Inflammatory caspases and their substrates

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Abstract

Inflammasomes are protein complexes comprised of a sensor, an adaptor, and a pro-caspase, activation of which leads to the cleavage and activation of Gasdermin D and the inflammatory cytokine IL-1β, resulting in the inflammatory form of cell death known as pyroptosis. Inflammasomes have been linked to a multitude of different conditions, including cancers and inflammatory diseases such as sepsis. Inflammasomes are divided into two categories, the canonical inflammasome that is activated through the detection of various detrimental stimuli, and the non-canonical inflammasome which is activated by LPS from Gram-negative bacteria. The aim of this project was to study the canonical and non-canonical inflammasomes in more detail though the use of various inhibitors, and then to identify novel inflammatory caspase substrates and study their role within the body. Through the use of proteomic analysis, several potential novel inflammatory caspase substrates were identified. After further analysis, IL-F3 was highlighted as a target for further study and validated in epithelial cell lines. This work provides the foundations for further study into the role of inflammatory caspases in the activation of this RNA-binding protein.

Author’s declaration

I declare that this thesis is a presentation of original work, and I am the sole author. This work has not previously been presented for an award at The University of York, or any other University. All sources are acknowledged as references, formatted as per the Harvard Referencing style.

Contents

Introduction

Cell death

Cell death is a process that occurs ubiquitously in all organisms. A significant factor in the homeostasis of multicellular organisms is the balance between cell survival and cell death. This process is vital, both as a defence mechanism against detrimental stimuli during an immune response, and in the clearance of damaged or infected cells. However, dysregulated cell death can lead to significant damage of the host, inducing inflammation in autoinflammatory diseases, cancers, metabolic diseases, and microbial infections (Gautheron et al., 2020; Place and Kanneganti, 2020; Samir et al., 2020; Wong, 2011). Cell death can be both cause and consequence of inflammation, this can be difficult to distinguish in chronic diseases (Anderton et al., 2020). Currently, cell death can be categorised into regulated cell death (RCD) or accidental cell death (ACD). ACD refers to unexpected and severe insults of a physical, chemical, or mechanical nature that overwhelm any control mechanisms in place, it is completely unregulated and promotes an inflammatory response in the host (Fink and Cookson, 2005; Galluzzi et al., 2018). In contrast to ACD, RCD involves tightly genetically controlled and precise signalling cascades, with specific biochemical and immunological results (Tan et al., 2021; Tang et al., 2019). A form of RCD that occurs in strictly physiological scenarios is known as programmed cell death (PCD). Programmed cell death is an umbrella term that encompasses multiple different pathways, including apoptosis, necrosis, and pyroptosis. Apoptosis is a non-inflammatory form of PCD, and arguably the most well studied to date, however the focus here will be on the lytic, inflammatory form of PCD, pyroptosis (Cookson and Brennan, 2001). Pyroptosis is a double-edged sword, it is significant in protecting multicellular organisms from pathogens, however like all forms of cell death, excessive activation is detrimental to the host. The excessive activation of this highly inflammatory cell death can induce pathological inflammation leading to tissue damage, organ failure, and potentially septic shock (Mulvihill et al., 2018). Pyroptosis is involved in the pathogenesis of a wide range of diseases, including cardiovascular disease (Chen et al., 2016), Alzheimer's disease (Heneka et al., 2013), traumatic brain injury (Yuan et al., 2021), as well as CD4+ T-cell depletion in HIV infection (Doitsh et al., 2014). Both apoptosis and pyroptosis can be initiated by members of the protease family, caspases (Fuentes-Prior and Salvesen, 2004).

Caspases

The term caspase is derived from the proteases use of a cysteine catalytic site, and their rare specificity for cleavage at the carboxy-terminal of aspartic acid residues; they are **c**ysteine-dependent **asp**artate specific prote**ases** (Siegel, 2006). After originally being discovered in the *ced-3* gene in *Caenorhabditis elegans (C. elegans)* in 1993*,* the roles of caspases in innate immunity and development have since been characterised in a wide range of multicellular organisms (Li and Yuan, 2008; Yuan, 1993). Caspases are synthesised as inactive zymogens that are tightly regulated by proteolytic activation or homodimerization. Caspases consist of an amino-terminal (or N-terminal) domain of varying length, which can contain either a caspase recruitment domain (CARD) or a death effector domain (DED) motif, followed by

large and small catalytic subunits of ~20kDa and ~10kDa respectively that contain residues essential for substrate recognition and catalysis (MacKenzie & Clark, 2012; Van Opdenbosch & Lamkanfi, 2019)

At present, 12 caspases have been identified in humans, 10 in mice. This has led to the development of a general classification system, dividing caspases into two main groups in accordance with their structure, function, and activation mechanism. The first of these two groups are the apoptotic caspases, which can be further categorised into initiator or executioner caspases depending on which stage of the apoptotic cascade they are involved with. The human initiator subgroup includes caspase-8, -9, and -10; initiator caspases are monomeric and contain a long homotypic N-terminal domain (>90 amino acids) that is required for recruitment to their activation platform (Xu et al., 2019). The initiator caspases can be further divided by their involvement in either the extrinsic or intrinsic apoptosis pathway. The extrinsic pathway involves human caspase-8 and -10, which are activated through dimerization, aided by the death-inducing signalling complex (DISC) (Shalini et al., 2015). Caspase-9 is characterised as an intrinsic apoptotic caspase, activated through recruitment to the apoptosome (Kuranaga, 2012). Once the initiator caspases are activated, via DISC or the apoptosome, they then cleave the executioner apoptotic caspases (caspase-3, -6, and -7). Executioner caspases have a shorter pro-domain (<30 amino acids) and are synthesised as inactive dimeric zymogens. The cleavage and subsequent activation of these caspases by the initiator caspases allows for the cleavage of specific substrates to execute apoptotic cell death (Fuentes-Prior and Salvesen, 2004).

The second of the two main classification groups are the inflammatory caspases, comprised of caspase-1, -4 and -5 in humans, encoded by *CASP1, CASP4*, and *CASP5* respectively, all clustered on a single locus chromosome 11q22. Mice express inflammatory caspase-1 and caspase-11 only, encoded by *casp1*, and *casp11* genes on chromosome 9A1 (Bateman et al., 2021). Caspase-1 is highly conserved in both humans and mice. Caspase-5 is absent in mice and exists in humans and higher apes as the result of tandem genetic duplication (McIlwain et al., 2013); caspase-11 is thought to be the ortholog of caspase-4. Caspase-12 is also considered an inflammatory caspase in mice, however roles of caspase-12 have been debated (Vande Walle et al., 2016); humans express a truncated, inactive form of caspase-12, so will not be discussed further (Martinon and Tschopp, 2004). The best characterised inflammatory caspase is caspase-1. This caspase, originally named IL-1β converting enzyme (ICE), was identified whilst studying the processing of the inflammatory cytokine pro-IL-1β (Kostura et al., 1989). Caspase-1 is activated by the canonical inflammasome, whilst caspase-4 and -5 are activated by the non-canonical inflammasome (Elizagaray et al., 2020).

The canonical inflammasome and caspase-1

The inflammasome pathways are essential for a multitude of different biological processes, however dysregulation can have detrimental results, including cancer development, and neurological and inflammatory diseases (Howrylak and Nakahira, 2017). Inflammasomes are high molecular weight protein complexes that typically

consist of a pattern recognition receptor containing an N-terminal pyrin domain and/or a CARD (NLRP/NLRC), which when stimulated sets off a series of downstream events that leads to inflammatory caspase activation (Próchnicki and Latz, 2017). The nucleotide-binding domain and leucine-rich repeat containing receptor (NLR) family was the first to be identified as inflammasome forming sensor proteins. Additional gene families that induce inflammasome formation have since been discovered, for example the absent in melanoma 2 (AIM2) receptor from the AIM2-like receptor (ALR) family (Kumari et al., 2020). Inflammasome-activating pattern recognition receptors (PRRs) are found predominantly in immune and inflammatory cells such as macrophages and neutrophils, responding to a diverse range of chemically different stimuli; some of the stimuli are highlighted in table 1 Whilst the study of inflammasomes has been done primarily in innate immune cells, there have been studies suggesting the presence of inflammasome complexes in epithelial cells throughout the body, including the skin, lungs, eyes, and intestines (Santana et al., 2016).

The nucleotide-binding oligomerization domain-like receptor 1 (NLRP1) was the first protein to assemble an inflammasome complex to be identified (Martinon et al., 2002). Mice carry three paralogs of NLRP1 (NLRP1a-c), of which NLRP1b is the best characterised. The NLRP1 (NLRP1b in mice) inflammasome is activated in response to *Bacillus anthracis* toxins, specifically the anthrax lethal toxin (LeTx), a causative agent of anthrax disease (Chavarría-Smith and Vance, 2015). NLRP1/NLRP1b has also been shown as an inflammasome sensor for eukaryotic toxoplasmosis-causing parasite *Toxoplasma gondii* (Ewald et al., 2014) and for a diverse range of proteases from the *Picornaviridae* family of viruses, including human rhinovirus (HRV), poliovirus 1 (PV1), and enterovirus D68 (EV68) (Bauernfried et al., 2021; Tsu et al., 2021).

The nucleotide-binding oligomerisation domain-like receptor 3 (NLRP3) responds to various pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), including ATP, monosodium urate crystals, nucleic acids, zymosan, β-glucan, and osmolarity or pH decrease, which are all signals of the presence of an infection, or a potentially harmful deviation from homeostasis (Al Mamun et al., 2021; Mariathasan et al., 2006; Martinon et al., 2006). Most studies focus on cells from myeloid lineage, however other cells use inflammasomes too, for example keratinocyte exposure to skin irritants or ultraviolet B irradiation triggers NLRP3 inflammasome (Fenini et al., 2020; Hasegawa et al., 2016). A ‘priming step’ for activation of the NLRP3 inflammasome has been described, in which the detection of stress stimuli by toll-like receptor-4 (TLR4) or other receptors, activates the nuclear factor kappa B (NF-κB) pathway, which in turn upregulates transcription of NLRP3 (Fenini et al., 2020; Hasegawa et al., 2016). The NLRP3 pathway is arguably the most characterised inflammasome in human disease (Huang et al., 2020), however despite several mechanisms involving homeostatic disruptions being proposed, a single unifying signal leading to NLRP3 activation has not yet been identified.

