Caspase-7, an inflammatory executioner

Timur Ozdenya

MSc By Research
University of York
Department of Biology
January 2024

Abstract

Caspase's functions in multicellular organisms are critical to homeostasis. Classically, caspase's functions have been organised based on cellular mechanisms they control, mainly apoptosis and inflammation. In recent years, evidence has suggested that apoptotic and inflammatory pathway's components have overlapping functions. One of these inflammatory pathways, the non-canonical inflammasome, activates caspases-4 in responses to intracellular infections, leading to cell death. Our understanding of the non-canonical downstream signalling is limited. In this project, we investigated the interplay between the non-canonical inflammasome and apoptotic caspases. We generated a comprehensive examination of the activation of Caspase-7 by Caspase-4, which expands from its established role in apoptosis. Our findings reveal that Caspase-7, typically regarded as a mediator of apoptotic cell death, is activated during inflammatory responses to infection, hence challenging the previously established understanding of its role. We show that Caspase-4 can directly cleave and activate caspase-7 within flexible linkers suggesting a specialised function for caspase-7 in regulating inflammatory processes. This activation may play a role in a subtle and detailed control of inflammation, which could lead to the identification of new targets for treating inflammatory illnesses. This study not only enhances our understanding of the function of Caspase7 in cellular processes but also presents novel opportunities for medical therapies in infections and illnesses associated with inflammation.

Author 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 this, or any other, University. All sources are acknowledged as References.

Introduction

Caspases are a family of aspartate-specific cysteine proteases [1].These signalling proteases cleaved specific proteins that define their functions. Due to their key cellular functions, caspases play significant roles in various physiologically crucial homeostatic processes in multicellular organisms, for example programmed cell death (apoptosis and pyroptosis), inflammation and cell differentiation [2]. Classically, the caspase family has been subdivided into 2 general classes: the apoptotic and the inflammatory caspases. Apoptotic caspases are themselves subdivided into two categories, the initiator caspases (caspase-2, 8,9,-10) which integrate the apoptotic cues and the executioner caspases (caspase-3,-6, -7), which execute cellular dismantling associated with apoptosis. Initiator caspases are expressed as monomers whereas executioner caspases are dimers. Initiator apoptotic caspases can be activated through numerous pathways but will not be reviewed here [3]. Apoptotic caspases are essential to life and deletion of numerous members of the family is embryonically lethal [4][5]. Caspase-3 and caspase-7 are believed to be partially functionally redundant and share many substrates [6][7]. Inflammatory caspases (caspase-1,-4,-5) are activated by large signalling platforms called inflammasomes[8]. Inflammatory caspases are expressed as monomers. Their activation leads to two critical cellular outcomes: the maturation and secretion of pro-inflammatory cytokines [9] and the execution of a pro-inflammatory form of cell death called pyroptosis [10].

Activation mechanism

As their activity needs to be tightly regulated, caspase are produced as inactive zymogens. In response to certain stimuli, caspases can activate and gain proteolytic activity to cleave protein substrates, leading to cellular signalling. Caspases are structurally organised into an N-terminal domain and a proteolytic domain located in C-terminal. The N-terminal domain of initiator and inflammatory caspases is used to recruit them on their activating platform. The catalytic domain of caspases is divided into a large and a small subunit. The large subunit contains the catalytic dyad (Cysteine and Histidine) whereas the small subunit contains most of the elements required for the formation of the substrate binding pocket and the dimerisation interface. Caspase activation required the formation of a dimer from two monomers. As inflammatory and initiator caspases are monomeric, their dimerization on their respective signalling platform triggers their activation. Upon dimerisation, these caspases undergo subsequent self-processing to generate a fully active and mature caspase. Executioner caspases are generated as a dimeric zymogen and require cleavage by initiator caspases for catalytic activity [1].

Caspase activity

Caspase recognize and define tetrapeptides on their substrates [11] (including self-cleavage). These sequences are cleaved within a flexible loop after an aspartic acid but also after glutamic acid and phosphorylated serine (Fig1C) [2][12]. Generally, the amino acid succeeding the cleavage site is small and aliphatic (e.g., serine, alanine) Although proteomic studies have investigated these substrates, most caspase substrate's function remain uncharacterised [13][14][15].

Caspase-7

Recent research has shown a compelling dynamic between Caspase-3 and Caspase-7, showing their redundant roles as well as distinct contributions within apoptotic pathways. Both executioner caspases, when activated in the apoptosis cascade, have been the subject of investigations that have shown subtle distinctions in their roles and the outcomes resulting from their absence.

Caspase-7 is frequently seen as redundant to Caspase-3, given to their comparable structures and overlapping functions. It has been demonstrated that Caspase-7 may effectively substitute for Caspase-3 in specific instances of apoptosis when the latter is absent. In support of this, caspase-7
deletion in mice results in minor developmental defects. Interestingly, caspase-7 deletion has been shown to be protected from endotoxic shock [25]. One example, an investigation conducted on Chinese hamster ovary (CHO) cells lacking Caspase-7 revealed a decrease in the rate of cell proliferation, accompanied by an elevation in the production of recombinant proteins. This outcome was attributed to the arrest of the cell cycle in the G2/M phase. It is noteworthy that the lack of Caspase-7 resulted in a notable amplification in caspase-3 activity, indicating the presence of a compensation mechanism within the apoptotic caspase cascade [26]. Importantly, deletion of caspase-3 is embryonically lethal on most genetic backgrounds, leading to the assumption that caspase-3 is functionally redundant to caspase-7 but more important [5].

In contrast, Caspase-7 exerts distinct regulatory control over certain physiological responses, irrespective of the involvement of Caspase-3. A study conducted on pulmonary microvascular endothelial cells infected with *Pseudomonas aeruginosa* unveiled a noteworthy occurrence in which the infection prompted the extracellular accumulation of active Caspase-7. This accumulation occurred independently of Caspase-3 and the process of apoptosis. The extracellular Caspase-7 has been observed to regulate transmissible cytotoxicity, suggesting a distinct role beyond its established intracellular actions [27]. Similarly, caspase-7 activity is also important in protecting against gram positive infection by bacteria such as *Listeria.* Caspase-7 is important to control inflammatory death, in a mechanism likely involving the Acid sphingomyelinase (ASM)[28][29]. In contrast, Caspase-7 exerts distinct regulatory control over certain physiological responses, irrespective of the involvement of Caspase-3. A study conducted on pulmonary microvascular endothelial cells infected with *Pseudomonas aeruginosa* unveiled a noteworthy occurrence in which the infection prompted the extracellular accumulation of active Caspase-7. This accumulation occurred independently of Caspase-3 and the process of apoptosis. The extracellular Caspase-7 has been observed to regulate transmissible cytotoxicity, suggesting a distinct role beyond its established intracellular actions [27].Similarly, caspase-7 activity is also important in protecting against gram positive infection by bacteria such as *Listeria*. Caspase-7 is important to control inflammatory death, in a mechanism likely involving the Acid sphingomyelinase (ASM)[28][29]. The study described in the article titled "Absence of Correlation between Caspase Activation and Caspase Activity Assays in MCF-7 Breast Cancer Cells Treated with Paclitaxel" offers valuable insights into the distinct functions of caspase-3 and caspase-7, specifically in relation to programmed cell death in cancerous cells [30]. The MCF-7 cell line, derived from human breast cancer, has a distinct lack of caspase-3, making it an exceptional system for investigating caspase functionality in the absence of any interfering effects from Caspase-3. The findings of this study indicate that the lack of Caspase-3 does not result in a noticeable increase in caspase-7 activity when cells are exposed to paclitaxel, a chemotherapeutic drug. The observed absence of enzymatic action is also evident in the hydrolysis of artificial caspase substrates, namely Leucine-Glutamic

Acid-HistidineAspartic Acid conjugated with 7-amino-4-trifluoromethyl coumarin, Aspartic Acid-Glutamic Acid-
Valine-Aspartic Acid conjugated with 7-amino-4-trifluoromethyl coumarin and Valine-Glutamic Acid-Isoleucine-Aspartic Acid conjugated with 7-amino-4-trifluoromethyl coumarin (LEHD-AFC, DEVDAFC, and VEID-AFC) , as well as in the cleavage of endogenous caspase substrates such as Lamin A, β- catenin, gelsolin, protein kinase Cδ, topoisomerase I, and caspases-6, -8, and -10. It is worth noting that the return of wild-type caspase-3 into MCF-7 cells results in partial restoration of substrate cleavage. However, this reintroduction does not provide measurable activity capable of cleaving synthetic caspase substrates. This observation implies that Caspase-3 performs distinct functions that do not overlap with those of caspase-7. In addition, the aggregation of cleaved caspase-3 in the cytoplasm suggests an intricate control of caspase activity, which might not be well captured by activity tests based on tetrapeptides.
Supporting the view of non-redundant roles for caspase-7 and caspase-3 are the distinct phenotypes of mice deficient in said caspases. While most mice lacking caspase-3 on the 129 genetic background experience prenatal death or perish shortly after birth due to an excessive number of brain cells, mice deficient of caspase-7 on the same genetic background are able to survive [5][31]. These results suggest that caspase-3 and caspase-7 serve distinct roles, nevertheless, a significant challenge in the interpretation of these findings lies in the considerable variation that may occur in the relative expression levels of caspase-3 and caspase- 7 among different mice strains, as well as within specific tissues.

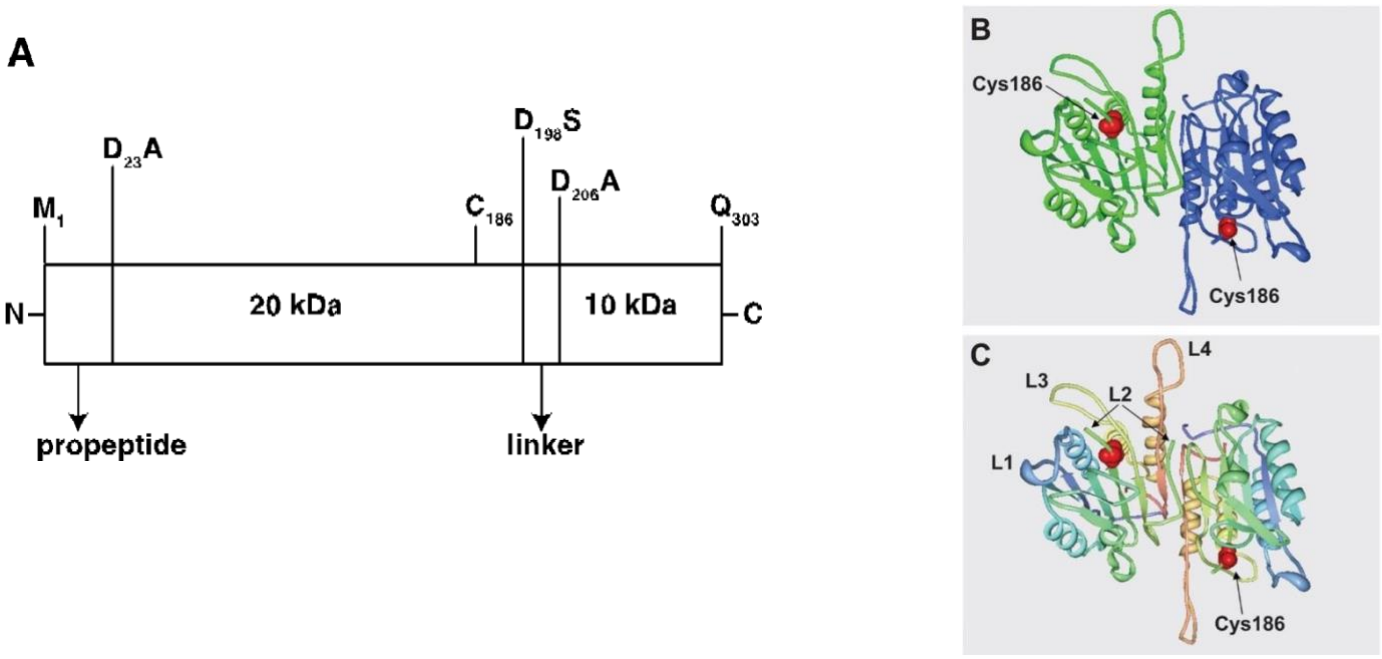

Figure 1. Caspase-7 structural organisation *(A)schematic representation of caspase 7 showing domain organisation and all cleavage sites. (B) Three- dimensional structure of procaspase-7 (PDB:1K88) with the monomers of the homodimer shown in green and blue, respectively. The active site Cys186 is shown in red and space fill. (C) Three-dimensional structure of procaspase-7 (PDB:1K88) with backbone colouring by secondary structure. The positions of the flexible L1-L4 loops, which form the catalytic groove and substrate binding pockets, are indicated. The active site Cys186 is shown in red and space fill. Adapted from: [20].*

