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DNA Cleavage During Pyroptosis

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A thesis

submitted in partial fulfillment of the

requirements for the degree of

Master of Science

University of Washington

2022

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Program Authorized to Offer Degree:

Laboratory Medicine and Pathology

University of Washington

Abstract

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Programmed cell death is a normal process in living organisms that removes damaged or infected cells. DNA fragmentation is an important aspect of some forms of programmed cell death, and is a well-recognized feature of apoptosis. DNA damage is also present during pyroptosis, a form of pro-inflammatory programmed cell death mediated by caspase-1. This enzyme cleaves and activates the pore-forming protein, gasdermin D, leading to cell swelling and plasma membrane rupture. The goal of this thesis was to better understand the mechanism and consequences of DNA damage during pyroptosis. We used *Salmonella* and lethal toxin as pyroptosis inducers and confirmed the presence of DNA damage. We found that release of the DNA-associated nuclear protein HMGB1 also occurs during pyroptosis. Extracellular calcium, which enters pyroptotic cells through gasdermin D pores, is required for both DNA damage and HMGB1 release from the nucleus. However, gasdermin D pores

alone are insufficient to stimulate DNA damage. Lastly, we found that DNA from cells undergoing pyroptosis does not appear to resemble apoptotic DNA fragmentation using agarose gel electrophoresis. Together, these results provide further insight into the process of DNA damage during pyroptosis.

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ACKNOWLEDGEMENTS

This project would not have been possible, and many of its ideas would not have been developed without the support of many people at the Department of Laboratory Medicine and Pathology.

Many thanks to my mentor Dr. Susan Fink, who accepted me to be a member in her laboratory, for offering guidance and support throughout my journey in this project, and for all the benefits that I received from her wealth of knowledge. I really appreciate that she found the time to read my numerous revisions and helped in enhancing my writing. I would also like to thank Dr. Andreas den Hartigh, for being an ideal teacher and supervisor in the laboratory, and for offering advice and encouragement with a perfect blend of insight and humor.

Special thanks to my committee members, Drs. Stephen Polyak, and Sean C. Murphy, who I am grateful to for being part of this project, for their encouraging words and thoughtful, detailed feedback which have been very important to me. I am also grateful that they took time out of their schedules to participate in my research and made this project possible. Thank you to Heather Eggleston, who is the manager of academic services and program advisor, for all of the kind words, support, and assistance she has provided.

Finally, I would like to thank the Saudi government and the Ministry of Education in Saudi Arabia for their financial support, and for giving me the opportunity to pursue my education and research interest. I would not also forget my family and friends, who endured this long process with me, always offering support and love.

1. INTRODUCTION:

Cell death is a natural and essential process that is responsible for maintaining and preserving homeostasis in the body. This important process plays pivotal roles in eliminating damaged and defective cells, and facilitating embryonic development (1). Cell death can occur via multiple pathways resulting from different stimulators, and molecular pathways, which produce distinct morphological changes (2). Apoptosis is the most understood form of cell death and has the best characterized mechanism, with a programmed non-inflammatory pathway, and very distinct morphological changes. Moreover, apoptosis is mediated by caspase enzymes, which are proteases that cleave cellular substrates to produce the features associated with apoptosis. Apoptotic cells are packaged into membrane-bound apoptotic bodies, which can be taken up by phagocytes and the cellular contents are degraded. Another form of cell death is called autophagic cell death. This form of cell death is characterized by the formation of large intracellular vesicles and plasma membrane blebbing (1).

Other more recently described forms of programmed cell death include pyroptosis, ferroptosis, and NETosis (3). Pyroptosis is a pro-inflammatory programmed cell death that requires caspase-1 activation (4). This programmed process is initiated by sensing of invading microorganisms or foreign products, and ends with cell rupture and the release of cellular components into the extracellular medium **(Figure 1)** (5). Caspase-1 is activated by inflammasomes, which are multiprotein complexes that contain innate immune sensors including Nod-like receptors (NLRs) (6).

In *Salmonella*-infected cells, the recognition of this microorganism by NLRC4 receptors activates the assembly and formation of NLRC4 inflammasomes (7,8). NLRC4 consists of a leucine-rich repeat domain, nucleotide-binding, and oligomerization domain, and caspase activation and recruitment domains that can interact directly with caspase-1 (5).

NLRP1b is another inflammasome receptor that can activate caspase-1 and is stimulated by anthrax lethal toxin (9).

Following activation within the inflammasome, caspase-1 cleaves the pore-forming protein, gasdermin D. The amino-terminal fragment, called gasdermin-N inserts into the cell membrane and oligomerizes, forming a pore that measures 180 Å in inner diameter (10). Caspase-1-mediated pore formation leads to water and ion influx, cell swelling and osmotic lysis (11). Caspase-1 also cleaves the inactive precursors of the inflammatory cytokines interleukin (IL)-1β and IL-18 to produce active cytokines that contribute to the inflammatory nature of pyroptosis (12).

Pyroptosis is an important protective response to infection (9). Several studies show that death of infected cells and secretion of the inflammatory cytokines represent an essential defense mechanism in controlling intracellular infection (13). This was supported by a study that showed the activation of caspase-1, and the maturation and release of the proinflammatory cytokines IL-1β and IL-18 are crucial in defending against *Salmonella* enterica serovar Typhimurium infection (14). In addition to its protective role, pyroptosis is also involved in several diseases, working as a trigger of inflammation and cause of organ dysfunction (5). Cardiovascular disease is an example where caspse-1 activation leads to inflammation and pyroptotic cell death, which then contributes to development of disease (15). Other examples of diseases involving pyroptosis are Parkinson's disease (16), inflammatory bowel disease (17), liver inflammation (18), and arthritis (19).

