# Investigating the Effect of Statins on the Human Non-Canonical Inflammasome

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Panagiotis Vezyrgiannis

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# Abstract

Inflammasomes are crucial components of the innate immune system, functioning as early detectors of various types of infections to produce an inflammatory response.
The non-canonical inflammasome (NCI) is activated by cytosolic lipopolysaccharide (LPS) from the outer cell membrane (OM) of Gram-negative bacteria. Cytosolic LPS is detected by guanylate-binding protein 1 (GBP-1) which forms a complex with other GBPs to recruit and oligomerize caspase-4/-5, resulting in pyroptosis, a form of inflammatory cell death accompanied by the release of interleukins (IL) 1β and 18. This pathway is important for immune responses to many pathogens including *Salmonella*. Recent studies suggest that GBP prenylation plays an important role as a "lipid anchor", stabilizing the GBP complex on the OM. This post-translational modification requires an isoprenoid group, a product of cholesterol metabolism via the mevalonate kinase pathway  Statins are some of the most prescribed drugs, mainly used to treat hypercholesterolaemia through inhibition the mevalonate pathway. However, their effect on the NCI and GBP prenylation is unknown. Through this thesis, I tested the hypothesis that statins impacted NCI activation.

My experiments with LPS transfections in IFNγ-primed HIEC-6 primary epithelial cells and THP-1 macrophages showed significant reduction of pyroptosis after overnight treatment with Mevastatin, supporting this hypothesis. In addition, I showed that GBP-1 might be more important for NCI activation in THP-1 cells than previously thought. Infecting these cells with *Salmonella* Typhimurium resulted in marked decrease of IL-1β secretion after Mevastatin treatment but the cell death response was varied; death of HIEC cells significantly increased, whereas THP-1 cells only exhibited the same effect when GBPs 1/-2 were absent or after treatment with the caspase-1/-4 inhibitor VX-765. Our findings suggest that inflammasome inhibition during *Salmonella* infection may result in the activation of alternative modes of cell death and that Mevastatin likely has pleiotropic effects on this phenomenon.

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# Author's Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged in the Bibliography.

# Introduction

## An Overview of the Inflammasome Pathways

The inflammasome signalling pathway is a crucial component of the innate immune system. Inflammasomes consist of cytosolic pattern recognition receptors (PRRs) which detect specific pathogen-associated molecular patterns (PAMPs) such as microbial nucleic acids, components of bacterial cell wall or proteins of the flagellar apparatus, or danger-associated molecular patterns (DAMPs) which are associated with damaged host cells, such as uric acid crystals or extracellular ATP. The main family of PRRs forming inflammasomes is the nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins, also known as NOD-like receptors (Takeuchi and Akira, 2010). Two other PRR that can form inflammasome are absent in melanoma 2 (AIM2)-like receptors (ALRs) and Pyrin, a member of the tripartite motif (TRIM) receptor family. Unlike most other types of PRRs, the inflammasome receptors don't activate transcriptional changes upon being triggered, but result in post-translational activity to produce an immune response by activating proteases called inflammatory caspases.(Broz and Dixit, 2016)

Inflammasomes function as early detectors of various types of infections by acting as caspase-activating platforms when triggered. These caspases then initiate an inflammatory response, typically via pyroptotic cell death and secretion of inflammatory cytokines, mainly interleukins -1β and -18 (IL-1β, IL-18) (Lamkanfi and Dixit, 2014a). Each of the inflammasome receptors recognize specific types of PAMPs and DAMPs.

Canonical inflammasome activation occurs when inflammasome receptors detect a PAMP/DAMP and recruit ASC (Apoptosis-associated Speck-like protein containing a CARD domain) molecules. This initial recruitment process occurs through homotypic interactions between pyrin domains (PYD) or caspase association and recruitment domains (CARD); caspases have a card domain and most NLRs have a PYD or CARD domain. ASC consists of both types of domains and as such can function as the perfect bridge between inflammasome PRRs and caspases(Lamkanfi and Dixit, 2014a). The recruited ASC molecules then fibrillate into a large protein complex, called the speck (Hayward *et al.*, 2018). The speck in turn recruits caspase-1 which is dimerised and cleaved via autoproteolysis (Boucher *et al.*, 2018). The active inflammasome sensor-ASC-Caspase-1 complex then induces maturation and secretion of pro-IL-1β and pro-IL-18 and cleaves Gasdermin D (GSDMD) resulting in membrane pore formation, disruption of osmotic regulation and eventually into a form of highly-inflammatory cell death called pyroptosis (Shi *et al.*, 2015).

Due to the wide range of PAMPs they can detect, inflammasomes are implicated in many types of illnesses (Lamkanfi and Dixit, 2014b) so understanding them and elucidating their exact mechanisms of function is of high medical importance. Inflammasomes are implicated with various diseases such as sepsis (Kumar, 2018), autoimmunity (Li, Guo and Bi, 2020; Jiang *et al.*, 2022), type 1 and 2 diabetes (Dixit, 2013; Sun *et al.*, 2020) and cancer (Van Gorp and Lamkanfi, 2019). Recent studies have found high inflammasome activity, particularly of NLRP3 and AIM2, during SARS-CoV-2 infections and a correlative relationship between inflammasome activity and severity of infection in COVID-19 patients (Rodrigues *et al.*, 2020). More importantly, NLRP3-triggered pyroptosis of macrophages was found to be a key driving factor of cytokine storms in the lungs of COVID-19 patients (Zhang *et al.*, 2021).

## Lipopolysaccharide and the Non-Canonical Inflammasome Pathway

A PAMP of particular interest is bacterial lipopolysaccharide (LPS), a key component in the outer cell membrane (OM) of Gram-negative bacteria. During infection, LPS is recognised by the membrane receptor Toll-like Receptor (TLR4) which induces expression of pro-inflammatory cytokines such as pro-IL-1β and TNF, type 1 interferons, as well as the NLRP3 inflammasome receptor via the NF-κB transcription factor complex (Lu, Yeh and Ohashi, 2008; Bauernfeind *et al.*, 2009). LPS activation of TLR4 therefore primes cells to produce an inflammatory response to infection.

LPS can also be detected in the cytosol independently of the TLR4 pathway through a complex called the non-canonical inflammasome (NCI). Within this pathway, LPS binds and oligomerises caspase-4/-5 via a guanylate binding proteins (GBPs) complex. Active caspase-4/5 cleave and activate GSDMD, leading to pyroptosis (Santos *et al.*, 2020a). Recent studies suggest that GBP1 acts as the first effector of the NCI pathway by recognising specific charges on the inner core of the polysaccharide domain of LPS. It then recruits GBPs 2- 4 to form the platform which oligomerises human caspase-4 (Santos *et al.*, 2020a; Wandel *et al.*, 2020).

GBPs are large GTPases belonging to the dynamin protein superfamily (Kutsch and Coers, 2020). Their expression is induced by both type I and type II interferons but mainly IFNγ. They are some of the most widely expressed interferon-stimulated genes (ISGs), found in many types of tissues in the immune system as well as the skin, respiratory, digestive and nervous systems (Tretina *et al.*, 2019). They play a variety of important roles in innate immunity and defence against pathogens.

Moreover, LPS is a common target of some antibiotics that can kill bacteria either by inhibiting LPS synthesis and disrupting OM formation or binding to LPS and disrupting the structure of the membrane (Epand *et al.*, 2016). Consequently, Gram-negative bacteria with modified, hidden or completely absent LPS can gain resistance to such antibiotics, including colistin, one of our most powerful bactericidal agents used as a last-resort antibiotic in severe infections (May and Grabowicz, 2018; Sabnis *et al.*, 2021). For example, colistin resistant strains of *Acinetobacter baumannii* have mutations in the genes involved in lipid-A production, the hydrophobic lipid compartment of LPS (Moffatt *et al.*, 2010). As a result, they utilise lipooligosaccharide (LOS), which is not recognized by the antibiotic, instead of typical LPS. A common mechanism of colistin resistance in *E. coli, Shigella, Salmonella* and many other pathogens is by modifying the lipid-A domain to reduce its net negative charge and thus its affinity to colistin and other polymyxin antibiotics (Nang, Li and Velkov, 2019). Since LPS is the key target of GBP1 and the NCI pathway, the question emerges of whether bacteria who gain antibiotic resistance by modifying or hiding their LPS are also able to more easily evade the NCI, compared to their wild-type counterparts. It has been previously shown that *Salmonella Enterica* serovar Paratyphi produces modified LPS containing an elongated O-antigen chain which helps reduce activation of canonical inflammasomes such as NLRC4 (Mylona *et al.*, 2021), hinting that there may be a similar effect on the NCI.

## Statins and their Potential Effects on the Non-Canonical Inflammasome

Statins are a class of drugs that are commonly used to treat cardiovascular diseases (CVDs), particularly hypercholesterolaemia (National Clinical Guideline Centre, 2014). Statins are mainly used to reduce blood cholesterol levels and mitigate the risk of atherosclerosis. They act as competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway, which is crucial for cholesterol production (Endo, 1992). Due to the increase of pollution, the expansion of the human lifespan and the rapid shift to hectic, stressful lifestyles and unhealthy dietary habits, CVDs have become humanity's leading cause of death, causing nearly 18 million

deaths each year (WHO, 2021). As a result, statins have become some of the most widely prescribed medications worldwide.

Besides cholesterol metabolism, the mevalonate pathway also produces isoprenoid groups, widely used for post-translational modifications called prenylations. This is relevant to our research because GBP-1 is farnesylated. Farnesylation is a type of prenylation where an isoprenoid called farnesyl pyrophosphate (FPP) is attached to the prenylation domain of a protein. Because statin medications inhibit this pathway (Manaswiyoungkul, De Araujo and Gunning, 2020), we hypothesized that statins may disrupt GBP-1 farnesylation and potentially have a negative effect on NCI function. GBP prenylation is important to their function, allowing them to translocate to lipid membranes or polymerise in the cytosol (Britzen-Laurent *et al.*, 2010; Kutsch and Coers, 2020). Specifically in the context of the NCI, the farnesylation domain of GBP-1 may act as a lipid anchor that allows GBP1 to "stick" on the bacterial OM after associating with its LPS in order to recruit the other GBPs and initiate the NCI (Santos *et al.*, 2020a).

Previous research on the potential effects of statins on other inflammasomes, namely NLRP3, has yielded contradictory results.

Current literature offers several examples of statins shown to inhibit the NLRP3 canonical inflammasome (Khalifeh et al., 2021). One particular study found that Mevastatin was able to suppress NLRP3 in endothelial cells by inhibiting the effects of oxidized low-density lipoprotein (ox-LDL) and TNFα signalling, which would otherwise be sufficient to activate it (Wang et al., 2017). Under normal conditions, ox-LDL can trigger NLRP3 by activating the pregnane X receptor (PXR). The researchers found that statins can inhibit PXR, independently of their cholesterol-limiting effects. They also showed that statins can prevent Nf-κB, a crucial regulatory signalling pathway, from binding on the NLRP3 promoter, thus limiting the expression of its inflammasome components.

On the other side of the argument, some studies reported increased activation of NLRP3 in response to statin treatment. There is evidence that inhibition of protein prenylation in adipose tissue by statins triggers NLRP3, resulting in inflammation and IL-1β – mediated insulin resistance (Henriksbo et al., 2019). Some researchers have shown that statins may activate p38 and mTOR signalling, which in turn may induce pro-IL-1b expression and thus potentiate NLRP3 activation (Henriksbo et al., 2020). Other studies suggest mevalonate kinase deficiency (MKD) or inhibition of the mevalonate pathway influences NLRP3 activity, specifically upregulating its expression and activation response (Pontillo, Paoluzzi and Crovella, 2010; Skinner *et al.*, 2019).

Notably, the effects of statins on the non-canonical inflammasome remain unexplored.

## Central Hypothesis and Project Aims

Although literature suggests that not all cell types may require GBPs for intracellular LPS detection (Fisch *et al.*, 2019; Santos *et al.*, 2020a),**we hypothesise that disruption of GBP prenylation by statins may negatively affect NCI activation**. In this research project we explored this hypothesis and found that statins indeed have a negative effect on NCI-mediated cell death in human primary epithelial cells after *in vitro* LPS transfection, whereas no such effect was reported when using the cancerous human monocyte cell line THP-1, differentiated into macrophages. Furthermore, we found the effect of statins on cell death, but not on IL-1β secretion, to be seemingly reversed when the same cell types were infected by live cultures of *Salmonella Enterica* serovar Typhimurium. However, no effect at all was reported upon exposure of these cells by outer membrane vesicles (OMVs) which were isolated by the same *Salmonella* strains.

# Methods

## Mammalian Cell Culture

For this project the following mammalian cell lines were used:

Human Intestinal Epithelial primary Cells (HIEC-6, ATCC CRL-3266™) were grown using Opti-MEM® (Gibco) supplemented with 4% Fetal Bovine Serum (FBS), 10 mM L-Glutamine and 10 ng/mL of human epidermal growth factor (hEGF). HeLa cancerous human epithelial cells (ATCC CCL-2™) were grown using Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS and 10 mM L-Glutamine. THP-1 cancerous human monocytes (ATCC TIB-202™) were grown in RPMI 1640 (Gibco) supplemented with 10% FBS and 10 mM L-Glutamine. They were differentiated into macrophages by addition of 50 ng/mL of Phorbol-12-myristate-13-acetate (PMA 100ug/mL re-suspended in DMSO, by Sigma-Aldrich) to their growth media for 48hrs. After a 48h incubation, the media were changed to fresh RPMI without PMA.

In preparation for use in experiments, cells were transferred from culture flasks to 96-well plates at a density of 15,000 cells per well for epithelial cells and 70,000 cells per well for THP-1 macrophages, using appropriate growth media. Where necessary, the cells were primed overnight with 10ng/mL of human interferon gamma (IFNγ) to induce expression of GBPs. Alternatively, Pam3CSK4 (Invivogen, 1mg/mL stock in $H_2O$) was used at a final concentration of 1ug/mL to activate TLR2 signalling and induce expression of inflammasome components.

Mevastatin treatment was done overnight by addition of 10 µM Mevastatin (10 mM re-suspended in ethanol, by Sigma-Aldrich) with fresh growth media. The following morning the growth media were changed to Opti-MEM®, regardless of cell type, and 10 mM Mevastatin was replaced on the treated cells.

Where necessary, 10 µM of the NLRP3 inhibitor MCC-950 (Sigma-Aldrich, 10mM stock in $H_2O$) or 25 µM of the caspase-1/-4 inhibitor VX-765 (Sellec, 25 mM stock in DMSO) were added to the cells on the morning of the experiments during the change to fresh Opti-MEM® and incubated for approximately 30min to 1h before commencing with the experiment.

All cell cultures were incubated at 37$^{o}$C with 5% $CO_2$.

## LDH Cell Death Assay

After treatment, 4µL of 10% Triton X- 100 were added to the "100% Lysis" wells (96-well plate) and incubated in room temperature for 5 minutes. 15 µL of supernatant were remove from each condition of treated cells and transferred into a new 96-well plate. 1.5 mL of LDH substrate solution (LDH Cytotoxicity detection kit by Takara Bio, according to manufacturer instructions) were prepared per plate of samples. 15 µL of substrate were added in each well of the new plate. The plate was then incubated for approximately 20 minutes away from light. The reaction was stopped by adding 15 µL of HCl stop solution (HCl 1N). The plates were measured at 490 nm using a spectrophotometer.

## Western Blotting

Western blotting was used to detect cleavage of Caspase-1/-4/-5, GSDMD and IL-1β/-18 as qualitative indicators of inflammasome activation and pyroptosis. After treatment, cell supernatant was gathered from each condition and precipitated using the methanol and chloroform method; Depending on the volume of the cell supernatant, 1 volume of methanol and 0.4 volumes of

chloroform were added and briefly vortexed to mix, then centrifuged at max speed for 12 min to separate the sample in three phases. The aqueous phase on top of the protein interphase was removed and discarded, and 1.6 volumes of methanol were added and centrifuged again to gather the protein pellet at the bottom of the tubes. The remaining liquid was then discarded, and the tubes were left open under a fume hood for approximately 30 min to allow the protein pellet to dry completely. SDS Lysis Buffer (1X TruPage™ LDS Sample Buffer by Sigma in 1.5% SDS 66mM Tris pH 8 buffer) was heated to 95°C and added to the emptied wells. The cell lysate (extract) was then harvested. The cell supernatant protein pellets were also re-suspended in 25 µL of SDS lysis buffer.

