Characterization of Thiomuscimol: A Novel Small Molecule Pyroptosis Inhibitor

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Abstract

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Pyroptosis, a form of pro-inflammatory programmed cell death mediated by caspase-1 and dependent on gasdermin D, is a crucial element of the innate immune response. Pyroptosis arises from activation of sensor proteins which respond to threats to host health, leading to the formation of multiprotein complexes known as inflammasomes that induce production of pro-inflammatory cytokines. The downstream effects of pyroptosis such as cell rupture and the release of inflammatory signals are implicated in the progression of a number of diseases, indicating that having the ability to control pyroptosis has great therapeutic potential. $GABA_A$ receptor agonist muscimol inhibits pyroptotic lysis. The goal of this thesis was to better understand the mechanism of the muscimol analog thiomuscimol, which inhibits pyroptosis in a unique manner. We used *Salmonella typhimurium* and anthrax lethal toxin as pyroptosis inducers, and we identified the target concentrations of thiomuscimol and that the effects of thiomuscimol were not due to cytotoxicity nor were boosted by a pretreatment. Then we determined that thiomuscimol binds in a reversible manner, and that it inhibits caspase-3 in addition to caspase-1.

Finally, we determined that thiomuscimol is capable of both diffuse active caspase-1 inhibition as well as foci-bound caspase-1 inhibition. Together, these results provide insight into thiomuscimol's mechanism, contributing to the understanding of small molecule pyroptosis inhibition.

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Acknowledgements

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Chapter 1. Introduction

1.1 Pathogen recognition in Innate Immunity

The immune system relies on a delicate balance of specific and nonspecific responses in order to maintain appropriate function and ensure the body's health. One of the body's first lines of defense lies in the innate immune system, where an initial cellular response responds broadly and quickly to invading pathogens. Sensor cells such as neutrophils, dendritic cells and macrophages use pattern recognition receptors (PRRs) in order to recognize molecular structure cues on pathogens known as Pathogen-Associated Molecular Patterns (PAMPs) (*Janeway's Immunobiology*, 9th edition). Different sensor proteins are found in different locations throughout the cell; for example, Toll-like receptors (TLR) are transmembrane proteins that detect PAMPS found on extracellular pathogens or pathogens taken into vesicles. Conversely, nucleotide-binding domain and leucine rich repeat containing (NLR) are cytoplasmic, sensing intracellular pathogens (*Janeway's Immunobiology*, 9th edition). Some sensor proteins are also capable of recognizing downstream consequences of pathogen invasion, or damage signals arising from aging or negative environmental effects (Zheng et al,2020).

1.2 Inflammasome Activation leads to Induction of Pyroptosis

After pathogen invasion has been recognized, there are different actions that can be taken to eradicate the threat. PRR activation from pathogen invasion and PAMP recognition can lead to the formation of inflammasomes, multi-protein signaling complexes comprised of oligomerized sensor proteins, adaptor proteins, and cysteine proteases called caspases that lead to pro-inflammatory cytokine cleavage and activation (Zheng et al., 2020). During the first phase of inflammasome formation, the sensor protein recognizes its stimulus and becomes activated, often leading to the recruitment of the ASC adaptor protein which is comprised of a pyrin domain and a CARD domain and serves as a crucial

interim for many inflammasomes in order to recruit pro-caspase-1. Activated caspase-1 is the main effector molecule during inflammasome activity and is recruited via its CARD domain (Liang et al., 2020). After pro-caspase-1 is recruited by the ASC adaptor protein to the inflammasome and is cleaved to become active caspase-1, it initiates proteolytic cleavage of pro-IL-1ß and pro-IL-18 into active IL-1ß and IL-18, as well as the cleavage of gasdermin D, leading to the formation of the gasdermin D pore. Upon pore formation, there is release of pro-inflammatory cytokines and plasma membrane rupture, leading to the perpetuation of inflammation (Sundaram & Kanneganti, 2021).

1.3 Pyroptosis versus Apoptosis

Pyroptosis differs significantly from the more well-characterized form of "silent" cell death, apoptosis, sometimes arising from different stimuli and enacting a different molecular pathway. Both pyroptosis and apoptosis are crucial for immune health and are forms of programmed cell death (contrasting from accidental cell death necrosis), though apoptosis is non-inflammatory and is reliant on different caspases for activation (Fink & Cookson, 2005). Apoptosis relies on two classes of caspases: initiator caspases (caspase-2,-8,-9, and -10), which are responsible for initiating the cascades responsible for caspase activation, and effector caspases (caspase-3,-6,and -7) which lead to the dismantling of the cell through the packaging of cellular compartments into apoptotic bodies. These two classes of apoptotic caspases differ in structure and in activation but are both typically reliant on the involvement of proapoptotic proteins such as the BCL-2 protein family (Bertheloot et al., 2021). As previously described, pyroptosis is reliant on caspase-1 for pro-inflammatory cytokine activation and cleavage. IL-18 and Il-1ß lead to the activation of other inflammatory pathways, with IL-1ß acting as a pyrogen and leading to fever and IL-18 leading to promotion of IFN-γ production through activated T cells (Garlanda et al., 2013; Nakanishi 2018). Pyroptosis results in cellular swelling and membrane rupture,

leading to the presence of cellular contents in extracellular space, contrasting to the preservation of membrane integrity through membrane blebbing seen in apoptosis (Bertheloot et al., 2021).

1.4 Different Inflammasome Structures lead to Pyroptosis Induction

Innate immune sensor proteins are present throughout the cell, with most of the inflammasome sensor proteins located within the cytosol. Inflammasomes are primarily named for the protein components found within their structures; for example, the NLR family have a C-terminus leucine rich repeat (LRR), and central NACHT domains. These are followed by a CARD domain (NLRC4), pyrin domain (NLRP3,6 and 7) or both (NLRP1) (Zheng et al., 2020). Different sensor proteins are activated by different pathogen signals. The mouse paralog of human NLRP1, NLRP1b, responds to *Bacillus anthracis* lethal toxin. Lethal toxin is a two-component toxin comprised of protective antigen protein which allows entry into the cell, and the lethal factor protease (Zheng et al., 2020). Conversely, NLRC4 is activated by gram-negative bacteria like *Salmonella enterica* serovar Typhimurium. During *Salmonella* infection, the bacteria use microneedle-like structures known as type 3 secretion systems (T3SSs) in order to inject protein effector molecules into the cell's cytosol. Murine macrophages have multiple NAIP proteins that are activated both by the T3SS needle and bacterial flagellin (Naseer et al., 2022). These NAIP proteins recognize the bacterial components, and activate NLRC4, leading to NLRC4 oligomerization, as well as ASC adaptor protein and caspase-1 activation and recruitment (Naseer et al., 2022; Zheng et al., 2020). Though different inflammasomes are activated by different stimuli, they largely lead to the same end result of caspase-1 recruitment and activation, proinflammatory cytokine release, and pyroptosis. It is important to mention that inflammasome sensors that contain CARD domains themselves are able to directly interact with caspase-1, activating the cysteine protease in an ASC-independent manner (Figure A).

Inflammasome sensors that lack CARD domains within their structure are reliant on ASC-dependent caspase-1 activation through inflammasome formation (Jorgensen & Miao, 2015).

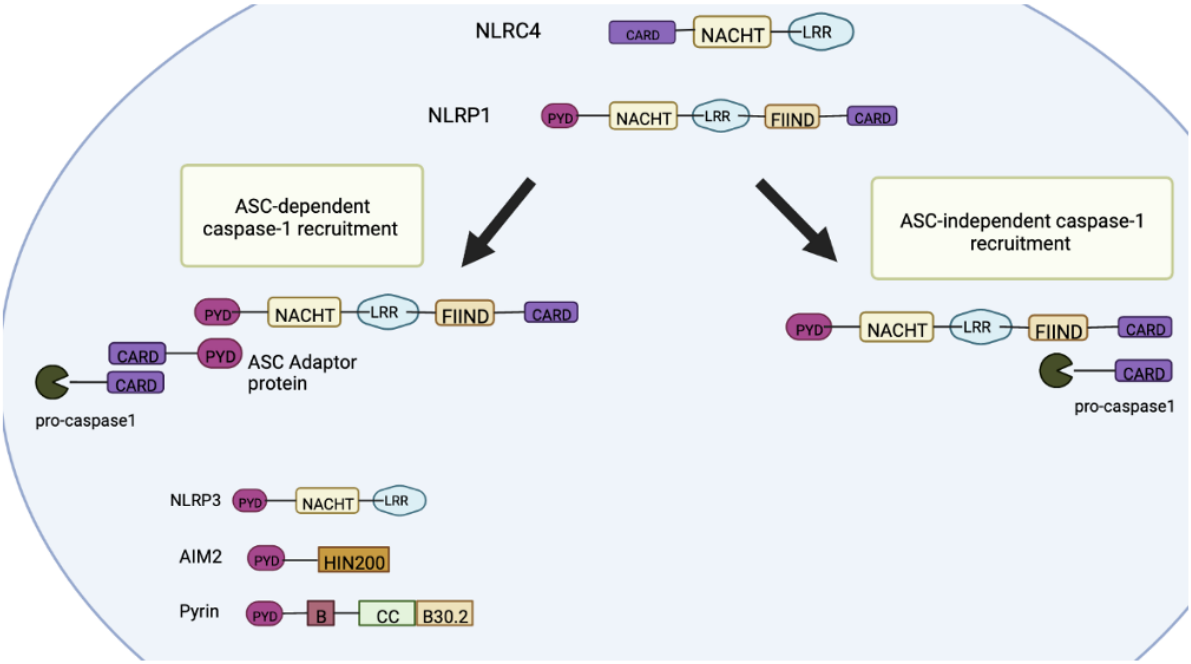

Figure A. Methods of Caspase-1 Recruitment. Inflammasomes that do not contain CARD domains within their structures must recruit ASC adaptor protein for caspase-1 recruitment. Inflammasomes that have CARD domains in their structures (NLRC4, NLRP1) are capable of direct caspase-1 recruitment without the ASC adaptor protein.

