-to-use mix of GoTaq polymerase, dNTPs (400 µM dATP, 400 µM dGTP, 400 µM dTTP, 400 µM dCTP) and 4mM MgCl₂. Volumes of each component are listed in Table 2.5 The entire PCR cycle consisted of 1 cycle at 94 °C for 3 mins which denatures the template DNA strands and disrupts the hydrogen bonds and base stacking interactions between complementary strands. This step is then followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 mins. This results in the annealing of primers and exponential extension of new DNA strands. The final stage of the PCR reaction is 72 °C for 10 mins to allow for the completion of any partially amplified strands and clearance of any replication machinery from the DNA (Table 2.6).

Table 2.4: PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SY21 (WT forward)</td>
<td>5’ GGCATGGAGTCAGAGATGAAAGAC 3’</td>
</tr>
<tr>
<td>SY22 (WT reverse)</td>
<td>5’ GCCCATGTGGCATTACCTGCCAGC 3’</td>
</tr>
<tr>
<td>SYKO (Mutant forward)</td>
<td>5’ AGATCTACACCTCTGCACAACTGG 3’</td>
</tr>
<tr>
<td>PJK ( Mutant reverse)</td>
<td>5’ TGGCGCTACCGGGTGGATGTGGAT 3’</td>
</tr>
</tbody>
</table>

Table 2.5: PCR Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA (100 ng)</td>
<td>2</td>
</tr>
<tr>
<td>GoTaq Hot Start Master Mix (2X)</td>
<td>10</td>
</tr>
<tr>
<td>Forward primer</td>
<td>0.4</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.4</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>7.2</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Table 2.6: PCR Reaction Conditions

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (Cycles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>3 mins (1)</td>
</tr>
<tr>
<td>94</td>
<td>1 min (35)</td>
</tr>
<tr>
<td>55</td>
<td>1 min (35)</td>
</tr>
<tr>
<td>72</td>
<td>1 min (35)</td>
</tr>
</tbody>
</table>
2.4.4 DNA Agarose Gel

2% agarose gels were prepared in 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and heated until dissolved. Once dissolved, 1 in 10,000 dilution of Sybr Safe DNA stain was added to the liquid agarose mix. The agarose was allowed to cool until cool enough to touch and subsequently poured into the cassette and allowed to set out of direct sunlight. Once set, Horizon 11.14 Gel Electrophoresis Apparatus rig was used to separate PCR products and Quick-Load 2-Log DNA ladder (0.1 kb – 10 kb). 20 µL of each PCR product and 7 µL of DNA ladder was loaded to gel and run at 120 V for 40 mins. DNA bands were then visualised under UV light using a GelDoc-it System (UVP, LLC, CA, USA).

2.4.5 Imiquimod-induced Psoriasis Model

Imiquimod-induced psoriasis was induced in both caspase-11 WT and KO mice. The Aldara treated group contained 5 mice and the control group contained 2 mice for each time analysed. 24 hours before trial start day, the back of all animals were shaved using an electric shaver and Telogen hair growth phase was confirmed. On day 0 of trial, animals were anaesthetised in a mouse hut using 4% isoflurane and 2 L/min oxygen. Once asleep, they were transferred to a face mask and 1.5% isoflurane and 2 L/min oxygen was used to maintain the sleep. 50 mg of Aldara cream was applied to the back of all treatment mice. Vasoline was applied to the back of all control mice. This process was repeated for a further 4 days. Mice were weighed and scored daily for disease severity. Erythema, scaling, and thickening were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. If at any point a mouse reached 20% or more weight loss, it would be humanely sacrificed immediately. Following four consecutive days of Aldara application, the experimental mice were sacrificed and skin backs sections were dissected. Small back skin sections for protein analysis were snap frozen in liquid nitrogen and later stored at -80 °C. A skin section was placed in a histology cassette and fixed in 10 % (v/v) Formalin. A skin section was also dissected and placed in 500 µl DMEM in a 24-well plate and cultured for 24 hours at 37 °C and 5% CO₂. Skin sections were then weighed for normalisation in further experiments (eg. ELISA, LDH assay).
2.4.6 Wound Healing Model

The wound healing model was used in both caspase-11 WT and KO mice. 24 hours prior to start date of trial, the hair on the back of the experimental mice were shaved using an electric razor. Telogen hair growth phase was then confirmed (pink skin with no evidence of hair in the hair shaft). On day 0 of the trial, the mice were anaesthetised with isoflurane, as described in section 2.4.5. The back of each mouse was wiped with an ethanol soaked cotton pad to sterilise. 2 x 3 mm punches were incised into the back of each mouse and each mouse was photographed. This process was repeated for a further 10 days and weight was monitored daily. If at any point a mouse reached 20% weight loss, it would be humanely sacrificed. After 10 days of observation of the wound closure, animals were sacrificed humanely. Each wound was removed from the back of the mouse and cut in two sections, resulting in 4 half wound sections. One section was stored in a histology cassette and fixed in 10% (v/v) Formalin and transferred to 70% ethanol after 24 – 48 hours. Another section was cultured in 500 µL DMEM for 24 hours, and the wound was then removed from the supernatants and weighed for normalisation of results. The final two sections were snap frozen in liquid nitrogen and stored at -80°C until further protein analysis.
2.4.7 Histology

2.4.7.1 Tissue Processing

As mentioned previously, skin samples were fixed for approximately 48 hours in 10% (v/v) Formalin, and then transferred to 70% ethanol for long term storage. Skin samples were then processed using the TP1020 Automatic Tissue Processor (Leica, Microsystems, Ireland). The tissue processor is an automated machine that subjects the tissue to the conditions listed in Table 2.7. The entire tissue processing listed results in dehydrated, clarified and paraffin infiltrated skin samples.

Once samples were processed, each skin sample was embedded individually in paraffin using Leica EG1150H Heated Paraffin Embedding Module (Leica, Microsystems, Ireland). Paraffin blocks were left to cool and stored at -20°C overnight. When completely set, 5 µm sections of skin was cut using the Leica RM2235 manual Rotary Microtome (Leica, Microsystems, Ireland). Sectioned skin was then placed in a water bath at 55°C and mounted on glass slides. Once mounted, sections were left at 55°C overnight to dry.

Table 2.7: Tissue Processing Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% Ethanol</td>
<td>1</td>
</tr>
<tr>
<td>80% Ethanol</td>
<td>1</td>
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<tr>
<td>95% Ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1</td>
</tr>
<tr>
<td>100% Ethanol</td>
<td>1</td>
</tr>
<tr>
<td>50% Ethanol/ 50% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% xylene</td>
<td>1</td>
</tr>
<tr>
<td>100% paraffin</td>
<td>1</td>
</tr>
<tr>
<td>100% paraffin</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>
2.4.7.2 Hematoxylin and Eosin (H&E) Staining

Standard H&E histological staining is a widely used staining technique to identify the nucleus and cytoplasm of connective tissue. Eosin is a negatively charged acidic dye that stains basic structure red/pink, therefore staining the cytoplasm a light pink colour. Haematoxylin acts as a basic dye that stains acidic structures a purple/blue colour. Haemotoxylin is composed of the dye called hematein and aluminium ions. The aluminium ions act as mordant and binds to the tissue and the hematein dye then binds to the aluminium ions. The complete staining process results in the cytoplasm being stained pink and the nucleus stained purple. The staining process was carried out as follows:

Rehydration:
- 10 immersions in xylene
- 10 immersions in xylene
- 10 immersions in 100% ethanol
- 10 immersions in 90% ethanol
- 10 immersions in 70% ethanol

Staining:
- 10 immersions in tap water
- 5 mins in Sellafields hemmatoxylin, occasionally dipping
- 30/40 immersions in running tap water
- 2 mins in Eosin
- 30/40 immersions in running tap water

Dehydration:
- 10 immersions in 70% ethanol
- 10 immersions in 90% ethanol
- 10 immersions in 100% ethanol
- Place entire rack of slides into xylene
- Remove slides one at a time. Using 2 drops of Surgipath sub-X mounting media (Leica, Microsystems, Ireland), mount cover slips on the slides.
2.4.7.3 Toluidine Blue Staining of Mast cells

Toluidine is a basic metachromatic thiazine dye that has high affinity for acidic components. Toluidine stains polysaccharides purple and nucleic acids blue. Mast cells are mainly found in connective tissue and consist of large metachromatic granules that are stained a purple/blue colour by toluidine blue. The dehydration process is the same as listed for H&E staining except instead of staining with H&E, the slides are stained with Toluidine blue (1% Toluidine blue in EtOH, 1% NaCl pH 2) for 3 mins and washed as in H&E staining listed previously. Every step thereafter is the same as described in section 2.4.7.2.

2.4.7.4 H&E and Mast Cell Histological Analysis

H&E and Toluidine blue slides of skin sections were viewed using the Olympus BX51 research microscope (Olympus Inc., Tokyo, Japan). Disease severity was assessed by the increased epidermal thickness. Epidermal thickness was measured (nm) using Image J software. Mast cell numbers were counted per field in the dermis. Average values were taken from three pieces of skin section per mouse. Three images were analysed per section. Scores were graphed using GraphPad Prism.

2.4.7.5 Immunofluorescence

Slides with 5 μm slices of skin tissue were rehydrated as listed in section 2.4.7.2. Once rehydrated, slides were boiled for 20 mins in Tris-EDTA (10mM Tris Base, 1mM EDTA pH 9.0) antigen retrieval buffer and allowed to cool overnight. Slides were washed in PBS for 5 min at RT. Tissue was then blocked for 1h rocking at RT in 20% FBS in PBS. Tissue was then probed with primary antibody for 2h (anti-PCNA used at 1 in 50 dilution; CD11b at1 in 200 dilution; or Langerin at 1 in 100 dilution; in blocking buffer). Tissue was then washed three times with PBS and secondary anti-rabbit alexaflour 488 was added to the tissue at a 1 in 500 dilution in blocking buffer for 1h at RT. Tissue was then washed with PBS three times and mounted using Prolong-mounting media with 10 μg/mL DAPI. Slides were subsequently viewed using Olympus BX51 research microscope (Olympus Inc., Tokyo, Japan). PCNA positive cells were counted per field in the epidermis in three images per mouse. Cd11b and Langerin positive cells were counted per field in the dermis in three images per mouse taken at 10X magnification.
2.4.8 Flow Cytometry

Flow cytometry is a widely used technique to analyse the expression of cell surface markers. Following two days of Aldara application, the mice were sacrificed and inguinal nodes were dissected from the hind legs of each mouse and stored in RPMI until digestion. Inguinal nodes were chopped with scissors and forceps and digested in digestion buffer (20 mg/mL Collagenase 1A and 100 μg/mL DNAse I in RPMI with no FBS) for 15 mins at 37 °C, 5% CO₂. Collagenase and DNAse was then inhibited by the addition of PBS and 5mM EDTA. Cells were then strained using a 70 μm cell strainer and washed with complete RPMI twice by centrifuging at 400 g for 5 mins. 1 mL of fresh RPMI was then added to resuspend the cells and the live cells were counted using Tryphan blue. Cells were normalised to contain 1 x 10⁶ cells in each sample.

Cells were spun at 400 g for 5 mins and resuspended in 1 mL PBS. Live/dead cells were stained at a 1 in 600 dilution in 100 μl PBS. Stained cells were incubated at RT in the dark for 20 mins. Cells were then stained for CD45 (FITC), Ly6C (APCγ7), F4/80 (BV421), CD3/B220 (PE), γδ (BV510), CD11c (BV605), Ly6G (BV650), MHC-II (BV711), CD103 (PE), CD24 (PE-Cy5) and Cd11b (PE-Cy7) with FCγ block. Stained cells were incubated at RT for 30 mins. 1 mL of PBS was added and cells were centrifuged at 400 g for 5 mins then washed twice with 1 mL PBS. Stained cells were then resuspended in 200 μL PBS and stored at 4 °C overnight.

2.4.8 Cell Death Assay (Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labelling (TUNEL) Staining)

During apoptosis, genomic DNA is cleaved, yielding double-stranded low molecular weight DNA-fragments or single strand breaks in high molecular weight DNA. TUNEL staining uses the enzyme terminal deoxynucleotidyl transferase to incorporate labelled dUTP into the free 3’OH termini that are generated during apoptosis. Cell death in caspase-11 WT and KO skin sections was analysed by fluorescence microscopy using an In situ Cell Death Detection Kit Fluorescein (Roche), using a modified version of the manufacturer’s protocol. In brief, skin sections were subjected to xylene for 2 x 1 min intervals and then rehydrated in decreasing concentration of ethanol (100%, 90%, 70%) for 5 mins each. Sections were then placed in Tris-EDTA buffer for antigen retrieval (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0), boiled for 20 min and left to cool on the bench overnight. Skin sections were then washed in PBS and
incubated in blocking buffer (0.1 M Tris-HCl pH 7.5, containing 3% BSA and 20% FBS) for 30 mins at RT. A positive control was then prepared by incubating one slide of both caspase-11 WT and caspase-11 KO in 200 U/mL DNAse I (Roche Diagnostics) which was diluted in a 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA buffer for 30 mins at RT. All slides were then washed for 3 x 5 min intervals in PBS. The TUNEL reaction mixture was prepared by adding the enzyme and label mix at a 1:9 dilution and 50 µL was added to each slide, including positive control and excluding negative control. The negative control received 50 µL of label mix only. Slides were incubated at 37 °C for 60 mins in the dark and then washed 3 times with PBS for 5 mins each time. Stained sections were then mounted using fluorescence mounting media with 10 µg/mL DAPI. Images were then taken using the Olympus BX51 Research Microscope (Olympus Inc., Tokyo, Japan).

2.4.9 Bone Marrow Derived Macrophage Scratch Assay

BMDMs were counted and seeded 2 x 10⁵ cells/mL in a 24-well plate. 24 hours later, a scratch through the monolayer was created using a sterile p200 tip applied horizontally across each well. Cells were then stimulated with either 1 µg/mL LPS, 20 ng/mL IFN-γ, 20 ng/mL IL-1β, 5 ng/mL IL-17A or skin conditioned media and cultured for a further 1-3 days. Wounds were photographed daily on Olympus IX81 inverted microscope. Migrated cells were counted each day and scores were graphed using GraphPad Prism.

2.5 Measurement of Cytokine Concentration by ELISA

2.5.1 Explant Culture of Skin samples

As mentioned previously, skin explant cultures from Aldara treated mice were set up on the final day of each trial. A small section of skin (approximately 1.5 cm²) was dissected from the shaved back of each mouse. Skin section was briefly placed in ethanol to sterilise skin and then PBS to wash off ethanol before being placed in 500 µL DMEM in a 24 well plate. The skin explant culture was cultured for 24 h in 5% CO₂ at 37 °C. Supernatants were then removed and stored at -20 °C until further use. Each skin section was weighed to normalise future results.

Alternatively, skin was dissected from healthy mice, cut in three 1 cm² pieces and weighed to ensure uniform weight. Each skin section was then washed three times with PBS and placed in 1.5 mL of DMEM in a well in a 12-well plate. Skin was then stimulated with either 17 mg Aldara or 20 µg Imiquimod and cultured for 24 h at 37 °C. Conditioned
media from skin was sterile filtered with a 0.45 μm filter and applied to BMDMs seeded at 0.5 x 10^6 cells/mL in 500 μL/well of a 24 well plate. Conditioned media was applied to cells at a 50:50 ratio with fresh DMEM and then cultured for 24 h. Supernatants and lysates were retained and used for further analysis.

2.5.2 ELISA Assay

Cytokine expression levels were determined by analysing the supernatants of skin explant cultures or the supernatant of cell line experiments where displayed. Commercially available ELISA kits were used according to manufacturer’s instructions and are listed in Tables 2.8-10. High-binding ELISA plates were coated with 50 μL per well of capture antibody and stored at 4 °C overnight. ELISA plates were washed x4 with PBS + 0.05% Tween-20. Non-specific binding was prevented by blocking the plate with 200 μL Assay diluent (1% BSA in PBS). Plates were washed again as previously described. 50 μL standards were then added to the plate with a two-fold concentration dilution between each standard. Concentrations varied per ELISA kit and standards were diluted in Assay diluent. 50 μL of samples were either added undiluted or also diluted in Assay diluent to each corresponding well. All samples and standards were analysed in duplicate. ELISA plates were then incubated overnight at 4 °C. Plates were washed again the next day before the addition of 50 μL per well of detection antibody, diluted in Assay diluent. In general, the ELISA plate was incubated for 1h at RT or according to manufacturer’s instructions. Plates were washed as before and incubated with 50 μL HRP-conjugated Streptavidin diluted in Assay diluent for 30 min at RT away from direct sunlight. Plates were subsequently washed with PBS x5 for 3 min each time, before 50 μL of substrate solution was added to each well and incubated for 15-30 mins (depending on kit) in the dark at RT. Once a sufficient colour developed, 50 μL stop solution (1 M H₂SO₄) was added to each well and OD values were determined by measuring absorbance at 450 nm using Spectramax Microplate Reader (Molecular Devices). Concentrations of cytokines were determined using the standard curve from each ELISA plate.
Table 2.8: Commercial Murine ELISA Kits

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier</th>
<th>Top working standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Biolegend/MSC</td>
<td>2000 pg/mL</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Biolegend/MSC</td>
<td>125 ng/mL</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Biolegend/MSC</td>
<td>2000 ng/mL</td>
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<tr>
<td>IL-6</td>
<td>Biolegend/MSC</td>
<td>500 pg/mL</td>
</tr>
<tr>
<td>IL-10</td>
<td>Biolegend/MSC</td>
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Table 2.9: Non Kit Murine ELISA - IL-18

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<tr>
<th>Step</th>
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<th>Supplier</th>
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<tbody>
<tr>
<td>Capture</td>
<td>Monoclonal rat anti-mouse IL-18</td>
<td>MBL</td>
</tr>
<tr>
<td>Detection</td>
<td>Monoclonal rat anti-mouse IL-18</td>
<td>MBL</td>
</tr>
<tr>
<td>Avidin-HRP</td>
<td>Avidin-HRP</td>
<td>Biolegend/MSC</td>
</tr>
<tr>
<td>Standard</td>
<td>Recombinant mouse IL-18</td>
<td>MBL</td>
</tr>
</tbody>
</table>

Table 2.10: Commercial human ELISA kits

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier</th>
<th>Top working standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>Biolegend/MSC</td>
<td>125 pg/mL</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Biolegend/MSC</td>
<td>250 pg/mL</td>
</tr>
<tr>
<td>IL-18</td>
<td>R&amp;D Biosystems</td>
<td>750 pg/mL</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Biolegend/MSC</td>
<td>500 pg/mL</td>
</tr>
<tr>
<td>IL-6</td>
<td>Biolegend/MSC</td>
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</tr>
<tr>
<td>IFN-γ</td>
<td>Biolegend/MSC</td>
<td>500 pg/mL</td>
</tr>
</tbody>
</table>

2.6 Western Blots

2.6.1 Protein Extraction from Skin Extracts

Skin extracts from Imiquimod-induced psoriasis trial or wound healing trial that were previously stored at -80 °C were removed and thawed on dry ice. A marble pestle and mortar was placed on dry ice and liquid nitrogen was poured in the mortar to fully chill. Skin sections were then placed in the chilled mortar and a small cup of liquid nitrogen was poured on top. As the liquid nitrogen evaporated, the pestle was used to grind the
skin to powder. Once skin was in complete powder form, it was scraped into an eppendorf and 200 µL RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 1% (v/v) NP-40) supplemented with protease inhibitors (cOmplete™ ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail) was added to each eppendorf. Lysates were then stored at -80 °C until further use.

2.6.2 BCA Assay

The BCA assay is used to determine protein concentrations in cell or tissue lysates and allows for the normalisation of all samples before further analysis (Western blot). The principles of the assay are based on two steps; initially, bicinechonic acid (BCA) detects cuprous cations (Cu¹⁺). Copper is then chelated with protein in an alkaline condition which results in the formation of a light blue complex. This is known as the biuret reaction and occurs when peptides containing three or more amino acid residues form a coloured complex with the cupric ions. BCA then reacts with the cuprous cations formed initially, which results in the chelation of two molecules of BCA with one cuprous ion, releasing an intense purple colour. The BCA/copper complex is water soluble and is absorbed at 562 nm at increasing protein concentrations. The BCA reagent is 100 times more sensitive than using the first step of the detection alone.

A top standard of 320 µg/mL of bovine serum albumin (BSA) was used and a two-fold serial dilution was then performed to create a standard curve. 50 µL of each standard and sample was added to corresponding wells in a 96-well plate. Samples were either diluted or tested neat. 50 µL of BCA working solution (25 parts Micro BCA Reagent MA, 24 parts Micro BCA Reagent MB and 1 part Micro BCA Reagent MC) was added to each well. The plate was then incubated at 37 °C for 30 mins in the dark. The absorbance was measured at 562 nm using Spectra Max 340 PC Microplate Reader (Molecular Devices, LLC, CA, US). The BSA standard curve was then used to determine protein concentration in each sample and samples were then normalised with PBS to ensure equal protein concentrations.

2.6.3 SDS-PAGE

SDS PAGE is a commonly used technique for the separation of proteins based on their molecular weight. SDS is an anionic detergent that when dissolved, its molecules has a net negative charge within a large range of pH. The negative charge on SDS causes it to
denature the proteins by destroying most of the complex structures of proteins. Due to the negative charge, the proteins are then strongly attracted toward an anode, when a charge is applied to the gel. Polyacrylamide gels allow smaller proteins to migrate faster through the gel than larger proteins, which results in the separation of proteins in a size gradient. The rate of protein separation is dependent on the pore sizes.

The components of each percentage of polyacrylamide gel is listed in Table 2.11. A 1.5 mm, 15-well Biorad comb was used to form 15 wells in each gel. Resolving and stacking gels were poured and allowed to set before loading of protein samples. Protein samples were prepared in 2 X SDS loading buffer with the addition of 1 M DTT at a 1 in 10 dilution. 20 µL of 1 µg/µL protein was loaded per well. 5 µL SeeBlue Plus2 pre-stained protein marker was loaded in the left lane of every gel. 1 X Running buffer (25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS) was added to completely submerged the rig and the gel was run at 90 V until the dye moved into the resolving gel. Voltage was then increased to 120 V until the dye was at the end of the resolving gel.

Table 2.11: Components of Polyacrylamide Gels

<table>
<thead>
<tr>
<th>Reagent</th>
<th>15% Resolving</th>
<th>12% Resolving</th>
<th>10% Resolving</th>
<th>5% Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>3.4 mL</td>
<td>4.9 mL</td>
<td>5.9 mL</td>
<td>3.4 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>3.8 mL</td>
<td>3.8 mL</td>
<td>5 mL</td>
<td>-</td>
</tr>
<tr>
<td>1 M Tris-HCl (pH 6.8)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>630 µL</td>
</tr>
<tr>
<td>Protogel (30% acrylamide, 0.8% bisacrylamide)</td>
<td>7.5 mL</td>
<td>6 mL</td>
<td>3.8 mL</td>
<td>830 µL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µL</td>
<td>150 µL</td>
<td>150 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>10% APS</td>
<td>150 µL</td>
<td>150 µL</td>
<td>150 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>6 µL</td>
<td>6 µL</td>
<td>6 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

2.6.4 Transfer

Once the proteins had run through the gel, the gel was removed from the rig and the transfer apparatus was set up. A Biorad wet transfer system was used. Nitrocellulose paper was soaked in water for approx. 5 mins. Nitrocellulose paper, filter paper and sponges were then soaked in 1 X transfer buffer (25 mM Tris-HCl, 187 mM Glycine, 10% (v/v) Methanol). The soaked membrane was placed on the gel and two filter papers
and one sponge were placed on either side to form a sandwich. The sandwich of gel and nitrocellulose was then orientated in the transfer rig so that the proteins would transfer from gel to nitrocellulose. 1 X transfer buffer was added to completely submerge the rig and an ice-pack was added to ensure no over-heating occurred. Transfer was set up at 200 mA for 1 hour.

2.6.5 Blocking, immune-blotting and visualisation of protein

Once the transfer was complete, the membrane was then blocked in 5% Non-fat milk in TBST (10 mM Tris, pH8, 150 mM NaCl, 0.05% Tween-20) for one hour, rocking at RT. For the use of phospho blots, membranes were blocked in 3% BSA in 1 X TBST. The blocking step prevents any non-specific binding of antibodies in later steps. The primary antibody was then added to the membrane at a dilution listed in Table 2.12 and was diluted in 5 mL of blocking buffer. The membrane was then left rolling in primary antibody at 4 °C overnight. Membranes were then washed in 1 X TBST for 3 X 10 min intervals. Once washed, secondary antibody was added to the membrane and was diluted in 5 mL of blocking buffer. Secondary antibody dilutions are listed below in Table 2.13. Membranes were incubated with secondary antibody for 1 hour at RT. Membranes were then washed as described before and proteins were detected using the Immobilon Chemiluminescent HRP Substrate (Millipore) and viewed using the BioRad ChemiDocTM MP Imaging System.
Table 2.12: Primary Antibodies for Western Blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat monoclonal anti-Caspase-11 (C1354)</td>
<td>Sigma</td>
<td>1/200</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-Caspase-1 (sc-514)</td>
<td>Santa Cruz</td>
<td>1/200</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-Caspase-1 (sc-622)</td>
<td>Santa Cruz</td>
<td>1/200</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-IL-1β (sc-7884)</td>
<td>Santa Cruz</td>
<td>1/200</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-Caspase-3 (9662)</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-cleaved Caspase-3 (9664)</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-Caspase-4 (M029-3)</td>
<td>MBL</td>
<td>1/1000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-Caspase-5 (M060-3)</td>
<td>MBL</td>
<td>1/1000</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-STAT1 (9172)</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-pSTAT1 (TY701) (7649)</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>Gasdermin D</td>
<td>Sigma</td>
<td>1/500</td>
</tr>
<tr>
<td>pERK1/2</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>tERK1/2</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>pIκBα</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>tIκBα</td>
<td>Cell Signalling</td>
<td>1/1000</td>
</tr>
<tr>
<td>p/tIκBα</td>
<td>Gift</td>
<td>1/500</td>
</tr>
<tr>
<td>Flag</td>
<td>Sigma</td>
<td>1/5000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-β-Actin</td>
<td>Sigma</td>
<td>1/10,000</td>
</tr>
</tbody>
</table>

Table 2.13: Secondary Antibodies for Western Blot

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP conjugated anti-mouse IgG</td>
<td>Jackson Immunolabs</td>
<td>1/5000</td>
</tr>
<tr>
<td>HRP conjugated anti-rabbit IgG</td>
<td>Jackson Immunolabs</td>
<td>1/5000</td>
</tr>
<tr>
<td>HRP conjugated anti-rat IgG</td>
<td>Jackson Immunolabs</td>
<td>1/5000</td>
</tr>
</tbody>
</table>

2.7 qPCR of skin Tissue

Quantitative PCR (qPCR) is a useful technique used to characterise and quantify nucleic acids. Initially, reverse transcription is required to reverse transcribe RNA transcripts into cDNA. qPCR then enables the collection of data as the PCR progresses by incorporating fluorescent labelling. SYBR green is a commonly used cyanine dye used to stain nucleic acids as it binds to double stranded DNA by intercalating between the DNA bases. SYBR
green can therefore be used to quantitatively measure DNA amplification at the end of each cycle as the fluorescence is measured after each cycle.

Skin sections were dissected from the shaved back of mice and snap frozen in liquid nitrogen prior to storage at -80°C. Tissue was then thawed using 500 μL Trizol and processed using a tissue homogeniser. Homogeniser was washed three times with PBS between each sample. Once tissue was sufficiently digested, 100 μL chloroform was added to each sample and centrifuged at 12,000 g for 15 mins at 4°C. The sample separated into a clear upper aqueous phase, an interphase and a lower red chloroform phase. The upper aqueous phase, containing the RNA was transferred to a fresh eppendorf and 250 μL high-grade isopropanol was added to the sample. Samples were incubated for 10 mins at RT before centrifugation at 12,000 g for 10 mins at 4°C. RNA forms a gel-like white pellet at the bottom of the Eppendorf. The supernatant was then removed and the RNA pellet was resuspended in 500 μL of 75% high grade ethanol. The RNA was then centrifuged at 7,500 g for 5 mins at 4°C. The supernatant was again discarded and the pellet was allowed to air dry for 5-10 mins. The RNA pellet was then resuspended in 30 μL nuclease free water and incubated in a waterbath at 55°C for 15 mins. RNA was then quantified using the NanoDrop ND-1000.

Following quantification, 1 μg of RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit as per manufacturer’s recommendations. cDNA was then diluted 10 times with nuclease-free water. Amplification of cDNA using specific primers listed below were performed using PowerUp™ SYBR™ Green Master Mix.

**Table 2.14: Primers used for qPCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-2</td>
<td>5’ TAGCATGCAGCCAACCAGGA 3’</td>
<td>5’ AAGGACACACATGCGTCAAAC 3’</td>
</tr>
<tr>
<td>Vegf</td>
<td>5’ AAGGATGAAAGGGCTGGAGTG 3’</td>
<td>5’ TGAGAGGTCTGTTCCCGA 3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’ CCAAGCCCTACCTACTTCC 3’</td>
<td>5’ CTCTGAGGGCTGACACAAGG 3’</td>
</tr>
<tr>
<td>eNOS</td>
<td>5’ TCCGGAAGGCGTTTGATC 3’</td>
<td>5’ GCCAAATGTGCTGTCACC 3’</td>
</tr>
<tr>
<td>TSP-1</td>
<td>5’ ACTTCACCTTTGCCACCTC 3’</td>
<td>5’ AGACTCTGGAATGCGGTTG 3’</td>
</tr>
<tr>
<td>IFN-α</td>
<td>5’ TGAAGGACAGAGAGACTTGG 3’</td>
<td>5’ GAATGAGTCTAGGAGGTGT 3’</td>
</tr>
<tr>
<td>IFN-β</td>
<td>5’ GGAGATGACGGAGAAGTGC 3’</td>
<td>5’ CCGAGTGCTGGAGAAATTGT 3’</td>
</tr>
<tr>
<td>IL-17A</td>
<td>5’ TTTTCAGCAAGGAATGTTGGA 3’</td>
<td>5’ TTCATTGTGGAGGAGGCAAGC 3’</td>
</tr>
<tr>
<td>IL-17F</td>
<td>5’ CAAGAAATCCCTGCTCCTCG 3’</td>
<td>5’ GAGCATCTTCTCCAACCTGAA 3’</td>
</tr>
</tbody>
</table>
2.8 Immunofluorescence for Cell Lines

Hek293T cells were plated on cover slips in 24-well cell culture plates at 2 x 10^5 cells/mL and cultured for 24 hours at 37 °C. Once cells were treated, supernatants were removed and each well was gently washed in 500 µL PBS. Cells were then fixed by the addition of 500 µL of 4% paraformaldehyde for 15 mins at RT. Once fixed, wells were washed with 500 µL PBS for 2 X 5 min intervals. PBS was then gently aspirated from the wells and the cells were then permeabilised by the addition of PBS + 0.2% triton x-100 for 10 mins at RT. Once permeabilised, wells were washed with PBS for 5 mins and then blocked with 200 µL 2% BSA in PBS for 1 hour at RT. Cover slips were then incubated in primary antibody at 1 in 50 dilutions in blocking buffer for 2 hours at RT, never drying out. Cells were then washed with PBS for 3 x 5 min intervals. Secondary antibody was then added to each corresponding slide at 1 in 500 dilution in blocking buffer and was incubated for 30 mins at RT. Cover slips were washed again (3 X 5 mins PBS) and then mounted with DAKO mounting media and DAPI (1 in 100 dilution).

2.9 Plasmid Preparation

2.9.1 Transformation of Competent Bacteria by Heat Shock

Competent E.coli bacteria, DH5α were stored at -80 °C and thawed on ice for 2 mins before use. 50 µL of competent bacteria was used for each transformation and was transferred to 13 mL bug culture tubes. 1-2 µL plasmid was added to bacteria and incubated on ice for 30 mins, allowing the plasmid to coat the bacterial cells. Bacteria was heat shocked in a water bath at 42 °C for 40 seconds, then returned to ice to recover for 2 mins. The increase in temperature disrupts the membrane, allowing the plasmid to enter the cells, where it can incorporate into the replication of the bacteria. 450 µL LB broth was added to each tube. The bug culture tubes were then incubated for one hour at 37 °C in a shaker at 220 rpm. 50 µL of each culture was plated on an LB + Amp agar plate and incubated upside down at 37 °C overnight. Growth would only occur if the plasmid (with ampicillin resistant gene) is successfully incorporated into the bacteria.
2.9.2 Starter/Midi/Maxi Culture

The following day, one individual, round colony was chosen and inoculated into 3 mL of LB broth with Ampicillin (100 µg/mL). The culture was then incubated at 37 °C in a shaker at 280 rpm overnight. 100 µL starter culture was then transferred to a fresh 100 mL LB broth with ampicillin. Midi cultures were incubated overnight at 37 °C rotating at 280 rpm. Cultures were then centrifuged at 5000 g for 10 mins in a Sorvall RC 5 C Plus rotor (Thermo Fischer Scientific inc.)