Another event that occurs during some forms of programmed cell death is chromosomal DNA fragmentation. DNA degradation during programmed cell death is important physiologically and lack of proper DNA digestion causes pathology (20). For example, nucleic acids are recognized by multiple innate immune sensors and failure to degrade DNA during apoptosis can lead to autoinflammatory and autoimmune diseases (20). DNA fragmentation

during apoptosis occurs as a result of a caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) (21), the latter of which is cleaved by the apoptotic caspase, caspase-3. Cleavage of ICAD leads to the activation of CAD, which causes double stranded DNA breaks between nucleosomes, resulting in a ladder of oligonucleosomal DNA fragments on gel electrophoresis. This DNA laddering is characteristic of apoptosis. Cells with DNA fragmentation can be identified using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which detects free 3'-hydroxyls in DNA strand breaks.

In addition to apoptotic cells, pyroptotic cells are also TUNEL-positive (22). TUNEL staining during pyroptosis is prevented by YVAD-CMK, a selective irreversible inhibitor of caspase-1, indicating that DNA damage requires caspase-1 activity (22,23). TUNEL positivity can result from non-enzymatic DNA damage, such as that caused by reactive oxygen species (24,25). However, the broad-spectrum nuclease inhibitor, aurintricarboxylic acid, prevents pyroptotic TUNEL staining (22), suggesting that DNA damage requires nuclease activity. The CAD inhibitor protein, ICAD, is not degraded during pyroptosis (22, 23).

Together, these findings suggest that DNA damage in pyroptosis is caused by a by an unidentified caspase-1-dependent nuclease that is different from the CAD nuclease. As a consequence of DNA fragmentation, a protein called high mobility group box chromosomal protein 1 (HMGB1) is released from the nucleus in apoptosis and necrosis (26). Intracellular HMGB1 binds to DNA and serves as a transcriptional regulator. HMGB1 was also identified extracellularly as a potent macrophage stimulant, activating inflammation, and serving as a damage-associated molecular pattern (27,28). In more recent findings, excessive extracellular HMGB1 and oxidation of HMGB1 were found to be responsible for leukocyte recruitment and activation of cytokine release (29,30). These findings suggest that DNA damage during pyroptosis may also mediate release of nuclear HMGB1 to act as an additional inflammatory signal.

The goal of this project was to better understand the mechanism and consequences of DNA damage during pyroptosis. We used *Salmonella* and lethal toxin, as pyroptosis inducers and confirmed the presence of DNA damage using TUNEL staining. Then we identified HMGB1 release from the nucleus in association with DNA damage in pyroptosis We found that extracellular calcium is required for both DNA damage and HMGB1 release from the nucleus. Calcium influx occurs through gasdermin D pores, but our results showed that gasdermin D pores are insufficient to cause DNA damage. Lastly, we found that DNA from cells undergoing pyroptosis does not appear to resemble apoptotic DNA fragmentation using agarose gel electrophoresis.

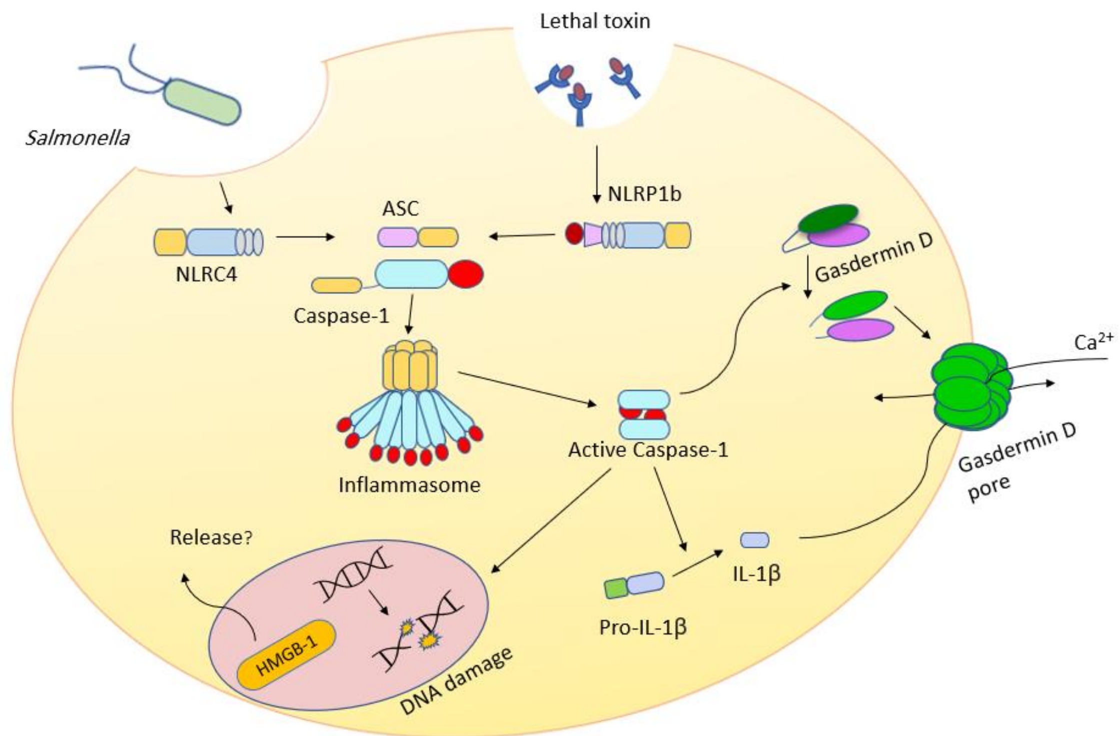

Figure 1. Mechanism of Pyroptosis.

