Bachelor Degree Project

UNIVERSITY OF SKÖVDE

ACTIVATION OF NLRP3 INFLAMMASOME LEADS TO THE DIFFERENTIAL SECRETION OF INTERLEUKINS 1β/18, POSSIBLY LINKED TO PYROPTOSIS IN THP-1 CELLS

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Jana Damiani
a17janda@student.his.se

Supervisor:
Mikael Ejdebäck
mikael.ejdeback@his.se

Matthew Herring
matthew.herring@his.se

Examiner:
Maria Algerin
maria.algerin@his.se

School of Bioscience
University of Skövde
Box 408
541 28 Skövde

Abstract

Organisms are constantly exposed to microorganisms, some of which can cause disease, and yet, sickness only rarely occurs. Upon infection, the immune system is mobilized to eliminate the threat and re-establish homeostasis. The innate immune system, or "first line of defense", is responsible for detecting infection and subsequently initiating inflammation. An important mediator of this process is the NLRP3 inflammasome, a molecular hub that coordinates the propagation of the immune response. Specifically, through the secretion of interleukins 1β and 18. These structurally related proteins are known to be secreted via unconventional pathways, however, the specificity of this matter remains inconclusive. One form of secretion suggested was "pyroptosis", a form of cell death intended to enhance inflammation. The aim of this project was to quantify the secretion of structurally related interleukins-1β/18 and evaluate the possible correlation with cell death over time. Samples were collected, interleukin release was quantified using sandwich ELISA and cell viability of stimulated cells was measured using the PrestoBlue assay. During prolonged inflammasome activation, IL-1β and IL-18 showed differences in their patterns of secretion and regulation. IL-1β was eventually downregulated whilst IL-18 maintained a constant increase over time. Strikingly, cell viability remained unchanged over the course of the experiment, demonstrating that pyroptosis is dispensable to the secretion of interleukins 1β and 18. Ultimately, the study of the relationship shared between these processes not only explains how immunity is founded, but also, uncovers the truths behind pathogenesis, how to prevent it and potentially find a cure for certain diseases.

List of abbreviations

AP-1	Activator Protein-1
ATP	Adenosine Tri-Phosphate
CAPS	Cryopyrin Associated Periodic Syndromes
Da	Dalton
DAMPs	Damage Associated Molecular Patterns
FCAS	Familial Cold Autoinflammatory Syndrome
GSDMD	Gasdermin-D
IFN	Interferon
IL-1β	Interleukin 1β
LPS	Lipopolysaccharide
LRR	Leucine Riche Repeat
LSD	Least Significant Difference
MWS	Muckle-Wells syndrome
NFκB	Nuclear Factor kappa light chain enhancer of activated B cells
NLRP3	NOD-like receptor 3
NOD	Nucleotide binding ad oligomerization domain
NOMID	Neonatal onset multi-systemic inflammatory disease
OMV	Outer Membrane Vesicles
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptor
PYD	Pyrin Domain
ROS	Reactive Oxygen Species
TLR	Toll-Like Receptor

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Introduction

The initiation of an immune response: the innate immune system

Living organisms are continuously exposed to the outside world and its risks. These have developed chemical and physical barriers, delimiting what is "self" (within the body) and what is "non-self" (exterior to the body). However, in the unlikely event during which a disease-causing microorganism breaches these barriers, then the immune system takes over to restore homeostasis. The surveillance mechanism that acts in the first instances of infection is the innate immune system, a rapid, efficient (but rather non-specific) method of tagging and eradicating the invader. The main effectors of innate immunity are specialised immune cells such as macrophages and neutrophils that can recognize foreign bodies by different means: through the complement system, soluble molecules or by pattern recognition receptors (PRRs). The latter identify molecules called pathogen-associated molecular patterns (PAMPs) that are very commonly found on or in microbes (Murphy & Weaver, 2017). These are "conserved motifs" that are not recurrently subjected to mutations, usually characteristic of pathogens thus allowing them to be targeted by innate immune cells (Aderem & Ulevitch, 2000). Later, the immune cells get activated and are charged with the task of eliminating the pathogen and recruiting other immune cells to the site of the infection (Murphy & Weaver, 2017). In other words, as Chen et al. (2017) wrote, an inflammatory response is specific to the nature of its stimulus and the location of it, but although this diversity is present, these responses share a common pattern: First, cell surface receptors detect the invader; Second, inflammatory pathways are triggered. Third, inflammatory markers are delivered to finally call upon inflammatory cells. The innate immune system interacts with another part of immunity called the adaptive immune system which is called upon via effector cells of the innate immune system (such as dendritic cells) and the actions of cytokines. The particularity of this mechanism is its specificity and its capacity to adapt to any type of threat that has evaded its precursor, the innate immune system (Clark & Kupper, 2005).

After evaluating the functionality and correspondence between both immune systems (innate and adaptive), it was thought that the innate immune system was somehow restricted with a modest capacity to recognize foreign invaders. However, after further research it was demonstrated that, although the innate immune system is less specific than its counterpart: the adaptive immune system; it still offers a relatively wide amount of recognition patterns. This confers a survival advantage to certain organisms that lack the adaptive immune response, for example: fruit flies. The initiation and organization of the innate immune response is directed by the information received and processed by different types of receptors, known as PRRs. The latter are grouped according to the location and function of the receptors, some are free, some are attached to the surface of the membrane and act as phagocytic or signalling receptors and some are found within the cell (Murphy & Weaver, 2017). One such group of pathogen receptors is the "Toll-like receptors" or TLRs, found on and within a variety of innate immune cells (macrophages, neutrophils, dendritic cells, mast cells etc.) and others: endothelial, epithelial and fibroblastic cells (Clark & Kupper, 2005). These receptors were initially discovered through the study of the immune system of the *Drosophila*; in 1997, Janeway and colleagues were responsible for forming the bridge with the human innate immune system and the first of those receptors found was the TLR4 or Toll-like Receptor 4 (Aderem & Ulevitch, 2000; Medzhitov, Preston-Hurlburt & Janeway, 1997). This receptor was found to react strongly to very low concentrations of lipopolysaccharide (LPS) and induces a cascade of signals that direct phagocytosis, a form of engulfment; the expression of inflammatory cytokines and finally the activation of the adaptive immune response (Takeda & Akira, 2005).

The inflammasome, a molecular hub for inflammation

Although "non-self" substances are differentiated from "self-substances" by receptors such as the surface Toll-like receptor 4, an "explanatory" and effector signal must be transmitted from the cell surface (where TLR4 is found) to the nucleus and further away to different extremities of the body. This signal follows a detailed pathway through the complex interactions of numerous mechanisms to finally reach its correct destination(s) and create a warning echo within the host's immune system. More specifically, macrophages, epithelial cells and other immune cells have the capacity to compose intracellular protein complexes known as inflammasomes, which are critical in the initiation and progression of the innate immune response. NLRP3 is one of the most commonly studied inflammasome, it is a tripartite protein formed of an N-terminal Pyrin Domain (PYD), a Nucleotide-binding and Oligomerization Domain (NOD), and a C-terminal Leucine-Rich Repeat (LRR) (Kelley, Jeltema, Duan, & He, 2019; Franchi, Warner, Viani, & Nuñez, 2009). The assembly of this protein complex occurs in two distinct steps by two distinct signals: signal one is referred to as priming, whilst signal two is referred to as activation (Kelley et al., 2019).