2.9.3 Plasmid midi preps

Plasmids were purified and prepared using the Qiagen QIAfilter plasmid Midi kit. The bacteria plasmids were resuspended in 4 mL of buffer P1. 4 mL of buffer P2 and then P3 were added. The Qiagen column was equilibrated with 4 mL buffer QBT. The DNA was then filtered and transferred directly to the Qiagen column. Once the lysates had run through the column completely, the column was washed twice with 10 mL buffer QC. The DNA was then eluted with 5 mL elution buffer into a Sorvall tube. 3.5 mL isopropanol was added to precipitate the DNA. The DNA was then centrifuged at 15,000 g for 30 mins at 4 °C. The supernatant was discarded and 2 mL 70% ethanol was added to centrifuge tube. Tubes were centrifuged for 15 mins at 15,000 g at 4 °C. The supernatant was again discarded and tubes were centrifuged for a further 10 mins to remove any residual ethanol. Once completed, tubes were allowed to air dry in a sterile hood and then DNA was resuspended in 50 µL nuclease free water and 50 µL TE. DNA concentration was then determined using the NanoDrop ND-1000. DNA concentration was then adjusted to 250 ng/µL with H₂O:TE.

2.9.4 Site-directed Mutagenesis

The QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent) was used to perform site-directed mutagenesis of NLRP6 plasmid. The mutagenic oligonucleotide primers were designed and then made by MWG Eurofins. Primers are listed below in Table 2.15. The reaction set up is listed below in Table 2.16. The cycle parameters are listed below in Table 2.17. Following amplification of mutated oligos, 2 µL of Dpm I restriction enzyme was added directly to each amplification reaction. Reaction was gently mixed and incubated at 37 °C for 10 min to digest the parental supercoiled dsDNA.
The XL10-Gold ultracompetent cells were gently thawed on ice. For each control and sample reaction, 45 µL ultracompetent cells were transferred to pre-chilled bug culture tubes. 2 µL of β-ME was added to the 45 µL of ultracompetent cells. Cells were incubated on ice for 2 mins. 2 µL of the Dpn I-treated DNA from both the control and sample reaction were added to individual tubes of ultracompetent cells. Cells and reactions were incubated together on ice for 30 mins. Bacteria was heat shocked at 42 °C in a water bath for 30 seconds. Tubes were then incubated on ice for 2 mins. 500 µL of LB broth was then added to each tube. Tubes were incubated for 1 hour at 37 °C rotating at 240 rpm. 10 µL of positive control was plated on LB agar plates with Kanamycin. 50 µL of sample mutagenesis was plated on LB agar plates with Kanamycin. The transformation plates were incubated at 37 °C overnight. One colony was obtained and transferred to 3 mL LB broth with Kanamycin. Culture was grown as discussed in section 2.9.2. Mini prep was performed to prepare DNA for sequencing.

Table 2.15: Site-directed mutagenesis primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP6 Mutant Forward</td>
<td>5’ GCGAGTGCGCTTCTGCGCAGTGACGGCCCTCCGGAGCAGCAGCGACTCGG 3’</td>
</tr>
<tr>
<td>NLRP6 Mutant Reverse</td>
<td>5’ GCCACGTCATGCCGACAGCGACGACTCGG 3’</td>
</tr>
</tbody>
</table>

Table 2.16: Site-directed Mutagenesis Reaction Components

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Mutagenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Reaction buffer</td>
<td>5 µl</td>
<td>5 µl</td>
</tr>
<tr>
<td>pWhitescript 4.5 kb control plasmid (5 ng/µl)</td>
<td>5 µl</td>
<td>-</td>
</tr>
<tr>
<td>NLRP6 plasmid</td>
<td>-</td>
<td>1 µl</td>
</tr>
<tr>
<td>Control forward primer</td>
<td>1.25 µl</td>
<td>-</td>
</tr>
<tr>
<td>Control reverse primer</td>
<td>1.25 µl</td>
<td>-</td>
</tr>
<tr>
<td>Mutagenesis forward primer</td>
<td>-</td>
<td>1.34 µl</td>
</tr>
<tr>
<td>Mutagenesis reverse primer</td>
<td>-</td>
<td>1.37 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>QuikSolution reagent</td>
<td>1.5 µl</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>34 µl</td>
<td>38.79 µl</td>
</tr>
<tr>
<td>QuikChange Lightning Enzyme</td>
<td>1 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
Table 2.17: Cycle Parameters for Site-directed mutagenesis

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>95 °C</td>
<td>2 mins</td>
</tr>
<tr>
<td>2</td>
<td>18</td>
<td>95 °C</td>
<td>20 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60 °C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>68 °C</td>
<td>3.75 mins</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>68 °C</td>
<td>5 mins</td>
</tr>
</tbody>
</table>

2.9.5 Sequencing of DNA

DNA concentration was determined using NanoDrop 1000 and 15 µl 100 ng/µl of plasmid DNA was sent to MWG Eurofins for sequencing with 2 µl of each of the primers listed in Table 2.18.

Table 2.18: Primers used for Sequencing of Site-directed Mutagenesis plasmid

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLRP6 Seq forward</td>
<td>5’ GGTTCAGAGCGTGTGAAGCAG 3’</td>
</tr>
<tr>
<td>NLRP6 Seq reverse</td>
<td>5’ GGATGAAGAAGGGAGGCTG 3’</td>
</tr>
</tbody>
</table>

2.9.6 Generation of Stable cell lines

WT and NLRP6<sup>R653G</sup> were cloned into the Pd1 lentiviral vector via restriction digest of Spe1 and Nde1. Primers for NLRP6 were designed to incorporate Spe1 and Nde1 into each end of the gene. The forward primer was designed to incorporate a clamp, Spe1, Kozak sequence and then 18 bp of the NLRP6 gene. The reserve primer was designed to incorporate a clamp, Nde1, a stop codon and the 18 bp of the other end of the NLRP6 gene. The gene was amplified using the exact primers listed below in Table 2.19 using ExTaq polymerase under the conditions listed in Table 2.16 and 2.17. Once amplified, 5 µL of product was run on a 1% agarose gel to confirm amplification and the remainder of the PCR product, along with the Pd1 vector, was digested for 1 hour at 37°C using 1 µL Spe1, 1 µL Nde1 and Buffer NEB 2.1. The ends of the vector were subsequently dephosphorylated to prevent religation. This treatment involved the addition of 1 ul of the enzyme alkaline phosphatase for one hour at 55°C. Once complete, the molar ratio of
the vector and inserts was calculated and a range of ligations were set up to include a vector to insert ratio of 1:2 – 1:10. The ligation was incubated overnight at 18°C using T4 ligase. The following morning, 5 μL of the ligation reaction was heat transformed into 50 μL of Stbl3 competent bacteria as described in section 2.9.1. The following day, round colonies were chosen and restreaked on fresh agar plates. A 200 μL culture was incubated for 3 hours at 37°C and subsequently amplified using the NLRP6 primers listed in Table 2.19. PCR products were run on a 1% agarose gel to confirm the presence of the NLRP6 insert. Colonies which were positive for the presence of the NLRP6 gene were maxi-prepped as described in section 2.9.3.

Hek293T cells were then seeded at 4 x 10^5 cells/mL in a 10 cm dish. 24 h later, 4μg empty vector (Pd1)/NLRP6<sup>WT</sup>/NLRP6<sup>R653G</sup>, along with 2 μg of each of the packaging vectors VSV-G (Dal) and GPP (Dal), were transfected into the cells. A further 24 h later the supernatant was retained and replaced with fresh medium. The next day, supernatant was again retained and pooled with supernatant from the previous day. Virus containing media was then filter sterilised and spinoculated into THP-1s and Hek293T cells using polybrene. 1 μg/mL Polybrene was used to spinoculate 1 x 10^6 cells with either 2.5 mL or 5 mL virus-containing media. Mixture was centrifuged at 800 g for 40 mins at 30°C. THP-1s were then gently resuspended in 1 mL of RPMI and seeded in a 12 well plate. Hek293Ts were gently resuspended in 10 mL of DMEM and seeded in a 10 cm dish. Cells were cultured overnight and the following day, 2 μg/mL puromycin was added to all cells to begin the selection process. Cells were monitored daily for survival and live cells were grown in 2 μg/mL puropmycin for two weeks to ensure sufficient selection.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’ GGGACTAGTGCCGCGCCTGCCATGGACCAGCCAGAGGCC 3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ CCCCATATGCTACTTTATCGTCGTCATCCTT 3’</td>
</tr>
</tbody>
</table>

2.10 Cell Transfection

2.10.1 Transfection of Plasmid DNA

The delivery of plasmid DNA into HEK293Ts was performed using a chemical transfection reagent, Lipofectamine 2000 (Invitrogen). Lipofectamine is a cationic lipid-
based reagent that has a positively charged surface, which interacts with the negatively charged phosphate backbone of the nucleic acid to be delivered into the cell. This forms the transfection complex. The positive charge of the transfection reagent also allows for interaction between the cell membrane, which is negatively charged. This mediates the entry of the transfection complex into the cell.

Plasmid transfections were performed in 6 well or 24 well plates. HEK293T cells were seeded at 2 x 10^5 cells/mL and cultured overnight. A master mix of Lipofectamine 2000 and Opti-MEM was prepared so that eventually 1 µl of Lipofectamine would be used for every 1 µg plasmid DNA in a total of 200 µl Opti-MEM. The initial mastermix of Opti-MEM and Lipofectamine was prepared originally to contain 1 µL of Lipofectamine in 100 µL Opti-MEM. The Lipofectamine was put directly into Opti-MEM to insure it is solubilised and does not coat the inner surface of the eppendorf. A second tube was prepared with the desired quantity of DNA in 100 µL Opti-MEM. The Lipofectamine mastermix and tube of DNA/optimem were allowed to stand for 10 mins and then 100 µL of the Lipofectamine mastermix was added to 100 µL of the DNA mix. This was allowed to site for 15 mins and then gently pipetted drop by drop onto the cells. Cells were then cultured for 24 hours to allow for plasmid uptake.

2.10.2 Preparation of Cell Lysates from Plasmid Transfected Cells for Western Blotting

After 24 hours, supernatants were removed and cells were lysed. Plates were incubated on ice for the duration of lysing and 150 µl RIPA buffer was added to each 6 well or 75 µl to 24-well. RIPA buffer was supplemented with 10 µg/mL Leupeptin, 1.7 µg/mL aprotinin, and 1 mM PMSF immediately prior to lysing. Once RIPA was added, cells were incubated on ice for 10 mins to ensure all cells had lysed. The lysed cells were then transferred to eppendorfs and then centrifuged at 20,000 g for 10 mins at 4 °C. Supernatants were removed, eliminating a pellet of cell debris, and then stored at -80 °C for further quantification of protein or analysed immediately after.

2.11 Assays

2.11.1 Griess Assay

The Griess assay is a widely used technique that is based on the chemical diazotisation reaction, originally described by Griess in 1879. The assay uses sulphanilamide and N-
1-naphthylendiamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Assay is used to detect the presence of nitrite ions in a variety of biological samples, such as serum, plasma, urine and culture medium. The limit of detection is 2.5 µM of nitrite.

The assay involves the use of a standard curve to quantify the concentration of nitrite ions in a solution. The top standard is 100 µM and is decreased two-fold for seven concentrations. 50 µL of standards and samples were added to corresponding wells in a 96-well plate and then 50 µL Griess Reagent (Enzo Life Sciences) was added to each well. The plate was left to incubate at RT for 15-30 mins and then absorbance was read at 540 nm using Spectramax Microplate Reader (Molecular Devices).

2.11.2 LDH Assay

The LDH assay is a colorimetric assay used to quantify the concentration of lactate dehydrogenase (LDH) released into the media from damaged cells, which is an indication of cellular damage. LDH released into culture supernatant is measured with a coupled enzymatic assay which results in the conversion of tetrazolium salt (INT) into a red formazan product. The amount of colour is directly proportional to the number of cells lysed.

50 µL of skin explant culture or cell supernatant is pipetted to 96-well plate. For skin explant cultures, a whole skin lysate was used as a positive control and DMEM was used as a negative control. 50 µL of diluted skin lysate and 50 µL of DMEM was also added to 96-well plate. 50 µL of the CytoTox 96 Reagent was added to each well and the plate was incubated at RT in the dark for 30 min. 50 µL of stop solution was then added to each well and absorbance was read at 490 nm using Spectramax Microplate Reader (Molecular Devices).

2.11.3 Luciferase Assay

The firefly luciferase is a 60 kDa protein that is produced by species of the lamyridae family of beetles. A bioluminescent signal from firefly luciferase occurs from the oxidation of D-luciferin. Light output can be used as a measure of the amount of firefly luciferase protein produced, which can determine the activity of the promotor driving firefly expression.
HEK293T cells were seeded at $2 \times 10^5$ cells/mL in a 96-well plate and 200µL per well. 24-hours later, reporters, substrates, activators and empty vectors were transfected into the cells so that each well received a total of 220 ng DNA. A mixture constitutively expressing Renilla luciferase plasmid and also inducible NFκB or ISRE responsive firefly luciferase plasmids were added to each well. 80ng of NFκB, 40 ng TK Renilla and 10-50 ng NLRP6/Mutant was added to each well and supplemented with empty vector to bring a total DNA concentration to 220 ng. Cells were transfected with 0.44 µl GeneJuice in 20 µL serum free media as described in section 2.9.1. 16 hours later, cells were lysed with 1 x lysis buffer and luminescence was measured using the Luciferase and Renilla mix. NFκB values were normalised against Renilla values and results were graphed using GraphPad prism.

2.11.4 alamarBlue® Cell Viability Assay

The alamarBlue® assay is a widely used assay for the determination of cell viability. The active ingredient of alamarBlue®, Resazurin, is a cell permeable chemical that is non-toxic, blue in colour and non-fluorescent. Due to the reducing environment in the cytosol of live cells, when Resazurin enters a live cell, it is reduced to resorufin, which has a red colour and is highly fluorescent. Viable cells continue to reduce resazurin to resorufin which can then be measured spectrophotometrically to quantify the viability of the cells.

THP-1 cells were seeded at $1 \times 10^6$ cells/mL and seeded in a 96-well plate with a total volume of 200 µL/well. 20 ng/mL of phorbol 12-myristate 13-acetate (PMA) was added to the cells to differentiate them to macrophages and adhere them to the bottom of the well. Additionally, Hek293T cells were seeded at $2 \times 10^5$ cells/mL with a total of 200 µL/well. 24 h later, 0, 50, 100 and 200 ng of either empty vector, NLRP6 WT or NLRP6R653G were transfected into the cells using 0.5 µL Lipofectamine® 2000 per well to deliver the DNA into the cells. The cells were incubated for 48 hours at 37 °C with 5% CO$_2$. 20 µL of alamarBlue® was added to each well and approximately 5 h later, the fluorescence was measured at 544 and 590 nm. Viability of the transfected cells were compared to cells transfected with an empty vector.

2.12 Statistical Analysis

Data obtained was analysed using Graphpad Prism 5 software. Error bars are indicative of SEM. Two-way Analysis of Variance (ANOVA) followed by Bonferroni post-tests
were used to compare all $Casp-11^{+/+}$ and $Casp-11^{-/-}$ groups during wound healing and psoriasis experiments. mRNA expression data mined from publically available datasets was analysed using Wilcoxon test to determine the significant differences between two groups and the Kruskall Wallis test to compare significant differences between three groups. Two-way ANOVA followed by Bonferroni post-tests were used to compare statistical differences between $NLRP6^{R653G}$ patient and healthy controls. p-values below 0.05 were considered statistically significant.
Chapter 3: Preliminary study investigating the role of caspase-11 in skin repair and disease
3. Preliminary study investigating the role of caspase-11 in skin repair and disease

3.1 Introduction

The skin is a seminal organ that acts as a physical barrier protecting internal organs from the external environment. The skin also plays important immunological roles in protection against invading PAMPS or DAMPs. Immune responses are mediated via extensive crosstalk between epithelial, stromal and immune cells to ensure maintenance of tissue homeostasis and host defence. Dysregulation of either innate or adaptive immune cell types present in the skin can result in the development of various skin disorders such as psoriasis, atopic dermatitis and contact dermatitis. Inflammatory caspases are believed to be crucial mediators of innate immune responses in the skin repair process (189, 190). Therefore, we aimed to investigate the role caspase-11 plays in skin health and repair.

Wound healing is a crucial aspect of perioperative care. If methods were discovered that accelerated the healing process of wounds post-surgery, it would result in less postsurgical stress and improve surgery outcome. The wound healing process occurs in three steps; inflammation, tissue formation and tissue remodelling. The basic sequence of events that occur during mammalian wound healing has been well established. Directly after wound incision a fibrin-rich clot forms to limit bleeding. In unwounded healthy skin, small numbers of leukocytes are present. However, shortly after wound infliction, neutrophils, and then macrophages and mast cells are rapidly recruited to the site of incision. Once leukocytes are recruited they phagocytose bacteria, clear matrix and debris from the site of incision, and release pro-inflammatory cytokines to recruit other immune cells to the site (191)(192). The second stage of wound healing occurs from day 2-10 and involves the formation of new tissue. Finally, the last stage of wound repair consists of tissue remodelling and can begin from 2-3 weeks after wound infliction and last for weeks to months (193).

During the initial inflammatory response phase, cells recruited to the wound site release pro-inflammatory cytokines and growth factors to aid in the subsequent tissue formation. While these are a crucial aspect of wound repair, excessive or dysregulated inflammation can result in increased scarring (194) or can contribute to impaired wound healing, as
seen in diabetes (195)(196). IL-1 is believed to be secreted by macrophages, neutrophils, keratinocytes and fibroblasts and has been shown to play an important role in the wound healing process (197)(198). Activity of IL-1 is mediated by the IL-1 receptor (IL-1R) and inhibited by IL-1R antagonist (IL-1Ra). IL-1R<sup>−/−</sup> mice displayed reduced inflammatory cell accumulation and reduced scarring following wound infliction (199). Moreover, IL-1Ra<sup>−/−</sup> mice displayed delayed wound healing and drastically increased inflammatory cell accumulation (200). Increased levels of IL-1β have also been observed in wounds of diabetic humans and mice (201)(202). Interestingly, NLRP3 is also believed to be implicated in the wound repair process. Weinheimer-Haus et al., showed that NLRP3<sup>−/−</sup> and Casp-1<sup>−/−</sup> mice display delayed wound healing and have a reduced accumulation of inflammatory cells in the incision site (203). Additionally, Abdullah et al., have recently reported that activation of NLRP3 promotes wound closure in WT mice (204). These findings suggest a definitive role for the canonical inflammasome in the initial stages of wound repair.

It is evident that the canonical inflammasome plays a crucial role in the initial inflammatory stages of wound repair in skin. However, whether the non-canonical inflammasome is also responsible for inflammation in the wound site is still unclear. Based on the clear role that caspase-1, NLRP3 and IL-1β play in this early stage, we hypothesise that caspase-11, or caspase-4 and -5 in humans, may in fact play a similar role. The aim of this study is to determine the role that caspase-11 plays in the wound healing process and in doing so, identify a possible area for further research. The significance of these findings could potentially identify a new aspect of innate immunity that could be targeted to improve wound healing. A new inflammatory mediator for clinicians to target (either activate or inhibit) could result in improved wound healing and in particular, increase the speed of post-surgery recovery.

Psoriasis is a chronic inflammatory skin disease that affects approximately 2% of the world population. The most common form of psoriasis is known as plaque psoriasis or psoriasis vulgaris. It is easily diagnosed as red plaques with well-defined borders and a silver/white scale. Plaques are usually present on the knee, scalp, elbows and the lumbosacral area. There are other less common forms of psoriasis such as pustular, guttate, inverse, erythrodermic, palmo-plantar and drug-associated psoriasis. The severity of the disease is defined by the amount of psoriasis that covers the body. The palm is used
to represent 1% of the total body surface and percentage of the affected area is calculated using this as a rough guide for measurement. Clinicians can also assess the disease severity using the Psoriasis Activity and Severity Index (PASI) score. The PASI scoring system ranks the severity of erythema (redness), desquamation (scale), induration (thickness) and percentage of area covered. Clinicians assign scores of 0-4 based on severity in four regions of the body (head and neck, upper limbs, trunk and lower limbs). The highest score that can be assigned is 72 (205).

The initiation of psoriasis occurs as a result of keratinocyte and innate cell activation, which subsequently results in the further recruitment of immune cells into the psoriatic lesion. Immune cell infiltration, in conjunction with increased and dysregulated keratinocyte proliferation and differentiation (acanthosis) results in an increase in the thickening of the dermis and epidermis. Abnormal keratinocyte differentiation also leads to thickening of the outer layer of the epidermis known as the stratum corneum (Figure 3.1.1). Thickening of the stratum corneum results in the thick scale often observed in psoriatic patients. Increased angiogenesis is another main characteristic observed in this disease. Increased vascularisation allows dispersal of oxygen and nutrients to the lesion to sustain the newly infiltrated immune cells.
As psoriasis affects a large proportion of the population, understanding the pathogenesis of the disease is crucial for adequate treatment of the disease. While there have been great developments in skin research and increased understanding of skin pathologies in recent years, there are still pitfalls to the available drugs designed to treat psoriasis. Research in this area is limited by practical factors, namely the availability of skin biopsy tissue. Compared to the relatively straightforward accessibility of blood sample collection, obtaining skin biopsies involves a painful procedure and they are not often easily obtained from patients for this reason. The availability of an animal model is a great tool that allows researchers to explore the mechanistic details of psoriasis. The discovery of new signalling pathways that play a role in the pathogenesis of the disease could lead to new drug targets and could eventually impact patients’ treatment plans and quality of life.
Psoriasis was originally believed to be a T-cell driven disease. However, recent developments have suggested dysfunction of innate immune components as drivers of psoriasis. The main research focus of our laboratory is to investigate the role of inflammatory caspases-1, 4, and 5 in various inflammatory diseases and cancers. Several publications suggest that the inflammasome is a key pathogen recognition mechanism in keratinocytes and other innate cells present in the skin (206). As a result of this, we sought to investigate the role of inflammatory caspases in the pathogenesis of psoriasis. There have been a number of publications in recent years suggesting that inflammasomes are key immune components affected in psoriasis. IL-18 was the initial inflammasome-related cytokine that was reported to be present at increased levels in psoriasis lesions (206)(207). An additional study showed that caspase-1 activity is increased in lesions of psoriasis patients (208) and was later confirmed in 2011 by Dombrowski et al. Collectively these findings suggest that inflammasome activity is most likely increased during psoriasis. An additional publication in 2012 by Sollberger et al., investigated the expression of inflammatory caspases in human keratinocytes (209) and reported that caspase-4 is responsible for inflammasome activation in keratinocytes in vitro. Additionally Salskov-Iverson et al., have shown that caspase-5 expression levels are significantly increased in both plaque and guttate psoriasis (210) although, the exact mechanism responsible for driving its up-regulation is still unclear. Another recent publication has proposed a mechanism for the up-regulation of caspase-5 expression in psoriasis (211). Zwicker et al., show that IFN-γ and cytosolic DNA are responsible for up-regulation and activation of caspase-5 in human epidermal keratinocytes, and suggest that a similar mechanism occurs during psoriasis. As caspases -4 and-5 are believed to be the human orthologs of caspase-11, the goal of this study was to gain better mechanistic insight regarding the role of caspase-11 during a murine Imiquimod-induced psoriasis model.
3.2 Results

3.2.1 Inflammatory caspase expression levels are increased in murine wounds and human psoriatic lesions

In addition to their role in mounting immune responses against pathogens, inflammatory caspases are implicated in a wide range of diseases. Inflammatory caspases may also be responsible for the immune dysregulation often observed during disease (212). Sollberger et al., reported that caspase-1, -4 and-5 are expressed in human keratinocytes and that caspase-4 is required for inflammasome activation and IL-1β cleavage (209). We therefore hypothesised that inflammatory caspases may play a role in the healing process following wound infliction and in the pathogenicity of an inflammatory skin disease, such as psoriasis.

The aim of this preliminary study was to investigate whether caspase-11 plays a role in skin health and repair, using two in vivo murine models. The more promising model from this study would subsequently be studied in more detail for the remaining duration of this PhD project. To determine if inflammatory caspase expression is increased at the site of a wound, a murine gene array dataset was mined and expression values were graphed using GraphPad Prism (213). Gene array datasets are a widely-used tool that can give an indication as to whether a particular gene is up-regulated or down-regulated in diseases. Figure 3.1 (A) shows that caspase-1 expression levels are significantly increased up to 24 hours after wound infliction. Additionally, caspase-11 expression is significantly increased from 12-24 hours after wound initiation (Figure 3.1 (B)). This suggests that both caspase-1 and caspase-11 may be involved in the initial stages of wound repair. Similarly, gene expression values from four widely-cited publically available datasets of human psoriatic skin lesions were analysed from Pubmed Geo Datasets (22-25). Caspase-1, caspase-4 and caspase-5 expression levels were found to be significantly increased in psoriatic lesions (Figure 3.2, 3.3 and 3.4, respectively). While the patient numbers varied in each study, it was conclusive in all four datasets that the expression levels of the inflammatory caspases were significantly higher in psoriatic lesions. The data mined from the gene array datasets suggests that the inflammatory caspases examined are likely playing a role in skin repair and the disease pathogenesis of psoriasis. To confirm that expression levels of all caspases were not elevated in an unspecific manner in the datasets examined, caspase-2 expression was mined as a negative control (Figure 3.5.). Caspase-
expression was not increased in three of four datasets examined (Figure 3.5 (A-C)). Additionally, in the dataset shown in Figure 3.5 (D), caspase-2 expression was significantly decreased in the patient’s psoriatic lesions. This suggests that the increase in expression of inflammatory caspases is not a phenomenon that is universally observed for all caspase genes. As mentioned previously, caspases-4 and -5 are human orthologs of murine caspase-11. Therefore, investigating the role of caspase-11 in a murine model of psoriasis should result in greater insight into the mechanistic impact of caspase-11 (or caspase-4 and -5) during this disease.

### 3.2.2 Genotyping of Casp-11+/+, Casp-11−/− and Casp-11+/−, C57BL/6J mice

The Casp-11−/− mice used for the purpose of this study were originally generated on C57BL/6J strain in the laboratory of Junging Yuan, Harvard Medical School, Massachusetts, US. Following arrival in Trinity College Dublin, mice were backcrossed on C57BL/6J strain bought from Harlan Laboratories. Homozygous Casp-11+/− mice and Casp-11+/− mice are now being bred in the Comparative Medicine Unit in Trinity Biomedical Sciences Institute, Dublin.

Each experimental mouse and breeding pair were genotyped to confirm the presence or absence of Casp-11. DNA was extracted from ear punches of mice and PCR amplified using WT and caspase-11 mutant specific primers (SY21/SY22 and PJK/SYKO respectively). Figure 3.6 shows representative genotyping results of Casp-11+/+, Casp-11−/− and Casp-11+/−. In Casp-11+/− mice, two alleles of the WT caspase-11 gene are amplified and are separated on an agarose gel. The amplification region of the Casp-11 WT gene is approximately 200bp. Casp-11+/− mice consist of two alleles of a truncated Casp-11 gene, due to the insertion of a cassette into the Casp-11. Amplification products of Casp-11+/− result in a 600 bp band on an agarose gel. Casp-11+/− mice have one WT allele and one caspase-11 KO allele resulting in the amplification of both a 200 bp WT band and a 600 bp KO band (Figure 3.6).

### 3.2.3 Casp-11+/− BMDMs when stimulated with IFN-γ or IL-17A are less able to migrate than Casp-11+/+ BMDMs in a scratch wound assay

Before a murine model of wound repair was established in the lab, we initially sought to investigate the rate of migration of BMDMs in a scratch assay using various immune stimulants. The scratch assay is a useful tool that allows scientists to investigate the
regrowth and migration of macrophages back into the scratch area. A key aspect of wound repair is the migration of immune cells into the wound to aid in the repair process via the release of inflammatory mediators and growth factors. We hypothesised that stimulation of BMDMs with known caspase-11 activators would induce migration of these cells. BMDMs were therefore isolated and cultured from both Casp-11+/+ and Casp-11−/− mice and seeded in a 24-well plate. Once cells had attached to the plate, a tip was used to induce a ‘scratch’ across the monolayer of cells. Cells were then stimulated with either LPS, IL-1β, IFN-γ or IL-17A and the rate of migration back into the scratch was photographed for three consecutive days, using an inverted microscope.

**Figure 3.7** (A) shows representative images of the scratch of both untreated Casp-11+/+ and Casp-11−/− BMDMs from day 0-3. Cells that migrated back into the scratch site were counted at each day. Results show that the migration of unstimulated macrophages back into the scratch occurs at a similar rate in both Casp-11+/+ and Casp-11−/− mice (**Figure 3.7** (B)).

As caspase-11 is not constitutively expressed in macrophages, it was decided to induce the up-regulation and activation of caspase-11 via the addition of various caspase-11 inducing ligands. **Figure 3.8** (A) shows representative images of the scratch site over 4 days following stimulation with LPS. It would appear that there are no significant differences in migration of Casp-11+/+ and Casp-11−/− BMDMs when stimulated with LPS (**Figure 3.8** (B)). This would suggest that caspase-11 is not playing a role in mediating the migration of macrophages in an LPS-dependent manner.

Additionally, BMDMs were stimulated with IFN-γ to induce caspase-11 up-regulation. Caspase-11 expression has been shown by our lab and others, to be induced in response to IFN-γ in both bacterial infection of *B. thailandensis* and in a model of acute experimental murine colitis (214)(128). **Figure 3.9** (A) shows representative images of Casp-11+/+ and Casp-11−/− BMDMs following scratch infliction. Cell counts suggest that a decrease in Casp-11−/− macrophage migration occurs over time, when compared to Casp-11+/+ (**Figure 3.9** (B)). These results suggest that IFN-γ mediated BMDM migration is dependent on caspase-11 signalling.

BMDMs were then stimulated with IL-1β following scratch initiation. Recent findings from our laboratory have shown that IL-1β robustly induces up-regulation of caspase-11
expression in BMDMs within 2 hours of stimulation (129). Therefore, we next sought to investigate whether IL-1β impacts the migration of Casp-11+/+ and Casp-11−/− macrophages. **Figure 3.10** shows that IL-1β did not induce a migratory phenotype in BMDMs, and there were no observable differences in migration between untreated and IL-1β treated BMDMs in Casp-11+/+ and Casp-11−/− mice. These findings suggest that IL-1β does not augment migration of macrophages via up-regulation of caspase-11.

Another stimulant used in the scratch wound healing analysis was IL-17A. IL-17A has been shown to play a role in skin health and immunity, however there are conflicting opinions on whether IL-17A promotes or hinders wound closure (215)(216). To investigate the effect of IL-17A in a BMDM scratch assay, Casp-11+/+ and Casp-11−/− BMDMs were seeded and a scratch was applied to the monolayer of cells 24 hours later. IL-17A stimulation resulted in an increased migration of BMDMs into the scratch over the three days observed (**Figure 3.11 (A)**). **Figure 3.11 (B)** shows significantly less migration of Casp-11+/+ macrophages when compared to Casp-11+/+. Interestingly, these results suggest that IL-17A promotes migration of macrophages into a wound site. It appears that this IL-17A-induced migration and proliferation of BMDMs may be mediated by caspase-11.

### 3.2.4 Wound repair is slower in Casp-11−/− mice, when compared to Casp-11+/+ mice

*In vitro* findings to date suggest a possible role for caspase-11 in migration and proliferation of BMDMs following stimulation with IFN-γ and IL-17A. A delay in migration and proliferation of Casp-11−/− BMDMs was evident when compared to stimulated Casp-11+/+ BMDMs. Additionally, gene array analysis suggests caspase-1 and caspase-11 expression levels are increased in murine skin up to 24 hours after wound initiation. As a result of these findings, it was decided to investigate the role caspase-11 plays in wound repair during a murine wound healing model.

The back skin of mice was shaved 48 hours prior to the experiment, to confirm that hair was in Telogen growth phase. Two 3 mm wounds were inflicted per mouse and the wound was photographed daily to record the rate of closure. **Figure 3.12 (A)** shows representative images of the closure of wounds from day 0-9 of both Casp-11+/+ and Casp-11−/− mice. Interestingly, it appears that Casp-11−/− mice heal wounds at a slower rate when compared to Casp-11+/+ mice. The circumference of each wound was measured and a graphical representation is displayed in **Figure 3.12 (B)**. While it appears there is a
visual delay in Casp-11+/− wound healing, statistical tests suggest there is no significant differences between the healing rate of both groups. However, the lack of significance is most likely due to insufficient power of the experiment with the small group numbers used. Further investigation of these findings is warranted and will be carried out by the lab in future studies.

To confirm that caspase-11 expression levels are increased in the wounds during this model, two-day old wounds were homogenised and probed for caspase-11 by Western blot. It appears that pro-caspase-11 expression (45 kDa and 38 kDa) is up-regulated in the wounds two days after wound infliction (Figure 3.13). There are no differences in expression levels of caspase-1 at this time in Casp-11+/+ and Casp-11−/− skin. The observed up-regulation of caspase-11 expression further supports the hypothesis that this enzyme has a role in the wound healing process.

3.2.5 Cytokine expression profiles are similar in both Casp-11+/− and Casp-11+/+ wound explant cultures

As the wound healing process requires the recruitment of multiple immune cells to the site of the wound, it was decided to investigate whether the production of various pro-inflammatory cytokines might give further insight into the cell types and mechanisms that are important in the this process. Wounds were inflicted and after 11 days, mice were humanely sacrificed. Wounds were excised and cultured in DMEM for 24 hours. Results displayed in Figure 3.14 suggest that cytokine production of IL-1β (A), IL-1α (B), IL-18 (C), TNF-α (D), IL-6 (E) and IL-10 (F) were similar in both Casp-11+/+ and Casp-11−/− skin. There are no significant differences in cytokine production between Casp-11+/+ and Casp-11−/− wounds taken 2, 3 and 11 days after wound infliction. While there were no significant differences observed in Casp-11+/+ and Casp-11−/− mice, this study has given insight into the optimum times to focus on for each cytokine in future trials.