1.5 Clinical Relevance of Pyroptosis Inhibition

Though pyroptosis plays an important role in immune defense and offers early protection against intracellular pathogens, a surplus of this proinflammatory cell death is associated in multiple disease states and negative clinical outcomes (Jorgensen & Miao, 2015). An example of one such implication is the role of pyroptosis within the progression of sepsis, a disease with high fatality arising from excessive host response and organ dysfunction following infection. Though moderate pyroptosis early in infection can be protective, excessive pyroptosis can lead to an uncontrolled inflammatory response, progressing the disease state and contributing to a poorer prognosis (Zheng et al., 2021).

Outside of sepsis, pyroptosis is associated with the pathogenesis of Alzheimer's disease (Venegas et al, 2017), irritable bowel disease, (Yuan et al., 2018), and cancers such as melanoma, colorectal cancer, and hepatocellular carcinoma (Yu et al., 2021). Because of the detrimental effects of excessive pyroptosis, there is great therapeutic potential for assisting in prevention and treatment of these disease states through a mechanism of controlled pyroptosis inhibition.

1.6. Small Molecule Pyroptosis Inhibition

We previously found that the amino acid glycine is capable of inhibiting the final lytic event in the pyroptosis cascade (Brennan & Cookson, 2000; Fink & Cookson,2006). Importantly, glycine does not impact upstream caspase-1 activation, gasdermin D pore formation, or cytokine secretion (Loomis et al., 2019). Glycine is an agonist for neuronal glycine receptors, and a small-scale, hypothesis-driven small-molecule screen revealed that the neuronal $GABA_A$ receptor agonist muscimol also inhibits pyroptotic lysis (Loomis et al., 2019). Interestingly, a conformationally restrained muscimol analog (4,5,6,7-tetra-hydroisoxazolo(5,4-c)pyridin-3-ol (THIP)) which is also active at the $GABA_A$ receptor was not protective against pyroptotic lysis, indicating that the $GABA_A$ receptor activity is not sufficient for prevention of pyroptotic lysis (Loomis et al., 2019).

1.7. Identification of Thiomuscimol's Unique Pyroptosis Inhibition

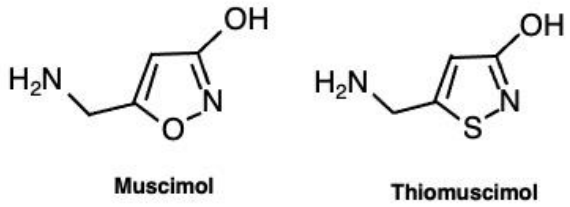

Figure B. Muscimol and Thiomuscimol Chemical Structures.
GABAA receptor agonists muscimol and thiomuscimol bear almost identical structures. Note the replacement of Oxygen with Sulfur at the base of the molecule's five membered ring.

Based on these findings, our group then tested muscimol analogs and identified thiomuscimol as inhibitor of pyroptotic lysis. Like muscimol, thiomuscimol is a $GABA_A$ receptor agonist, and is identical in structure to muscimol with the exception of a sulfur replacement within the five-membered ring (Figure B). Outside of its activity related to the $GABA_A$ receptor, thiomuscimol also inhibits protein disulfide isomerases (PDI), an enzyme family located primarily in the endoplasmic reticulum that functions as chaperone proteins to reconstruct misfolded proteins (Zhao et al.,2015).

To analyze thiomuscimol's ability to inhibit pyroptotic lysis, our group compared thiomuscimol and muscimol's ability to inhibit pyroptotic lysis by measuring the release of the large cytoplasmic protein, lactate dehydrogenase (LDH) as a marker of lysis in the presence of *Salmonella* and anthrax lethal toxin. We found that thiomuscimol was similarly able to inhibit LDH release for both pyroptotic stimuli. Interestingly, when ASC-citrine expressing bone marrow derived macrophages (BMDM) were exposed to *Salmonella* in the presence of thiomuscimol or muscimol, thiomuscimol was uniquely able to inhibit ASC-citrine foci formation and the uptake of membrane impermeant nuclear dye To-PRO-3 (where uptake serves as a marker of gasdermin D pore formation). Muscimol, like glycine, only inhibited the final lytic event, allowing ASC-citrine foci formation and To-PRO-3 uptake to occur. These results were similarly observed during exposure to anthrax lethal toxin, with thiomuscimol treated cells showing a significant decrease in To-PRO-3 uptake, while muscimol did not.

To ensure that thiomuscimol was not blocking *Salmonella's* ability to translocate T3SS effector proteins and thus activate NAIP proteins, BMDM were infected with *Salmonella* containing a plasmid encoded T3SS adenylate cyclase fusion protein (SspA-Cya), in the presence or absence of thiomuscimol or muscimol. Translocation efficiency was assessed by measuring cAMP production by ELISA. cAMP levels were not decreased in the

presence of thiomuscimol, indicating that thiomuscimol was not blocking *Salmonella* type III secretion system effector protein translocation. Taken together, these results indicate that thiomuscimol has a unique and upstream mechanism to muscimol in prevention of pyroptosis, alluding to inhibition during the formation of inflammasomes.

1.8 Aim of Thesis

Because of the identification of thiomuscimol as a small molecule inhibitor of pyroptosis through a unique mechanism, the goal of this thesis project was to further elucidate the mechanism of action of thiomuscimol's unique inhibition of pyroptosis, specifically inflammasome formation (Figure C). We used *Salmonella* and lethal toxin as pyroptosis inducers, and we identified the target concentrations of thiomuscimol and that the effects of thiomuscimol were not due to cytotoxicity nor were boosted by a pretreatment. Then we determined that thiomuscimol binds in a reversible manner, and that it inhibits caspase-3 in addition to caspase-1. Finally, we determined that thiomuscimol is capable of both diffuse active caspase-1 inhibition as well as foci-bound caspase-1 inhibition.

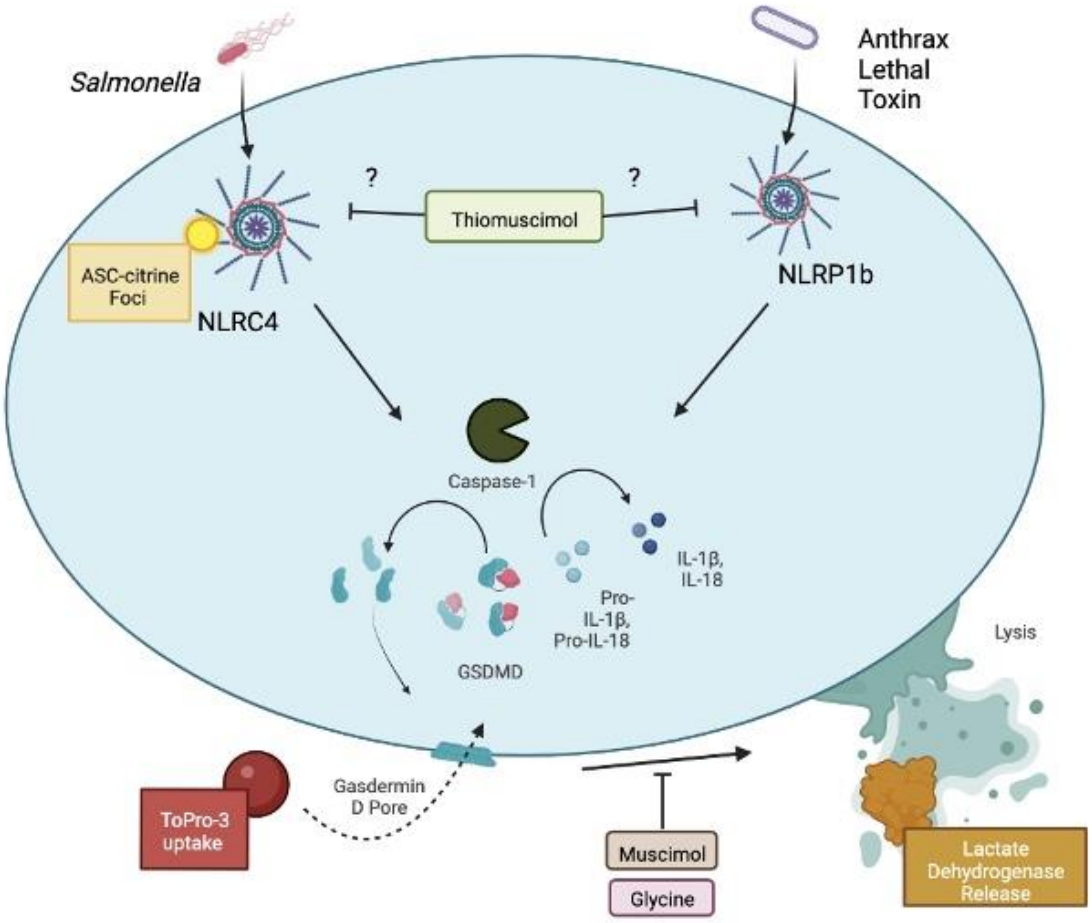

Figure C. Proposed Thiomuscimol Inhibition during Pyroptosis Cascade.
Thiomuscimol was found to act upstream of both muscimol and glycine, which prevent pyroptotic lysis without inhibition of inflammasome activation, caspase-1 activation, and gasdermin d pore formation. Thiomuscimol inhibits ASC citrine foci formation (inflammasome activation), caspase-1 during both *Salmonella* infection and anthrax lethal toxin exposure, To-PRO-3 uptake, and LDH release (as a marker of pyroptotic lysis).