Priming is an essential first step in inflammasome formation, it is usually initiated by the recognition of pathogen associated molecular patterns (PAMPs) that are specific to exogenous compounds (Bauernfeind et al., 2009). Lipopolysaccharide (LPS), a molecule found in Gram-negative bacteria is an example of PAMP that is detected via the interaction with TLR4 found on the surface of macrophages (Gurung et al., 2012; Murphy & Weaver, 2017). Upon, recognition of such foreign compounds, a signalling cascade is directed towards the nucleus of the cell, prompting the transcription of a multitude of protein encoding genes. Transcription factors known as NF-κB and Activator Protein-1 (AP-1) are subsequently localized in the nucleus and direct the transcription of cytokine genes (Silverman & Maniatis, 2001; Murphy & Weaver, 2017). Levels of NLRP3, pro-interleukin-1β (pro-IL-1β), pro-IL-18 as well as procaspase-1 are upregulated at this instance, preparing for the activation and propagation of the inflammatory response (Latz, Xiao, & Stutz, 2013; Kelley et al., 2019).

Following priming of the inflammasome, a second signal is required to complete the formation of the NLRP3 inflammasome. The signal leading to the activation of NLRP3 remains uncertain as there is a wide variety of triggers (Lamfanki & Dixit, 2014). Damage Associated Molecular Patterns (DAMPs) are mostly the source of inflammasome activation, however PAMPs are also recognized to influence this process. As opposed to PAMPs, "damage" molecules are generally "self-associated" and secreted by cells within the surrounding. Examples of such molecules are Adenosine Triphosphate (ATP) and hyaluronan as DAMPs (Lamfanki & Dixit, 2012). Also, crystals, aggregates (example: β-amyloid), bacterial, viral and fungal pathogens act as potent PAMPs for inflammasome activation (Lamfanki & Dixit, 2012). With ongoing research on the functionality of the NLRP3 inflammasome, it was determined that the detection of such a variety of inducing agents occurs indirectly that remains unclear. A few hypotheses were proposed, such as NLRP3 is activated in response to a potassium efflux (Muñoz-Planillo et al., 2013); translocation to mitochondria (Misawa et al., 2013; Subramanian, Natarajan, Clatworthy, Wang, & Germain, 2013), reactive oxygen species (Harijith, Ebenezer, & Natarajan, 2014), disruption of mitochondria and lysosomal integrity (Iyer et al., 2013; Shimada et al., 2012; Hornung et al., 2008).

Another particularity of NLRP3 activation is its capacity to be formed in two distinct manners: through the canonical (classical) way and the non-canonical pathway. The canonical pathway is said to be dependent on PRRs such as Toll-like receptors or cytokine receptors which in turn order the assembly of NLRP3 and the synthesis of immature forms of cytokines through the NFκB transcription factor. This pathway also centres around the cleavage of pro-caspase-1 into its active form and subsequently the maturation of interleukins IL-1β, IL-18 (Pellegrini, Antonioli, Lopez-Castejon, Blandizzi & Fornai, 2017). Some of the stimuli that trigger this pathway are: an increase in extracellular Adenosine Triphosphate (ATP), the exposure to Gram-positive bacteria, a

potassium efflux, the generation of Reactive Oxygen Species (ROS), lysosomal disruption, calcium imbalance induced by silica crystals and many more (Vanaja, Rathinam, & Fitzgerald, 2015). Secondly, the non-canonical pathway is initiated by the presence of LPS (Gram-negative bacteria) without the need for receptors such as TLR4. This allows immune cells to detect pathogens that have created means to bypass receptor recognition. The main effectors of this pathway are caspases-4 and 5, responsible for the detection of cytosolic LPS, more specifically the lipid A moiety of it (Kelley et al., 2019). Also, Gram-negative bacteria such as *Escherichia coli* have been shown to produce Outer Membrane Vesicles (OMVs) that transport LPS into the cytosol through endocytosis. Caspases 4 and 5 are then capable to directly recognize the stimulus and act by activating caspase 1. Both these systems work in conjunction with the aim of activating caspase-1, in different manners and stimulate the production and release of messenger cytokines to promote an adequate response (Yi, 2018).

Although there is uncertainty and diversity in the induction of NLRP3 inflammasome, it is agreed that the activation of this molecular complex occurs via two signals. Priming is essential for inflammasome activation, presenting a first step in its regulation. Activation of NLRP3 is initiated in response to a wide variety of signals, ensuring an inflammatory response for all possible threats.

Interleukin production and the secretory pathway linked to pyroptosis

The capacity of sensor cells to recognize different types of pathogens, involves the development of different messenger molecules targeted for a specific response. In the case of NLRP3, its activation results in the production and maturation of molecules known as: interleukins or cytokines (Groslambert & Py, 2018). Cytokines are small proteins released by macrophages (and other immune cells) and are responsible for expanding the inflammatory response towards other parts of the body. These molecules can act in an autocrine, paracrine or endocrine manner affecting one cell, adjacent cells or distant cells, respectively (Murphy & Weaver, 2017).

Upon inflammasome activation, caspase-1 is recruited with the aim of cleaving interleukins 1β and 18 (IL-1β/IL-18) into their mature forms. These pro-inflammatory interleukins are by-products of inflammasome activation and possess different roles in the propagation of inflammation (Yang, Wang, Kouadir, Song, & Shi, 2019). IL-1β, is a pyrogen meaning it induces fever, that is solely synthesized in response to a pro-inflammatory stimulus (Brough & Rothwell, 2006). It is also an activator of lymphocytes as well as the vascular endothelium, allowing other immune cells to make their way to the site of infection (Murphy & Weaver, 2017). On the other hand, IL-18 is classified as a "multifunctional" protein, capable of inducing interferon-gamma (IFN-γ) production from Natural Killer cells (NK cells) and enhance cytotoxicity of T-cells (Dinarello, 2006; Volin & Koch, 2011). Although IL-1β and IL-18 are distinct in their functionality, they share structural homology by being "leaderless" proteins, secreted in via unconventional pathways (Dubyak, 2012). The secretion of these key regulatory molecules during inflammation, remains to this day a controversial matter (Tsutsumi et al., 2019). Numerous mechanisms of secretion have been proposed, but one seemed to be of great interest: pyroptosis.