Caspase-7 substrates and specificity

Caspase-7 has an optimal substrate peptide recognition sequence of DEVD, exerting its regulatory influence by cleaving a wide array of specific substrates, each with distinct functional consequences [2]. Caspase-7 exhibits a distinct preference for specific substances it acts upon, which is closely related to its structural arrangement at the active site and the methods by which it becomes activated. The preferred substrate for caspase-7 generally is determined by the four critical positions within the peptide chain, P1 to P4, according to the Schechter-Berger nomenclature [32]. The binding pocket of Caspase-7 has a distinct affinity for substrates possessing a DEXD motif at the P4 to P1 locations [33]. In this context, the letter D indicates aspartic acid, E represents glutamic acid, and X represents a variable amino acid. The substrate recognition and processing efficiency of the enzyme are mostly determined by the characteristics of its binding pocket. Although caspase-7 exhibits some substrate preferences similar to other caspases, its different substrate specificity can be attributed to its unique structural characteristics and interactions with substrates or inhibitors. Comprehending these structural intricacies is crucial in the development of specific inhibitors and elucidating the exact function of caspase-7 in the control of cell death.

One of caspase-7's main substrates is poly(ADP-ribose) polymerase 1 (PARP1). When PARP1 is cleaved by caspase-7, it is rendered inactive, which interferes with its vital function in DNA

repair. The apoptotic cell's distinctive DNA fragmentation is caused by this event, which also stops cell survival mechanisms from mending damaged DNA. Caspases use sites located outside their active sites called exosites to provide additional specificity toward substrates, providing a novel drug-targeting interface for the development of more specific inhibitors. These exosites were previously identified by Boucher and colleagues in the Nterminal domain of caspase-7 and were found to improve the cleavage rate of the PARP1 and the hsp90 co-chaperone p23 thus increasing the specificity of the caspase [7]. Key lysine residues (K38KKK) within the N-terminal domain of caspase-7 were identified as critical elements for the efficient proteolysis of PARP1 and p23, and further proved this by expressing caspase-7 lacking these residues which was less- efficient at PARP and p23 cleavage in comparison with cells expressing the wild-type peptidase [7]. The significance of this study is that it questions the prevailing belief that due to having almost identical activity towards certain synthetic peptide substrates, caspase-3 and caspase-7 serve functionally redundant roles within the cellular death mechanism. Even though caspase-7 is intrinsically less active than caspase-3, it is shown to be better at cleaving certain substrates, suggesting a novel role for caspase-7.

Structural proteins found in cells are the targets of caspase-7. For example, during apoptosis, caspase-7 cleaves Lamin A, a part of the nuclear envelope [34]. The dissolution of the nuclear envelope caused by this cleavage aids in nuclear condensation, which is a characteristic feature of apoptotic morphology. Moreover, the actin-binding protein gelsolin is broken down by caspase-7. This cleavage event has a significant effect on the cytoskeleton, causing actin filament disintegration and morphological alterations in the cell that ultimately aid in the cell's disintegration.

Another notable substrate of caspase-7 is RhoGDI, a regulatory protein that plays a crucial role in controlling the activity of Rho GTPases [6]. Rho GTPases are key regulators of cellular processes such as cytoskeletal dynamics, cell motility, and cell adhesion. RhoGDI functions by sequestering inactive Rho GTPases in the cytoplasm, preventing their activation and downstream signalling [35]. Caspase-7 cleaving RhoGDI during apoptosis, would render this inhibitor inactive. The consequences of this cleavage event on cellular and apoptotic mechanisms are profound. The sequestered Rho GTPases are released during RhoGDI cleavage, hence enabling their activation. Subsequently, activated Rho GTPases start signalling cascades that affect cytoskeletal dynamics. RhoGDI cleavage by caspase-7 can result in cytoskeletal rearrangements, such as modifications to actin filament organisation and morphology of the cell. Furthermore, other cellular processes including cell adhesion and migration may be impacted by the release of RhoGTPases from RhoGDI's inhibitory regulation.

Through its interaction with Rho GTPases, ROCK1, a serine/threonine kinase, is essential for controlling the dynamics of the actin cytoskeleton, cell contraction, and cell motility[36]. ROCK1 is cleaved by caspase-7 during apoptosis, which has important ramifications for both the morphology of cells and the course of programmed cell death. The kinase activity of ROCK1 is activated upon cleavage by caspase-7 as caspase-7 cleavage removes an inhibitory domain. Normally, ROCK1 phosphorylates downstream targets to facilitate actinmyosin contraction and cell adhesion, such as myosin light chain (MLC) [36]. The proteolytic cleavage of ROCK1 by Caspase-7 leads to the generation of an active fragment of ROCK1, which facilitates the phosphorylation of myosin light chain (MLC). This phosphorylation event subsequently triggers the assembly of contractile actin-myosin

filaments. This mechanism plays a pivotal role in the development of membrane blebs, a distinctive morphological alteration observed during the execution phase of apoptosis.
Membrane blebbing is a biological phenomenon that contributes to the disintegration of a cell into apoptotic bodies. These entities are then engulfed by neighbouring cells by phagocytosis, therefore preventing the release of intracellular contents and the induction of inflammation. The activation of ROCK1 and the accompanying cellular alterations are integral components of the systematic and tightly controlled apoptosis. The involvement of Caspase-7 in the cleavage of ROCK1 contributes to the physical reconstruction of a deteriorating cell, hence preventing the initiation of an inflammatory response during apoptosis and preserving tissue homeostasis. Caspase-activated DNase or CAD, a protein sometimes referred to as DFF45 (DNA fragmentation factor 45 kDa subunit), is essential for preventing the deterioration of cellular DNA. ICAD prevents the endonuclease CAD (caspase-activated DNase) from cleaving genomic DNA into nucleosomal fragments during apoptosis when it is active [37]. Caspase 7 targets ICAD for cleavage during apoptosis. The release of CAD from its inhibitory complex and the deactivation of ICAD are the outcomes of this cleavage event. After being released from ICAD's inhibition, CAD enters the nucleus and becomes active [37]. The hallmark of apoptotic DNA fragmentation, nucleosomal fragments are created when active CAD cleaves genomic DNA. By ensuring that the DNA inside the apoptotic cell is broken down into smaller, more manageable pieces, this process helps to stop inflammatory reactions from occurring when the DNA is released into the extracellular environment.

An essential enzyme in the control of sphingolipid metabolism and the conversion of sphingomyelin to ceramide is called acid sphingomyelinase, or ASM. Ceramide, in turn, is important for cellular stress responses, apoptosis, and signalling in cells. It has been documented that Caspase-7 affects ceramide synthesis and ASM activity via cleaving ASM during apoptosis [28]. The cleavage of ASM by caspase-7 has the potential to affect ceramide levels and sphingolipid metabolism in cells. Ceramide is a bioactive lipid that takes part in several different biological functions, such as programmed cell death. Elevated levels of ceramides have the potential to stimulate pro-apoptotic signalling pathways, mitochondrial malfunction, and caspase activation, all of which are factors that contribute to the programme of apoptosis. Thus, caspase-7's cleavage of ASM establishes a connection between caspase7 activation and sphingolipid metabolism, emphasising the complex interplay between lipid signalling and the control of apoptosis. Elevated levels of ceramide, triggered by the cleavage and activation of ASM by caspase-7 have been shown to maintain cellular integrity by opposing the formation of gasdermin D pores via enhancing membrane repair [26]. This provides more time for the extrusion of intestinal epithelial cells (IECs), which is a consequence of pyroptosis [38]. Additionally, findings from this same study demonstrate that the cleavage of caspase-7 and ASM is necessary for the elimination of *Chromobacterium violaceum* and *Listeria monocytogene*s after the perforin pore-induced attack by natural killer cells or cytotoxic T lymphocytes. Caspase-7 acting as a “death facilitator that delays poredriven lysis” is yet another example of it not carrying out a typical executioner’s role [28].

Cell Death

The growth and maintenance of multicellular organisms rely not only on controlled cell proliferation but also on the elimination of cells that are no longer necessary or may constitute a risk to the organism. This encompasses the elimination of cells that are at risk of undergoing neoplastic transformation or have been adopted by microorganisms for the purpose of pathogen proliferation. Programmed cell death (PCD) serves as the principal mechanism by which the organism orchestrates the removal of these cells [39][40]. Cell death can be initiated by developmental programmes and stress-induced signals that activate proteins located in the cell membrane and cytoplasm. These proteins then trigger a complex series of transcriptional alterations and post-translational modifications, ultimately leading to cell death. Apoptosis is the most recognized form of PCD, characterised by the systematic disassembly and typically immunologically silent removal of cells. In contrast, pyroptosis and necroptosis are more violent forms of cell death, marked by the rupture of the cell membrane, leading to the release of potent inflammatory inducers such as cytokines and alarmins. This release is particularly significant in infected cells, where it includes the expulsion of pathogens and their components, thereby alerting and activating the immune system to the presence of danger.

Apoptosis is a form of inflammation-free, silent programmed cell death, that eliminates individual cells within an organism while preserving the structure of surrounding tissue, primarily driven by caspase action [41]. Initiation of apoptosis occurs via either cell- intrinsic or cell-extrinsic pathways [42]. The cell death signal for the extrinsic pathway emanates at the cell surface/plasma membrane, at which a cell surface transmembrane death receptor is activated via the binding of its complimentary ligand (e.g., Fas by FasL/Fas ligand), inducing oligomerization of the receptor. As a result, clustering of proteins that bind to the intracellular domain of the receptor, for example FADD (Fas-associated death domain) is promoted. FADD recruits pro-caspase -8/-10 via interactions between its death effector domains (DED) and the caspase respectively, binding its pro-domain to form the death induced signalling complex (DISC), which catalyses caspase dimerization and activation [42]. The now active caspase 8/-10 can induce cell death by either direct cleaving of effector caspases -3/-7 or indirectly via proteolytic activation of BH-3 only proteins (pro- apoptotic members of the BCL-2 family), thus engaging the intrinsic pathway [2].