A study by Schmid-Burgk *et al* involved a genomic-wide CRISPR Cas-9 screen to identify gene products involved in NLRP3 inflammasome-dependent cell death. This

study identified the mitotic Ser/Thr NIMA-related kinase 7 (NEK7) as an essential component of NLRP3 inflammasome activation. To further this discovery, He *et al* were able to conclude that Nek7 knock-out prevents caspase-1 activation and IL-1β release in NLRP3 but has no effect on the NLRC4 or AIM2 inflammasomes (He et al., 2016). They then further concluded that potassium efflux promotes the NLRP3/Nek7 interaction that induces the NLRP3 inflammasome and subsequent caspase-1 activation. NEK7 has been shown to interact with the LRR domain of NLRP3 downstream of the reactive oxygen species (ROS) induction (Sharif et al., 2019; Shi et al., 2016).

The nucleotide-binding oligomerisation domain-like receptor family CARD containing protein4 (NLRC4) responds to more specific activation stimuli, binding to the inner rod, flagellin, or needle proteins of Type III secretion systems of bacteria (Kortmann et al., 2015; Shi et al., 2017). Unlike other inflammasomes, NLRC4 requires the involvement of an intermediate, the NLR neuronal apoptosis inhibitory protein (NAIP), to become active (Vance, 2015; Zhao et al., 2011). NAIP acts as a sensor for the PAMPs listed above, before associating with NLRC4 to induce NLRC4 inflammasome oligomerisation.

The absent in melanoma 2 (AIM2) inflammasome-forming protein was originally discovered in the study of cancers (DeYoung et al., 1997). AIM2 is composed of a N-terminal PYD and a C-terminal hematopoietic interferon-inducible nuclear protein 200 (HIN-200) (Xue et al., 2019). The AIM2 inflammasome is activated via direct binding to cytosolic double-stranded DNA of microbial or host origin; the positively charged HIN-200 domain forms an electrostatic attraction with the negatively charged sugar-phosphate backbone of double-stranded DNA without sequence specificity, liberating AIM2 autoinhibition (Hornung et al., 2009; Matyszewski et al., 2018).

Finally, the pyrin inflammasome. Pyrin contains an N-terminal PYD followed by the B-box, α-helical coiled coil, and B30.2 domains, and is classed as a member of the tripartite motif (TRIM) protein family (Heilig and Broz, 2018). Pyrin is held in an inactive state via RhoA effector serine-threonine kinase PKN1 and PKN2 phosphorylation of pyrin, which promotes the interaction between pyrin and regulatory proteins 14-3-3ε and 14-3-3 (Jéru et al., 2005). The inactivation of RhoA through bacterial toxin-induced modifications inhibits PKN1 and PNK2 phosphorylation of pyrin, preventing inhibition via the regulatory proteins and allowing activation of the pyrin inflammasome (Schnappauf et al., 2019). Interestingly, pathogens have found a way to overcome activation of the pyrin inflammasome. For example, the pathogenic bacteria *Yersinia pestis* and *Yersinia pseudotuberculosis* inject an effector called YopM that, through direct phosphorylation of the PKNKs, is able to ensure pyrin autoinhibition (Chung et al., 2016).

Table 1:

Inflammasome sensors and their stimuli

Pattern Recognition Receptor	Examples of Activating Stimuli
NLRP1	*Toxoplasma gondii* (Levinsohn et al., 2012) LeTx (Boyden and Dietrich, 2006) Viral dsDNA (Bauernfried et al., 2021) *Picornavirus* proteases (Tsu et al., 2021)
NLRP3	ATP (Mariathasan et al., 2006) Monosodium urate crystals (Martinon et al., 2006) Nigericin (Pelegrin and Surprenant, 2007) Mitochondrial dysfunction/ROS production (Zhou et al., 2011) Ionic flux (including potassium, calcium, chloride and sodium) (Muñoz-Planillo et al., 2013)
NLRC4	T3SS needle protein & inner rod protein (Grandjean et al., 2017) Bacterial flagella (Kay et al., 2020)
AIM2	Cytosolic DNA (Hornung et al., 2009)
Pyrin	Rho GTPase inactivation (Xu et al., 2014)

Following PRR activation and oligomerisation via their specific stimuli, inflammasome sensors undergo homotypic interactions that lead to the activation of caspase-1, with or without the recruitment of the adaptor protein ASC. ASC is a 22kDa adaptor protein composed of both a PYD (N-terminal) and CARD (C-terminal) domain, initially named PYCARD (Fernandes-Alnemri et al., 2007). Originally thought to be just a linker between PYD-containing receptors and CARD-containing caspase-1, it has become clear that ASC has a more complex role. Upon activation of the inflammasome receptors, ASC-PYD oligomerisation leads to the formation of ASC filaments, and the interactions between these ASC filaments through ASC-CARD interactions leads to the formation of the ASC speck, with a single cellular focus of around 1µm (Dick et al., 2016). The ASC speck recruits caspase-1 monomers, increasing local caspase concentration and promoting proximity-induced dimerization; however, ASC is not essential for caspase-1 activation. NLRC4 can activate caspase-1 through direct CARD homotypic interactions, as NLRC4 does not contain a PYD for ASC recruitment, however this is less efficient than in the presence of ASC (Boucher et al., 2018; Broz et al., 2010). The ASC speck has also been shown to persist outside the cell after pyroptosis, where it can function as an inflammatory signal in the circulation/peripheral tissue (Baroja-Mazo et al., 2014., Liston & Masters, 2017).

The non-canonical pathway

The non-canonical inflammasome activates caspase-4 and -5 in humans, and caspase-11 in mice. This occurs in response to lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria. LPS is a PAMP significant in mediating sepsis and septic shock; LPS is the most common cause of sepsis, which accounts for 19.7% of deaths globally (Pérez-Hernández et al., 2021; Rudd et al., 2020). LPS is the major component present in the outer membrane of Gram-negative bacteria, which is also made up of phospholipids, and is formed of three counterparts; lipid A, the core oligosaccharides, and the O-antigen (Candelli et al., 2021). Extracellular LPS is detected by Toll-like receptor 4 (TLR4) along with its co-receptors MD2 and cluster of differentiation 14 (CD14), which triggers a protein-protein interaction cascade that results in the induction of pro-inflammatory genes such as IL-1β (Lin et al., 2015; Medzhitov et al., 1997). Cytosolic LPS however is detected by the non-canonical inflammasome. Upon binding to LPS, inflammatory caspase-4 and -5 undergo dimerization to become active (Karki et al., 2007; Rathinam et al., 2019). Until recently, it was believed that the caspases were directly binding to the lipid A portion of LPS via their CARD to trigger this pathway as this was shown *in vitro* (Shi et al., 2014), however recent studies *in vivo* and *in cellulo* identified guanylate binding proteins (GBPs) that facilitate the presentation of hydrophobic bacterial LPS to these caspases (Elizagaray et al., 2020; Meunier et al., 2014; Santos et al., 2020). Interferon-inducible GBPs recognise the outer portion of LPS, allowing for the assembly of the inflammasome. In humans, GBP1 initiates the assembly of the platform, GBP2 and 4 facilitate caspase-4 recruitment, and GBP3 controls activation of caspase-4 (Wandel et al., 2020). In mice, GBPs facilitate the recruitment and localisation of the IFN-inducible protein immunity-related GTPase family member b10 (IRGB10), destroying the invading pathogens membrane and causing the release of LPS and dsDNA, activating the non-canonical and AIM2 inflammasome respectively (Man et al., 2016; Shrivastava et al., 2017). However, humans do not express a functional IRGB10 (Ha et al., 2021).

Inflammatory caspase functions are defined by the substrates they cleave; however, these substrates are not currently well defined.

Inflammatory caspase substrates

Inflammatory caspases are highly specific proteases which follow defined determinants for substrate cleavage. The cleavage site of a caspase substrate is usually located within a flexible solvent exposed loop (Coombs et al., 1998). Caspases were thought to have a strict requirement for the presence of an aspartate in the P1 position, however recent studies have suggested the presence of glutamic acid or phosphorylated serine are also viable, expanding the potential for substrate identification (Seaman et al., 2016). Each caspase has a preferred sequence of amino acids at positions P2 to P4 (Timmer et al., 2009), for example inflammatory caspases prefer hydrophobic or aromatic amino acids at P4, glutamic acids at P3, and aliphatic amino acids at P2 (Seaman et al., 2016).

A well-studied substrate of inflammatory caspases is gasdermin D (GSDMD), a member of the pore-forming gasdermin family. Gasdermins are highly conserved in an array of organisms, ranging from fungi (Daskalov et al., 2020), coral (Jiang et al., 2020), to higher vertebrae (Ramos-Junior and Morandini, 2017). Mammalian GSDMD contains around 480 amino acids, divided into two domains, the N-terminal and C-terminal, which are separated by a linker loop (Kuang et al., 2017). The cleavage of GSDMD at the $FLTD_{275}$ site ($LLSD_{276}$ in mice) by caspase-1, -4 and -5 releases the N-terminal from its C-terminal inhibitory counterpart; the oligomerisation of the free N-terminals form pores within the plasma membrane, which induces a highly inflammatory form of cell death known as pyroptosis (Wang et al., 2020). The N-terminus alone can induce pyroptosis when expressed ectopically, whilst an over-expression of the suppressive C-terminus can block GSDMD-dependent pyroptosis (Mulvihill et al., 2018). The formation of 1.1-2.4nm pores by N-terminal oligomerisation disrupts the integrity of the cell membrane, causing a loss of ionic gradient and therefore an increase in osmotic pressure, resulting in swelling and subsequent plasma membrane rupture (PMR). This rupture releases intracellular components such as inflammatory cytokines and lactate dehydrogenase (LDH), along with a variety of other DAMPs, further eliciting an immune response (Rayamajhi et al., 2013). Originally, PMR was thought to be a passive event that occurred following pore formation, however this is not the case. A recent study by Kayagaki *et al*., (2021) showed that the pores formed in response to GSDMD oligomerisation can release IL-1β, however the pore is too small for the release of LDH. It was demonstrated that the 16kDa protein NINJ1 is required for PMR, acting downstream of GSDMD, and that a NINJ1 deletion reduces the amount of protein released in pyroptotic cell death. The mechanism behind this is currently unknown. GSDMD pore formation also leads to a potassium efflux, enabling NLRP3 inflammasome activation downstream under certain circumstances (Baker et al., 2015). It is important to note that not all stimuli that initiate inflammasome assembly elicit pyroptosis, and not cell types undergo pyroptotic cell death after the activation of caspase-1, however the molecular mechanism behind this is not yet understood (Chen et al., 2014; Di Gioia et al., 2020; Wolf and Underhill, 2018).