As all processes function in nature, homeostasis is of prime importance to maintain integrity and balance in the "circle of life". Cells are subjected to a cycle of growth, division and eventually death, through which there is regeneration of new tissue. Cell death exists in different versions, with some programmed, predetermined such as apoptosis or even traumatic and inflammatory such as pyroptosis (Nirmala & Lopus, 2019). Pyroptosis is a form of cell death that resembles apoptosis in its dependence on caspase activation and is similar to necrosis in the cell's rupture (Nirmala & Lopus, 2019; Kroemer et al., 2008). More specifically, pyroptosis is dependent on caspase-1 and the subsequent cleavage of Gasdermin-D (GSDMD), a pore forming molecule (He et al., 2015). This interaction is followed by cell swelling, rupture and release of intracellular components creating

local inflammation (Bergsbaken, Fink, & Cookson, 2009; Kroemer et al., 2008). It has been debated, whether pyroptosis is essential to the secretion of IL-1β and IL-18 into the extracellular environment. According to some research, the quantity of IL-1β is "proportional" to the amount of dying cells, making these processes dependent on each other (Cullen, Kearny, Clancy, & Martin, 2015) Other research suggests that a cell's commitment to pyroptosis depends on the intensity of the stimulus, its duration and the type of cell in question (Stoffels et al., 2015). The relationship between inflammasome activation, cell death and interleukin secretion remains to this day unclear.

NLRP3 in health and disease

NLRP3 is the best-characterized Nod-like Receptor (NLR), owing to its capacity to recognize a wide variety of stimulators. Under normal conditions, the inflammasome responds to cellular stress and is activated with the purpose of eradicating any type of threat (Menu & Vince, 2011). However, this complex molecule is extensively studied for its implication in pathogenesis of several human inflammatory diseases (Schroder & Tschopp, 2010). More specifically, NLRP3 has been recognized as the main cause of a category of inflammatory diseases known as Cryopyrin-Associated Periodic syndromes (CAPS) (Menu & Vince, 2011). This family of diseases is composed of three closely related conditions: Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS) and Neonatal Onset Multi-Systemic Inflammatory disease (NOMID), with increasing severity (Menu & Vince, 2011). Not only is this molecular complex involved with these inherited diseases but it also plays a role in pathologies such as gout (So, 2008; Menu & Vince, 2011), type II diabetes (Maedler et al., 2002), cancer (Murphy, Morales, Scott, & Kupper, 2003), Alzheimer's (Halle et al., 2008; Menu & Vince, 2011) and much more.

Thus, the study of such processes gives a better understanding of the function of NLRP3 inflammasome in health and in diseased states. The sequence of inflammasome formation, interleukin secretion, cell death and everything in between reflects the importance of understanding these mechanisms. As this will lead to the efficient development of drugs for all pathologies of the innate immune system.

Research question

Finally, a question arises: how do levels of IL-1β and IL-18 change before, during and after NLRP3 activation and how does this affect cell viability?

Aim and objectives

The aim in this project was to determine the effects of inflammasome activation in THP-1 cells (monocyte-like cells pre-differentiation), over time as measured by secretion of two interleukins. Samples were collected at varying time points along the process of NLRP3 inflammasome priming/activation to measure the secretion levels of IL-1β , IL-18 as well as their correspondence with cell viability. These were quantified using specific sandwich Enzyme Linked Immunosorbent Assays (ELISA) to assess and compare the time kinetics of IL-1β and IL-18. Cell viability was measured using the PrestoBlue® assay by Thermofisher to potentially find a timely correlation between the three factors: activation of NLRP3, release of cytokines and cell death. A global understanding of these interconnecting pathways will engender an awareness of the function of NLRP3 inflammasome in macrophages, its role in the formation of an inflammatory response and its potential de-regulation in auto-inflammatory diseases. As Xie et al. (2014) formulated: "The significance of the innate immunity lies not in comprising the first line of defence against pathogenic and non-pathogenic insults, but also in developing an efficient adaptive immune response".

Materials and methods

Cell culture

THP-1 cells containing an ASC-GFP construct (Invivogen) were grown in RPMI 1640 media (Sigma Aldrich) with L-glutamine; Heat inactivated Premium Grade Foetal Bovine Serum 10% (FBS; Biowest); 58 mM Sodium pyruvate (Sigma Aldrich); 0.45% Glucose solution (Sigma Aldrich), 10 mM HEPES (Sigma Aldrich) and 1X Penicillin-streptomycin (Sigma Aldrich). Media was changed every two days to keep cells at a concentration between 5 x 10^5 cells/mL and 1.6 x 10^6 cells/mL. In addition, selection pressure was maintained by adding Zeocin™ (Invivogen) at 0.4 μg/mL. The cells were incubated at 37 °C, 5 % CO_2.

Stimulation and Activation of THP-1 cells

For the collection of samples for IL-1β and IL-18 ELISA, cells were grown in a T-175 flask to accommodate a maximal medium volume of 120mL containing 1x10^6 cells/mL. Differentiation of THP-1 monocyte-like cells into macrophage-like cells was done by the addition of Phorbol Myristate Acetate (PMA; Invivogen) at a concentration of 0.5 μg/mL. Cells were incubated with PMA for four hours and subsequently PMA was removed by replacing with fresh media followed by incubation for another 18 hours.

The first sample was taken 10 minutes before priming Lipopolysaccharide (LPS; Invivogen); 1000 μL of the medium was taken and allocated equally into two Eppendorf tubes: one for each ELISA. Then, 100 ng/mL LPS was added to the culture for 3h and 30min. The second sample was taken in the same manner as the previous one, just before activation of cells by addition of 5mM Adenosine Triphosphate (ATP; Sigma) diluted in cell culture medium. Following this step, samples were taken every ten minutes for 2 hours after ATP addition and then every 30 minutes until 5h 30 min after ATP addition. Later, samples were also taken 22, 24 and 48 hours following ATP addition. In total, 24 samples were collected for each ELISA.

PrestoBlue cell viability assay

Cell viability assay - Suspension cells: Optimal incubation time and cell density was determined. Suspension cells were seeded at different densities ranging between 10 000 to 160 000 cells/well/100 μL and incubated for 20 minutes, one hour and two hours with PrestoBlue™ Cell Viability Reagent (Invitrogen). **Cell viability assay - Adherent cells:** Adherent cells, differentiated with PMA (0.5 μg/mL) for four hours, were subjected to LPS and ATP for the respective time points: 10, 30 and 50 minutes (PrestoBlue™ was added simultaneously with ATP). The cell densities were ranging between 5000 to 40 000 cells/well/100 μL.

PrestoBlue assay - Proceeding with the cell viability assay, the cells were placed in a 96 well-plate at 10 000 cells per well (100 000 cells/mL), and PMA (0.5 μg/mL) was added to all wells simultaneously. Secondly, after incubation overnight, LPS and ATP were added at times that corresponded with the time of LPS-priming required (3 hours 30 minutes) and the time of ATP addition which was adjusted to fit the time points selected. Finally, PrestoBlue™ Cell Viability Reagent (Invitrogen) was left to incubate for 30 minutes and absorbance values were read at 571 nm and corrected by subtraction of values determined at 595 nm. The assay was run in duplicate (one of each biological replicate) and each time point was divided into three technical replicates.

Quantification of Interleukins: IL-1β and IL-18

Cytokine secretion in cell culture supernatants was measured using sandwich ELISA kits for IL-1β (Biolegend-ELISA MAX™) and IL-18 (Total IL-18 DuoSet ELISA, R&D Systems). Each ELISA was performed in duplicate, each a biological replicate and every sample run in a technical triplicate.