The intrinsic pathway can be triggered by numerous stresses (e.g., DNA damage or growth factor deficiency), involves transcriptional and/or post translational increases of pro-apoptotic BH3-only proteins that bind with high affinity to members of the pro-survival BCL-2 protein family [41]. The BCL-2 proteins maintain critical effectors of apoptosis, BAK and BAX in their inactive states however binding of BH3-only proteins to all the BCL-2 proteins neutralises the BCL-2 proteins and triggers the release of BAK and BAX. The now liberated BAK and BAX are free to assemble into complexes that cause a breach in the outer mitochondrial membrane, thus promoting mitochondrial outer membrane permeabilization (MOMP) and subsequent release of various mitochondrial proteins, notably cytochrome c which binds to apoptotic peptidase-activating factor 1 (APAF-1); forming the heptameric backbone of the apoptosome complex. Caspase -9 is recruited to and activated at this newly formed complex via dimerisation and cleaving, in turn activating downstream effector caspases (-3,-6,-7) [41]. The characteristic hallmarks of apoptosis, including as nuclear condensation, phosphatidylserine exposure and genomic DNA fragmentation, are produced as a result of mature caspases-3 and -7 cleaving a broad range of substrates.

Unlike apoptosis, pyroptosis is an inflammatory, lytic form of cell death. Pyroptosis is dependent on and executed by members of the gasdermin family [10]. Gasdermin D, one of its members, is cleaved by inflammatory caspases. There are two distinct yet complementary mechanisms regulating inflammatory caspase activation; canonical (caspase-1 dependant), or non-canonical (caspase-4/5 dependent) inflammasomes[31].In the canonical inflammasome (CI), Pattern recognition receptors (PRR) sense damage-associated molecular markers (DAMPs), pathogen-associated molecular markers (PAMPs), or homeostatic alteration which can stimulate defined PRR such as the NLR (nucleotide-binding domain and leucine-rich repeat containing) family receptors, for example NLRP3 (NLR family pyrin domain-containing 3) or NLRC4 (NLR family CARD domain-containing).[43]. Following PRR sensing, activation and oligomerization, the adaptor protein ASC is recruited, forming the inflammasome complex, which in turn recruit's caspase-1 monomers, increasing local caspase-1 concentration and thereby caspase-1 dimerization and activation. Active caspase1 cleaves several targets, with gasdermin D (GSDMD) being essential for pyroptosis, being cleaved into an N-terminal and C-terminal fragment. The now free N-terminal GSDMD fragments are recruited to the cell membrane, where they form transmembrane pores that result in potassium efflux and water influx, weakening the plasma membrane and rupturing the cell [39]. The process of terminal membrane rupture occurring downstream of GSDMD pores has been demonstrated to be facilitated by the membrane protein NINJ1 [44]

The non-canonical inflammasome (NCI) activates caspase-4 and -5 (caspase-11 in mice) upon lipopolysaccharide (LPS) recognition directly from Gram-negative bacteria in the cytoplasm via their caspase activation domain receptor (CARD) [18]. The process of presenting hydrophobic bacterial LPS to the caspases is facilitated by Interferon- inducible guanylate binding proteins (GBPs). These GBPs possess the ability to recognize charges within the outer region of LPS on cytosolic bacteria, so facilitating the construction of an inflammasome directly on the bacterial surface [34]. These caspases now act in a similar manner to caspase-1 in the canonical pathway, cleaving GSDMD and beginning the cascade of events which ultimately lead to the cell rupturing [39]. This also contributes to the maturation and secretion of inflammatory cytokines (IL-1B, IL-18) [40][6].

In addition to being activated during apoptosis, caspase-7 proteolytic maturation has also been observed under inflammatory circumstances. In contrast to the mechanism by which caspase8 and -9 protein complexes involved in apoptosis activate caspase- 7, macrophages stimulated with lipopolysaccharides (LPS) and ATP or infected with the Gram-negative pathogens *Salmonella typhimurium* and *Legionella pneumophila* can also achieve this activation via employment of the inflammasome complex [25]. Biochemical studies demonstrated that caspase-7 is a direct substrate of caspase-1 with cleavage occurring after the canonical activation sites Asp23 and Asp198 [20], however caspase-7 was not previously shown to have a defined role within the non- canonical inflammasome. My study demonstrates that caspase-7 is a direct substrate of caspase-4.

A novel role for caspase-7

Caspase-7 functioning as a downstream effector of caspase-4 in the context of inflammation implies that, akin to the apoptotic route, the NCI pathway employs downstream proteases to mediate subsequent signalling events. Possessing the ability to finely modulate inflammatory signals within the human intestinal epithelium by the precise cleavage of specific protein substrates suggests caspase-7, an inflammatory executioner. The activation of the NCI in most cells leads to the induction of pyroptosis, which involves the cleavage of GSDMD and is followed by well-documented alterations in cellular shape and loss of membrane integrity. Caspase-7 has been shown to cleave GSDMD as well as oppose the formation of GSDMD pores via cleavage of ASM [28] further demonstrating not only its versatility but further roles it could play within inflammation. The intricate and precise cleavage of substrates, as well as its dual role in regulating pyroptosis, suggests that caspase-7 may have significant influence in controlling inflammation in the human intestinal epithelium. Comprehending the mechanisms and consequences of this cleavage process is crucial for elucidating the complexities of the non-canonical inflammasome pathway and its function in preserving cellular homeostasis when confronted with inflammatory stimuli. Additional investigation has the potential to provide new treatment targets for disorders characterised by disrupted inflammation in the intestinal epithelium and likely in other cell types.

Hypothesis

I hypothesise that caspase-7 is specifically activated as a result of the non-canonical inflammasome pathway initiated by caspase-4, deviating from its conventional activation methods (apoptosis). This alternate activation pathway leads to specific substrate selectivity and functional consequences, mainly affecting inflammation and cellular death. The objective of my study is to clarify the different mechanisms involved in the activity of caspase-7 resulting from non-canonical inflammasome activation.

In order to test this hypothesis, I focused on determining the exact locations where caspase4 triggers the activation of caspase-7, as well as the following impacts of these cleavage events on cellular activities. I employed site-directed mutagenesis to create mutant forms of caspase-7 that contain targeted mutations at critical cleavage sites, specifically Asp23, Asp198, and Asp206. The selection of these residues was chosen as they play a crucial role in the conversion of caspase-7 to its active homodimer state. Replacing Aspartate with Alanine at these specific locations prevents self-cleavage, effectively isolating the impact of external caspase activity. In addition, to prevent self-activation and focus on the modifications induced by caspase-4, a catalytic mutant of caspase-7 (C285A) was employed as the reference point for the tests. This method enables a regulated investigation of caspase-7 activation, offering valuable understanding of its involvement in non-canonical inflammasome pathways.

Results

To study how caspase-4 activates caspase-7, I started by generating a stock of recombinant caspase-4. Caspase-4 was expressed in *E.coli* BL21 pLyS overnight at 21 ° C and purified as described [45]. Caspase-4 was expressed from 4 litres of bacteria and produced proteins that were not efficiently observed on gel upon Coomassie staining (data not shown). This is likely due to the low level of expression and lack of sensitivity for the Coomassie staining used. Caspase-4 has been shown to be toxic and expressed at low levels in *E.coli* (Boucher lab, personal communication). For accuracy, I performed an active site-titration to confirm the presence of active caspases, as described previously [45]. Caspase-4 was efficiently titered (Fig2) using zVAD-fmk. The titration shows a complete inhibition curve (Fig2A) as displayed for the absence of caspase activity when the inhibition (zVAD-fmk) was above 100nM. The linear section of the curve (Fig2B) was used to determine a titer of 2.21µM. The samples were aliquoted and frozen to be used for subsequent *in vitro* experiments. As caspase-4 purification (Fig 3)(Fig 5) was not as clean as caspase-7, I chose western blot to measure caspase-7 cleavage using an antibody against caspase-7 to reduce ambiguity.

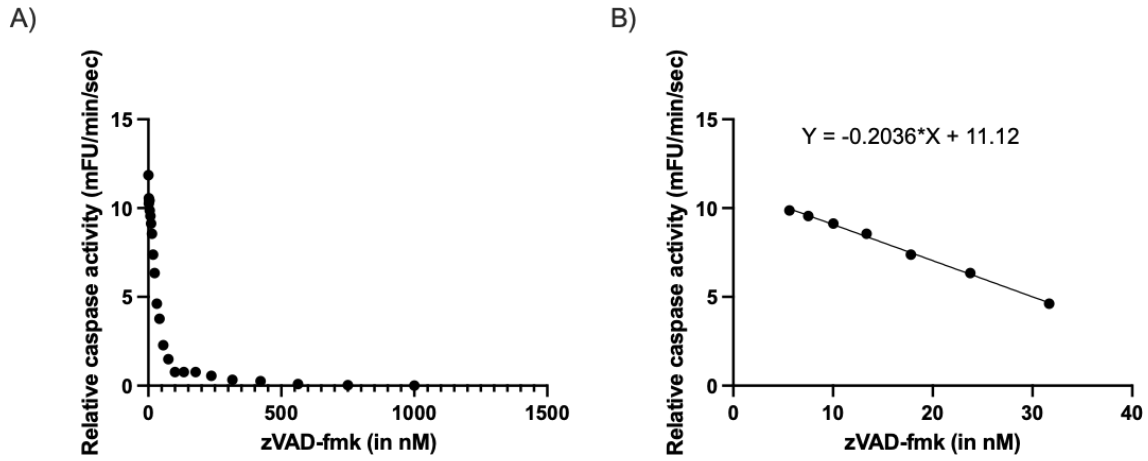

Figure 2: Active site titration of caspase-4. *Recombinant caspase-4 produced was active site titrated using the pan-caspase inhibitor zVAD-fmk in high salt caspase buffer. A) Full curve of inhibition B) Linear region used to determine the titer. The titer corresponds to the concentration of inhibitor (zVAD-fmk) at which the caspase activity was null, following extrapolation of the linear region. A titer of 54.62 nM (before dilution corrections) was calculated with an r2=0.99 goodness value.*

During apoptosis, initiator caspases (e.g., Caspase-8) cleave the executioner caspase-3, -6 and -7 [1]. To test whether caspase-4 has the ability to cleave these proteins, I developed an in vitro assay. I incubated decreasing concentrations of the recombinant caspase-4 I expressed and titrated (Fig2), with catalytic mutants (C285A) of human caspase-3, -6 and -7 expressed in *E.coli*. Caspase-4 and its putative substrate (Caspase-3, -6 and -7) were incubated at 37 ℃ for 2 hrs before being resolves on a 12% SDS_PAGE(Sodium dodecyl-

sulfate polyacrylamide gel electrophoresis), transferred on membranes and probed for the substrates with their respective caspases. If caspase-3, -6 and -7 are cleaved by caspase-4, I would expect the generation of a cleaved fragment of about 20
kDa. From this experiment, I observed that caspase-3 was minimally cleaved by caspase-4 whereas caspase-6 was not cleaved at all. When analysing the caspase-7 reaction, I found that caspase-4 was cleaving it efficiently. As only caspase-4 and caspase-7 (catalytic mutant) are present in my essay, I can conclude that caspase-7 is a direct substrate of caspase-4. Therefore, I chose to continue my project investigating caspase-7 cleavage by caspase-4.