Inflammasomes stimulated by *Salmonella* (NLRC4) and anthrax lethal toxin (NLRP1b) activate caspase-1. Caspase-1 cleaves gasdermin D, releasing the N-terminal pore-forming domain, which inserts into the plasma membrane. Caspase-1 also cleaves the precursor of interleukin 1β (IL-1β) to generate the mature inflammatory cytokine, which is released through gasdermin D pores. Gasdermin D pores also allow influx of water and ions including calcium. In addition to these events, caspase-1-dependent nuclease-mediated DNA damage has been described, but the mechanism and consequences of DNA damage during pyroptosis have not been studied.

2. RESULTS:

2.1. DNA Damage and HMGB1 Release Occur During Pyroptosis

DNA degradation during pyroptosis is caspase-1- and nuclease-dependent. These dependencies were reported after treating *Salmonella*-infected macrophages with a specific caspase-1 inhibitor (YVAD) or a nuclease inhibitor (ATA), both of which eliminated DNA damage (22). During apoptosis, HMGB1 release from the nucleus is a consequence of DNA fragmentation (31). Based on these findings, we hypothesized that DNA damage and HMGB1 release could be associated with pyroptosis.

To confirm the presence of damaged DNA in cells undergoing pyroptosis, we infected murine bone-marrow derived macrophages (BMDMs) with *Salmonella* or treated them with anthrax lethal toxin to trigger pyroptosis, as previously described. We performed TUNEL staining to visualize DNA damage and found that both stimuli stimulated DNA damage (**Figure 2**), consistent with previous findings (22). We then performed

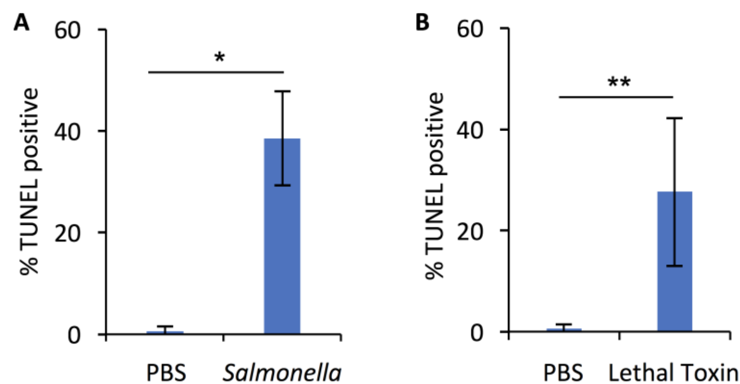

Figure 2. DNA Damage Occurs During Pyroptosis.
BMDM were infected with *Salmonella* or treated with lethal toxin to stimulate pyroptosis. Untreated controls received PBS alone. DNA damage was assessed using TUNEL staining and the percentage of TUNEL positive cells was quantified by fluorescence microscopy. Data are means ± SD, $n = 10$ replicates, pooled from five independent experiments. $*P < 0.05$, $**P < 0.001$, by unpaired t test.

immunostaining for HMGB1 (**Figure 3**). In control cells that received PBS only, HMGB1 was uniformly present in the nucleus. We found that HMGB1 was absent from the nucleus in pyroptotic cells infected with *Salmonella*. In addition, HMGB1 release from the nucleus was observed in lethal toxin-treated cells undergoing pyroptosis. In summary, our results show that HMGB1 release from the nucleus and DNA damage both occur during pyroptosis in macrophages.

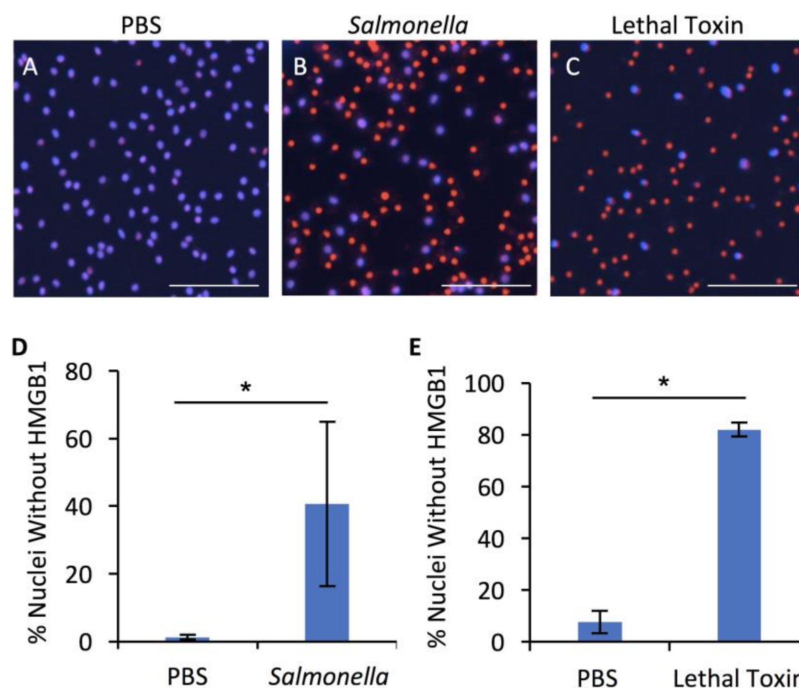

Figure 3. HMGB1 is Released from the Nucleus During Pyroptosis.
BMDM were treated with PBS alone (A), infected with Salmonella (B) or treated with lethal toxin (C) to stimulate pyroptosis. HMGB1 (blue) was visualized using immunofluorescence and nuclei were counterstained using TO-PRO-3 (red). Scale bar = 100µm. The percentage of cells demonstrating absence of nuclear HMBG1 was quantified (D and E); data are means ± SD, n = 6 replicates, pooled from three independent experiments. *$P < 0.05$, by unpaired t test.