One of the IL-1β replicates, the samples were diluted five-fold and the standard concentrations were modified by increasing concentrations. All other steps were also performed according to the instructions in the given manual.

Samples for the IL-18 ELISA, were diluted two-fold (for both replicates) and analysed as described in the protocol.

Statistical analysis

All data were analysed using IBM SPSS Statistics® software. Statistical significance of the hourly change in interleukin-18 secretion was measured using the repeated measures ANOVA test followed by a Bonferroni post-hoc test. Significant difference is illustrated by $p<0.05$ (*), $p<0.01$ (**) and $p < 0.001$ (***).

Results

Inflammation results from the activation of the innate immune system and aids with its amplification through the adaptive immune system. Simply put, inflammation encompasses four symptoms: heat, pain, redness and swelling. Each of these features is representative of the effects of messenger molecules such as cytokines, also known as interleukins. These are essential in the formation of a bridge between both immune systems.

Quantification of interleukins: IL-1β and IL-18

The formation of the NLRP3 inflammasome in response to foreign particles involves, directly and indirectly, the maturation of messenger molecules such as the interleukin IL-1β and IL-18.

Time kinetics of IL-1β secretion:

In THP-1 cells, exposure to LPS and ATP is believed to induce the secretion of IL-1β, the time kinetics of its secretion was studied in a sandwich ELISA. The extrapolation of absorbance into concentration values by the use of a standard curve, showed discrepancies between high absorbance values and low standard concentrations (see Figure 1 in Appendix 1). Thus, absorbance values were plotted in Figure 1, where the change in absorbance was representative of the change in concentration over time.

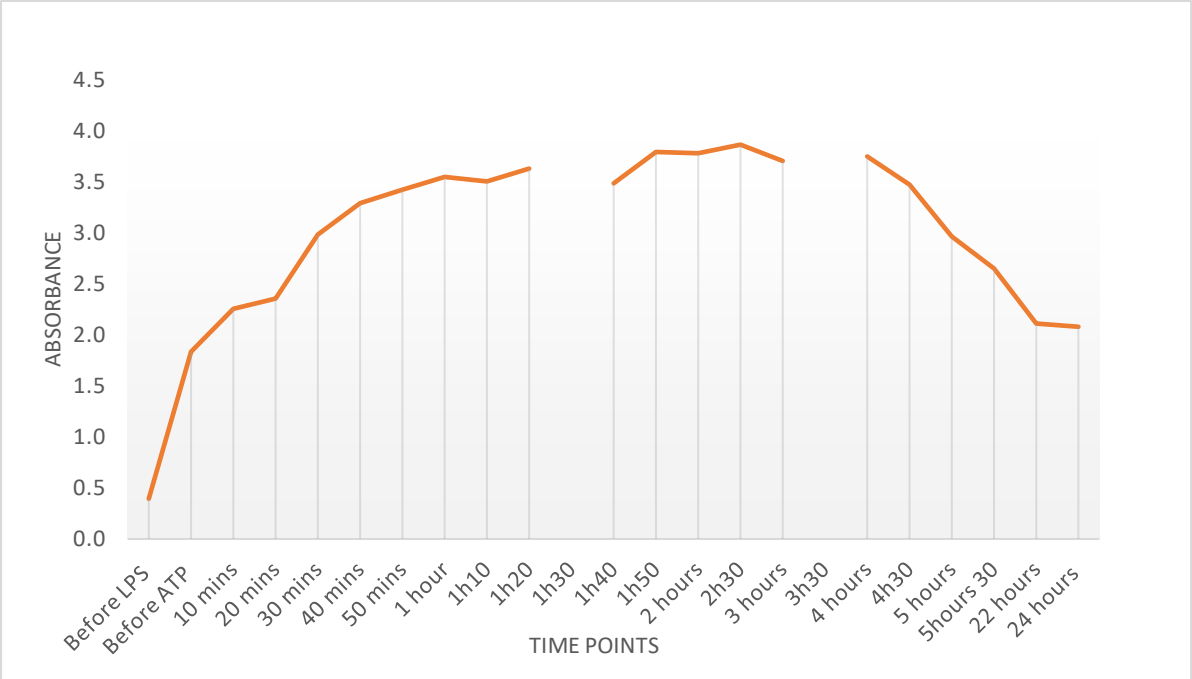

Figure1. Graphical representation of the change in absorbance of IL-1β over different ATP incubation times-Biological Replicate 1. Each time point or sample was represented by the average of its technical triplicate. The missing values reflect the absorbance values that were not read by the plate reader.

The change in IL-1β "concentration" over time (as seen in Figure 1) is an increase directly after LPS addition with absorbance values varying from: 0.4 before LPS to 1.8 after LPS showing a 350% increase. A second increase was observed after the addition of ATP and which was maintained over a period of 2 hours and 30 min, with absorbance values reaching a maximum of 3.9 and an increase of 72% as compared to after 10 minutes of ATP addition (absorbance: 2.25). After reaching peak value at 2 hours and 30 min after ATP addition, the beginning of a decrease can be seen three hours after ATP addition that remains throughout the experiment without reaching a "close to zero" value. Some data points are missing in the graph because the values were too high to be accurately determined and thus, were not included in the graph. Nevertheless, a similar trend of the curve was also matched by plotting the change in absorbance values over time seen for the second biological Replicate 2, Figure 2.

In this second sandwich ELISA, higher standard concentrations were used (see Figure 2 in Appendix 1) and the samples were diluted to accommodate the capacity of the plate reader. The standard curve generated was however deemed un-reliable to predict concentration values. Change in absorbance was plotted in Figure 2 and is considered to reflect the change in secreted IL-1β.