Both the cell extract and supernatant samples were boiled for 5 min at 95°C to ensure protein denaturation. SDS-PAGE was run using 12% polyacrylamide gels and Tris-glycine running buffer at 160V for approximately 70 minutes. The protein bands were then transferred onto a nitrocellulose membrane using BioRad TURBOBlot transfer system, set for mixed molecular weight at 2.5A – 25V for 7 min. The membranes were blocked in milk solution (5% w/v skimmed milk powder in 1X TBS-T) for 30 minutes. Mouse anti-caspase-4 (1:2000, Protein tech), anti-H3 (1:3000, BioLegend), rabbit anti-caspase-5 (1:500, Cell Signalling Technology ), anti-caspase-1 (1:2000, Protein tech), GSDMD (1:3000, Cusabio) and anti-IL-18 (1:2000, Protein tech) primary antibodies were used overnight at 4°C or at room temperature with addition of sodium azide solution ($NaN_3$ at 0.2% final concentration), when necessary. Membranes were then washed three times 1X TBS-T on the orbital shaker for 10min, discarding the buffer and briefly rinsing the membranes with TBS-T between each wash. The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000, Invitrogen) for 2h at room temperature. Chemiluminescence was measured for 180 to 300 sec with addition of 400 µL of HRP substrate (Immobilon® Crescendo Western HRP Substrate by EMD Millipore or SuperSignal™ West Femto Maximum Sensitivity Substrate by ThermoFischer Scientific), using the BioRad Chemidoc MP™ imaging system.

## ELISA

ELISAs (Human IL-1β Uncoated ELISA Kit by Invitrogen) were used to quantify release of IL-1β from cell samples. ELISA plates were coated overnight with h. IL-1β capture antibody (1:250 in PBS) and washed 3 times with PBS-T. 250 µL of ELISA blocking buffer were then added in each well and incubated at 4°C overnight, or at room temperature for 1h. Afterwards, 40 µL of undiluted supernatant from HIEC-6 cultures or of diluted (1:50 in PBS) supernatant from THP-1 cultures were added to each well, along with human IL-1β standard solution (prepared according to kit instructions, using a 12-fold serial dilution of 2/3). The plates were incubated overnight at 4°C. The following day, they were washed thoroughly three times with PBS-T before adding 40 µL of hIL-1β detection antibody (1:250 in blocking buffer) and incubating for 1h at room temperature. They were then washed five times and 40 µL of Avidin-HRP (1:250 in blocking buffer) were added in each well and incubated for 1h at room temperature. After six washes with PBS-T, 40 µL of TMB solution were added in each well and incubated at room temperature for approximately 20 min before stopping the reaction with 40 µL of $H_2SO_4$ solution. The plates were measured at 450 nm using a spectrophotometer.

## Bacterial Cultures

For this project, we used *Salmonella Enterica* serovar Typhimurium strains ST4174 and SL1344. Two knockout mutant strains of SL1344 were also used, ΔOrgA and ΔSSaV, deficient for SPI-1 and SPI-2 functionality respectively (Lawrence *et al.*, 2021). All cultures used for live infection assays were grown using a single colony in 3mL LB broth, in an orbital shaker overnight at 37 °C, 170 rpm. In the morning, 40 µL from each culture was introduced to 3 mL of fresh LB broth and grown under the same conditions for 2.5 hours to ensure all cultures were in logarithmic growth phase ($OD_{600}$ = 0.5 –

1.2) prior to infection. In experiments where cultures in stationary phase were needed, bacteria from the overnight culture were used directly.

### LPS Transfection in Human Cell Lines

On the morning of the experiment, growth media were changed to 90 µL fresh Opti-MEM® per well on the 96-well cell culture plates. LPS transfection mix was prepared by diluting LPS EK or EB (Invivogen) in Opti-MEM® to an appropriate concentration based on each experiment (typically 0.02 - 0.5 µg/mL), and briefly vortexing for 30sec. Lipofectamine LTX (Invitrogen) was then added to the solution to a concentration of 3% v/v. The solution was gently mixed and left to incubate at room temperature for 15min. 10 µL of the solution were then added into each well with a gentle "zig-zag" motion to ensure even distribution. The plate was than centrifuged at room temperature at 500 x g. Where necessary, a plain 3% LTX solution in Opti-MEM® was used as a negative control. As a positive control of canonical inflammasome activation, the NLRP3 trigger Nigericin (Nigericin salt by Sigma-Aldrich, re-suspended in ethanol) was used by diluting it to a concentration of 2% v/v in Opti-MEM® and adding 10 µL per control well. Two columns of wells, one for the untreated control and one for the 100% lysis control, were left untreated, only supplementing 10 µL of Opti-MEM® to ensure all wells on the plate contain 100 µL of total media.

### Bacterial Growth Assay with Mevastatin

The $OD_{600}$ of 4 different concentrations of Salmonella that has been cultured in LB broth overnight was measured. We prepared a 2X culture master mix with an approximate $OD_{600}$ of 0.2 once diluted to 1X. 500 µL each of culture and LB were mixed to achieve a final consistency of ¼ pure culture and ¾ LB. The treatment master mixes for the different experimental conditions were then prepared:

1. Untreated: Pure LB.
2. Mev (1): Add 10 µL Mevastatin (10 mM re-suspended in ethanol, by Sigma-Aldrich) to 500 µL LB.
3. Mev (1/10): Take 50 µL of Mev (1) and mix with 450 µL LB for a ten-fold dilution.
4. Mev (1/100): Repeat with Mev (1/10) as above.
5. Gentamycin (Gen): Add 2 µL of Gen 500X (50 mg/mL stock) to 500 µL LB.
6. Pure LB (Negative Control).

The culture and treatment master mixes were then plated on a 24-well tissue culture plate using the following layout:

| Untreated | Mev (1) | Mev (1/10) | Mev (1/100) | Gen | LB |
|---|---|---|---|---|---|
| 50 µL Culture + 50 µL LB | 50 µL Culture + 50 µL Mev(1) | 50 µL Culture + 50 µL Mev(1/10) | 50 µL Culture + 50 µL Mev(1/100) | 50 µL Culture + 50 µL Gen | 100 µL LB |
| >> | >> | >> | >> | >> | >> |
| >> | >> | >> | >> | >> | >> |

The plate was incubated at 37°C with $OD_{600}$ measurements taken at 0, 30, 60, 120 and 180 mins to observe the growth progression of the culture.

## OMV Isolation and Infection in Human Cell Lines

Cultures used for OMV isolation were grown in 26 mL 2YT broth overnight under the same conditions. OMVs were extracted using the ExoBacteria™ OMV isolation kit (Systems Biosciences). The OMV concentration was then calculated by quantifying their protein content with a nanodrop reader (set for A280 Protein readout). The OMV solution was finally diluted to a concentration of 0.4 mg/mL. 10 µL of OMV solution were added to each well containing 90 µL of cell culture, on a 96-well plate (Final concentration 4 µg/mL). The plate was then centrifuged at

## Live Bacterial Infection in Human Cell Lines

After preparation of the cell lines and bacteria, 1 mL of each strain culture was centrifuged at 6000 x g for 2 min and the supernatant was discarded. The pellet was resuspended in 1 mL of sterile PBS, pre-warmed at 37°C and centrifuged again. This process was repeated twice to wash the culture and ensure no LB remains. After the final re-suspension in PBS, 500 µL of bacteria were diluted further in 500 µL PBS and their $OD_{600}$ was measured to confirm their growth phase and quantify them. 40 µL of bacterial solution were added to the cell lines after being diluted in Opti-MEM® to achieve final concentrations that would result in multiplicities of infection (MOI) 10, 50 and 100. The MOI were calculated as such:

- For a *Salmonella* culture in log phase, **an $OD_{600}$ of 1 corresponds to $1x10^6$ bacteria/µL.**
- Since every well has 15k cells, for MOI 10 we **will need 150k bacteria, or 0.15 x $10^6$**.
- For our experimental set-up we will need **40 µL of culture per well x 15 wells per MOI** => 600 µL "infection mix" per MOI
- Final calculation (for MOI 10) => [0.15 x $10^6$ x 15]/$1x10^6$ = 2.25 µL culture added to 600 µL Opti-MEM®. Multiply by 5 and 10 to get the numbers for MOI 50 and 100, respectively

After 45 min of infection, 10 µL of gentamicin (1 mg/mL stock) was added in all wells, to ensure any bacteria that had not successfully invaded the cells were killed. The cultures were incubated for 6h in total, with cell supernatant harvested at 3h and 6h for LDH and ELISA.

| No Treatment | | | | | | IFNγ | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Untreated | MOI 10 | MOI 50 | MOI 100 | LPS Trans. | 100% Lysis | Untreated | MOI 10 | MOI 50 | MOI 100 | LPS Trans. | 100% Lysis |
| 90 µL OptiMEM +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 80 µL OptiMEM +10 µL LPS Mix +10 µL Gen | 90 µL OptiMEM +10 µL Gen | 90 µL OptiMEM +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 80 µL OptiMEM +10 µL LPS Mix +10 µL Gen | 90 µL OptiMEM +10 µL Gen |
| 90 µL OptiMEM +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 80 µL OptiMEM +10 µL LPS Mix +10 µL Gen | 90 µL OptiMEM +10 µL Gen | 90 µL OptiMEM +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 50 µL OptiMEM +40 µL Mix +10 µL Gen | 80 µL OptiMEM +10 µL LPS Mix +10 µL Gen | 90 µL OptiMEM +10 µL Gen |
| Mevastatin | | | | | | IFNγ + Mevastatin | | | | | |

## Statistical Analysis

Prism 9 by GraphPad Software was used to perform all statistical analysis and generate all of the graphs for this project. I used two-way analysis of variance (ANOVA) since my experiments explored how multiple factors affected the inflammasome response (e.g. how Mevastatin treatment with and without IFNγ priming, as well as different MOIs or strains of *Salmonella,* affect the inflammatory cell death response).

To interpret which factor was responsible for the statistically significant differences in each experiment, appropriate multiple comparisons tests were used, following the recommended settings of the software; if the dataset was separated in two groups (e.g. Mevastatin vs Untreated), then the Šídák method was the default test. In most cases there were four groups per dataset and as such Tukey's multiple comparisons test was used. Both of these tests compare each mean with every other mean of the same row across columns, and can calculate confidence intervals. For the experiments exploring whether Mevastatin had any effect on the growth of the *Salmonella* cultures themselves (Figure 4), the means of the cultures with different Mevastatin concentrations were compared with the mean of the negative control (Untreated culture), using the Dunnett test for multiple comparisons.

Normality tests were also used for each type of dataset, to confirm whether the use of parametric statistics was supported. In all cases, the data was shown to be normally or approximately normally distributed, making the use of ANOVA tests suitable.

# Results

## Statin Treatment Inhibits LPS Detection in Human Primary Epithelial Cells

Current research shows that GBP-1 acts as the primary receptor that detects cytosolic LPS to activate caspase-4 (casp-4) via non-canonical inflammasome (Santos *et al.*, 2020a). GBP-1 has a farnesylation domain (CAAX box), which has been shown to play the role of a lipid anchor, stabilising the protein on the outer cell membrane of cytosol-invading bacteria so that it can better attach to their LPS and form a platform with other GBPs to recruit casp-4. Since the mevalonate pathway plays a role in GBP post-translational modifications like farnesylation and geranylgeranylation (Hooff *et al.*, 2010), statin treatment might influence the non-canonical inflammasome as well.

To determine whether inhibition of the mevalonate kinase pathway affects the non-canonical inflammasome's response to intracellular LPS in human primary epithelial cells, we treated HIEC-6 cells with Mevastatin (10 µM, Campia *et al.*, 2009), a statin used in clinics, for 16h. We then transfected these cells with ultrapure *E.coli* LPS EB at a final concentration of 0.5 µg/mL, using lipofectamine LTX vesicles and incubated for 6h, to trigger the NCI. To measure cell death, we harvested supernatant samples from our cultures and performed an LDH assay as well as ELISAs to measure the levels of IL-1β inflammatory cytokine that were released during pyroptosis. For our controls, we left cells untreated and treated others with just lipofectamine without LPS, to ensure that lipofectamine does not interfere with the NCI or trigger any non-specific cell death or IL-1β release itself (Figure 1, "UT" and "LTX" respectively).

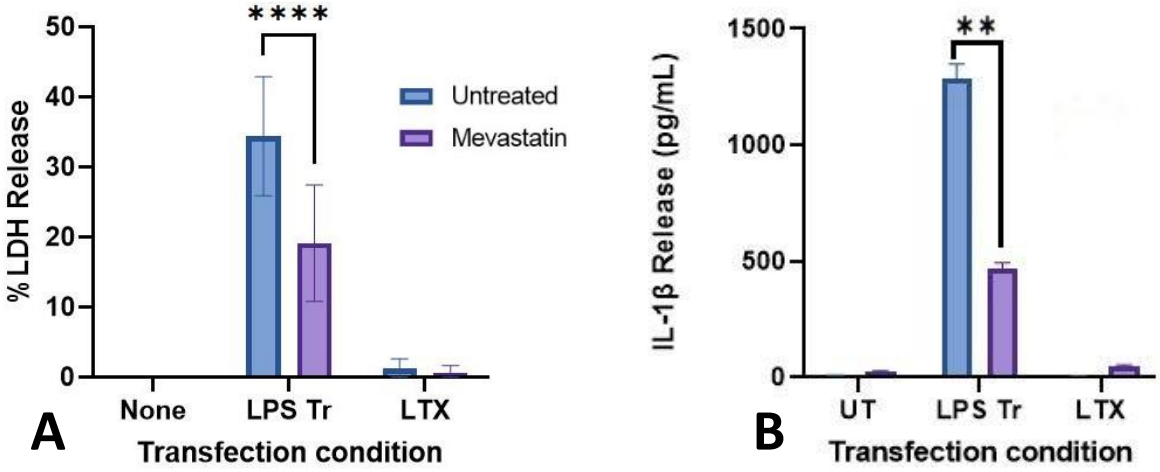


**Figure 1 Mevastatin reduces LPS-induced NCI response in HIEC-6 cells. A**) *Cell death indicated by % of LDH release after LPS transfection for 6h in HIEC-6. Mevastatin treatment caused a significant decrease in cell death (P-value < 0.0001) compared to untreated cells.* ***B)****. ELISA measuring release of the inflammatory cytokine IL-1β (pg/mL). IL-1β release due to NCI activation by LPS transfection was significantly decreased by Mevastatin treatment (P-value = 0.0043). Plain LTX included as negative control. Summarised results from 5 experiments*

Averaged results from 5 experiments showed that cells transfected with LPS but not treated with Mevastatin exhibited approximately 30% of cell death (Figure 1A), when data was normalised to remove non-specific background death, and released around 1.2 ng/mL of IL-1β (Figure 1B). After overnight treatment with Mevastatin, cell death caused by LPS EB transfection was significantly decreased (2-way ANOVA with Šídák's multiple comparisons test, P-value < 0.0001, Figure 1A). IL-1β release data support these results, showing a significant decrease of cytokine secretion of over 1000pg/mL in Mevastatin-treated cells (2-way ANOVA with Šídák's multiple comparisons test, P-value <0.005, Figure 1B). The release of this inflammatory cytokine and its correlation to the LDH

results in figure 1 confirm pyroptosis, associated with the activation of NCI by cytosolic LPS. These results show that disruption of the mevalonate kinase pathway has an inhibitory effect on the function of the NCI, supporting our hypothesis that GBP prenylation plays a crucial role for its function as primary LPS detector.