Chapter 2. Materials & Methods

2.1 Cell Culture

Murine bone marrow derived macrophages (BMDM) were isolated from Balb/c, ASC knockout (C57BL/6 background), ASC Citrine (C57BL/6 background) mice (Jackson labs). BMDMs were grown for seven days at 37°C in 5% CO2 in Dulbecco's Minimal Essential Medium (DMEM) containing 10% Serum Plus medium supplement, 5 mM Hepes, 100 U/mL penicillin and streptomycin, and 30% L929 cell supernatant for differentiation. Cells were collected with PBS containing 1 mM EDTA, and seeded in DMEM 5% Serum without phenol red.

2.2 Pyroptosis and Apoptosis Inducers

Salmonella Typhimurium (SL1344) were cultured in Luria Bertani Broth containing 0.3M NaCl overnight, before a 1:15 dilution three hours prior to infection. An MOI of 10:1 was used to infect macrophages for 90 minutes to 2 hours for pyroptosis induction. Anthrax lethal toxin was made utilizing 1 ug/mL protective antigen and 1 ug/mL lethal factor (List Biological), and cells were exposed to anthrax lethal toxin for three hours for pyroptosis induction. Staurosporine (Sigma Aldrich) was used in a concentration of 1 uM for 90 minutes at 35°C in 5% CO_2 for apoptosis induction. In the case of the time-delay apoptosis induction experiment, thiomuscimol (1mM) or media alone was added to cells after the 90 min staurosporine exposure.

2.3 Reagents

Thiomuscimol (Cayman Chemicals, Santa Cruz) was reconstituted at 10 mM in serum free (SF) DMEM and stored as frozen aliquots at -20°C until use. Muscimol hydrobromide (Sigma Aldrich) was reconstituted at 200 mM in SF DMEM and was stored at 4°C. PACMA31 (Cayman Chemicals) was reconstituted 50 mM in 100% DMSO and CCF642 (Cayman Chemicals) was reconstituted at 13 mM in 100% DMSO. Both PACMA31 and CCF642 were stored as frozen aliquots at -20°C until use. Both were then diluted to

target concentrations (2.25 uM for PACMA31, 5 uM for CCF642) in a standardized 1% DMSO.

2.4 Cellular Assays

2.4.1 Cellular Viability Assays

Cellular viability was assessed by looking at cellular ATP production using Cell Titer Glo 2.0 Cell Viability assay (Promega). After pyroptosis induction was completed, the Cell Titer Glo 2.0 reagent was added to the wells in triplicate, with a media only control. The luminescent signal was read after a ten-minute stabilization period, within thirty minutes of reagent addition to the plate.

2.4.2 LDH Release Assay

After pyroptosis induction in the presence or absence of drug conditions, cell rupture was analyzed by measuring LDH present in the supernatant using the Cytotox96 Kit (Promega). Maximum LDH release was obtained by exposing cells to the detergent-based lysis buffer included in the Cytotox96 kit for 30 minutes. LDH release was determined from triplicate samples and calculated as 100 x (experimental LDH – spontaneous LDH)/(maximum LDH- spontaneous LDH).

2.4.3 Caspase-3 Activity Assay

After pyroptosis or apoptosis induction, caspase-3 activity was measured by utilization of the SensoLyte Homogenous AMC Caspase-3/7 Fluorometric Assay Kit (Anaspec).

2.5 Kinetic Microscopy

BMDM were seeded on 96 well optic plates and were exposed to pyroptosis inducers in presence or absence of drugs to measure pyroptosis inhibition. In experiments where ASC foci formation was assessed to analyze NLRC4 inflammasome formation, ASC-citrine macrophages were utilized. Nuclear dye To-PRO-3 was used to measure formation of the

gasdermin D pore. Cellular morphology, ASC foci formation and To-PRO-3 uptake were all observed over time by utilization of a Cytation1 imaging system running Gen5 version 3.11 (Biotek).

2.6 Immunofluorescence Microscopy

BMDM were seeded on poly-D-lysine coated coverslips in optical 24 well plates and were stained with the FLICA FAM-YVAD-FMK activity probe (ImmunoChemistry Technologies) for caspase-1 following pyroptosis activation with inducers and drugs in media containing 5 mM glycine. After FLICA staining, cells were fixed with BD Cytofix/Cytoperm (Fisher), and To-PRO-3 was used as a nuclear stain for cell quantification. Imaging of active caspase-1 was performed on a Leica SP8X confocal system running the LasX imaging software using a 63x NA 1.4 oil immersion objective.

2.7 Quantification and statistical analysis

Statistical tests were run using Excel or GraphPad Prism. Statistical tests used are indicated in each figure legend.

Chapter 3. Results

3.1 Thiomuscimol does not Inhibit Inflammasomes through Cytotoxicity

We first sought to determine whether thiomuscimol causes cellular toxicity, which could nonspecifically inhibit inflammasome activation. To determine if the cells exposed to thiomuscimol are metabolically compromised, we treated cells with muscimol and thiomuscimol using a range of previously established inhibitory concentrations. We assessed metabolic activity by quantifying cellular ATP content (Figure 1). Thiomuscimol at the highest concentration of 2 mM caused a significant decrease in cellular ATP to about 60%, and the second highest concentration of 0.67 mM to about 80% (Figure 1). We did not observe significant decrease in cellular ATP at 0.22 mM, where we previously observed ASC-citrine foci formation inhibition. We also infected cells with *Salmonella* to induce NLRC4-dependent pyroptosis and found a reduction in cellular ATP (Figure 1) in infected pyroptotic cells compared to uninfected cells. We also used another cellular viability assay that quantified cellular NAD/NADPH, but we found that during infected conditions NAD/NADPH concentrations were significantly altered by bacterial metabolic activity by including a media and bacteria control (not shown). We found that ATP activity was not significantly impacted by bacterial metabolism by using the same control and proceeded with this viability assessment for following experiments. We observed a dose-dependent effect of thiomuscimol, but not muscimol, to rescue cellular ATP (Figure 1) in *Salmonella*-infected cells. The rescue of cellular ATP in pyroptotic cells was statistically significant at a concentration where thiomuscimol alone did not greatly impact cellular viability, leading to preserved cellular ATP content in *Salmonella*-infected cells when compared to muscimol and vehicle controls (Figure 1). For future examination of thiomuscimol, concentrations below 2mM were selected due to the reduction in cellular ATP at 2 mM.

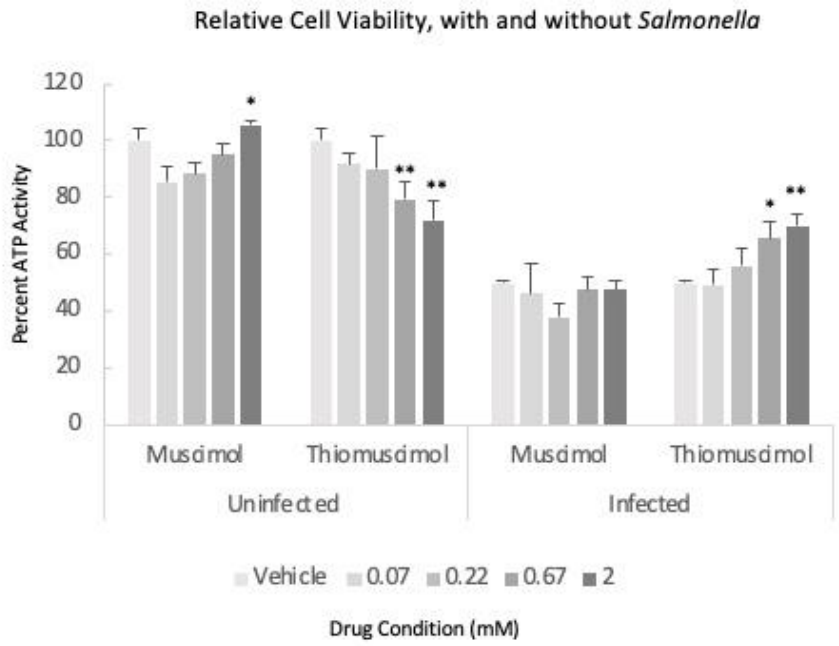

Figure 1. Thiomuscimol, unlike Muscimol, significantly rescues cell viability during Salmonella infection.

Bone Marrow Derived Macrophages (BMDM)were infected with *Salmonella* to stimulate pyroptosis through the NLRC4 inflammasome or exposed to drug conditions in DMEM alone. Vehicle controls in both conditions received DMEM alone. Cellular viability was assessed in the presence or absence of thiomuscimol or muscimol using the cellular Viability Assay Cell Titer Glo 2.0. Data are means +- SD, n=3 replicates, representative of two independent experiments. Stars (*) indicate statistical significance against infected or uninfected vehicle condition, respectively. The presence of thiomuscimol at the two highest concentrations allowed for a significantly higher percent ATP production in *Salmonella* infected cells, in a dose-dependent manner. No muscimol + *Salmonella* conditions were found to be significant compared to *Salmonella* alone. In uninfected conditions, the lowest concentration of muscimol had significantly more ATP activity, while the two highest concentrations of thiomuscimol were found to have significantly lower. Infected vehicle control cells had significantly lower ATP activity than uninfected ($P<0.01$). *$P < 0.05$, **$P < 0.01$ (One way ANOVA, Dunnett's Post-hoc test).