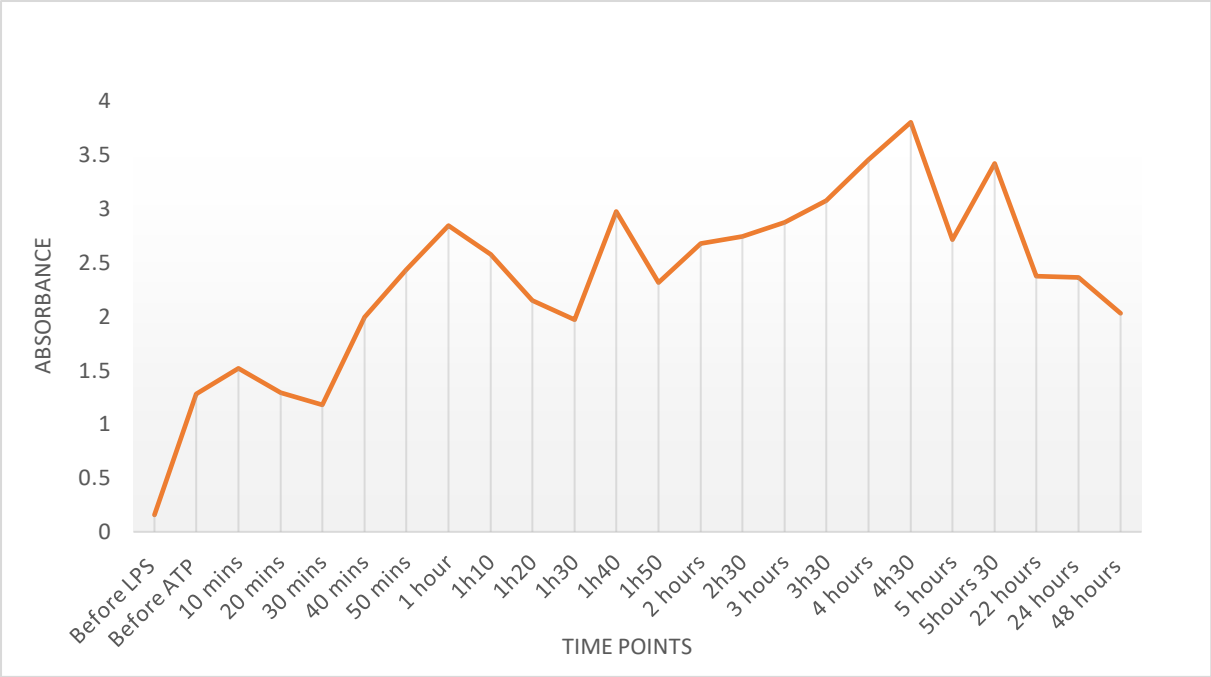

Figure 2. Graphical representation of the change in absorbance of IL-1β over different ATP incubation times- Biological Replicate 2. Each time point or sample was represented by the average of its technical triplicate.

The absorbance values showed larger variations between the samples over time compared with those observed in Figure 1. Similarly to Figure 1, there was a distinct increase in absorbance after LPS addition with absorbance values varying from: 0.2 before LPS and 1.3 after LPS accounting for a 550% increase between time points. This increase remained until reaching a peak value of 3.8 after 4 hours 30 from ATP addition, followed by a relative decrease in absorbance reaching an absorbance of 2 after 48 hours. Although there are differences in individual time points between both replicates, there is a global resemblance in trend of IL-1β secretion throughout time.

Data within both biological replicates were combined see Figure 3, which allowed a visual representation of the "expected" progression of IL-1β from hour to hour. The differing sample dilutions within both replicates, were compensated by multiplying concentration with the dilution factor.

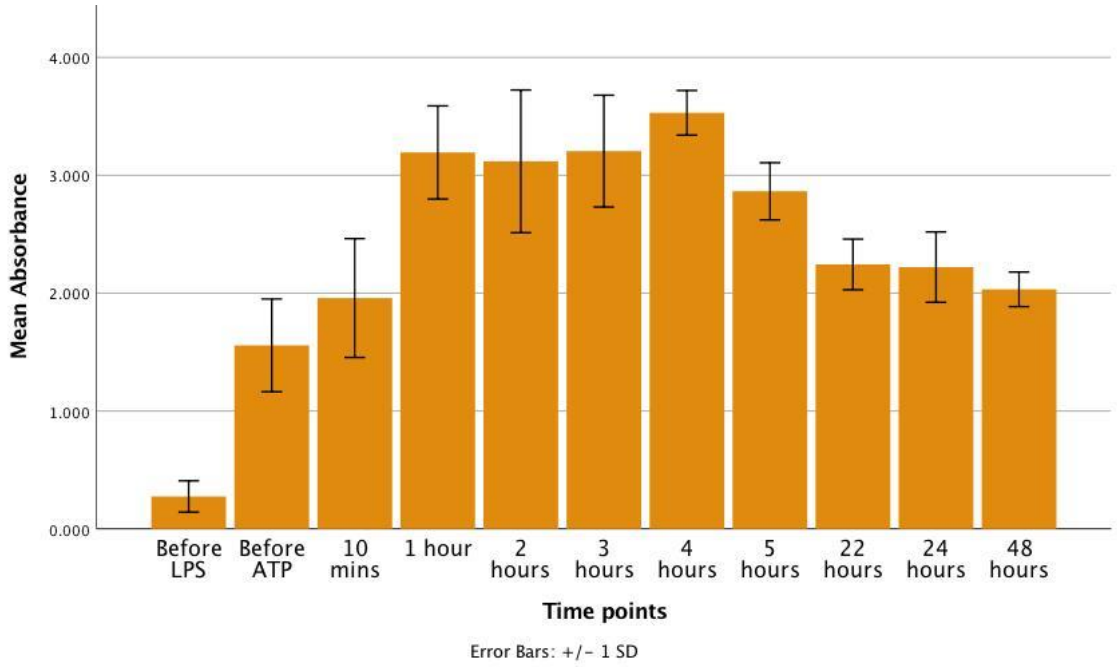

Figure 3. Bar chart of the hourly change in absorbance of secreted IL-1β within the combined replicates. Bars represent ± 1 Standard Deviation (SD).

To summarize, the overall change in absorbance within both replicates shows first a strong increase after LPS addition, an increase after ATP addition and a gradual decrease after at least 3 hours and 30 minutes of ATP exposure (Figure 3).

Time kinetics of IL-18 secretion:

Another product of NLRP3 inflammasome formation is interleukin 18 or IL-18. Secreted interleukin 18 was also measured in cell culture supernatant. Standards of known concentrations were used to obtain standard curve, allowing concentrations of the samples to be determined as seen in Figure 4.

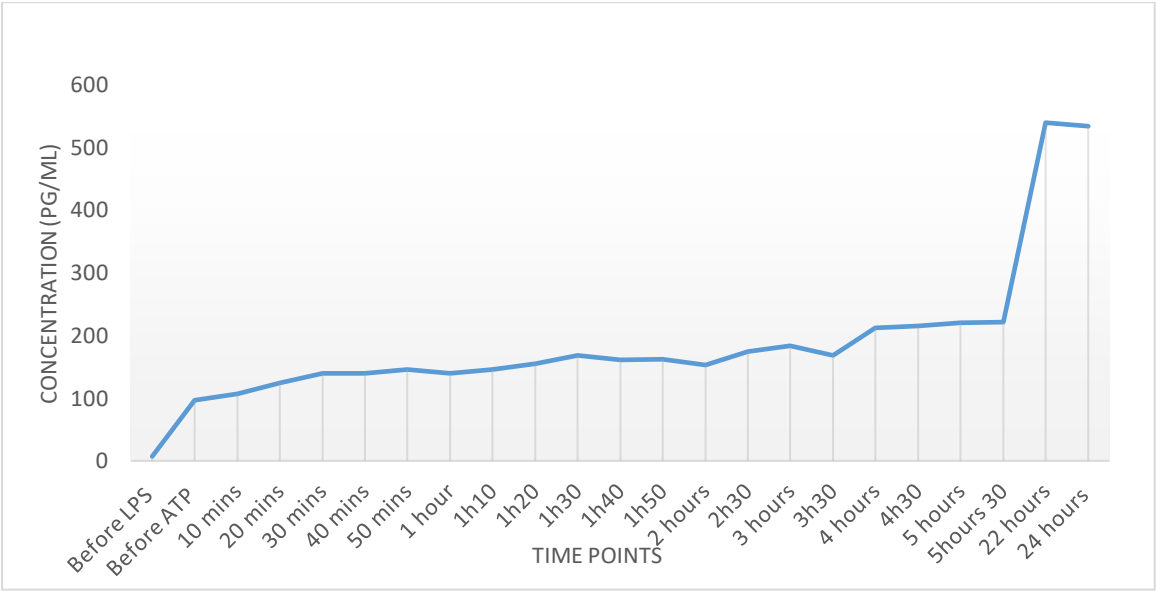

Figure 4. Graphical representation of the change in IL-18 concentration over time after ATP addition- Replicate 1.