2.2. DNA Damage During Pyroptosis Requires Extracellular Calcium

Many cellular DNases require calcium, which is normally kept at a very low concentration inside of cells ~10^{-7}M (32,33). In pyroptosis, calcium influx from the

extracellular medium follows plasma membrane pore formation (34). This led us to the hypothesis that DNA fragmentation in pyroptosis could be calcium-dependent. To test this hypothesis, *Salmonella*-infected macrophages were used to assess DNA damage using medium both with and without calcium. Previous experiments showed that caspase-1-dependent calcium influx occurs in *Salmonella*-infected macrophages, and caspase-1 activation is unaffected in the absence of extracellular calcium (35). Extracellular calcium is also not required for *Salmonella*-induced capase-1-dependent cytokine processing, pore formation, or plasma membrane rupture (32,35).

We infected BMDMs with *Salmonella* in standard medium containing calcium or calcium free medium and assessed DNA damage using TUNEL staining. We found that *Salmonella*-induced DNA damage was significantly reduced in calcium-free medium (**Figure 4**). This demonstrates that DNA damage during pyroptosis is extracellular calcium-dependent.

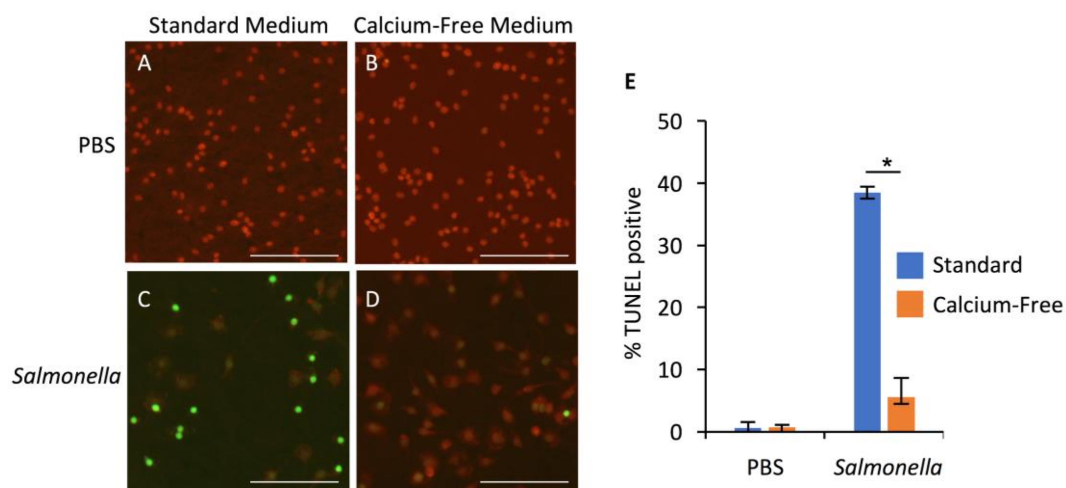

Figure 4. DNA Damage During Pyroptosis Requires Extracellular Calcium. BMDM were treated with PBS alone (A and B) or infected with *Salmonella* (C and D) in standard calcium-containing medium (A and C) or calcium-free medium (B and D). DNA damage was assessed using TUNEL staining (green) and nuclei were counterstained using TO-PRO-3 (red). Scale bar = 100µm. The percentage of TUNEL positive cells was quantified (E); data are means ± SD, $n = 4$ replicates, pooled from two independent experiments. *$P < 0.05$, by unpaired t test.

2.3. HMGB1 Nuclear Release During Pyroptosis Requires Extracellular Calcium

Since we found that extracellular calcium is required for DNA damage, we next tested the hypothesis that HMGB1 release from the nucleus might also be dependent on extracellular calcium. To test this hypothesis, we infected BMDMs with *Salmonella* in standard medium with calcium or medium without calcium, and assessed nuclear HMGB1 using immunofluorescence. We found that *Salmonella* infection stimulated HMGB1 release from the nucleus in the standard medium, but there was no release in the calcium-free medium (**Figure 5**). This leads us to the conclusion that calcium is important in release of HMGB1 from the nucleus, as well as DNA damage.