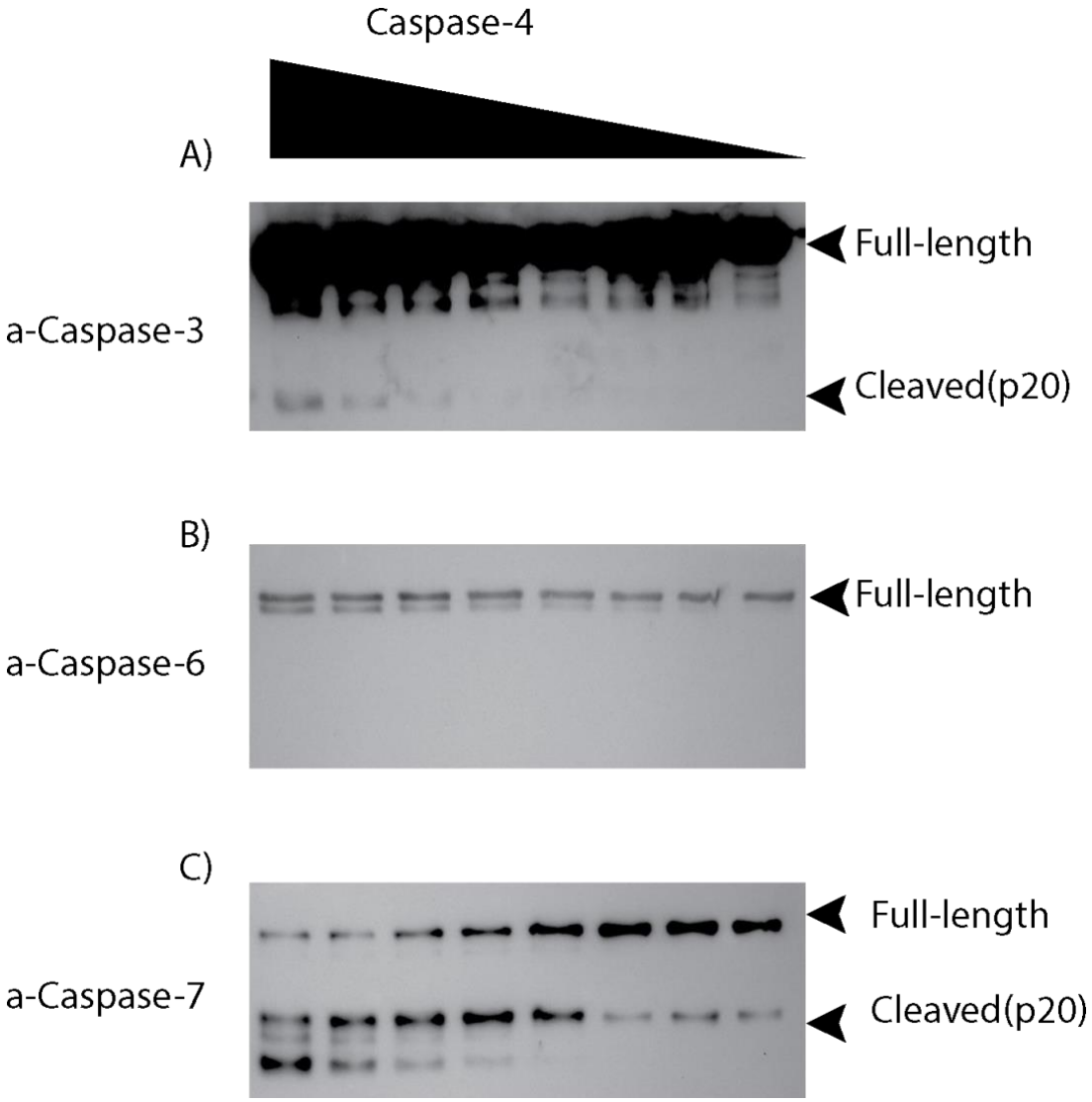

Figure 3: Caspase-4 preferentially cleaves caspase-7*. In vitro assays showing caspase-4 cleavage on the human executioner caspase-3 (A), caspase-6 (B) and caspase-7(C). Caspase-4 dilution (From 1 µM, ½ dilution) was incubated with 2 µM of recombinant executioner caspases (caspase-3, -6 or -7) for 2hrs in high salt caspase buffer. Samples were run on a 12% gel SDS-PAGE before being transferred on a nitrocellulose membrane and imaged using appropriate antibodies. (N=2).*

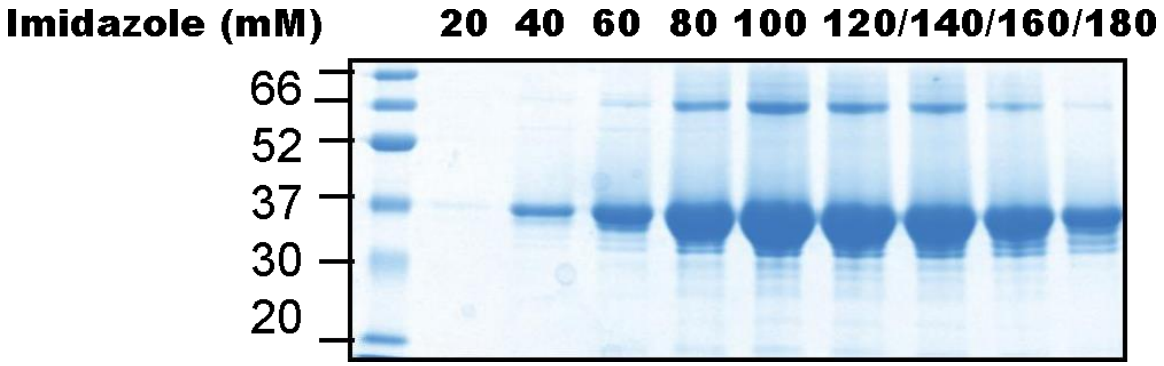

Figure 4: Caspase-7 purification fractions. *Example of a purification of caspase-7 upon expression in E. coli. Caspase-7 C285A 6His, expressed in E. coli BL21 pLyS and purified on nickel Sepharose resin was eluted with various concentrations of imidazole (20-180 mM imidazole). Imidazole fractions were analysed on a 12% SDS-PAGE and stained with Coomassie staining. Full length caspase-7 appears at around 35 kDa with a purity of 95% estimated. Fractions 100mM-160 mM were used for further experimentation.*

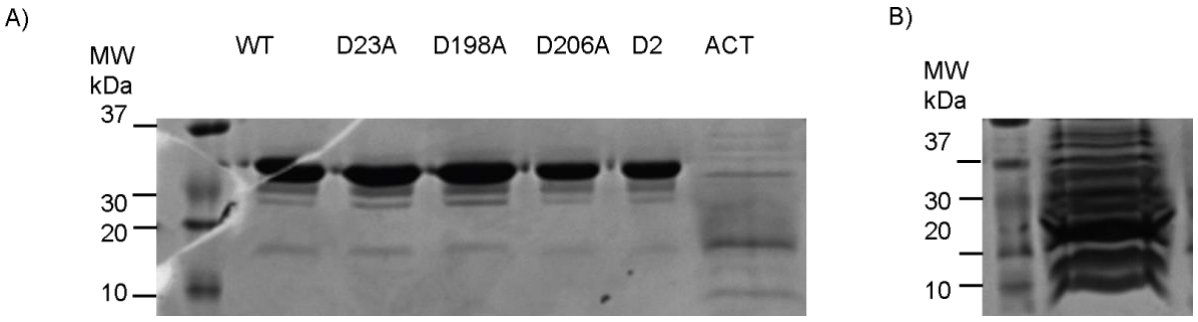

Figure 5: Caspase mutants employed within experiments. *Coomassie staining of the recombinant caspase-7 (A) and caspase-4 (22kDa/10 kDa) (B) used during this study. Caspase-7 mutants (N-terminal peptide mutant (D23A), interdomain linker site 1 mutant (D198), interdomain linker site 2 (D206A) and the double interdomain mutant linker D198A/D206A mutant (D2) as catalytic mutants (34 kDa), with active caspase-7 (ACT) are shown on the gel as a control (20kDa and 10 kDa). Purified proteins were run on a 12% acrylamide gel and was stained with Coomassie.*

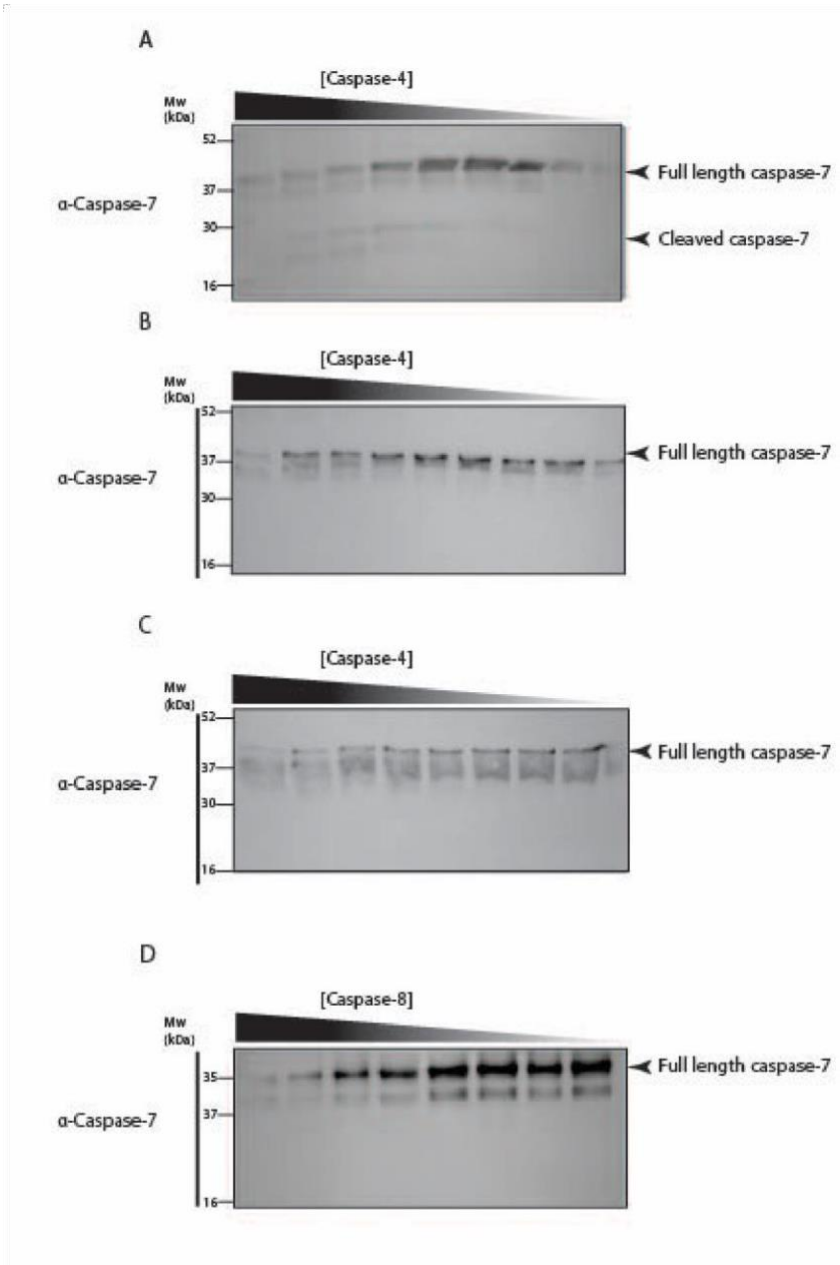

Figure 6: Caspase-4 may cleave caspase-7 at D23 and D198*. SDS/PAGE analysis of cleavage assays of various caspase-7 mutants with full-length caspase-4. Caspase-4 (starting concentration of 1μM, across a ½ dilution) was incubated with caspase-7(WT (**A**), D23A (**B**), D198A (**C**) and control caspase-8 (**D**) in high salt activity buffer for 2 hours at 37 ℃ after dimerization to test for cleavage. Samples were run on a 12% gel SDS-PAGE before being transferred on a nitrocellulose membrane and imaged using appropriate antibodies. Bands detected at 34kDa display full length caspase-7 detection. The detection of a smaller subunit (20 kDa) suggests caspase-4 has cleaved caspase-7.*