## Statin Treatment does not impact LPS responses in THP-1 Macrophages

The activity of different inflammasomes is often dependent on cell type and as such there are noted differences on how the NCI operates between macrophages and epithelial cells. To test whether statins have different effects on the NCI – if any – across these two cell types, we performed our LPS transfection experiment using THP-1 human cancerous macrophages. Relevant literature suggests that although GBPs are important for NCI function in macrophages, GBP-1 is not crucial for its activation (Santos *et al.*, 2020) and thus IFNγ-priming was not necessary in this case.

On average, we observed approximately 20% - 30% cell death after 6h of LPS transfection in THP-1 macrophages (Figure 2A) and IL-1β release concentrations of about 20 ng/mL (Figure 2B). Transfection of statin-treated THP-1 macrophages with LPS EB revealed no significant difference in cell death or IL-1β release compared to the untreated cells (2-way ANOVA with Šídák's multiple comparisons test, P-value > 0.9, Figure 2).

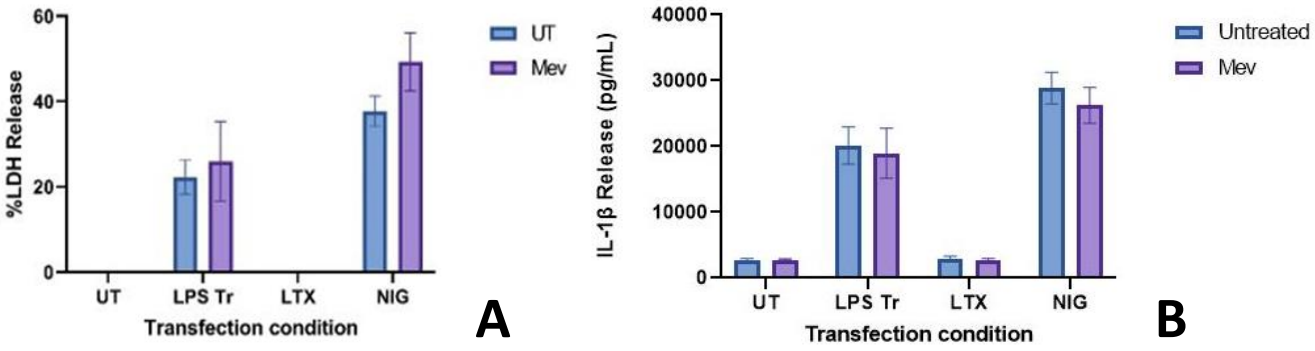


**Figure 2. Mevastatin does not impact NCI function in THP-1 Macrophages during LPS transfection. A.** Cell death indicated by % of LDH release after LPS transfection (LPS Tr) for 6h in THP-1. **2B**. IL-1β (pg/mL) release by "UT" (Untreated) and "Mev" (Mevastatin treatment). No significant difference in cell death or IL-1β release was found (2-way ANOVA with Šídák's multiple comparisons test, P-value > 0.9). LTX control is included as negative control. Treatment with the NLRP3 activator nigericin (Nig) included as positive control for NLRP3 activation. Summarised data from 4 experiments, normalised to untreated samples (UT).

## IFNγ Priming Potentiates LPS-Dependent Cell Death in THP-1 Macrophages in a Dose-dependent Manner

After several repeats, there seemed to be minor differences on the overall level of cell death between LPS EK transfection experiments. We hypothesized that it was due to using different LPS concentrations (*0.5 or 0.25 µg/mL respectively*). To test this, I set up a dose-response experiment using 5-fold serial dilutions of LPS EK in THP-1 macrophages which had been primed overnight with IFNγ. Across 4 repeats of this experiment, IFNγ priming significantly increased pyroptosis. (Figure 3, *paired t-test, adjusted P-value: 0.022*). Moreover, this data points at an optimal LPS concentration (0.0008 µg/mL) where IFNγ priming becomes integral for sufficient NCI activation. This suggests that THP-1 macrophages might become saturated with LPS at higher doses, hiding the effect of GBPs on NCI activation that becomes evident at these lower concentrations.

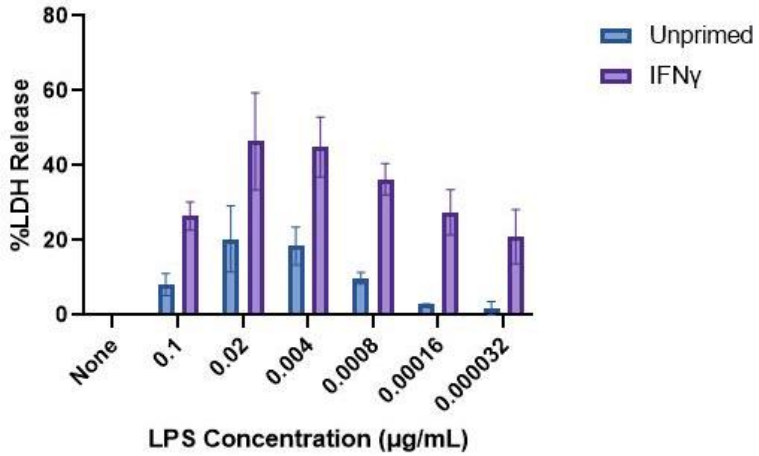


***Figure 3**. LPS transfection dose-response study with serial dilution in THP-1 macrophages. Cell death is indicated by the percentage of LDH release compared to a 100% lysis control. IFNγ priming resulted in significantly higher cell death across all LPS concentrations. Summarised data from 4 independent experiments.*

## Mevastatin does not interfere with *Salmonella* growth

In preparation for live *Salmonella* infection experiments, it was important to ensure that Mevastatin does not interfere with normal growth of the bacteria, which may affect our results. We first performed a growth assay; *Salmonella* ST 4174 overnight culture was mixed with either pure LB broth or 3 different concentrations of Mevastatin (100 µM, 10 µM and 1 µM) solution in a 96-well plate. A Gentamycin control (100 µg/mL) was included to have a scale of comparison in case Mevastatin was found to have a toxic effect on the culture, since statins are known to be bacteriostatic at certain concentrations (Thangamani *et al.*, 2015). LB broth alone was also included as a control to indicate any potential contamination of the culture that would have affected the results. The experiment was performed 4 times.

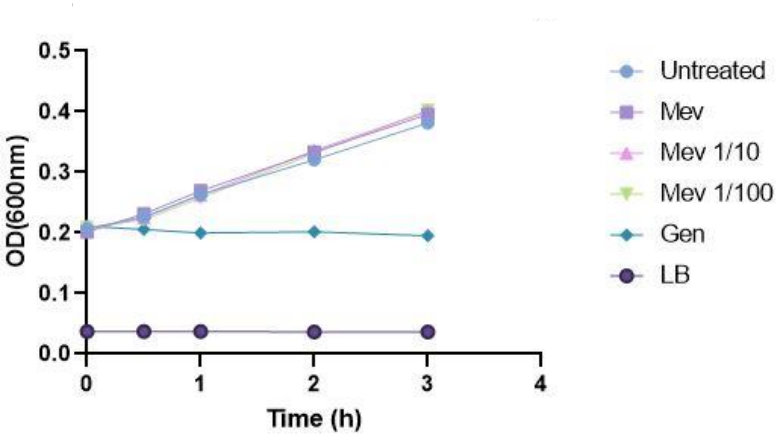


***Figure 4. Mevastatin does not interfere with normal growth of Salmonella ST 4174**. Growth curve measured at OD600 over 3h. Mevastatin treatment had no significant effect in bacterial growth at any concentration used (P-value = 0.9), compared to untreated cultures. Pure LB broth included as negative control. Gentamycin control included to compare potential Mevastatin toxicity. Summarised data from 4 repeats.*

My results indicate no significant difference in *Salmonella* growth with Mevastatin at any concentration and time point, compared to the untreated control (2-way ANOVA with Dunnett's

multiple comparisons test, P-value = 0.9). This suggests that Mevastatin toxicity itself would not affect the results of the infection experiments we performed in our cells later on.

## Cell Death of THP-1 Macrophages Is Affected by LPS Structure

Throughout our experiments in THP-1 cells, two different types of LPS have been used: LPS EK and EB. LPS EB is derived by E. coli 0111:B4, has a longer O-antigen chain at the end of the polysaccharide chain and is considered a smooth LPS (Coleman, Goebel and Leive, 1977). LPS EK is derived from *E. Coli* K12, which is considered a rough strain that doesn't normally express the O-antigen on the LPS (Kuhnert, Nicolet and Frey, 1995). Transfection experiments in THP-1 macrophages revealed a variation in the level of cell death triggered by the different types of LPS, at the same concentration of 0.5 µg/mL (Figure 5). Specifically, transfection with LPS EK resulted in significantly higher levels of cell death compared to LPS EB (Paired t-test, P-value <0.0001).

This data suggests that shorter O-antigen chains significantly correlate with increased NCI activation in THP-1 macrophages.

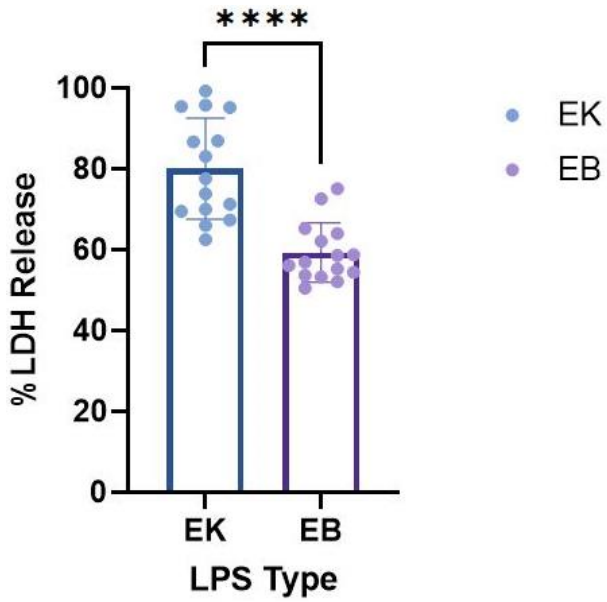


***Figure 5****. Percentage of cell death measured by LDH release after transfection of THP-1 macrophages with LPS EK and EB. LPS EK caused significantly higher levels of cell death compared to EB (P-value <0.0001). Summarised data from 5 repeats.*

## Overnight Mevastatin Treatment Potentiates Cell Death in Human Primary Epithelial Cells during *Salmonella* Infection

After numerous transfections in THP-1 and HIEC-6 using purified LPS EK and EB, we aimed to explore the wider effect of Mevastatin during actual infection of the cells with live *Salmonella enterica* Typhimurium. Untreated and statin-treated HIEC-6 cells were primed overnight with IFNγ and infected with *S.* Typhimurium strain ST 4174 at logarithmic phase growth, using 2 different multiplicities of infection (MOI 10, 50). A LPS transfection (0.0625 µg/mL) condition was included as a control, to compare potential difference in NCI activation between infection and transfection.

In line with our previous findings, Mevastatin treatment significantly reduced cell death during ultrapure LPS transfection (2-way ANOVA with Tukey's multiple comparisons test, P-value < 0.0001). However, it consistently increased the cell death response after 3 hours of infection. In the summarised 4 experimental datasets, this effect was only significant when comparing between the untreated or the Mevastatin-treated cells and the ones threated with both Mevastatin and IFNγ (Figure 6A, P-values: 0.0044, 0.0081 respectively*).*

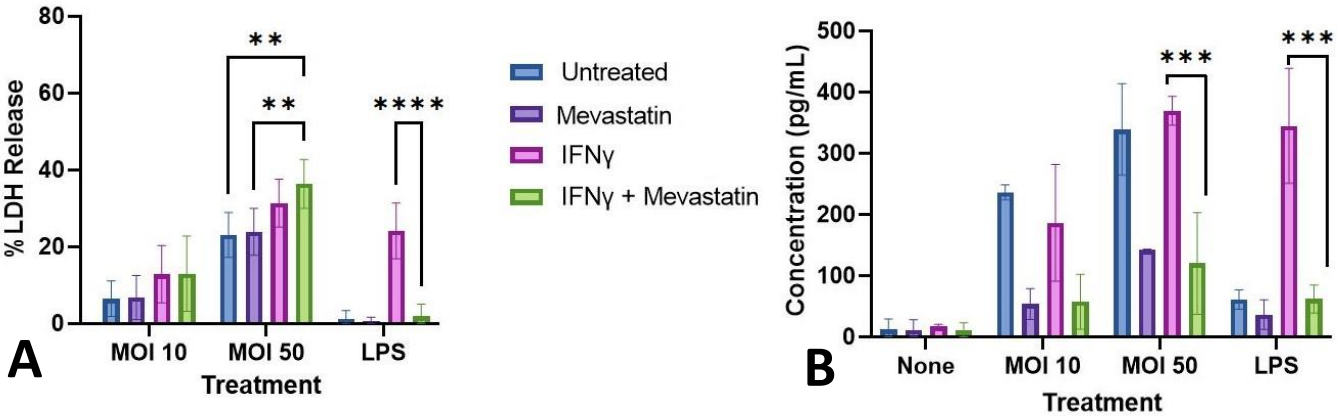


***Figure 6. Effect of Mevastatin on HIEC-6 epithelial cells after infection by Salmonella ST 4174.*** ***A****. Cell death at 3h post-infection, measured by percentage of LDH release compared to 100% Lysis control. We noted a significant increase in cell death at MOI 50 after treatment with both IFNγ and Mevastatin, compared to no treatment or just statin-treatment without priming (P-values: 0.0044, 0.0081 respectively). Summarised data from 4 experiments, normalised for clarity.* ***B****. IL-1β release concentration at 6h-post infection of HIEC-6 by Salmonella ST 4174 was significantly decreased at MOI 50 after treatment with both IFNγ and Mevastatin (P-value = 0.0037). Summarised data from 2 experiments. LPS transfection included as a positive control of NCI activation showed a significant decrease in death of primed cells and IL-1β release after Mevastatin treatment (P-value < 0.0001).*

It is worth noting however, that within each of these experiments IFNγ and Mevastatin individually increased cell death significantly (P-value < 0.05*)* at MOI 50 and IFNγ produced a significant cell death increase in MOI 10 samples as well (P-value = 0.0001*).* Although the exact measurements of LDH release slightly differed from experiment to experiment, this pattern was consistently observed. Additional repeats may help reduce the statistical difference within the summarised data sample, therefore increasing significance overall.

ELISA data of IL-1β release post-infection at MOI 50 (Figure 6B) revealed that Mevastatin treatment significantly reduces cytokine release (P-value = 0.0004), hinting that the NCI is not responsible for the observed increase in cell death. Moreover, although there was no difference in cell death with Mevastatin treatment of un-primed cells (Figure 6A), we do observe a significant decrease in IL-1β secretion (P-value = 0.0037). Similarly, IL-1β levels of cells not treated with Mevastatin were not

affected by IFNγ priming. Comparing with the data from the LPS control, we notice a smaller decrease of IL-1β secretion in IFNγ-primed, Mevastatin-treated cells infected at MOI 50 (Figure 6B). This all may suggest that the observed increase in cell death is due to the activation of multiple Inflammasomes by *Salmonella* PAMPs, besides the NCI, such as NLRC4 and NLRP3. Whether Mevastatin plays a role in the function of these canonical inflammasomes remains unclear and is beyond the scope of this project. It should also be mentioned that the ELISA data were extrapolated from only 2 experiments, in contrast to 5 experiments for the LDH data, so further experiments are required before definite conclusions can be drawn.