3.2.6 Casp-11−/− mice are less susceptible to Imiquimod-induced psoriasis after 5 days of Aldara treatment

The second murine model used in this preliminary study was the Imiquimod-induced psoriasis model. The imiquimod-induced psoriasis model is widely used as a short and effective model to study the initiation of psoriasis in mice (181). This model was therefore used to investigate the potential role of caspase-11 in the pathogenesis of psoriasis.
Female mice were bred until both treatment and control groups reached the age of 8-12 weeks or until they entered the Telogen hair growth phase.

The back hair of each mouse was shaved at least 48 hours prior to the trial start date. Aldara cream was applied to the back of each mouse for 5 days in the treatment groups. Representative photos of mouse back are shown in Figure 3.15 (A) taken at day 5. Disease severity was monitored daily and scored using a scoring system similar to the PASI scoring system for humans, except the entire surface of the mouse skin is not taken into consideration. Casp-11+/ mice displayed significantly less scaling when compared to Casp-11+/+ mice after 3, 4 and 5 days of Aldara treatment Figure 3.15 (B). Similarly, Casp-11+/ mice display significantly less erythema in the skin when compared to Casp-11+/+ mice (Figure 3.15 (C)). Weight was measured daily and percentage weight loss was calculated from the starting weight of each mouse. As shown in Figure 3.15 (E), Casp-11+/ mice lose significantly less weight than Casp-11+/+ mice. On the final day of the trial, mice were harvested and spleen was weighed to determine the systemic impact of the disease. Each spleen weight was normalised to the original weight of each mouse. As seen in Figure 3.15 (D), Aldara treatment caused a large increase in spleen size, however, there were no significant differences between the weights of Casp-11+/+ and Casp-11−/− spleens.

3.2.7 Aldara cream induces the up-regulation of caspase-11 in murine skin

As it appeared that caspase-11 may play a role in the pathogenesis of Imiquimod-induced psoriasis, caspase-11 expression was then analysed to confirm that caspase-11 is up-regulated following treatment with Aldara. As mentioned previously, caspase-11 is not constitutively expressed, cellular stimulation is required for its transcriptional up-regulation (123)(217). Skin sections were dissected from the back of each mouse, following their humane sacrifice, at the end of the trial. Western blot analysis of whole skin homogenates showed that caspase-11 is significantly up-regulated in Casp-11+/+ whole skin sections after five days of Aldara treatment (Figure 3.16). Both pro (45, 38 kDa) and active (25 kDa) caspase-11 were identified, suggesting that both upregulation and activation of caspase-11 occurs during the IMQ-induced psoriasis model. There were no observable differences in caspase-1 protein expression between Casp-11+/+ and Casp-11−/− mice. As caspase-11 is up-regulated and activated in this model, it would suggest that caspase-11 most likely plays a role in the pathogenesis of this disease.
3.2.8 Decreased proliferation in Casp-11−/− mice following 5 days of Aldara treatment

As psoriasis is a disease largely characterised by the increased and dysfunctional proliferation of keratinocytes in the epidermal layer, increased epidermal thickness can be used as another readout of disease activity. Skin sections from treated mice were H&E stained to observe the histological changes induced by Aldara (Figure 3.17 (A)). A slight but not significant increase of epidermal thickness was observed in Casp-11−/− skin when compared to Casp-11+/+ skin, suggesting that caspase-11 does not likely impact epidermal thickening at this stage of the model (Figure 3.17 (B)).

One of the key characteristics of psoriasis is the increased and dysregulated proliferation of keratinocytes in the skin. Studies have shown that during the initiation of psoriasis, signals sent to the stem cells in the basal layer of the epidermis, induce a dysregulated hyper-proliferation of keratinocytes (218). While there were no observable differences in the thickness of the epidermis at this late stage of the model, we next sought to examine the proliferation of keratinocytes in the epidermis. Proliferating cell nuclear antigen (PCNA) is a widely used marker of cell proliferation. PCNA is a nuclear protein, required for DNA synthesis and replication (219). Immunofluorescent staining of Aldara treated skin sections for PCNA positivity showed significantly less PCNA positive cells in the epidermis of Casp-11−/− mice (Figure 3.18). A decrease in proliferation in Casp-11−/− skin suggests that caspase-11 is required for the increased and dysregulated proliferation of keratinocytes observed in this model.
3.3 Discussion

Results from this chapter suggest that caspase-11 plays a key role in skin homeostasis and that a deficiency of caspase-11 results in a protective phenotype during a murine Imiquimod-induced model of psoriasis. Absence of caspase-11 also results in a delayed wound repair in stimulated BMDMs and in a murine wound repair model. While these studies are not yet complete, they suggest an important role for caspase-11 in the skin and provide sufficient evidence for the continuation of these investigations.

Investigation of the expression of inflammatory caspases in a publically available gene array dataset from murine skin wounds suggest that both caspase-1 and caspase-11 are up-regulated in the wound after 24 hours. In addition, increased expression of caspases-1/4 and 5 in human psoriatic lesion led us to hypothesise that the inflammatory caspases most likely play an important role in skin maintenance and repair. Using in vitro scratch assay experiments, this study showed that caspase-11 is required for the migration of BMDMs in response to specific stimuli. Casp-11+/− BMDMs stimulated with IFN-γ and IL-17A display delayed migration and proliferation in a scratch wound assay when compared to Casp-11+/+ BMDMs. This observation suggests that both IFN-γ and IL-17A require caspase-11 signalling to stimulate BMDM proliferation and migration.

As IFN-γ is a known inducer of caspase-11, we sought to determine whether LPS may have similar effects, which would suggest that proliferation/migration effects were solely due to caspase-11 activation. However, LPS-stimulated caspase-11 did not result in increased proliferation of BMDMs, suggesting that the caspase-11 dependent effects are specific to IFN-γ and IL-17 signalling mechanisms. Administration of IFN-γ has been shown in a number of studies to impair wound repair (220)(221). A study investigating the role of endogenous IFN-γ in wound healing has shown that IFN-γ−/− wounds were reduced by 40% after 3 days, in contrast to IFN-γ+/+ wounds which were only reduced by 50% 6 days following wound infliction (221). Interestingly, it appeared that while neutrophil and T-cell recruitment was significantly lower in IFN-γ−/− wounds, macrophage recruitment was only slightly reduced at days 3-6 after wound infliction (221). Our study used a scratch assay to examine the effects of IFN-γ on macrophage proliferation/migration, and is therefore a very different system to an in vivo wound, where IFN-γ is likely to play multiple different roles in response to wound infliction. Interestingly, although we saw no effect of LPS stimulation on macrophage recruitment.
in the scratch assay, topical LPS application has been previously shown to increase the *in vivo* wound healing process (222). It is most likely that these inflammatory mediators play cell specific roles, therefore a more conclusive answer can only be obtained when the wound healing process is examined more thoroughly *in vivo*.

There have been conflicting opinions as to the role of IL-17A in wound repair. IL-17A is a member of the IL-17 family of cytokines and is recognised by the IL-17R receptor. Activation of IL-17R via IL-17A results in NFκB and MAPK activation and subsequent production of pro-inflammatory cytokines, chemokine, metalloproteinases (MMPs) and AMPs (223). Some reports suggest that IL-17A hinders wound repair (216). Whereas, another study has shown that IL-17A+/− mice displayed a delayed healing after wound infliction (215). Our study suggests that IL-17A is capable of stimulating BMDM migration in a scratch assay, which appears to be dependent on caspase-11. However, a more conclusive result would be obtained if IL-17A was administered to both Casp-11+/− and Casp-11+/+ groups in an *in vivo* wound model. Administration of IL-17A *in vivo* would determine if IL-17A increases the rate of wound repair and if it is mediated by caspase-11. While this study has not elucidated the mechanism of IL-17A induced proliferation, it is most likely mediated by up-regulation of expression of caspase-11 via NFκB. Stimulation of BMDMs with IL-17A and analysis of caspase-11 expression would determine if IL-17A mediates up-regulation of caspase-11.

While the BMDM scratch assay is a useful assay to determine preliminary differences in primary macrophages migration patterns, it is a single cell type taken out of context of an *in vivo* situation. It was therefore more beneficial to study the wound repair process *in vivo* in murine skin. Findings from our study reveal that caspase-11 deficiency results in an impaired wound repair response *in vivo* and that caspase-11 is up-regulated in wounds two days after wound infliction. While there have been no previous studies investigating the role of caspase-11 during the wound healing process, a recent study has reported that, in the absence of both NLRP3 and caspase-1, a delayed wound repair response is also observed (203). Interestingly, both NLRP3 and caspase-1 deficient groups display reduced angiogenesis. With this in mind, it would be interesting to investigate the levels of angiogenesis in Casp-11+/− mice during this wound healing model. Conversely, sustained inflammasome activation in diabetic humans and mice has been suggested to
impair the wound healing process (224). It would therefore appear that the role of the inflammasome is complex, and tightly regulated, during the repair process.

Our study has also suggested that caspase-11 deficiency does not impact inflammatory cytokine production (IL-1β, IL-1α, IL-18, TNF-α, IL-6, IL-10) during this wound healing model. However, measurements taken within the first 24-hours may give more insight into the impact of caspase-11 on cytokine secretion. The earliest time point measured in this study was taken at 2 days after wound infliction. While caspase-11 expression is still present at this time, it is possible that other inflammatory mediators have begun to compensate for the absence of caspase-11 during this process. Gene array expression data has suggested that caspase-11 is significantly increased within 24 hours, therefore it would be necessary to investigate the cytokine production of both groups at this time before one can conclusively say that caspase-11 does not impact pro-inflammatory cytokine production during wound repair. As IFN-γ and IL-17A both require caspase-11 to induce proliferation in this scratch assay, measuring their production levels from in vivo wound sections may identify if they play a role in this mechanism.

The Imiquimod-induced model of psoriasis is a widely used model to study the disease pathogenesis of psoriasis. The increased expression levels of human caspase-4 and caspase-5 observed in gene array datasets was confirmed by the murine IMQ-induced psoriasis, as findings presented in this chapter have shown that caspase-11 expression is increased in murine skin lesions. It was additionally shown that in the absence of caspase-11, the disease severity of IMQ-induced psoriasis is less severe. Increased levels of caspase-1 and caspase-5 have been reported in human lesions of psoriasis however, the exact mechanism of their up-regulation is still unclear (208)(210). An interesting study suggests that caspase-1 processing of Lyn kinase may be linked to psoriasis. Marchetti et al, reported that transgenic mice expressing cleaved Lyn display severe spontaneous psoriasis-like symptoms (225). These findings suggest a definite link between inflammatory caspases and psoriasis.

While there have been no studies to date investigating the role of caspase-1 and caspase-11 in murine IMQ-induced psoriasis, a study using SHARPIN-deficient mice, which exhibit chronic proliferative dermatitis (cpdm) has identified a possible role for inflammatory caspases-1 and -11 in skin disorders. Cpdm is a spontaneous multi-organ disease with similar characteristics to atopic dermatitis and human psoriasis. Douglas et
al. reported that caspase-11 and caspase-1 expression is significantly increased in cpdm mice (226). Additionally, genetic ablation of both caspase-11 and caspase-1 in cpdm mice resulted in significantly less skin inflammation and delayed disease onset but no differences in spleen size or systemic immunological disease severity (226). Our study presents very similar observations in the murine IMQ-induced psoriasis model, as we show that Casp-11<sup>-/-</sup> mice undergo less skin inflammation (erythema and scaling), but no differences in spleen weights compared to WT mice, suggesting that systemic inflammation is occurring at a similar level in both groups. Thus, our findings suggest that caspase-11 has a clear role in the pathogenesis of psoriasis locally, in the skin, but has no major impact on the systemic effects of this disease model. The exact mechanism of the up-regulation and activation of caspase-11 in Imiquimod-induced psoriasis is still unclear.

As psoriasis is a chronic inflammatory skin disease that occurs as a result of increased and dysregulated proliferation of keratinocytes, it was hypothesised that Casp-11<sup>+/+</sup> mice would display less thickening of the epidermis. However, this was not the case when epidermal thickness was measured following 5 days of Aldara treatment. In contrast, Douglas et al. have shown that in a cpdm model of skin disease, Casp-11<sup>+/+</sup>Casp-1<sup>-/-</sup> mice display a decrease in epidermal thickening when compared to cpdm alone (226). This would suggest that either the combined deficiency of caspase-11 and caspase-1 or that caspase-1 deficiency alone is responsible for the decreased epidermal thickening in cpdm mice. Although our findings suggest that Casp-11<sup>+/+</sup> mice do not display a decrease in epidermal thickening, there was an observable difference in the proliferation of keratinocytes of the epidermis of Casp-11<sup>+/+</sup> mice at this late time observed. Lower levels of PCNA positive cells in Casp-11<sup>+/+</sup> skin suggest that caspase-11 may be responsible for driving the dysregulated proliferation of keratinocytes in this model or alternatively responsible for the secretion of an inflammatory mediator that is capable of driving this proliferation.

Collectively, results from this preliminary study have shown that caspase-11 is an important inflammatory mediator in both a murine wound healing model and Imiquimod-induced psoriasis model. Delayed healing in Casp-11<sup>+/+</sup> mice suggests that caspase-11 is most likely important in driving the wound healing process, however further research must be performed to confirm the mechanism and purpose of its upregulation during this
process. Additionally, the protected phenotype observed in caspase-11 deficient mice during Imiquimod-induced psoriasis has highlighted caspase-11 as a potential therapeutic target for the treatment of psoriasis. As the role of caspase-11 in driving the pathogenesis of psoriasis has significant translational impact, it was decided that further work would focus on elucidating the mechanistic impact of caspase-11 deficiency on the murine psoriasis phenotype.
Figure 3.1: Murine Casp1 and Casp11 mRNA expression levels are increased in wound tissue up to 24 hours following injury.

(A) Casp1 and (B) Casp11 mRNA expression levels in murine skin following wound incision were analysed using the dataset GDS4602. Graphs are shown to indicate the gene expression values in individual mice. Two-way Anova found ** p < 0.01, *** p < 0.001.
Figure 3.2: Caspase-1 mRNA expression levels are increased in human psoriatic lesions in comparison to non-lesion and control areas. Relative caspase-1 mRNA expression levels in normal, non-lesional and lesional skin were analysed using the following datasets: GDS4602 (Nair et al, 2009), GDS3539 (Yao et al, 2008), GDS2518 (Reischl et al, 2007), GDS4600 (Suarez-Farinas et al, 2012). Graphs indicate the gene expression values in individual patients (GDS4602 n=88, GDS3539 n=28, GDS2518 n=13, GDS4600 n=85). Wilcoxon test was used to evaluate significant differences between two groups and Kruskall Wallis test was used to test between three groups. ** p < 0.01, *** p < 0.001.
Figure 3.3: Caspase-4 mRNA expression levels are increased in human psoriatic lesions in comparison to non-lesional and control areas.

Relative caspase-4 mRNA expression levels in normal, non-lesional and lesional skin were analysed using the following datasets: GDS4602 (Nair et al, 2009), GDS3539 (Yao et al, 2008), GDS2518 (Reischl et al, 2007), GDS4600 (Suarez-Farinas et al, 2012). Graphs indicate the gene expression values in individual patients (GDS4602 n=88, GDS3539 n=28, GDS2518 n=13, GDS4600 n=85). Wilcoxon test was used to evaluate significant differences between two groups and Kruskall Wallis test was used to test between three groups. ** p < 0.01, *** p < 0.001.
Figure 3.4: Caspase-5 mRNA expression levels are increased in human psoriatic lesions in comparison to non-lesional and control areas.

Relative caspase-5 mRNA expression levels in normal, non-lesional and lesional skin were analysed using the following datasets: GDS4602 (Nair et al, 2009), GDS3539 (Yao et al, 2008), GDS2518 (Reischl et al, 2007), GDS4600 (Suarez-Farinas et al, 2012). Graphs indicate the gene expression values in individual patients (GDS4602 n=88, GDS3539 n=28, GDS2518 n= 13, GDS4600 n=85). Wilcoxon test was used to evaluate significant differences between two groups and Kruskall Wallis test was used to test between three groups. ** p < 0.01, *** p < 0.001.
Figure 3.5: Caspase-2 mRNA expression levels are similar in human psoriatic lesions in comparison to non-lesional and control areas.

Relative caspase-2 mRNA expression levels in normal, non-lesional and lesional skin were analysed using the following datasets: GDS4602 (Nair et al, 2009), GDS3539 (Yao et al, 2008), GDS2518 (Reischl et al, 2007), GDS4600 (Suarez-Farinas et al, 2012). Graphs indicate the gene expression values in individual patients (GDS4602 n=88, GDS3539 n=28, GDS2518 n=13, GDS4600 n=85). Wilcoxon test was used to evaluate significant differences between two groups and Kruskall Wallis test was used to test between three groups. *** p < 0.001.
Figure 3.6: Genotyping of Casp-11+/+, Casp-11-/- and Casp-11+/- C57BL/6J mice.

Genomic DNA of Casp-11+/+, Casp-11-/- and Casp-11+/-, C57BL/6J mice, was isolated and amplified using primers designed to distinguish the WT caspase-11 gene and the insert, knocking out caspase-11. DNA was amplified and separated on a 2% Agarose gel, with Syber Safe DNA stain. Agarose gels were run for 1 hour at 120V and DNA bands were visualised under Transillumination using GelDoc-it system. Caspase-11 in WT mice is observed at 200 bp and disrupted caspase-11 in KO mice is observed at 600 bp. Heterozygous mice display both 200 bp and 600 bp bands.
BMDMs were isolated from *Casp-11<sup>-/-</sup>* and *Casp-11<sup>+/+</sup>* mice and cultured in GM-CSF for 7 days. On day 7, BMDMs were seeded at 2 x 10<sup>5</sup> cells/mL in 24 well-plates. 24 hours later, a scratch wound was applied to each well using p200 tip. Photos were taken on this day and for a further three days and representative images are shown in (A). Magnification was 4X. Average cell migration into wound was graphed using Prism (B). Data represents mean ± SEM n=3. Two-way ANOVA found no significant differences between *Casp-11<sup>+/+</sup>* and *Casp-11<sup>-/-</sup>* groups.
Figure 3.8: LPS stimulated *Casp-11*−/− and *Casp-11*+/− BMDMs proliferate and migrate at the same rate in a scratch wound assay. BMDMs were isolated from *Casp-11*−/− and *Casp-11*+/− mice and cultured in GM-CSF for 7 days. On day 7, BMDMs were seeded at 2 x 10^5 cells/mL in 24 well-plates. 24 hours later, a scratch wound was applied to each well using p200 tip. Cells were stimulated with 1 µg/mL LPS. Photos were taken on this day and for a further three days and representative images are shown in (A). Magnification was 4X. Average cell migration into wound was graphed using Prism (B). Data represents mean ± SEM n=3. Two-way ANOVA found no significant differences between *Casp-11*+/+ and *Casp-11*−/− groups.
Figure 3.9: IFN-γ stimulated Casp-11−/− BMDMs proliferate and migrate at a slower rate than Casp-11+/+ BMDMs in a scratch wound assay.

BMDMs were isolated from Casp-11−/− and Casp-11+/+ mice and cultured in GM-CSF for 7 days. On day 7, BMDMs were seeded at 2 x 10⁵ cells/mL in 24 well-plates. 24 hours later, a scratch wound was applied to each well using p200 tip. Cells were stimulated with 20 ng/mL IFN-γ. Photos were taken on this day and for a further three days and representative images are shown in (A). Magnification was 4X. Average cell migration into wound was graphed using Prism (B). Data represents mean ± SEM n=3. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 3.10: IL-1β stimulated Casp-11−/− and Casp-11+/+ BMDMs proliferate and migrate at the same rate in a scratch wound assay.

BMDMs were isolated from Casp-11−/− and Casp-11+/+ mice and cultured in GM-CSF for 7 days. On day 7, BMDMs were seeded at 2 x 10^5 cells/mL in 24 well-plates. 24 hours later, a scratch wound was applied to each well using a 200 μl pipette tip. Cells were stimulated with 20 ng/mL IL-1β. Photos were taken on this day and for a further three days and representative images are shown in (A). Magnification was 4X. Average cell migration into wound was graphed using Prism (B). Data represents mean ± SEM n=3. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 3.11: IL-17A stimulated Casp-11⁻/⁻ BMDMs proliferate and migrate at a slower rate than Casp-11⁺/+ BMDMs in a scratch wound assay. BMDMs were isolated from Casp-11⁻/⁻ and Casp-11⁺/+ mice and cultured in GM-CSF for 7 days. On day 7, BMDMs were seeded at 2 x 10⁵ cells/mL in 24 well-plates. 24 hours later, a scratch wound was applied to each well using p200 tip. Cells were stimulated with 5 ng/mL. Photos were taken on this day and for a further three days and representative images are shown in (A). Magnification was 4X. Average cell migration into wound was graphed using Prism (B). Data represents mean ± SEM n=3. Two-way ANOVA found *** p < 0.001.
Figure 3.12: *Casp-11*⁻/⁻ mice are less efficient at healing wounds than *Casp-11*⁺/⁺ mice following punch incisions to skin.

2 x 3 mm punch incisions were excised into the back of *Casp-11*⁺/⁺ and *Casp-11*⁻/⁻ mice. Photographs were taken daily to measure healing of the wounds (A). Image J software was used to measure circumference of wound at each day and percentage of the original wound was calculated. Scores were graphed using GraphPad Prism (B). N=3 for both groups. Two-way ANOVA found no significant differences between *Casp-11*⁺/⁺ and *Casp-11*⁻/⁻ groups.
Figure 3.13: Caspase-11 expression is up-regulated in two day old skin wounds of Casp-11+/+ mice.
2 x 3 mm punch biopsies were inflicted to the backs of Casp-11+/+ and Casp-11-/- mice. Wounds after two days were dissected and snap frozen. Wounds were then homogenised and protein concentration was normalised using BCA assay. Western blots were probed for caspase-11, capsase-1, and IL-1β as shown. Control groups = 2 and Wound groups = 3.
Figure 3.14: Similar levels of pro-inflammatory cytokine production were observed in Casp-11\textsuperscript{+/+} and Casp-11\textsuperscript{-/-} skin explant cultures 2, 3 or 11 days after wound infliction.

2 x 3 mm punch biopsies were inflicted to the backs of Casp-11\textsuperscript{+/+} and Casp-11\textsuperscript{-/-} mice. Wounds after two, three or eleven days were dissected and wound explant cultures were cultured for 24 hours in 0.5 mL DMEM. The weight of the wound was then measured and all data was normalised against weight. IL-1\textbeta\ (A), IL-1\alpha\ (B), IL-18 (C), TNF-\alpha\ (D), IL-6 (E) and IL-10 (F) production was measured by ELISA and graphed using GraphPad Prism. All groups consisted of n=3. Two-way ANOVA found no significant differences between Casp-11\textsuperscript{+/+} and Casp-11\textsuperscript{-/-} groups.
Figure 3.15: Reduced disease severity in Casp-11+/− mice following 5 days of Aldara treatment.

Disease severity scoring of Imiquimod-induced psoriasis in Casp-11+/+ and Casp-11−/− mice. Mice were treated with Aldara cream (5% Imiquimod), each mouse receiving 50mg per day. Back skin was photographed daily and representative images are shown in figure (A). Scaling (B) and Erythema (C) were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. Spleen weight was measured after 4 days of Aldara treatment (D). Weight loss was monitored each day (E). Control N=2 and Imiquimod treatment N=5. Two-way ANOVA was used to analyse statistical significance between Casp-11+/+ and Casp-11−/− groups. *p<0.05, ** p < 0.01, *** p < 0.001.
Figure 3.16: Caspase-11 is up-regulated in Casp-11\textsuperscript{+/+} mice back skin following 5 days of treatment with Aldara.

Mice were sacrificed and whole skin extracts were dissected after 5 days of Aldara treatment. Skin extracts were snap frozen on the day of dissection in liquid nitrogen and homogenised and normalised with BCA assay at a later date. Untreated and Aldara treated Casp-11\textsuperscript{+/+} and Casp-11\textsuperscript{-/-} lysates were run on 12% SDS-PAGE gels, and probed for caspase-11, caspase-1 and Actin as a loading control. Control (N=2) and Imiquimod (N=5) for both Casp-11\textsuperscript{+/+} and Casp-11\textsuperscript{-/-} groups.
Figure 3.17: Histological H&E staining of back skin shows no differences in epidermal thickness between Casp-11+/+ and Casp-11−/− mice after five days of Imiquimod treatment.

Mice were treated with 50 mg Aldara for five days. Mice were sacrificed and back skin was excised. Back whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of control and Imiquimod treated back skin taken at day 5 were stained using Haemotoxylin and Eosin (at 10X magnification) (A). Epidermis thickness was measured using an average of three images per piece of tissue and three pieces of tissue per mouse. Control (N=2) and Imiquimod (N=5). Thickness was measured in nm using Image J software (B). Scale bar = 20 μm. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 3.18: PCNA staining of back skin shows less proliferation in the epidermis of Casp-11+/ mice after five days of Aldara treatment.

Mice were treated with 50 mg Aldara for five days. Mice were sacrificed and back skin was excised. Back whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of control and Aldara treated back skin taken at day 5 were probed using anti-PCNA, a proliferation marker (A). PCNA positive cells were counted per field in epidermis in an average of three images per piece of tissue and three pieces of tissue per mouse (at 10X magnification) (B). Control (N=2) and Aldara (N=5). Two-way Anova found *p<0.05. Scale bar = 20 μm
Chapter 4: Investigating the role of Caspase-11 in Imiquimod-induced Psoriasis
4. Investigating of the role of Caspase-11 in Imiquimod-induced Psoriasis

4.1 Introduction

Psoriasis is the chronic inflammatory skin disease that affects 2% of the population and has an unclear aetiology. The skin is the largest organ of the body and typically encompasses approximately 20 square feet. The skin is comprised of three main compartments. The hypodermis is the inner most layer and is composed of adipose and connective tissue which acts as insulation. The next layer of the skin is the dermis and consists of collagenous connective tissue, sweat glands, blood vessels and a range of immune cells. The epidermis is the outermost layer and is largely comprised of keratinocytes but also plays host to the DC-like subtype of cells known as LCs. The epidermis forms a waterproof barrier protecting internal organs from the environment.

Psoriasis is typically characterised by the formation of demarked erythematous plaques on the surface of the skin with the presence of scaling. Scaling occurs as a result of hyperproliferation of the keratinocytes in the epidermis with premature maturation and incomplete cornification of the keratinocytes and retention of nuclei in the outer most layer, the stratum corneum. In comparison to healthy skin, the growth rate of basal keratinocytes is dramatically increased. As a result, the epidermis increases in thickness (acanthosis) with the development of finger-like, elongated rete ridges, protruding into the dermis. The granular layer of keratinocytes, the first layer of terminally differentiating keratinocytes, becomes much smaller or non-existent. In addition, there is an infiltration of neutrophils and activated CD8+ T cells into the epidermis and an increase in infiltrating macrophages, dendritic cells, mast cells, neutrophils and CD3+ T cells into the dermis. An increase in the dilation and elongation of the blood vessels in the dermal papillae is also typically observed during psoriasis (227).

The antimicrobial peptide, LL37, is proposed to be released from keratinocytes during psoriasis and is believed to contribute to the initiation of psoriasis by forming complexes with self-nucleic acids in psoriasis lesions (170). The LL37/nucleic acid complexes are believed to enter into plasmacytoid (pDCs) and conventional DCs (cDCs) where they are recognised by the intracellular sensors, TLR7, 8 and 9 (170, 228). Activation of TLR7, 8 and 9 result in the production of type-I IFNs. Activation of dermal cDCs by Type I IFNs
have been reported to induce the production of IL-23 which further acts on cells to induce IL-17 and other inflammatory cytokine production. Interestingly, the murine Imiquimod-induced psoriasis model mimics the initiation process of psoriasis by using Imiquimod to activate TLR7/8 in place of the LL37/nucleic acid complexes discussed (229). The murine Imiquimod-induced psoriasis model is now a widely used model in the study of psoriasis in mice.

Caspase-11, the murine ortholog of caspase-4 and -5, has been implicated in the development of psoriasis during the initial focus of this project. This chapter will therefore focus on further characterisation of the role caspase-11 plays during this chronic disease.
4.2 Results

4.2.1 A deficiency of caspase-11 results in a significantly lessened psoriasis phenotype when compared to littermate controls

Findings presented in Chapter 3 suggest that caspase-11 is implicated in the pathogenesis of psoriasis. However, to confirm that these results were not due to strain, breeding or housing differences, we sought to repeat the trial using littermate Casp-11+/+ and Casp-11−/− mice. As performed before, the back skin of all mice were shaved 48 hours in advance and Aldara or Vaseline, as control, was applied daily for four consecutive days to induce psoriasis symptoms (Figure 4.1).

Similar to the results shown in Figure 3.15, Casp-11−/− mice underwent significantly less erythema and scaling when compared to littermate Casp-11+/+ mice in this experimental model of psoriasis (Figure 4.1 (A-C)). However, in littermate Casp-11−/− and Casp-11+/+ mice there were no differences in weight loss over the course of the four days of Aldara treatment (Figure 3.7 (E)). Additionally, there were no significant differences in spleen enlargement between Casp-11+/+ and Casp-11−/− mice (Figure 4.1 (D)). These findings show that a deficiency in caspase-11 has a localised, protective effect in the skin, but does not appear to affect the systemic inflammation that occurs during this model.

4.2.2 Caspase-11 deficient mice display less angiogenesis in the skin during Imiquimod-induced psoriasis

As we have seen that Casp-11−/− mice display a less severe phenotype to Imiquimod-induced psoriasis, we wanted to investigate the classical hallmarks of psoriasis in order to determine which of these are altered in the absence of caspase-11. There are typical characteristics of psoriasis that are observed in human psoriatic lesions and are mimicked during the murine Imiquimod-induced psoriasis model. The first characteristic we aimed to examine was angiogenesis. Angiogenesis is defined as the formation of new capillaries from pre-existing blood vessels. Migration of endothelial cells are directed by the gradient of angiogenic markers, therefore we aimed to analyse angiogenic makers present in the skin during this disease. In addition, angiogenesis is tightly regulated by both pro- and anti-angiogenic proteins, therefore it was important to investigate both types of markers (230).
Vascular endothelial growth factor (VEGF) is one of the key pro-angiogenic factors implicated in the pathogenesis of psoriasis, with patient serum VEGF levels correlating with disease severity (231, 232). mRNA VEGF expression levels were examined in the skin of Casp-11+/+ and Casp-11−/− skin treated with Aldara for 4 days (Figure 4.2 (A)) and 24 hours (Figure 4.2 (D)). Interestingly, at 24 hours there is significantly more VEGF mRNA in Casp-11−/− skin when compared to Casp-11+/+, however an inverse trend is observed after 4 days of Aldara treatment. Less VEGF mRNA suggests that lower levels of pro-angiogenic signalling are occurring in Casp-11−/− skin at 4 days of Aldara treatment, which correlates with the disease severity observed at this time. The increased VEGF expression observed in Casp-11−/− skin at 24 hours may suggest that a deficiency of caspase-11 results in a misbalance in angiogenesis in the skin. This suggests that caspase-11 likely plays a role in the kinetics of VEGF expression in the skin, and a deficiency of caspase-11 results in alteration of its expression.

The angiopoietins, Ang-1 and Ang-2, along with their receptor Tie-2 are also crucial mediators in driving angiogenesis in psoriasis (233). While Ang-2 destabilises blood vessels, acting as an antagonist for Tie2, in the presence of other pro-angiogenic markers Ang-2 acts to drive angiogenesis. Targeting Ang-2 has been successful in the treatment of psoriasis, highlighting its importance in driving its pathogenesis (233). We therefore sought to determine the mRNA expression levels of Ang-2 in the skin of Casp-11+/+ and Casp-11−/− skin after 4 days (Figure 4.2 (B)) and 24 hours (Figure 4.2 (E)) of Aldara treatment. Similar to VEGF, analysis of Ang-2 expression displayed significantly less Ang-2 in Casp-11−/− skin when compared to Casp-11+/+ after 4 days of Aldara treatment. However, conversely to VEGF, there were no apparent differences between the expression of Ang-2 in Casp-11+/+ and Casp-11−/− skin after 24 hours of Aldara treatment (Figure 4.2 (E)). The mRNA expression levels of Ang-2 observed in caspase-11 deficient skin after 4 days of Aldara treatment are significantly lower than Casp-11+/+ skin and even slightly lower than untreated skin (Figure 4.2 (B)). This suggests that VEGF and Ang-2 undergo different kinetics of expression in the skin during this model, and are significantly altered in the absence of caspase-11.

As angiogenesis is tightly regulated by both pro- and anti-angiogenic markers, we next sought to investigate an anti-angiogenic marker that is implicated in psoriasis. Thrombospondin-1 (TSP-1) is an endogenous suppressor of angiogenesis that inhibits
endothelial cell proliferation and migration (234). In addition, keratinocytes isolated from lesions of psoriatic patients display significantly reduced expression of TSP-1 (235). Examination of TSP-1 mRNA expression levels in Casp-11+/+ and Casp-11−/− skin analysed after 4 days and 24 hours of Aldara treatment is shown in Figure 4.2 (C) and (F) respectively. Expression of TSP-1 is significantly higher in Casp-11−/− skin when compared to Casp-11+/+ skin after 4 days of Aldara treatment, consistent with the reduced angiogenesis observed in Casp-11−/− mice during this model.