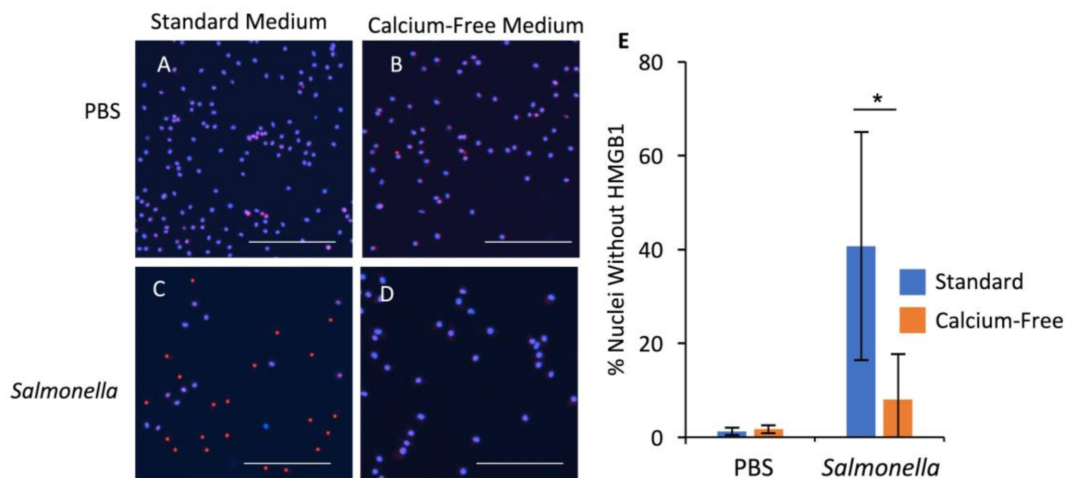

Figure 5. HMGB1 Nuclear Release During Pyroptosis Requires Extracellular Calcium.

BMDM were treated with PBS alone (A and B) or infected with *Salmonella* (C and D) in standard calcium-containing medium (A and C) or calcium-free medium (B and D). HMGB1 (blue) was visualized using immunofluorescence and nuclei were counterstained using TO-PRO-3 (red). Scale bar = 100µm. The percentage of cells demonstrating absence of nuclear HMBG1 was quantified (E); data are means ± SD, $n = 4$ replicates, pooled from two independent experiments. *$P < 0.05$, by unpaired t test.

2.4. *Salmonella* Infection Stimulates DNA Damage in the Absence of Gasdermin D

Gasdermin D membrane pores are generated after the cleavage of full-length gasdermin D protein by active caspase-1 (9). This cleavage generates the N-terminal fragment, allowing its binding and insertion into the plasma membrane and formation of the pores. It also separates the N-terminal fragment from the C-terminal fragment, which act as an inhibitor of pore formation (36). We found that extracellular calcium is required for DNA damage and HMGB1 release, and calcium influx occurs through gasdermin D pores. This suggests the hypothesis that gasdermin D pore formation is essential to DNA damage during pyroptosis. To test this hypothesis, we used gasdermin D knockout cells and infected them with *Salmonella*.

We first measured release of the large cytoplasmic enzyme lactate dehydrogenase (LDH) in the supernatant, as an indicator of cell lysis. We found that there was no LDH release from *Salmonella*-infected gasdermin D knockout cells, while LDH release was detected from wildtype cells under the same experimental conditions (**Figure 6A**). These results confirm previous observations that gasdermin D is required for lysis during pyroptosis (9). After confirming that cells did not release LDH, we performed TUNEL staining to determine if we could detect DNA damage in gasdermin D knockout cells. Surprisingly, we found TUNEL-positive cells with DNA damage in both wildtype and gasdermin D knockout cells after *Salmonella* infection (**Figure 6B**).

These results suggest that in the absence of gasdermin D, *Salmonella* infection induces DNA damage, while the plasma membrane remains intact. Cell death with DNA damage and an intact plasma membrane represents a pattern similar to apoptosis. Recently published findings from another research group demonstrate that caspase-1 activation stimulates apoptosis in gasdermin D-deficient cells (37). Therefore, based on our observations and these

published findings, we hypothesize that gasdermin D knockout cells undergo apoptotic DNA fragmentation during *Salmonella* infection.

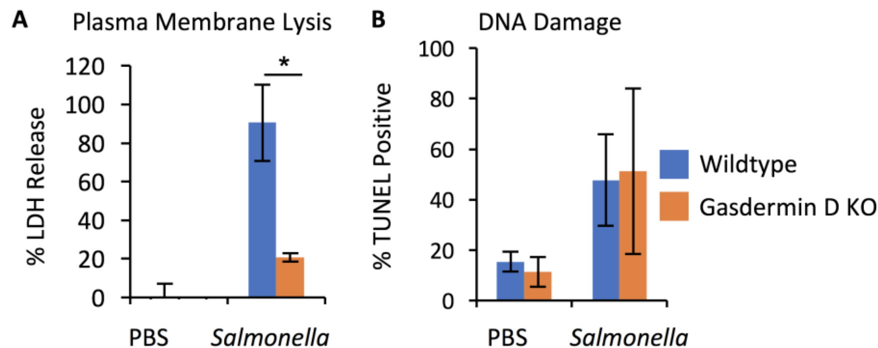

Figure 6. *Salmonella* Infection Stimulates DNA Damage in the Absence of Gasdermin D.
Wildtype or gasdermin D KO BMDMs were treated with PBS or infected with *Salmonella.* Release of the cytoplasmic enzyme LDH into the supernatant during plasma membrane lysis was quantified (A). DNA damage was assessed using TUNEL staining and the percentage of TUNEL positive cells was quantified (B). Data are means ± SD, $n = 3$ replicates in (A) and $n = 4$ replicates, pooled from two independent experiments (B). *$P < 0.05$, by unpaired t test.

2.5. Gasdermin D Pores Alone are Insufficient to Cause DNA Damage

Since caspase-1 triggers apoptosis in gasdermin D knockout cells, we were unable to experimentally determine if gasdermin D is required for pyroptotic DNA damage. In order to determine whether gasdermin D pores are associated with DNA damage, we changed our approach to ask whether gasdermin D pores are sufficient to stimulate DNA damage.

To test this hypothesis, we used immortalized BMDMs transduced to express doxycycline-inducible N-terminal gasdermin D or full-length gasdermin D (38). In the cells expressing inducible N-terminal gasdermin D, doxycycline treatment causes gasdermin D membrane pore formation, in the absence of inflammasome activation. As a control, we used cells with doxycycline-inducible expression of full-length gasdermin D, which does not induce membrane pores.