It has been previously shown that NLRP3 and NLRC4 inflammasomes are redundant following infection by *S.* Typhimurium knock-out strains that lack function of the *Salmonella* pathogenicity island 2 (SPI-2) T3SS (Bierschenk *et al.*, 2019). This particular study showed that SPI-2 may "mask" the effects of SPI-1 virulence that would otherwise lead to premature detection by the host. When SPI-2 is deleted both pyroptosis and IL-1β release are markedly increased. The SPI-2 deletion mutant also increased cleavage of both caspase-1 and caspase-4, the main effector of the NCI. SPI-2 plays a crucial role in the function and maintenance of the *Salmonella*-containing vacuole (SCV)(Jennings, Thurston and Holden, 2017). Dysfunctions in SPI-2 may therefore result in early vacuolar escape, allowing inflammasome receptors to detect the bacteria more readily.

Crucially, the SPI-2 core effector protein *SifA* is also known to be prenylated (Reinicke *et al.*, 2005), which may explain the curious effect of Mevastatin on pyroptosis following *Salmonella* infection; We hypothesised that Mevastatin prevents *SifA* prenylation, disrupting proper function of SPI-2, which results in a heightened detection of SPI-1 T3SS components by the NAIP-NLRC4 inflammasome and of LPS by the NCI due to premature escape from the SCV. To test this hypothesis, we acquired *S.* Typhimurium mutant strains; SL 1344 ΔSSaV and ΔOrgA, which do not allow for secretion of SPI-2 and SPI-1 effectors, respectively, and repeated the infection experiments at MOI 50 only (Figure 7).

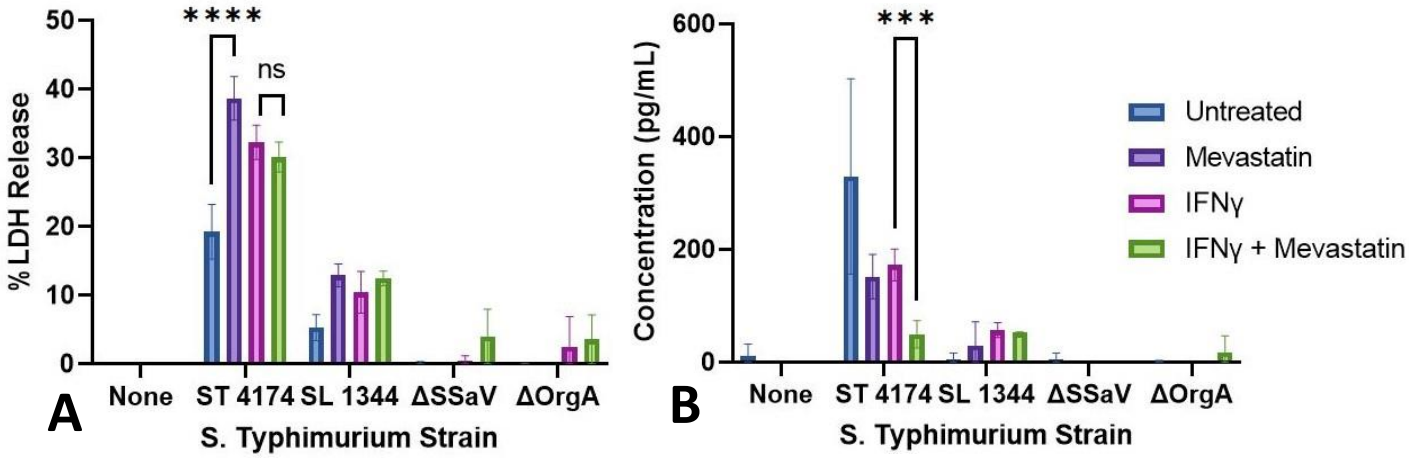


***Figure 7. Infection of HIEC-6 epithelial cells by Salmonella ST 4174, SL 1344, and mutants ΔSSaV and ΔOrgA, deficient for SPI-2 and SPI-1 function respectively.*** ***A****. Cell death at 3h post-infection, measured by percentage of LDH release compared to 100% Lysis control.* ***B****. IL-1β release concentration at 6h-post infection of HIEC-6 by Salmonella ST 4174, SL 1344, and the mutants ΔSSaV and ΔOrgA.*

Preliminary data from one experiment seem to disprove this hypothesis; if it were true, we would expect that cell death increase in Mevastatin-treated cells infected with the WT strains to be equivalent to that of cells infected with the SPI-2 knockout strain, ΔSSaV, which is very clearly not the case here (Figure 7A). Moreover, although we do see a significant increase in cell death of un-primed, Mevastatin-treated cells infected with ST 4174 compared to untreated (2-way ANOVA with

Tukey's multiple comparisons test, P-value < 0.0001), there is no significant difference among IFNγ-primed cells. We found no significant difference in cell death triggered by any of the other strains. It is worth noting that previous research reported increased levels of cell death after infection with ΔSSaV strain compared to WT *S.* Typhimurium (Bierschenk *et al.*, 2019), something we did not observe in our findings (Figure 7A).However, that particular research utilised different cell types than our experiments, so a direct comparison would not be accurate. In contrast to cell death, IL-1β release from un-primed cells was higher without Mevastatin treatment (Figure 7B), whereas Mevastatin caused a significant reduction of IL-1β release from IFNγ-primed cells (P-value < 0.004). Overall, this data strongly suggests a rejection of this new hypothesis, although more repeats and troubleshooting are required to reach a definitive conclusion. Contrarily, the near-total lack of cell death after infection with the SPI-1 deficient strain ΔOrgA is to be expected; epithelial cells are not phagocytic and since SPI-1 encodes the *T3SS* necessary for Salmonella to invade the host cells, it is unlikely they would infect HIEC cells at all.

Efforts to recreate these infection experiments in HeLa cells produced mixed results. Although established research (Giannella *et al.*, 1973) suggests sufficient invasion and cell death levels after *Salmonella Typhimurium* infection of HeLa cultures, our HeLa cells showed inconsistent levels of cell death and infection across 4 attempts using the same experimental protocol as our HIEC-6 cells. One of the experiments showed no cell death at all, suggesting the infection had failed completely. The other 3 repeats showed very low mean cell death levels of 19% at 6h post-infection with ST4174 (Figure 8A) with IFNγ priming, compared to nearly 40% that we observed in IFN-primed HIEC-6 cells infected under the same conditions (Figure 7A). Previous research performing similar experiments on HeLa also reported overall lower cell death (Santos *et al.*, 2020a), suggesting this particular cell line might be less sensitive than the HIEC-6 when it comes to *Salmonella* infection. Similar to our results in HIEC cells however, IFNγ priming significantly increased cell death in HeLa (2-way ANOVA with Šídák's multiple comparisons test, P-value = 0.0002, 0.037 for ST4174 and SL1344 respectively, Figure 8A), even at these low levels. This agrees with our prior findings in epithelial cells and further cements the important role of IFNγ for inflammasome function.

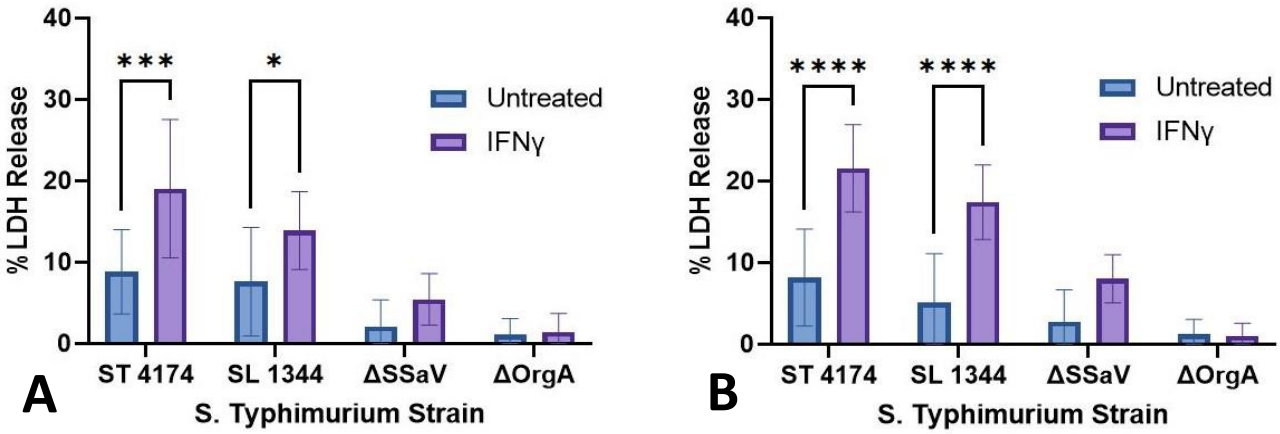


***Figure 8. Infection of HeLa cells by Salmonella ST 4174, SL 1344, and mutants ΔSSaV and ΔOrgA, deficient for SPI-2 and SPI-1 function respectively.*** ***A****. Cell death at 6h post-infection with cultures in logarithmic growth phase, measured by percentage of LDH release compared to 100% Lysis control.* ***B****. Cell death at 6h post-infection with cultures in stationary growth phase. Priming the cells with IFNγ potentiates the inflammasome response resulting to significantly higher levels of cell death. Summarised data from 3 experiments, normalised to remove non-specific background death.*

Interestingly, we observed the same amount of cell death when using bacteria cultures in stationary phase instead of logarithmic growth (Figure 8B). This finding is somewhat curious, since epithelial cells typically are non-phagocytic and *Salmonella* is known to lose its invasive ability in stationary phase due to a lower expression level of SPI1 effectors (Lee and Falkow, 1990). A few early studies using HeLa cells claimed that they can phagocytose certain types of bacteria (Shepard, 1955, 1960) but more recent research has shown that HeLa can only perform actions similar to phagocytosis under very specific conditions (Hanawa-Suetsugu *et al.*, 2019; Uribe-Querol and Rosales, 2020). IFN-priming had the same significant effect on cell death observed previously (P-value <0.0001) and as with the HIEC infections, the mutant strains caused little to no cell death.

We then performed an experiment infecting IFN-primed HeLa cells with *Salmonella* cultures in logarithmic growth phase and adding Mevastatin treatment (Figure 9). In contrast to both the LPS transfection (Figure 1) and the *Salmonella* infection experiments (Figure 7) in HIEC-6 cells, Mevastatin had no effect on cell death in HeLa, regardless of priming status (2-way ANOVA with Tukey's multiple comparisons test, P-value > 0.7).

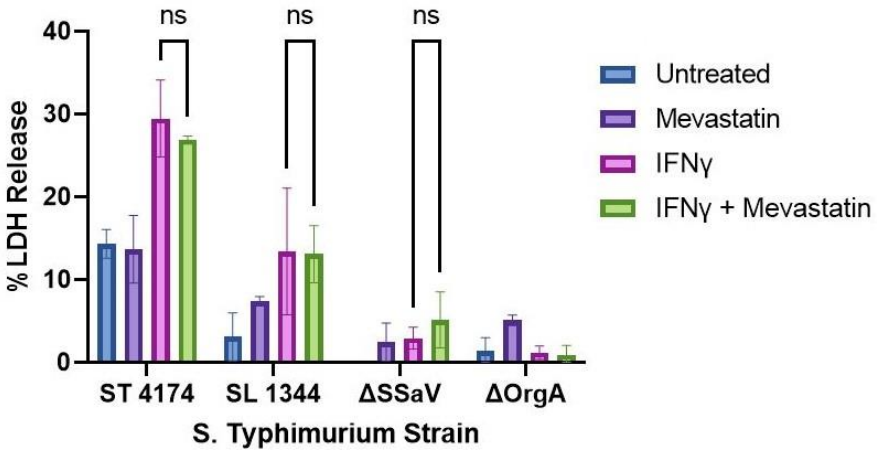


***Figure 9. Mevastatin has no effect on cell death after infection of HeLa cells by Salmonella ST 4174, SL 1344, and mutants ΔSSaV and ΔOrgA.*** *Cell death at 6h post-infection with cultures in logarithmic growth phase, measured by percentage of LDH release compared to 100% Lysis control. No difference was observed with Mevastatin treatment, regardless of priming status. Normalised to remove non-specific background death.*

Due to the low levels of cell death and the overall lack of consistency between the datasets of individual experiments, made obvious by the large statistical error bars on the graph from the summarized dataset (Figure 8), we decided it would be best to not pursue further experiments with HeLa for this stage of the project and focus instead on our findings in HIEC-6 epithelial cells.

## Mevastatin treatment increased death of THP-1 Cells during Salmonella Infection when GBP-1 is absent or Caspase-1/-4 were inhibited.

A preliminary infection experiment in THP-1 cells using the same setup as HIEC-6 cells revealed that priming with IFNγ significantly increased pyroptosis 3h post-infection with Salmonella at MOI 10 (2-way ANOVA with Tukey's multiple comparisons test, P-value < 0.005) and MOI 50 (P-value < 0.001), as well as during LPS transfection (P-value < 0.0001). Interestingly, Mevastatin does not appear to have the same effect of increasing cell death as in HIEC-6. Contrarily, in this particular experiment, Mevastatin significantly reduced cell death in IFNγ-primed cells infected with *Salmonella* Typhimurium ST 4174 at both MOI (P-value < 0.005).

We then performed this experiment on a mutant THP-1 strain with deletions for GBP-1/-2/-5 via CRISPR-Cas9 (ΔGBP). These GBPs all have prenylation domains, making them potentially sensitive to statin inhibition. GBP-1 and -2 in particular play a crucial role during NCI activation by forming a complex around LPS to recruit caspase-4 (Santos *et al.*, 2020a). As a result, the ΔGBP line is functionally deficient for NCI activation. The cell death by LPS transfection was almost completely ablated, with IFNγ having no effect (P-value < 0.0001), highlighting the importance of GBPs for NCI activation in macrophages. More importantly, the effect of Mevastatin seemed to be restored, compared to the WT cell line: at both MOI Mevastatin significantly increased pyroptosis compared to the untreated control (P-value < 0.005). Notably, IFNγ also had a small yet significant increase in cell death (P-value = 0.004), hinting at further involvement in inflammasome activation independently of GBPs.

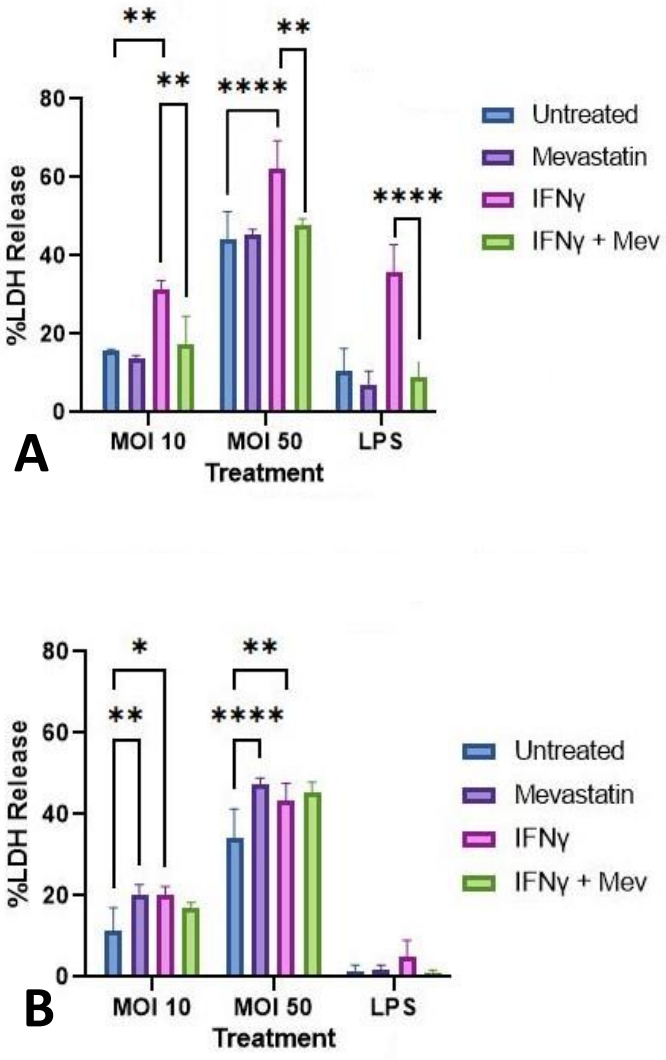


*Figure **10A**. Cell death of wild type and **B** ΔGBP1-2-5 THP-1 macrophages at 3h post-infection by Salmonella ST 4174. Measured by percentage of LDH release compared to 100% Lysis control. LPS transfection included as a positive control of NCI activation. Normalised for clarity.*

These results seem to contradict our earlier LPS transfection experiments in THP-1 cells (Figure 2), suggesting both a significant role of (prenylated) GBPs during inflammasome activation and a significant reduction in cell death caused by Mevastatin priming.