Next, I tried to identify the cleavage site used by caspase-4 to cleave caspase-7. Caspase-7 has three pre-identified cleavage sites (Fig1A) that have been described previously [24][19]. The N-terminal domain site D23 has been shown to be cleaved by caspase-3 whereas caspase-8 and -9 cleave the interdomain linker site D198. Under rare conditions, a second

cleavage site, D206A, can also be cleaved. Using site-directed mutagenesis, I generated mutants of these sites to alanine to prevent caspase cleavage (respectively D23A; D198A; D206A) as catalytic mutants (to prevent self-cleavage) and expressed these constructs in *E.coli*. I purified each of them using a nickel affinity column and I achieved a purity above 95% for each construct expressed and purified (Fig4). The mutants generated migrate according to a similar pattern (Fig5A). An example of the purified and concentrated caspase-4 used for the cleavage assays is also displayed (Fig5B).

To test the location of the cleavage site, I incubated WT (Fig6A), D23A (Fig6B), D198A (Fig6C) caspase-7 with decreasing concentration of caspase-4 (as in Fig3). As a control, I use caspase-8 (Fig4D), which has been shown to cleave caspase-7 efficiently. Depending on the cleavage site location, I am expecting a defined set of fragments generated, based on our antibody which recognised caspase-7 large subunit (Table 1). We hypothesised that mutations occurring at critical cleavage sites would lead to modified cleavage patterns, perhaps causing a decrease in caspase-7 activation compared to the wild type. The western blot analysis shows differences in the cleavage pattern between the wild-type (WT) caspase-7, which displayed more complete processing, but mutants D23A and D198A exhibited decreased, suggesting that these residues are crucial for efficient caspase-4-mediated activation (Fig6). For these experiments, the level of cleaved caspase-7 was not clearly detected but a decrease in the generation of caspase-7 full length suggests that D23A and WT caspase-7 are cleaved by caspase-4. Caspase-8 cleavage was also unclear but again, a decrease in the abundance of caspase-7 full length suggests that cleavage was occurring. The results highlight the crucial functions of D23 and D198 in the activation pathway of caspase-7, indicating that any changes

to these sites could potentially affect the enzyme's role.

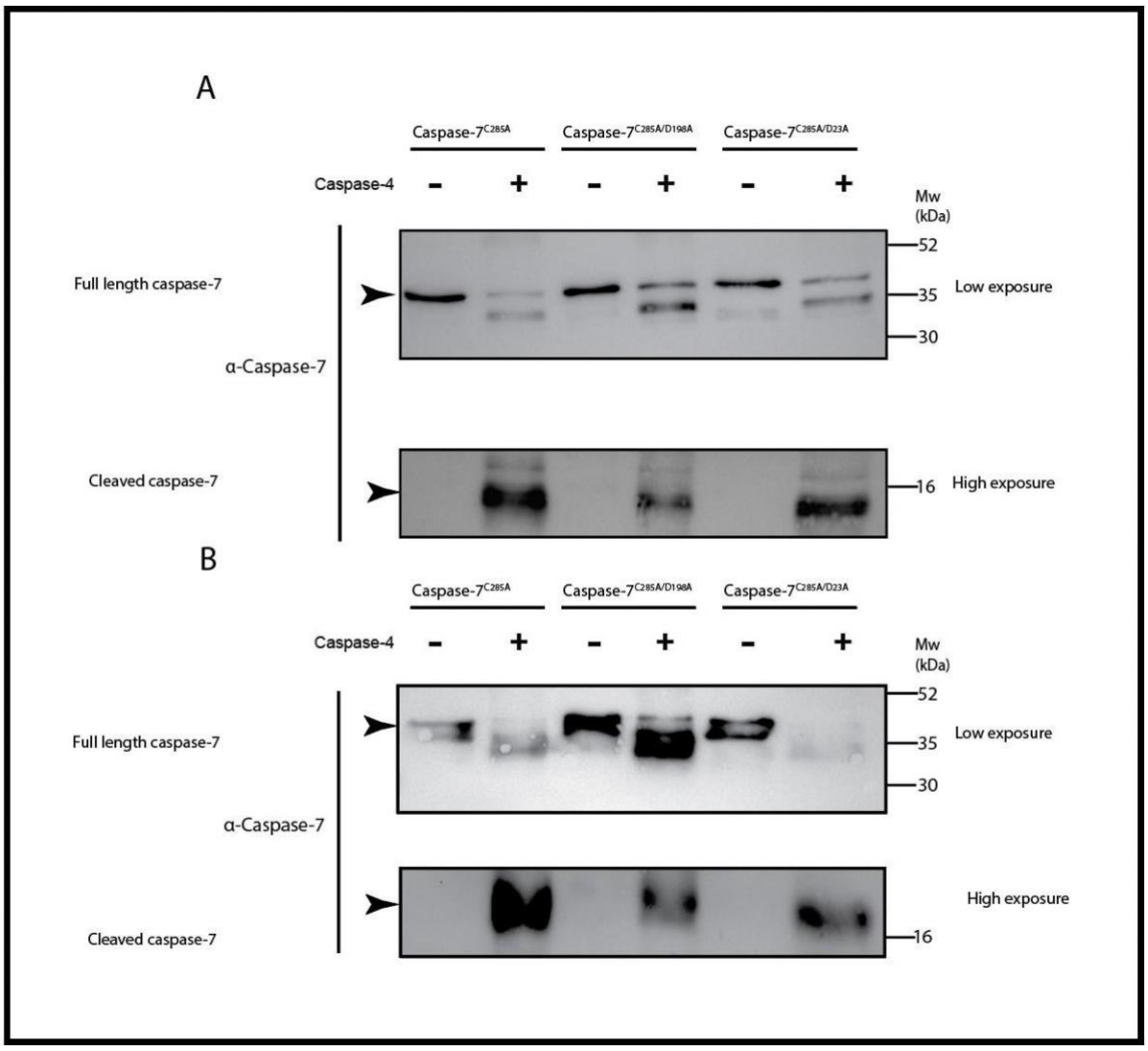

Figure 7: Caspase-4 cleaves preferentially Caspase-7 at D198*. WT caspase-7, caspase7 D198A and caspase-7 D23A (2μM) were incubated with 1μM of caspase-4 for 2 hrs at 37 ℃ to access their cleavage. A cleavage band around 20kDa suggests activation by caspase-4. Two repeats are presented (A and B). Samples were run on a 15% gel SDS-PAGE before being transferred on a nitrocellulose membrane and imaged using appropriate antibodies.*

To uncover the exact cleavage site of caspase-7 by caspase-4, we repeated the experiment and ran the samples on a 15% acrylamide gel and performed a shorter transfer to observe the cleaved products (Fig7). As I had difficulties observing the cleave fragment, I expected that a shorter transfer would allow for a better visualisation of this fragment. A shorter transfer did improve the visualisation of the bands. The results of this experiment showed that mutation at D198 to an alanine reduces the generation of the p20 fragment of caspase-7 but did not

completely block it. These results indicate that the altered residues may play a crucial role in the structural and functional framework required for the activation of caspase-7 by caspase-4. However, other amino acids, potentially also located in the interdomain linker like D198, may be alternate sites. The site D206 has been reported previously as accessible for cleavage [19].

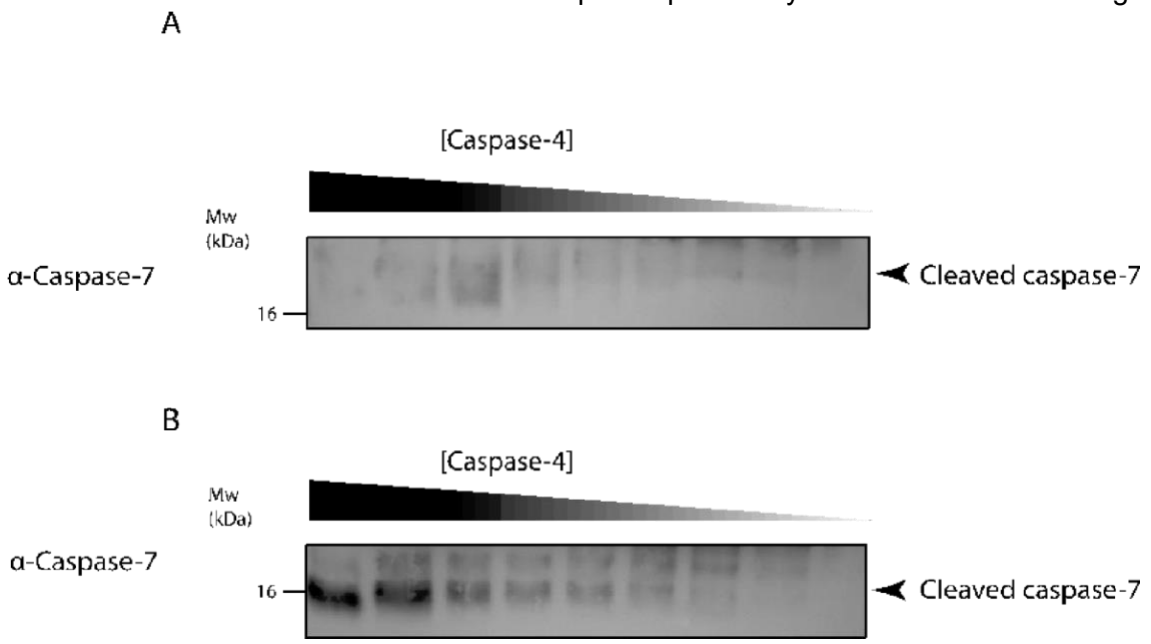

Figure 8: Mutation D206 has minimal effect on caspase-7 cleavage by caspase-4. (A) Western blot analysis showing the effect of decreasing concentrations of caspase-4 on recombinant caspase-7 D198A (A) and caspase-7 D206A (B). Membranes are probed against anti-caspase7 and have been overexposed to highlight the large subunit cleaved fragment (20 kDa).

To test whether the caspase-7 D206 site was cleaved by caspase-4, I mutated the amino acid to alanine and compared with the caspase-7 D198A mutants. As in Fig7, caspase-7 D198A displays a minimal (yet apparent) cleavage. However, the caspase-7 D206A mutant was efficiently cleaved (Fig8B), in a similar manner as WT caspase-7 (Fig8a)(Fig3C). This suggests that alone, D206A and D198A mutations are not sufficient to prevent caspase-7 activation by caspase-4. Previous reports [19] suggested that both cleavages can be used for cleavage. I next asked whether these sites could be redundant. I generated a mutant of caspase-7 mutated at both sites (D198A/D206A) and tested the impact of these mutations on caspase-7 activation by caspase-4. Caspase-7 D198A/D206A, which display uncleavable interdomain linker, is still sensitive to proteolysis by caspase-4 (Fig9). The cleavage is minimal and like what has been observed with Caspase-7 D198A. This suggests two things. First, D206A is not redundant with D198A and is likely not a cleavage site used by caspase-4. Secondly, caspase-7 can be processed at alternative sites within the interdomain linker. My results do not allow to confirm this. This will require further investigations that were not possible during my master by research.