First, we tested plasma membrane pore formation, by measuring the uptake of the small membrane-impermeant nuclear stain, TO-PRO-3. We verified that addition of doxycycline led to gasdermin D pore formation and observed TO-PRO-3 positive cells with expression of N-terminal, but not full-length gasdermin D (**Figure 7A**). Next, we used TUNEL staining as an indicator for DNA fragmentation. There was no TUNEL staining after doxycycline treatment of either cell line, indicating that no DNA damage occurred (**Figure 7B**). We also used *Salmonella*-infected cells as a positive control for TUNEL staining (**Figure 7C**). Based on these results, we concluded that gasdermin D pores alone are not sufficient to cause DNA damage.

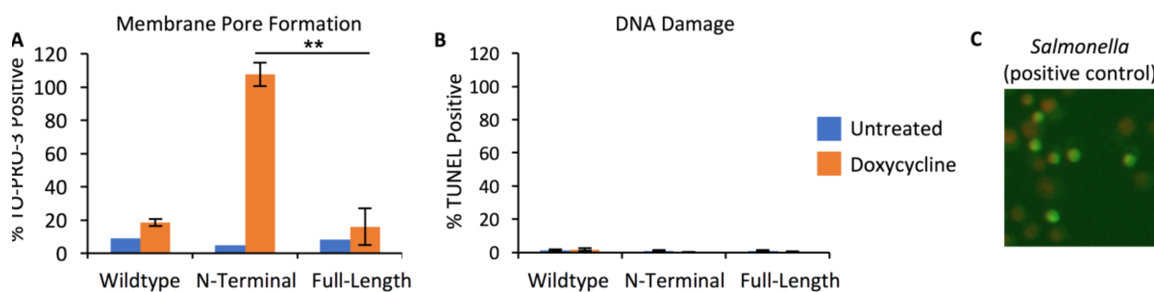

Figure 7. Gasdermin D Pores Alone are Insufficient to Cause DNA Damage. Wildtype BMDMs or immortalized BMDMs transduced to express doxycycline-inducible N-terminal gasdermin D or full-length gasdermin D were treated with doxycycline. Uptake of the membrane-impermeant nuclear stain, TO-PRO-3, by unfixed cells was measured as an indication of plasma membrane pore formation (A). DNA damage was assessed using TUNEL staining and the percentage of TUNEL positive cells was quantified (B). *Salmonella* was used as a positive control in this experiment (C). Data are means ± SD, $n = 4$ replicates in (A) and $n = 6$ replicates in (B), each from one experiment representative of three independent experiments. $^{**}P < 0.001$, by unpaired t test.

2.6. Agarose Gel Electrophoresis of DNA from Pyroptotic and Apoptotic Cells Appears Different

From previous studies, caspases have been identified as the executioners of both apoptotic and pyroptotic processes. Moreover, DNA degradation is one of the downstream effects of caspase activity and is a common hallmark that occurs in both apoptosis and pyroptosis (22, 39). During apoptosis, DNA cleavage between nucleosomes produces double-stranded fragments in multiples of 180-200 base pairs that produce a laddering pattern when separated on agarose gels (39, 40). To determine whether DNA from pyroptotic cells produces a similar pattern, we performed agarose gel electrophoresis of DNA isolated from cells induced to undergo pyroptosis via *Salmonella* infection or lethal toxin treatment (**Figure 8**). Staurosporine was used as a control to stimulate apoptosis, and DNA isolated from staurosporine-treated macrophages demonstrated the expected oligonucleosomal DNA laddering fragments (**Figure 8, lane 4**). DNA from *Salmonella*-infected macrophages, (**Figure 8, lane 2**) and lethal toxin treated macrophages (**Figure 8, lane 3**) demonstrated only one prominent band, similar in appearance to large genomic DNA from PBS-treated controls (**Figure 8, lane 1**). TUNEL staining was also performed in the same experiment as DNA isolation to confirm that DNA damage was present in the pyroptotic cells. These results indicate that DNA damage during pyroptosis does produce oligonucleosomal DNA laddering fragments, as is characteristic of apoptosis.