The levels of secreted IL-18, similarly to the secretion of IL-1β showed a large increase after addition of LPS with concentrations of: 7 pg/mL before LPS and 97 pg/mL after LPS. Following this initial increase, and after ATP addition, the concentration of IL-18 detected within the supernatants, presented a slow and relatively stable increase up until 5 hours and 30 min after ATP addition. On the contrary to the observed changes recognized in the secretion of IL-1β, the pattern of IL-18 release into the media escalated at a slower rate and reached high levels after an extended time of ATP exposure (22 hours; 538 pg/mL). A decrease in IL-18 was not observed within the given time frame of sampling. Finally, the relevance of these results was tested again by performing the same method with a second replicate (Figure 5).

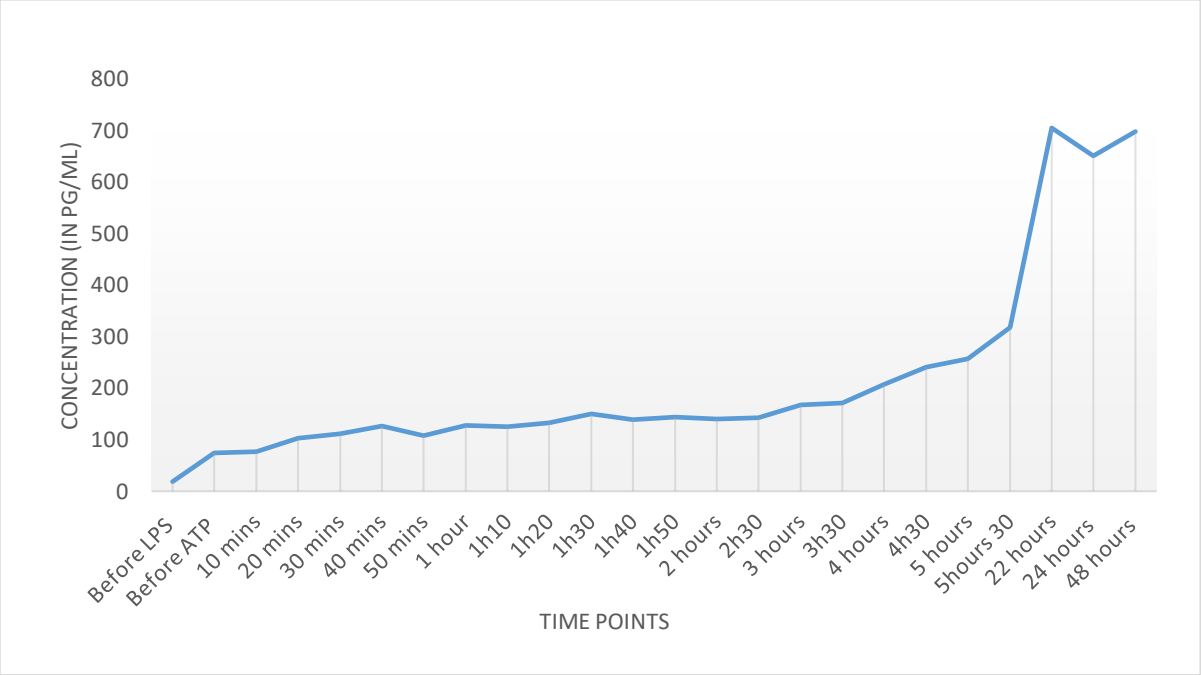

Figure 5. Graphical representation of the change in IL-18 concentration over time after ATP addition- Replicate 2.

In agreement with the results found in Figure 4, Replicate 2 also showed an increase in secretion of IL-18 after LPS addition with concentrations of: 18.5 pg/mL before LPS to 74 pg/mL after LPS. Subsequently, this was followed by a steady, slow increase which was maintained after 22 hours of ATP with a peak concentration of: 704 pg/mL and no signs of a "return to normal" or decrease after the time elapsed. As data sets were closely related and presented the same tendency, these were combined and the results were visually depicted as a bar chart seen in Figure 6 (see Table 1 Appendix 2).

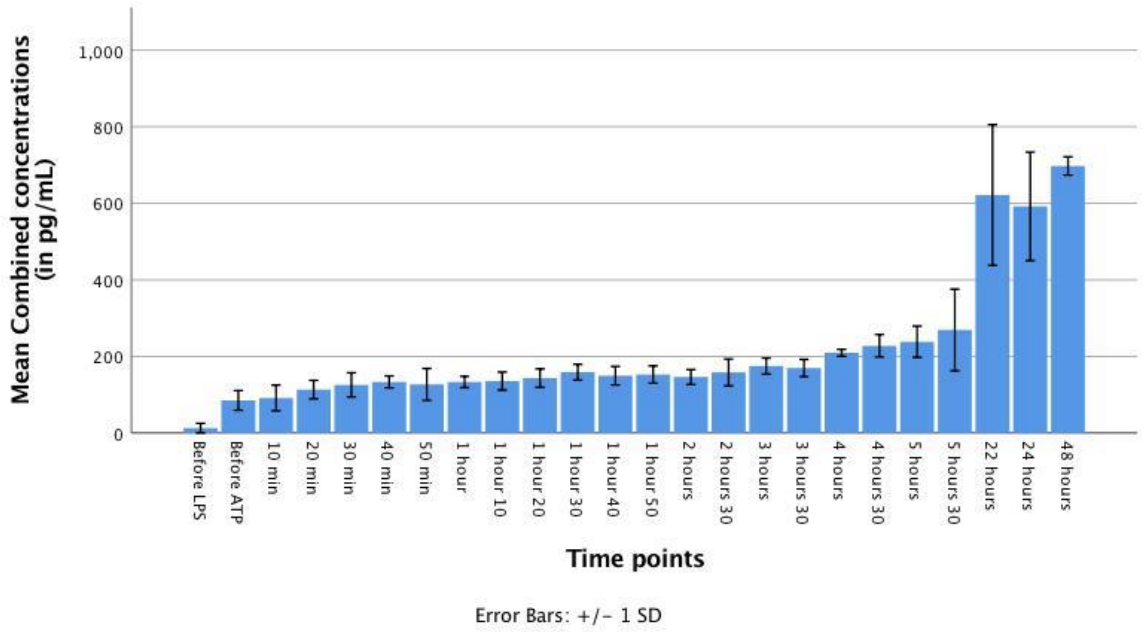

Figure 6. Chart representative of the temporal changes in concentrations of IL-18 over time, within both replicates. Bars represent mean value ± 1 SD and mean was calculated using both technical triplicates of each time point (six samples in total, three from each replicate).