Table 1: Expected fragments generated upon human caspase-7 activation *– caspase-7's expected fragment sizes based on current literature [19][24]. Expected molecular weights for the large (L) and small subunits (S) are presented.*

Potential Cleavage Site(s)	**Expected Molecular Weight (kDa) L/S**
Uncleaved	34
D23	31.8
D198	22.2/12.1
D206	23/11.3
D198 + D206	22.2/11.3
D23 + D198	19.7/12.1
D23 + D206	20.5/11.3
D23 + D198 + D206	19.7/11.3/0.5

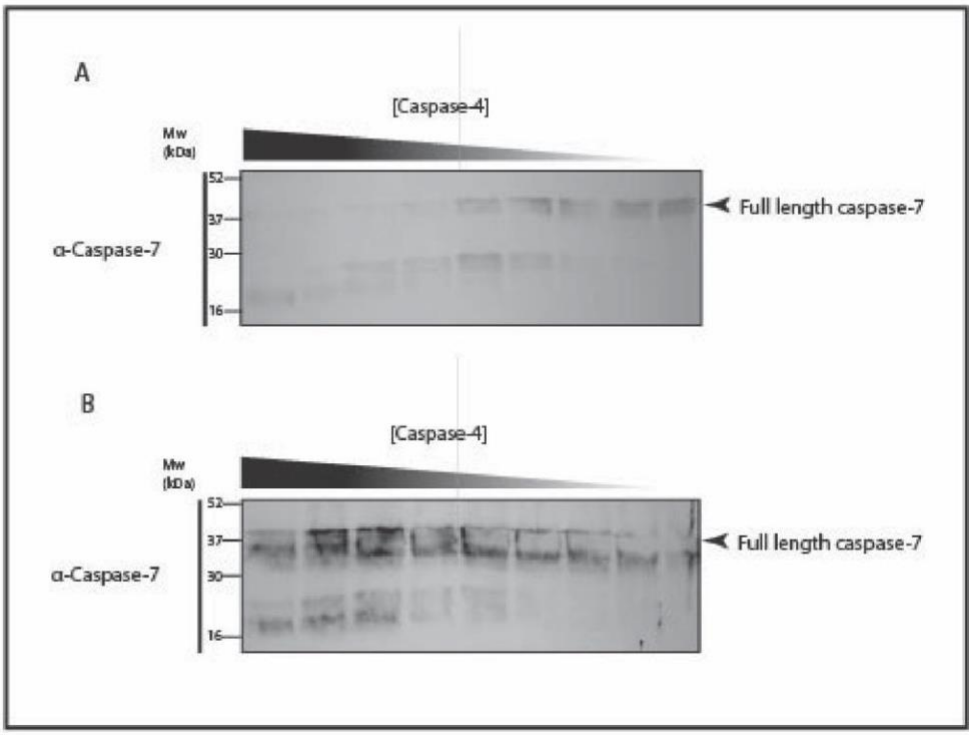

Figure 9: The impact of D206A and D206A/D198A mutations on caspase-7 cleavage by caspase-4*. (A) Recombinant caspase-7 D206A is cleaved by caspase-4, showing cleaved product at about 20 kDa, with higher concentrations of caspase-4 show caspase-7 D198A/D206A processing is taking place. (B) Full length caspase-7 bands are prominent throughout with faint cleavage bands at 20 kDa still visible at high concentrations of caspase-4, potentially pointing towards alternative sites of cleavage. In vitro cleavage assays were analysed on a 15% SDS-PAGE and subject to western blot using an anti-caspase-7 antibody. The result still supports a model in which the IDL residue D198 is the primary cleavage target by caspase-4.*

Discussion

Understanding caspase-7 function downstream of inflammasome is important. My work set a better understanding of the signalling pathways controlling inflammation. By using an in vitro system, I was able to demonstrate that caspase-7 is directly cleaved by caspase-4. Caspase7 was cleaved at D198 (cleavage site IQAD), which is different from the naturally preferred site for caspase-4 (LEHD). The system I used however was not perfect. In some experiments, I had difficulties in detecting cleavage products. This is a common problem encountered in the proteolytic signalling field. In the future, usage of alternative approaches, such as proteomic (e.g., Edman degradation analysis), may facilitate the detection and characterisation of the cleaved fragments obtained. As the reaction was performed in a buffer concentration in salt, it is also possible that the detection of caspase product was slightly affected. I have observed that mixing the recombinant in vitro reaction (made in high-salt buffer to favour caspase-4 activity) with the loading dye created a very viscous solution, which was difficult to load on SDS-PAGE. It is possible that this viscosity impaired my ability to load the sample equally. Additionally, this may have impacted the solubility of caspse-7, impacting the loading of my gels and explaining the variations I observed in certain gels. Usage of desalting column or diluting my sample post-reaction to reduce salt concentrations may be used in the future to facilitate this. Usage of desalting column may be used in the future to facilitate this.
Additionally, I did not investigate the kinetics of caspase-7 activation in primary cells (e.g., macrophages or epithelial cells). This would in the future provide important information as to when caspase-7 gets activated. Our study focussed on caspase-7 activation by caspase-4 but caspase-1 has also been suggested to activate caspase-7. Comparison of the roles of caspase-7, identification of substrates and evaluation of the activation kinetics would be of interest and may shed lights on differences between the canonical and non-canonical inflammasome pathways.

Understanding the distinction between the various forms of cell death, which all play different roles in maintaining health and combating disease is essential for comprehending cellular responses to environmental stimuli and stressors. Nevertheless, the data from our research suggests a more cohesive model in which these various pathways intersect, with a particular emphasis on the role of caspase-7. Unlike its conventional role in apoptosis, caspase-7 seems to actively contribute to inflammatory processes via its interaction with caspase-4. This interaction demonstrates a functional versatility, going beyond the known boundaries of apoptotic mechanisms, implying that caspase-7 may serve as a bridge between apoptosis and inflammation.

A new, regulatory role for caspase-7 within inflammatory pathways could be crucial when the immune system must not only quickly eliminate pathogens but also precisely control the inflammatory response to prevent excessive damage to surrounding tissues.

The activation of caspase-7 during pathogenic attacks, not only by apoptotic signals but also by pathways typically associated with immune response to infection, can result in the processing of specific substrates essential to both the implementation of apoptosis and regulation of inflammation. For instance, caspase-7 possesses the ability to cleave GSDMD, resulting in the inactivation of GSDMD, blocking the formation of pores in the cell membrane and subsequent release of inflammatory cytokines. This suggests that caspase-7 may somehow modulate caspase-4 and that it may have an important role in controlling infection outcomes. As mentioned above, caspase-7 activation kinetics, relative to GSDMD activation

by caspase-4, may impact the final outcomes of its signalling. This function facilitates the equilibrium between cellular apoptosis and the secretion of signals essential for immunological activation and recruitment.

The promotion of apoptosis in order to facilitate the clearance of infected cells, further highlights this regulatory role for caspase-7. This is significant when discussing the crosstalk between apoptosis and pyroptosis as the efficient and regulated clearance of pathogens ensures dying cells do not release their contents abruptly and trigger an uncontrolled inflammatory response. Interruption of the removal of said pathogens can result in sepsis or chronic inflammation, a hallmark of many autoimmune disorders. That said, it is also possible that caspase-7 is activated by caspase-4 as a backup mechanism when pyroptosis is highjacked by pathogens. *Shigella* is a good example of a pathogen that can subvert pyroptosis[46]. As discussed above, this crosstalk between cell death modalities is important for responses to infection. It has been well characterised with canonical inflammasome but not with the non-canonical inflammasome. Activation of caspase-7 downstream of caspases may allow for apoptosis execution to eliminate pathogens when pyroptosis is inhibited upon noncanonical inflammasome activation.

These intricate roles emphasise the significance of caspase-7's role within host defence. The ability to switch between promoting cell death and regulating inflammatory signals provides a strategic advantage in managing infections and essential for maintaining long-term tissue stability whilst preventing the development of chronic inflammatory diseases. By engaging in both pathways, caspase-7 offers a crucial albeit complex target for therapeutic solutions. Modulating caspase-7 activity can potentially allow for the precise calibration of cellular responses, like enhancing apoptosis in malignant cells and reducing excessive inflammation seen in disorders like rheumatoid arthritis or inflammatory bowel disease. Modulating the activity of caspase-7 in the context of sepsis or viral infections might avoid excessive immune responses and promote the clearance of pathogen-infected cells. This is important since incorrect inflammation in these conditions can cause severe tissue damage and organ failure. This is why understanding caspase-7 function in such signalling pathway is important to develop novel therapies.

The advancement of specific inhibitors or enhancers of caspase-7 signifies a hopeful frontier in therapeutics. Drugs could be engineered to selectively modulate the activity of caspase-7 in a targeted manner. These drugs could function as activators to enhance apoptosis in malignant tissues, or as inhibitors to decrease excessive inflammation in autoimmune illnesses. Utilising advanced drug delivery methods, such as carriers based on nanoparticles or targeted delivery technologies, has the potential to improve the accuracy and effectiveness of these interventions. This can reduce unintended consequences and optimise the desired therapeutic results.

Future work

My study did not manage to clarify what was the second site cleaved by caspase-4. It is possible that caspase-4 cleave not only aspartic acid but also glutamic acid, as reported for other caspases [2]. Investigation of addition sites within the interdomain linker may identify the site cleaved when D198 is uncleavable.

Further investigation into the activation of caspase-7 and its complex involvement with multiple cellular death pathways is crucial. Future research should prioritise investigating the precise structural and biochemical processes via which caspase-7 is triggered, namely by caspase-4, in the context of inflammatory responses and infections. Advanced techniques such as cryoelectron microscopy and X-ray crystallography can provide important information about the structural changes that occur in caspase-7 when it becomes activated. To date, no structural information guides our understanding of the activation of caspase-7 by apoptotic or inflammatory caspases. Such studies will be crucial in understanding how alterations in its structure influence interactions with substrates and other caspases. Recent structure of caspases with their substrates suggest that this would be technically possible [47][48]. Design of inhibitors based on the interaction interfaces between caspase-4 and -7 could allow to specifically develop caspase-7 activation inhibitors. This would not only support our understanding of the roles of caspase-7 during inflammation but may also provide new strategies to develop immunomodulators.

The impact of caspase-4 cleaving caspase-7 in the context of infection is significant as it can enhance the cell's capacity to rapidly execute inflammatory cell death. This procedure can inhibit the spreading of infection by facilitating prompt cell lysis and the secretion of cytokines which can trigger additional immune responses. Studying this interplay in cellular models of infection will offer valuable insights into how manipulating these pathways can strengthen the body's defence mechanisms while avoiding excessive tissue harm. A main question remaining is how caspase-7 signals during inflammation. As we have limited information on this, identifying novel substrates for caspase-7 will be critical. Recently, ASM has been identified as one of such substrates that may control inflammation downstream of caspase-4 [28]. Caspase-7 was also suggested to have functions in the nucleus and may therefore influence transcription [49]. Others have attempted to identify caspase-7 substrates, but these remain to be validated in an inflammatory context. A proteomic approach using caspase-7 knockout cells would help us understand which proteins are cleaved downstream of the inflammasome activation. This is likely an optimal approach as the identify of these substrates will be correlated with the potential role of caspase-7 in such process. As caspase-7 has been shown to be activated in epithelial cells and macrophages, a comparison of those substrates in different cell types may also identify cell-specific substrates and functions for caspase-7 [6][7][20][25].