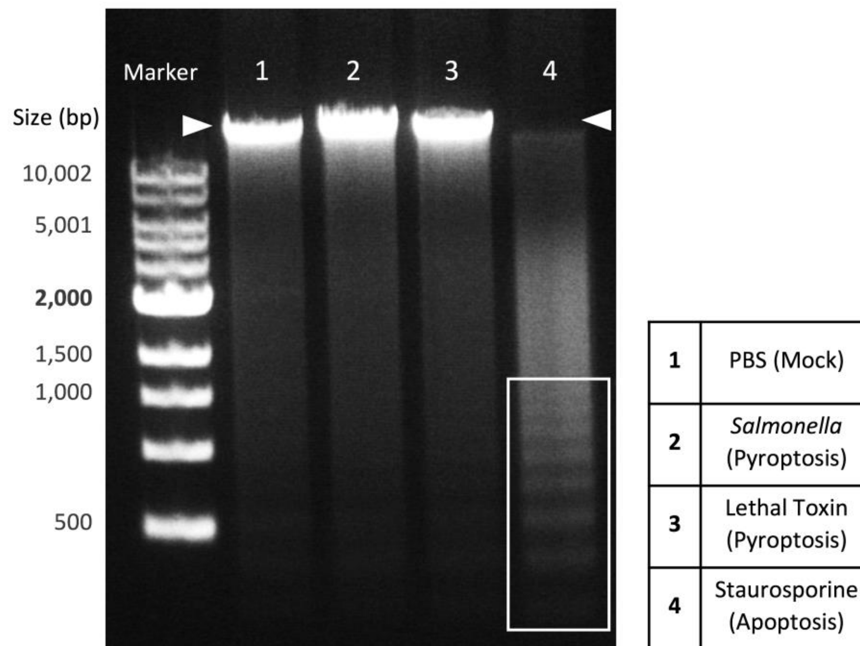

Figure 8. Agarose Gel Electrophoresis of DNA from Pyroptotic and Apoptotic Cells Appears Different.
BMDM were infected with *Salmonella* or treated with lethal toxin to stimulate pyroptosis. Staurosporine was used to induce apoptosis. Control cells received PBS alone. DNA was isolated and separated using agarose gel electrophoresis. Prominent loss of large genomic DNA (white arrow) and appearance of oligonucleosomal DNA laddering (white box) was observed during apoptosis, but not pyroptosis. Results from one experiment representative of four independent experiments are shown.

3. MATERIALS AND METHODS:

Cell culture:

Mice from Jackson (www.jax.org) were used to isolate BMDMs. Cells were cultured from wild type Balb/c, C57Bl/6, and gasdermin D knockout (on the C57Bl/6 background) mice. Mouse stock numbers were C57Bl/6: 000664, Balb/c: 000651, and gasdermin D knockout: 032663. Cells were differentiated at 37°C in 5% CO_2 to allow them to grow for a week, using Dulbecco's minimal essential medium (DMEM) with 10% serum, and 30% L929 supernatant. Cells were seeded using 5% serum DMEM.

Doxycycline inducible macrophages:

Immortalized BMDMs transduced to express doxycycline-inducible gasdermin D were a kind gift from Charles L Evavold et.al. Two types of cells were used: those expressing N-terminal and full-length gasdermin D. N-terminal gasdermin D expressing cells induce gasdermin D pores after doxycycline treatment, whereas full-length gasdermin D expressing cells were used as a negative control and do not form gasdermin D pores.

Pyroptosis inducer:

Salmonella enterica serovar Typhimurium*:*

Salmonella enterica serovar Typhimurium strain SL1344 was used in all experiments as an inducer of pyroptosis. Bacteria were cultured overnight in Luria-Bertani (LB) broth containing + 0.3M NaCl, then diluted 1:15 and grown for three hours before infection. Cells were infected at a multiplicity of infection of 10:1 for 90 minutes at 37°C in 5% CO_2.

Anthrax Lethal Toxin:

Lethal toxin was used as an inducer of pyroptosis and consisted of anthrax lethal factor and anthrax protective antigen, both at a final concentration 1μg /mL. Anthrax lethal factor and

anthrax protective antigen were obtained from List Biologicals. Cells were treated for two hours at 37°C in 5% CO_2.

Reagents:

Staurosporine was used in 1 μM as apoptosis inducer. Cells were treated for six hours at 37°C in 5% CO_2. Staurosporine was obtained from Sigma.

TUNEL staining:

DNA degradation was detected using terminal deoxynucleotidyl transferase dUTP nick end labeling in *Salmonella*, lethal toxin, and staurosporine treated cells. This procedure was performed in 96 well plate, and glass coverslips to improve cells' attachment for two hours, then washed with PBS. DNA-breaks were detected using In Situ Cell Death Detection Kit (Roche).

Lactate dehydrogenase release:

Cell membrane rupture was measured using the levels of LDH release in the supernatant. LDH was measured using Cytotox96 non-radioactive assay (Promega). Absorbance at 490 nm was read at 490 nm by a plate reader (Molecular Devices).

Calculation of the cytotoxicity percentage:

Percent cytotoxicity = ((OD_{490}-experimental − OD_{490}-spontaneous)/(OD_{490}-total lysis − OD_{490}-spontaneous)) × 100

TO-PRO-3 staining:

TO-PRO-3 (Invitrogen) is a membrane impermeable dye that stains nucleic acids and uptake by unfixed cells serves as an indicator of plasma membrane damage, including the formation of gasdermin D pores. TO-PRO-3 was also used in fixed cells as a nuclear stain.

Antibody staining:

After seeding and infecting cells, antibodies labeling was used to detect HMGB1 release from the nucleus. Primary antibody was used against nuclear HMGB1 (Rabbit Ab for HMGB1 from Abcam) in 1:500 dilution. After washing, secondary antibody (goat anti-rabbit IgG from Invitrogen) was used in 1:500 dilution against primary antibody with a fluorescent dye. TO-PRO-3 also was added to label unstained nuclei.

Fluorescence microscopy:

Macrophages were seeded in Greiner 96 Black Flat Bottom Fluotrac plates, 24 well plate, and glass coverslips prior to treatment with staurosporine, anthrax lethal toxin, or *Salmonella*. In all experiments, multiple fields were examined and imaged using a cytation-1 microscope and Gen5 imaging software (BioTek). Five random images were taken for each well in all experiments, including positive and negative controls. The percentage of positive cells was calculated for each separate well.

Agarose gel electrophoresis:

Agarose gels (2% agarose) were used to detect DNA double stranded breaks in DNA from pyroptotic or apoptotic cells. DNA was collected and extracted using Quick-DNA Microprep Ki (Zymo Research). Gels were run for two hours at 80 V, and DNA was visualized using GelRed Staining Solution. Gels were imaged using Gel Doc EZ imager (Bio-Rad).

Statistical analysis:

Calculations, statistical tests, and graphs were all done using Excel. Replicates consisted of separate wells as detailed in the individual figure legends. Unpaired student's t-test was used for comparisons between two groups. *P* values of less than 0.05 were considered statistically significant.