The changes seen between the different time points were further analyzed to determine where were the significant changes occurring before (after LPS) and after ATP addition or in other words after activation of NLRP3 inflammasome. The comparison between time points, in an hourly fashion, simplified the visible changes in time kinetics of IL-18 secretion in stimulate THP-1 cells. After ensuring normality (see Tale 2 Appendix 2), another graph was plotted with the significant differences between before the addition of ATP and all the samples following that time point (Figure 7).

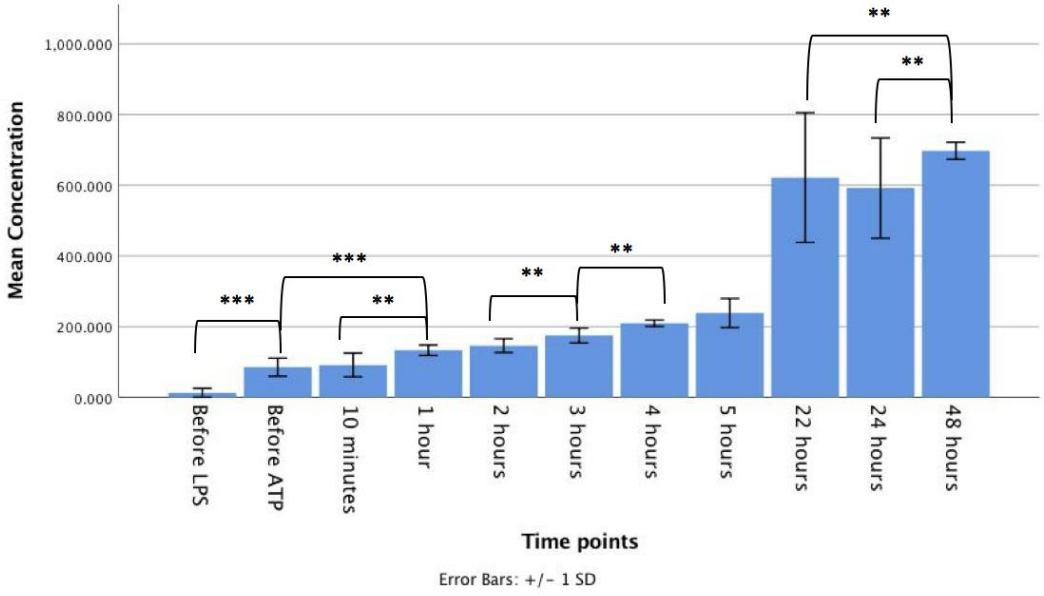

Figure 7. Bar chart representative of the significant, hourly changes in concentrations between time points. Bars represent mean value ± 1 SD (Standard Deviation). Statistical significance was determined by repeated measures ANOVA, followed by Bonferroni post-hoc test. One asterisk denotes significant difference with $p< 0.05$; two asterisks, $p< 0.01$ and three asterisks, denotes a significant difference with $p< 0.001$.

In Figure 7, specific time points were selected to show differences in IL-18 secretion over time. Firstly, there was a significant change in secreted IL-18 after LPS addition (p-value: 0.001) and concentrations that went from 13 pg/mL (before LPS) to 85 pg/mL (After LPS/Before ATP). This is of importance as substantial amounts of IL-18 are found within the supernatant before activation of the NLRP3 inflammasome using ATP. In addition, there is no immediate detectable change after ATP (10 minutes) addition, rather the change happened one hour after ATP exposure where the p value was: 0.005 with a concentration of: 133 pg/mL. There were noticeable variations between most consecutive hours such as between: two, three and four hours of ATP incubation with p values of: 0.003 for both intervals. Finally, the obvious increase in IL-18 secretion after 22 hours of ATP incubation, was maintained and showed significant difference between time points 22 hours and 48 hours with p= 0.018 and a peak concentration of 697 pg/mL. The secretion of IL-18 presents variations over time, where changes were not always significant in an hourly fashion (see Table 3 Appendix 2). Nonetheless, a steady increase was preserved throughout long periods of ATP exposure, as opposed to its counterpart: IL-1β which showed a decrease after a few hours of ATP incubation. The continuity of IL-18 secretion and its evolution between large time gaps remains undetermined.

PrestoBlue cell viability assay

A side effect of inflammasome activation and interleukin secretion is a certain type cell death, known as pyroptosis. It is thought that through the actions of NLRP3 activation, pores are formed within the membrane inducing cell lysis occurs and enhancing cytokine secretion. Pyroptosis is only one of the proposed mechanisms of cytokine secretion and the interdependence of both processes has yet to be determined.

Cell viability assay: Suspension cells

Thus, cell viability was measured using the PrestoBlue™ reagent, by observing the change in absorbance over time which is indicative of the metabolic capacity of cells. The benefits of this assay are highlighted by its capacity to measure “live” metabolic activity of cells indicated by a change in color, for which the intensity of the latter is proportional to the amount of “live” cells. A few tests were performed to determine what parameters suit the cell type (THP-1) that is used and that yields the best results. In Figure 8, different cell densities: 10 000, 20 000, 40 000, 80 000 and 160 000 cells (in suspension) were seeded per well and subjected to different incubation times with PrestoBlue™.

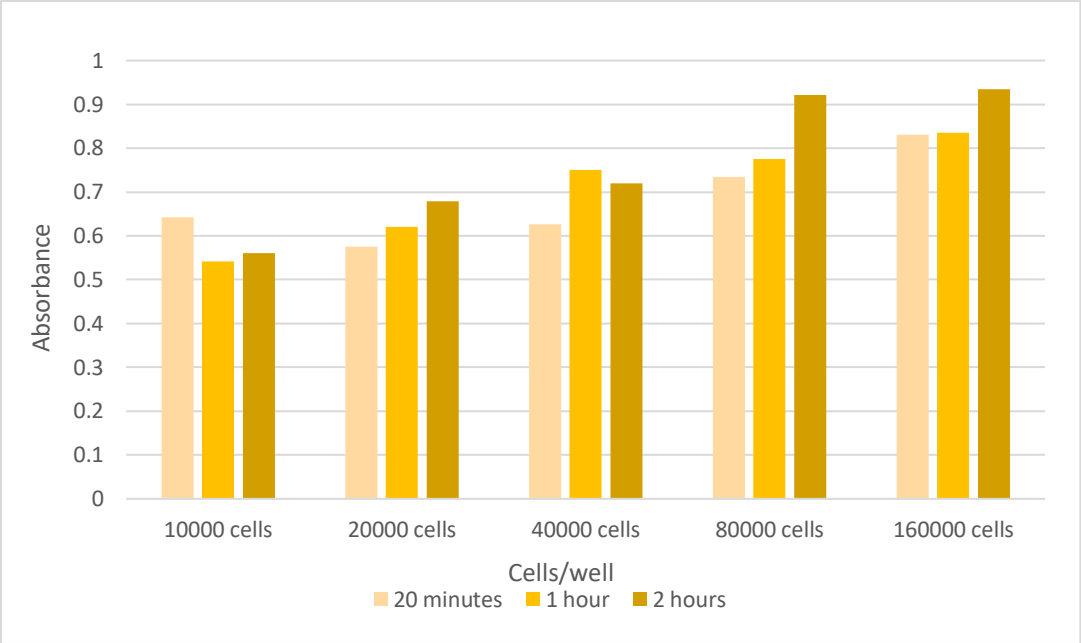

Figure 8. Graphical representation of the change in absorbance depending on cell density and incubation time with PrestoBlue™ in suspension cells. Volume per well was: 100 µL.