3.2 Thiomuscimol's Active Concentrations are from 1 mM to 0.25 mM

Upon determining that thiomuscimol does not inhibit inflammasome formation via cytotoxicity, we conducted a dose response curve to elucidate the ideal thiomuscimol concentration for maximum inflammasome inhibition with limited cytotoxicity. Cells were exposed to a two-fold dilution of thiomuscimol, ranging from 1 mM to 0.03 mM, in the presence and absence of *Salmonella.* In order to observe inflammasome formation and pyroptotic consequences over time, we used macrophages expressing ASC tagged with the fluorescent protein, citrine (Tzeng et al., 2016) and used live cell fluorescence microscopy to monitor ASC-citrine foci formation as an indicator of NLRC4 inflammasome activation. We simultaneously assessed uptake of the membrane impermeant dye To-PRO-3 as an indicator of gasdermin D pore formation. During *Salmonella* infection, thiomuscimol potently inhibited ASC-citrine foci formation from 0.125 mM to its highest concentration, 1 mM (Figure 2A). Thiomuscimol also inhibited To-PRO-3 uptake moderately at 0.25 mM to potently at 1 mM (Figure 2C). Unlike thiomuscimol, muscimol did not inhibit either ASC-citrine foci formation or To-PRO-3 uptake (Figure 2B and 2D). Together, these results indicate that thiomuscimol inhibits both gasdermin D pore formation and upstream NLRC4 inflammasome activation in a dose-dependent manner.

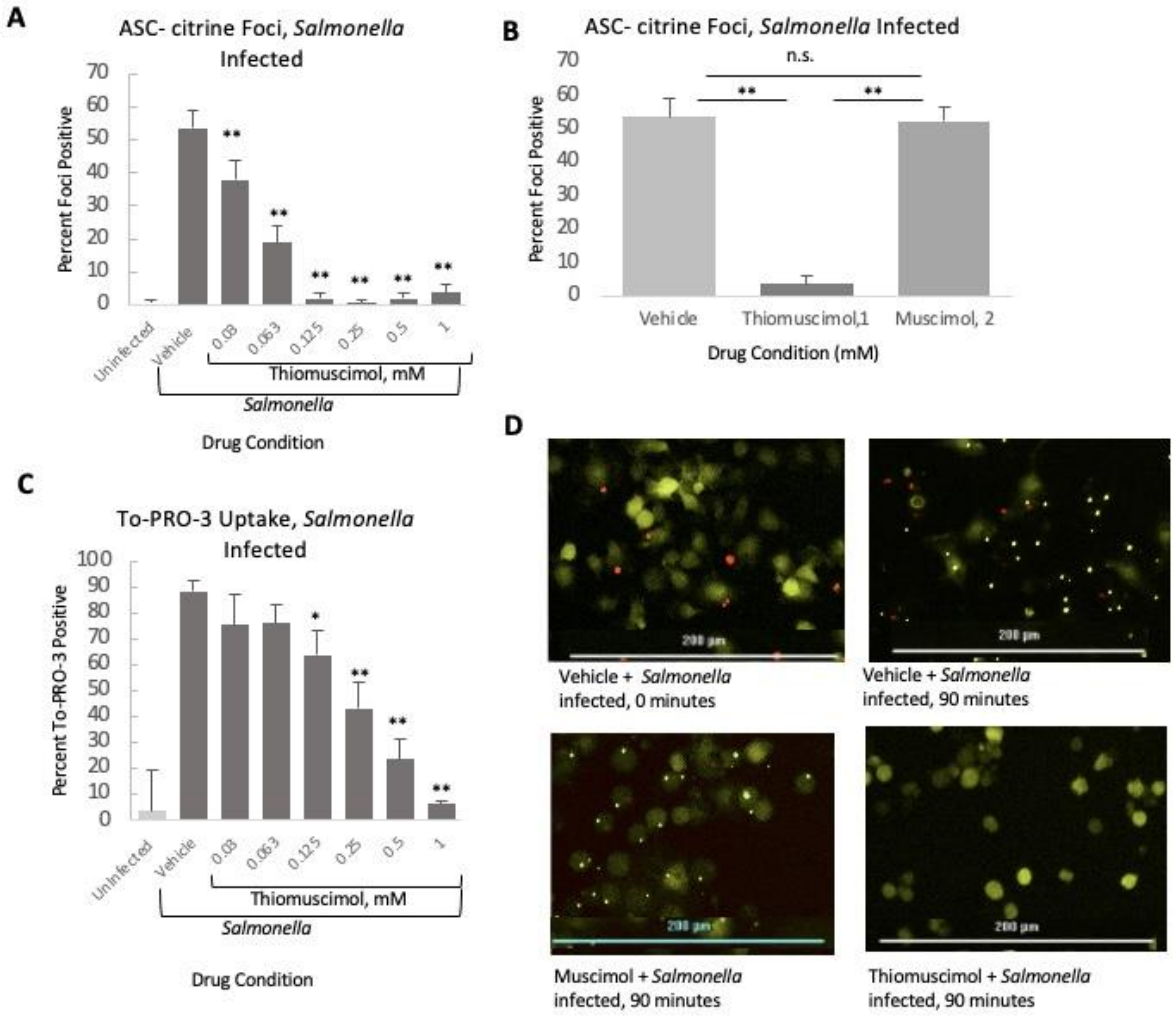

Figure 2. Thiomuscimol inhibits ASC-citrine foci formation and To-PRO-3 uptake in a dose-dependent manner.

Bone Marrow Derived Macrophages (BMDM)were infected with *Salmonella* to stimulate pyroptosis through the NLRC4 inflammasome. Untreated controls received DMEM alone. Inflammasome activation was assessed in the presence or absence of muscimol and thiomuscimol using fluorescent citrine tagged ASC adaptor proteins, visualized over time via kinetic microscopy(D). Data are means +- SD, n=3 replicates, representative of two independent experiments. Thiomuscimol at the highest concentration was found to significantly decrease foci formation (B) when compared to muscimol and to vehicle control. Thiomuscimol's inhibition of Foci formation and To-PRO-3 uptake was observed in a dose dependent manner (A, C, D). Stars (*) indicate statistical significance against Infected vehicle control group (A, C). *P < 0.05, **P < 0.01 (A-C) , n.s. nonsignificant(A) (unpaired t-test(A))(One-way ANOVA Dunnett's Post-hoc test(A,C)).

3.3 Pretreatment does not Optimize Thiomuscimol's Inflammasome Inhibition

To determine if the protective effects of thiomuscimol against both ASC-citrine foci formation and To-PRO-3 uptake could be further optimized by pretreatment, we exposed cells to thiomuscimol for 1 hour prior to *Salmonella* infection. We found that pretreatment did not lead to a significant difference in foci formation when compared to the same concentration of thiomuscimol without pretreatment (Figure 3A), nor for To-PRO-3 uptake (Figure 3B). Additionally, increased cellular exposure to thiomuscimol did not match the rescuing effects previously seen during *Salmonella* infection, with thiomuscimol not significantly rescuing ATP production under pretreatment conditions (Figure 3C). Pretreatment was therefore not included in subsequent experiments.