It has also been well documented that caspase-1 cleaves the interleukin family members IL-1β and IL-18. The interleukin-1 family members are a group of cytokines that are involved in a variety of innate immune processes, currently comprising of 11 members: IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36α, IL-36β, IL-36γ, IL-36Ra IL-37, and IL-38 (Kaneko et al., 2019). The inflammatory cytokine interleukin-1β (IL-1β) is potent inflammatory cytokine that is crucial in infection and injury (Dinarello, 2018). This cytokine is found in multiple cell-types but is studied primarily in the context of inflammatory cells of myeloid lineage. Most cytokines contain a signal sequence leading to release from the cell via secretory vesicles, however IL-1β does not contain this sequence and instead follows a less-conventional secretion pathway. This cytokine is also unusual in that it is expressed as a precursor to its active form. Inflammasome activation is responsible for both the maturation and secretion of IL-1β (Monteleone et al., 2018). After cell exposure to PAMPs or DAMPs, an inactive 31kDa precursor, pro-IL-1β, is produced; cells containing this precursor are said to be ‘primed’. Once cells are primed, the encounter of additional PAMPs or DAMPs will allow inflammasome activation and subsequent caspase-1 activation (Lopez-

Castejon and Brough, 2011; Martín-Sánchez et al., 2016). The processing of pro-IL-1β by active caspase-1 allows the release of mature IL-1β from the cell through GSDMD pores (Kaneko et al., 2019b).

Inhibition of the inflammasome

The role of inflammasomes in a plethora of inflammatory diseases evokes a significant interest in the development of inhibitors that can work either directly on the inflammasome complex or prevent consequent substrate activation. Below is an outline of the inhibitors used throughout this work, with a brief description of their mechanism of action.

VX-765 is a well understood caspase-1 inhibitor that acts through covalent modification of the catalytic cysteine residue in the active site of the caspase, and has previously been linked to a reduction in IL-1β and IL-18 inflammatory cytokine release in a multitude of inflammatory models both *in vitro* and *in vivo* (Doitsh et al., 2014; Flores et al., 2020; Van Opdenbosch and Lamkanfi, 2019; Wannamaker et al., 2007). MCC950 is another inhibitor that has been validated in multiple *in vitro* and *in vivo* disease models. This small-molecule inhibitor acts directly on the NLRP3 inflammasome to prevent downstream caspase-1 activation (Coll et al., 2019). The production of intracellular reactive oxygen species (ROS) has been previously shown to induce NLRP3 inflammasome formation and subsequent caspase-1 activation. Ebselen is an antioxidant that has been previously shown to inhibit ROS production through peroxide scavenging, therefore preventing induction of the NLRP3 inflammasome (Jabaut et al., 2013).

Inhibitors can also work to prevent pyroptosis through direct interactions with the pore forming protein GSDMD. Dimethyl fumarate (DMF), disulfiram (DS), and Necrosulfonamide (NSA), all work to prevent the oligomerisation of p30-GSDMD, prevent both pyroptosis and the release of inflammatory cytokines (Hu et al., 2020; Humphries et al., 2020; Rathkey et al., 2018).

The importance of finding new substrates of inflammatory caspases is significant. A broader knowledge of the roles of inflammatory caspases in the body could provide new insight into potential biomarkers for infections, as well as in sepsis and other inflammatory-related diseases. Alongside this, finding new substrates provides targets for inhibitors of proteolytic cleavage of inflammatory caspase substrates, potentially providing new therapeutic options for a multitude of inflammatory diseases. In this study, the aim was first to optimise detection of inflammatory caspase-4 in various cell types. The efficiency of the previously discussed inhibitors were then tested in THP-1 cells to further understand the inflammasome pathway. Finally, through the use of proteomic analysis potential novel inflammatory caspase substrates were identified. This work provides an insight into the activation of inflammatory caspases, and the identification of new substrates opens a wide-range of new therapeutic targets.

Methods

Cell Culture

All cells were kept at 37°C with 5% CO_2 unless stated otherwise.

The human monocytic cancerous cell line THP-1 was maintained in Roswell Park Memorial Institute Medium (RPMI-1640 – Gibco) with 10% fetal calf serum (FCS), supplemented with penicillin-streptomycin at 1% concentration and 10mM L-Glutamine. The addition of 50ng/mL of Phorbol-12-myristate-13-acetate (PMA – Sigma-Aldrich) to the media of the THP-1 line allowed the differentiation of these cells into macrophage-like cells. After 48 hours of PMA treatment at 37°C, 5% CO_2, the media was replaced with fresh RPMI without PMA. The human intestinal epithelial cell line HIEC-6 was maintained in Opti-MEM® reduced serum media (Gibco) supplemented with 20mM HEPES, 10mM GlutaMAX™, 10ng/mL EGF, 4% FCS, and 1% Pen/Strep. The immortalised human keratinocyte cell line (HACAT) was maintained in calcium-free DMEM supplemented with 10% FSC, Pen/Strep, and GlutaMAX™. HeLa were maintained in Dulbecco's Modified Eagle Medium (DMEM – Gibco), with 10% FCS, supplemented with penicillin-streptomycin and L-glutamine.

Cells were primed with 10ng/mL human interferon gamma (IFNɣ) or Pam3CSK4 (1µg/mL H_2O – Invitrogen) overnight when necessary to induce expression of various canonical and non-canonical inflammasome components.

Cell treatments

Inhibitors were diluted with Opti-MEM®, which was added to the cells on the morning of intended use. After the addition of fresh Opti-MEM®/Opti-MEM®+inhibitors the cells were incubated for 30 mins to 1 hour before transfection.

Inhibitor	Abbreviations used throughout text	Final concentration	Target/s of inhibitors
VX-765	VX	25 µM	Caspase-1 and -4 inhibitor
MCC950	950	10 µM	NLRP3 inflammasome inhibitor
Dimethyl fumarate	DMF	10 µM	Oligomerisation of GSDMD
Disulfiram	DS	10 µM	Oligomerisation of GSDMD
Necrosulfonamide	NSA	10 µM	Oligomerisation of GSDMD
Ebselen	Eb	10 µM	ROS

Nigericin was used at a final concentration of 10µM and was added directly to the cells where necessary after incubation with the selected inhibitors.

LPS transfection

The morning the cells were intended for use, the old media was discarded and replaced with fresh Opti-MEM® media (with or without inhibitors). LPS from *E. coli* K12 (LPS EK, InvivoGen) was diluted with Opti-MEM® to 10µg/mL LPS concentration. The LPS + Opti-MEM® was vortexed and Lipofectamine LTX (Invitrogen) was added at a concentration of 3%. The LPS mix was then left for 15 minutes to incubate at room temperature. After incubation, the LPS 3% lipofectamine

mix was added directly to the cells, whilst the negative controls were supplemented with Opti-MEM® to ensure all wells were at the same volume. Cells were spun for 5mins at 500 x g at room temperature before being incubated at 37°C, 5% CO_2 for 6 hours.

Lactate Dehydrogenase Assay

The level of LDH released from cells was used to assess pyroptotic cell death in each sample. The LDH cytotoxicity detection kit from Takara Bio was used for this assay, and everything was performed via the manufacturer's instructions. After incubation with/without relevant inhibitors for 6hours, 4µL of 10% triton X-100 was added to the 100% lysis control wells and incubated for 5mins at room temperature to lyse the cells. All samples were transferred to new 96-well plates (15 µL each) and 15µL of LDH substrate (LDH Cytotoxicity detection kit, Takara Bio) was added to each well. The plates were incubated at room temperature in the dark until a clear colour change was visible (roughly 25 mins), at this point 15 µL of HCL stop solution (1N) was added to stop the reaction. A spectrophotometer was used at 490 nm to read the plates. Three repeats were obtained for each experiment.

Enzyme-Linked Immunosorbent Assay (ELISA)

IL-1β secretion from cells was measured by ELISA; everything was prepared via the manufacturer's instructions (Human IL-1β Uncoated ELISA kit, Invitrogen). The ELISA plates were coated with human IL-1β capture antibody (1in250 PBS dilution) overnight. Plates were washed three times with PBST and blocked for 1 hour at room temperature (250µL ELISA blocking buffer per well). Sample supernatants (10µL) were diluted with 240µL DPBS. Samples (40µL) were then added to each blocked well overnight along with the human IL-1β standards, prepared via manufacturer's instructions (12-fold serial dilution of 2/3, with an initial concentration starting at 2ng/mL). After overnight incubation of samples and standards at 4°C on the blocked plates, the plates were washed three times with PBST as before and 40µL/well of human IL-1β detection antibody was added (1in250 blocking buffer dilution). The plates were washed after a 1hour incubation at room temperature, and the avidin-HRP (1in250 blocking buffer dilution) was added (40µL/well) for 30 mins. After washing the plate thoroughly 5 times with PBST, TMB solution was added at 40µL/well; the stop solution (H_2SO_4 1N) was added after a clear colour change was detected (roughly 25 mins). A spectrophotometer was used at 450nm to read the plates. Three repeats were obtained for each experiment.

Protein Precipitation

Methanol and chloroform:

Cellular protein extraction was done via the methanol and chloroform method. After the extraction of the cell supernatant from the well plate, methanol (250µL) and chloroform (100µL) were added to the extracted supernatant (250µL). The samples were vortexed and centrifuged at 16.1 x 10^3g and the upper phase subsequently removed. An additional 400µL methanol was added, samples were centrifuged again as before, the supernatant was removed, and the protein pellet was left to dry in the fume hood for roughly 30 mins. After 30 mins, the pellets were resuspended in 30µL SDS lysis buffer (1X TruPage™ LDS sample buffer – Sigma, in 1.5% SDS 50mM

Tris buffer [pH 8]) boiled to 95°C, which was also added to the now-empty well plate (extract - 25µL/well). The cell extract was then harvested by thoroughly scrapping each well and removing the content into fresh 1.5ml tubes.

Acetone:
For the acetone method of protein precipitation, 30µL of 10% IGEPAL® was added to each well and swirled to mix. After two minutes, the liquid was transferred to fresh 1.5ml tubes and diluted with cold acetone (1in5 dilution). Samples were kept in at -80°C until use. On the day the samples were required, they were centrifuged at -4°C at 16.1 x 10^3g for 10 mins whilst still cold, the supernatant was removed, and the pellet re-suspended in 25µL lysis buffer boiled to 95°C.