We then proceeded to test the *SifA* prenylation disruption hypothesis in THP-1 cells as we did with HIEC-6 cells earlier. In the combined dataset of 4 such experiments we observed overall high levels of cell death after 3h of infection by wild type ST 4174 and SL 1344 strains at MOI 50 (Figure 11). Interestingly, there was no significant change in cell death caused by Mevastatin treatment or IFNγ-priming (2-way ANOVA with Tukey's multiple comparisons test, P-value > 0.999). The SPI-2 deficient strain, ΔSSaV, caused overall less cell death compared to the wild type strains (P-value < 0.0001),

which goes against previous reported findings (Bierschenk *et al.*, 2019) that reported increased cell death from the SPI-2 mutants. However, within that sample, double treatment with IFNγ and Mevastatin seemed to significantly increase cell death compared to no treatment at all (P-value = 0.0405) but even in that case we must concede that death of untreated cells was again abnormally low compared to what the literature suggests we should expect under these conditions. Similarly, levels of cell death triggered by infection with the SPI-1 deficient strain ΔOrgA were low but still present. Unlike HIEC cells, THP-1 macrophages are phagocytic, which could explain how the two mutant strains were able to infect them and trigger some cell death, even at low amounts.

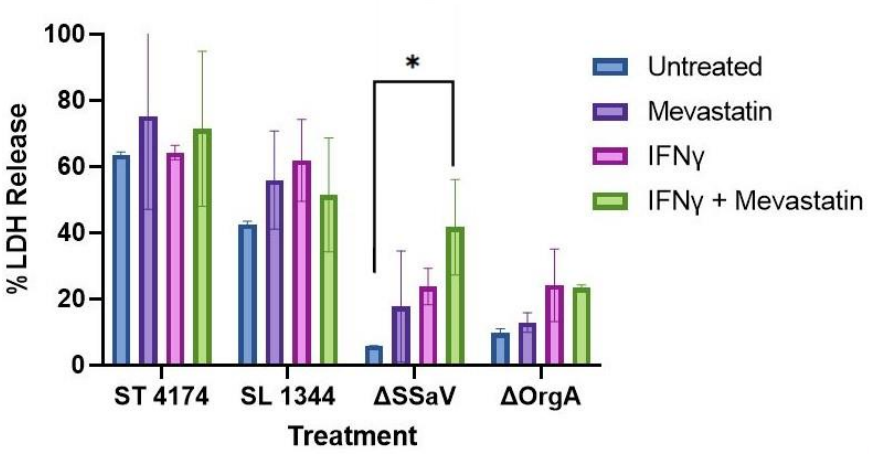


***Figure 11. Cell Death of THP-1 Macrophages at 3h post-infection with Salmonella ST 4174, SL 1344, and mutants ΔSSaV and ΔOrgA, deficient for SPI-2 and SPI-1 function respectively.*** *There was no significant change in cell death caused by Mevastatin treatment or IFNγ-priming when infected with WT strains. Death triggered by ΔSSaV infection was overall lower than previous research reports, but Mevastatin-treatment significantly increased death of IFNγ-primed cells compared to untreated/un-primed cells (P-value = 0.0405). Summarised data from 4 experiments, normalised for clarity.*

To elucidate how much of this cell death is directly caused by inflammasome activation, canonical or non-canonical, we also performed this experiment with additional treatment with VX-765, an inhibitor of casp-1/-4 (Figure 12). If the cell death was solely pyroptotic, we would expect to see almost total ablation regardless of treatment, since no inflammasome should be functional in the absence of active caspase-1 or caspase-4, based on current scientific consensus at least (Downs *et al.*, 2020).

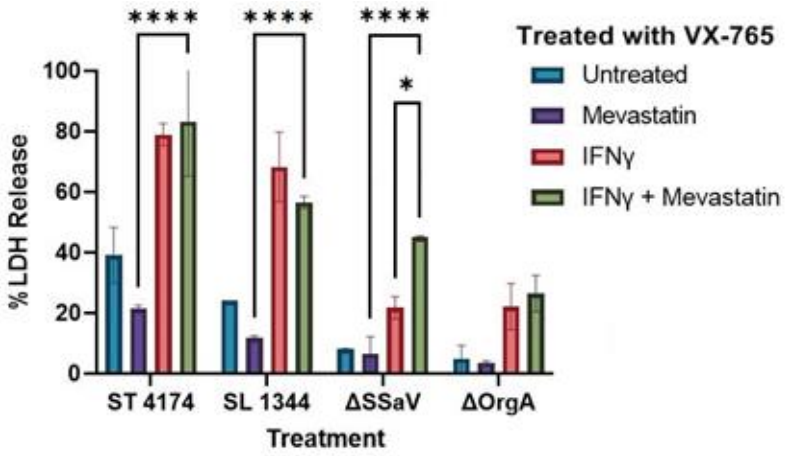


***Figure 12. Cell Death of THP-1 Macrophages at 3h post-infection with Salmonella ST 4174, SL 1344, and mutants ΔSSaV and ΔOrgA, deficient for SPI-2 and SPI-1 function respectively. The cells had been treated with the caspase-1/-4 inhibitor VX-765 prior to infection.*** *Death of primed cells was significantly higher than that of un-primed cells infected with either one of the WT strains and the ΔSSaV mutant (P-value < 0.0001). Summarised data from 4 experiments, normalised for clarity.*

However, although death was seemingly reduced after VX treatment (nearly halved in completely untreated cells) this fall was only significant when combined with Mevastatin treatment in un-primed cells infected with ST 4174 (2-way ANOVA with Tukey's multiple comparisons test, P-value = 0.0002 for Mevastatin-treated vs Mev + VX, P-value = 0.0055 for Untreated vs Mev + VX). Similarly, cell death was only significantly reduced by VX-765 after infection with SL 1344 when comparing between un-primed, Mevastatin-treated cells (P-value = 0.003).

Crucially, death of primed cells was significantly higher than that of un-primed cells infected with either one of the WT strains (P-value < 0.0001), regardless of Mevastatin treatment. A significant increase in cell death was also observed in IFNγ-primed, Mevastatin-treated cells infected with

ΔSSaV compared to un-primed ones (P-value < 0.0001). In this case we also noted a slightly significant effect of Mevastatin between primed cells, resulting in an increase of cell death (P-value = 0.0118).

Since VX-765 evidently has no effect in cell death of IFNγ-primed THP-1 macrophages after infection with S. Typhimurium, it is unlikely that the majority of this death is caused by inflammasome activation whether it be the NCI or any canonical inflammasome.

## Mevastatin Does Not Affect Inflammasome Activation of THP-1 Macrophages Infected with *Salmonella* Typhimurium OMVs

Untreated and statin-treated THP-1 macrophages, primed with IFNγ to induce GBP expression, were treated with OMVs (4 μg/mL) extracted from *Salmonella* Typhimurium ST 4174 for 6h. This experiment was repeated 6 times. Summarised LDH assay data revealed no significant difference in cell death between the untreated and Mevastatin-treated cells (Figure 13A, Paired t-test, P-value = 0.86). ELISA data of IL-1β release after cell death confirm no significant effect of Mevastatin treatment (Figure 13B, Paired t-test, P-value = 0.6181).

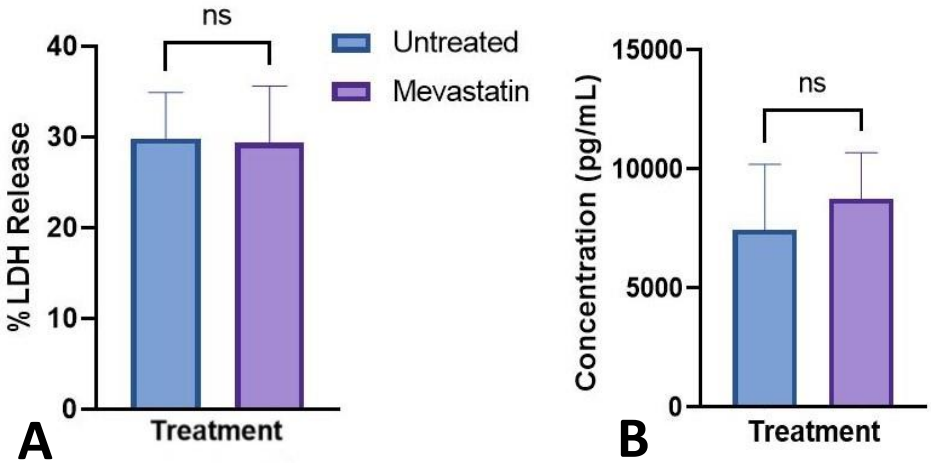


***Figure 13A**. Death of THP-1 macrophages and **7B**. release of inflammatory cytokine IL-1β at 6h post-infection by OMVs extracted from Salmonella ST 4174. Summarised data from 3 experiments, normalised for clarity.*

We also performed this experiment with OMVs extracted from two *Escherichia Coli* strains, TOP10 ("Top") and NEB10-beta ("Neb"), a derivative of *E.Coli* DH10Band another strain of *Salmonella* Typhimurium, SL 1344. Results agreed with our previous findings, showing no difference in cell death or IL-1β release due to Mevastatin treatment (Figure 14).

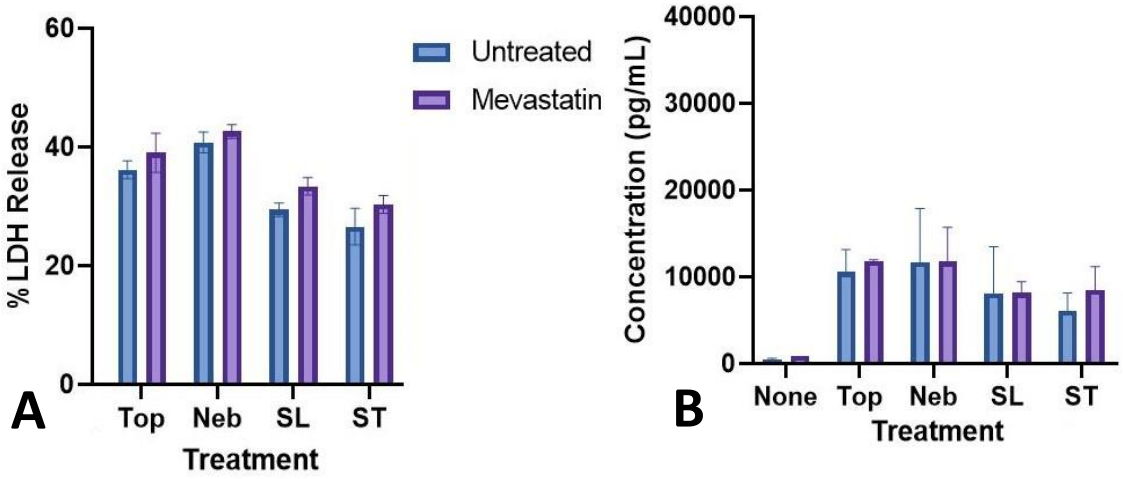


***Figure 14A**. Cell death of THP-1 macrophages and **B**. release of inflammatory cytokine IL-1β at 6h post-infection by OMVs extracted from Salmonella Typhimurium ST 4174 (ST), SL 1344 and E. Coli Neb and Top. Summarised data from 2 experiments. LDH data normalised for clarity.*

To check whether the inhibition of NCI activation by Mevastatin treatment that we observed with LPS transfections in epithelial cells extends to OMV infection, we performed this experiment in HIEC-6 primary epithelial cells. We observed minimal amounts of cell death induced by OMV infection, compared to the LPS transfection control in primed cells. This might be due to the lack of phagocytosis by HIEC cells which renders them able to be only passively infected by OMVs instead of actively uptaking them, contrarily to macrophages. Moreover, we found no significant difference in cell death between statin-treated and untreated cells after 6h of incubation, regardless of whether they had been primed with IFNγ or not (Figure 15).

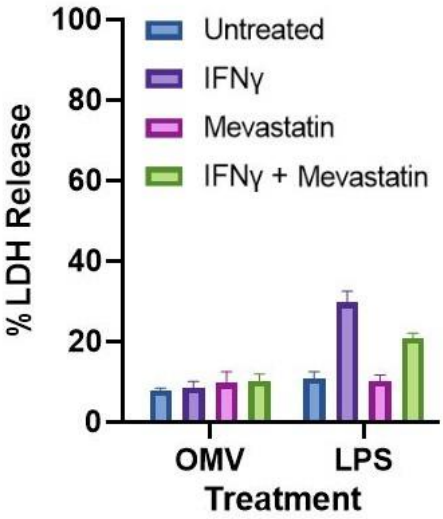


***Figure 15****. Cell death of HIEC-6 primary epithelial cells at 6h post-infection by OMVs extracted from Salmonella ST 4174. Transfection of LPS EK included as control. Normalised for clarity.*

Finally, to ensure our macrophage findings are consistent, we also infected bone marrow-derived murine macrophages (BMDMs) with OMVs from the two *E.Coli* and *S*. Typhimurium strains. Due to unavailability of murine IFNγ, we primed the cells with Pam3CKS, a synthetic triacylated lipopeptide which induces expression of GBPs and other inflammasome components through the TLR-2/NF-κB signalling pathway (Brandt *et al.*, 2013; Lamkanfi and Dixit, 2014b). Otherwise, the BMDMs were treated with the same concentration of OMVs (4 mg/mL) and incubated for 6h under the same conditions as the THP-1 macrophages.

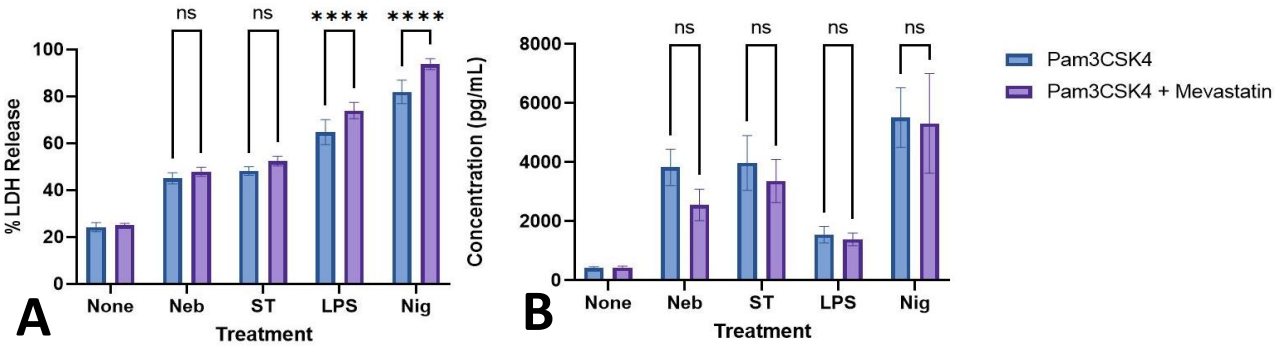


***Figure 16A****. Cell death of BMDMs and **10B** release of inflammatory cytokine IL-1ϐ at 6h post-infection by OMVs extracted from Salmonella Typhimurium ST 4174 (ST) and E. Coli (Neb). Nigericin treatment (Nig) and transfection of LPS EK included as positive controls for NLRP3 and NCI activation respectively. Summarised data from 2 experiments.*

Our LDH data (Figure 16A) revealed no significant difference of death between statin-treated and untreated cells after OMV infection (2-way ANOVA with Šídák's multiple comparisons test, P-value > 0.1). ELISA data concur (Figure 16B), showing no difference in IL-1β release concentration with Mevastatin treatment (P-value > 0.06). Notably, Mevastatin caused a significant increase in cell death after LPS transfection and Nigericin treatment (P-value < 0.0001). However, there was no difference in cytokine release (P-value > 0.99). This might hint that the increased cell death was not pyroptotic and thus not associated with inflammasome activity. However, further experiments utilising the inhibitors MCC950 and VX-765 are necessary to confirm this. On this regard our results remain inconclusive, but we hypothesise that this increase may have been necroptosis, perhaps caused by non-specific toxicity of the LPS and Nigericin reagents to the BMDMs. More experiments would be required to determine the causes behind this finding and whether Mevastatin plays an actual role.