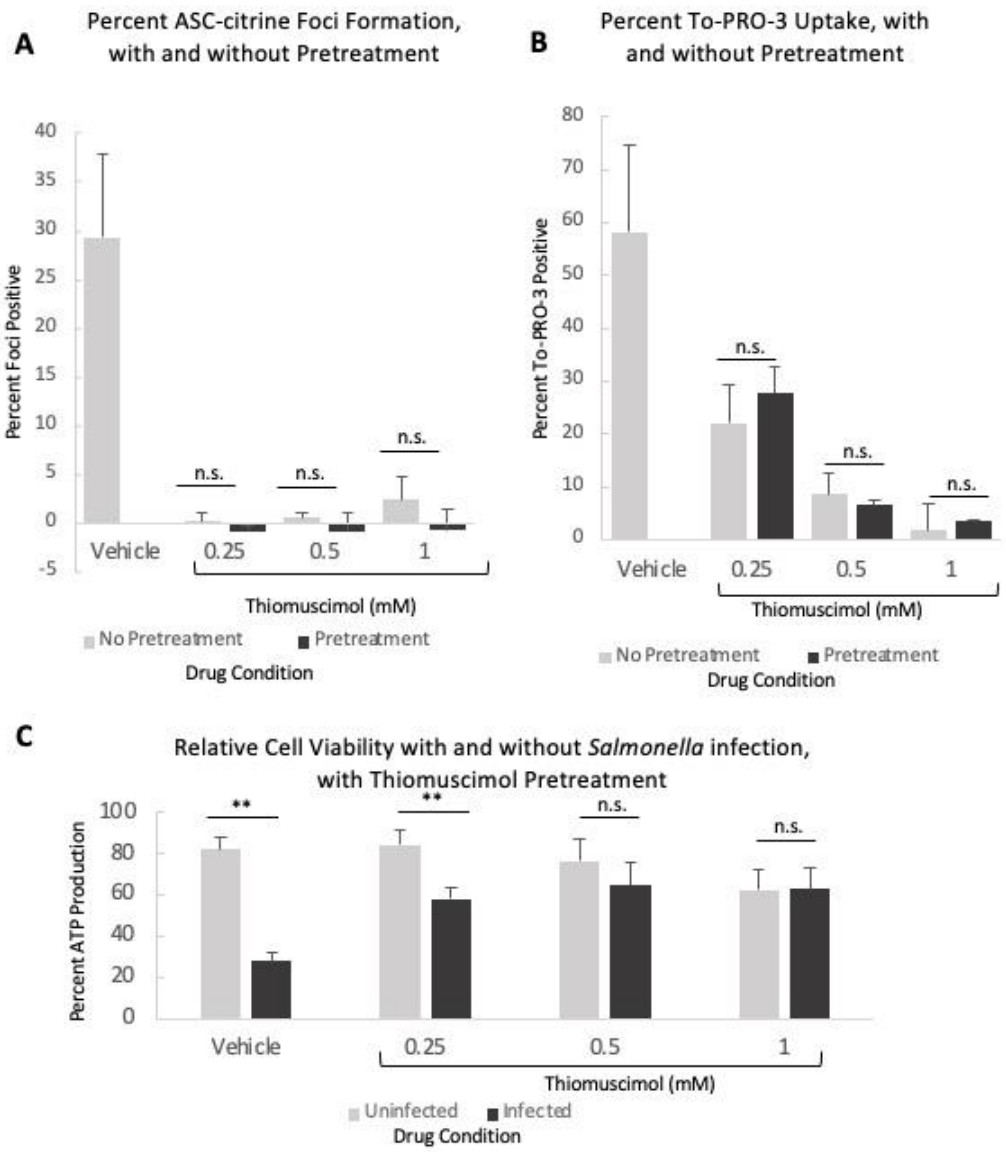

Figure 3. Pretreatment does not significantly assist in Thiomuscimol's protective effects.
Bone Marrow Derived Macrophages (BMDM) were infected with *Salmonella* to stimulate pyroptosis through the NLRC4 inflammasome. Untreated controls received DMEM alone. Inflammasome activation was assessed in the presence or absence of muscimol and thiomuscimol using fluorescent citrine tagged ASC adaptor proteins, visualized over time via kinetic microscopy for 1h (A, B). Cellular viability was assessed in the presence or absence of thiomuscimol or muscimol using the cellular Viability Assay Cell Titer Glo 2.0 (C). Data are means +- SD, n=3 replicates, representative of two independent experiments. *P < 0.05, **P < 0.01, n.s. nonsignificant (unpaired t-test).

3.4 Thiomuscimol Binds in a Reversible Mechanism

To further understand the mechanism of thiomuscimol's inhibition of ASC foci, we sought to understand if thiomuscimol was binding to its target in a reversible or irreversible fashion. Cells were infected with *Salmonella* for one hour in the presence or absence of thiomuscimol at the target concentrations while ASC-citrine foci formation and To-PRO-3 uptake were assessed. Then, the medium was removed and replaced with fresh DMEM lacking thiomuscimol, and ASC-citrine foci and To-PRO-3 uptake were again assessed. We calculated the percent of cells that had formed ASC-citrine foci or taken up To-PRO-3 before removal and after removal, assessing the change in foci formation and To-PRO-3 uptake between the two conditions (Figure 4). As expected, *Salmonella* infected cells treated with vehicle alone formed ASC-citrine foci and demonstrated To-PRO-3 uptake in the first hour, with minimal additional foci formation and To-PRO-3 between the 1h and 2h time points (Figures 4A,4B). However, after thiomuscimol was removed, there was a significant increase in ASC-citrine foci formation at all tested concentrations (Figure 4A, 4C). This effect was also observed in To-PRO-3 uptake (Figure 4B,4C). Taken together, these data indicate that thiomuscimol reversibly inhibits inflammasome inhibition and gasdermin D pore formation.

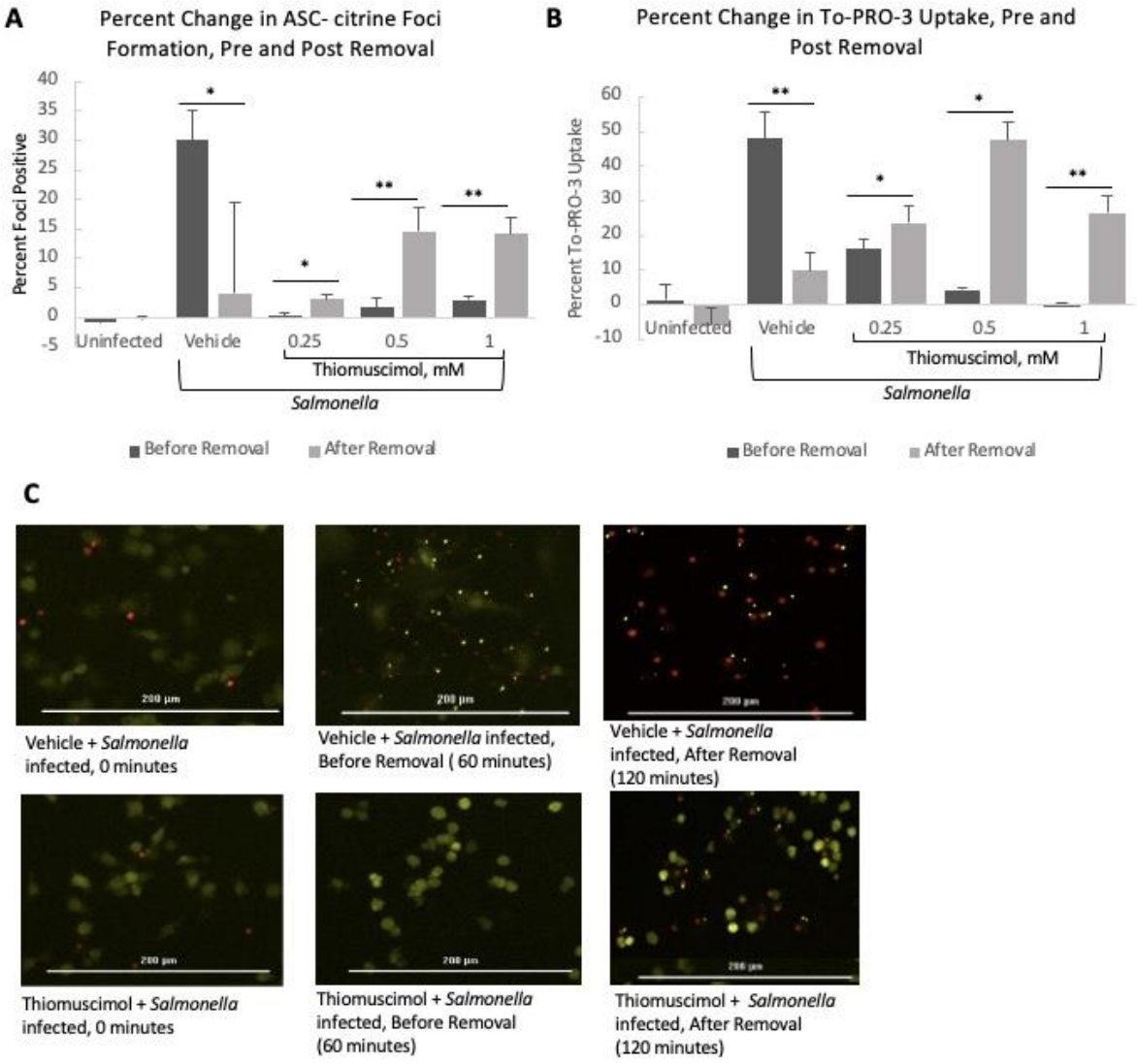

Figure 4. Removal of thiomuscimol after *Salmonella* infection allows for significant ASC-citrine foci formation and To-PRO-3 Uptake.

Bone Marrow Derived Macrophages (BMDM) were exposed to *Salmonella* and three concentrations of Thiomuscimol (0.25 mM, 0.5 mM, 10 mM) for one hour. Media containing drug and *Salmonella* was removed and replaced with fresh DMEM containing To-PRO-3 (1 uM). Inflammasome activation was assessed in the presence or using fluorescent citrine tagged ASC adaptor proteins, visualized over time via kinetic microscopy (A-C). Data are means +- SD, n=3 replicates, representative of two independent experiments. *P < 0.05, **P < 0.01, n.s. nonsignificant (paired t-test).

3.5 Thiomuscimol does not Redirect from Pyroptosis to Apoptosis, and Blocks Caspase-3 Activation

We previously found that thiomuscimol blocks caspase-1 activation in response to lethal toxin exposure and *Salmonella* infection by utilizing the FLICA (FAM-YVAD-FMK) activity probe. Prior studies demonstrate that in the absence of caspase-1, inflammasomes are redirected from pyroptosis to apoptosis and caspase-3 is activated instead (Bast et al., 2014). We therefore hypothesized that thiomuscimol-mediated inhibition of caspase-1 activation may also redirect *Salmonella*-infected macrophages away from pyroptosis and toward apoptosis. To test this hypothesis, we examined caspase-3 activity during *Salmonella* infection in the presence of thiomuscimol by using a fluorescent substrate cleavage assay. Cells were exposed to a known apoptosis inducer, staurosporine, to provide a baseline level of caspase-3 activity during apoptosis (Figure 5A). Uninfected cells exhibited a background level of caspase-3 activity, which was not affected by any concentration of muscimol. *Salmonella*-infected cells had slightly lower levels of caspase-3 activity, though those that were treated with muscimol had slightly higher caspase-3 activity at the highest concentrations. Since we have previously shown that muscimol prevents pyroptotic lysis, the observed shift in caspase-3 activity could represent redirection from pyroptotic lysis to apoptosis. However, both *Salmonella*-infected and uninfected cells treated with thiomuscimol exhibited a dose-dependent decrease in caspase-3 activity (Figure 5A). This result indicates that thiomuscimol does not redirect *Salmonella*-infected cells from pyroptosis or apoptosis, contrasting with both caspase-1 genetic knockout and the modest effect of muscimol on caspase-3. Additionally, these results suggest the possibility that thiomuscimol has an effect to limit caspase-3 activation. To test this hypothesis, BMDMs were exposed to the apoptosis inducer staurosporine, in the presence or absence of muscimol or thiomuscimol (Figure 5A). Staurosporine exposed cells exhibited an increase in caspase-3 activity, which was not inhibited by muscimol at any

concentration. Thiomuscimol treated staurosporine exposed cells exhibited a dose-dependent decrease in caspase-3 activity, where all concentrations of thiomuscimol led to significantly lower caspase-3 activity. (Figure 5A). To determine whether thiomuscimol interferes with the caspase-3 assay, we induced apoptosis with staurosporine before adding thiomuscimol (1mM) or DMEM alone immediately prior to assessing caspase-3 activity. Notably, there was no difference in caspase-3 activity in cells that received medium alone or thiomuscimol, indicating that thiomuscimol does not interfere with the caspase-3 activity assay (Figure 5B). Taken together, these results indicate that thiomuscimol does not redirect from pyroptosis to apoptosis in *Salmonella* -infected cells. In addition, thiomuscimol is capable of inhibiting activation of caspase-3 during staurosporine exposure.