Western Blot

Western blotting was used to study cleavage of various proteins as an indication of canonical or non-canonical inflammasome activation. After protein precipitation, both the cell extract and supernatant were boiled to 95°C for 5 minutes to denature the proteins. After denaturation, 10µL of sample per lane was run on a 12% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) in Tris-glycine running buffer at 160V for approximately 1.5 hours, then transferred to a nitrocellulose membrane using a Trans-blot Turbo system (Bio-Rad), for 7 minutes at 25V. The BlueEye pre-stained protein molecular weight ladder was used as a marker (Sigma-Aldrich). The membranes were then blocked with 1X tris-buffered saline-Tween 20 (TBST: P1379) containing 5% skimmed-milk powder for 1 hour at room temperature, then incubated overnight with 5% milk TBST and the following polyclonal antibodies: Caspase-4 at 1:2000 (Protein Tech, 11856-1-AP), GSDMD at 1:3000 (Cusabio, CSB-PA00956LA01HU), IL-18 at 1:2000 (Protein Tech, 10663-1-AP), IL-F3 at 1:2000 (Protein Tech, 19887-1-AP), G3BP1 at 1:2000 (Protein Tech, 13057-2-AP), U2AF2 at 1:2000 (Invitrogen, PA5-102524), and anti-histone C-terminus antibody as the loading control at 1:3000 (BioLegend, 819409). Membranes were incubated with antibody at 4°C or room temperature depending on antibody; antibodies incubated at room temperature had the addition of sodium azide solution at a final concentration of 0.02% to prevent bacterial growth. After overnight incubation the membranes were washed with TBST for 15 mins on an orbital shaker three times and incubated for a further 2 hours with either horseradish peroxidase-conjugated goat anti-rabbit (Invitrogen, A16096) or donkey anti-mouse (Invitrogen, A16017) IgG antibodies at 1:3000 concentrations at room temperature. The membranes were washed as before, then imaged using the BioRad Chemidoc MP™ imaging system. Immobilon™ Crescendo Western HRP substrate (Millipore) or SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific) were used to develop the blots. After imaging, HRP inactivation was performed (30 min incubation at 37°C with 30% hydrogen peroxide) and re-probed with other antibodies.

All illustrated figures were made using Biorender.com software.

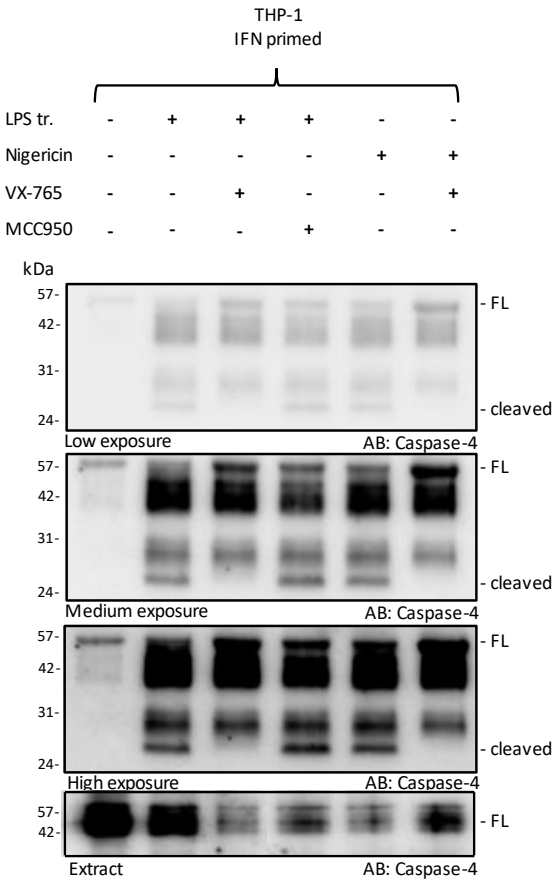
Results

Caspase-4 activation in the monocytic THP-1 cell line:

Caspase-4 cleavage has previously been used as a readout of its activation. Firstly, we wanted to study caspase-4 cleavage, and optimise this experiment specifically in the monocytic THP-1 cell line. To detect caspase-4 cleavage, cells were first transfected with LPS to initiate the non-canonical inflammasome pathway or treated with nigericin to initiate the NLRP3 canonical inflammasome pathway. Nigericin is a toxin derived from *Streptomyces hygroscopicus* known to activate the NLRP3 inflammasome via potassium efflux (Armstrong et al., 2019). All cells were primed with IFNɣ 16 hours before use to amplify the cell response (Brubaker et al., 2020). Active caspase-4 was detected via Western blot analysis, as shown in figure 1a. To ensure the optimum image was obtained from this experiment, three exposures were used, labelled high, medium, and low relating to their exposure. This was to ensure the full-length band could be seen clearly alongside the caspase-4 isoforms. In the untreated cells, the full-length caspase-4 band can be clearly seen at 43kDa, whereas in the cells transfected with LPS and those treated with nigericin, the active p20 caspase-4 cleavage product was also detected. This was to be expected as the LPS transfection induced the non-canonical inflammasome, while nigericin activated the NLRP3 canonical inflammasome, both activating caspase-4. Traditionally, the NLRP3 inflammasome activates caspase-1, however here it can be seen that caspase-4 is activated as well (this has previously been shown in unpublished data). The addition of the caspase inhibitor VX-765 alongside LPS transfection blocked the detection of the p20 fragment, suggesting VX-765 prevented caspase-4 activation. The addition of the inhibitor MCC950 had no effect on caspase-4 cleavage after LPS transfection, which was also to be expected as this inhibitor should only affect the NLRP3 canonical inflammasome, and not the non-canonical inflammasome. This is further supported in the nigericin treated cells, in which MCC950 prevents caspase-4 cleavage, suggesting it did have an effect when used in the context of the canonical inflammasome. The caspase-4 cleaved products were detected in the supernatants, while the cellular extract was used as a loading control in this experiment, as this provided clear full-length caspase-4 bands but no cleaved product.

The results obtained from the LDH assay, shown in figure 1b, supported the caspase-4 activation seen in the Western blot experiments. LDH release was significantly reduced in the LPS transfected cells and the nigericin treated cells with the addition of VX-765. As seen previously in figure 1a, MCC950 did not affect LPS transfected cells as NLRP3 is downstream of caspase-4, and not required for the execution of cell death in this set up.

1a.



1b.

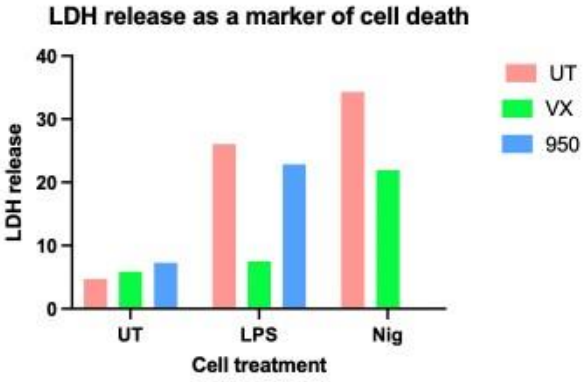


Figure 1: ***Activation of caspase-4 in the canonical and non-canonical inflammasomes in THP-1 cells.*** *Panel 1a: an immunoblot of LPS transfected (LPS tr.) or nigericin treated THP-1 cells, all primed with IFNɣ with the addition of either VX-765 or MCC950 inhibitors. All caspase-4 immunoblots are from cell supernatant and are shown at various exposures to ensure the optimum image was obtained. Full length caspase-4 is denoted as FL. Panel 1b: LDH release in THP-1 cells primed with the same treatments as previously stated. The unit of LDH release is nmol/min/ml. Abbreviations used are: UT – untreated, VX – VX765, 950 – MCC950, nig – nigericin treated. All data shown is the result of three experimental repeats.*

Effect of canonical inflammasome inhibition on known caspase-4 substrate GSDMD and pyroptotic cell death:

Caspase inhibition has the potential to significantly affect the prognosis of a multitude of different inflammatory conditions. Based on this, six inhibitors were chosen to study in the context of both the canonical and non-canonical inflammasomes. The inhibitors chosen and their inhibitory targets have been previously stated in the methods section; where each inhibitor reacts with the canonical pathway was shown in figure 2a. Many of the inhibitors chosen are thought to target cysteines in an unspecific manner, so it was hypothesised that these compounds could inhibit the activity of inflammatory caspases, as they are cysteine proteases (Hu et al., 2020; Humphries et al., 2020; Rathkey et al., 2018). To study these inhibitors in the canonical inflammasome pathway, the human monocytic cell line THP-1 was treated with nigericin before treatment with each inhibitor. The activation of the NLRP3 inflammasome by nigericin and subsequent activation of caspase-1 was shown in figure 2b. The immunoblot shows full-length caspase-1 (FL), caspase-1 activation is evident by the p20 fragment, whilst the intermediate fragment is the transient p30/p10 active species (Boucher et al., 2018). Nigericin treatment alone caused both caspase-1 and GSDMD cleavage, whilst the inhibitors had varied results. Caspase-1 activation was inhibited by MCC950, however the level of full-length protein is lower than the other samples, which could be why the p20 fragment is not visible in this lane. VX-765, DS, and NSA treatment, however this is not mirrored in the GSDMD immunoblot. In the GSDMD immunoblot, two cleaved fragments are present, the p30 fragment labelled is caspase-1 dependent cleavage, while the lower band is thought to be caspase-3-dependent cleavage (Chen et al., 2019). Under certain conditions nigericin can activate caspase-8, which in turn activates caspase-3, which has been shown to cleave GSDMD at a different site to the inflammatory caspases, D84. This could explain why GSDMD was cleaved in the VX-765 treated samples despite no caspase-1 activation, as VX-765 is specific to caspase-1 and -4, so should have minimal effect on caspase-3 and -8 activity. Caspase-3 cleaves a smaller, inactive GSDMD fragment, explaining the lower band labelled p20.

Caspase-1 cleavage and GSDMD activation after nigericin treatment was further supported through lactate dehydrogenase release to quantify the level of cell death in these cells, as shown in figure 2c. LDH release was inhibited in cells treated with MCC950, whilst VX-765, DS, and DMF showed reduced LDH release compared to the untreated sample. NSA and Eb had no significant effect on the level of LDH released from the cells. An ELISA was done to detect IL-1β cytokine release, a known caspase-1 substrate, with treatment of inhibitors, shown in figure 2d. IL-1β was blocked in VX-765, MCC950, and NSA treated samples, in accordance with the inhibition of caspase-1 activation shown in fig 2b. Ebselen reduced IL-1β secretion by around 50%, suggesting it was able to partially inhibit the NLRP3 inflammasome. While disulfiram did block caspase-1 activation, there was a significant level of LDH and IL-1β secretion, even in untreated samples, suggesting that disulfiram could promote toxicity in cells.