Studying the role of caspase-7 in other pathological conditions, such as cancer, autoimmune diseases, and neurodegenerative disorders, could offer a better understanding of its roles beyond its involvement in apoptosis. Furthermore, comprehending the activity of immune cells

in autoimmune reactions could aid in developing targeted medicines that address abnormal inflammation while preserving essential immune activities.

Working with human cells to investigate these mechanisms present many challenges. Human cellular models frequently fall short in replicating the intricate *in vivo* setting, where various cell types interact and communicate amidst a multitude of signalling molecules. Furthermore, ethical and logistical restrictions restrict the degree to which human cells can be modified in laboratory environments. These constraints highlight the importance of developing *in vitro* models that can mimic the physiological circumstances of the human body, such as organ-ona-chip technologies and 3D bioprinting of tissue models [50]. These would facilitate our understanding of the physiological role of caspase-7 during cellular process.

Another direction for future research involves investigating whether this activation mechanism of caspase-7 by caspase-4 is conserved across species, in particular between humans and mice. Studies have suggested that this may also happen in mice through caspase-11 but it is unclear if this happens in other contexts [51]. In addition, humans and higher primates also expressed caspase-5, highly similar to caspase-4[39]. Whether caspase-5 can also activate caspase-7 remains to be clarified. This investigation would provide insight into the evolutionary stability of cell death mechanisms and enhance the translatability of preclinical findings to human therapies. Caspase-5 is only conserved in higher primates and its roles have not been fully described yet. Techniques such as CRISPR/Cas9 gene editing can be utilised to create mouse models that either overexpress or knock out these caspases, allowing researchers to observe the resultant effects on cell death and inflammatory responses under various pathological conditions, such as infection, cancer, or autoimmune diseases. Additionally, cross-species studies using primary cell lines derived from both humans, mice and chimps, can help differentiate any subtle differences or similarities in the caspase activation cascades and their downstream effects.

Such findings will not only confirm the significance of mouse models in studying human diseases but also potentially provide new treatment targets within these pathways. If significant similarities are found, these pathways could then be targeted more confidently in drug development, knowing that results observed in mouse models are likely to be applicable to human conditions. On the other hand, discovering distinctions can assist in clarifying how diseases appear in specific species and result in the creation of more precise models or customised therapies.

Conclusion

This work has enhanced our comprehension of caspase-7 activation, specifically emphasising its involvement in the non-canonical inflammasome pathway triggered by caspase-4. The findings of this study provide evidence that caspase-7 can be activated in a different way than its usual role in apoptosis. This activation involves a specific cleavage by caspase-4, resulting in a unique selection of target molecules and functional effects that have a significant impact on both inflammation and cell death. Although I have not addressed these yet, this confirmation of caspase-7 activation directly downstream of caspase-4 suggest that caspase7 may have direct targets in inflammation.

Through application of site-directed mutagenesis, we designed variants of caspase-7 with targeted mutations at Asp23, Asp198, and Asp206, which play a crucial role in the conversion of caspase-7 to its active state. These modifications were necessary to investigate the hypothesis that by preventing self-cleavage at these sites, we could isolate the effects of caspase-4-mediated activation. This would enable a controlled investigation into the specific roles and mechanisms of caspase-7 within the non-canonical inflammasome pathway. The use of a catalytic mutant of caspase-7 (C285A) further refined our insights into these interactions, underscoring the specificity of caspase-4's influence on caspase-7 beyond its usual context.

By conducting a sequence of cleavage assays illustrated in Figures 2 and 3, I have proven that caspase-4 can efficiently cleave both the WT form of caspase-7 and several altered versions, even though these modifications were intended to prevent self-cleavage. Significantly, the existence of cleavage bands in mutants with the C285A mutation, which causes caspase-7 to lose its catalytic activity, clearly indicates that caspase-4 is directly responsible for activating caspase-7. This suggests alternative sites of cleavage to those previously predicted, which are not affected by the mutations at Asp23 and Asp198. These mutations were first thought to stop the activation caused by caspase-4.

The results of this investigation not only confirm the suggested concept, but also provide fresh opportunities for comprehending how the participation of caspase-7 in non-canonical pathways could be utilised for therapeutic advantages. The capacity of caspase-7 to participate in inflammatory reactions by means of selective activation by caspase-4 implies possible regulatory roles that could be focused on in order to modify inflammation-related disorders. This discovery has significant implications for the advancement of innovative treatment approaches targeting diseases characterised by excessive or dysregulated inflammation, including autoimmune disorders, chronic inflammatory diseases, and specific types of cancer [52][53][54][55].

Given these findings, future research should focus on identifying these alternative cleavage sites and understanding their structural and functional implications. Additionally, expanding these studies to *in vivo* models will be crucial for validating the physiological relevance of these findings and for exploring the potential of targeting caspase-7 in therapeutic strategies. Such

research could ultimately lead to the development of novel treatments that more effectively manage pathological conditions by precisely modulating caspase activity. Moreover, it is imperative to extend this research by doing comparative analyses in various model organisms, such as mice, to validate the preservation of these pathways across different species and improve the applicability of these findings in clinical settings.

To summarise, our study not only provides an understanding of the intricate activation of caspase-7 in non-canonical inflammasome pathways, but also emphasises its potential as a new target for therapeutic intervention. By gaining a more profound comprehension of the complex molecular interactions of caspases, we create opportunities for developing more efficient therapies that can specifically regulate cellular processes related to human well-being and illness.

Materials and Methods

All caspase substrates and inhibitors were sourced from Sigma-Aldrich (see appendix). All other chemicals were acquired from Sigma–Aldrich or ThermoFisher, with detailed specifications provided in the supplementary materials.

Expression and Purification of Recombinant Caspases

Caspases were expressed in Escherichia coli BL21(DE3) pLysS strain in 2YT media (1 L): 16 g tryptone, 10 g Bacto yeast extract, 5 g NaCl (autoclave-sterilised) and 50 µg/mL ampicillin) using a pET-23(+) vector modified to include a C-terminal Histidine (6X) epitope to enable purification by Immobilised-Metal affinity chromatography (IMAC) using a Sepharose highbinding Nickel resin (ThermoFischer, cat A50584). Plasmids used for expression were obtained through Addgene and are a kind gift of Dr Guy Salvesen. Mutations were inserted by site-directed mutagenesis using overlapping PCR techniques using the CloneAmp PCR mastermix (Takara) and verified with Sanger sequencing (EuroFin). Purification was conducted via nickel affinity chromatography (FastFlow Nickel Sepharose resin, ThermoFischer), with the elution of proteins monitored through Coomassie staining on a 12% acrylamide gel, as described previously by our group [45]
Caspase-7 C285A and its mutants were expressed in 2YT media for 6 hrs with induction at 30 °C using 200µM IPTG. Pellet was then harvested and resuspended in a resuspension buffer (150 mM NaCl, 50 mM Tris pH 7.6) and frozen at -80 °C until purification. The Nickel resin was washed with washing buffer (500 mM NaCl, 50 mM Tris pH 7.6) before elution in elution buffer (150 mM NaCl, 50mM Tris pH 7.6, 200mM Imidazole). Caspase-4 was expressed in 2YT media for 16 hrs at 21 °C and purified as caspase-7. Once purified, caspase4 was concentrated using an Amicon concentrator (30kDa cutoff) [45]. Caspase-4 was activesite titrated using zVAD-fmk (ChemMedChem), as described here [45].

Cleavage Assays

Caspase-4 was preactivated in a high salt buffer (60 mM 4-(2-hydroxyethyl)piperazine-1ethanesulfonic acid (HEPES) at pH 7.4 (NaOH), 1.2 M sodium citrate, 60 mM NaCl, 0.012 % w/v CHAPS, and 12 mM DTT (freshly added) (filter-sterilised)) at 37°C for 1 hour before the addition of caspase-7 C285A and related mutants (2µM) for 2 hrs. The reaction was stop by the addition of 4X Nupage buffer (Novex) following 5 min of boiling at 95 °C.

SDS-PAGE and Immunoblotting

Proteins resolved from cleavage assays were subjected to SDS-PAGE using tris-glycine buffer system (25 mM Tris, 192 mM glycine, 0.1%(w/v) SDS, pH 8.3). Following electrophoresis, proteins were transferred to nitrocellulose membranes and probed using specific antibodies (detailed in Appendix). Proteins were detected using the chemiluminescence substrate

Immobilon Forte (Millipore), and images captured and analysed using the iBright CL1500 Imaging System.

Coomassie staining

GelCode (ThermoFischer, cat 24596) was used to stain acrylamide gels upon purification of the caspases.

Appendix

Table 2: Reagents and chemicals used during this study

Description	Manufacturer	Catalogue No.	Use
Acrylamide	Sigma-Aldrich	A7168	Making SDS gels
Hydrogen peroxide 35%	Merck	K53724700141	Stripping Western blot membranes
Immobilon Crescendo western HRP substrate	Millipore	WBLUR0500	Western blot HRP substrate
Immobilon Forte western HRP substrate	Millipore	WBLUF0500	Western blot HRP substrate
LB	Millipore	51208	Growing bacteria
Nitrocellulose membranes	Bio-RAD	1620094	Membranes for Western Blot
LDS sample buffer	Novex	84788	Lysis buffer used in preparation of Western blot samples
Opti-MEM	Gibco	11058-02[DB1]	Media for cell culture and treatments
Prime-step pre-stained broad range protein ladder	Biolegend	773302	Protein ladder for SDS page Western blot
Skim milk powder	Sigma Aldrich	70166-500G	Making 5% milk in TBST for western blot blocking buffer and making of antibodies
Superscript™ IV Firststrand synthesis system	Invitrogen	18091050	Reverse transcription of RNA to cDNA
Temed	Sigma-Aldrich	T22500	Making SDS acrylamide gels
Trans-Blot Turbo 5X Transfer buffer	Bio-RAD	10026938	Transfer buffer used in semi-dry transfer of Western blots
2x YT Medium	Sigma-Aldrich	91079-40-2	Microbial growth medium
VX-765	Selleckchem	S2228	Caspase inhibitor used in treatment of cells

Table 2: Antibodies used during my studies.

Name	Manufacturer	Catalogue No.
Caspase-7 (mouse)	Cell Signalling	D2Q3L
Caspase-4 (mouse)	Proteintech	11856-1-AP
Caspase-3 (rabbit)	Proteintech	66470-2-16
Anti-Rabbit HRP	Proteintech	SA000001-2
Anti-Mouse HRP	Proteintech	SA000001-1

Bibliography

1. Fuentes-Prior P, Salvesen GS. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J. 2004 Dec 1;384(2):201–32.

2. Julien O, Wells JA. Caspases and their substrates. Cell Death Differ. 2017 Aug;24(8):1380–9.

3. Vitale I, Pietrocola F, Guilbaud E, Aaronson SA, Abrams JM, Adam D, et al. Apoptotic cell death in disease—Current understanding of the NCCD 2023. Cell Death Differ. 2023 May;30(5):1097–154.