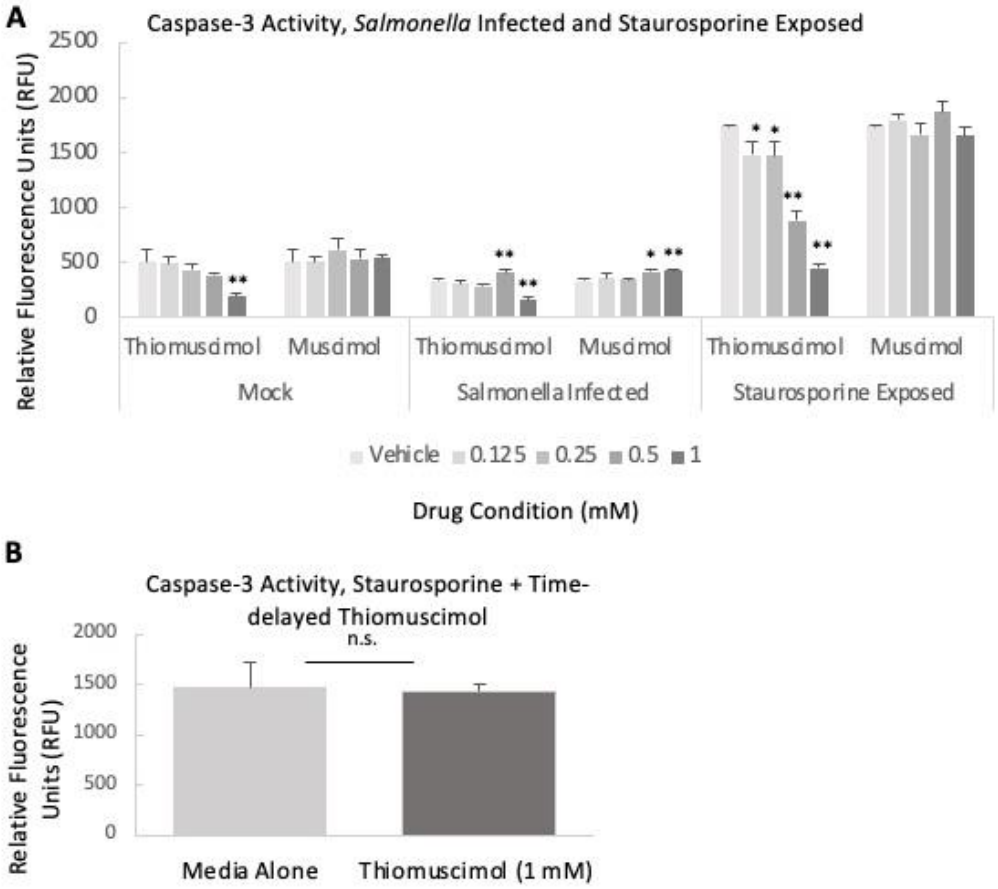

Figure 5. Thiomuscimol inhibits activation of Caspase-3 and does not redirect from apoptosis to pyroptosis.

Bone Marrow Derived Macrophages (BMDM)were infected with *Salmonella* to stimulate pyroptosis through the NLRC4 inflammasome, with staurosporine as an apoptosis inducer control(A). BMDMs were separately exposed to staurosporine to analyze apoptosis activity in the presence or absence of drug conditions(B). Untreated controls in both conditions received DMEM alone. Caspase-3/7 activity was assessed in the presence or absence of thiomuscimol or muscimol (A-C). Data are means +- SD, n=3 replicates, representative of two independent experiments. Stars (*) indicate statistical significance against vehicle control within each drug group. Thiomuscimol significantly inhibited caspase-3 production in mock-infected/exposed cells at the highest concentration (A). In *Salmonella* infected conditions, thiomuscimol significantly inhibited caspase-3 at the highest condition. There was a significant increase in caspase-3 in the thiomuscimol 0.5 mM, muscimol 0.5- and muscimol 1.0-mM *Salmonella* infected conditions (A). In staurosporine exposed cells, thiomuscimol significantly inhibited caspase-3 production at all concentrations (A). *P < 0.05, **P < 0.01, n.s. nonsignificant (One- way ANOVA, Dunnett's Post-hoc test.) A time-delay addition of thiomuscimol was not significantly different than the addition of media after an initial staurosporine exposure (B), (unpaired t-test).

3.6 PDI inhibition is not Sufficient for Inflammasome Inhibition

Hoffstrom et al. identified thiomuscimol as a PDI inhibitor during a screen of small molecules that effectively suppress polyglutamine-induced apoptosis through inhibition of PDI enzymatic activity (2010). We hypothesized that thiomuscimol may be inhibiting inflammasome formation by inhibiting PDI, potentially by regulating or inhibiting downstream effects. To test this hypothesis, we identified two other PDI inhibitors and assessed ASC-citrine foci formation and To-PRO-3 uptake in *Salmonella* infected-cells that were treated with each inhibitor at the concentrations where they have been described to inhibit PDI (Gao et al., 2021; Young et al., 2020). These two inhibitors, PACMA31 and CCF642, bear some structural resemblance to thiomuscimol with the presence of a sulfur group within a five-membered ring, though both molecules are bulkier in structure (Figure 6A). Neither PDI inhibitor significantly inhibited ASC-citrine foci formation (Figure 6C) or To-PRO-3 uptake (Figure 6D) during *Salmonella* infection. Both inhibitors significantly decreased cellular ATP activity in uninfected conditions when compared to a media only control and did not offer protection in the form of rescuing ATP production during *Salmonella* infection when compared to *Salmonella* infected media only control (Figure 6B). These data indicate that inhibition of PDI is not sufficient to recapitulate the effect of thiomuscimol to inhibit inflammasome formation and pyroptotic gasdermin D pore formation.

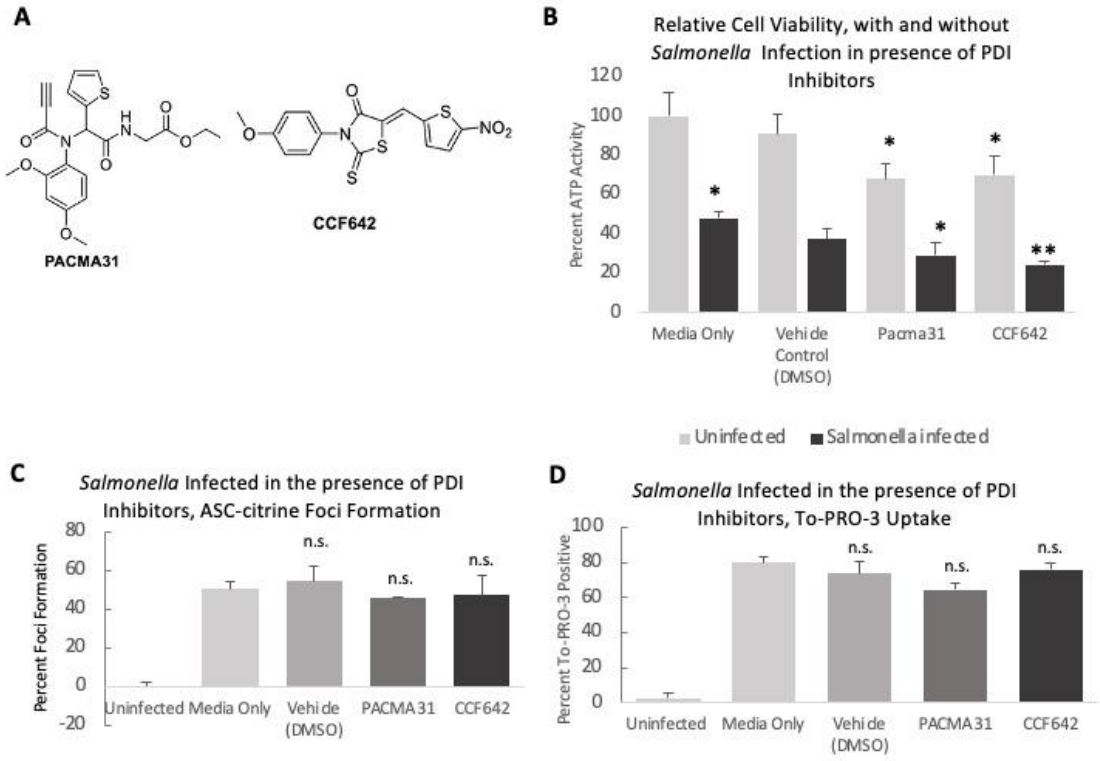

Figure 6. Other PDI Inhibitors do not appear to inhibit inflammasome formation during *Salmonella* infection.