4. DISCUSSION:

Pyroptosis is important in the pathogenesis of human diseases, yet the events occurring during pyroptosis are not completely understood.

In this thesis, we demonstrated that HMGB1 release from the nucleus is present together with DNA damage in pyroptosis after *Salmonella* and lethal toxin treatments. We also showed that both DNA damage and HMGB1 release in pyroptosis are dependent on extracellular calcium. Using doxycycline-inducible cells, we demonstrated that gasdermin D pores are not sufficient to cause DNA damage. Lastly, we found preliminary evidence that the oligonucleosomal double stranded DNA fragments, characteristic of apoptosis, are not produced during pyroptosis.

DNA fragmentation is an important feature of programmed cell death. As extracellular DNA can act as a self-antigen, it can trigger chronic inflammation and autoimmune diseases by activating antigen-presenting cells, pro-inflammatory cytokines, and autoantibody production (41). DNA fragmentation during apoptosis is critically important for preventing inflammation and autoimmunity (34), but the role and mechanism of DNA fragmentation during pyroptosis has not been well-studied. Here we found DNA damage during pyroptotic cell death is combined with HMGB1 release from the nucleus, therefore, acting as a potential potent inflammatory mediator when released from the cell. Additionally, several studies now show that HMGB1 release is sex-associated in pulmonary endothelial cells (42,43). This opens even more questions about whether HMGB1 release is sex-associated with infectious and/or autoimmune diseases.

Apoptotic cells release small DNA fragments into the circulation and these DNA fragments can serve as a diagnostic tool for prenatal testing and diseases such as metabolic disorders and cancer (44,45). The fragment sizes of apoptotic DNA in circulation are not random but rather reflect the length of DNA associated with nucleosomes. Our findings

indicate that, unlike apoptosis, pyroptosis does not produce oligonucleosomal-length double stranded fragments. Future experiments may reveal a novel fragmentation pattern characteristic of pyroptosis. Pyroptotic DNA may someday be similarly measured in circulation and used as a biomarker for diseases.

Our experiments also show calcium dependency of DNA damage and HMGB1 release from the nucleus. Pore formation in the cell membrane allows water and calcium influx into the cell from the extracellular space, which causes DNA degradation. Calcium is well-known for its roles in apoptotic cell death, and activation of caspases and nucleases (46,47). This brought up the question of whether calcium is crucial in pyroptotic DNA damage, which we demonstrated here (Figures 4, 5). Thus, discovery of the calcium-dependent nuclease(s) would be one of the next steps for this project. To narrow the list of potential nucleases, it is also important to know that Dnase1L3 (Dnase γ) is calcium dependent (48), and may contribute to DNA degradation during pyroptosis. Moreover, DNase I, DNase II, cyclophilins, and Endonuclease G are all possible candidates that could contribute to DNA damage during pyroptosis. In the future, an experiment to address this could use gene-specific nuclease knockout cells, to investigate nuclease-specific functions in causing DNA damage using TUNEL to identify the nuclease that causes DNA damage in pyroptosis.

Subsequent experiments in this thesis demonstrated that cell membrane pores, which are caused by the cleavage of gasdermin D, are insufficient on their own to provoke nuclear DNA degradation. Another study confirmed that mitochondrial damage and DNA release from mitochondria to the cytosol require both gasdermin D and E in both pyroptosis and intrinsic apoptosis with caspase activation (49). This suggests that nuclear DNA damage could be included by a mechanism that requires caspase-1 activation but not gasdermin D. In addition, our results here do not exclude the role of other gasdermin family members like gasdermin E in pyroptotic nuclear DNA damage. Another possibility is that another substrate could cause

nuclear membrane damage after nuclease activation. To further investigate the role of gasdermin E in pyroptosis and to characterize similarities in DNA damage in both mitochondria and nuclei, additional caspases like caspase-3 and -9 should be studied as well. This is based in part a study demonstrating that gasdermin E has a role as a promotor of pyroptosis, after its cleavage by caspase-3 (50).

The last experiment in this thesis, is the separation of DNA from pyroptotic cells and comparison between the resulting DNA patterning and apoptotic DNA damage using agarose gel. DNA breaks can be either single or double-stranded breaks, and each has its independent signaling pathway, DNA damage response, clinical effects, and outcomes (51). Thus, it is important to identify the type of DNA breaks in the cells undergoing different processes of cell death in response to varying conditions. Our result here show that DNA appears different in apoptosis than pyroptosis using agarose gel electrophoresis. This finding is consistent with the previously published observation that the CAD inhibitor protein, ICAD remains uncleaved in pyroptotic cells (22,23), suggesting that CAD nuclease activation does not occur during pyroptosis.

One of the future directions for this project is to find the appropriate conditions to separate single-stranded DNA breaks, which could be occurring in pyroptotic cells. One approach would be to use denaturing gels to separate single-stranded DNA breaks. Another option could be the Comet Assay (single-cell gel electrophoresis).

Another way to approach this hypothesis is to use the nucleic acid sequencing-based methods to help in identifying the length and the type of lesions in DNA. This method was used in the quantification of DNA double-strand breaks by a quantitative double strand breaks sequencing (qDSB-Seq) (52).

5. CONCLUSION:

DNA damage in pyroptosis has not previously been well-understood. In this thesis, we found that HMGB1 release from the nucleus is combined with DNA damage in pyroptosis. Additionally, nuclear DNA damage and HMGB1 release are both calcium dependent. We also found that gasdermin D pores are insufficient to cause DNA damage alone in pyroptosis.

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