Findings presented in Figure 4.2 confirm that a deficiency in caspase-11 results in less angiogenesis during the later stage of Imiquimod-induced psoriasis, which is consistent with the lower erythema scores assigned to Casp-11−/− skin during this model. However, examination of angiogenic marker expression at earlier stages in Imiquimod-induced psoriasis suggests that caspase-11 may in fact be required for regulation of VEGF expression prior to the progression of the disease.

4.2.3 Less differentiation and proliferation occurs in the epidermis of Casp-11−/− mice during Imiquimod-induced psoriasis

Analysis of angiogenic markers has identified a clear reduction in one of the typical characteristics of psoriasis in Casp-11−/− skin, therefore we next sought to determine whether another typical characteristics of psoriatic lesions were affected. Epidermal thickening occurs in psoriatic lesions due to the increased differentiation and proliferation that occurs during the disease. H&E stained skin sections were analysed to determine the thickening of epidermis over time in this model. Findings presented in Figure 4.3 show that significantly less epidermal thickening occurred in Casp-11−/− skin after 24 hours (B and D) of Aldara treatment. While the trend appears to continue after 4 days of Aldara treatment, no significant difference was observed at this time point (A and C). These findings suggest that reduced keratinocyte differentiation and proliferation occurs during the early stages of IMQ-induced psoriasis in caspase-11 deficient mice.

To examine the effects of caspase-11 expression on keratinocyte proliferation in more detail, proliferating nuclear cell antigen (PCNA) was used as a marker to analyse the differences in keratinocyte proliferation between Casp-11+/+ and Casp-11−/− skin during this model. PCNA is a widely used marker to investigate proliferation, and its expression is primarily observed in the epidermis of the skin. Findings presented in Figure 4.4 show that caspase-11 deficient mice display significantly less proliferation in the basal layer of
the epidermis after 4 days of Aldara treatment (A and C). While a similar trend is observed in the skin examined at 24 hours post Aldara treatment, there are no significant differences at this time point (B and D).

Collectively these results confirm that a deficiency of caspase-11 results in less epidermal thickening and proliferation during Imiquimod-induced psoriasis. Differentiation and proliferation of keratinocytes leads to cornification of the outermost epidermal layer, resulting in scaling which is typically observed in psoriasis. We have shown that caspase-11 deficient mice display significantly less scaling during the Imiquimod-induced psoriasis model, and we have now confirmed that less epidermal thickening and proliferation also occurs at a histological level during this model.

4.2.4 Less cell death is observed in the skin of Casp-11−/− mice during Imiquimod-induced psoriasis

During psoriasis, keratinocytes undergo increased proliferation and differentiation that begins in the basal epidermal layer and results in an increase in epidermal thickening. However, in the outermost layers of the epidermis, keratinocytes begin to terminally differentiate and die. Dead keratinocytes build up during psoriasis and the increase in dead cells results in a layer of scaling on the surface of the lesion. The decreased scaling, epidermal thickness and proliferation observed in Casp-11+/− mice following Aldara treatment would lead us to hypothesise that there will most likely be differences in the level of cell death occurring between Casp-11+/− and Casp-11−/− mice. Caspase-11 is responsible for mediating the inflammatory form of cell death, pyroptosis. Therefore we next aimed to investigate the level of cell death occurring in the skin of both groups. To investigate this, the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) staining technique was used. TUNEL staining labels the blunt ends of DNA that occurs during DNA fragmentation of both apoptosis and pyroptosis (236).

Fragmented DNA was probed with TdT and viewed microscopically. Untreated and Aldara treated back skin sections of Casp-11+/− and Casp-11−/− were stained and TUNEL positive cells in the epidermis were counted. Scores were graphed using GraphPad prism. Significantly less cell death was observed in the epidermis of Casp-11−/− mice following four days (Figure 4.5 (A and C)) and 24 hours (Figure 4.5 (B and D)) of Aldara treatment. This result implies that caspase-11 contributes significantly to the pathogenic levels of cell death that occur during this model. However, as TUNEL positive cells can
either be undergoing apoptosis or pyroptosis, it was necessary to further distinguish between the two forms of cell death.

To investigate protein cleavage of mediators of both apoptosis and pyroptosis, western blots were probed for Gasdermin D, a pore forming protein which is processed by caspases to result in pyroptosis, and caspase-3, a crucial mediator of apoptosis (130)(36). Immunoblot of Gasdermin D suggest there is less full length Gasdermin D and less cleaved Gasdermin D (30 kDa) in Casp-11+/ skin sections after 4 days of Aldara treatment (Figure 4.6 (A)). There does not appear to be any difference in caspase-3 cleavage between Casp-11+/+ and Casp-11−/− Aldara treated groups. As caspase-3 is the final caspase to be cleaved during both the intrinsic and extrinsic apoptosis pathways, it would appear that apoptotic pathways are occurring similarly in both Casp-11+/+ and Casp-11−/− skin (237). Immunoblots of Gasdermin D and caspase-3 at 24 hours of Aldara treatment suggest there is no determinable cleavage of either protein at this time (Figure 4.6 (B)). Immunoblots presented in Figure 4.6 also confirm that caspase-11 expression is induced at both 4 days (A) and 24 hours (B) of Aldara treatment confirming the involvement of caspase-11 in the pathogenesis of this disease.

In addition to Gasdermin D investigation, another method was used to investigate pyroptosis. Lactate dehydrogenase (LDH) is normally present in the cytosol of healthy cells. During pyroptosis and necrosis, cells lose membrane integrity and become lysed, releasing LDH (237). Contrasting, apoptotic cells form blebs when they undergo cell death and do not release any cellular components unless they undergo a secondary necrosis. Therefore, measuring the release of LDH is a widely used technique to quantify levels of pyroptosis (237). Skin explant cultures of both Casp-11+/+ and Casp-11−/− mice were cultured after four days and 24h of Aldara treatment. Findings displayed in Figure 4.6 reveal that Casp-11−/− mice produce significantly less LDH than Casp-11+/+ following treatment with Aldara at 4 days (C) but not 24 hours (D). Collectively these findings suggest that a deficiency of caspase-11 results in less Gasdermin D cleavage and less LDH production, confirming that there is less pyroptosis occurring in the skin of Casp-11−/− mice at both early and late stage Imiquimod-induced psoriasis.
4.2.5 There are no differences in cytokine production of Casp-11+/+ and Casp-11−/− skin and explant cultures

Crosstalk between immune cells is a crucial aspect of the immune response that occurs during psoriasis. Keratinocytes, dendritic cells, neutrophils and other innate-like immune cells are responsible for the production of pro-inflammatory cytokines and anti-microbial peptides that are released in order to recruit other innate and adaptive cells. The pro-inflammatory cytokine expression profiles in skin explant cultures from Aldara treated Casp-11+/+ and Casp-11−/− mice were measured by ELISA to address whether the protected phenotype of Casp-11−/− mice to Aldara was due to dysregulated inflammatory cytokine production. Following five days of Aldara treatment, skin explants were cultured for 24 hours. Supernatants were analysed by ELISA to determine pro-inflammatory cytokine secretion levels. As shown in Figure 4.7, there were no differences in production of IL-1β (A), IL-18 (B), IL-1α (C), IL-10 (D) and IL-6 (E) expression levels by Casp-11+/+ and Casp-11−/− mice. These results would suggest that inflammasome activation, cell death, and NFκB activation are seemingly unaffected by the absence of caspase-11 during Imiquimod induced psoriasis, and are not likely the cause of the protected phenotype of Casp-11−/− mice observed during this model. However, it is very possible that measuring cytokine expression levels at this point in the trial may be too late. As cytokines are released within hours, it was next decided to examine cytokine production following 24 hours of Aldara treatment. Figure 4.8 shows that at 24 hours post Aldara treatment, there were no significant differences in the secretion of IL-1β (A), IL-18 (B), IL-1α (C), IL-10 (D) and IL-6 (E) by Casp-11+/+ and Casp-11−/− mice. This would conclusively suggest that a deficiency of caspase-11 does not result in altered inflammasome-mediated and NFκB-mediated cytokine secretion profiles.

There are a range of additional cytokines known to play important roles in mediating psoriasis. IL-17A and F are two IL-17 family members implicated in psoriasis and share the greatest homology of all IL-17 subtypes. Both IL-17A and IL-17F bind to the IL-17RA and IL-17RF receptor complex. IL-17A has been reported to induce increased proliferation and uncontrolled differentiation of keratinocytes (238). This subsequently contributes to skin-barrier disruption via downregulation of expression molecules, such as filaggrin, that are responsible for keratinocyte differentiation (239). Findings presented
in Figure 4.9 (A) and Figure 4.10 (A) suggest that IL-17A mRNA expression levels are not affected by the ablation of caspase-11 at either 4 days or 24 hours (respectively). IL-17F has also been implicated in psoriasis, with expression increasing up to 33-fold in psoriatic lesions (240). Similarly to IL-17A, IL-17F acts directly on keratinocytes to induce increased production of pro-inflammatory cytokines and anti-microbial peptides that are implicated in psoriasis (241). As a result, we sought to investigate the mRNA expression levels of IL-17F to determine if the ablation of caspase-11 affects the expression of IL-17F. However, analysis of IL-17F mRNA expression levels showed no significant differences between Casp-11<sup>+/+</sup> and Casp-11<sup>−/−</sup> mice after either 4 days (Figure 4.9 (B)) or 24 hours (Figure 4.10 (B)). Collectively these findings suggest that caspase-11 is not required for the induction of IL-17A and IL-17F expression during Imiquimod-induced psoriasis.

Type I IFNs (α and β) are primarily produced by pDCs and are typically induced in response to viral infection. However, their expression has also been implicated in triggering the initiation of psoriasis due to the influx of the plasmacytoid DCs into the psoriatic lesions (242). Following 4 days (Figure 4.9) and 24 hours (Figure 4.10) of Aldara treatment, Casp-11<sup>+/+</sup> and Casp-11<sup>−/−</sup> mice express similar levels of IFN-α (C) and IFN-β (D) mRNA, with no significant differences observed between the two groups. These findings suggest that ablation of caspase-11 does not affect the expression of type-I IFNs during Imiquimod-induced psoriasis.

IL-22 is an inflammatory cytokine that has been implicated in the pathogenesis of psoriasis. Expression of IL-22 is reported to be elevated in the plasma and skin of psoriasis patients (243). IL-22 induces hyperplasia in the epidermis and inhibits epidermal differentiation (243). The pro-inflammatory cytokine IL-23 is primarily produced by inflammatory DCs within the skin during inflammation (244), with keratinocytes and macrophages also contributing to the production of IL-23 (245, 246). IL-23 has been shown to result in expansion and maintenance of the T helper 17 subset of T cells (Th17) which typically produce the inflammatory cytokine IL-17. As a result, it was decided to investigate the mRNA expression levels of IL-23 and IL-22. While there appears to be higher expression of IL-23 in untreated mRNA samples from Casp-11<sup>−/−</sup> mice, there are no significant differences in mRNA expression of the cytokines following 4 days (Figure 4.9) or 24 hours (Figure 4.10) of Aldara treatment. Similarly, there are
no significant differences in the mRNA expression levels of IL-22 following 4 days of Aldara treatment (Figure 4.9 (F)) with no IL-22 production determined as early as 24 hours following Aldara treatment. These findings suggest that caspase-11 deficient mice express similar levels of IL-23 and IL-22 during Imiquimod-induced psoriasis.

4.2.6 Decreased leukocyte numbers are observed in Casp-11+/+ skin when compared to Casp-11−/− following three days of Aldara treatment

Infiltration of immune cells into both the dermis and epidermis is another key characteristic of psoriasis. It was therefore decided to investigate whether differences in the infiltration of immune cell populations occur in the skin in the absence of caspase-11. Mast cells are innate, tissue resident cells that were originally believed to be the main effector cell in allergy. However, now they have been identified as a key cell population that infiltrate into a psoriatic lesion. Mast cells have been determined to localise in the subepidermal connective tissue in psoriatic lesions and are major IL-22 producers in both psoriasis and atopic dermatitis (247). Considering this, it was decided to investigate if the mast cell infiltration differed between Casp-11+/+ and Casp-11−/− Aldara treated skin. Toluidine blue was used to stain mast cell granules in both Casp-11+/+ and Casp-11−/− skin. Figure 4.11 shows representative images of mast cell staining of both Casp-11+/+ and Casp-11−/− back skin following four days (A) and 24 hours (B) of Aldara treatment. Mast cell counts suggest there are no significant differences in mast cell number in the dermis of Casp-11+/+ and Casp-11−/− mice at either time points examined (Figure 4.11 (C) and (D) respectively). These results suggest that, at both advanced and early stages of the psoriasis model, caspase-11 deficiency does not impact the number of mast cells present in the dermis.

As there were no differences in mast cell infiltration in Casp-11+/+ and Casp-11−/− back skin, we next sought to investigate expression levels of innate cell populations, using CD11b. CD11b is an integrin family member protein that is expressed on the surface of leukocytes such as macrophages, neutrophils, granulocytes, monocytes and natural killer cells. CD11b is therefore used as a general leukocyte marker. As caspase-11 is primarily expressed in innate cells such as macrophages and DCs, we aimed to determine if the migration of CD11b+ cells was affected in the absence of caspase-11 during this model. Findings presented in Figure 4.12 show that there were less CD11b+ cells in the lower dermis layer of the skin after 4 days of Aldara treatment (A and C). While there was no
significance, there was a similar trend observed after 24 hours of Aldara treatment. Less CD11b+ cell infiltration in Casp-11−/− skin suggests that caspase-11 is required for infiltration of this cell population during Imiquimod-induced psoriasis.

LCs are an important DC-like cell type that are expressed in the epidermis of healthy skin and play an important role in sensing pathogens and danger signals. There are two distinct populations of LCs – short term and long term (248). Short term LCs are similar to monocytes and have high Cd11b expression. As we have observed significantly less Cd11b+ cells in the skin of Casp-11−/− mice after 4 days of Aldara treatment, we next aimed to determine if a deficiency in caspase-11 alters the presence of LCs in the skin. Figure 4.13 shows that Casp-11+/+ and Casp-11−/− mice display similar levels of Langerin positive cells in 4 day (A, C) and 24 hours (B, D) of Aldara treated skin.

As we have seen less infiltrating CD11b+ in the skin of Casp-11−/− mice, we next aimed to further investigate the infiltration of innate cells using antibody markers combined with flow cytometric analysis. However, as the skin is a very difficult tissue to manipulate and extract cells from, researchers often use spleens or skin draining lymph nodes to analyse the variation of cell populations affected in this model (229, 249–251). As there were only localised differences observed in the skin and no differences in the spleen weight of Casp-11+/+ and Casp-11−/− mice during this model, inguinal (skin-draining) nodes were chosen to analyse for flow cytometry. Casp-11+/+ and Casp-11−/− mice were treated with Aldara for two days and on the third day, inguinal lymph nodes were removed and digested using DNase I and Collagenase IA. Once digested, tissue was filtered and stained for various cell markers.

Figure 4.14 (C) shows that there are significantly less CD11b+ cells, as a percentage of viable CD45+, CD3/B220- cells, in Casp-11−/− inguinal nodes when compared to Casp-11+/+ following three days of Aldara treatment. The gating strategy that was used is shown in (A) and representative images are shown in (B). Findings presented here confirm that caspase-11 regulates migration of leukocytes towards the psoriatic lesion.

As we have seen less infiltrating CD11b+ cells in both the skin and the skin-draining nodes in caspase-11 deficient mice, we aimed to elucidate the exact cell population that may be causing the decreased number of CD11b+ cells. Neutrophils are a cell type that express CD11b, have been implicated in the pathogenesis of psoriasis and migrate into
the skin during early stages of the disease (252). We therefore sought to use flow cytometric analysis to investigate if neutrophil infiltration is affected by the absence of caspase-11 during this disease. Findings presented in **Figure 4.15** show that neutrophils (viable CD45⁺, CD3/B220⁻, CD11b⁺ and Ly6G⁺ cells) are not present in the skin-draining lymph nodes of untreated mice and small numbers of neutrophils are recruited to the area following induction of psoriasis (**Figure 4.15 (C)**). Gating strategies for determining neutrophil populations are shown in (A) and representative populations are shown in (B). As neutrophils play an important early role in mediating psoriasis, it may explain the low levels of neutrophils observed at this later time. Nevertheless, there are no observable differences in the recruitment of neutrophils to the inguinal nodes of Casp-11⁺/⁻ and Casp-11⁻/⁻ mice following three days of Aldara treatment.

Another important innate cell type that express CD11b and migrate towards the skin during psoriasis is macrophages. Macrophages also express caspase-11 once induced, therefore, we next aimed to compare the number of macrophages in Casp-11⁺/⁻ and Casp-11⁻/⁻ skin draining nodes. As can be seen in **Figure 4.16**, there are no differences in the number of viable macrophages (CD45⁺, CD3/B220⁻, CD11b⁺, Ly6G⁻, F4/80⁺) in the skin-draining nodes of Casp-11⁺/⁻ and Casp-11⁻/⁻ mice after 3 days of Aldara treatment. This would suggest that a deficiency of caspase-11 does not impact the number of macrophages present in the draining nodes after three days of this model.

Another innate cell type that are believed to play important roles in driving the pathogenesis of psoriasis are dendritic cells (DCs). The skin and draining nodes play host to two different DC populations, dermal DCs and LCs. Unfortunately the most widely used marker to distinguish DC populations, CD11c, was not successfully used to identify DCs during this experiment as it was digested from the cells during the digestion process. While enzymatic digestion is required to ensure the innate surface markers are accessible to antibodies, it has also been found to digest CD11c (and other markers) from the surface of cells explaining the problem encountered in this situation (253). Therefore, we have used gating out strategies to investigate possible DC populations. As we have gated out keratinocytes (keratinocytes do not express CD45), T and B cells (CD3/B220⁺ cells were gated out), macrophages, monocytes and neutrophils, (F4/80, Ly6C and Ly6G were gated out) it is plausible that the populations identified are likely DCs. Therefore, the populations examined hereafter are likely but not conclusively DC populations.
Skin researchers have identified CD24a as a marker that clearly distinguishes between dermal DCs and LCs without the use of CD11c (254). CD24a expression has been found to strictly occur with the expression of CD207 (Langerin) making it a useful marker to identify LCs (254). Findings shown in Figure 4.17 suggest there are no significant differences between the number of LCs in Casp-11+/+ and Casp-11− skin-draining nodes. While there is no statistical significance, there does appear to be slightly less LCs in Casp-11− nodes suggesting that caspase-11 may be required for the migrating of LCs during this model.

In addition, we sought to investigate alternative “DC populations”. By gating on CD24 negative cells, we next aimed to identify the two clear populations that emerged – CD11b+, CD103− and CD103+, CD11b−. The CD103+, CD11b− and CD11b+, CD103− DC populations are both classical DC populations (255). Based on the findings shown in Figure 4.18, it does not appear that either of these “DC” populations are affected by the absence of caspase-11 in the draining nodes during this model. Gating strategies are shown in (A) and representative images are shown (B). While we cannot conclusively report that DC populations are not affected by the absence of caspase-11, we can suggest that caspase-11 is not likely responsible for the recruitment of the DC populations examined. Further investigation is therefore required to conclusively determine if caspase-11 affects the migration of DCs to the skin during this model.

While we have seen clear differences in the migration of CD11b+ cells to the skin and draining lymph nodes, we have not yet been able to identify an exact CD11b+ cell population that is affected by the absence of caspase-11.

4.2.7 Casp-11− BMDMs produce significantly less Nitric Oxide

Having addressed the typical characteristics that occur during psoriasis, we next wanted to mechanistically determine how caspase-11 is contributing to this disease. To do so, we looked at inflammatory mediators that have been reported to play important roles in the skin and may be implicated in the pathogenesis of psoriasis. Nitric oxide (NO) is an extraordinary chemical that has been shown to have a diverse range of functions including a role in defence against pathogens, neurotransmission and smooth muscle relaxation. However, in recent years, a clear role for NO in the skin has been discovered. While NO is crucial for various key processes responsible for maintaining skin homeostasis, it can
also play harmful roles in skin physiology and result in skin pathologies. It is believed that dysfunctional NO regulation is linked to many skin disorders (256).

Before it was decided to investigate the role of NO in Imiquimod-induced psoriasis, a macrophage polarisation experiment was performed with a fourth year student in our lab. The aim of this experiment was to polarise BMDMs isolated from Casp-11+/- and Casp-11-/- mice into M1 and M2 macrophages and investigate the function of caspase-11, the non-canonical inflammasome, in both phenotypes. To confirm M1 polarisation, NO secretion was indirectly measured using Griess reagent. Results displayed in Figure 4.19 (A) suggest that caspase-11 deficiency results in a decreased production of nitrite (NO2-), a stable metabolite of NO. This observation led us to question whether a deficiency in caspase-11 would have an impact on NO production in Imiquimod-induced psoriasis.

To investigate this further, Casp-11+/+ and Casp-11-/- BMDMs were isolated and transfected with either Imiquimod or Aldara, with or without previous LPS priming. Interestingly, Casp-11-/- BMDMs primed with LPS and subsequently transfected with either Imiquimod or Aldara, produced significantly less NO than Casp-11+/+ BMDMs (Figure 4.19 (B)). These findings suggest that the “psoriasis-inducing” ligands are stimulating an increased production of nitric oxide, and that this production of nitric oxide may be mediated by caspase-11. A link between caspase-11 and nitric oxide production has not yet been reported in the literature.

Inducible Nitric Oxide Synthase II (NOS2) is the enzyme responsible for the production of NO in macrophages following exposure to LPS, cytokines and other factors. Genome wide association studies have identified a SNP in NOS2 as a susceptibility loci in psoriasis (257). Therefore, we next sought to investigate the gene expression of the inducible NOS isoform (NOS2) in psoriasis lesions. NOS2 expression can be induced in many cell types, including keratinocytes, melanocytes and fibroblasts. Interestingly, in all four datasets analysed, NOS2 expression levels are significantly higher in psoriatic lesions compared to non-lesions or healthy control skin (Figure 4.20 A-D).

Based on these findings, protein expression levels of NOS2 were analysed in Casp-11-/- and Casp-11+/+ Aldara treated skin homogenates. Interestingly, Western blot of skin homogenates revealed that Casp-11+/- Aldara treated homogenates had significantly less NOS2 expression when compared to Casp-11+/+ homogenates (Figure 4.21 (A)).
However, no observable differences were observed in mRNA expression levels of NOS2, or NOS3, the endothelial nitric oxide synthase, between Casp-11−/− and Casp-11+/+ skin at 4 days of Aldara treatment (Figure 4.21 (B and C) respectively) and 24 hours (data not shown). Furthermore, whole skin explant cultures of Casp-11+/+ and Casp-11−/− mice produced similar levels of NO, when measured spectrophotometrically using the Griess assay (Figure 4.21 (D)).

While there are no observable differences in the production of nitric oxide from skin explants and no differences in mRNA expression levels of NOS2 and NOS3, we have seen that Casp-11−/− BMDMs are hindered in their ability to produce NO and there is also an observable difference of NOS2 in the skin, at the protein expression level. We therefore hypothesised that caspase-11 may play a role in NOS2 induction in some but not all cell types. As dendritic cells are a cell type that are important in mediating psoriasis, we next aimed to investigate if in vitro transfection of Imiquimod or Aldara resulted in altered NOS2 expression or NO production in Casp-11−/− BMDCs. Findings presented in Figure 4.22 suggest that Casp-11−/− BMDCs may have slightly less NOS2 upregulation, however no significant differences in the production of NO were detectable. These findings suggest that DCs are not significantly affected by the absence of caspase-11 in mediating NO production.

The examination of specific cell types has not led to increased mechanistic insight regarding the role of caspase-11 during the pathogenesis of psoriasis. Therefore we tested an alternative experimental set-up to further investigate the role of caspase-11 during psoriasis. We previously showed that a deficiency in caspase-11 results in decreased migration of CD11b+ cells to both the skin and inguinal nodes during this model (Figure 4.12 and Figure 4.14). We aimed to determine whether Imiquimod or Aldara could induce the secretion of inflammatory mediators from the skin which were responsible for the recruitment of CD11b+ cells. To investigate this, skin explants were dissected from healthy Casp-11+/+ and Casp-11−/− mice. Skin explants were weighed to ensure uniform weight and cultured for 24 hours with either Imiquimod or Aldara. This conditioned media (CM) was subsequently sterile filtered and applied to BMDMs (Figure 4.23 (A)). To determine whether inflammatory mediators secreted into CM, from the skin of Casp-11+/+ and Casp-11−/− mice, could enhance macrophage migration rates, a scratch was generated in BMDM monolayers and migration over time was measured. Findings
presented in Figure 4.23 (B-H) show that there are no differences in the migration of either Casp-11+/+ and Casp-11−/− macrophages stimulated with CM from either Casp-11+/+ or Casp-11−/− skin. In addition, it appears that Imiquimod and Aldara-treated skin CM does not result in enhanced migration when compared to CM from untreated skin.

While CM from Aldara-treated skin did not appear to enhance macrophage migration, we next aimed to determine whether CM from treated skin is capable of driving inflammasome activation in BMDMs. Caspase-11 is involved in the regulation of non-canonical inflammasome activation (Figure 1.4). CM from Imiquimod and Aldara-treated skin was applied to BMDMs for 24 hours. IL-1β and IL-18 production by BMDMs were measured by ELISA (Figure 4.24 (A) and (B) respectively). Casp-11+/− BMDM secreted less IL-1β when compared to Casp-11+/+ BMDMs, but more notably, CM from Aldara-treated Casp-11+/− skin was not capable of inducing similar levels of IL-1β secretion induced by WT BMDM (Figure 4.24 A). In addition, CM from Aldara treated Casp-11+/− skin induced less secretion of IL-18 when compared to CM from Casp-11+/+ skin (Figure 4.24 (B)). These findings suggest that the absence of caspase-11 in the skin results in reduced activation of macrophages, resulting in less inflammatory cytokine secretion. We hypothesise that the reduced secretion of inflammatory mediators contributes to the reduced pathogenesis of Aldara-treated Casp-11−/− mice.

To further investigate the effect of inflammatory mediators released from Aldara-treated skin, we sought to investigate the ability of CM from treated skin to mediate NO production and cell death. Experimental set up was similar to Figure 4.23 (A). Immunoblots show that less NOS2 upregulation occurs in Casp-11−/− BMDMs, when compared to Casp-11+/+ BMDMs (Figure 4.25 (A)). It also appears that CM from Casp-11−/− Aldara-treated skin induces less NOS2 expression than CM from Casp-11+/+ skin. Reduced NO secretion from WT BMDMs occurred following incubation with CM from Casp-11−/−, compared to Casp-11+/+ Aldara-treated skin (Figure 4.25 (B)). These results suggest that impaired NOS2 induction and NO production occurs in Casp-11−/− mice during the Aldara-induced psoriasis.

Western blot analysis of Gasdermin D showed that no detectable processing occurred in either Casp-11+/+ or Casp-11−/− BMDMs following stimulation with CM from Aldara treated skin (Casp-11+/+ or Casp-11−/−) (Figure 4.25 (A)). In addition, no significant
differences in LDH secretion from Casp-11+/+ or Casp-11−/− BMDMs was observed, regardless of incubation with CM from Casp-11+/+ or Casp-11−/− skin (Figure 4.25 (C)).

Collectively these findings suggest that CM from Aldara-treated skin from Casp-11−/− mice is impaired in its ability to induce IL-1β, IL-18 and NO secretion, but has no observable impact on cell death or migration in macrophages. Results suggest that impaired inflammasome activation and NO production in macrophages contribute to the decreased psoriasis phenotype observed in Casp-11−/− mice.

It is evident from the findings presented in this study that caspase-11 plays an important role in the pathogenesis of Imiquimod-induced psoriasis as a deficiency in caspase-11 results in a protected phenotype. Additionally, caspase-11 is up-regulated and activated during this model, suggesting a role for caspase-11 in this disease. Lower expression levels of angiogenic markers likely explain the reduced erythema observed in Casp-11−/− skin. Less infiltrating leukocytes, epidermal thickness, proliferation and cell death may explain the reduced thickening and scaling of the skin observed in Casp-11−/− mice. CM from Casp-11−/− skin induces less IL-1β, IL-18 and NO production in BMDMs, suggesting that caspase-11 expression in skin mediates the secretion of inflammatory mediators which drive the pathogenesis of this disease.
4.3 Discussion

Results from this chapter suggest that caspase-11 plays a key role in mediating the pathogenesis of psoriasis, as a deficiency of caspase-11 resulted in a protective phenotype during a murine experimental model of psoriasis. While expression of inflammatory caspases have been reported to be increased in psoriatic lesions of patients, an exact role for inflammatory caspases has not yet been elucidated. This study utilised the murine Imiquimod-induced psoriasis model to investigate the role caspase-11 plays in this disease. This study has reported that caspase-11 is implicated in all of the typical hallmarks of psoriasis. This study initially demonstrated that a deficiency of caspase-11 resulted in decreased angiogenesis, keratinocyte proliferation, pyroptosis and decreased leukocyte infiltration. However, it is not clear what exact role caspase-11 plays in driving these characteristics. One must question whether caspase-11 plays a direct role in all of these characteristics or if a deficiency in caspase-11 has knock on affects within this disease.

This study has reported that caspase-11 deficient mice display less angiogenic markers in the skin during this model. Endothelial cells are the key cell type present in the lining of blood vessels and are crucial in attracting leukocytes through the production of chemokines. Leukocytes anchor to the surface of endothelial cells through adhesive molecules, mediating their migration. In the case of CD11b+ cells, ICAM-1 is the surface adhesion molecule responsible for mediating the migration of leukocytes (258). Inhibition of the canonical inflammasome has previously been reported to result in decreased CD11b+ cell infiltration with significant reductions observed in mRNA levels of ICAM-1 and VCAM-1 (259). In addition, a recent publication has proposed a role for the non-canonical inflammasome in mediating leukocyte migration. Cheng et al. reported that caspase-11 is responsible for mediating pyroptosis in endothelial cells (ECs) and a deficiency of caspase-11 in endothelial cells results in decreased migration of leukocytes during endotoxemia-induced lung injury (260). This study presents a direct link between caspase-11, pyroptosis and leukocyte migration. An earlier study presented by Li et al, has also reported that caspase-11 deficient leukocytes are defective at migrating in both in vitro and in vivo settings. They suggest that caspase-11 regulates cell migration by promoting Actin depolymerisation mediated by Aip1 and Cofilin (261). This mechanism may explain the differences in leukocyte infiltration observed in our study. We have seen
significantly less pyroptosis in the skin of caspase-11 deficient mice at both early (24h) and late (4 days) time points in our study. It is likely that inflammatory mediators released during pyroptosis, ie IL-1β, GSDMD, are responsible for mediating the recruitment of these leukocytes. Interestingly, lower expression levels of angiogenic markers are only prominent at the later stages of the model, suggesting this is most likely a secondary effect. While we have not shown in this study that caspase-11 deficiency results in less pyroptosis specifically in endothelial cells, we propose this as a likely explanation for the decrease in leukocyte migration to the skin and suggest it as an area of further research.

While it is evident that CD11b+ cell migration into the skin was decreased in the absence of Caspase-11 during IMQ-induced psoriasis, the exact CD11b+ cell population has not yet been identified. This study reported low levels of neutrophils present in the skin draining nodes of both Casp-11+/+ and Casp-11−/− groups. However, neutrophils function by migrating to the lesion and acting within the skin. As they are not primarily antigen-presenting cells, they would not likely enter into the skin-draining nodes. Therefore, it would be wise to examine the presence of neutrophils via immunofluorescence or flow cytometry in the skin of both Casp-11+/+ and Casp-11−/− groups. In addition, it may also be prudent to examine the presence of macrophages in the skin and not just the skin-draining nodes of Casp-11+/+ and Casp-11−/− mice during this model. While there were no evident differences in the presence of neutrophils and macrophages in skin-draining nodes, there may be differences in these populations within the skin itself. Therefore it is possible that neutrophils or macrophages may in fact be the CD11b+ cell populations affected in the absence of Caspase-11 during this model.

This study also reported an increase in VEGF expression in Casp-11−/− skin at early stage psoriasis but less VEGF at the later stage of psoriasis. VEGF is reported to mediate the pathogenesis of psoriasis in two ways. One is via keratinocyte-derived VEGF production acting directly on the endothelium to induce angiogenesis (262, 263). The other is via indirect VEGF acting on the epidermis in a paracrine manner to induce increased proliferation of keratinocytes in the epidermis (264, 265). Caspase-11 may be required at a later stage to mediate the increased development of new blood vessels but in the initial development of psoriasis it may act in an inhibitory manner to prevent the over expression of VEGF. Analysis of VEGF expression at each day of the Imiquimod-induced psoriasis
model may give more of an insight into the mechanistic role of VEGF and caspase-11 in driving this disease.

We have also seen significantly less proliferation in the epidermis of caspase-11 deficient mice during this model. Canonical inflammasome activation has been reported to drive proliferation in myeloid cells which proposes a role for inflammasome activation in mediating proliferation (266). In addition, inhibition of IL-1β has been reported to prevent proliferation in muscle cells (267). Pyroptosis in the muscle is reported to be responsible for driving proliferation of surrounding muscle cells via secretion of IL-1β (267). In our study, CM from Aldara treated Casp-11−/− skin induces less secretion of IL-1β from BMDMs when compared to Casp-11+/− skin CM. We hypothesise that early caspase-11-dependent IL-1β secretion from myeloid cells may be responsible for mediating the increased keratinocyte proliferation observed in the skin. IL-1β secretion has been reported to be upregulated in psoriatic lesions and inhibition of IL-1β has been reported to be a successful therapy in the treatment of the disease (268) which is comparable to the differences we observe in caspase-11 deficient mice during this model.