## The NLRP3 Canonical Inflammasome Is Not Functional in HIEC-6 Primary Epithelial Cells

For the majority of our experiments, Nigericin was used as a positive control of canonical inflammasome activation. Nigericin is a potent trigger of the NLRP3 inflammasome; it functions by forming pores in the cell membrane which results in potassium cation efflux, activating NLRP3(Muñoz-Planillo *et al.*, 2013). In macrophages, Nigericin causes markedly higher amounts of pyroptosis compared to LPS transfection (Figure 2) or treatment with OMVs (Figure 16). Although there doesn't seem to be extensive research on the particular matter, current literature suggests that HIEC-6 epithelial cells express NLRP3. Importantly, NLRP3 is thought to be activated downstream of the NCI to induce maturation of IL-18 and IL-1β by cleaving their unprocessed forms(Baker *et al.*, 2015a).

In a few of our HIEC-6 transfections, we have seen small but persistent amounts of cell death with Nigericin treatment. To elucidate whether that death is a result of canonical inflammasome activation, we set up an LPS transfection experiment using Nigericin in both un-primed and IFNγ–primed cells as well as two inhibitors: the NLRP3 inhibitor MCC-950 and the Caspase-1/-4 inhibitor VX-765. If the observed death was indeed NLRP3-dependent, then both inhibitors would completely ablate it.

After 6 repeats of the experiment, our results indicate virtually no difference in Nigericin- induced cell death regardless of treatment or priming status (2-way ANOVA with Tukey's multiple comparisons test, P-value > 0.99, Figure 17A). Similarly, there was no difference in IL-1β secretion (P-value > 0.99, Figure 17B). In contrast, death and cytokine secretion caused by LPS transfection were dependent on IFNγ priming and significantly decreased by VX-765, as expected in the case of pyroptosis caused by NCI activation.

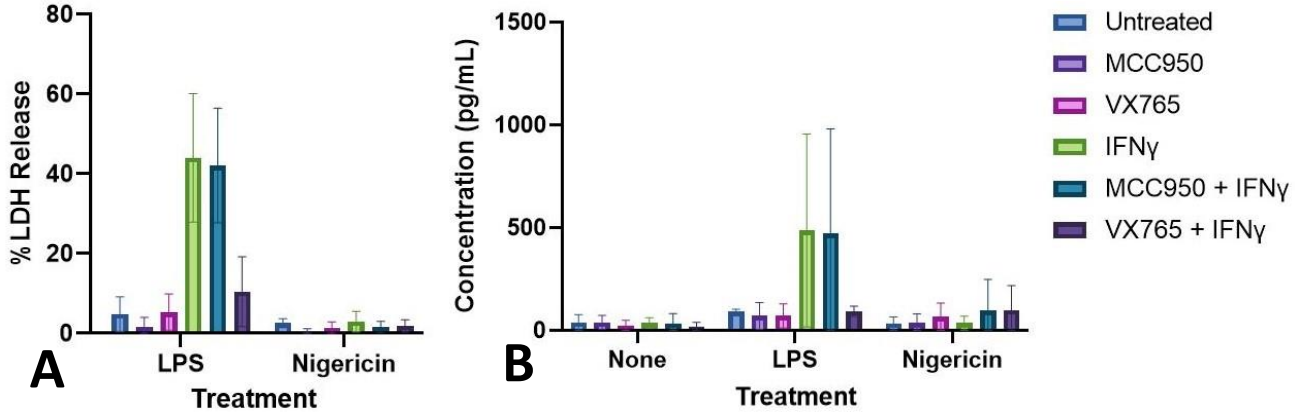


***Figure 17. Nigericin treatment does not trigger NLRP3 inflammasome activation in HIEC-6 cells. A****. Cell death of HIEC-6 cells and* ***B.*** *release of inflammatory cytokine IL-1ϐ after Nigericin treatment (Nig) and transfection of LPS. Both death and cytokine secretion are nearly non-existent after Nigericin treatment, regardless of priming conditions. In contrast, LPS transfection triggered a strong pyroptotic response, dependent on IFNγ priming. Inhibition of NLRP3 by MCC950 showed no significant difference, whereas Casp-1/-4 inhibition by VX765 significantly ablated pyroptosis, confirming NCI activity. Summarised data from 6 experiments.*

Overall, Nigericin caused very little cell death compared to LPS, implying our observations from earlier experiments were not new findings but simply non-specific necroptosis, possibly caused by general toxicity, stress, or minor discrepancies in our experimental technique. As such, our findings

seem to agree with the general consensus that HIEC-6 human primary epithelial cells do not have a functional NLRP3 inflammasome.

# Discussion

## LPS Transfections

Our LPS transfection experiments in IFN-primed HIEC-6 primary cells showed that overnight Mevastatin treatment causes a significant loss of pyroptosis, associated with reduced function of the NCI. Previous studies have established the critical role of GBP-1 as the upstream receptor of the NCI pathway and have suggested that its farnesylation domain may fulfil the part of a "lipid anchor", stabilising GBP-1 on the bacterial outer membrane during the formation of the GBP-caspase-4 complex (Santos *et al.*, 2020b; Wandel *et al.*, 2020). This is also supported by earlier work on the regulation and intracellular localisation of GBPs, which established that prenylation of GBP-1/-2/-5 is necessary for their association with lipid membranes (Britzen-Laurent *et al.*, 2010). Our findings provide further evidence towards this hypothesis, showing that widely prescribed statins are able to disrupt the function of the NCI by inhibiting GBP prenylation.

Other studies have shown that statins are able to prevent protein prenylation, even recommending them as potential cytotoxic and cytostatic treatment options against cancer, in combination with farnesyl-transferase inhibitors (Wojtkowiak, Gibbs and Mattingly, 2009). Moreover, prenylation was shown to be necessary for the translocation of other GTPases, such as Rac and Rho, on the plasma membrane (Cordle *et al.*, 2005). This further supports observations that farnesylation of GBP-1 serves a similar function. To test this hypothesis, future research could clone and express recombinant forms of the farnesylation enzyme responsible to modify GBP-1 (encoded by the FNTA and FNTB genes in humans) tagged with a green-fluorescent protein (GFP) marker. These recombinant proteins could be mixed *in vitro* with GBP-1 and purified LPS or *Salmonella* and E.Coli cultures and then observed using fluorescent microscopy. Association of the GFP-tagged farnesylation domains with the outer membranes of the bacteria would show up as localised fluorescence, thereby confirming our hypothesis.

In THP-1 macrophages the effect of Mevastatin was not as clear. Out initial LPS transfection experiments in THP-1 cells showed virtually no difference in NCI activation after Mevastatin treatment. This was somewhat expected, as there are reports that GBP-1, although important, is not crucial for NCI activation in THP-1 cells in response to cytosolic LPS (Fisch *et al.*, 2019; Santos *et al.*, 2020b), unlike HIEC. For that same reason, we did not prime the THP-1 macrophages with IFNγ prior to transfection.

Minor inconsistencies between cell death levels of individual transfection experiments led us to perform LPS dose response experiments, transfecting both IFNγ - primed and unprimed THP-1 cells with 6 different concentrations of LPS EK. We found that IFNγ significantly increased the pyroptotic cell death across all different concentrations. This effect became more pronounced when using lower concentrations of LPS and we identified 0.004 μg/mL and not the higher 0.1 or 0.02 μg/mL as the ideal concentration where cell death peaked. This suggests that THP-1 and HIEC-6 cells have different sensitivity levels of LPS and that the former may become "saturated" by it at higher concentrations, which enables pyroptosis even without GBP-1. At lower concentrations however, GBP-1 becomes crucial to NCI function. Even at LPS concentrations as low as 0.00016 μg/mL, we observed more than 20% cell death in primed cells, compared to nearly no death if unprimed ones.

When we included LPS transfection controls using that ideal concentration in our infection experiments in Mevastatin-treated, primed THP-1 cells (Figure 10A), we saw the same significant reduction of cell death and IL-1β release that we observed in our HIEC-6 experiments. We conclude that Mevastatin has an inhibitory effect on the NCI on both cell types and that GBP-1 is a lot more important to NCI function in THP-1 cells than previously reported.

Another interesting result was that the structure of LPS itself played a role in NCI activation. The much sorter polysaccharide chain of LPS EK must make more identifiable or accessible by the NCI since it caused about 20% more cell death in THP-1 cells, compared to the same concentration of LPS EB. This was a statistically significant difference.

Structural differences of LPS have already been established as key factors in pathogen virulence, antibiotic resistance and innate immunity (John, Liu and Jarvis, 2009; Chilton, Embry and Mitchell, 2012; Matsuura, 2013). Research on different species of the *Bordetella* genus has shown that species that utilise LOS or rough LPS (shorter polysaccharide chain) do not activate TLR4 as potently as those with smooth LPS (longer polysaccharide), seem to not activate MyD88, which are important for inflammasome priming, at all and are generally more pathogenic (Mann *et al.*, 2005; Fedele *et al.*, 2008; Chilton, Embry and Mitchell, 2012). This is relevant to our findings because LPS EK is rough (Kuhnert, Nicolet and Frey, 1995), whereas LPS EB, which elicited a much lower cell death response, is smooth (Coleman, Goebel and Leive, 1977).

A lot of the focus of relevant literature is placed on the ionic charge and the acetyl chain length of the lipid-A component of LPS, since this is considered to be the part which interacts with the aforementioned pathways. Although this is most likely the case for GBP1 as well (Santos *et al.*, 2020b), we hypothesise that the length of the O-antigen chain might have an indirect but important effect; Perhaps the shorter chain of rough LPS makes lipid-A more accessible GBPs and/or easier for the formation of the GBP-caspase-4 complex. A recent study reported that *Salmonella* Paratyphi, a bacterium that produces LPS with elongated O-antigen chains, caused significantly lower activation of the inflammasomes, resulting in reduced pyroptosis levels compared to *Salmonella* Typhimurium, whose LPS O-chain is much shorter. (Mylona *et al.*, 2021). This effect was directly linked to the *FepE* gene, which regulates the length of the O-antigen. The researchers demonstrated that *FepE* is expressed at much higher levels in Paratyphi compared to Typhimurium, resulting to the observed inflammasome resistance. Deleting or disabling *FepE* caused a significant increase of the pyroptotic response. Moreover, this study reported that it was the NCI in particular whose activation was hindered by the very long o-antigen chains, providing further support towards our own hypothesis.

## Infections with *Salmonella* Typhimurium

We infected IFNγ-primed HIEC-6 primary epithelial cells and THP-1 macrophage-like cells with *Salmonella* Typhimurium and found that overnight Mevastatin treatment had interesting but contradictory effects on each cell type's death response 3 hours post infection;

Mevastatin significantly increased *Salmonella*-induced cell death of HIEC cells, regardless of priming condition. However, the LPS transfection control we included showed the opposite effect, consistent with our previous transfection experiments.

It seemed likely that Mevastatin could also be disrupting the function SPI-2 by inhibiting prenylation of its core effector protein *SifA.* Since SPI-2 is crucial for the maintenance of the SCV, its disruption could result in early vacuolar escape of the bacteria, which would trigger a heightened cell death response, mainly through redundant activation of NCI and NLRC4. We used SPI-1 and SPI-2

*Salmonella* knockout strains to explore this hypothesis but contrary to the literature (Bierschenk *et al.*, 2019), we observed much lower cell death levels compared to our WT strains. It is worth mentioning that Bierschenk *et al* used different cell types for their infection experiments, namely BMDMs and human macrophages isolated from blood samples, as well as THP-1 cells. This could potentially explain why our observed cell death levels were so much lower, since these observations were made on HIEC cells.

More importantly, Mevastatin treatment resulted in significantly reduced IL-1β release in both cases. This suggests that the increased cell death is not pyroptotic and further supports our original hypothesis on Mevastatin's inhibitory effects on the NCI.

Our THP-1 infections provided additional evidence towards this original hypothesis; Interferon priming showed a marked increase in pyroptosis, whereas Mevastatin significantly decreased it. In our un-primed WT THP-1 macrophages we didn't observe the cell death increase that Mevastatin caused in HIEC. However, this phenomenon was restored in our GBP knockout THP-1 strain (ΔGBP). Moreover, infections using the SPI-2 knockout *Salmonella* strain and the caspase-1/-4 inhibitor VX-765 also resulted in significantly increased cell death, further suggesting that the observed cell death is likely not pyroptotic.

We speculated that this response might be a result of caspase-5 dependent LPS recognition, which would not have been inhibited by VX-765. In a recent publication, Baker *et al.* hypothesise caspases 4 and 5 serve a redundant role during NCI activation and, more importantly, lead to downstream activation of NLRP3 to induce IL-1β maturation (Baker *et al.*, 2015b). The researchers found that the effect of caspase-5 on IL-1β secretion was especially significant during *Salmonella* infection, compared to LPS transfection. It is therefore likely that in the absence of functional caspase-4, the cells compensate with caspase-5 as a back-up mechanism of inducing cell death to minimise the effect of the infection. However, NLRP3 still depends on caspase-1 for its function, and Baker *et al* report that caspase-5 didn't respond to LPS in absence of caspase-4. Thus, it is unlikely -but not impossible- that caspase-5 would be responsible for the observed cell death in our case.

An entirely different theory would be that the cell death increase we observed after Mevastatin treatment and caspase-1/-4 inhibition is not at all inflammasome-mediated but rather a result of a different programmed death pathway, such as apoptosis or necroptosis, which may act as a backup mechanism when pyroptosis fails. This has been previously observed in mouse macrophages (Doerflinger *et al.*, 2020). Our results from *Salmonella* infections in HIEC cells seem to support this hypothesis as well, due to the combination of cell death increase but reduction in cytokine release, which signifies non-pyroptotic cell death.

During bacterial infection, macrophages and other phagocytic cells perform a crucial function known as respiratory burst, when high amounts of reactive oxygen species such as superoxides and hydrogen peroxide are synthesised to form antimicrobial compounds and kill bacteria captured within the phagosomes (Segal, 2005). Researchers recently found that Cytochrome b-245 chaperone 1 (CYBC1), also known as EROS (Essential for Reactive Oxygen Species), plays an integral role to this process and innate immunity in general (Thomas et al., 2017). EROS functions as a chaperone to recruit and stabilise the different components of NADH oxidase. It has been also implicated with human pathology, as a key factor in chronic granulomatous disease (CGD), which is an autosomal recessive disorder caused by mutations that lead to EROS deficiency (Arnadottir et al., 2018). CGD is a primary immunodeficiency characterised by an inability to make reactive oxygen species, which results to severe opportunistic infections and auto-inflammation. Moreover, EROS has also been linked to the function of the NLRP3 canonical inflammasome via the P2X7 ion channel (Ryoden et al.,

2020). P2X7 binds extracellular ATP (a common PAMP) and allows influx of calcium ions resulting to NLRP3 activation. EROS – deficient macrophages exhibit a significant decrease in P2X7 expression and reduction of caspase-1 and IL-1β cleavage and overall ATP-induced cell death.

Crucially for our own research, EROS is high in macrophages and further up-regulated by IFNγ or a combination of IFNγ and infection by *Salmonella* Typhimurium (Thomas et al., 2017). Our infections in IFNγ-primed THP-1 macrophages with VX-765 treatment could perhaps result in high cell death not because of any inflammasome function but due to a high expression of EROS leading to increased production of free radicals and therefore increased ER stress.