4. Zheng T, Hunot S, Kuida K, Flavell R. Caspase knockouts: matters of life and death.

5. Lakhani SA, Masud A, Kuida K, Porter GA, Booth CJ, Mehal WZ, et al. Caspases 3 and 7: Key Mediators of Mitochondrial Events of Apoptosis. Science. 2006 Feb 10;311(5762):847–51.

6. Walsh JG, Cullen SP, Sheridan C, Lüthi AU, Gerner C, Martin SJ. Executioner caspase-3 and caspase-7 are functionally distinct proteases. Proc Natl Acad Sci. 2008 Sep 2;105(35):12815–9.

7. Boucher D, Blais V, Denault JB. Caspase-7 uses an exosite to promote poly(ADP ribose) polymerase 1 proteolysis. Proc Natl Acad Sci. 2012 Apr 10;109(15):5669–74.

8. Ross C, Chan AH, Von Pein JB, Maddugoda MP, Boucher D, Schroder K. Inflammatory Caspases: Toward a Unified Model for Caspase Activation by Inflammasomes. Annu Rev Immunol. 2022 Apr 26;40(1):249–69.

9. Chan AH, Schroder K. Inflammasome signaling and regulation of interleukin-1 family cytokines. J Exp Med. 2020 Jan 6;217(1):e20190314.

10. Broz P, Pelegrín P, Shao F. The gasdermins, a protein family executing cell death and inflammation. Nat Rev Immunol. 2020 Mar;20(3):143–57.

11. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, et al. A Combinatorial Approach Defines Specificities of Members of the Caspase Family and Granzyme B. J Biol Chem. 1997 Jul;272(29):17907–11.

12. Timmer JC, Zhu W, Pop C, Regan T, Snipas SJ, Eroshkin AM, et al. Structural and kinetic determinants of protease substrates. Nat Struct Mol Biol. 2009 Oct;16(10):1101–8.

13. Dix MM, Simon GM, Cravatt BF. Global Mapping of the Topography and Magnitude of Proteolytic Events in Apoptosis. Cell. 2008 Aug;134(4):679–91.

14. Crawford ED, Julien O, Mahrus S, Nguyen H, Yoshihara HAI, Zhuang M, et al. The DegraBase: A Database of Proteolysis in Healthy and Apoptotic Human Cells*□S.

15. Agard NJ, Maltby D, Wells JA. Inflammatory Stimuli Regulate Caspase Substrate Profiles*□S.

16. Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, et al. A Unified Model for Apical Caspase Activation. Mol Cell. 2003 Feb;11(2):529–41.

17. Riedl SJ, Fuentes-Prior P, Renatus M, Kairies N, Krapp S, Huber R, et al. Structural basis for the activation of human procaspase-7. Proc Natl Acad Sci. 2001 Dec 18;98(26):14790–5.

18. Chai J, Wu Q, Shiozaki E, Srinivasula SM, Alnemri ES, Shi Y. Crystal Structure of a Procaspase-7 Zymogen: Mechanisms of Activation and Substrate Binding.

19. Boucher D, Blais V, Drag M, Denault JB. Molecular determinants involved in activation of caspase 7. Biosci Rep. 2011 Aug 1;31(4):283–94.

20. Lamkanfi M, Kanneganti TD. Caspase-7: A protease involved in apoptosis and inflammation. Int J Biochem Cell Biol. 2010 Jan;42(1):21–4.

21. Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, et al. Mechanism of XIAPMediated Inhibition of Caspase-9. Mol Cell. 2003 Feb;11(2):519–27.

22. Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, et al. A conserved XIAPinteraction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature. 2001 Mar 1;410(6824):112–6.

23. Choi YE, Butterworth M, Malladi S, Duckett CS, Cohen GM, Bratton SB. The E3 Ubiquitin Ligase cIAP1 Binds and Ubiquitinates Caspase-3 and -7 via Unique Mechanisms at Distinct Steps in Their Processing. J Biol Chem. 2009 May;284(19):12772–82.

24. Denault JB, Salvesen GS. Human Caspase-7 Activity and Regulation by Its N-terminal Peptide. J Biol Chem. 2003 Sep;278(36):34042–50.

25. Lamkanfi M, Moreira LO, Makena P, Spierings DCJ, Boyd K, Murray PJ, et al. Caspase-7 deficiency protects from endotoxin-induced lymphocyte apoptosis and improves survival. Blood. 2009 Mar 19;113(12):2742–5.

26. Safari F, Akbari B. Knockout of caspase-7 gene improves the expression of recombinant protein in CHO cell line through the cell cycle arrest in G2/M phase. Biol Res. 2022 Jan 11;55(1):2.

27. Renema P, Kozhukhar N, Pastukh V, Spadafora D, Paudel SS, Tambe DT, et al. Exoenzyme Y induces extracellular active caspase-7 accumulation independent from apoptosis: modulation of transmissible cytotoxicity. Am J Physiol-Lung Cell Mol Physiol. 2020 Aug 1;319(2):L380–90.

28. Cassidy SKB, Hagar JA, Kanneganti TD, Franchi L, Nuñez G, O'Riordan MXD. Membrane Damage during Listeria monocytogenes Infection Triggers a Caspase-7 Dependent Cytoprotective Response. Philpott DJ, editor. PLoS Pathog. 2012 Jul 12;8(7):e1002628.

29. Nozaki K, Maltez VI, Rayamajhi M, Tubbs AL, Mitchell JE, Lacey CA, et al. Caspase-7 activates ASM to repair gasdermin and perforin pores. Nature. 2022 Jun 30;606(7916):960–7.

30. Kottke TJ, Blajeski AL, Meng XW, Svingen PA, Ruchaud S, Mesner PW, et al. Lack of Correlation between Caspase Activation and Caspase Activity Assays in Paclitaxel-treated MCF-7 Breast Cancer Cells. J Biol Chem. 2002 Jan;277(1):804–15.

31. Kuida K, Zheng TS, Na S, Kuan CY, Yang D, Karasuyama H, et al. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature. 1996 Nov;384(6607):368–72.

32. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun. 1967 Apr;27(2):157–62.

33. Wei Y, Fox T, Chambers SP, Sintchak J, Coll JT, Golec JM, et al. The structures of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. Chem Biol. 2000 Jun;7(6):423–32.

34. Okinaga T, Kasai H, Tsujisawa T, Nishihara T. Role of caspases in cleavage of lamin A/C and PARP during apoptosis in macrophages infected with a periodontopathic bacterium. J Med Microbiol. 2007 Oct 1;56(10):1399–404.

35. Cho HJ, Kim JT, Baek KE, Kim BY, Lee HG. Regulation of Rho GTPases by RhoGDIs in Human Cancers. Cells. 2019 Sep 5;8(9):1037.

36. Shi J, Wei L. Rho kinase in the regulation of cell death and survival. Arch Immunol Ther Exp (Warsz). 2007 Apr;55(2):61–75.

37. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspaseactivated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature. 1998 Jan 1;391(6662):43–50.

38. Churchill MJ, Mitchell PS, Rauch I. Epithelial Pyroptosis in Host Defense. J Mol Biol. 2022 Feb;434(4):167278.

39. Bateman G, Hill B, Knight R, Boucher D. Great balls of fire: activation and signalling of inflammatory caspases. Biochem Soc Trans. 2021 Jun 30;49(3):1311–24.

40. Demarco B, Chen KW, Broz P. Cross talk between intracellular pathogens and cell death. Immunol Rev. 2020 Sep;297(1):174–93.

41. Bedoui S, Herold MJ, Strasser A. Emerging connectivity of programmed cell death pathways and its physiological implications. Nat Rev Mol Cell Biol. 2020 Nov;21(11):678–95.

42. Parrish AB, Freel CD, Kornbluth S. Cellular Mechanisms Controlling Caspase Activation and Function. Cold Spring Harb Perspect Biol. 2013 Jun 1;5(6):a008672–a008672.

43. Gauthier AE, Rotjan RD, Kagan JC. Lipopolysaccharide detection by the innate immune system may be an uncommon defence strategy used in nature. Open Biol. 2022 Oct;12(10):220146.

44. Kayagaki N, Kornfeld OS, Lee BL, Stowe IB, O'Rourke K, Li Q, et al. NINJ1 mediates plasma membrane rupture during lytic cell death. Nature. 2021 Mar 4;591(7848):131–6.

45. Boucher D, Duclos C, Denault JB. General In Vitro Caspase Assay Procedures. In: V. Bozhkov P, Salvesen G, editors. Caspases,Paracaspases, and Metacaspases [Internet].

New York, NY: Springer New York; 2014 [cited 2023 Jul 12]. p. 3–39. (Methods in Molecular Biology; vol. 1133). Available from: https://link.springer.com/10.1007/978-1-4939-0357-3_1

46. Kobayashi T, Ogawa M, Sanada T, Mimuro H, Kim M, Ashida H, Akakura R, Yoshida M, Kawalec M, Reichhart JM, Mizushima T, Sasakawa C. The Shigella OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection. Cell Host Microbe. 2013 May 15;13(5):570-583.

47. Liu Z, Wang C, Yang J, Chen Y, Zhou B, Abbott DW, et al. Caspase-1 Engages FullLength Gasdermin D through Two Distinct Interfaces That Mediate Caspase Recruitment and Substrate Cleavage. Immunity. 2020 Jul;53(1):106-114.e5.

48. Devant P, Dong Y, Mintseris J, Ma W, Gygi SP, Wu H, et al. Structural insights into cytokine cleavage by inflammatory caspase-4. Nature. 2023 Dec 14;624(7991):451–9.

49. Erener S, Pétrilli V, Kassner I, Minotti R, Castillo R, Santoro R, et al. InflammasomeActivated Caspase 7 Cleaves PARP1 to Enhance the Expression of a Subset of NF-κB Target Genes. Mol Cell. 2012 Apr;46(2):200–11.

50. Leung, C.M., de Haan, P., Ronaldson-Bouchard, K. et al. A guide to the organ-on-a-chip. Nat Rev Methods Primers 2, 33 (2022)

51. Julien O, Zhuang M, Wiita AP, O'Donoghue AJ, Knudsen GM, Craik CS, et al. Quantitative MS-based enzymology of caspases reveals distinct protein substrate specificities, hierarchies, and cellular roles. Proc Natl Acad Sci [Internet]. 2016 Apr 5 [cited 2024 Aug 10];113(14). Available from: https://pnas.org/doi/full/10.1073/pnas.1524900113

52. Yao J, Sterling K, Wang Z, Zhang Y, Song W. The role of inflammasomes in human diseases and their potential as therapeutic targets. Signal Transduct Target Ther. 2024 Jan 5;9(1):10.

53. Dahiya R, Sutariya VB, Gupta SV, Pant K, Ali H, Alhadrawi M, et al. Harnessing pyroptosis for lung cancer therapy: The impact of NLRP3 inflammasome activation. Pathol - Res Pract. 2024 Aug;260:155444.

54. Lin Z, Chen Q, Ruan HB. To die or not to die: Gasdermins in intestinal health and disease. Semin Immunol. 2024 Feb;71:101865.

55. Si Y, Liu L, Fan Z. Mechanisms and effects of NLRP3 in digestive cancers. Cell Death Discov. 2024 Jan 5;10(1):10.