This study also investigated mRNA expression levels of typical cytokines that have previously been reported to play a role in psoriasis. mRNA expression levels of IL-17A, IL-17F, IFN-α, IFN-β, IL-23 and IL-22 were not affected by the absence of caspase-11, suggesting that these cytokines are not affected in the absence of caspase-11. Dysregulation of NFκB has also been linked to the pathogenesis of psoriasis and is mediated by the release of pro-inflammatory cytokines (269). It was hypothesised that a deficiency in caspase-11 would affect the expression of NFκB mediated inflammatory cytokine expression. However, inflammatory cytokine secretion (IL-1β, IL-1α, IL-18, IL-10 and IL-6) from Casp-11+/+ and Casp-11−/− skin explant cultures after 5 days or 24 hours of Aldara treatment were similar. Interestingly, we reported that skin stimulated with Aldara in vitro and conditioned media then applied to BMDMs resulted in a more significant secretion of IL-1β. This would suggest that investigating the skin explants as a whole may be misleading and analysis of individual cell types may give more insight into the role of the non-canonical inflammasome in mediating this disease. IL-1β is secreted following inflammasome activation and during pyroptosis and therefore may be an important inflammatory mediator in driving this disease.
The role of nitric oxide (NO) during Imiquimod-induced psoriasis was also examined during this study. Unpublished findings from our lab identify a role for caspase-11 in NO production following LPS/IFNγ-mediated polarisation of BMDM to M1 macrophages. While NO is known for its antimicrobial and tissue damaging effects and is produced by M1 macrophages during bacterial infection, it has many versatile signalling functions (270). Nitric oxide has been found at higher levels in psoriatic lesions and serum of psoriasis patients, and has been linked to the potent effects of IL-8 in the formation of psoriatic lesions (271)(272). Findings in this study suggest a role for caspase-11 in mediating NO production via the inducible nitric oxide synthase, NOS2. Links between inflammasomes and regulation of NOS2 expression have been previously reported. One study reports that IL-1β contributes to an NLRP3 inflammasome-mediated response to *Leishmania* infection which is mediated through NO (273). A recent study used the NLRP3 inhibitor, MCC950, to inhibit the effects of LPS-induced inflammatory hyperalgesia in mice (11). In addition to inhibiting the inflammasome and caspase activation, MCC950 caused increased eNOS, iNOS and nNOS expression levels in heart, brain and lung tissue of LPS-treated mice, suggesting that the NLRP3 inflammasome inhibits NOS expression, and NO production, during a model of inflammatory hyperanalgesia (274). In addition, studies from the Bortoluci group have reported that caspase-1 is required for Flagellin-induced iNOS expression, and that epigenetic regulation of iNOS expression is mediated by NLRC4 (275). Interestingly, the *Casp-1*−/− mice used in these studies were 129-derived mice that were later found to have incorporated a caspase-11 passenger mutation in their genome, thus caspase-11 may have a role on the observed effects on NOS2. Our findings are contradictory to those reporting that NLRP3 inflammasome inhibition induces NOS expression (274). However, our research supports those published by the Bortoluci group as we observed a significant decrease in NOS2 expression in caspase-11-deficient skin during Imiquimod-induced psoriasis, and decreased NOS2 induction and NO production in *Casp-11*−/− BMDMs. This suggests that caspase-11 is responsible for regulation of NOS2 expression. However it is likely that caspase-11 plays a cell specific role in NOS2 regulation as there was no observable differences in NO production from DCs. The skin is comprised of a range of cell types, and caspase-11 might regulate NOS2 expression in only some of these cells. Interestingly, *Casp-11*−/− skin explants treated with Aldara and then applied to BMDMs induced less IL-1β secretion when compared to *Casp-11*+/+ skin CM. IL-1β secretion has
been reported to act in a paracrine manner and induce upregulation of NOS2 expression (276, 277). This may explain the increased expression of NOS2 and increased production of NO produced by BMDMs following stimulation with CM from WT treated skin.

Nitric oxide is also an important modulator of angiogenesis via VEGF activation. In addition, TSP-1 has also been reported to inhibit endothelial-mediated nitric oxide production via CD34 activation and subsequent VEGF and eNOS inhibition. Interestingly, in this study we have seen significantly less VEGF in the skin of caspase-11 deficient mice. Again, mRNA expression levels of TSP-1 in the skin of caspase-11 deficient mice were significantly higher at the later stage of this model. Inflammatory cells, in particular macrophages, are believed to make significant contributions to vascular remodelling (278). Our study has shown that there were no significant differences in nitric oxide production from the skin of Casp-11+/+ and Casp-11−/− skin during this model. However, we have seen that macrophages deficient in caspase-11 produce less nitric oxide when primed with LPS and stimulated with Aldara. In addition, CM from skin explants stimulated with Aldara, which was subsequently applied to BMDMs, induced more nitric oxide production when caspase-11 was present. The absence of caspase-11 in BMDCs did not impact the induction of NOS2 and production of nitric oxide. These findings would propose a macrophage specific role for caspase-11 in mediating the induction of nitric oxide. Nitric oxide production from macrophages may also be responsible for mediating the increased angiogenesis observed during the model. As we have seen less nitric oxide produced from Casp-11−/− BMDMs and lower expression levels of angiogenic markers in Casp-11−/− skin during the later stage of this model, we hypothesise that caspase-11 in macrophages is responsible for mediating the induction of NOS2 and subsequent nitric oxide production. This increase in nitric oxide may act further on endothelial cells in the skin to induce angiogenesis.

Another key characteristic of the pathogenesis of psoriasis is the increase in keratinocyte cell death that results in the formation of the outer stratum corneum layer. An increase in dysregulation and proliferation of keratinocytes results in an increase in cell death and the formation of dead skin cells on the outer surface (scaling). Findings presented in this study suggest that Casp-11−/− mice display less scaling in the Imiquimod-induced psoriasis model and significantly less epidermal thickening in the beginning stages of the model. Interestingly, TUNEL staining suggests that caspase-11 deficiency results in less
epidermal cell death at both earlier and later stages of the psoriasis model. In support of this, Douglas et al., have reported that Casp-1+/Casp-11−/− murine skin in a cpdm skin model also display less cell death (226). Collectively, these findings suggest that caspase-11 has a role in cell death in skin disorders such as psoriasis and dermatitis. As TUNEL stains both pyroptotic and apoptotic cells, it was important to identify the type of cell death occurring during this model. Findings in this chapter display similar levels of caspase-3 cleavage in both Casp-11+/+ and Casp-11−/− skin. As caspase-3 cleavage occurs during apoptosis, this suggests that defective pyroptosis, rather than apoptosis, may explain the differences in cell death observed. In support of this, we show that LDH release is decreased in IMQ-treated skin explants from Casp-11−/−, compared to Casp-11+/+, mice. We also reported that Casp-11−/− skin displays less Gasdermin D cleavage, which is a key mechanism responsible for driving pyroptosis. This study has suggested a clear role for caspase-11 in mediating pyroptosis at both the earlier and later stages of cell death. As it is widely established that caspase-11 drives pyroptosis in a range of cell types, we hypothesise that the inflammatory mediators released during pyroptosis may be responsible for propagating this disease by acting further on other immune cells, keratinocytes and endothelial cells.

Collectively, this study has reported that caspase-11 plays an important role in mediating this experimental model of psoriasis. We have reported a role for caspase-11 in mediating epidermal thickening, proliferation and cell death. In addition, we have reported that decreased leukocyte infiltration occurs in the absence of caspase-11 with decreased angiogenic markers also present in the skin of Casp-11−/− mice. While we have not fully elucidated the exact role caspase-11 plays in mediating this disease, we propose that caspase-11 is required for driving pyroptosis in both myeloid cells and keratinocytes. Inflammatory mediators released during pyroptosis such as IL-1β, may act further on cells within the skin to induce proliferation and recruitment of leukocytes. We have also proposed a macrophage specific role for caspase-11 in mediating the induction of NOS2 and subsequent nitric oxide production. NO has been widely implicated in driving angiogenesis and therefore may explain the decreased angiogenic markers observed in the skin of caspase-11 deficient mice during this model. While an exact mechanism is not yet fully clear, this study has outlined a clear role for caspase-11 in driving the pathogenesis of psoriasis.
Figure 4.1: Reduced disease severity in Casp-11-/- mice during Imiquimod-induced psoriasis.

Mice were treated with Vaseline (Control) or Aldara cream (5% Imiquimod, IMQ), each mouse receiving 50mg per day. Back skin was photographed daily and representative images are shown in figure (A). Scaling (B) and Erythema (C) were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. Spleen weight was measured after 4 days of Aldara treatment (D). Weight loss was monitored each day (E). Control N=2 and Aldara treatment N=5. Two-way ANOVAs were performed for scaling, erythema, and weight loss. *p<0.05, *** p < 0.001.
Figure 4.2: Significantly less angiogenic marker expression occurs in *Casp-11*−/− skin during late stage Imiquimod-induced psoriasis.

Mice were sacrificed and whole skin extracts were dissected after 4 days and 24 hours of Aldara treatment. Skin extracts were snap frozen on the day of dissection in liquid nitrogen. Skin was homogenised and RNA was isolated using Trizol. RNA was converted to cDNA and qPCR was performed to determine relative expression levels of Vegf (A, D); Ang-2 (B, E); and TSP-1 (C, F) at 4 days and 24 h, respectively. Control (N=2) and Aldara (N=5) for both *Casp-11*+/+ and *Casp-11*−/− groups at both time points. Two way ANOVA found * p < 0.05,
Figure 4.3: Histological H&E staining of skin sections show that less epidermal thickening occurs in Casp-11−/− mice at early, but not late, stage Imiquimod-induced psoriasis.

Mice were treated with 50 mg Aldara for four days or 24 hours. Mice were sacrificed and back skin was excised. Back whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of control and Aldara treated back skin taken at day 4 (A) and 24 hours (B) were stained using Haemotoxylin and Eosin. Epidermal thickness was measured (average of three 10X images per skin section, and three pieces of skin sections per mouse), shown in (C) for 4 day and (D) for 24 hour treated skin. Scale bar = 20 μm. Control (N=2) and Aldara (N=5). Thickness was measured in nm using Image J software. Two-way ANOVA found

* p<0.05
Figure 4.4: PCNA staining of FFPE skin sections reveals that less cell proliferation occurs in Casp-11\(^{-/-}\), compared to Casp-11\(^{+/+}\) epidermis, during late stage Imiquimod-induced psoriasis.

Representative images of PCNA stained control and Aldara treated back skin sections taken at day 4 (A) and 24 hrs (B). PCNA positive cells were counted per 10X field in the epidermis in an average of three images per skin section and three skin sections per mouse, as shown in (C) 4 days and (D) 24 hrs Aldara treatment. Scale bar = 20 \(\mu\)m. Control (N=2) and Aldara (N=5). Two-way Anova found ** p<0.001
Figure 4.5: Less cell death is observed in FFPE skin of Casp-11/- mice at early and late stages of experimental psoriasis.

Mice were treated with 50 mg Aldara for four days. Mice were sacrificed and back skin was excised. Back whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of control and Aldara treated back skin taken at day 4 (A) and 24 hrs (B) were stained for TUNEL positivity. TUNEL positive cells were counted per field in the epidermis in an average of three images per piece of tissue and three pieces of tissue per mouse as shown in (C) for 4 days and (D) for 24 hrs of Aldara treatment. Magnification was 10X. Scale bar = 20 μm. Control (N=2) and Aldara (N=5). Two-way Anova found * p < 0.05.
Figure 4.6: Skin explant cultures from *Casp-11−/−* mice produce less LDH than those from *Casp-11+/+* mice, following 4 days of Aldara treatment. *Casp-11−/−* and *Casp-11+/+* mice were treated with Aldara for four days or 24 hours before mice were sacrificed and back skin was harvested. Whole skin back extracts were homogenised and protein concentration was normalised. Lysates from 4 day (A) and 24 hour (B) treated skin were run on 12% SDS-PAGE gels, and probed for Gasdermin D, caspase-11, caspase-3 and Actin as a loading control. After four days or 24 hours of Vasoline or Aldara treatment, *Casp-11−/−* and *Casp-11+/+* mice skin explant cultures were set up for 24 hours. Percentage cytotoxicity of skin explant cultures for 4 days (C) and 24 hours (D) was measured by LDH assay. Homogenised skin lysates were used as a positive control. Control (N=2) and Aldara (N=5). Two-way ANOVA found *p*<0.05.
Figure 4.7: Similar levels of pro-inflammatory cytokine production in $Casp-II^{+/+}$ and $Casp-II^{-/-}$ skin explant cultures at late stage Imiquimod-induced psoriasis.

Following 5 days of Vasoline or Aldara treatment, mice were sacrificed and skin extracts were dissected from the back of each mouse. Skin explant cultures were cultured in 0.5 mL DMEM for 24 hours in a 24-well plate. The weight of the skin was then measured and all data was normalised against weight. IL-1β (A), IL-18 (B), IL-1α (C), IL-10 (D) and IL-6 (E) production was measured by ELISA and graphed using GraphPad Prism. No significant differences were observed. Control group size was 2 and Aldara group size was 5 for both $Casp-II^{+/+}$ and $Casp-II^{-/-}$ mice. Two-way ANOVA found no significant differences between $Casp-II^{+/+}$ and $Casp-II^{-/-}$ groups.
Figure 4.8: Similar levels of inflammation-related cytokine production in Casp-11+/+ and Casp-11/- skin explant cultures at early stage Imiquimod-induced psoriasis.

Following 24 hours of Vasoline or Aldara treatment, mice were sacrificed and 4 x 3mm punch biopsies were dissected from the back of each mouse. Skin explant cultures were cultured in 0.5 mL DMEM for 24 hours in a 24-well plate. IL-1β (A), IL-18 (B), IL-1α (C), IL-10 (D) and IL-6 (E) production was measured by ELISA and graphed using GraphPad Prism. No significant differences were observed. Control group size was 2 and Aldara group size was 5 for both Casp-11+/+ and Casp-11/- mice. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11/- groups.
Figure 4.9: No differences in psoriasis-associated cytokines during the late stage of Imiquimod-induced psoriasis.

Mice were sacrificed and whole skin extracts were dissected after 4 days of Aldara treatment. Skin extracts were snap frozen on the day of dissection in liquid nitrogen. Skin was homogenised and RNA was isolated using Trizol. RNA was converted to cDNA and qPCR was performed to determine relative expression levels of IL-17A (A), IL-17F (B), IFN-α (C), IFN-β (D), IL-23 (E) and IL-22 (F). Control (N=2) and Aldara (N=5) for both Casp-11+/+ and Casp-11−/− groups at both time points. Two way ANOVA found *** p < 0.001.
Figure 4.10: No differences in psoriasis-associated cytokines during early stages of Imiquimod-induced psoriasis.

Following 24 h Aldara treatment, mice were sacrificed and whole skin extracts were dissected. Skin extracts were snap frozen on the day of dissection in liquid nitrogen. Skin was homogenised and RNA was isolated using Trizol. RNA was converted to cDNA and qPCR was performed to determine relative expression levels of IL-17A (A), IL-17F (B), IFN-α (C), IFN-β (D), IL-23 (E) and IL-22 (F). Control (N=2) and Aldara (N=5) for both Casp-11+/+ and Casp-11−/− groups at both time points. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.11: Toluidine blue staining of affected skin shows no differences in Mast cell numbers between Casp-11<sup>+/+</sup> and Casp-11<sup>-/-</sup> mice during Imiquimod-induced psoriasis.

Mice were treated with 50 mg Aldara for (A, C) four days and (B, D) 24 hrs. Mice were sacrificed and back skin was excised. Whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of toluidine blue stained control and Aldara treated skin sections taken at day 4 (A) and 24 hours (B). Mast cells were counted in dermis (Average of three (10X magnification) images per piece of tissue and three pieces of tissue per mouse) in sections from 4 days (C) and 24 hrs (D). Scale bar = 20 μm. Control (N=2) and Aldara (N=5). Two-way ANOVA found no significant differences between Casp-11<sup>+/+</sup> and Casp-11<sup>-/-</sup> groups.
Figure 4.12: Less CD11b+ cells are observed in Casp-11−/− skin at early and late stage Imiquimod-induced psoriasis.

Mice were treated with 50 mg Aldara for four days. Mice were sacrificed and back skin was excised. Representative images of control and Aldara treated FFPE skin sections from day 4 (A) and 24 hrs (B) were probed using anti-CD11b. CD11b positive cells were counted per field in the dermis in an average of three images per piece of tissue and three pieces of tissue per mouse as shown in (C) for 4 days and (D) for 24 hrs of Aldara treatment. Control (N=2) and Aldara (N=5). Images taken at 10X magnification. Scale bar = 20 μm. Two-way Anova found ** p < 0.001.
Figure 4.13: Deficiency of Caspase-11 does not alter the Langerhans cell population in the skin.

Mice were treated with 50 mg Aldara for four days. Mice were sacrificed and back skin was excised. Back whole skin tissue was fixed in Formalin and paraffin embedded. Representative images of control and Aldara treated FFPE skin sections from day 4 (A) and 24 hrs (B) were probed using anti-Langerin. Langerin positive cells were counted per field in the epidermis in an average of three images per piece of tissue and three pieces of tissue per mouse as shown in (C) for 4 days and (D) for 24 hrs of Aldara treatment. Control (N=2) and Aldara (N=5). Images taken at 10X magnification. Scale bar = 20 μm. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.14: Decreased migration of CD11b+ cells into skin draining lymph nodes of Casp-11−/− mice during mid-stage Imiquimod-induced psoriasis.
Mice were treated with 50 mg Aldara for 3 days. Mice were sacrificed and inguinal nodes were dissected and digested with 30 mg/mL collagenase 1A. Cells were stained for flow cytometric analysis. Gating strategy for acquiring CD11b+ cells is shown in (A). Representative images of CD11b+ cells in untreated and Aldara treated Mice are shown in (B). Number of live CD11b+ cells are shown in (C). Two way Anova found * p<0.05.
Figure 4.15: No differences in Neutrophils in Casp-11+/+ and Casp-11−/− inguinal nodes during mid-stage Imiquimod-induced psoriasis.
Mice were treated with 50 mg Aldara for 3 days. Mice were sacrificed and inguinal nodes were dissected and digested with 30 mg/mL collagenase 1A. Cells were stained for flow cytometric analysis. Gating strategy for acquiring Neutrophils is shown in (A). Representative images of Neutrophils in untreated and Aldara treated mice are shown in (B). Number of live Neutrophil cells are shown in (C). Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.16: No differences in Macrophages in Casp-11+/+ and Casp-11−/− inguinal nodes during mid-stage Imiquimod-induced psoriasis.
Mice were treated with 50 mg Aldara for 3 days. Mice were sacrificed and inguinal nodes were dissected and digested with 30 mg/mL collagenase 1A. Cells were stained for flow cytometric analysis. Gating strategy for acquiring Macrophages is shown in (A). Representative images of Macrophages in untreated and Aldara treated mice are shown in (B). Number of live Macrophage cells are shown in (C). Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.17: No differences in Langerhans cells in Casp-11+/+ and Casp-11−/− inguinal nodes during mid-stage Imiquimod-induced psoriasis.

Mice were treated with 50 mg Aldara for 3 days. Mice were sacrificed and inguinal nodes were dissected and digested with 30 mg/mL collagenase 1A. Cells were stained for flow cytometric analysis. Gating strategy for acquiring Langerhans is shown in (A). Representative images of Langerhans in untreated and Aldara treated mice are shown in (B). Number of live Langerhan cells are shown in (C). Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.18: No differences in Dendritic cell populations in *Casp-11**/+ and *Casp-11**/- inguinal nodes during mid-stage Imiquimod-induced psoriasis.

Mice were treated with 50 mg Aldara for 3 days. Mice were sacrificed and inguinal nodes were dissected and digested with 30 mg/mL collagenase 1A. Cells were stained for flow cytometric analysis. Gating strategy for acquiring DC populations is shown in (A). Representative images of DC populations in untreated and Aldara treated mice are shown in (B). Number of live CD11b⁺, CD103⁻ cells are shown in (C). Number of live CD103⁺, CD11b⁻ cells are shown in (D). Two-way ANOVA found no significant differences between *Casp-11**/+ and *Casp-11**/- groups.
Figure 4.19: Casp-11−/− BMDMs produce significantly less Nitric Oxide following M1 polarisation and stimulation with psoriasis inducing ligands.

BMDMs were isolated and cultured for 7 days in M-CSF. Cells were seeded at 2 x 10^5 cells/mL. (A) BMDMs were treated with IFN-γ (10 ng/mL), LPS (100 ng/mL) or primed with LPS (100 ng/mL) for 4 hours and then treated with IFN-γ (10 ng/mL) for 24 hours. Nitric Oxide production in supernatants was assessed indirectly by measuring Nitrite concentration, using Griess reagent. (B) BMDMs were transfected with 7 μg/mL Imiquimod or 160 μg/mL Aldara cream dissolved in DMSO for 24 hours (B). Nitric Oxide production in the supernatants was measured using Griess reagent. Two-way ANOVA found ** p < 0.01, *** p < 0.001.
Figure 4.20: NOS2 mRNA expression levels are increased in human psoriatic lesions in comparison to non-lesional and control areas.

Relative NOS2 mRNA expression levels in normal, non-lesional and lesional skin were analysed using the following datasets GDS4602 (Nair et al, 2009), GDS3539 (Yao et al, 2008), GDS2518 (Reischl et al, 2007), GDS4600 (Suarez-Farinas et al, 2012). Graphs are shown to indicate the gene expression values in individual patients (GDS4602 n=88, GDS3539 n=28, GDS2518 n=13, GDS4600 n=85). Wilcoxon test was used to evaluate significant differences between two groups and Kruskall Wallis test was used to test between three groups. ** p < 0.01, *** p < 0.001.
Figure 4.21: Nitric Oxide Synthase expression and nitric oxide production in Casp-11−/− skin.

Casp-11−/− and Casp-11+/+ mice were treated with Vasoline (control) or Imiquimod for four days. On day four, mice were sacrificed and whole skin extracts were snap frozen. Skin extracts were homogenised and protein concentration was normalised using BCA assay. Untreated and Imiquimod treated Casp-11+/+ and Casp-11−/− samples were run on 12% SDS-PAGE gels, and probed for NOS2 and Actin as a loading control (A). RNA was isolated from skin using trizol and converted to cDNA. Relative expression levels of Skin explant cultures were also set up for 24 hours in 0.5mL of DMEM. Nitric Oxide levels in skin explant cultures were measured by Griess assay (D). Control (N=2) and Aldara (N=5). Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.22: Casp-11<sup>−/−</sup> BMDCs treated with LPS, IMQ and Aldara produce similar levels of Nitric oxide when compared to Casp-11<sup>+/+</sup>.

BMDCs were isolated and cultured for 10 days in GM-CSF. Cells were seeded at 6.25 x 10<sup>5</sup> cells/mL. (A) BMDCs were primed with LPS (100 ng/mL) and transfected with 7 μg/mL Imiquimod or 160 μg/mL Aldara cream dissolved in DMSO for 24 hours. Lysates were run on 12% SDS PAGE gels and probed for NOS2, Casp-11 and Actin (B). Nitric Oxide production in supernatants was assessed indirectly by measuring Nitrite concentration, using Griess reagent (B). n=4 for (A) and (B). Two-way ANOVA found no significant differences between Casp-11<sup>+/+</sup> and Casp-11<sup>−/−</sup> groups.
A

Dissect skin from mice

Stimulate skin with Imiquimod/Aldara cream for 24 hours

Add conditioned media from skin to WT and Casp-11 KO BMOMs

B Conditioned media from WT untreated skin

C Conditioned media from WT Imiquimod treated skin

D Conditioned media from WT Aldara treated skin

E Conditioned media from KO untreated skin
Figure 4.23: Conditioned media (CM) from Aldara treated Casp-11+/+ and Casp-11−/− skin extracts do not induce enhanced macrophage migration.

Skin explants were dissected from the back of shaved mice. One whole skin section per mouse was cut in three pieces and weighed to ensure uniform weight. Skin was then cultured overnight with either 20 μg Imiquimod or 17 mg Aldara cream in a total volume of 1.5 mL DMEM culture media. 24 hours later, conditioned media from the skin explants were sterile filtered and applied (in a 50:50 ratio with fresh DMEM) to BMDM seeded at 0.5 x 10^6 cells/mL (A). A scratch was applied and 24 hours later, the number of cells that migrated back into the scratch site were counted. Representative images for conditioned media from WT control (B), Imiquimod (C) and Aldara (D) treated skin and KO control (E), Imiquimod (F) and Aldara (G) treated skin on WT and Casp-11−/− BMDMs are shown. Graphical representation of the BMDMs that migrated back into the scratch site are shown in (H). Images were taken at 4X magnification. Scale bar = 200 μm. Two-way ANOVA found no significant differences between Casp-11+/+ and Casp-11−/− groups.
Figure 4.24: CM from Casp-11^{-/-} psoriatic skin causes WT BMDMs to produce less IL-1β and IL-18 than media from Casp-11^{+/+} psoriatic skin.

Experimental set-up was carried out as described in Figure 4.23 (A). 24 hours later, supernatants from BMDMs were retained along with conditioned media from skin prior to addition to BMDMs. Secretion of IL-1β (B) and IL-18 (C) was determined by ELISA. Two-way ANOVA found that * p<0.05 and ***, ** p<0.001.
Figure 4.25: CM from Aldara-treated Casp-11−/− skin explants induces less Nitric Oxide production from WT BMDM, when compared to CM from Casp-11+/+ skin explants.

Experimental set-up was carried out as described in Figure 4.23 (A). 24 hours later, lysates and supernatants from BMDMs were retained. Lysates were run on 12% SDS-PAGE gels and probed for NOS2, Gasdermin D, Casp-11 and Actin. Production of nitric oxide was measured by Griess Assay (B) and Percentage Cytotoxicity (C) was measured by LDH assay. Two-way ANOVA found that * p<0.05 and *** p<0.001.
Chapter 5: Linking the effects of a novel NLRP6 Mutation to an Autoinflammatory Syndrome
5. Linking the effects of a novel NLRP6 Mutation to an Autoinflammatory Syndrome

5.1 Introduction

The innate immune system is a complex and diverse system that is responsible for the recognition of a broad array of pathogens and microbial insults. Dysregulation of the innate immune system can result in the development of autoinflammatory syndromes. The term “Autoinflammatory Syndrome” was first coined in 1999 by Kastner and Colleagues in Cell, where it was described as a set of clinical disorders which contrasted to those associated with autoimmune syndromes (279). Autoinflammation is characterised by inflammation arising as a result of dysregulation of the innate immune system, without the presence of autoimmune T cells, B cells, or high titre autoantibodies. The classical features of autoinflammation include fevers and skin rashes, although symptoms vary between specific disorders. Similarly, the pathological mechanisms responsible for these autoinflammatory symptoms are varied, many of which are still being elucidated. However, all autoinflammatory syndromes arise from an aberrant activation of innate immune components, resulting in systemic involvement of multiple systems and organs.

The most common monogenic autoinflammatory disorder is Familial Mediterranean Fever (FMF). FMF is an autosomal recessive inherited syndrome that effects people of Mediterranean descent and is characterised by periods of fever that can last for any length of time between 3 hours and 4 days. Typically patients develop intense serositis (i.e. inflammation of serous tissue, such as those which line the lungs, heart, abdomen, and inner abdominal organs). FMF occurs as a result of a mutation in the MEFV gene, which codes for the Pyrin protein, and has a prevalence of 1-5 in 10,000 people. Another commonly diagnosed group of autoinflammatory disorders that arise as a result of mutations in the inflammasome component, NLRP3, are the Cryopyrin-associated periodic syndromes (CAPS). Muckle-wells syndrome (MWS), Familial cold autoinflammatory syndrome (FCAS) and chronic infantile neurological cutaneous and articular syndrome (CINCA) are three syndromes that are collectively known as CAPS, as they arise from mutations in the NLRP3 gene (previously known as Cryopyrin). CAPS are dominantly inherited disorders that typically present with fevers, increased acute
phase reactants, and urticarial rash. Uncontrolled production of the potent inflammatory cytokine, IL-1β, is responsible for the symptoms of CAPS. However, the CAPS subtypes differ in severity, the range of multi-organs affected, and long-term morbidity and mortality. An additional dominantly inherited autoinflammatory disorder arises as a result of a mutation in NLRP12, known as NLRP12-associated periodic fever syndrome (280). Interestingly, a subset of patients that present with CAPS-like symptoms (recurrent fever and cold sensitivity) have a mutation in the NLRP12 gene as opposed to the NLRP3 gene and are therefore diagnosed with Familial Cold Autoinflammatory Disorder-2. The prevalence of this disorder is not known. The autoinflammatory disorders mentioned are very similar in that they all arise as a result of a mutation in an inflammasome component and all result in recurrent fevers and skin rashes, among other symptoms.

A patient at Our Lady’s Children’s Hospital, Crumlin presented with a range of autoinflammatory symptoms such as panniculitis, granulomatous hepatitis, arthritis, microcytic hypochromic anaemia. Panniculitis is a term used to describe a broad range of inflammatory skin conditions that results in inflammation of the adipose tissue underneath the skin. It results in a thickening of the skin and the development of nodules just below the surface. Granulomatous hepatitis is the accumulation of inflammatory cells in the liver that results in severe inflammation in and around granulomas in the liver. Arthritis is inflammation in a joint and microcytic hypochromic anaemia is a condition where the patient has fewer and smaller red blood cells present in their blood which usually occurs as a result of inflammation, and lower iron levels. In addition to these symptoms, the patient has had consistently high inflammatory markers such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).

As the patient was displaying such extreme autoinflammatory symptoms, the treating consultant, Dr. Ronan Leahy, arranged for gene sequencing to be performed on the patient’s genome using a targeted next-generation sequencing gene panel for autoinflammation and vasculitis (281). This next generation sequencing was designed by targeting a panel of 168 genes believed to be linked to autoinflammation and vasculitis. The sequencing results for this patient identified four rare heterozygous mutations in the patient’s genome; CECR1 (I313V), SH3BP2 (X562W), C6 (D696G) and NLRP6 (R653G). Mutations in the genes CECR1, SH3BP2 and C6 were ruled out by clinicians as being unlikely to be contributing to the phenotype of the patient (eg ADA functioning
was normal, no signs of Cherubism). Therefore it was decided to investigate the role of \textit{NLRP6}^{R653G} as a contributor to the patient’s symptoms. NLRP6 has not yet been associated with an autoinflammatory syndrome. Dr. Leahy subsequently contacted our laboratory, and we initiated a collaboration, aiming to determine the functional effect of the NLRP6 mutation, and to hypothesise its impact on the patient’s immune system. As the functional role of NLRP6 during innate immunity is still unclear, investigating this patient’s immune responses would also give insight into the functional importance of NLRP6 during innate immunity.

The symptoms displayed by the patient, are clinically defined as being ‘Blau-like’. Blau syndrome is a rare autoinflammatory syndrome that generally presents itself within the first few months of life and can be characterised by the presence of noncaseating granulomas that mainly affect the skin, joints and uveal tracts. Histological analysis of the affected tissues show epithelioid and multinucleated giant cells organised in granulomas (282). Blau syndrome is the only identified autoinflammatory disease where the gain-in-function mutation presents with granulomatous inflammation. The majority of patients that present with Blau syndrome contain a mutation in their \textit{NOD2} gene (also known as \textit{CARD15}), located on chromosome 16q12. Specifically, the majority of mutations presented occur in and around the NOD/NACHT domain of the protein. NOD2 is a cytosolic Nod-Like protein that recognises muramyldipeptide (MDP), a bacterial cell wall component, resulting in self oligomerisation and subsequent recruitment of the adaptor protein kinase receptor interacting protein 2 (RIP2)(103). Active RIP2 then mediates the activation of NFκB (283). Mutations in the NACHT domain of NOD2 result in an increased basal activation of NFκB and hyper-activation of NFκB when stimulated (284). Conversely, mutations in the LRR domains are strongly linked with Crohn’s disease and are believed to result in a loss of function mutation (285).

While there certainly appears to be links between the patient’s symptoms and the characteristic Blau syndrome symptoms, sequencing has shown that the patient does not have any mutation in their \textit{NOD2} gene. NLRP6 is another NLR protein that has been reported to activate NFκB (104). However, the physiological function of NLRP6 has yet to be elucidated. Studies published to date suggest that NLRP6 plays an important role in intestinal homeostasis (286). However, other publications propose a role for NLRP6 in the regulation of NFκB (104)(103). NLRP6 is structurally very similar to NLRP3,
consisting of a LRR domain that is responsible for sensing of ligands, a NACHT domain, and an N-terminal Pyrin domain.

There have been two SNPs identified in the NLRP6 gene that are associated with a physical phenotype, and neither SNPs are located within the LRR domain. A SNP (rs17655730) has been linked to mean platelet volume in a large GWAS study. However, the significance of this in humans has not yet been determined (287). Additionally, a sex-specific link has been correlated in a Sardinian population of a single SNP (rs7948797) with decreased susceptibility to hypertension in males (288). There have been no other defined SNPs in the NLRP6 gene that have been associated with a phenotype or disorder. We hypothesise that the identified NLRP6\textsuperscript{R653G} mutation in this patient associates NLRP6 with a novel autoinflammatory phenotype. While this mutation could lead to the characterisation of a novel autoinflammatory syndrome, it will also help us to identify the nature of the functional interactions between NLRP6 and NFκB.

**Hypothesis:** The NLRP6\textsuperscript{R653G} mutation impacts innate immune signalling pathways to cause an autoinflammatory disease.