Additionally, it has been shown that P2X7, which would also be upregulated by EROS in this scenario, can result in apoptosis or necroptosis independently of NLRP3, upon activation by ATP (Kanellopoulos and Delarasse, 2019). A recent study found that membrane lipid composition is especially important for P2X7 function, which was inhibited by cholesterol binding to its transmembrane domain (Karasawa *et al.*, 2017). This hypothesis could be further supported by the fact that in both these THP-1 and the HIEC-6 infection experiments, we observed a link between this cell death increase and the presence of Mevastatin, which disrupts cholesterol synthesis. That being said, EROS can only account for the upregulation of P2X7 but not its activation.

In either case, since a deficiency in EROS has been implicated with decreased cell death and immunodeficiency (Thomas *et al.*, 2017; Arnadottir *et al.*, 2018), it is reasonable to assume that its upregulation would have the opposite result, but this is all just speculative at this stage and further experimentation would be required to test this hypothesis. Performing these infection experiments using P2X7 inhibitors or THP-1 knockout strains deficient for EROS could help elucidate its potential role in programmed cell death of cells lacking functional inflammasomes.

The wider literature hints at other potential explanations for our THP-1 results, besides EROS; It has been shown in murine macrophages (BMDMs) that caspase-8 could initiate apoptosis in response to *Salmonella* infection, when caspases -1 and -11 (the murine orthologue of casp-4) were absent (Doerflinger *et al.*, 2020). The researchers highlighted that the observed cell death presented with the key hallmarks of apoptosis and not necroptosis. Other studies have suggested that the NLRP1b and NLRC4 canonical inflammasomes can cause apoptotic-like cell death via caspase-8 when caspase-1 is absent (Van Opdenbosch *et al.*, 2017).

A potential caveat arises however; although VX-765 is formally known to only inhibit caspases-1 and -4, empirical data from our lab suggest that in the concentration we used (25 μM) it might affect the function of caspase-8 as well. If true, then our observations could not be explained by caspase-8-mediated apoptosis. It's worth noting that embryonic fatality via RIPK3 and MLKL mediated necroptosis has been observed in mice lacking caspase-8 (Alvarez-Diaz *et al.*, 2016), which could offer an additional explanation for our findings in THP-1 cells, if caspase-8 is indeed inhibited by VX-765.

Finally, it should be pointed out that most of these scenarios cannot immediately explain the role Mevastatin plays in increasing cell death in particular. Besides P2X7, another potential link could be found in the Rac and Rho protein families. Like GBPs, they are small prenylated GTPases and fulfil many roles important for innate immunity across multiple cell types (Greenwood, Steinman and Zamvil, 2006). Interestingly, Rac is directly involved in the production of superoxides by inducing activation of the NADPH oxidase in phagocytes (Pick, 2014), for which EROS is also a chaperone. Rac1 is crucial for the assembly of the NADPH oxidase complex in macrophages and its prenylation domain may act as a lipid anchor to stabilise the complex formation on the phagosome or cell

membrane, similarly to our hypothesised role of GBP prenylation (Cordle *et al.*, 2005). About 0.5% of functional proteins are known to be prenylated and some of these, like Rac and Rho, fulfil multiple crucial functions which could be disrupted by Mevastatin (Goldstein and Brown, 1990; Tamanoi *et al.*, 2001; Graaf *et al.*, 2004). Perhaps the disruption of multiple significant proteins would result in increased basal stress level, further contributing to this cell death increase we observed to be associated with Mevastatin treatment.

## Experiments with Outer Membrane Vesicles

Outer membrane vesicles (OMVs) are nanoparticles that derive from the outer membrane of gram-negative bacteria. They consist of a combination of proteins and lipids found in the OM and are often secreted both in culture and during infection (Cecil *et al.*, 2019). They can provide us with a way to study cell responses to LPS under more natural circumstances, instead of delivering purified LPS to the cells directly via transfection, while avoiding triggering the more complicated range of cell responses seen during infection with live bacteria.

We isolated OMVs from two *Salmonella* Typhimurium and two *Escherichia Coli* wild type strains. Then, similarly to the transfection experiments, we used the OMVs to treat IFNγ-primed HIEC-6 cells and THP-1 macrophages as well as BMDMs primed with Pam3CKS to induce GBP expression. As usual, some of our cells were treated overnight with Mevastatin. Our results indicated no significant difference of Mevastatin on pyroptosis among all three cell types.

Previous studies have established that GBPs are required for the inflammasome response to OMVs (Finethy *et al.*, 2017). The exact mechanism by which OMVs deliver LPS into the cytosol is not clearly defined for all cell types. In macrophages, they are most likely uptaken via endocytosis, but opinions differ regarding the exact manner of LPS delivery; one key study reported that LPS is released into the cytosol after the OMVs have entered the early endosome and is subsequently detected by the NCI (Vanaja *et al.*, 2016). Contrarily, some researchers postulated that prenylated GBPs associate with LPS of OMVs that have escaped the endosome, instead of free LPS in the cytosol, to activate the NCI pathway (Santos *et al.*, 2018). This posits the question: why would Mevastatin not affect the recognition of OMVs by the NCI, when prenylation of GBPs seems to be directly involved? The proposed experiment plan using GFP-tagged GBP farnesyl domains as outlined above could potentially help elucidate this question as well.

### HIEC-6 Cells do not have a functioning NLRP3 Inflammasome

The HIEC-6 primary epithelial cell line is not widely used. In fact, ATCC, the supplier of HIEC-6, listed just 28 publications in total that cited the particular cell line, even though it was first discovered nearly 30 years ago. Among this limited literature it is assumed that NLRP3 is expressed and functional in HIEC cells like other epithelial cell lines.

Our results using the known NLRP3 trigger Nigericin on HIEC cells suggest the opposite: We observed nearly no cell death or IL-1β release with Nigericin treatment (Figure 17). Using the caspase-1/-4 inhibitor VX-765 and the NLRP3-specific inhibitor MCC-950 made no difference, strongly suggesting that the minimal levels of cell death observed were inflammasome independent, likely due to toxicity of Nigericin. In contrast, pure LPS transfection in IFNγ -primed cells resulted in high levels of pyroptosis which were ablated when using VX-765, but MCC-950 had no effect. Consequently, we believe NLRP3 to be absent, or at the very least non-functional, in HIEC-6. Transcriptomic and

proteomic analyses could help clarify whether NLRP3 is expressed at all in that particular cell line and, if so, which factors render it inactive.

Besides the immediate relevance for this particular cell line, our findings have significant broader implications for the function of the non-canonical inflammasome. NLRP3 is widely believed to be activated downstream of the NCI and induce maturation of pro- IL-1β and pro-IL-18 into their active forms (Lamkanfi and Dixit, 2014a; Baker *et al.*, 2015a; Schmid-Burgk *et al.*, 2015; Mathur, Hayward and Man, 2017). This is mainly because an alternative mechanism whereby the caspase-4 inflammasome activates these crucial interleukins has not yet been found, yet they are both released upon NCI-mediated pyroptosis. Our data shows that despite Nigericin having no effect and the NLRP3 inhibitor being present, there is IL-1β release after LPS transfection (Figure 17). As a result, we believe there must be an NLRP3-independent mechanism that cleaves pro-IL-1β and pro-IL-18 into their active forms during NCI-driven pyroptosis.

## Limitations

The key limitation of this study is that, due to the potential pleiotropic effects Mevastatin can have on intracellular mechanisms and pathways, it remains unclear whether it disrupts the NCI through GBP-1 or via some other, possibly undiscovered mechanism. This was particularly highlighted by the OMV and *Salmonella* infection experiments. Further work is required in order to elucidate the specific mechanisms through which Mevastatin interferes with cell death post-infection and to answer the questions of whether GBPs are involved, and to what extent. Repeating the infection experiments using the ΔGB-1/-2/-5 THP-1 strain or different pathogens such as *E.Coli* or *Shighella*, and using OMVs isolated from these bacteria, would be a good way to begin answering some of these questions, or at the very least provide further context to our current findings.

Our few attempts to confirm our findings using HeLa cells have produced inconsistent results. Further optimisation and repeats using HeLa or other human epithelial cell lines could provide more evidence and confirm the validity of our findings.

In an effort to further support the findings of the transfection and infection experiments, multiple Western blots were performed. The aim was to detect cleavage of Caspase-1/-4/-5, GSDMD and IL-1β/-18 as qualitative indicators of inflammasome activation and pyroptosis. However, due to consistent technical difficulties which required extensive troubleshooting, the majority of these blots were deemed too unclear and hard to interpret to be presented in this thesis. Thus, several repeats of these blots are needed in order to cement the accuracy of our results.

Finally, our work on HIEC-6 cells revealed that they most likely do not have a functional NLRP3 inflammasome, but did not examine if this is due to some regulatory element or modification, or if the cells simply do not express NLRP3 at all. Genetic sequencing can be used to test whether HIEC-6 encode the NLRP3 gene and transcriptomic analysis or even Western and Northern blotting can be used to check its expression.

# Bibliography

Alvarez-Diaz, S. *et al.* (2016) 'The Pseudokinase MLKL and the Kinase RIPK3 Have Distinct Roles in Autoimmune Disease Caused by Loss of Death-Receptor-Induced Apoptosis', *Immunity*. Cell Press, 45(3), pp. 513–526. doi: 10.1016/J.IMMUNI.2016.07.016.

Arnadottir, G. A. *et al.* (2018) 'A homozygous loss-of-function mutation leading to CYBC1 deficiency causes chronic granulomatous disease', *Nature Communications*. Nat Commun, 9(1). doi: 10.1038/s41467-018-06964-x.

Baker, P. J. *et al.* (2015a) 'NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5', *European Journal of Immunology*. John Wiley & Sons, Ltd, 45(10), pp. 2918–2926. doi: 10.1002/EJI.201545655.

Baker, P. J. *et al.* (2015b) 'NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5', *European Journal of Immunology*. Eur J Immunol, 45(10), pp. 2918–2926. doi: 10.1002/eji.201545655.

Bauernfeind, F. G. *et al.* (2009) 'Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression', *The Journal of Immunology*. The American Association of Immunologists, 183(2), pp. 787–791. doi: 10.4049/jimmunol.0901363.

Bierschenk, D. *et al.* (2019) 'The Salmonella pathogenicity island-2 subverts human NLRP3 and NLRC4 inflammasome responses', *Journal of Leukocyte Biology*. John Wiley and Sons Inc., 105(2), pp. 401–410. doi: 10.1002/JLB.MA0318-112RR.

Boucher, D. *et al.* (2018) 'Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity', *Journal of Experimental Medicine*. The Rockefeller University Press, 215(3), pp. 827–840. doi: 10.1084/jem.20172222.

Brandt, K. J. *et al.* (2013) 'TLR2 Ligands Induce NF-κB Activation from Endosomal Compartments of Human Monocytes', *PLOS ONE*. Public Library of Science, 8(12), p. e80743. doi: 10.1371/JOURNAL.PONE.0080743.

Britzen-Laurent, N. *et al.* (2010) 'Intracellular trafficking of guanylate-binding proteins is regulated by heterodimerization in a hierarchical manner', *PLoS ONE*. PLoS One, 5(12). doi: 10.1371/journal.pone.0014246.

Broz, P. and Dixit, V. M. (2016) 'Inflammasomes: mechanism of assembly, regulation and signalling', *Nature reviews. Immunology*. Nat Rev Immunol, 16(7), pp. 407–420. doi: 10.1038/NRI.2016.58.

Campia, I. *et al.* (2009) 'Geranylgeraniol prevents the cytotoxic effects of mevastatin in THP-1 cells, without decreasing the beneficial effects on cholesterol synthesis', *British Journal of Pharmacology*. Wiley-Blackwell, 158(7), pp. 1777–1786. doi: 10.1111/j.1476-5381.2009.00465.x.

Cecil, J. D. *et al.* (2019) 'Outer Membrane Vesicle—Host Cell Interactions', *Microbiology spectrum*. NIH Public Access, 7(1). doi: 10.1128/MICROBIOLSPEC.PSIB-0001-2018.

Chilton, P. M., Embry, C. A. and Mitchell, T. C. (2012) 'Effects of differences in lipid A structure on TLR4 pro-inflammatory signaling and inflammasome activation', *Frontiers in Immunology*. Frontiers, 3(JUN), p. 154. doi: 10.3389/FIMMU.2012.00154/BIBTEX.

Coleman, W. G., Goebel, P. J. and Leive, L. (1977) 'Genetic analysis of Escherichia coli O111:B4, a strain of medical and biochemical interest.', *Journal of Bacteriology*. American Society for

Microbiology (ASM), 130(2), p. 656. doi: 10.1128/jb.130.2.656-660.1977.

Cordle, A. *et al.* (2005) 'Mechanisms of Statin-mediated Inhibition of Small G-protein Function', *Journal of Biological Chemistry*. Elsevier, 280(40), pp. 34202–34209. doi: 10.1074/JBC.M505268200.

Dixit, V. D. (2013) 'Nlrp3 Inflammasome Activation in Type 2 Diabetes: Is It Clinically Relevant?', *Diabetes*. American Diabetes Association, 62(1), pp. 22–24. doi: 10.2337/DB12-1115.

Doerflinger, M. *et al.* (2020) 'Flexible Usage and Interconnectivity of Diverse Cell Death Pathways Protect against Intracellular Infection', *Immunity*. Cell Press, 53(3), pp. 533-547.e7. doi: 10.1016/J.IMMUNI.2020.07.004.

Downs, K. P. *et al.* (2020) 'An overview of the non-canonical inflammasome', *Molecular Aspects of Medicine*. Elsevier Ltd, p. 100924. doi: 10.1016/j.mam.2020.100924.

Endo, A. (1992) 'The discovery and development of HMG-CoA reductase inhibitors', *Journal of Lipid Research*. Elsevier, pp. 1569–1582. doi: 10.1016/s0022-2275(20)41379-3.

Epand, R. M. *et al.* (2016) 'Molecular mechanisms of membrane targeting antibiotics', *Biochimica et Biophysica Acta (BBA) - Biomembranes*. Elsevier B.V., 1858(5), pp. 980–987. doi: 10.1016/j.bbamem.2015.10.018.

Fedele, G. *et al.* (2008) 'Lipopolysaccharides from Bordetella pertussis and Bordetella parapertussis Differently Modulate Human Dendritic Cell Functions Resulting in Divergent Prevalence of Th17-Polarized Responses', *The Journal of Immunology*. American Association of Immunologists, 181(1), pp. 208–216. doi: 10.4049/JIMMUNOL.181.1.208.

Finethy, R. *et al.* (2017) 'Inflammasome activation by bacterial outer membrane vesicles requires guanylate binding proteins', *mBio*. American Society for Microbiology, 8(5). doi: 10.1128/MBIO.01188-17/ASSET/79B3E663-C12A-4AE2-B746-F9AE90029475/ASSETS/GRAPHIC/MBO0051735220004.JPEG.

Fisch, D. *et al.* (2019) ' Human GBP 1 is a microbe-specific gatekeeper of macrophage apoptosis and pyroptosis ', *The EMBO Journal*. EMBO, 38(13). doi: 10.15252/embj.2018100926.

Giannella, R. A. *et al.* (1973) 'Invasion of HeLa Cells by Salmonella typhimurium: A Model for Study of Invasiveness of Salmonella', *The Journal of Infectious Diseases*. Oxford Academic, 128(1), pp. 69–75. doi: 10.1093/INFDIS/128.1.69.

Goldstein, J. L. and Brown, M. S. (1990) 'Regulation of the mevalonate pathway', *Nature*. Nature, 343(6257), pp. 425–430. doi: 10.1038/343425A0.

Van Gorp, H. and Lamkanfi, M. (2019) 'The emerging roles of inflammasome-dependent cytokines in cancer development', *EMBO reports*. EMBO, 20(6). doi: 10.15252/embr.201847575.