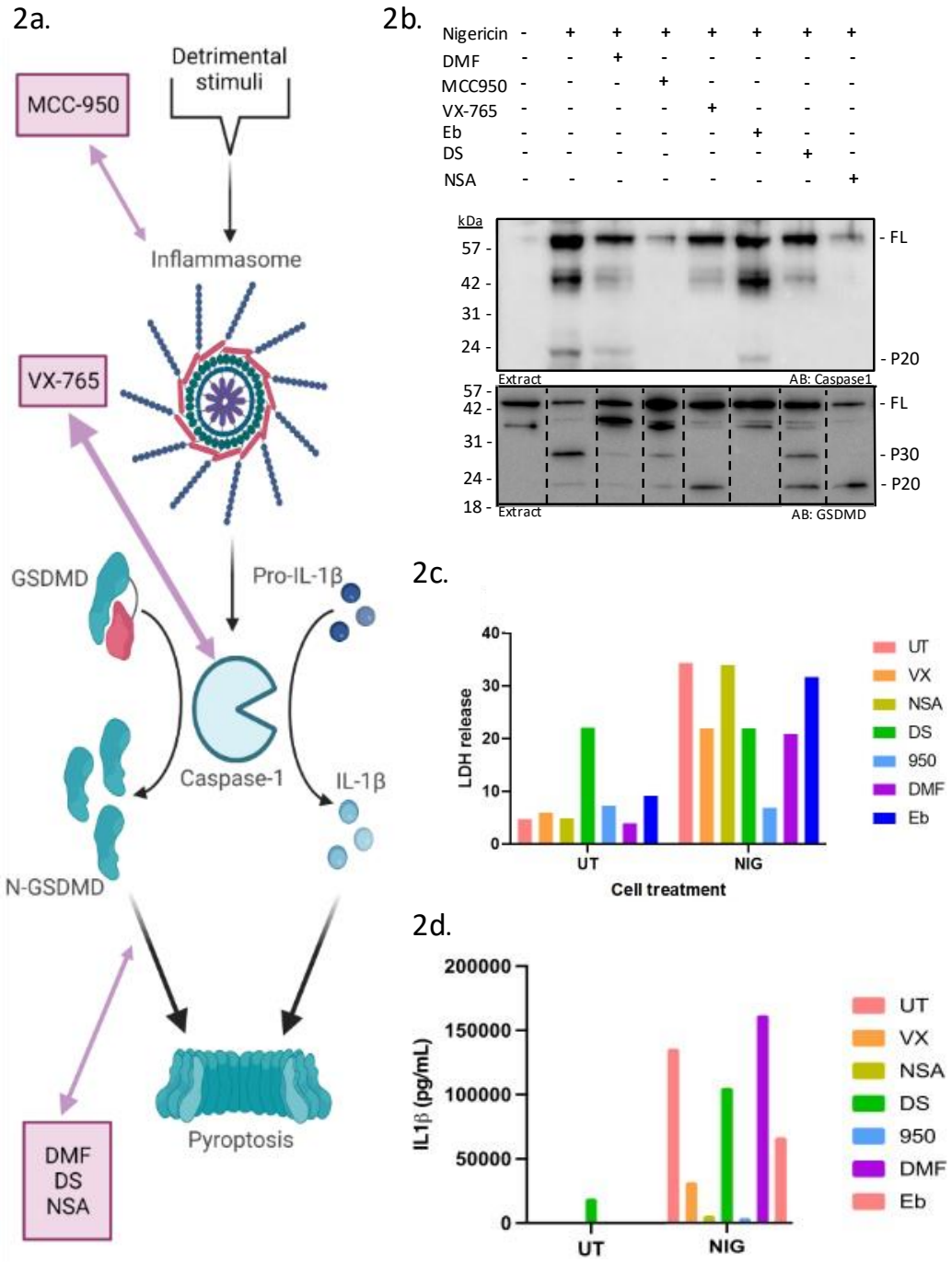


Figure 2: ***Canonical inflammasome inhibition by various inhibitors and the effects on known substrate GSDMD.*** *Panel 2a: a basic schematic of the canonical inflammasome and where the inhibitors interact with this pathway. Panel b: Nigericin-treated THP-1 cells primed with IFNɣ and treated with 6 inhibitors. Caspase-1 and GSDMD immunoblotted in cell extracts. GSDMD western is presented with dotted lines between lanes to show loading order has been changed for consistency with caspase-1 membrane. Full-length proteins are labelled FL, cleaved fragment labelled P20. The lowest fragment on the GSDMD immunoblot, labelled P20, is a caspase-3 cleaved inactive fragment. Panel c: LDH release in nigericin-treated (labelled NIG), IFNɣ-primed THP-1 cells compared to untreated cells with addition of same inhibitors. Panel d: IL-1β secretion by nigericin-treated (labelled NIG), IFNɣ-primed THP-1 cells compared to untreated THP-1 with addition of same inhibitors. The data shown is the result of three experimental repeats.*

Effect of non-canonical inflammasome inhibition of the known substrate GSDMD and pyroptotic cell death:

We next wanted to assess the impact of the same inhibitors on the non-canonical inflammasome. To induce the non-canonical inflammasome, THP-1 cells were transfected with LPS to activate caspase-4. A schematic of the non-canonical pathway and where the inhibitors interact with this pathway are shown in figure 3a. The detection of caspase-4 cleavage in THP-1 cells with inhibitors was extremely difficult to obtain. The addition of the inhibitors gave inconclusive results in which the repeats contradicted each other, so the experimental data is not shown for caspase-4 detection in LPS treated THP-1 cells. GSDMD was cleaved in the samples treated with LPS as shown by the presence of the p30 cleaved fragment in figure 3b. This fragment was not present in the samples treated with VX-765, DMF, or NSA, suggesting these inhibitors successfully blocked GSDMD activation. The LDH assay shown in figure 3c mirrored this result, as LDH release was significantly reduced in VX-765, DMF, and NSA treated samples, suggesting GSDMD has not been able to induce pore formation in these cells. This is again suggested in the ELISA shown in figure 3d, in which IL-1β release was significantly reduced in the VX-765, DMF, and NSA treated cells. MCC950, DS and Ebselen showed a reduction in IL-1β release, suggesting these inhibitors were able to partially block IL-1β release.

MCC950 did not inhibit GSDMD cleavage or LDH release when transfected with LPS but did show partial inhibition in the IL-1β release. MCC950 inhibits the NLRP3 inflammasome, which is assumed to be required for IL-1β cleavage. This suggests that caspase-4 could be responsible for the IL-1β cleavage present in this experiment despite a caspase-4 Western blot not being obtained. Caspase-4 was activated via the non-canonical inflammasome by LPS transfection. As before, disulfiram seemed to induce toxicity in THP-1 cells suggested through LDH and IL-1β secretion in untreated samples.

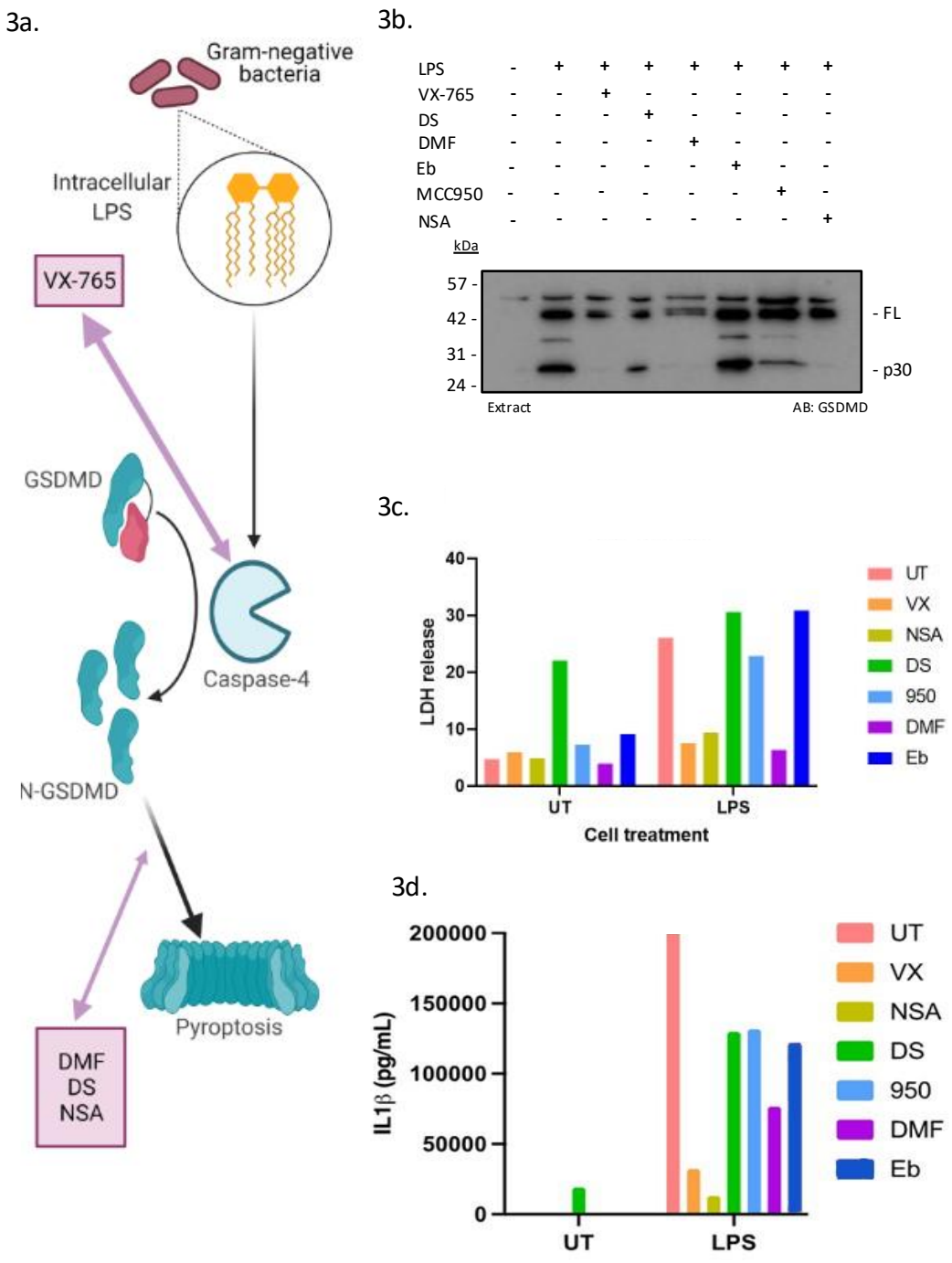
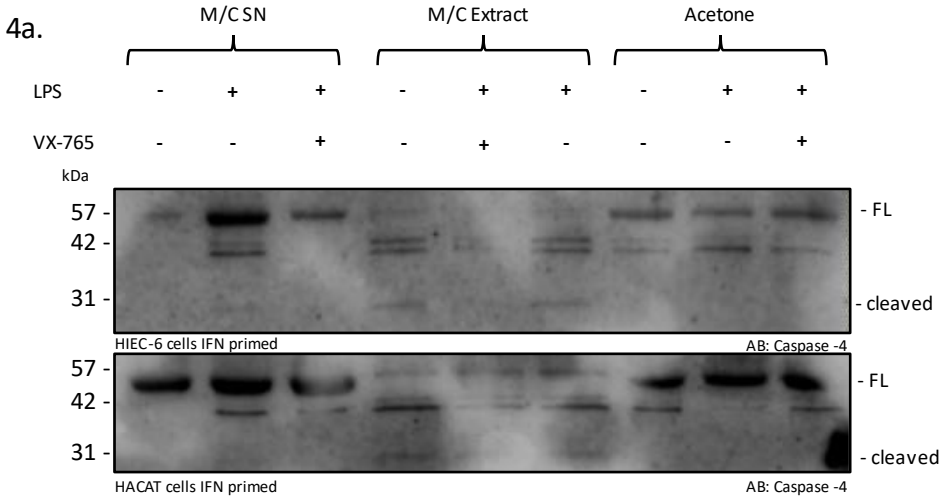