**Aims:** To determine the impact of the NLRP6\textsuperscript{R653G} mutation on innate immune signalling pathways, and in doing so elucidate the functional role of NLRP6 during innate immunity.

Approaches taken to achieve this aim include: (i) Comparing NLRP6\textsuperscript{R653G} patient and control PBMC responses to TLR/NLR ligands; and (ii) developing *in vitro* assays to determine the impact of the mutation on NFκB and other inflammatory signalling pathways.
5.2 Results

5.2.1 Inflammatory markers in a patient displaying autoinflammatory symptoms are lowered by administration of Adalimumab

A paediatric patient was admitted to OLCHC under the care of a consulting immunologist. On each visit to the clinic, the patient’s inflammation markers were measured and compared over time. The patient displayed severe autoinflammatory symptoms from a very young age, with C reactive protein (CRP) levels reaching as high as 100 mg/L, almost 10 fold higher than the acceptable range (10 mg/L) (Figure 5.1 (A)).

CRP is an acute phase protein produced by the liver in response to inflammation. CRP levels higher than 2 mg/L can cause serious long term damage and an increased risk of heart disease. Similarly, the patient’s erythrocyte sedimentation rate (ESR) reached levels as high as 100 mm/h (Figure 5.1 (B)). An ESR test is an indirect way to measure inflammation in the body by measuring the sedimentation rate of red blood cells in a tall, thin tube over time. In a healthy individual, red blood cells will settle relatively slowly, leaving a relatively small level of plasma (clear fluid), which is measured in mm after one hour. However, in a person with high levels of inflammation, high concentrations of acute phase reactants such as CRP and fibrinogen, will cause the red cells to settle out of the plasma quicker, leaving a larger volume of plasma after one hour. The acceptable range of ESR for children is 0-10 mm/hr, which is significantly lower than the patient’s ESR levels, highlighting the unregulated inflammatory state of the patient. An additional inflammatory marker used by clinicians to identify inflammation is the measurement of serum amyloid A levels. Amyloid A is another acute phase protein, released during increased inflammation in response to inflammatory cytokines such as IL-6 and TNF-α. The patients Amyloid A levels reached as high as 180 mg/L, with typical reference ranges below 10 mg/L (Figure 5.1 (C)). Collectively, these findings suggest the patient was in an extreme level of inflammation prior to treatment.

Due to the excessive inflammation observed in the patient, a range of treatment plans were tested for their efficacy over the course of a number of years (Table 5.1). Due to consistently low iron levels, the patient has been prescribed iron supplements since 2006. A growth hormone deficiency has enlisted the requirement for a growth hormone, Genotropin, since 2015. Low levels of Vitamin D have resulted in the requirement of vitamin D supplements daily, starting from 2014. Prednisolone was administered in 2012.
for the treatment of extreme erythema nodosum skin rashes. Prednisolone is a steroid used for the treatment of diseases with significant inflammation such as panniculitis, as seen in the patient. Administration of prednisolone reduced the panniculitis symptoms but had no effect on the elevated inflammatory markers (Figure 5.1). Sulphasalazine was trialled in March 2012 for the treatment of arthritis but was unsuccessful. An immunosuppressant, Azathioprine was also trialled in 2012 but resulted in worsening liver functioning so was not continued. In 2013, Colchicine, which is an effective treatment for several autoinflammatory diseases, was also tested for its efficacy in reducing inflammation and the treatment of erythema nodosum. However, due to worsened neutropenia, colchicine was not continued. Ciproheptadine was administered in 2014 to increase the patient’s appetite.

In March of 2013, the patient was administered a high dose of Adalimumab (80 mg) followed by 20 mg weekly doses. Adalimumab is an anti-TNF-α antibody used to block the inflammatory effects of the cytokine TNF-α. Adalimumab is typically used to treat patients with psoriatic and rheumatoid arthritis, Crohn’s disease and ankylosing spondylitis. Following the incorporation of Adalimumab into the patient’s treatment plan, there was an observed decrease in CRP and ESR levels (Figure 5.1 (A) and (B) respectively). While the levels of these inflammatory markers were dramatically lower, all levels were still far higher than the acceptable ranges for a female of her age. Therefore, the Adalimumab concentration was increased to 40 mg weekly. The increased Adalimumab concentration again significantly reduced the patients CRP, ESR and Amyloid A levels. However, to this date, the patient’s CRP, ESR and Amyloid A levels have not yet been reduced to an acceptable limit (red lines in Figure 5.1 (A-C)), highlighting the requirement for a more efficient treatment plan.

5.2.2 Sequencing results of the patient with mutation NLRP6\textsuperscript{R653G}

Due to the severe inflammatory phenotype observed, it was hypothesised that the patient may have a mutation in one of the genes typically linked to autoinflammation such as NLRP3, NLRP12, Pyrin or NOD2. As the patient displayed typical symptoms observed during Blau syndrome, which arises as a result of mutations in NOD2, this was the primary candidate gene which was hypothesised to harbour a mutation. However, next generation sequencing identified mutations in 4 genes: CECR1, SH3BP2, C6 and NLRP6. As mentioned previously, the mutations in CECR1, SH3BP2 and C6 were ruled out as
the causative mutations, and the focus of this project was placed on investigating the mutation in the NLRP6 gene.

To determine whether the NLRP6R653G mutation was a sporadic mutation or inherited from either of the parents, NLRP6 in the patient and parents were sequenced. Interestingly, sequencing results determined that the mother also contains the heterozygous mutation R653G in the NLRP6 gene. As the mother does not display any extreme inflammation or have any typical autoinflammatory symptoms, it is possible that the patient encountered an environmental trigger at a young age that initiated the aberrant inflammatory responses. This phenomenon is not uncommon for autoinflammation. For example, asymptomatic parents and other family members of CAPS patients have also been found to harbour the CAPS-associated mutation in NLRP3 (154).

5.2.3 Mutation R653G is predicted to be a “probably damaging” mutation

As was previously discussed, whole genome sequencing (WGS) of the patient presenting with autoinflammatory symptoms identified a mutation in the NLRP6 gene. NLRP6 is located on chromosome 11, position 278,365-285,359 with eight exons responsible for the translation of the NLRP6 protein (Figure 5.3). The C>G mutation was identified at position 281691 on chromosome 11, which has been previously identified as a nonsynonymous missense mutation (Single Nucleotide Polymorphism (SNP) rs146018617), and validated by independent submissions to refSNP as being a rare heterozygous mutation (289). For example, one submission from Agilent Europe reported that WGS performed on 1264 chromosomes has genotype frequencies CC-0.998; CG-0.002 and GG-0.00 at the 281691 position (290). The C>G mutation at position 281691 is within exon 4, and translates to the amino acid mutation R653G, located between LRR1 and LRR2. As mentioned previously, LRRs in TLR/NLR proteins are considered to be responsible for sensing of ligands, although LRR-containing proteins are also involved in cellular processes including apoptosis, autophagy, ubiquitin related processes and nuclear mRNA transport (291). The mutation may therefore have an impact on NLRP6’s ability to sense or recognise one or more particular PAMPs or DAMPs.

The next question we wanted to address was whether the mutation was present on an external surface of the protein or within the folded hydrophobic regions of NLRP6. If a mutation is located on the surface of a protein it may disrupt protein-protein interactions or ligand binding. Alternatively if mutations occur within the internal regions of a protein,
a bulky amino acid change may prevent sufficient protein folding, rendering the protein ineffective. Unfortunately, the crystal structure of NLRP6 has not yet been determined. There are, however, useful protein structure prediction software tools that can be used to predict the likely structure of a particular protein.

**Figure 5.4 (A)** shows an example of the predicted structure of NLRP6 as designed by PHYRE2. The predicted 3D structure was then modelled using RasTop. The PYRIN domain, NACHT domain and LRRs were highlighted and displayed in the predicted 3D structure. As mutation R653G is located between LRRs 1 and 2, and as the main role of NLR LRRs is considered to be in ligand recognition, it is possible that this mutation will interrupt the protein's ability to recognise a ligand. Based on this example of the predicted NLRP6 3D structure, amino acid residue 653 is located on the external surface of NLRP6, suggesting that it may in fact be interrupting the ability of the LRR domain to interact with ligand/proteins or may be causing new functions for the protein.

Another bioinformatic approach used for this study was to investigate the likelihood that the NLRP6\textsuperscript{R653G} mutation will impact the protein’s function and structure. The programme used for this prediction was Polyphen2 (292). Polyphen2 uses the known or predicted 3D structure of a protein to determine whether the amino acid substitution will likely destroy the electrostatic interactions, interactions with ligands or the hydrophobic core of the protein. Polyphen2 uses probability algorithms to determine if this mutation is damaging. The mutation is also scored qualitatively and graded as “benign”, “possibly damaging” or “probably damaging”. Polyphen2 predicts that mutation R653G will be “probably damaging” (**Figure 5.4 (B)**). The score assigned by Polyphen2 for mutation R653G was 0.993 with a sensitivity of 0.7 and specificity of 0.9. Therefore, this programme would suggest that the mutation would most likely cause an interruption to the 3D structure of NLRP6.

Collectively, this bioinformatics analysis suggests that mutation R653G is located on the external surface of the protein, which may hinder or improve its ability to interact with other proteins. It is also predicted to have a “probably damaging” impact on NLRP6 structure.
5.2.4 Overexpression of NLRP6 drives NFκB activation in an ASC-dependent manner in vitro

As the patient displayed excessive inflammation but was responsive to anti-TNF-α therapy, it was hypothesised that an innate signalling pathway was affected as a result of this mutation. Previous studies have suggested that NLRP6 is a regulator of NFκB signalling. However, as mentioned earlier, there are conflicting reports within the literature regarding the role that NLRP6 plays in regulating NFκB. In a murine study, Anand et al suggested that NLRP6 is a negative regulator of NFκB in vivo (103). However, a previous in vitro study showed that NLRP6 was capable of driving NFκB activation (104). Therefore, we next sought to confirm whether NLRP6 is capable of regulating NFκB in vitro, using a luciferase reporter assay.

HEK293T cells were transfected with ASC, increasing concentrations of NLRP6 and an NFκB-luciferase reporter plasmid. HEK293T cells are a useful cell line for the purpose overexpression studies, as they are easily transfectable. This embryonic kidney cell line does not endogenously express inflammasome components, and therefore represents a convenient way to study inflammasomes in isolation. As the cells do not express NLRP6, one can transfec in the mutant NLRP6 plasmid and be confident that there is no endogenous WT NLRP6 already present. Our findings are in agreement with the previously published in vitro assays (8), showing that NFκB activity increases with increasing concentrations of NLRP6, in an ASC-dependent manner (Figure 5.5 (A)).

MC005 is a Molluscum Contagiosum Virus Protein that targets NEMO-regulated IκB kinase activation, therefore inhibiting NFκB (293). In the presence of MC005, NLRP6 was incapable of activating NFκB (Figure 5.5 (B)). Transfection efficiency for luciferase assays was confirmed by Western blot (Figure 5.5 (C)). It therefore appears that, in an in vitro setting, NLRP6 is capable of driving NFκB activation. However, it must be taken into account that this is an overexpression system, involving transfection of non-physiological levels of ASC and NLRP6 into HEK293T cells. Thus, it may not be giving an accurate indication of what occurs in an in vivo setting.

5.2.5 Mutation R653G disrupts the ability of NLRP6 to activate NFκB

Having confirmed the ability of NLRP6 to drive NFκB activity in vitro, we next sought to investigate the impact of the NLRP6 mutation on NFκB activation. Site-directed mutagenesis was performed to introduce a Cytosine to Guanine mutation at position
2985bp of the NLRP6 plasmid, to mimic the C>G mutation identified at position 281691 of the NLRP6 gene. The C>G change at this position results in a change of Arginine to Glycine when the protein is translated. The mutated NLRP6\textsuperscript{R653G} plasmid was sequenced to confirm that the site had been successfully mutated (Figure 5.6 (A)). NFκB activation was again measured following transfection of ASC, NFκB-luciferase reporter plasmid and either wild-type (WT) NLRP6 or NLRP6\textsuperscript{R653G}. While WT NLRP6 drove NFκB activation in an ASC-dependent manner, NLRP6\textsuperscript{R653G} was not capable of activating NFκB (Figure 5.6 (B)). These findings therefore suggest that this single base-pair mutation has a functional impact on the NLRP6 protein.

5.2.6 NLRP6 is a cytoplasmic protein

Greiner et al have previously shown that overexpressed NLRP6 is localised within the cytoplasm (104). We wanted to confirm this finding, and to determine whether the NLRP6\textsuperscript{R653G} mutation changes the cellular localisation of the protein. HEK293T cells were therefore transfected with either caspase-1, WT or R653G NLRP6 plasmids for 24 hours. Immunostaining for caspase-1, a known cytoplasmic protein, was used as a cytosolic positive control. Flag antibody was used to immunostain for WT/R653G NLRP6 plasmids. Both WT and NLRP6\textsuperscript{R653G} appear to have a cytosolic localisation (Figure 5.7 (A)).

We next decided to investigate whether WT and NLRP6\textsuperscript{R653G} could form cytosolic specks, indicative of inflammasome formation, when co-transfected with ASC, as has been previously shown for NLRP6 (104). Therefore, the inflammasome was reconstructed in vitro by transfecting HEK293T cells with caspase-1, ASC and either WT or R653G NLRP6 (294). Interestingly, following transfection of both WT and NLRP6\textsuperscript{R653G} and in the presence of caspase-1, IL-1β and ASC, expression of both WT and mutant NLRP6 appears to form ASC-like specks within the cytosol of some cells (Figure 5.7 (B-C)). This findings suggest that while the mutation affects the ability of NLPR6 to activate NFκB, there was no impact on the proteins ability to form an inflammasome. Further suggesting that NLRP6 may use different regions of the protein to function in different ways.
5.2.7 Mutation NLRP6<sup>R653G</sup> has no significant impact on production of IL-1 family members following LPS stimulation for up to 4 hours

Another approach taken to gain further insight into the impact of the NLRP6<sup>R653G</sup> mutation was to compare the responsiveness of PBMCs from the NLRP6<sup>R653G</sup> patient and healthy controls. Before any patient samples could be obtained, ethics were applied for and granted by OLCHC Medical Research Ethics Committee. As the patient is under 18, both parent/guardian consent and patient assent forms were designed and given to the patient and parent/guardian along with an information sheet describing the details of the study and any possible repercussions that may occur as a result. Once consent was obtained, blood was drawn and PBMCs were isolated.

An experimental plan was designed to determine the general responsiveness of the patient’s PBMCs, examining inflammatory cytokine production and intracellular signalling pathways. Our in vitro assays and previous literature all suggest that NLRP6 plays a role in regulating NFκB. Therefore it was decided to activate NFκB in the patients PBMCs, and to compare the responses to similarly treated healthy controls. Additionally, NLRP6 has been implicated in forming an inflammasome with ASC and caspase-1 (104). Therefore, it was decided to investigate the ability of the patient’s PBMCs to form an inflammasome in response to inflammasome stimulants. We also sought to investigate the response of the patient’s PBMCs to two forms of heat-killed bacteria, both of which activate NFκB signalling pathways. These experiments were designed to gain an initial insight into the possible effect that mutation R653G may have on the protein.

LPS is a component of Gram negative bacteria that is universally used as a TLR4 stimulant (14). Ligand binding to TLR4 results in activation and translocation of NFκB into the nucleus, promoting the upregulation of pro-inflammatory cytokines (295). To induce NFκB activation, PBMCs were treated with LPS over a time course of 0, 15, 30, 60, 120 and 420 minutes. Supernatants were subsequently analysed for cytokine secretion by ELISA. Although transcriptional upregulation of proIL-1β is NFκB dependent, secretion of mature IL-1β is inflammasome dependent. Therefore LPS alone would not be expected to stimulate IL-1β secretion from healthy PBMCs. However, it has been shown in CAPS patients (MWS) that LPS stimulation alone can cause IL-1β secretion from PBMCs, without the need for a second, inflammasome activating, signal (296). ELISA analysis revealed that minimal IL-1β secretion occurred in both the NLRP6<sup>R653G</sup>
patient and healthy control PBMCs following 0-420 minutes of LPS stimulation (Figure 5.8 (A)). This suggests that the NLRP6 mutation does not lead to inappropriate inflammasome activation following an initial, priming signal.

Interestingly, on one occasion over the course of the year (and 4 separate PBMC isolations), the patient was secreting high basal levels of IL-18. However, overall there were no significant differences in the secretion of IL-18 between the patient and healthy controls (Figure 5.8 (B)). IL-18 is a member of the IL-1 family of cytokines. Similarly to IL-1β, the processing and secretion of IL-18 is mediated by caspase-1, which is dependent upon inflammasome activation. IL-1α is an additional IL-1 family member that was examined. In contrast to IL-1β, transcription of proIL-1α is NFκB-independent and its processing, which is not essential, is independent of caspase-1. IL-1α release is thought to occur during inflammatory forms of cell death (297). IL-1α ELISA results show that little IL-1α release occurred in patient and healthy control PBMCs, with no differences in minimal levels of secretion following LPS stimulation (Figure 5.8 (C)). This result suggests that the secretion of danger signals, such as IL-1α, are not overtly affected in NLRP6<sup>R653G</sup> patient PBMCs. Findings from investigation of these three IL-1 family members would suggest that mutation NLRP6<sup>R653G</sup> has no observable effect on the secretion of inflammasome mediated cytokines (IL-1β, IL-18) or secretion of cytokines released during cell lysis (IL-1α).

5.2.8 The NLRP6<sup>R653G</sup> patient produces significantly higher levels of IL-6 from PBMCs

To measure the effect of the NLRP6<sup>R653G</sup> mutation on NFκB signalling, IL-6 levels were determined in both the NLRP6<sup>R653G</sup> patient and healthy controls following stimulation with LPS for 0-420 minutes. IL-6 is a pro-inflammatory cytokine whose expression is up-regulated following activation of NFκB. IL-6 production has been implicated in a wide range of inflammation-associated disease states and is primarily up-regulated at sites of acute and chronic inflammation (298)(299)(300). Interestingly, the NLRP6<sup>R653G</sup> patient produced significantly higher levels of IL-6 basally and at early time points following LPS stimulation, when compared to healthy controls. (Figure 5.9 (A)). The human IL-6 promoter contains multiple regulatory elements, including those binding transcription factors belonging to the NF-κB, C/EBP and AP-1 families. The consistently elevated
basal levels of IL-6 indicates that NLRP6$^{R653G}$ is inducing a chronic inflammatory state via uncontrolled activation of one of these transcription factors in the patients PBMCs.

TNF-α was an additional NFκB-dependent pro-inflammatory cytokine that was analysed (**Figure 5.9 (B)**). TNF-α is a potent cytokine that is involved in systemic inflammation and also contributes to the acute phase reaction. While the patient displayed higher basal levels of IL-6, there were no TNF-α differences observed between the patient and healthy controls at basal levels and the patient responded similarly to healthy controls after longer LPS stimulations (120 and 420 minutes). However, it must be noted that the patient was receiving Adalimumab, the anti-TNF-α antibody, for the entire duration of this study. While it is not likely that this drug would interfere with the patient’s ability to respond to stimulants and subsequent ability to produce TNF-α, it is possible that this drug is preventing basal production of TNF-α from the patients PBMCs.

IL-8 is a chemotactic factor which attracts neutrophils, basophils and T cells to sites of inflammation. The IL-8 promoter sequence contains DNA binding sites for the inducible transcription factors AP-1, NF-IL-6, and NFκB, and functional cooperativity among these factors appears to be critical for optimal IL-8 production. As shown in **Figure 5.9 (C)**, the patient produced similar levels of IL-8 when compared to healthy controls, suggesting that the NLRP6$^{R653G}$ mutation does not interfere with basal production of IL-8 by PBMCs, or in response to LPS. Ultimately, these observations support the theory that NLRP6 may play a role in regulating IL-6, but not IL-8 or TNF-α, production and that the NLRP6$^{R653G}$ mutation impedes the ability of NLRP6 to regulator IL-6.

### 5.2.9 Enhanced pro-IL-1β expression in NLRP6$^{R653G}$ patient PBMCs following LPS stimulation

Intracellular signalling proteins were also examined in the PBMC lysates following stimulation with LPS for 0-240 minutes. Extracellular Signal-Regulated Kinase-1 and -2 (ERK1/2) are members of the MAP kinase family, a primordial signalling family that play important roles in regulating proliferation, differentiation, apoptosis and survival. While ERK can play important roles in a range of pathways, the focus of this study was to investigate its changes in response to TLR stimulants (301). Like most kinases, ERK activation can be measured by its phosphorylation status. ERK1 is directly phosphorylated by MEK at Thr202/Tyr204 and ERK2 at Thr185/Tyr187 following stimulation of TLR4. **Figure 5.10** reveals that increased expression of phosphor-ERK1/2
proteins occurs in PBMCs from the NLRP6^{R653G} patient, compared to a representative healthy control, following 15, 30 and 60 minutes LPS stimulation. Additionally, ERK phosphorylation is observed in the untreated control of the patient with NLRP6^{R653G} suggesting that a low basal activation of ERK1/2 is occurring in the patient with mutation NLRP6^{R653G}.

IκBα is an inhibitory protein that functions by binding to NFκB in the cytosol, blocking the ability of the transcription factors to translocate to the nucleus. Following TLR activation, a series of intermediary proteins act in a signalling cascade that eventually culminate in the activation of IκKα/β. IκKα/β phosphorylates IκB, which causes its degradation, allowing NFκB to translocate to the nucleus. Measuring the levels of IκBα, and its degradation, can be used as an indirect readout of NFκB activation. Immunoblot of IκBα in Figure 5.10 shows degradation of IκBα at 60 and 120 minutes in the representative healthy control and the return of IκBα at 240 minutes. Interestingly, there is less expression of IκBα in PBMCs from the NLRP6^{R653G} patient prior to LPS stimulation, and a possible delay in the kinetics of IκBα degradation following LPS stimulation. This finding suggests that enhanced NFκB activation may be occurring in the NLRP6^{R653G} patient PBMCs prior to activation of cells with LPS.

Examination of intracellular caspase-1 expression levels following PBMC stimulations with LPS show similar levels of expression in healthy controls and the NLRP6^{R653G} patient (Figure 5.10). This suggests that the NLRP6^{R653G} mutation does not alter the expression of the inflammatory caspase which is activated within the canonical inflammasome. Pro-IL-1β expression levels appeared to be higher in the NLRP6^{R653G} patient PBMCs, when compared to healthy controls (Figure 5.10). As mentioned previously, pro-IL-1β expression is upregulated following NFκB activation and its up-regulation is independent of inflammasome activation. Collectively these findings suggest that PBMCs from the patient with mutation NLRP6^{R653G} display an enhanced NFκB and ERK activity, consistent with the inflammatory phenotype of the patient.

5.2.10 Mutation NLRP6^{R653G} has no observable impact on NLRP3 activation

Findings in this study have so far suggested a role for NLPR6 in regulating NFκB-mediated signalling pathways. Therefore, we next sought to investigate the impact of the NLRP6^{R653G} mutation following inflammasome activation. As mentioned previously, the key aim of this study was to investigate the impact of the NLRP6^{R653G} mutation to
TLR/NLR ligands. NLRP6 has been shown to form an inflammasome with ASC and caspase-1 within the intestine (104), and the inducer of NLRP6 inflammasome has very recently been proposed to be lipoteicoic acid (LTA) from Gram positive bacteria (102). However, during the research for this project, a ligand/stimulator for the NLRP6 inflammasome had yet to be identified. As discussed previously, inflammasome activation occurs via two signals. LPS or other TLR agonists are generally used to activate the first ‘priming’ signal in vitro. Two activators that are commonly used as 2nd signals, to activate the NLRP3 inflammasome in vitro are ATP and Nigericin. ATP is an endogenous danger signal that is widely used as a NLRP3 canonical activator. Both ATP and Nigericin cause an unspecific conductance of K⁺ across the cell membrane. A reduction of intracellular K⁺ has been found to be crucial for NLRP3 inflammasome activation in monocytes and macrophages (80). Similar to ATP and Nigericin, Poly(I:C) activates NLRP3, however its mechanism of activation occurs in a different manner. Poly(I:C) is a double stranded RNA that is responsible for activating NLRP3 in a MAVS dependent manner that also results in a decrease in intracellular K⁺ (302). Additionally, Poly(dA:dT) is a double stranded DNA that is responsible for activating the AIM2 inflammasome (303).

To activate the inflammasome in the patient, PBMCs were either primed with LPS for 12 hours and stimulated with ATP or Nigericin for the final 30 minutes of the experiment or primed with LPS for 4 hours and transfected with Poly(dA:dT) or Poly(I:C) for a further 8 hours. Figure 5.11 display IL-1β (A), IL-18 (B) and IL-1α (C) expression levels following stimulation with LPS in conjunction with ATP, Nigericin, Poly(dA:dT) or Poly(I:C) from the patient and healthy control PBMCs. Findings shown in Figure 5.11 suggest that mutation NLRP6^R653G does not impact the patient’s ability to respond to inflammasome stimulation, as similar levels of IL-1β, IL-18 and IL-1α were observed. However it cannot be conclusively stated that this mutation does not affect inflammasome activation as these stimulants are most likely not activating the NLRP6 inflammasome. It does however, suggest that the mutation does not affect the patient’s ability to activate other inflammasomes in response to these stimuli.
5.2.11 Production of NFκB-regulated cytokines is unaffected following inflammasome stimulation

Our findings to date suggest that the NLRP6\textsuperscript{R653G} mutation leads to a possible dysregulation of NFκB signalling in PBMCs (Figure 5.10) and increased basal secretion of IL-6 (Figure 5.9). Collectively, these results suggest that NLRP6 may play a role in regulating NFκB. To determine whether any differences in NFκB-mediated cytokine production occurred following inflammasome stimulation, IL-6, TNF-α and IL-8 levels were also measured. As was seen with the IL-1 family of proteins, the patient produced similar levels of IL-6, TNF-α and IL-8 (Figure 5.12 (A-C) respectively) following stimulation with known inflammasome stimulants, suggesting that the mutation is affecting NFκB alone and has no impact on inflammasome functioning. Findings from these experiments suggest that activation of NLRP3 and AIM2 inflammasomes are not affected by the NLRP6\textsuperscript{R653G} mutation. Experiments should be repeated with LTP (as carried out in recent Cell paper (102)) before any conclusion regarding the impact of the mutation on NLRP6 inflammasome formation can be made.

The expression levels of intracellular proteins involved in inflammasome complexes were also investigated following inflammasome activation. Caspase-1 is a crucial component of canonical inflammasomes and, once activated, it directly mediates IL-1β and IL-18 cleavage. No cleaved, active caspase-1 fragments were detectable in either the patient or healthy control PBMCs following stimulation with LPS and ATP or Nigericin (Figure 5.13). However, caspase-1 expression does appear to be induced in the patients and not healthy controls PBMCs following stimulation with LPS. Additionally, as was seen in Figure 5.10, pro-IL-1β levels were significantly higher in the patients PBMCs when compared to healthy controls (Figure 5.13). Increased expression of pro-IL-1β is indicative of signal 1 ‘priming’ and NFκB activation, as this drives the expression of pro-IL-1β. This suggests that the NLRP6\textsuperscript{R653G} mutation is either inducing aberrant activation of NFκB or preventing NLRP6 from efficiently acting as a negative regulator of NFκB activity. To distinguish between the two proposed mechanisms, the exact role of NLRP6 must first be elucidated.
5.2.12 Patient with mutation NLRP6<sup>R653G</sup> produces significantly higher levels of Caspase-1 and pro-IL-1β following infection with heat-killed bacteria

Developments in science have led to a much greater understanding of the exact components in bacteria or viruses that are detected by the host immune response. For example, the discovery that TLR4 recognises the LPS component of gram-negative bacteria has led scientists to use LPS alone as a means of TLR4 activation. While these developments have made the analysis of signalling pathways much more straightforward, there are also various downfalls to this use of experimental setup. By eliminating the whole bacterium or virus, one is also eliminating other possible bacterial ligands that are still unknown. Stimulation of cells with a single ligand will not result in phagocytosis of a bacterium or induce any cellular damage within. Therefore, a more accurate means of stimulating cells can be to use whole bacteria or viruses.

*Lactobacillus rhamnosus* is a gram positive bacteria that is responsible for the activation of TLR2. *L. rhamnosus* is a non-pathogenic inhabitant of the human microflora and is used as a preservative in various dairy products to extend their shelf life. Heat-killed *L. rhamnosus* (HKLR) has been shown to induce a potent TNF-α response in mouse mononuclear cells (304). PBMCs from the patient and healthy controls were stimulated with HKLR for 24 hours at increasing concentrations. **Figure 5.14** shows that patient NLRP6<sup>R653G</sup> produced similar levels of IL-1β (A), IL-1α (B) and IL-18 (C) following stimulation with HKLR. These results suggest that mutation NLRP6<sup>R653G</sup> does not affect inflammasome activation and increased cell death in response to HKLR. In addition, no significant differences in the production of NFκB mediated pro-inflammatory cytokines, IL-6, TNF-α, and IL-8, were observed in response to HKLR (**Figure 5.14 (D-F)**). This suggests that the NLRP6<sup>R653G</sup> mutation does not affect the patient’s ability to respond to sustained NFκB stimulation.

Western blot analysis of cell lysates stimulated with HKLR show increased expression of pro-caspase-1 and pro-IL-1β in the patient with NLRP6<sup>R653G</sup> when compared to healthy controls following HKLR stimulation (**Figure 5.15**). As mentioned previously, expression of pro-IL-1β and caspase-1 are induced following NFκB activation, which again highlights the increased activation of NFκB occurring in the PBMCs of the patient.

Heat-killed *Salmonella typhimurium* (HKST) is a flagellated gram-negative bacteria that is a potent agonist of TLR2 and TLR4. TLR2 recognises peptidoglycan components of
HKST cell wall and TLR4 recognise the LPS component of the cell wall that results in the production of pro-inflammatory cytokines such as TNF-α and IL-6 (305).

As the TLR2 agonist, HKLR, produced an increased expression of caspase-1 and pro-IL-1β in the patients PBMCs, we next sought to investigate the patient’s response to a combined TLR2 and TLR4 agonist. As was seen when the patients PBMCs were stimulated with HKLR, stimulation with HKST resulted in the production of similar levels of IL-1β, IL-18, IL-1α, IL-6, TNF-α and IL-8 (Figure 5.16 (A-F) respectively). These findings suggest that mutation NLRP6<sup>R653G</sup> does not affect cytokine production in response to these bacterial ligands.

Intracellular protein expression levels of inflammasome components were also examined following HKST stimulation. Similarly to HKLR stimulation, expression of pro-caspase-1 and pro-IL-1β is increased in the PBMCs of the patient with mutation NLRP6<sup>R653G</sup> (Figure 5.17). Increased expression of pro-IL-1β has been observed at all time-points and treatments examined so far, and would suggest that mutation NLRP6<sup>R653G</sup> significantly impacts the ability of NFκB to up-regulate the expression of pro-IL-1β.

Collectively, the stimulation of PBMCs with both HKLR and HKST have suggested there is no impact in response to bacterial infection when mutation NLRP6<sup>R653G</sup> is present. While an exact mechanism has yet to be elucidated, there appears to be a link between NLRP6 and NFκB. Whether NLRP6 is acting as a positive or negative regulator of NFκB is still unclear, as it is still unknown if mutation NLRP6<sup>R653G</sup> is a gain or loss of function mutation.

5.2.13 Generation of Stable Cell lines expressing NLRP6 results in cell death

As the patient with mutation NLRP6<sup>R653G</sup> appeared to display an elevated basal activation of NFκB, we next sought to generate THP-1 cells that express either the WT NLRP6 or NLRP6<sup>R653G</sup>. The generation of stably-expressing cell lines are a useful way to study the effect of expression of a particular gene and, in this case, determine if a mutation alters the function of the gene. The generation of a cell line stably-expressing either WT or mutant NLRP6 would allow us to conclusively determine if the mutation in NLRP6 is attributing to the increased basal activation of NFκB and increased production of IL-6.

Therefore, it was decided to integrate both the WT and mutant NLPR6 gene into a lentiviral vector, PD1 and spinoculate the lentiviral vectors, along with packaging
vectors, into THP-1s. After multiple cloning attempts, the NLRP6 WT and R653G genes was successfully cloned into PD1 vectors. To confirm that cloning was successful, primers specific to the NLRP6 gene product were used to amplify the cloned regions within the vectors. 7 WT and 7 NLRP6\textsuperscript{R653G} clones were tested to determine if the WT and mutant genes were successfully ligated into the plasmid. Figure 5.18 (A) shows that all clones appeared to be successful, and clone 1 (WT) and clone 7 (R653G) were chosen for subsequent transfections. Transfection of lentiviral WT and mutant NLRP6 plasmids were performed 3 times and on each transfection, cells transfected with NLRP6 WT were visibly dead. Interestingly, cells expressing empty vector or NLRP6\textsuperscript{R653G} did in fact survive. Western blot analysis of Flag expression showed that cells expressing NLRP6\textsuperscript{R653G} express Flag-NLRP6 (Figure 5.18 (B)). However, no flag expression was observed in cells transfected with WT NLRP6. As cells transfected with WT NLRP6 did not survive, one can conclude that NLPR6 is most likely toxic to THP-1s. While actin was still observable in WT NLRP6 lysates, this was likely due to the presence of dead but not degraded cells still present in the culture. Observation of cultures confirmed there was no growth in cells stably-transfected with WT NLRP6. Interestingly, mutant NLRP6 did not induce cell death to the same extent, suggesting that the single base pair mutation is most likely altering the function of the protein.