Graaf, M. R. *et al.* (2004) 'Effects of statins and farnesyltransferase inhibitors on the development and progression of cancer', *Cancer treatment reviews*. Cancer Treat Rev, 30(7), pp. 609–641. doi: 10.1016/J.CTRV.2004.06.010.

Greenwood, J., Steinman, L. and Zamvil, S. S. (2006) 'Statin therapy and autoimmune disease: From protein prenylation to immunomodulation', *Nature Reviews Immunology*. Europe PMC Funders, pp. 358–370. doi: 10.1038/nri1839.

Hanawa-Suetsugu, K. *et al.* (2019) 'Phagocytosis is mediated by two-dimensional assemblies of the F-BAR protein GAS7', *Nature Communications 2019 10:1*. Nature Publishing Group, 10(1), pp. 1–13. doi: 10.1038/s41467-019-12738-w.

Hayward, J. A. *et al.* (2018) 'Cytosolic Recognition of Microbes and Pathogens: Inflammasomes in

Action', *Microbiology and Molecular Biology Reviews*. American Society for Microbiology, 82(4). doi: 10.1128/mmbr.00015-18.

Henriksbo, B. D. *et al.* (2019) 'Statins Promote Interleukin-1β-Dependent Adipocyte Insulin Resistance Through Lower Prenylation, Not Cholesterol', *Diabetes*. Diabetes, 68(7), pp. 1441–1448. doi: 10.2337/DB18-0999.

Henriksbo, B. D. *et al.* (2020) 'Statins activate the NLRP3 inflammasome and impair insulin signaling via p38 and mTOR', *American journal of physiology. Endocrinology and metabolism*. Am J Physiol Endocrinol Metab, 319(1), pp. E110–E116. doi: 10.1152/AJPENDO.00125.2020.

Hooff, G. P. *et al.* (2010) 'Isoprenoids, small GTPases and Alzheimer's disease', *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. Biochim Biophys Acta, 1801(8), pp. 896–905. doi: 10.1016/j.bbalip.2010.03.014.

Jennings, E., Thurston, T. L. M. and Holden, D. W. (2017) 'Salmonella SPI-2 Type III Secretion System Effectors: Molecular Mechanisms And Physiological Consequences', *Cell Host and Microbe*. Cell Press, pp. 217–231. doi: 10.1016/j.chom.2017.07.009.

Jiang, Q. *et al.* (2022) 'Inflammasome and Its Therapeutic Targeting in Rheumatoid Arthritis', *Frontiers in Immunology*. Frontiers Media S.A., 12, p. 5861. doi: 10.3389/FIMMU.2021.816839/BIBTEX.

John, C. M., Liu, M. and Jarvis, G. A. (2009) 'Profiles of structural heterogeneity in native lipooligosaccharides of Neisseria and cytokine induction', *Journal of Lipid Research*. Elsevier, 50(3), pp. 424–438. doi: 10.1194/JLR.M800184-JLR200.

Kanellopoulos, J. M. and Delarasse, C. (2019) 'Pleiotropic Roles of P2X7 in the Central Nervous System', *Frontiers in Cellular Neuroscience*. Frontiers Media S.A., 13, p. 401. doi: 10.3389/FNCEL.2019.00401/BIBTEX.

Karasawa, A. *et al.* (2017) 'The P2X7 receptor forms a dye-permeable pore independent of its intracellular domain but dependent on membrane lipid composition', *eLife*. eLife Sciences Publications Ltd, 6. doi: 10.7554/ELIFE.31186.

Khalifeh, M. *et al.* (2021) 'Statins as anti-pyroptotic agents', *Archives of Medical Science*. Termedia Publishing House, 17(5), pp. 1414–1417. doi: 10.5114/AOMS/141155.

Kuhnert, P., Nicolet, J. and Frey, J. (1995) 'Rapid and Accurate Identification of Escherichia coli K-12 Strains', *APPLIED AND ENVIRONMENTAL MICROBIOLOGY*, 61(11), pp. 4135–4139.

Kumar, V. (2018) 'Inflammasomes: Pandora's box for sepsis', *Journal of Inflammation Research*. Dove Press, 11, p. 477. doi: 10.2147/JIR.S178084.

Kutsch, M. and Coers, J. (2020) 'Human guanylate binding proteins: nanomachines orchestrating host defense', *FEBS Journal*. Blackwell Publishing Ltd. doi: 10.1111/febs.15662.

Lamkanfi, M. and Dixit, V. M. (2014a) 'Mechanisms and Functions of Inflammasomes', *Cell*. Cell Press, 157(5), pp. 1013–1022. doi: 10.1016/j.cell.2014.04.007.

Lamkanfi, M. and Dixit, V. M. (2014b) 'Mechanisms and Functions of Inflammasomes', *Cell*. Cell Press, 157(5), pp. 1013–1022. doi: 10.1016/j.cell.2014.04.007.

Lawrence, A. L. E. *et al.* (2021) 'Salmonella enterica Serovar Typhimurium SPI-1 and SPI-2 Shape the Global Transcriptional Landscape in a Human Intestinal Organoid Model System', *mBio*. American Society for Microbiology (ASM), 12(3). doi: 10.1128/MBIO.00399-21.

Lee, C. A. and Falkow, S. (1990) 'The ability of Salmonella to enter mammalian cells is affected by

bacterial growth state (bacterial invasion/Madin-Darby canine kidney celis/oxygen/stationary phase/oxrA)', *Proc. Natl. Acad. Sci. USA*, 87, pp. 4304–4308.

Li, Z., Guo, J. and Bi, L. (2020) 'Role of the NLRP3 inflammasome in autoimmune diseases', *Biomedicine & Pharmacotherapy*. Elsevier Masson, 130, p. 110542. doi: 10.1016/J.BIOPHA.2020.110542.

Lu, Y. C., Yeh, W. C. and Ohashi, P. S. (2008) 'LPS/TLR4 signal transduction pathway', *Cytokine*. Academic Press, pp. 145–151. doi: 10.1016/j.cyto.2008.01.006.

Manaswiyoungkul, P., De Araujo, E. D. and Gunning, P. T. (2020) 'Targeting prenylation inhibition through the mevalonate pathway', *RSC Medicinal Chemistry*. Royal Society of Chemistry (RSC), 11(1), pp. 51–71. doi: 10.1039/c9md00442d.

Mann, P. B. *et al.* (2005) 'Comparative toll-like receptor 4-mediated innate host defense to Bordetella infection', *Infection and Immunity*. American Society for Microbiology, 73(12), pp. 8144–8152. doi: 10.1128/IAI.73.12.8144-8152.2005/ASSET/0EB4EAF4-B53F-4CAE-A938-6E07D581048E/ASSETS/GRAPHIC/ZII0120555090008.JPEG.

Mathur, A., Hayward, J. A. and Man, S. M. (2017) 'Molecular mechanisms of inflammasome signaling', *Journal of Leukocyte Biology*. Wiley, 103(2), p. jlb.3MR0617-250R. doi: 10.1189/jlb.3mr0617-250r.

Matsuura, M. (2013) 'Structural modifications of bacterial lipopolysaccharide that facilitate gram-negative bacteria evasion of host innate immunity', *Frontiers in Immunology*. Frontiers, 4(MAY), p. 109. doi: 10.3389/FIMMU.2013.00109/BIBTEX.

May, K. L. and Grabowicz, M. (2018) 'The bacterial outer membrane is an evolving antibiotic barrier', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, pp. 8852–8854. doi: 10.1073/pnas.1812779115.

Moffatt, J. H. *et al.* (2010) 'Colistin resistance in Acinetobacter baumannii is mediated by complete loss of lipopolysaccharide production', *Antimicrobial Agents and Chemotherapy*. American Society for Microbiology Journals, 54(12), pp. 4971–4977. doi: 10.1128/AAC.00834-10.

Muñoz-Planillo, R. *et al.* (2013) 'K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter', *Immunity*. NIH Public Access, 38(6), pp. 1142–1153. doi: 10.1016/j.immuni.2013.05.016.

Mylona, E. *et al.* (2021) 'Very long O-antigen chains of Salmonella Paratyphi A inhibit inflammasome activation and pyroptotic cell death', *Cellular Microbiology*. John Wiley & Sons, Ltd, 23(5), p. e13306. doi: 10.1111/CMI.13306.

Nang, S. C., Li, J. and Velkov, T. (2019) 'The rise and spread of mcr plasmid-mediated polymyxin resistance', *Critical Reviews in Microbiology*. Taylor and Francis Ltd, pp. 131–161. doi: 10.1080/1040841X.2018.1492902.

National Clinical Guideline Centre, U. (2014) *Statins for the primary and secondary prevention of CVD*, *NICE Clinical Guidelines, No. 181.* National Institute for Health and Care Excellence (UK). Available at: https://www.ncbi.nlm.nih.gov/books/NBK268908/ (Accessed: 8 December 2020).

Van Opdenbosch, N. *et al.* (2017) 'Caspase-1 Engagement and TLR-Induced c-FLIP Expression Suppress ASC/Caspase-8-Dependent Apoptosis by Inflammasome Sensors NLRP1b and NLRC4', *Cell Reports*. Cell Press, 21(12), pp. 3427–3444. doi: 10.1016/J.CELREP.2017.11.088.

Pick, E. (2014) 'Role of the Rho GTPase Rac in the activation of the phagocyte NADPH oxidase: Outsourcing a key task', *Small GTPases*. Taylor & Francis, 5(MAR). doi: 10.4161/SGTP.27952.

Pontillo, A., Paoluzzi, E. and Crovella, S. (2010) 'The inhibition of mevalonate pathway induces upregulation of NALP3 expression: New insight in the pathogenesis of mevalonate kinase deficiency', *European Journal of Human Genetics*. Nature Publishing Group, 18(7), pp. 844–847. doi: 10.1038/ejhg.2010.9.

Reinicke, A. T. *et al.* (2005) 'A Salmonella typhimurium effector Protein SifA is modified by host cell prenylation and S-acylation machinery', *Journal of Biological Chemistry*. J Biol Chem, 280(15), pp. 14620–14627. doi: 10.1074/jbc.M500076200.

Rodrigues, T. S. *et al.* (2020) 'Inflammasomes are activated in response to SARS-cov-2 infection and are associated with COVID-19 severity in patients', *Journal of Experimental Medicine*. Rockefeller University Press, 218(3). doi: 10.1084/JEM.20201707/211560.

Ryoden, Y. *et al.* (2020) 'Functional Expression of the P2X7 ATP Receptor Requires Eros', *The Journal of Immunology*. American Association of Immunologists, 204(3), pp. 559–568. doi: 10.4049/jimmunol.1900448.

Sabnis, A. *et al.* (2021) 'Colistin kills bacteria by targeting lipopolysaccharide in the cytoplasmic membrane', *eLife*. bioRxiv, 10. doi: 10.7554/eLife.65836.

Santos, J. C. *et al.* (2018) 'LPS targets host guanylate-binding proteins to the bacterial outer membrane for non-canonical inflammasome activation', *The EMBO Journal*. John Wiley & Sons, Ltd, 37(6), p. e98089. doi: 10.15252/EMBJ.201798089.

Santos, J. C. *et al.* (2020a) 'Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria', *Nature Communications*. Springer US, 11(1). doi: 10.1038/s41467-020-16889-z.

Santos, J. C. *et al.* (2020b) 'Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria', *Nature Communications*. Nature Research, 11(1), pp. 1–15. doi: 10.1038/s41467-020-16889-z.

Schmid-Burgk, J. L. *et al.* (2015) 'Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells', *European Journal of Immunology*. John Wiley & Sons, Ltd, 45(10), pp. 2911–2917. doi: 10.1002/EJI.201545523.

Segal, A. W. (2005) 'How neutrophils kill microbes', *Annual Review of Immunology*. Annu Rev Immunol, pp. 197–223. doi: 10.1146/annurev.immunol.23.021704.115653.

Shepard, C. C. (1955) 'Phagocytosis by HeLa Cells and Their Susceptibility to Infection by Human Tubercle Bacilli. (22043)', *Proceedings of the Society for Experimental Biology and Medicine*. SAGE PublicationsSage UK: London, England, 90(2), pp. 392–396. doi: 10.3181/00379727-90-22043.

Shepard, C. C. (1960) 'Phagocytosis of Microorganisms by Hela Cells', *The Journal of Immunology*, 85(4).

Shi, J. *et al.* (2015) 'Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death', *Nature*, 526(7575), pp. 660–665. doi: 10.1038/nature15514.

Skinner, O. P. *et al.* (2019) 'Lack of protein prenylation promotes NLRP3 inflammasome assembly in human monocytes', *Journal of Allergy and Clinical Immunology*. Mosby Inc., 143(6), pp. 2315-2317.e3. doi: 10.1016/j.jaci.2019.02.013.

Sun, X. *et al.* (2020) 'The NLRP3 Inflammasome and Its Role in T1DM', *Frontiers in Immunology*. Frontiers Media S.A., 11, p. 1595. doi: 10.3389/FIMMU.2020.01595/BIBTEX.

Takeuchi, O. and Akira, S. (2010) 'Pattern Recognition Receptors and Inflammation', *Cell*. Elsevier, 140(6), pp. 805–820. doi: 10.1016/J.CELL.2010.01.022.

Tamanoi, F. *et al.* (2001) 'Farnesylated proteins and cell cycle progression', *Journal of cellular biochemistry. Supplement*. J Cell Biochem Suppl, Suppl 37(SUPPL. 37), pp. 64–70. doi: 10.1002/JCB.10067.

Thangamani, S. *et al.* (2015) 'Exploring simvastatin, an antihyperlipidemic drug, as a potential topical antibacterial agent', *Scientific Reports*. Nature Publishing Group, 5. doi: 10.1038/SREP16407.

Thomas, D. C. *et al.* (2017) 'Eros is a novel transmembrane protein that controls the phagocyte respiratory burst and is essential for innate immunity', *Journal of Experimental Medicine*. J Exp Med, 214(4), pp. 1111–1128. doi: 10.1084/jem.20161382.

Tretina, K. *et al.* (2019) 'Interferon-induced guanylate-binding proteins: Guardians of host defense in health and disease'. doi: 10.1084/jem.20182031.

Uribe-Querol, E. and Rosales, C. (2020) 'Phagocytosis: Our Current Understanding of a Universal Biological Process', *Frontiers in Immunology*. Frontiers Media S.A., 11, p. 1066. doi: 10.3389/FIMMU.2020.01066/BIBTEX.

Vanaja, S. K. *et al.* (2016) 'Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation', *Cell*. Cell Press, 165(5), pp. 1106–1119. doi: 10.1016/J.CELL.2016.04.015.

Wandel, M. P. *et al.* (2020) 'Guanylate-binding proteins convert cytosolic bacteria into caspase-4 signaling platforms', *Nature Immunology*. Nature Research, 21(8), pp. 880–891. doi: 10.1038/s41590-020-0697-2.

Wang, S. *et al.* (2017) 'Statins Attenuate Activation of the NLRP3 Inflammasome by Oxidized LDL or TNFa in Vascular Endothelial Cells through a PXR-Dependent Mechanism s', *Molecular Pharmacology*. American Society for Pharmacology and Experimental Therapy, 92(3), pp. 256–264. doi: 10.1124/MOL.116.108100/-/DC1.

WHO (2021) *Cardiovascular diseases (CVDs)*, *World Health Organisation Fact sheets*. Available at: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (Accessed: 4 March 2022).

Wojtkowiak, J. W., Gibbs, R. A. and Mattingly, R. R. (2009) 'Working together: Farnesyl transferase inhibitors and statins block protein prenylation', *Molecular and cellular pharmacology*. NIH Public Access, 1(1), p. 1. doi: 10.4255/mcpharmacol.09.01.

Zhang, J. *et al.* (2021) 'Pyroptotic macrophages stimulate the SARS-CoV-2-associated cytokine storm', *Cellular & Molecular Immunology 2021 18:5*. Nature Publishing Group, 18(5), pp. 1305–1307. doi: 10.1038/s41423-021-00665-0.