Figure 3: ***Study of the non-canonical inflammasome and how inhibition effects known substrate GSDMD****. Panel 3a: A schematic of the non-canonical inflammasome and where the inhibitors used interact with the pathway. Panel 3b: LPS-transfected THP-1 cells treated with 6 inhibitors. GSDMD immunoblots in the cell extracts shown. The full-length GSDMD protein is labelled FL, whilst the active fragment is labelled p30. Panel 3c: LDH release in LPS-transfected, IFNγ-primed THP-1 cells compared to untreated cells with addition of same inhibitors. Panel d: IL-1β secretion by LPS-transfected, IFNγ-primed THP-1 cells compared to untreated THP-1 with addition of same inhibitors.*

Optimisation of caspase-4 detection in other cell types:

Caspase-4 was extremely difficult to detect via Western blot in cell types other than THP-1. For this reason, a different protein precipitation method, the acetone method, was included alongside the methanol and chloroform (M/C) method used previously for direct comparison to ensure optimal caspase-4 detection. Figure 4a shows the comparison of these methods in HIEC-6 and HACAT cells. The M/C method involved separating the cell extract and supernatant of the samples, whereas the acetone method allowed the supernatant and extract to be concentrated and run together. The M/C method of protein precipitation provided slightly clearer results in both cell types, and faint cleavage bands in the extract, however the acetone method showed only full length caspase-4. To further optimise this experiment, the M/C and acetone comparison was done again, but using two different caspase-4 antibodies, one from SantaCruz (clone 4B7) at a concentration of 1:500, and one from ProteinTech at 1:2000 concentration. There is a significant difference in these results, shown in HACAT cells in figure 4b. The ProteinTech antibody provided clear full-length bands, with evidence of activation through a reduction of the full-length LPS transfected band in the extract of the M/C method. Despite these optimisation steps, a clear caspase-4 cleaved fragment could not be detected in these cells. The SantaCruz antibody gave very little signal at all, despite being used at a very high concentration of 1:500, so this antibody was not used in any experiments going forward.



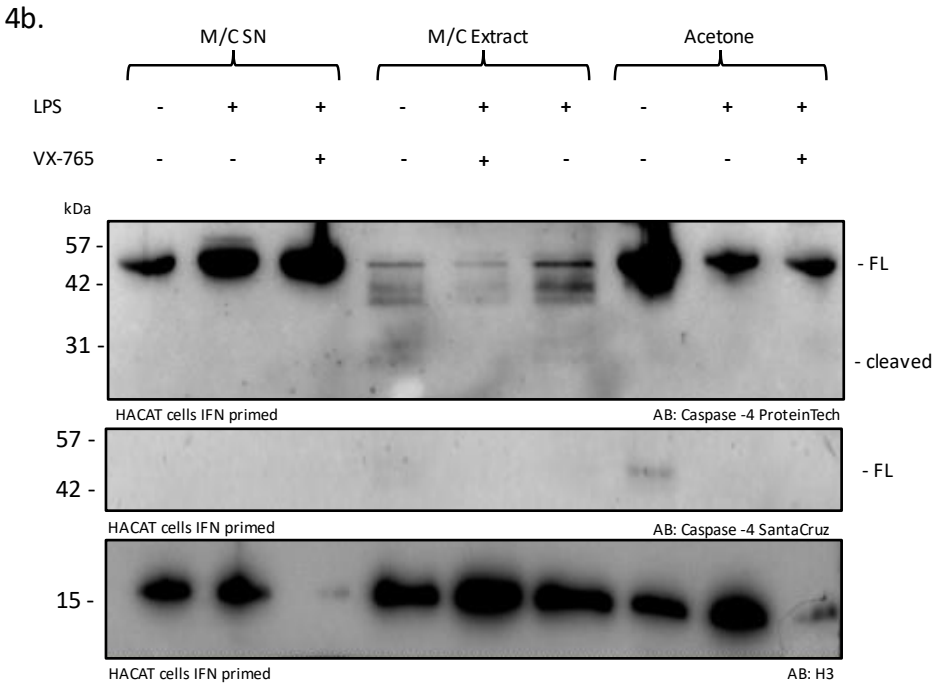
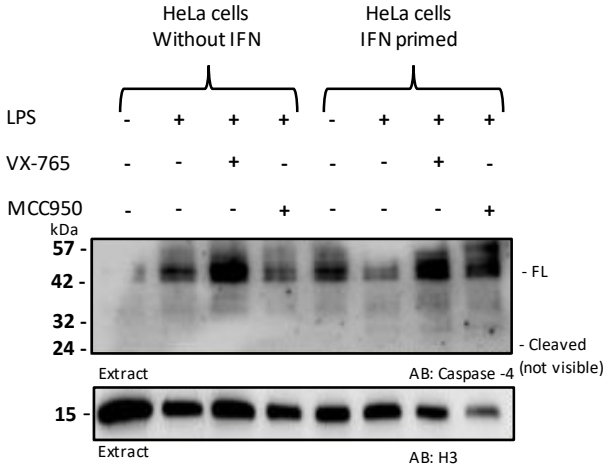


Figure 4: ***Optimisation of caspase-4 detection.*** *Panel 4a: comparison of protein precipitation methods in HIEC-6 and HACAT cells primed with IFNɣ. Cells were transfected with LPS and treated with VX-765 where labelled. Samples from both protein precipitation methods were run on one gel for a direct comparison. M/C denotes the methanol and chloroform method. The antibody used was ProteinTech caspase-4. Panel 4b: comparison of the ProteinTech and SantaCruz antibodies in HACAT cells primed with IFNɣ. Histone H3 is included as a loading control. Full-length proteins are labelled FL.*

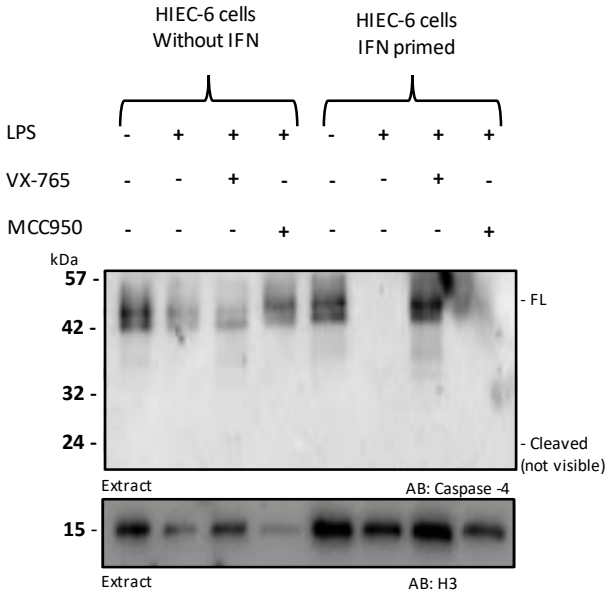
Caspase-4 activation in epithelial cells:

The active fragment of caspase-4 was extremely difficult to detect via Western blot in epithelial cells lines, even after multiple optimisation steps. The methanol and chloroform method of protein precipitation was used in all epithelial cell lines, with the ProteinTech caspase-4 antibody as previously optimised. We next wanted to further optimise caspase-4 detection by assessing the effect of priming with IFNɣ on these cells, and how this affected caspase-4 detection via Western blot. IFNɣ priming induces the expression of GBPs, which facilitate the recognition of LPS by caspase-4 in the non-canonical inflammasome (Tretina et al., 2019). The cleaved caspase-4 band was difficult to detect in HeLa and HIEC-6 cells, therefore a decrease in the amount of full-length fragment was taken as activation. In HeLa cells transfected with LPS, a reduction in the full-length caspase-4 band was seen in the IFNɣ-primed cells, but no reduction was seen in the cells without priming, as shown in figure 5a. VX-765 treatment after priming in the HeLa cells prevented the reduction of the full-length caspase-4 band, suggesting caspase-4 activation was blocked in these cells. Interestingly, MCC950 also seemed to block caspase-4 activation. The IFNɣ-primed HIEC-6 cells showed slightly different results; IFNɣ priming alongside LPS transfection and VX-765 treatment prevented full-length caspase-4 disappearance, while LPS transfection with the addition of MCC950 treatment caused a complete disappearance of the full-length caspase-4 band, shown in figure 5b. All cell types showed less, or no reduction of the full-length caspase-4 band in the samples without prior IFNɣ priming, suggesting IFNɣ is required for caspase-4 activation and for optimum detection via Western blot. However, we could not detect the caspase-4 cleaved fragment in the cells, even after optimisation. IFNɣ priming was used from here on when studying caspase-4. Nigericin treatment was not done in these cells as it had been seen in previous experiments that HIEC-6 cells did not respond to nigericin treatment. An LDH assay was done to accompany the HIEC-6 cell Western blot, as shown in figure 6b. LDH release was inhibited in LPS transfected cells when treated with the VX-765 caspase inhibitor in all cell types. As previously shown, MCC950 had no inhibitory effect in cells transfected with LPS. The 100% bars refer to the cells lysed with triton X-100 used as a control. The LDH results mirror those seen in the Western blot.

5a.



5b.



5c.