To determine whether transient transfection of the WT and NLRP6\textsuperscript{R653G} plasmids also induces cell death in THP-1s, a plasmid concentration range (0-200 ng) was transfected into THP1s for 48 hours. An alamarBlue was used to determine the viability of the transfected THP-1 cells. Figure 5.19 (A) shows that both the WT and NLRP6\textsuperscript{R653G} plasmids induce a significant amount of cell death, with 25-35% cell viability being observed after 48 hours. Hek293T cells, which are much more resistant to cell death, were similarly transfected with WT and NLRP6\textsuperscript{R653G} plasmids for 48 hours and cell viability also decreased following transfection (Figure 5.19 (B)). Collectively, these findings confirm that NLRP6 is toxic to THP-1 and Hek293T cells. Therefore, the use of an inducible expression plasmid (such as Tetracycline (Tet) inducible expression) may be more successful to study the role of mutation NLRP6\textsuperscript{R653G}. 

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5.3 Discussion

This study has investigated the impact of a novel mutation in NLRP6 and its association with an autoinflammatory syndrome. As previously discussed, the literature surrounding NLRP6 has been very limited to date and there have been no reports linking NLRP6 and autoinflammation. Findings from this study support the evidence that this novel mutation, NLRP6<sup>R653G</sup>, is responsible for an autoinflammatory disease observed in a paediatric patient attending Crumlin Children’s hospital. The consistently elevated inflammatory markers observed in the patient are indicative of the constant inflammatory status of the patient. As is seen in this study, administration of Adalimumab (anti-TNF-α), reduced CRP, ESR and amyloid levels. However, these markers have not yet been reduced to an acceptable level in the patient, and a more effective treatment is required.

This study also identified the patient’s mother as a carrier of mutation NLRP6<sup>R653G</sup>. As was briefly mentioned, CAPS-related mutations in NLRP3 have also been identified in asymptomatic relatives of CAPS patients (154). In FMF patients, studies have investigated the effect of environmental triggers on the phenotype of the disease and report that there is a stronger correlation of phenotype with the country of origin as opposed to the genetic mutation (306). Another study showed that Turkish children born and raised in Turkey have a greater disease severity than Turkish children raised in Germany, suggesting that environmental factors play an important role in the severity of an autoinflammatory disease (306). The explanation for the patient, but not the patient’s mother, displaying autoinflammatory symptoms is therefore likely due to the different environments they experienced at a young age.

The majority of research published thus far has focused on the role NLRP6 plays in maintaining intestinal homeostasis (100)(94). However, recent research is allowing us to slowly gain a greater understanding of the role NLRP6 plays in innate immunity, and its interaction with NFκB (104)(103). Findings from this study suggest that NLRP6 is capable of regulating NFκB activity. However, the exact mechanism underlying this regulation is still unknown. As discussed previously, published reports are in contradiction as to whether NLRP6 acts as a positive or negative regulator of NFκB. Grenier <i>et al.</i>, showed that overexpressed NLRP6 activates NFκB in an ASC-dependent manner in HEK293T cells (104). While a later publication by Anand <i>et al.</i>, used NLRP6 deficient mice to show that it plays a role in dampening NFκB activation during <i>L.</i>
monocytogenes infection, both in vitro and in vivo (103), showing that Nlrp6−/− BMDM stimulated with TLR2/TLR4 agonists secrete elevated levels of TNFα and IL-6 (11). In contrast, another study by Seregrin et al. have shown that NLRP6 expression in Ly6Chi monocytes drives TNFα production, mediated by its ability to enhance IL-18 production (101). Similar to the report by Greiner et al. in 2002, we have shown that NLRP6 overexpression is capable of activating NFκB in an ASC-dependent manner in HEK293T cells. The functional variance of NLRP6 that is suggested by these studies may be explained by cell-specific roles of NLRP6 or alternatively, the lack of other immune components expressed in the cell line examined. HEK293Ts are a cell line derived from human embryonic kidney cells that do not express most inflammatory proteins. It is therefore an artificial means of studying interactions within intracellular immune pathways. In a physiological setting, it is more likely that multiple proteins are interacting with each other intermittently over periods of time. Studying the functional role of NLRP6 during NFκB activation is in fact more complex than just two isolated proteins coming into contact without the presence of the myriad of other immune mediators. Nevertheless, HEK293Ts and NFκB reporter genes still represent a useful tool to tease out mechanisms of NFκB activation, using various inhibitors designed to target proteins along the pathway.

Our bioinformatic analysis suggests that mutation NLRP6R653G is most likely damaging the NLRP6 protein. Arginine is a large, polar amino acid that is often found at the active site of proteins and enzymes due to its amine-containing side chain. However, glycine is a smaller, less bulky amino acid. It is likely that the drastic change in amino acids at this site has altered the 3-dimensional structure of NLRP6 in this patient. This, combined with the fact that introduction of the NLRP6R653G mutation into an NLRP6 expression plasmid renders it incapable of activating NFκB in HEK293T cells, suggests that mutation R653G has a significant impact in protein function, causing a loss-of-function mutation.

A loss of function mutation as a result of R653G in NLRP6 would support the functional role for wild-type NLRP6 as a negative regulator of NFκB, which was previously suggested by Anand et al (103). Further support for this hypothesis comes from a recent publication which showed that perihematomal brain tissue from NLRP6−/− mice produce significantly higher levels of IL-1β, IL-6 and TNF-α following intracerebral haemorrhage (307), suggesting that TLR4 regulates NLRP6 expression following intracerebral
haemorrhage. Similarly, another recent report has suggested that NLRP6 negatively regulates NFκB and acts in a protective role during alkali burn to the cornea. This study used NLRP6 siRNA to ablate NLRP6 and reported a significant increase in NFκB activation, corroborating the observations we have made in this study regarding NLRP6 negatively regulating NFκB (308). Likewise, research investigating the role of NLRP6 in the intestine have reported that \( \text{NLRP6}^- \) mice are extremely susceptible to colitis, which suggests that NLRP6 may also be dampening inflammation in the intestine (94)(100). This study proposes a similar role for NLRP6. An increase in basal expression levels of pro-IL-1β and IL-6 production as well as dysregulated NFκB signalling in the patient with a mutation in NLRP6 would suggest that under normal conditions NLRP6 may in fact act as a negative regulator of NFκB. However, when the mutation is present, NLRP6 may be no longer capable of NFκB inhibition, resulting in unregulated basal activation of NFκB.

Although NFκB is the main transcription factor associated with pro-IL-1β expression, its transcriptional expression is also mediated by two alternative transcription factors, Spi-1/PU.1 and nuclear factor IL-6 (NFIL-6) (309, 310). It is therefore possible that either of these transcription factors may mediate the increased expression of pro-IL-1β observed in the NLRP6\(^{R653G}\) PBMCs, which proposes an area of future investigation. Nevertheless, the majority of research to date suggests that NLRP6 regulates NFκB in an inhibitory capacity and it is evident that our research investigating the patients PBMCs suggests the same.

Findings from this study suggest that mutation NLRP6\(^{R653G}\) is causing the patient to secrete high basal levels of IL-6. Transcription of IL-6 is tightly regulated and increased expression of IL-6 has been implicated in a range of inflammatory diseases. The transcriptional regulation of the IL-6 gene is complex and involves a range of different transcription factors, i.e., NFκB, specificity protein 1 (SP1), NF-IL-6 (also known as CAAT/enhancer- binding protein b), interferon regulatory factor 1 (IRF1) and activator protein 1 (AP-1). While this study has investigated NFκB activation, it is possible other transcription factors may mediate the increased production of IL-6 that we observed in the patient during our study. Future experiments should therefore focus on the other transcription factors responsible for IL-6 induction.
As mentioned previously, the patient presented with symptoms such as panniculitis, granulomatous hepatitis, arthritis, and microcytic hypochromic anaemia. Interestingly, high levels of IL-6 may explain some of the patient’s symptoms. Once synthesised, IL-6 transits via the blood stream to the liver, where it induces subsequent recruitment of acute phase proteins such as C-reactive protein, serum amyloid, fibrinogen etc. IL-6 has been shown to induce hepcidin production, which blocks the action of iron transporter, ferroportin 1 on the gut (298). A blockade of Ferroportin 1 results in decreased serum iron levels as iron cannot be efficiently absorbed. The IL-6-hepcidin axis is considered to be responsible for the hypoferremia and anaemia symptoms which are associated with chronic inflammation (298). Microcytic hypochromic anaemia is mainly induced as a result of reduced circulating iron levels. Therefore, we hypothesise that the patient’s anaemia may be explained by the increased basal production of IL-6 observed during this study. IL-6 has also been found to induce excess VEGF, which is reported to lead to increased vascular permeability and angiogenesis. These are two typical pathological features of inflammatory lesions and are seen in synovial lesions of rheumatoid arthritis patients (311). High basal secretion of IL-6 from the patient’s PBMCs may explain the development of arthritis in this patient. Additionally, IL-6 has been reported to aid in keratinocyte proliferation (312) and the generation of collagen in dermal fibroblasts, which is believed to account for changes in the skin of systemic sclerosis patients (313). In theory, treatment of the patient with an anti-IL-6 therapy would address whether IL-6 was responsible for the patients symptoms, however that would be a clinical decision.

Until very recently, there was no known ligand responsible for the activation of NLRP6. In vitro studies had previously suggested that NLRP6 forms an inflammasome with ASC and caspase-1 (104). However, the Nunez group recently presented evidence of inflammasome formation in haematopoetic cells, identifying lipoteichoic acid (LTA) as a novel activator of an NLRP6 inflammasome (102). Our findings suggest that typical inflammasome stimulants such as ATP, Nigericin, Poly(dA:dT) and Poly(I:C) do not activate the NLRP6 inflammasome. Or if they do, there are no differences in the patient’s IL-1β or IL-18 responses. Additionally, in vitro experiments suggested that similar levels of ASC specks form in both NLRP6 WT and mutant transfectected Hek293T cells. However, as the gram-positive bacterial component, LTA has now been identified as an NLRP6 activator in a murine study (102), it would be wise to infect PBMCs with LTA to examine the formation and activation of the NLRP6 inflammasome in the patient.
While *in vitro* evidence suggests that mutation NLRP6$^{R653G}$ does not affect the ability to form an inflammasome, until this is re-examined with the newly discovered ligand of NLRP6, one cannot conclusively report that inflammasome formation is not affected.

Interestingly, as the study presented by Hara *et al.*, has now identified LTA as an NLRP6 activator, it would be expected that another gram-positive bacteria containing LTA would also be capable of activating NLRP6. In this study, *L.rhamnosus* was used to infect PBMCs of the patient. *L.rhamnosus* is a non-pathogenic gram-positive bacteria that inhabits the human microbiome and also contains LTA in its structure. However, this study did not observe any differences in inflammasome activation following infection with *L.rhamnosus* which would suggest that the inflammasome formation of the patient is not affected by the presence of mutation R653G. *S.typhimurium* is a TLR2 and TLR4 stimulating bacteria that is also recognised by NLRC4 and typically results in the formation of an inflammasome. However, again no differences in inflammasome activation were observed between the patient and the healthy controls. The data presented in this study did however suggest that in the presence of both HKLR and HKST, the patient produced higher expression levels of pro-IL-1β and caspase-1 when compared to healthy controls, which is indicative of NFκB activation and not inflammasome activation.

As this study has proposed a novel mutation as causative of an autoinflammatory syndrome, it was important to conclusively determine that mutation NLRP6$^{R653G}$ is responsible for the observations presented during this study. Unfortunately, while attempting to generate stably-expressing THP-1 cells, it was determined that NLRP6 is in fact toxic to this human monocyctic cell line. However, due to time constraints it was not possible to stably express both WT and mutant NLRP6 in an alternative cell type that did allow expression. Therefore, the future direction of this project will focus on confirming these findings in a cell type capable of expressing NLRP6.

Findings from this study suggest that the patient is displaying a severe dysregulated immune phenotype. There appears to be a definite link between NLRP6 and NFκB, however the exact nature of their interaction has yet to be discovered. Once the findings from this study have been confirmed in a qualified medical laboratory, these results will help the patients treating clinician to design a personalised treatment plan for the patient.
As the patient is at a young age, this could potentially have a significant impact on their quality of life for years to come.
Figure 5.1: Clinical profile of paediatric patient being treated at Our Lady’s Children Hospital, Crumlin.

The patient underwent phlebotomy at each clinic visit and blood was analysed for typical inflammatory markers. CRP (A), ESR (B) and Amyloid A (C) were measured from 2012 – 2017 and levels were graphed using Graphpad prism. Reference levels of each marker are displayed with a red line on each graph. In March of 2013, patient was administered 80 mg of Adalimumab followed by weekly doses of 20 mg. In 2016, the weekly dose of Adalimumab was increased to 40 mg.
### Table 5.1: List of drugs administered to patient during course of treatment.
The drugs listed in this table were administered to the patient from 2006 to 2018.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Start Date</th>
<th>End Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron supplements (1 Galfer BD)</td>
<td>Jan 2006</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>Feb 2012</td>
<td>Feb 2013</td>
</tr>
<tr>
<td>Sulphasalazine (250 mg BD)</td>
<td>Mar 2012</td>
<td>Apr 2012</td>
</tr>
<tr>
<td>Azithioprine (25-37.5 mg)</td>
<td>Apr 2012</td>
<td>Apr 2012</td>
</tr>
<tr>
<td>Colchicine (20 mg/kg)</td>
<td>Jan 2013</td>
<td>Jan 2013</td>
</tr>
<tr>
<td>Adalimumab (80 mg followed by 20 mg weekly. Increased to 40 mg subcutaneously weekly)</td>
<td>Mar 2012</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Ciproheptadine</td>
<td>Feb 2014</td>
<td>Mar 2014</td>
</tr>
<tr>
<td>Vitamin D (1000 units daily)</td>
<td>May 2014</td>
<td>Jul 2015</td>
</tr>
<tr>
<td>Genotropin (1.6 mg daily)</td>
<td>Jul 2015</td>
<td>Ongoing</td>
</tr>
</tbody>
</table>
Figure 5.2: Sequencing results of the NLRP6 mutation present in patients genome. Genomic DNA was isolated from the blood of the patient and both parents. Once quantified, a region of NLRP6 was amplified and the previously determined mutation site was sequenced by MWG Eurofins.
Figure 5.3: NLRP6 is coded by eight exons to form the PYRIN, NACHT and 5 LRR domains.

The DNA and protein sequence of NLRP6 were aligned and the exon that codes for each protein domain was linked in a schematic. NLRP6 is located from 278,365-285,359 on chromosome 11 and has eight exons that code for two transcripts. The mutation identified in the patient was C>G at position 281691 (SNP rs146018617, highlighted by red arrow).
Figure 5.4: Mutation R653G is predicted to be a ‘probably damaging’ mutation and located on the external surface of NLRP6. 3D protein structure prediction software, PHyre 2 was used to predict the 3D structure of NLRP6$^{R653G}$ and a predicted structure was modelled and visualised using RasTop (A). Mutation R653G is highlighted in red. Polyphen has predicted mutation R653G to be a ‘probably damaging’ mutation with a probability score of 0.993, sensitivity 0.7 and specificity 0.9 (B).
Figure 5.5: NLRP6 activates NFκB in the presence of ASC, which is inhibited when IKK inhibitor (MC005) is co-transfected.

HEK293T cells were seeded at a density of 2 x 10^5 cells/mL in 200 µl/well in a 96-well plate. 24 hours later NLRP6, ASC and NFκB Luciferase Reporter gene (κB/TK) were transfected into cells. The luciferase activity was measured 16 hours later (A). NLRP6, ASC, κB/TK and IKK inhibitor (MC005) were transfected into cells. The luciferase activity was measured 16 hours later (B). Western blot confirms overexpression of NLRP6 plasmid in HEK293T cells (C). Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6^{R653G}. 

Figure 5.6: Mutation R653G disrupts the ability of NLRP6 to activate NFκB.
Site-directed mutagenesis was performed to mutate the amino acid residue 653 from Arginine to Glycine. Sequencing of site-directed mutagenesis NLRP6 plasmid vs WT NLRP6 plasmid is shown (A). HEK293T cells were seeded at a density of 2 x 10^5 cells/mL in 200 µl/well in a 96-well plate. 24 hours later NLRP6, NLRP6R653G, ASC and NFκB Luciferase Reporter gene (κB/TK) were transfected into cells. The luciferase activity was measured 16 hours later (A). Luciferase assay of WT and NLRP6R653G was graphed using GraphPad Prism (B). Two-way ANOVA found *** p < 0.001.
Figure 5.7: NLRP6 WT and NLRP6\textsubscript{R653G} are equally capable of forming inflammasome-associated ‘specks’.

HEK293T cells were seeded at 2 x 10\textsuperscript{5} cells on coverslips in a 24-well plate. Cells were transfected for 24h with either NLRP6\textsubscript{WT}, NLRP6\textsubscript{R653G} or Caspase-1/IL-1\beta (A) and then subsequently transfected as in (A), but with the addition of ASC (B). Cells were fixed with 4\% paraformaldehyde and stained for FLAG (NLRP6 WT and R653G), Caspase-1 and DAPI, to visualise nuclei. Immunofluorescence was viewed on Olympus BX51 and images were taken at 10 X magnification (A)(B). ASC Specks were counted per field and graphed on GraphPad Prism (C). Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6\textsubscript{R653G}. 
Figure 5.8: PBMCs from healthy donors and the NLRP6^{R653G} patient secrete similar levels of IL-1β, IL-18 and IL-1α both basally, and when primed with LPS. PBMCs were isolated at three month intervals over the course of one year from a patient with NLRP6 mutation R653G and healthy age and sex-matched controls. Three healthy controls were used at each time point. PBMCs were seeded at 1 x 10⁶ cells/mL and stimulated with 1 μg/mL LPS for 0, 15, 30, 60, 120, 240 minutes. IL-1β (A), IL-18 (B) and IL-1α (C) expression levels of PBMCs from the NLRP6 mutated patient and healthy controls were determined by ELISA. n=4 for NLRP6^{R653G} patient and n=12 for healthy controls. Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6^{R653G}.
Figure 5.9: PBMCs from the NLRP6<sup>R653G</sup> patient secrete elevated levels of IL-6, both basally and when primed with LPS.

PBMCs were isolated from the NLRP6<sup>R653G</sup> patient and healthy age and sex-matched controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 1 µg/mL LPS for 0, 15, 30, 60, 120, 420 minutes. IL-6 (A), TNF-α (B) and IL-8 (C) expression levels of PBMCs from the NLRP6 mutated patient were determined by ELISA and compared to healthy controls. n=4 for NLRP6<sup>R653G</sup> patient and n=12 for healthy controls. Two-way Anova statistical tests determined * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5.10: Dysregulated NFκB signalling is observed in NLRP6<sup>R653G</sup> patient PBMCs.

PBMCs were isolated from the NLRP6<sup>R653G</sup> patient and healthy age and sex-matched controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 1 μg/mL LPS for 0, 15, 30, 60, 120, 240 minutes. Cells were lysed in 150 μL RIPA buffer and lysates were run on 12% SDS-PAGE gels. Western blots were probed for phospho-ERK1/2, total ERK1/2, IκBα, Caspase-1, pro-IL-1β and Actin. Blot is representative of results obtained for N=9 healthy controls and N=3 NLRP6<sup>R653G</sup> patient.
PBMCs from the NLRP6R653G patient and healthy controls secrete similar levels of IL-1β, IL-18 and IL-1α following inflammasome activation.

PBMCs were isolated from a patient with mutation NLRP6R653G and healthy age and sex-matched controls. PBMCs were seeded at 1 x 10⁶ cells/mL and stimulated with 1 μg/mL LPS for 12 hours and stimulated with 5 mM ATP and 3.4 μM Nigericin for the last 30 minutes of the 12 hour stimulation. Alternatively, PBMCs were stimulated with LPS for 4 hours and transfected with 1 μg/mL Poly(dA:dT) or Poly(I:C). Secreted IL-1β (A), IL-18 (B) and IL-1α (C) were determined by ELISA and compared to healthy controls. n=4 for NLRP6R653G patient and n=12 for healthy controls. Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6R653G.
PBMCs from the NLRP6<sup>R653G</sup> patient and healthy controls secrete similar levels of IL-6, TNF-α and IL-8 following inflammasome activation. PBMCs were isolated from a patient with mutation NLRP6<sup>R653G</sup> and healthy age and sex-matched controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 1 μg/mL LPS for 12 hours and stimulated with 5 mM ATP and 3.4 μM Nigericin for the last 30 minutes of the 12 hour stimulation. Alternatively, PBMCs were stimulated with LPS for 4 hours and transfected with 1 μg/mL Poly(dA:dT) or Poly(I:C). IL-6 (A), TNF-α (B) and IL-8 (C) expression levels of PBMCs from patient NLRP6<sup>R653G</sup> were determined by ELISA and compared to healthy controls. n=4 for NLRP6<sup>R653G</sup> patient and n=12 for healthy controls. Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6<sup>R653G</sup>.
Figure 5.13: LPS stimulates increased up-regulation and expression of pro-IL-1β in PBMCs from the NLRP6<sup>R653G</sup> patient, compared to healthy controls.

PBMCs were isolated from a patient with mutation NLRP6<sup>R653G</sup> and two healthy age and sex-matched controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 1 µg/mL LPS for 12 hours and stimulated with 5 mM ATP and 3.4 µM Nigericin for the last 30 minutes of the 12 hour stimulation. Cells were lysed in 150 µL RIPA buffer and lysates were run on 12% SDS-PAGE gels. Western blots were probed for Caspase-1, pro-IL-1β and Actin. N=9 for healthy controls and N=3 for NLRP6 mutated patient.
Figure 5.14: Cytokine expression profiles from NLRP6<sup>R653G</sup> patient and healthy control PBMCs following stimulation with heat killed *Lactobacillus rhamnosus*. PBMCs were isolated from a patient with mutation NLRP6<sup>R653G</sup> and healthy controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 10<sup>6</sup>, 20<sup>6</sup> and 10<sup>7</sup> heat-killed *Lactobacillus rhamnosus* for 24 hours. IL-1β (A), IL-18 (B) and IL-1α (C), IL-6 (D), TNF-α (E) and IL-8 (F) expression levels of PBMCs from the NLRP6 mutated patient were determined by ELISA and compared to healthy controls. N=10 for healthy controls and N=3 for NLRP6 mutated patient. Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6<sup>R653G</sup>.
Figure 5.15: PBMCs from a patient with NLRP6<sup>R653G</sup> mutation show enhanced upregulation of pro-IL-1β following stimulation with heat killed *Lactobacillus rhamnosus*.

PBMCs were isolated from a patient with mutation NLRP6<sup>R653G</sup> and four healthy controls. PBMCs were seeded at 1 x 10<sup>6</sup> cells/mL and stimulated with 10<sup>6</sup>, 20<sup>6</sup> and 10<sup>7</sup> heat-killed *Lactobacillus rhamnosus* for 24 hours. Cells were lysed in 150 μL RIPA buffer and lysates were run on 12% SDS-PAGE gels. Western blots were probed for NLRP6, Caspase-1, pro-IL-1β and Actin. N=10 for healthy controls and N=3 for NLRP6 mutated patient.
Figure 5.16: Cytokine expression profiles from patient with NLRP6\textsuperscript{R653G} following stimulation with heat killed \textit{Salmonella typhimurium}. PBMCs were isolated from a patient with mutation NLRP6\textsuperscript{R653G} and healthy controls. PBMCs were seeded at $1 \times 10^6$ cells/mL and stimulated with $10^6$, $20^6$ and $10^7$ heat-killed \textit{Salmonella typhimurium} for 24 hours. IL-1$\beta$ (A), IL-18 (B) and IL-1$\alpha$ (C), IL-6 (D), TNF-$\alpha$ (E) and IL-8 (F) expression levels of PBMCs from the NLRP6 mutated patient were determined by ELISA and compared to healthy controls. N=10 for healthy controls and N=3 for NLRP6 mutated patient. Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6\textsuperscript{R653G} groups.
Figure 5.17: NLRP6^{R653G} patient PBMCs display enhanced caspase-1 and pro-IL-1β upregulation following stimulation with heat killed Salmonella typhimurium. PBMCs were isolated from a patient with mutation NLRP6^{R653G} and healthy controls. PBMCs were seeded at 1 x 10^6 cells/mL and stimulated with 10^6, 20^6 and 10^7 heat-killed Salmonella typhimurium for 24 hours. Cells were lysed in 150 μL RIPA buffer and lysates were run on 12% SDS-PAGE gels. Western blots were probed for Caspase-1, pro-IL-1β and Actin. N=10 for healthy controls and N=3 for NLRP6 mutated patient.
Figure 5.18: Successful cloning of NLRP6 WT and NLRP6\textsuperscript{R653G} into Pd1 vector.

(A) WT NLRP6 and NLRP6\textsuperscript{R653G} were amplified and amplified products were purified and digested with SpeI and NdeI. Inserts and vector were ligated overnight (using a molar ratio of 1 vector: 4 insert) and heat transformed into competent bacteria. Seven colonies of both NLRP6 WT and NLRP6\textsuperscript{R653G} were chosen to amplify to determine if the inserts had successfully ligated into Pd1 vector. Sequencing confirmed successful cloning. (B) THP-1 cells stably transfected with NLRP6 WT and NLRP6\textsuperscript{R653G} were generated and western blot analysis of Flag expression and Actin is shown in (B).
Figure 5.19: NLRP6 WT and NLRP6^{R653G} induce significant levels of cell death when transfected into THP-1 cells, compared to HEK293T cells.

THP-1 cells were seeded at 1 x 10^6 cells/mL in a 96 well plate with 200 µL/well and 20 ng/mL PMA for 24 hours. Cells were then transfected with 0, 50, 100 or 200 ng WT NLRP6 or NLRP6^{R653G} for 48 hours. Empty vector was added to each well to ensure total DNA concentration added was 200 ng. An alamarBlue assay was then performed to determine cell death (A). Hek293T cells were seeded at 2 x 10^5 cells/mL in a 96 well plate with 200 µL/well and transfected similarly for 48 hours. An alamarBlue assay was performed to determine cell death (B). Two-way ANOVA found no significant differences between WT NLRP6 and NLRP6^{R653G} groups.
Chapter 6: General Discussion and Future Work
6.1 Discussion

6.1.1 The role of Caspase-11 in skin immunity

As previously discussed during this thesis, skin immunity is crucial in protecting the host against an onslaught of invading pathogens and danger signals. The skin harbours a large variety of innate and adaptive cells in order to mount an efficient immune response. Inflammatory caspases are believed to play an important role in maintaining skin immunity, mainly via activation of canonical and non-canonical inflammasomes. However, there is a fine line between controlled activation of caspases in response to infection and over-activation of caspases observed in inflammatory diseases. This study has proposed a role for caspase-11 in mediating psoriasis in an experimental psoriasis model.

The initiation of psoriasis is driven by the presence of pathogens, trauma or chemicals on the skin, leading to activation of keratinocytes and effector leukocyte populations such as pDCs, myeloid DCs and macrophages. Once activated, innate cells release a cytokine milieu that elicits a significant adaptive immune response which is believed to be mainly driven by T cell populations. Sustained inflammation in the skin subsequently drives the typical hallmarks commonly observed during this disease, such as epidermal thickening, infiltration of immune cells and angiogenesis. This study investigated the involvement of caspase-11 in this disease-associated inflammation, and in doing so determined caspase-11 as a crucial mediator of psoriasis in murine skin. Determining the exact mechanistic role that caspase-11 plays in mediating this disease is difficult, however, it is likely that caspase-11 plays an important role in the initial sensing process of psoriasis by innate immune cells present in the skin. This study has therefore identified caspase-11 as a successful target for the treatment of psoriasis. Other innate immune genes have been reported to play important roles in psoriasis. A meta-analysis of three genome-wide association studies and two independent datasets has identified 15 novel psoriatic susceptibility loci as causative of psoriasis (314). 11 of these genes have been associated with mediating innate immune responses; DDX58, KLF4, ZC3H12C, CARD14, CARM, IL28RA, LCE3D, NOS2, FBXL19, NFKBIA and RNF114 (314). This study has highlighted the important role the innate immune system plays in mediating this disease. Many of the genes identified in this study are implicated in the TNF-α, IL-23 and IL-17
signalling pathways, which have been successfully targeted for the treatment of psoriasis (315). Interestingly, our study has also identified NOS2 as a potential driver of psoriasis and has shown that caspase-11 is required for the induction of NOS2 in LPS-stimulated macrophages. Thus our study implicates an additional innate immune gene, Casp-11, in mediating this disease. It is clear that the innate immune system plays a very important role in psoriasis, and targeting innate immune signalling pathways has proven very successful in the treatment of psoriasis. For example, the use of the JAK1/JAK3 inhibitor, Tofacitinib, to target the JAK/STAT signalling pathway is now FDA approved for psoriatic arthritis and proving successful in the treatment of this disease (316). As the Imiquimod-induced psoriasis model was established less than 10 years ago (229), there has not yet been a murine target identified from the Imiquimod-induced psoriasis model that has been translated into treating human psoriasis. However, the dramatic increase in use of this model in recent years will certainly lead to an increase in human psoriatic treatments in the near future.

This study reveals that caspase-11 is required for the pathogenesis of psoriasis during the Imiquimod-induced psoriasis model. However, we must question how this can be translated to human psoriasis. Chromosomal clustering of Casp-4/5 and -11 and sequence similarity has led to the belief that Casp-4 and -5 are the human orthologs of Casp-11. Functionally, there are also similarities between the human and murine orthologs. Regulation of caspase-4/5 and 11 expression is also mediated through similar mechanisms. Expression of caspase-4/5 and 11 is upregulated following stimulation with LPS and subsequent NFκB/STAT translocation into the nucleus. Caspasess-4/5 and -11 can also be strongly induced following stimulation with IFN-γ which is mediated by the transcription factor STAT1 (127, 317). In addition, all three inflammatory caspases act as intracellular sensors of LPS to mediate non-canonical inflammasome activation (318). Recent evidence also describes similar mechanisms of GSDMD cleavage by caspase-4/5 and -11 to mediate IL-1beta processing/pyroptosis (29, 31). Thus, based on the structural and functional similarities that exist between murine caspase-11 and human caspases-4 and -5, it is likely that targeting caspase-4/5 in human skin may alleviate the inflammatory symptoms of psoriasis and prevent disease progression. Use of the Imiquimod-induced psoriasis model has helped researchers increase their understanding of human psoriasis. Currently used human psoriatic treatments, such as Dexamethasone, Clobetasol and Etanercept are also effective at reducing disease severity in murine skin during the
Imiquimod-induced psoriasis model, highlighting the similarities between murine and human psoriasis (319). Another study has used the Imiquimod-induced psoriasis model to identify a clinical psoriatic target, and then subsequently used the model to test the solubility of treatments in cream (320). This model is therefore allowing researchers to optimise existing treatments and screen for novel effective treatments for human psoriasis. We therefore consider it to be a useful model to generate preliminary insight into the role of caspase-11 during psoriasis, with significant potential to apply our findings to human caspases-4 and 5.

Based on the literature published to date, caspase-11 has been reported to play two main roles in mediating innate immunity. The first of which is the recognition of intracellular LPS, resulting in non-canonical inflammasome activation (123, 321). Intracellular recognition of bacteria is a crucial mechanism designed to protect the host from bacterial infection. In addition, caspase-11 is responsible for mediating the inflammatory form of cell death, pyroptosis (29, 130). Our study has shown that caspase-11 contributes to the pathogenic processes that result in psoriasis, including angiogenesis, cellular proliferation, cell death and infiltration of innate immune cells to the skin. We propose that caspase-11 mediates its effects via its previously defined roles- activation of the non-canonical inflammasome and induction of pyroptosis. However, we identify a novel role for caspase-11 in mediating nitric oxide production in macrophages, which is also likely to contribute to its pathogenesis during this disease model. Caspase-11 has been implicated in playing roles in skin immunity previously, with a deficiency of caspase-1 and -11 proving to be protective in a murine model of dermatitis (226). In addition, expression of human caspases-4 and -5 are significantly increased in psoriatic lesions (210). Caspase-4 expression was also found to be required for the release of IL-1β in keratinocytes (209).

The findings presented in this study present caspase-11 (and caspase-4/5 in humans) as a target for the treatment of psoriasis. While there are a range of psoriasis treatments currently on the market, there is still a requirement for novel therapeutics that could be applied topically and have greater success with a wider range of patients (322). Topical creams are the standard of care for mild to moderate cases of psoriasis. However, if they are not effective, or in the treatment of moderate to severe cases, patients progress to the use of systemic therapies such as phototherapy, or biologics and non-biologics.
Phototherapies are often effective in the treatment of psoriasis, however the inconvenience in accessing phototherapy clinics and frequent requirement proves a significant hindrance. Biologics such as Adalimumab (anti-TNF-α), Etanercept (anti-TNF-α), Infliximab (anti-TNF-α), Secukinumab (anti-IL-17A), Ustekinumab (anti-IL-12/23) are often the next line of treatment for moderate-severe cases of psoriasis. Targeting pro-inflammatory cytokines has proven to be successful in alleviating the symptoms of psoriasis, however, their method of administration is severe. There is therefore a gap in the market for a more specific and effective topical therapy for the treatment of psoriasis – one that may also allow for the treatment of moderate and severe cases. The development of a specific caspase-4/5 inhibitor may prevent the initiation of psoriasis by targeting the gene before it mediates inflammation in the skin. The Imiquimod-induced psoriasis model could be used to test current inflammasome inhibitors such as MCC950 (NLRP3 inhibitor) to preliminarily determine the effectiveness of inflammasomes as a target. Ideally, if an effective short peptide caspase-4/5 inhibitor could be prepared and delivered as a topical treatment, it could reduce or eliminate the requirement of invasive, needle-based delivery of biologics.