Bone Marrow Derived Macrophages were exposed to PDI Inhibitors PACMA31 (2.25 uM) or CCF642(5 uM) at their active PDI inhibitive concentrations in the presence or absence of *Salmonella typhimurium.* DMSO was standardized at 1%, and a 1 % DMSO vehicle control was included (B-D). The structure of both drugs is provided here (A). NLRC4 inflammasome activation was assessed by measuring ASC Foci formation (C) and To-PRO-3 uptake (D) over time via kinetic microscopy. Relative cell viability was assessed by measuring ATP production (B) through the Cell Titer Glo Assay kit. Data are means +- SD, n=3 replicates, representative of two independent experiments. Stars (*) indicate statistical significance against media only or infected media only conditions, respectively. Uninfected conditions had significantly lower foci formation (C) and To-PRO-3 uptake (D) than media only, *Salmonella* infected conditions. Both PACMA31 and CCF642 had significantly lower ATP production when compared to media alone. These results were similarly observed during *Salmonella* infection, indicating no protective effect from either drug during infection, and some cytotoxicity in uninfected conditions. *P < 0.05, **P < 0.01. For C and D, n.s. indicates no significant difference between drug conditions and Media Only. (Unpaired t-test).

3.7 Thiomuscimol Blocks ASC Independent Caspase-1, but Inhibition is Lost at Lower Concentrations

NLRC4 and NLRP1b both contain CARD domains within their structure, which enable them to interact with caspase-1 outside of an ASC-dependent mechanism. In the absence of ASC, active caspase-1 cleaves gasdermin D to initiate pore formation and IL-1ß secretion (Miao et al., 2011). As we found that thiomuscimol prevents ASC- citrine foci formation at lower concentrations than it protected against To-PRO-3 uptake (Figures 2A and 2C), we hypothesized that ASC-independent diffuse caspase-1 activation may not be blocked at lower thiomuscimol concentrations. To determine if diffuse active caspase-1 and foci-bound active caspase-1 are both inhibited by thiomuscimol, we induced pyroptosis by exposing cells to anthrax lethal toxin in the presence or absence of thiomuscimol, and then staining for active caspase-1 by using the FLICA caspase-1 activity probe. Vehicle cells that were anthrax lethal toxin exposed had both foci-bound and diffuse active caspase-1 (Figure 7A). Thiomuscimol significantly inhibited foci-bound caspase-1 at all concentrations, but only significantly inhibited diffuse active caspase-1 at the two highest concentrations (Figure 7B). Taken together, these data indicate that there is likely cytoplasmic caspase-1 that is activated during anthrax lethal toxin exposure, that is not able to be inhibited by lower concentrations of thiomuscimol.

To determine whether thiomuscimol was able to inhibit true ASC-independent caspase-1 activation, we exposed ASC-/- and C57BL/6 wildtype (WT) control cells to *Salmonella* to induce pyroptosis. This experimental design had a second function: to confirm that the differential of protection between ASC-dependent and independent caspase-1 activation was not inflammasome-type dependent. Thiomuscimol significantly inhibited both diffuse active caspase-1 and foci-bound active caspase-1 in *Salmonella* -infected WT cells (Figures 8A, 8B). Thiomuscimol inhibited diffuse active caspase-1 at 0.5 mM but not at 0.25 mM within these cells, again as was previously observed during

NLRP1b activation (Figure 8B). Surprisingly, there was a significant increase in foci-bound caspase-1 activity at 0.5 and 0.25 mM thiomuscimol in C57BL/6 WT cells (Figure 8A). In ASC -/- cells, there was no significant foci-bound caspase-1 activation (Figure 8A). However, thiomuscimol significantly inhibited diffuse active caspase-1 activity in ASC -/- cells at all concentrations (Figure 8B). Together, these results indicate that thiomuscimol blocks foci-bound active caspase-1 at all concentrations but requires a higher concentration in order to inhibit diffuse active caspase-1.

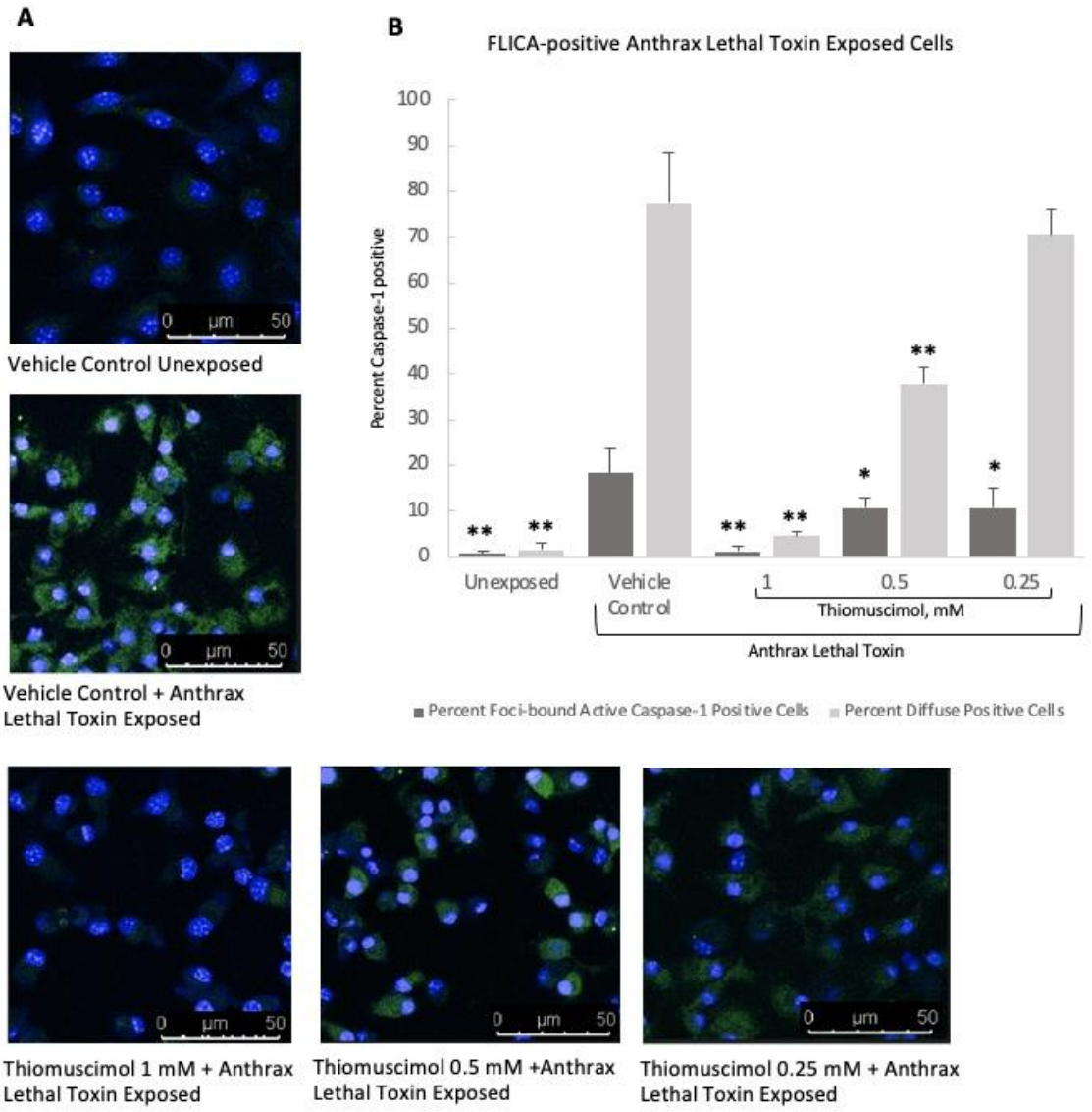

Figure 7. Thiomuscimol inhibits cytoplasmic and inflammasome-bound caspase-1 during Anthrax Lethal Toxin exposure of BALB/C cells.

Bone marrow derived macrophages were exposed to Anthrax lethal toxin for three hours in the presence or absence of Thiomuscimol at its active concentrations. Caspase-1 activity was assessed by the addition of FLICA activity probe, (A, B). Foci-bound caspase-1 positivity and diffuse active caspase-1 were quantified individually. Data are means +- SD, n=3-5 visual fields, representative of two independent experiments. Stars (*) indicate statistical significance against Vehicle control, Anthrax lethal toxin exposed condition, for foci-bound positivity and diffuse positivity, respectively. Thiomuscimol was seen to significantly lower percent foci positive LT exposed cells across concentrations. Thiomuscimol significantly inhibited diffuse caspase-1 at the two highest concentrations, but not at the lowest concentration (0.25 mM) (B). The inhibitive effects of thiomuscimol at the highest concentration on both diffuse and foci-bound caspase-1 was also observed qualitatively (A) . *$P < 0.05$, **$P < 0.01$.