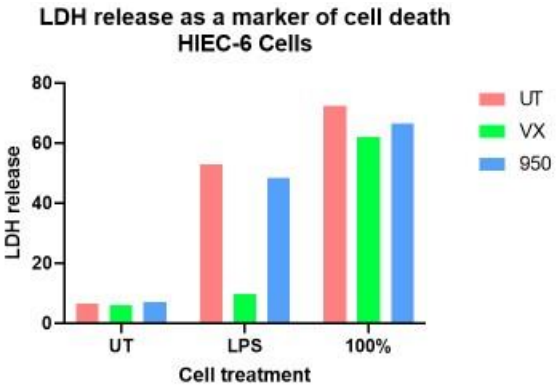
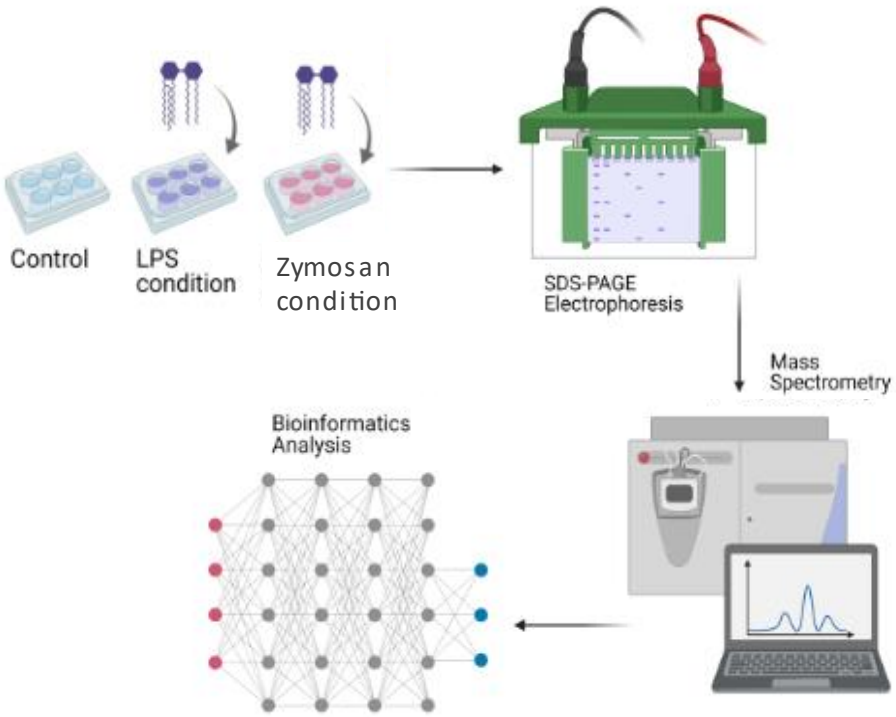


Figure 5: ***Effect of interferon gamma priming on caspase-4 activation.*** *Panel a: HeLa cells transfected with LPS and treated with VX-765 or MCC950, with or without prior IFNɣ priming. H3 was included as a loading control. Panel b: same experimental set up as previously stated, in HIEC-6 cells. HeLa cells showed a reduction in full-length (FL) caspase-4 band in IFNɣ primed LPS transfected cells, while HIEC-6 showed a complete disappearance. MCC950 also caused a complete disappearance of full-length caspase-4 in HIEC-6 cells only. Images include the space the cleaved caspase-4 fragment would be if it was visible, labelled 'cleaved (not visible). The above data are all in the cellular extract, no bands were detected in the cellular supernatant in these experiments. Panel c: LDH release in HIEC-6 cells with the same treatment as previously stated. Bars labelled 100% refer to the lysed control cells, VX refers to VX-765 treatment, 950 to MCC950 treatment.*

Identification of novel inflammatory caspase substrates:

After studying the activation of caspase-4 in multiple different cell lines, the next step was to identify possible substrates. Proteomic analysis was done in HIEC-6 cells treated with either LPS to activate the non-canonical inflammasome, or zymosan to induce the NLRP3 canonical inflammasome (Joly and Sutterwala, 2010). A schematic of this process is shown in figure 6a. The experimental process of mass spectrometry was carried out by Dr Adam Dowle from the Biology Technology facility at the University of York, then returned to our lab for analysis. From this data, potential substrates were analysed based on their cleavage stimuli, LPS or zymosan. The substrates that presented interesting cleavage patterns were then further analysed with the use of STRING. This allowed the visualisation of the pathways each potential substrate was known to be involved with, as shown in figure 6b. A list of potential substrates was developed with a focus on proteins involved with RNA binding, as this mechanism encompassed multiple substrates detected via mass spectrophotometry.

6a.



6b.

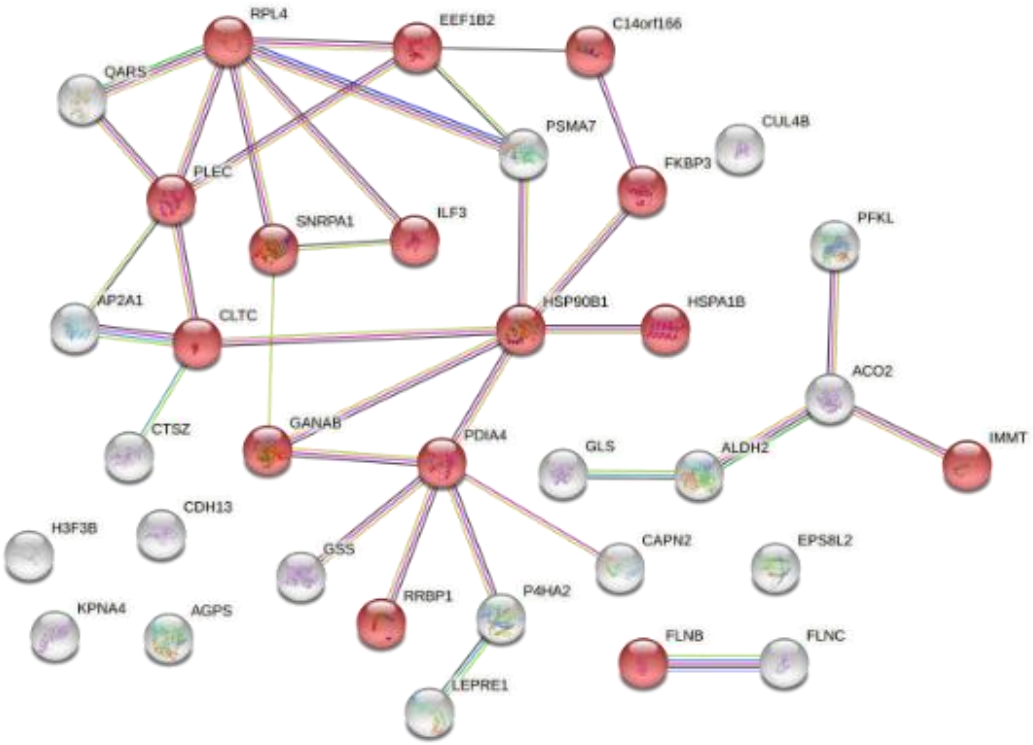


Figure 6: ***Proteomic analysis to identify novel inflammatory caspase substrates.*** *Panel 7a: a schematic of the process of generating the proteomic data. Panel 7b: STRING network of all potential substrates identified in LPS transfected samples (Szklarczyk et al., 2021). Substrates highlighted in red are all proteins that have been previously shown to be involved in RNA binding.*

IL-F3 as a novel inflammatory caspase substrate:

Proteomic analysis provided a list of potential inflammatory caspase substrates. After further analysis, interleukin enhancer binding factor 3 was the first substrate to be tested. An IL-F3 antibody was purchased from ProteinTech and used at a concentration of 1in2000. Cells were transfected with LPS to induce the non-canonical inflammasome and activate caspase-4.

In HeLa cells primed with IFNɣ, the cleaved IL-F3 fragment was detected when the cells were transfected with LPS, but not visible in the untreated samples. The cleaved fragment was also visible in the un-primed samples, but to a lesser extent. The addition of VX-765 reduced the level of the cleaved fragment, however this did not completely prevent cleavage. It is possible that the cleavage of IL-F3 in the presence of VX-765 was due to caspase-5, as this would not be inhibited by VX-765 which is specific to caspase-1 and caspase-4 only. In HIEC-6 cells, although the active fragment wasn't visible, there was a disappearance of the full-length band when transfected with LPS. VX-765 prevented this disappearance, suggesting IL-F3 cleavage was blocked by VX-765 in this cell type. MCC950 had no inhibitory effect on the cleavage of IL-F3 in either HeLa cells or in HIEC-6 cells when transfected with LPS, highlighting that this substrate was not NLRP3/caspase-1-dependent.

This experiment was also performed in THP-1 cells, however again these results provided conflicting data when repeated so have not been included as we could not provide conclusive results in this cell line.

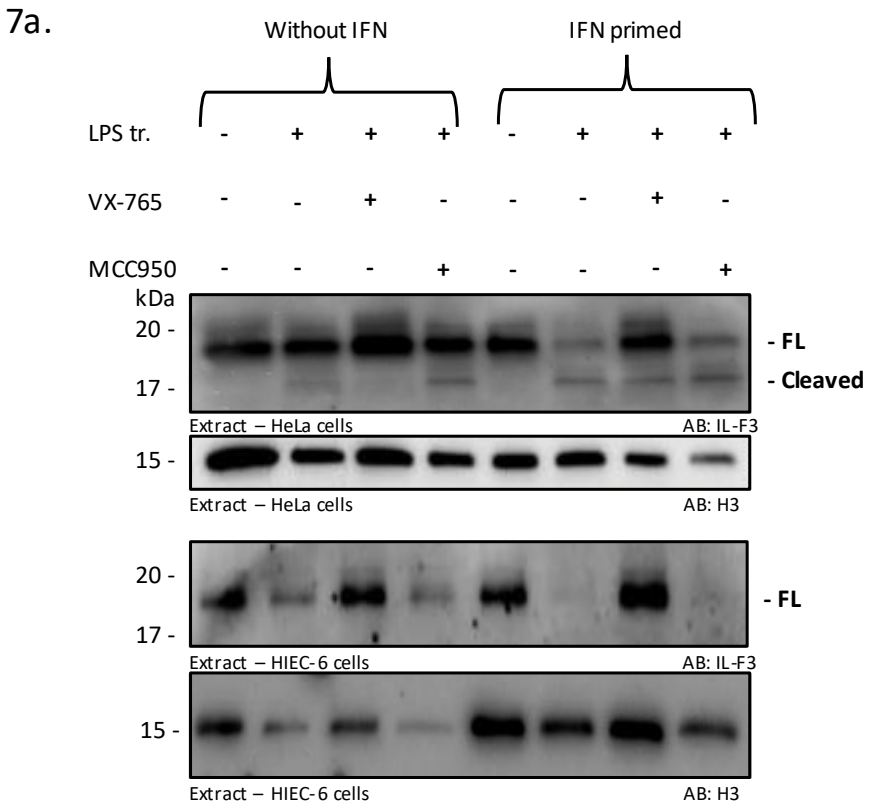


Figure 7: ***IL-F3 as an inflammatory caspase substrate.*** *IL-F3 immunoblots in two different cell types. In HeLa cells (top), IL-F3 cleaved fragment was visible in both primed and un-primed cells when transfected with LPS, labelled cleaved. The addition of VX-765 reduced the cleaved product but did not completely inhibit cleavage. The disappearance of full-length (FL) IL-F3 in HIEC-6 cells transfected with LPS suggests cleavage and was blocked by VX-765 both in primed and un-primed cells. MCC950 had no inhibitory effect in HIEC-6 cells. H3 was used as a loading control for both cell types*

Discussion

Since the discovery of the inflammasome by Martinon, Burns and Tschopp in 2002, inflammasomes have been implicated in a wide range of diseases (Martinon et al., 2002). The inhibition of caspases and GSDMD, both directly and indirectly, presents a new approach for therapeutics in inflammatory diseases. Part of this study included testing known caspase and GSDMD inhibitors to further understand inflammasomes. Despite their involvement in a multitude of inflammatory diseases, the substrates targeted by inflammatory caspases activated by inflammasomes have not been well outlined compared to their apoptotic counterparts. Here we identified multiple potential substrates via proteomics, highlighting IL-F3 as a promising target for further investigation.