The findings from this study therefore propose caspase-11 as an important innate immune mediator in the pathogenesis of psoriasis. This may have a significant impact on the future treatment of human psoriasis, with inhibition of human caspase-4/5 warranting further exploration as a novel psoriasis therapy.

6.1.2 The role of a novel NLRP6 mutation in autoinflammation

Findings in this study attribute a novel mutation in NLRP6 to an autoinflammatory disease. This is the first reported mutation of NLRP6 ever identified in a patient with autoinflammatory symptoms. Autoinflammatory diseases were first described twenty years ago. Now, this area has greatly expanded, with the discovery of thirty innate genes associated with autoinflammatory diseases (323). The ability to genetically identify mutations in innate immune genes has greatly enhanced the field of autoinflammation and led to the classification of novel autoinflammatory disorders. We propose NLRP6 as the newest addition to the list of innate genes associated with autoinflammation.

While this study has identified one patient with this novel mutation, globally there are likely to be other undiagnosed patients with similar mutations in NLRP6, as
autoinflammation is recognised as being under-diagnosed at present (324). The publication of our findings may lead to the inclusion of NLRP6 in gene sequencing panels for autoinflammation. We have already established a collaboration with the Lackmann/Hawkins groups (National Amyloidosis Centre, UCL, UK) who have incorporated the NLRP6 mutation into their Autoinflammatory disease screening process. This should lead to an increase in the number of patients identified with mutations in NLRP6. We are currently drafting a manuscript for submission to JACI to report our novel mutation in this case study. Once published, we aim to approach two additional groups that focus on the identification of autoinflammatory mutations. The Kastner group (NIH, Bethesda, MD) and the Simon group (NCIA, The Netherlands) are both linked to autoinflammatory disease alliances such as autoinflammatory.org (323). We aim to collaborate with both groups to include this novel mutation in NLRP6 in their screening process and in doing so identify more patients with mutation NLRP6R653G. This study has reported that a loss-of-function R653G mutation in the LRR domain of NLRP6 results in increased basal production of IL-6. Treatment of the NLPR6R653G patient with Tocilizumab, anti-IL-6R, may alleviate the autoinflammatory symptoms. Tocilizumab is an immunosuppressive therapy which is often effective in the treatment of Rheumatoid arthritis patients.

As previously discussed, NLRP6 is a member of the Nod-like receptor family of proteins that plays an important role in regulation of innate immunity. While this study focused primarily on the role NLRP6 plays in the innate immune system, a multitude of murine studies have suggested that a deficiency of NLRP6 results in significant intestinal dysbiosis. NLRP6 has been reported to regulate intestinal homeostasis through microbiota sensing (93). In addition, a deficiency of NLRP6 has been reported to result in inefficient mucus granule exocytosis, highlighting its importance in the regulation of intestinal homeostasis (325). However, whether information gathered from murine NLRP6 studies can be applied to the function of human NLRP6 is still unknown. Direct evidence of the role of human NLRP6 remains limited. The lack of intestinal symptoms in the NLPR6R653G patient involved in this study suggest that either significant functional differences exist between murine and human NLRP6, or that this mutation does not affect the function of NLRP6 in the intestine. In support of the first theory, expression of murine NLRP6 has been determined in both the small intestine and colon, however in humans, expression of NLPR6 is primarily located in the small intestine only (92). In addition,
NLRP6 was reported to play an important protective role in murine colorectal cancer (100), while expression of NLRP6 was not affected in human colorectal cancer patients when compared to healthy controls (326). This may suggest there are differences between NLRP6 functioning in humans and mice. Our study has however presented similar findings to those reported by Anand et al, where they investigated the role of murine NLRP6 in mediating NFκB regulation (103). Anand et al reported that a deficiency of NLRP6 results in significant increase in NFκB and ERK signalling in BMDMs following *Listeria* infection (103). Our study has reported dysregulated NFκB and ERK signalling in PBMCs with mutation NLRP6^R653G_. It is therefore likely that human and murine NLRP6 may play similar roles in innate immune regulation in myeloid cells. This may suggest that studying the role of NLRP6 in murine myeloid cells may in fact help to further define the role NLRP6 plays in regulation, however the role human NLRP6 plays in epithelial cells is still in question.

In order to further explore the immunological consequence of this novel mutation in NLRP6, and bring this project forward, we established a collaboration with researchers in St Vincent’s Hospital, Dublin; Dr. Dina Danso-Abeam and Dr Liz Ryan. They characterised the immune phenotype of the patient and discovered that the patient displays dysregulation in almost all immune cell populations examined. Dr Danso-Abeam established that the patient displayed an inverted proportion of CD4:CD8 T cells, among which there was a significant reduction of Mucosal-associated invariant T cells in the patient. The frequency of all B cell subsets examined were also affected (Naïve, Class-switched memory, unswitched memory, double negative memory, CD5^+^). The frequency of pDCs were significantly increased in the patient with higher expression of the migratory molecule, CCR5. In addition, the proportion of neutrophils within leukocyte populations was significantly reduced within the patient. However, the most prominent difference observed in the patient’s immune cell populations was the significant increase in total monocytes when compared to healthy controls. More specifically, the patient displayed significantly higher frequencies of the intermediate subset and decreased frequency of the non-classical subset. In addition, all monocyte subsets in the patient displayed increased activation, measured by higher fluorescence intensity of HLA-DR.

The patient’s dysregulated immune cell phenotype may be explained by the increased inflammatory makers observed in the patient’s blood and PBMCs, such as CRP, Amyloid
A and IL-6. IL-6 has been reported to drive systemic inflammation through diverse mechanisms. Increased production of IL-6 has been responsible for acting on the liver to induce increased CRP, Serum amyloid A and fibrinogen levels (327). Increased production of acute phase proteins results in the development of serious chronic inflammatory diseases (328). In addition, IL-6 has been reported to play an important role in linking innate and adaptive immunity through differentiation of naïve CD4+ T cells to Th17 cells (329). IL-6 has also been reported to inhibit T-regulatory cell differentiation (330). The imbalance between Th17 and Treg cells has been reported to be responsible for the disruption of immune tolerance that has been implicated in the pathogenesis of autoimmune and autoinflammatory diseases (331). Therefore an increased production of IL-6 from the patients PBMCs may explain the dysregulated proportion of T cell populations. The most significant differences observed in the patient’s immune cell profile were the increased frequency of intermediate monocytes. Monocytes are an important innate cell type responsible for mediating an immune response against a range of pathogens. Upon stimulation, the intermediate population of monocytes are the main cells responsible for the release of inflammatory cytokines such as IL-1α, TNF-α and IL-6 (332). Expansion of the intermediate population of monocytes has been implicated in a range of inflammatory diseases such as rheumatoid arthritis, chronic kidney disease, coronary heart disease and type-2 diabetes (333–336). Interestingly, increased expression of intermediate populations have been reported to be driven by IL-6 and TNF-α in rheumatoid arthritis patients (324). Inhibition of IL-6 and TNF-α with tocilizumab and adalimumab reduced the frequency of intermediate monocytes and correlated with reduced disease severity (324). As our study has reported increased basal production of IL-6, we propose that it is likely responsible for mediating the expansion of this population in the patient. The collective dysregulated immune cell populations observed in the patient’s blood are therefore likely explained by the increased inflammatory markers such as IL-6. Characterisation of these immune cell populations have increased our understanding of the impact of this novel mutation. In addition, it has highlighted the important role NLRP6 plays in regulation of immunity. The generation of a transgenic mouse with this exact mutation may allow us to further explore the impact of this mutation both systemically and intracellularly.

In summary, the identification of this novel mutation in NLRP6 has increased our understanding of the role NLRP6 plays in regulating the innate immune system and also
added a new gene to the list of innate genes implicated in autoinflammation. We propose this mutation as the first mutation of NLRP6 associated with autoinflammation however we expect this identification will lead to the identification of further patients with mutations in NLRP6.

6.2 Future work

6.2.1 Future Work to further investigate the role Caspase-11 plays in skin immunity.

Findings from this study have suggested that caspase-11 plays an important role in skin immunity. This study has reported that caspase-11 is involved in the wound healing response. In addition, caspase-11 plays an important role in driving the pathogenesis of an experimental murine model of psoriasis.

The initial focus of this study was to preliminarily investigate if caspase-11 plays a role in driving healing following wound infliction. Findings suggested that mice with a caspase-11 deficiency displayed slower wound healing when compared to WT mice, however there was no significant statistical difference between the two groups. We hypothesise that an increase in experimental numbers will give an increase in power and likely result in significant differences between the two groups. It would therefore be prudent to examine the wound healing process using larger group numbers.

This study also reported that a deficiency of caspase-11 results in a delayed migration of BMDMs in response to IFN-γ and IL-17A but not other caspase-11 activators such as LPS and IL-1β. IL-17A has not been previously reported to induce expression or activate caspase-11, therefore it would be interesting to determine if IL-17A has any impact on the induction of caspase-11 expression. Findings from this study have also highlighted that caspase-11 most likely plays cell specific roles in mediating inflammation. It would therefore be interesting to examine other innate cell populations to determine if a deficiency of caspase-11 results in delayed migration under the same conditions. As we have seen that caspase-11 is required for the migration of CD11b+ cells to the skin during Imiquimod-induced psoriasis, it would also be interesting to investigate if caspase-11 is required for the recruitment of CD11b+ cells following wound infliction. A decreased
migration of CD11b+ cells to the skin may potentially explain the delay in the healing process observed in Casp-11−/− mice.

We have reported that caspase-11 is responsible for the increased epidermal thickening and proliferation of keratinocytes observed in the skin during the murine psoriasis model. In addition, we reported that caspase-11 is required for mediating pyroptosis, CD11b+ infiltration and the increase in angiogenic markers in the skin during this model. As caspase-11 is primarily responsible for mediating the inflammatory form of cell death, pyroptosis, we hypothesise that caspase-11 mediated pyroptosis may be responsible for mediating this disease. Pyroptosis of endothelial cells has previously been reported to act on surrounding cells, driving the recruitment of leukocytes (260). Therefore it would be interesting to determine if pyroptosis is also decreased in endothelial cells of caspase-11 deficient mice. Co-staining for TUNEL positivity and endothelial cell positivity would give a clear indication if pyroptosis is also decreased in the endothelium of caspase-11 deficient mice. This may explain the decrease in leukocytes migrating to the skin of caspase-11 deficient mice during this model. Staining for endothelial cells using a marker such as Mucin, may also confirm that the presence of caspase-11 results in an increase in angiogenesis in the skin during this inflammatory skin disorder.

This study has reported no significant effect on cytokine production in the absence of caspase-11 during Imiquimod-induced psoriasis. However, stimulation of skin explants with Aldara and subsequent application of CM to BMDMs resulted in a significantly less secretion of IL-1β, IL-18 and nitric oxide in the absence of caspase-11. It is therefore likely that the presence of caspase-11 is impacting different cell types present in the skin in different ways. It would therefore be enlightening to investigate the effect of skin CM in other innate cells such as DCs, neutrophils and LCs, and determine whether IL-1β, IL-18 and nitric oxide secretion is also affected in these cell populations. In addition, it would be interesting to investigate the presence of all innate cell populations in the skin, in particular DCs, neutrophils and macrophages. This may be performed by optimisation of flow cytometry or immunofluorescence.

In addition, it would be interesting to determine the role caspase-11 plays in keratinocytes. Isolation of primary keratinocytes from caspase-11 deficient mice and stimulation with CM from Aldara treated skin may determine what role keratinocytes are playing in driving the pathogenesis of psoriasis in the presence of caspase-11.
6.2.2 Future work to further characterise the impact of a novel mutation in NLRP6 in mediating autoinflammation

Findings in this study have identified a novel mutation in NLRP6 that is attributed to an autoinflammatory disorder. *In vitro* experiments have shown that mutation NLRP6\textsubscript{R653G} affects the ability of NLRP6 to activate NFκB but has no impact on inflammasome formation. In addition, this study has shown that a patient with mutation NLRP6\textsubscript{R653G} has uncontrolled systemic inflammation, with high basal levels of IL-6, and dysregulated NFκB activation. There was also no impact on inflammasome-mediated cytokine secretion from the patient’s PBMCs.

This study has determined that mutation NLRP6\textsubscript{R653G} is also present in the mother’s genome. It would therefore be interesting to investigate the mother’s inflammatory markers to determine if dysregulated inflammation is also occurring in the mother’s immune cells.

Transcription of IL-6 is not just regulated by NFκB but also by other transcription factors, such as SP-1, NF-IL-6, AP-1 and IRF1. It would therefore be interesting to investigate if NLRP6 regulates these pathways and subsequently determine if mutation NLRP6\textsubscript{R653G} affects the signalling of these pathways.

This study has also reported that stable expression of NLRP6 in THP-1s and HEK293T cells result in cell death, suggesting that this gene is toxic to these two cell types examined. To further explore the effect of this mutation on NFκB signalling, it is necessary to identify a cell line that can stably express NLRP6. Once a cell line is identified, stable expression of NLRP6 and NLRP6\textsubscript{R653G} will determine if increased IL-6 production occurs in the presence of the mutation. In addition, using NFκB inhibitors, one can tease out the mechanistic impact NLRP6 has on regulating NFκB.

Based on the similarity between murine studies of caspase-11 in myeloid cells and the findings in our study, we propose that a transgenic mouse with the same novel mutation incorporated into its genome may increase our understanding of the increased systemic inflammation occurring in the patient. The availability of a mouse model to study this mutation will greatly enhance our understanding of the role NLRP6 plays in regulating NFκB and further explore the immunological impact of this mutation.
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Autoinflammatory Diseases: Consequences of Uncontrolled Inflammasome Activation

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Abstract

Inflammasomes are sensors within the innate immune system that are responsible for the regulation of caspase-1 activation and the initiation of inflammatory responses following cellular infection or damage. A significant number of chronic inflammatory and metabolic diseases have recently been identified to have inflammasome-mediated inflammation as a key driver of their pathogenesis; this area of research is under intense investigation at present. This review focusses on autoinflammatory diseases (AD), a rapidly expanding group of debilitating diseases that are associated with severe systemic inflammation. AD commonly arise as a result of mutations to genes that encode inflammasome components. Monogenic AD are relatively rare because they require fully penetrating mutations; however, they often present at birth and last a lifetime. Clinical awareness of AD is lacking and it is believed that, at present, many cases go undiagnosed. This review specifically discusses a number of inflammasome-associated AD and metabolic disorders that provide significant insight into our understanding of inflammasome signalling pathways. These AD highlight the potency of inflammasomes in their ability to initiate and sustain systemic inflammation. The debilitating symptoms of AD also reveal the extensive consequences of uncontrolled inflammasome activity. Clinical therapies that target the inflammasome and interleukin-1β, a product of its activation, in the successful management of AD and certain metabolic diseases will also be discussed.

INTRODUCTION

The immune system is an evolutionarily conserved system that has evolved to protect the host from invading pathogens and cellular damage. While the immune system is crucial in protecting the host from a variety of insults, the dysregulation of immune components is strongly linked to the development of both autoinflammatory and autoimmune diseases. Autoinflammatory diseases (AD) are a relatively new category of immunological disease. The clinical term AD was proposed in 1999, when only two genes (MEFV and NLRP3) had been genetically associated with this disease category. Today, 30 genes have been linked to AD, which is the term still used to describe this expanding group of diseases, caused by the overactivation of the innate immune system. As this is a relatively new group of diseases, with new clinical subtypes being identified on an ongoing basis, there are limited statistical analyses
available on AD. In 2013, a study estimated the incidence of AD to be 2.83 patients per million people in Sweden. Owing to their relatively recent identification and their low incidence rates, it is believed that clinical cases of AD are currently underdiagnosed and increased clinical awareness of AD is required.

Although both autoinflammatory and autoimmune diseases result from the immune system attacking the body’s own tissues, AD are characterised by intense episodes of inflammation, driven by innate immune cells, and are caused by mutations in genes that regulate innate immunity. The classical symptoms of autoinflammation are recurrent fever attacks, skin rash, and abdominal pain. However, AD symptoms vary greatly across clinical subtypes, and patients can present with a range of physical manifestations, including mouth ulcers, pyogenic skin or bone lesions, joint swelling, serositis, and granulomatous lesions. The number of gene mutations associated with AD is rapidly increasing. Examples of AD include cryopyrin-associated periodic syndrome (CAPS), Blau syndrome, familial mediterranean fever (FMF), tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS), and inherited hyperimmunoglobulinaemia D with periodic fever syndrome (HIDS).

The prevalence of a given disease can vary from 1:1,000 people (Sweet's syndrome) to 1:1,000,000 (Marshall's syndrome, also known as periodic fever adenitis pharyngitis aphthous [PFAPA] ulcers), and vary between populations. McGonagle and McDermott proposed a ‘continuum model’ for immunological diseases in 2006, integrating AD into a spectrum ranging from monogenic autoinflammatory disorders to monogenic autoimmune diseases, with polygenic autoinflammatory/autoimmune diseases and other diseases that may have both an autoinflammatory and an autoimmune component included within this spectrum. The majority of mutated genes identified to date linked to monogenic AD represent critical innate sensor or receptor proteins involved in inflammatory responses, such as NLRP3 for CAPS, NOD2 for Blau, and TNFRI for TRAPS. Both NLRP3 and NOD2 proteins belong to the Nod-like Receptor (NLR) protein family, a group of cytosolic sensor proteins that are capable of detecting intracellular infection or damage. A number of the NLR proteins initiate inflammatory pathways by their formation of multiprotein complexes, termed inflammasomes. The majority of AD identified to date are in fact linked to mutations in inflammasome components.

### INFLAMMASOMES: CRITICAL MEDIATORS OF INFLAMMATION

Inflammasomes are large complexes of proteins that form to mediate the activation of an inflammatory enzyme, termed caspase-1. Caspase-1 is transcribed as the inactive precursor protein pro-caspase-1, which requires proteolytic processing before the generation of its active form. Once active, caspase-1 is responsible for the maturation and secretion of interleukin (IL)-1β and IL-18, two potent proinflammatory cytokines that induce fever and interferon (IFN)γ secretion, respectively. In addition to the activation of the cytokines IL-1β and IL-18, inflammasome activation also results in a type of cell death, termed pyroptosis. Pyroptosis is an inflammatory form of cell death, mediated by caspase-1-dependent cleavage of an executioner protein, Gasdermin D (GSDMD). Cleaved GSDMD is responsible for forming pores in the cell membrane, mediating the release of proinflammatory cytokines IL-1β and IL-18. Thus, the ultimate outcome of inflammasome activation in cells is acute inflammation, driven by the secretion of potent inflammatory mediators IL-1β and IL-18, and pyroptotic cell death, which also contributes to local tissue inflammation in addition to eliminating damaged and infected cells.

Inflammasomes are composed of a sensor protein, such as certain NLR proteins; an adaptor protein, usually the ASC protein; and the enzyme caspase-1. The sensor protein is responsible for recognising intracellular pathogens, such as bacteria or viruses, and/or intracellular danger or stress signals, such as detection of nuclear factors (e.g., DNA or high motility group box 1 protein [HMGB1]) in the cytosol. Once activated following the recognition of a pathogen or danger signal, the sensor protein oligomerises and triggers the formation of an inflammasome. Inflammasomes generally require a priming step before they can become activated, which is termed Signal 1. This priming step is mediated by NFκB signalling, which occurs following...
extracellular pathogen recognition by a toll-like receptor (TLR) or intracellular recognition by certain types of NLR, such as NOD1/2. Activation of NFκB during Signal 1 results in the transcriptional upregulation of inflammasome components, such as NLRP3 and pro-IL-1β, the inactive precursor form of IL-1β. Signal 2 involves activation and formation of the inflammasome complex via ligand binding to a sensor protein (Figure 1). An alternative method of NLRP3 inflammasome activation, known as the noncanonical inflammasome, requires a Signal 3, mediated by inflammatory caspases-4/5 in humans (caspase-11 in mice). Caspase-4, 5, and 11 are responsible for direct recognition of intracellular lipopolysaccharides, which results in their subsequent cleavage and activation. In addition to regulation of the noncanonical inflammasome, active caspases-4, 5, and 11 can also initiate pyroptosis, because they are capable of directly processing GSDMD.

Several inflammasome complexes have been identified to date, including those that consist of NLR sensor proteins (NLRP1, NLRP3, NLRC4, NLRP6, and NLRP12) and other sensors, such as AIM2 and IFI16, which are members of the PYHIN protein family. There have been >23 distinct NLR genes identified in the human genome, several of which have been implicated in the regulation and activation of inflammasome complexes, which subsequently lead to the activation and secretion of the proinflammatory cytokines IL-1β and IL-18.

Figure 1: Inflammasome activation results in IL-1β mediated inflammation.

The initial priming step of inflammasome activation is mediated by pathogen recognition receptors, such as TLR, which recognise pathogen or danger signals during infection or injury. TLR activation results in translocation of NFκB into the nucleus to promote transcription and translation of inflammasome components and their targets, including IL-1β and NLR proteins (Signal 1). There are a number of mechanisms proposed to mediate Signal 2 activation; however, most occur via potassium (K+) efflux induced by pathogen or danger signals (e.g., ATP, ROS, MSU) and subsequent activation of NLRP3. NLRP3 oligomerisation initiates the formation of a multimeric inflammasome complex. The inflammasome complex facilitates the proteolytic cleavage and activation of caspase-1, allowing it to cleave pro-IL-1β into its mature and active form, which is secreted from the cell to mediate inflammation.

ATP: adenosine triphosphate; IL: interleukin; IκBα: inhibitor of NFκB; MSU: monosodium urate; NFκB: nuclear factor kappa B; NLRP3: NACHT, LRR, and PYD domains-containing protein 3; ROS: reactive oxygen species; TLR: toll-like receptor.
The most widely studied and best characterised of all inflammasomes is that of NLRP3. Extensive research has elucidated a range of microbial and nonmicrobial activators of the NLRP3 inflammasome. NLRP3 has been implicated in detecting a plethora of microbial pathogens, including the Influenza A virus, *Vesicular stomatitis* virus, bacterial *Staphylococcus aureus*, *Escherichia coli*, fungal *Candida albicans*, *Aspergillus fumigatus*, and parasitic *Schistosoma mansoni* and *Dermatophagoides pteronyssinus*.16-22 Additionally, phagocytosis of particulates, such as monosodium urate (MSU) crystals, amyloid-β, silica, calcium pyrophosphate dehydrate, asbestos, and alum have all been reported to activate NLRP3.23-27 These nonmicrobial agonists induce potassium efflux that results in subsequent NLRP3 activation. Recent studies28,29 have reported NEK7 as a novel NLRP3 inflammasome regulator. NEK7, a member of the NIMA-related kinase family, was originally found to be responsible for regulating mitotic progression and response to DNA damage but has since been reported to control NLRP3 oligomerisation, formation of an ASC speck, and subsequent caspase-1 activation downstream of potassium efflux and reactive oxygen species (ROS).28,29 Following their phagocytosis by innate immune cells, intracellular particulates are thought to damage the lysosomal membrane, resulting in the release of the lysosomal enzyme, Cathepsin B, into the cytosol, resulting in NLRP3 activation.30

**INFLAMMASOME-MEDIATED AUTOINFLAMMATORY DISEASES**

While inflammasome activation is a key mechanism responsible for mediating the host innate response following infection and injury, inappropriate inflammasome activity can lead to AD. As outlined previously, some of the well-characterised AD occur as a result of mutations in inflammasome-associated genes.

Mutations in *NLRP3* have been linked to a group of disorders collectively known as CAPS, including familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome, and neonatal-onset multisystem inflammatory disorder (also known as chronic infantile neurologic, cutaneous, and articular syndrome).31-33 All three CAPS subphenotypes occur as a result of dominantly inherited gain-of-function mutations in the *NLRP3* gene, which result in systemic inflammation with blood neutrophilia and fever.32 Localised neutrophilic inflammation is also observed in various tissues, such as skin, muscles, joints, and cerebrospinal fluid. Symptoms common to all CAPS patients are rash, periodic fevers, headaches, joint pain, conjunctivitis, and general malaise. FCAS is the least severe of the CAPS and symptoms, which occur from early infancy, are triggered within 2 hours after cold exposure and generally subside within 24 hours.34 FCAS is distinct from cold urticaria, which is caused by an allergic response to cold and generally develops later in life. Symptoms in Muckle–Wells syndrome patients, triggered by cold, stress, or other unknown factors, are similar to those of FCAS but may also be accompanied by progressive hearing loss and the development of amyloidosis, due to excessive serum amyloid production.34 Neonatal-onset multisystem inflammatory disorder has the highest degree of chronic inflammation of all CAPS, with symptoms including aseptic meningitis, papilloedema, joint problems, hearing loss, and often mental and physical developmental delays.

Both *in vitro* and *in vivo* data support the hypothesis that CAPS-associated *NLRP3* mutations result in enhanced responsiveness of the NLRP3 sensor protein, leading to inappropriate inflammasome activation and subsequent secretion of the potent inflammatory mediators, IL-1β and IL-18.35-38 Downstream markers of inflammation, such as IL-6, are also consistently elevated in patients with FCAS after a mild cold even when significant increases in IL-1β and IL-18 are undetectable.39 Additionally, pretreatment with anti-IL-1 therapy can prevent the FCAS response to a mild cold, suggesting a causative role for IL-1β in mediating the response.39 Approved and effective treatment options for CAPS patients now exist, as blocking the action of IL-1β using anakinra, rilonacept, or canakinumab are effective therapies for all CAPS patients.40 Therefore, the prognosis for all CAPS patients is greatly improved if the AD is diagnosed early and treated with the appropriate therapy before the damage caused by chronic inflammation has any permanent effect on the body. Mutations in the *LPIN2* gene, encoding the lipin-2 protein,
result in another NLRP3-associated AD, termed Majeed syndrome. Lipin-2 has been shown to regulate both the priming and activation steps of the NLRP3 inflammasome, and Majeed-associated LPIN2 mutations result in elevated pro-IL-1β and enhanced potassium efflux in macrophages, leading to aberrant NLRP3 activation.\(^{41}\)

The most common AD is FMF, which occurs as a result of mutations in the \(\text{MEFV}\) gene, encoding the pyrin protein. Patients with FMF display longer periods of fever and can have a range of other symptoms, including skin rash, arthritis, and serositis.\(^ {42}\) As the name suggests, FMF affects populations of Mediterranean descent, particularly Armenian, Turkish, Arabic, and some Jewish-Israeli populations, in which the carrier rates can be as high as 1:5.\(^ {43-45}\) The high frequency carrier rates suggest that a selective advantage may exist, and previous reports have suggested that the mutated pyrin protein could provide increased protection against infection, asthma, or allergy.\(^ {46-49}\)

The pyrin protein, named due to the presence of a PYRIN domain in its protein structure, is thought to be responsible for protein–protein interactions. NLRP3 and other NLRP proteins are also characterised by the presence of a PYRIN domain, which is crucial for its ability to recruit ASC and other adaptor proteins into inflammasome complexes. \textit{In vitro} pyrin overexpression studies reveal that, similar to NLRP3, pyrin oligomerises with ASC resulting in subsequent caspase-1 activation and release of IL-1\(\beta\).\(^ {50,51}\) To identify the impact of the \(\text{MEFV}\) Met694Val mutation, the most commonly found mutation in FMF patients, the Kastner group engineered a transgenic mouse strain that harboured the equivalent mutation in the murine \(\text{Mefv}\) gene.\(^ {52}\) The genetically altered mice displayed FMF-like symptoms and also secreted high levels of IL-1\(\beta\) in an ASC-dependent manner, but \(\text{Mefv}\) deficient mice did not, suggesting that the FMF-associated mutations are gain-of-function, and cause enhanced and inappropriate inflammasome activation.\(^ {52}\)

Additional support for this hypothesis comes from a more recent study, which suggested that the pyrin inflammasome is negatively regulated by its phosphorylation of the pyrin protein, which is mediated by the RhoA signalling pathway.\(^ {53}\) Under normal circumstances, the pyrin inflammasome is proposed to be selectively activated following RhoA GTPase inhibition by bacterial toxins;\(^ {54}\) however, in FMF patients, mutated pyrin proteins are not efficiently phosphorylated by RhoA-dependent kinases, resulting in a lowered threshold for the activation of the pyrin inflammasome.\(^ {53}\) This hypothesis is further supported by the fact that the antimitotic drug, colchicine, which inhibits microtubule polymerisation and activates RhoA, is an effective prophylactic treatment for FMF patients.

Inappropriate activation of the pyrin inflammasome is also linked to another unrelated AD: HIDS, also known as mevalonate kinase deficiency. HIDS is caused by mutations in the \(\text{MVK}\) gene, which encodes for the mevalonate kinase enzyme, an enzyme responsible for an early step in the isoprenoid synthesis pathway, catalysing the phosphorylation of mevalonic acid.\(^ {55}\) RhoA signalling is dependent upon its translocation to the plasma membrane, which is regulated by the isoprenylation of RhoA.\(^ {56}\) Defective isoprenoid synthesis occurs in the presence of mutations in the mevalonate kinase enzyme, resulting in loss of RhoA activity. Therefore, the molecular mechanism underlying the inflammatory symptoms of HIDS is also proposed to be mediated via the pyrin inflammasome.\(^ {53}\) In contrast to FMF patients, colchicine is ineffective at preventing HIDS flares, most likely due to its inability to activate RhoA, which is not tethered to the membrane due to the absence of isoprenylation. Anti-IL-1\(\beta\) therapies are the main treatment option for HIDS patients, although not all patients respond. Other treatment options include nonsteroidal anti-inflammatory drugs (NSAID), glucocorticoids, and other biologics, such as TNF-\(\alpha\) or IL-6 blocking agents.\(^ {57}\)

**INFLAMMASOME ACTIVATION IN THE PATHOGENESIS OF METABOLIC DISEASE**

The pathogenesis of many metabolic disorders, including atherosclerosis, Type 2 diabetes mellitus, obesity, and gout, is strongly associated with chronic inflammation. The inflammasome, and products of inflammasome activation (active IL-1\(\beta\) and IL-18), have recently been identified as key mediators of this inflammation, and thus are being intensively studied for
their ability to modulate the pathogenesis and progression of metabolic disease. For example, results from the recent CANTOS trial\textsuperscript{58} reveal that targeting IL-1β-mediated inflammation reduces the risk of adverse cardiac events in patients with a previous history of myocardial infarction and high sensitivity C-reactive protein level (>2 mg/L). Additional analysis from the CANTOS study\textsuperscript{59} suggests that inhibition of IL-1β in these patients is also associated with reduced incidences of lung cancer. This suggests that further investigation into the use of anti-IL-1β and inflammasome targeting therapies for cancers with an established inflammatory component is warranted. However, the potential adverse effects that may arise when blocking such a potent inflammatory mediator must also be considered, as patients receiving canakinumab during the CANTOS trial had an increased occurrence of potentially fatal infections and sepsis.\textsuperscript{58}

The contribution of the NLRP3 inflammasome to metabolic disease has been reviewed in great detail elsewhere;\textsuperscript{60,61} however, the proposed role of the inflammasome in the molecular pathogenesis of gout is summarised here as an example. Gout is a chronic inflammatory disease characterised by deposition of MSU crystals in joints, which form when high concentrations of urate are present. The clinical symptoms of gout arise as a result of the inflammatory response that occurs following recognition of the MSU crystals. Gout is believed to be the most common cause of inflammatory arthritis, with an increasing prevalence in both developing and developed countries.\textsuperscript{62} Activation of the NLRP3 inflammasome in gout has been well investigated and it is believed that TLR activation most likely acts as the first priming signal in the response to MSU crystals.\textsuperscript{63} Phagocytosis of MSU crystals by macrophages, which causes lysosomal damage and subsequent activation of the NLRP3 inflammasome constitutes Signal 2 (Figure 1). IL-1β has been implicated as a key inflammatory mediator responsible for driving the development of gout by promoting an influx of neutrophils into the synovium and joint fluid, which is a typical hallmark of an inflammatory bout in this disease.\textsuperscript{64} Anti-inflammatory therapies such as NSAID and glucocorticoids are effective in reducing the symptoms of gout. Colchicine is also prescribed as a prophylactic treatment or to relieve gouty flares. As described previously, colchicine inhibits microtubule polymerisation and, in contrast to the pyrin inflammasome, has been shown to disrupt NLRP3 inflammasome activation. Colchicine also inhibits microtubule-based inflammatory cell chemotaxis, secretion of chemokines and cytokines, and phagocytosis. Many of these cellular processes can be found in other diseases involving chronic inflammation, suggesting the potential efficacy of low-dose colchicine in other comorbid conditions associated with gout, such as osteoarthritis and cardiovascular disease.\textsuperscript{65}

**CONCLUSION**

Anti-IL-1β and inflammasome targeting therapies are emerging as important clinical treatments for the management of AD, metabolic diseases, and certain cancers. Although inflammasome activation may not be the primary cause or major pathogenic factor for many metabolic diseases, recent evidence suggests that targeting the inflammatory contribution to these diseases may limit their progression. In contrast, certain monogenic AD, including CAPS, FMF, and HIDS, have been reported to arise directly as a result of defective and uncontrolled inflammasome activation. The fact that many AD are effectively treated by IL-1β blockade and drugs that target inflammasome activity highlights the potency of inflammasomes in driving chronic inflammation. As the mechanisms governing inflammasome regulation continue to evolve, so too will additional targets and therapies to regulate inflammasome activity during disease. However, the importance of controlled, functional inflammation for homeostasis cannot be ignored. Thus, therapeutic inflammasome inhibition needs to be balanced against the beneficial contribution of inflammasomes to innate immunity.
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