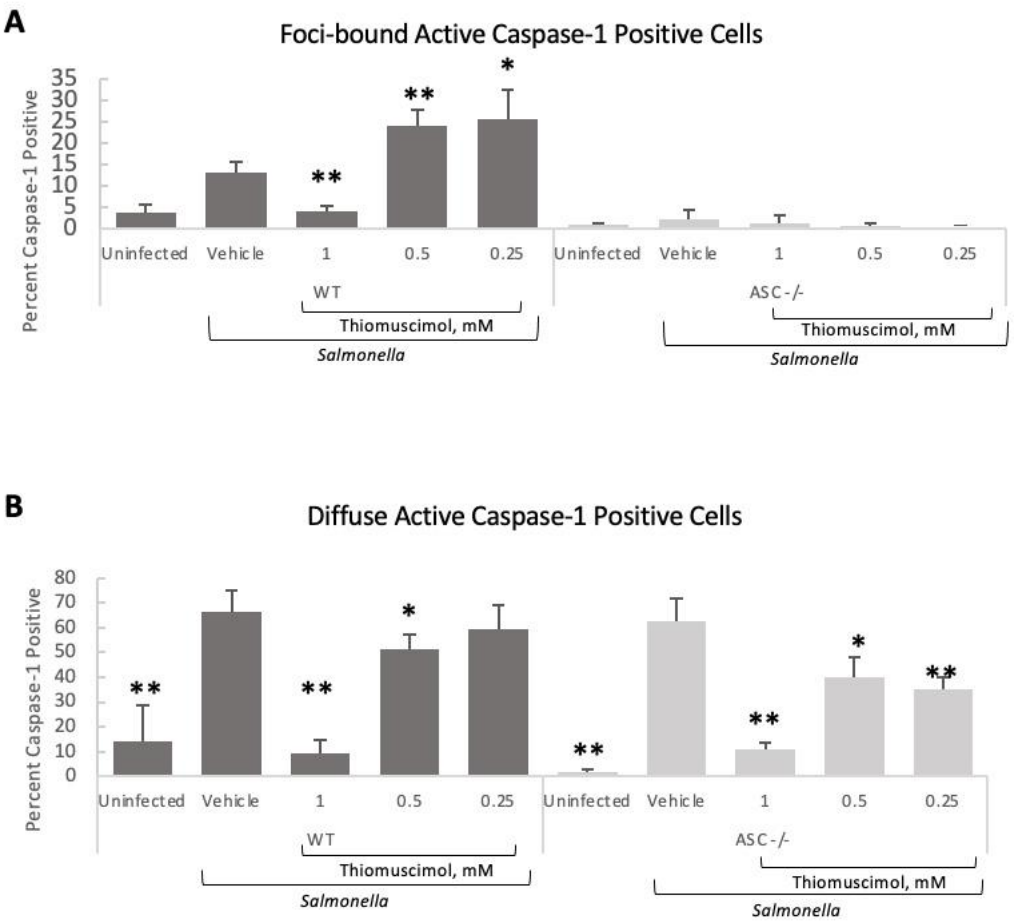

Figure 8. Preliminary Data indicates that Thiomuscimol inhibits caspase-1 in ASC Knockouts and in C56BL6 WT cells during *Salmonella* infection.
Bone marrow derived macrophages (C57BL/6 WT and ASC -/-) were exposed to *Salmonella typhimurium* for two hours in the presence or absence of Thiomuscimol at its active concentrations. Caspase-1 activity was assessed by the addition of FAM-YVAD-FMK activity probe (A, B). Foci-bound active caspase-1 and diffuse active caspase-1 were quantified individually. Data are means +- SD, n=3-5 visual fields, representative of one experiment. Stars (*) indicate statistical significance against Vehicle control, *Salmonella* infected condition, for Foci-bound positivity and diffuse positivity for each cell type, respectively. In WT cells, thiomuscimol was seen to significantly lower percent foci positive cells at the highest concentration and had a statistically significantly higher percent foci formation than the vehicle at 0.5 and 0.25 mM. There was no significant foci formation in any of the ASC KO conditions. Thiomuscimol significantly inhibited cytoplasmic caspase-1 at all concentrations in ASC -/- cells, and in all but the lowest concentration in C57BL/6 cells(B). *P < 0.05, **P < 0.01, n.s. nonsignificant (unpaired t-test)

Chapter 4. Discussion

4.1 Summary of Findings

In this thesis project, we have made several key observations that inform our understanding of the mechanism by which thiomuscimol inhibits inflammasome activation. First, we identified that thiomuscimol was not inhibiting pyroptosis through inducing cellular damage by assessing cellular viability through cellular ATP content in the presence of the molecule. We identified 1.0 to 0.25 mM as the ideal concentration range for inhibition of pyroptosis, while noting a concentration differential between inhibition of ASC-citrine foci formation and To-PRO-3 uptake. We determined that pre-treatment does not significantly boost thiomuscimol's efficacy at inflammasome activation inhibition nor cellular protection. We found that the concentration differential between ASC-citrine foci formation and To-PRO-3 uptake inhibition was indicative of ASC- independent caspase-1 activity. When we exposed cells to anthrax lethal toxin, we found that thiomuscimol blocked foci-bound active caspase-1 at all tested concentrations. Diffuse active caspase-1 was not blocked by lower concentrations of thiomuscimol. These results were echoed in ASC -/- cells infected with *Salmonella,* indicating that thiomuscimol is able to inhibit caspase-1 that is not ASC- reliant, though not as robustly as when caspase-1 is foci-bound. Taken together, these results suggest that structural components found both in ASC- dependent and -independent caspase-1 activation such as the CARD domain may play a role in thiomuscimol's binding mechanism, and that perhaps the context of foci-bound caspase-1 is more sensitive to thiomuscimol's inhibition.

We found that inhibition of inflammasome activation and gasdermin D pore formation by thiomuscimol is reversible, suggesting that inhibition may occur via a noncovalent interaction with its target. When we asked if thiomuscimol redirects from pyroptosis to apoptosis through caspase-1 inhibition, we found that thiomuscimol does not

redirect toward apoptosis, and additionally inhibits activation of caspase-3 in staurosporine exposed and unexposed cells. Since NLRC4 activates apoptotic caspases during caspase-1 KO conditions, the lack of caspase-3 activation in thiomuscimol-treated cells may indicate that thiomuscimol is acting to inhibit NLRC4 activation as well. We found that delayed addition of thiomuscimol following staurosporine exposure did not lead to inhibition of caspase- 3, which indicates that thiomuscimol is likely inhibiting the activation of caspases, as opposed to acting to inhibit already activated caspases. Interestingly, in previous research about thiomuscimol's ability to inhibit PDI, it was found that thiomuscimol was also able to decrease caspase-3/7 activation in MEF cells induced by actinomycin D, thapsigargin, and tunicamycin, while muscimol did not (Zhao et al., 2015). This indicates that thiomuscimol may in fact be acting to block caspase-3 activation, as opposed to an upstream event such as staurosporine entry into the cell. Finally, we found that other PDI inhibitors did not directly mimic the inflammasome activation inhibition effects of thiomuscimol, indicating that PDI inhibition itself is not sufficient to inhibit inflammasome activation.

4.2 Future Directions

A number of future directions could elucidate the mechanism by which thiomuscimol inhibits inflammasomes. One of these directions is analyzing which inflammasomes (and in response to what stimuli) thiomuscimol is able to inhibit. Some preliminary experiments have indicated that thiomuscimol is able to inhibit NLRP3; continuing these experiments and conducting others on inflammasomes that lack structural components like the NACHT domain, may indicate what elements are required for thiomuscimol's activity. We could examine thiomuscimol's other potential targets (such as CARD domain interactions), such

as analyzing thiomuscimol's actions in caspase-1 knockouts to see if the presence of caspase-1 is required for thiomuscimol's inflammasome inhibition.

The similarity in structure and yet difference in activity between muscimol and thiomuscimol suggests that the sulfur group within thiomuscimol plays a key role in its unique activity. Sulfur-containing and cysteine group interactions play an important role within inflammasome biology; as a specific example, higher superoxide production (like what can be seen in high reactive oxygen species environments) can lead to the reversible inhibition of caspase-1 through oxidation and glutathionylation of the cysteine residues within caspase-1's structure (Meissner et al., 2008). To analyze if thiomuscimol is reliant on a cysteine reactive mechanism, we could try quenching the sulfur molecule that we hypothesize is crucial for the molecule's mechanism of action. To do this, we propose to expose thiomuscimol to a molecule that inactivates reactive cysteines, such as N-acetyl cysteine, and assessing if thiomuscimol's inflammasome activation inhibition is halted under these conditions. We also have acquired a panel of molecules related to thiomuscimol in structure, with key differences that will allow us to determine if there are chemical group requirements (or conversely, or size constraints) for thiomuscimol's inflammasome inhibition.

Additionally, we found that thiomuscimol does not block the ability of *Salmonella* to inject its type III secretion system effector proteins, and plan to determine whether lethal factor entry and staurosporine entry are similarly not affected. To determine if staurosporine entry is affected, we could analyze upstream events of caspase-3 activity such as cytochrome C release after collapse of mitochondrial membrane potential (Malsy et al., 2019). This approach would also allow us to analyze where in the apoptosis cascade thiomuscimol may be acting. To analyze if lethal toxin entry is being inhibited by thiomuscimol, we could similarly look at upstream targets of inflammasome activation,

such as cleavage of mitogen-activated protein kinase kinases (MEKs) like MEK3 to ensure that lethal factor is successfully translocated into the cell (Fink et al., 2008).

Understanding how thiomuscimol inhibits inflammasome activation will hopefully allow for the eventual discovery of a small molecule (or required small molecule components) that may be used therapeutically for targeted inflammasome inhibition. Controlling inflammasome activation would in turn allow for more control over inflammation, which in turn would hopefully assist in decreasing the progression of the disease states that are aggravated by an uncontrolled inflammatory response.

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