Inhibition of caspase activation and subsequent substrate cleavage is a topic of investigation. Currently most inflammatory caspase inhibitors are poorly characterised, however further studies into potential caspase inhibitors could provide treatment to a significant number of diseases including cancers and sepsis. The caspase inhibitor VX-765 was taken to clinical trial after it showed promising results in the inhibition of caspase-1, and therefore inhibition of IL-1β and IL-18 release, both in monocytes *in vitro*, and in animal models of inflammatory and skin diseases *in vivo* (Wannamaker et al., 2007; Yang et al., 2017). This inhibition was seen throughout this project. Despite its success in animal models, multiple clinical trials involving VX-765 have been terminated and there has been no recent reports of VX-765 after it was found to induce hepatic toxicity in animals after long-term exposure (Zahid et al., 2019). MCC950 is a direct NLRP3 inhibitor that was shown by Coll *et al.* to work both *in vivo* in multiple species disease models, and *ex vivo* in human cells (Coll et al., 2015). MCC950 has been shown to work by directly interacting with the NACHT domain's Walker B motif of NLRP3 in mouse bone marrow-derived macrophages, inhibiting hydrolysis of ATP and therefore NLRP3 formation (Coll et al., 2019). This inhibitor was used as a control to successfully block NLRP3 and therefore caspase-4 activation throughout this project in the THP-1 cell line stimulated with PMA to mimic 'macrophage-like' cells.

While VX-765 and MCC950 were consistent throughout this work, other inhibitors used during this project were less reliable. Disulfiram has been previously reported to inhibit pyroptosis by preventing GSDMD pore-formation (Hu et al., 2020), however the level of LDH released when treated with disulfiram in this project did not suggest that pyroptosis was reduced significantly compared to controls. In this study, the results seem to suggest that disulfiram induced toxicity in cells. This has been suggested in various other contexts, including Lyme disease and alcohol dependence during clinical trial, in which titration of disulfiram was used to optimise and overcome this (Gao et al., 2020; Petersen, 1992). This could explain the toxicity and increased LDH release of cells treated with disulfiram; this was concluded not to be within the scope of this project but is something that could be studied further.

Necrosulfonamide (NSA) has been reported to directly bind to GSDMD to inhibit N-terminal oligomerisation and therefore pore formation (Rathkey et al., 2018), however again this inhibitor had mixed results when used on THP-1 cells. When treated with LPS, NSA blocked GSDMD p30 cleavage, alongside LDH and IL-1β release, however when treated with nigericin the results were less clear. We found that NSA blocked caspase-1 cleavage in THP-1 nigericin-treated cells but did not block the cleavage of GSDMD. NSA also significantly reduced the release of IL-1β from our THP-1 cells but did not significantly reduce the level of LDH released from the cells. This suggests that the cells were still undergoing GSDMD-induced cell death, despite a lack of IL-1β release. This would have to be studied further to be explained as this was not the expected outcome of this experiment. Ebselen and dimethyl fumarate both provided mixed results throughout the experiments and would have to be studied in more detail before a definite answer could be provided for the efficiency as inflammasome inhibitors.

Detection of caspase-4 in both monocytic and epithelial cell lines proved difficult throughout this work and required a significant amount of optimisation. Optimisation was done in the monocytic THP-1 cell line. This first involved comparing two different protein precipitation techniques. The methanol and chloroform method of protein precipitation was used throughout this project, however when difficulties with caspase-4 detection in THP-1 occurred the acetone method was suggested. The acetone method has previously been shown to yield a higher protein precipitation efficiency with a lower loss of protein when compared to the methanol and chloroform method in a comparative study in the context of rat brain structures (Fic et al., 2010). The acetone method also keeps both the cellular extract and supernatant together, which was thought to provide optimum caspase-4 detection with minimum protein loss. When compared however, the acetone method of precipitation did not allow for clear caspase-4 detection, so it was not used further. The second stage of optimisation was the antibody itself; it was thought that a different brand of antibody may allow for clearer detection of cleaved caspase-4, however the SantaCruz antibody produced no clear bands, even full-length caspase-4. We then compared the priming effect of IFNɣ in both HIEC-6 and HeLa cells, in which we found that priming allowed clearer visualisation of the full-length caspase-4, however we were still unable to clearly detect the cleaved fragment. The difficulty to detect caspase-4 was seen throughout both our lab, and in other studies. For example, Lagrange *et al.,* found that they could not detect pro-caspase-4 in the supernatant of their monocytic human myeloid leukaemia cell line, U937 (Lagrange et al., 2018). They overcame this by overexpression of 3xFLAG caspase-4, which allowed for the detection of processed caspase-4 in the supernatant, shown in their supplementary data. Due to time restraints this would not have been possible in this project, however this could be considered if problems persist with caspase-4 detection in further studies.

The identification of inflammatory caspase substrates could provide potential for the development of new therapeutic options for a wide range of diseases, as well as provide further insight into inflammasomes roles within the body and disease. After extensive analysis of proteomic data retrieved from HIEC-6 cells treated with LPS to

stimulate the non-canonical inflammasome, and Zymogen to stimulate the canonical inflammasome, a list of potential substrates was developed. Due to time-restrictions, only one of these substrates was validated via Western blot, interleukin enhancer binding factor 3 (IL-F3). IL-F3 (also known as NF90/NF110) encodes a double stranded RNA binding protein that regulates transcription, translation, mRNA stability, and microRNA processing (Li et al., 2020). The gene expression regulatory properties of IL-F3 have linked this protein to multiple different cellular functions depending on the RNA it binds too. IL-F3 is a ubiquitous protein expressed in animal organisms, but not in eubacteria, archaea, or plants; this protein has been recovered from the nucleus and cytosol of mammalian cells and are able to travel freely between these spaces (Castella et al., 2015), which suggests that this protein is available for caspase cleavage. Currently, IL-F3 is most commonly associated with its role in the cellular anti-viral response within the innate immune system, through promoting the translation of multiple antiviral proteins including type I interferons (IFNs), which are vital in the prevention of viral replication (Watson et al., 2019). Abnormal expression of IL-F3 has also been linked to a number of malignancies through tumour proliferation, invasion, and metastasis (Liu et al., 2019). For example, it has been shown *in vivo* in hepatocellular carcinoma that knockdown of IL-F3 lowers mRNA and protein levels of cyclin E1, delaying cell-cycle progression and proliferation, therefore reducing tumorigenic capacity of cells (Jiang et al., 2015). Finally, it has also been shown that IL-F3 is vital for development, as IL-F3 -/- mice suffered fatal neuromuscular respiratory failure within 12 hours of being born. Despite the wide range of research into multiple functions of IL-F3, there is little to no literature available on potential links of inflammatory caspases to IL-F3. This was outlined as a promising inflammatory caspase substrate as it was cleaved in HIEC-6 cells when stimulated with both LPS, and with zymogen, however there was not time to further validate this protein, or to study the precise function of this protein within the context of the inflammasome.

Originally, the specific protein-protein interactions involved in caspase-substrate binding were going to be studied in more detail. The use of the Split-TEV system was going to be used to study this aspect of the project. This method utilises the tobacco etch virus (TEV), split into two fragments which are fused to the proteins predicted to interact. If there is an interaction between the proteins of interest, the TEV protease is able to reform its proteolytic activity, cleaving either fluorescent or luminescent reporters (Alonso-Gardón and Estévez, 2021; Wehr et al., 2008, 2006). This was originally suggested to study caspase-substrate binding as it is a highly specific assay that would allow the validation of individual novel substrates, and has been previously shown to work in apoptotic caspases (Gray et al., 2010). However, the COVID-19 pandemic and subsequently reduced laboratory time available, paired with the additional optimisation involved with caspase-4 detection that was not originally planned, it was not possible to develop this system in the context of inflammatory caspase-substrate binding. Primers were designed to incorporate the TEV and caspase binding sites, but this was as far as this progressed regarding this element of the project. The future of this project would involve first developing the TEV system using known substrates GSDMD and IL-1β, before applying this system

to novel substrates identified by proteomic and Western blot analysis. While not developing this system is a limitation of this project, as this would have provided clear information on the interactions between caspases and their substrates, it lays the foundations for the group to study this further in the future.

While this study was done in the context of monocytic THP-1 cells, as well as HeLa, HIEC-6 and HACAT epithelial cell lines, it was not possible to do primary cell work in this timeframe. This is a limitation of this study, as despite having both biological and technical replicates, often cell lines produced conflicting results between repeats. Given more time, the results of this study would have been validated further in primary monocytes.

Conclusion

The study of inflammasomes is an emerging field of research that has made significant progress since its discovery in the early 2000s. Recent studies have highlighted the important of this homeostatic mechanism within the body, including proliferation, metabolism, and cellular repair. Inflammasomes are essential in the host response to pathogens, however dysregulation of this mechanism can result in the development of inflammatory diseases. The discovery of inhibitors such as MCC950 have vastly progress the understanding of inflammasomes, however there is still significant research to be done before other inhibitors become readily available. The identification and characterisation of novel inflammatory caspase substrates could open significant opportunity for the further understanding of inflammasomes. GSDMD and IL-1β have already provided the basis for a multitude of studies both into treatment and further understanding of inflammatory caspases. This work highlights IL-F3 as a potential inflammatory caspase substrate through Western blot, providing the foundation for further validation via the TEV system once developed. The discovery of inflammatory caspase substrates provides the targets for novel therapies for diseases related to inflammasomes, including inflammatory diseases and cancers, and is a significant pathway to be followed in the field of inflammasome research.

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