As it is generally observed in Figure 8, higher absorbance values were obtained with higher cell densities and increased incubation time with PrestoBlue. A second test was performed to determine whether these patterns were also valid on adherent and differentiated cells, Figure 9.

Cell viability assay: Adherent cells

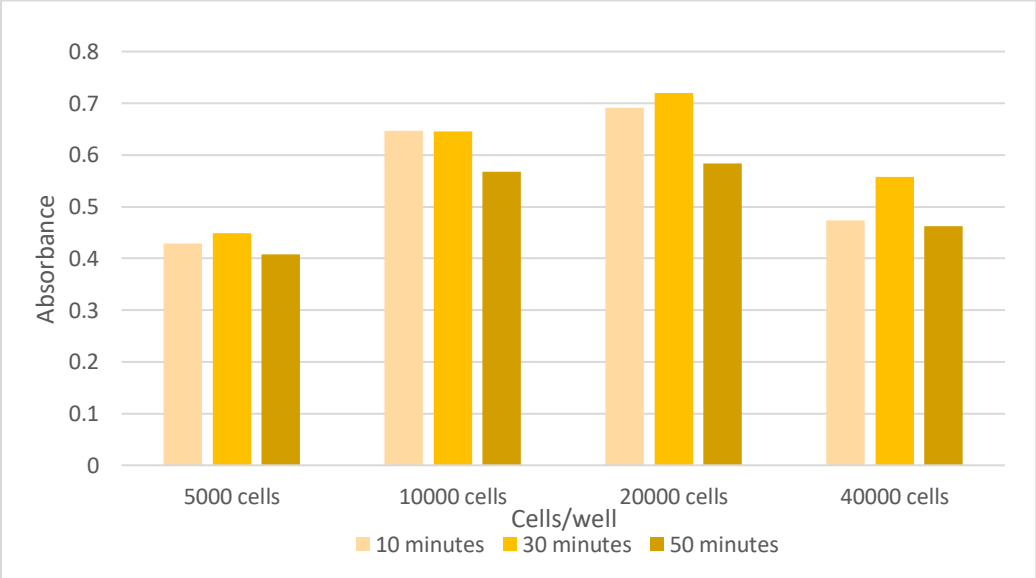

Figure 9. Graphical representation of the change in absorbance depending on cell density and incubation time with PrestoBlue for adherent and differentiated cells. Volume per well was: 100 µL.

For adherent cells, absorbance values also increased depending on cell density, however it could be seen that with an increased time of PrestoBlue incubation (50 minutes) and cell densities of 40000 cells, absorbance values were lower. This could be explained by the differentiation of cells and their activation with ATP caused some cells to die, explaining the reduced absorbance at 50 minutes of PrestoBlue (with ATP).

PrestoBlue Assay:

Based on the results obtained in both tests, and the aim of this project, it was decided that the smallest cell density and the shortest incubation time that yielded the most accurate absorbance values would be chosen as parameters for the biological replicates. Thus, replicates were incubated for 30 minutes with PrestoBlue and 100 000 cells/mL (or 10 000 cells/well/100µL) were seeded per well (Figure 10).

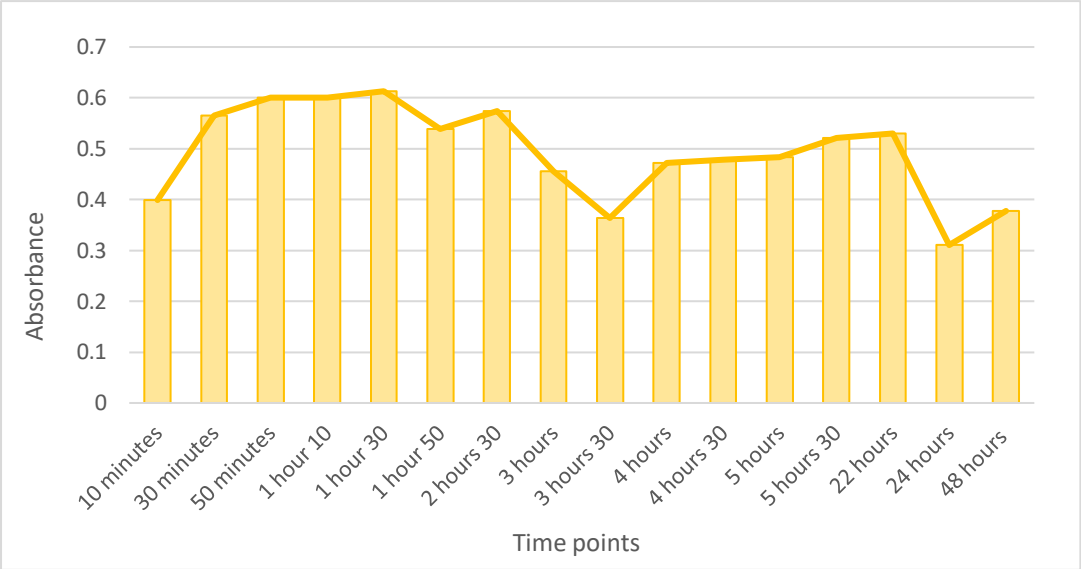

Figure 10. Plot representing the change in metabolic activity (cell viability) in relation to the time of ATP incubation. Absorbance values for each sample was averaged using both technical replicates from the combined biological replicates. Volume per well was: 100 µL.

The change in absorbance over time or, in other words, cell viability, slightly decreased with increased time of ATP incubation. These changes were not extreme as values fluctuated between approximately 0.6 and 0.3, this could possibly be explained by the insufficient time of PrestoBlue incubation and its reduction by live cells, but also by the "wash off" after removal of PMA containing media. Thus, Cell viability seemed quite constant over time despite the increased time of ATP, with absorbance remaining around 0.55.

Finally, the combination of all data was plotted in Figure 11, to visualize the "correlation" between interleukin secretion and cell viability over time. The scale of the graph did not allow the optimal observation of cell viability fluctuation, however, the trends of all variables in correspondence to each other shows a sustained increase for the release of IL-18; an increase and a following decrease in release of IL-1β and at last, slight fluctuations of cell viability that remain relatively "unchanged" over time (Figure 11).

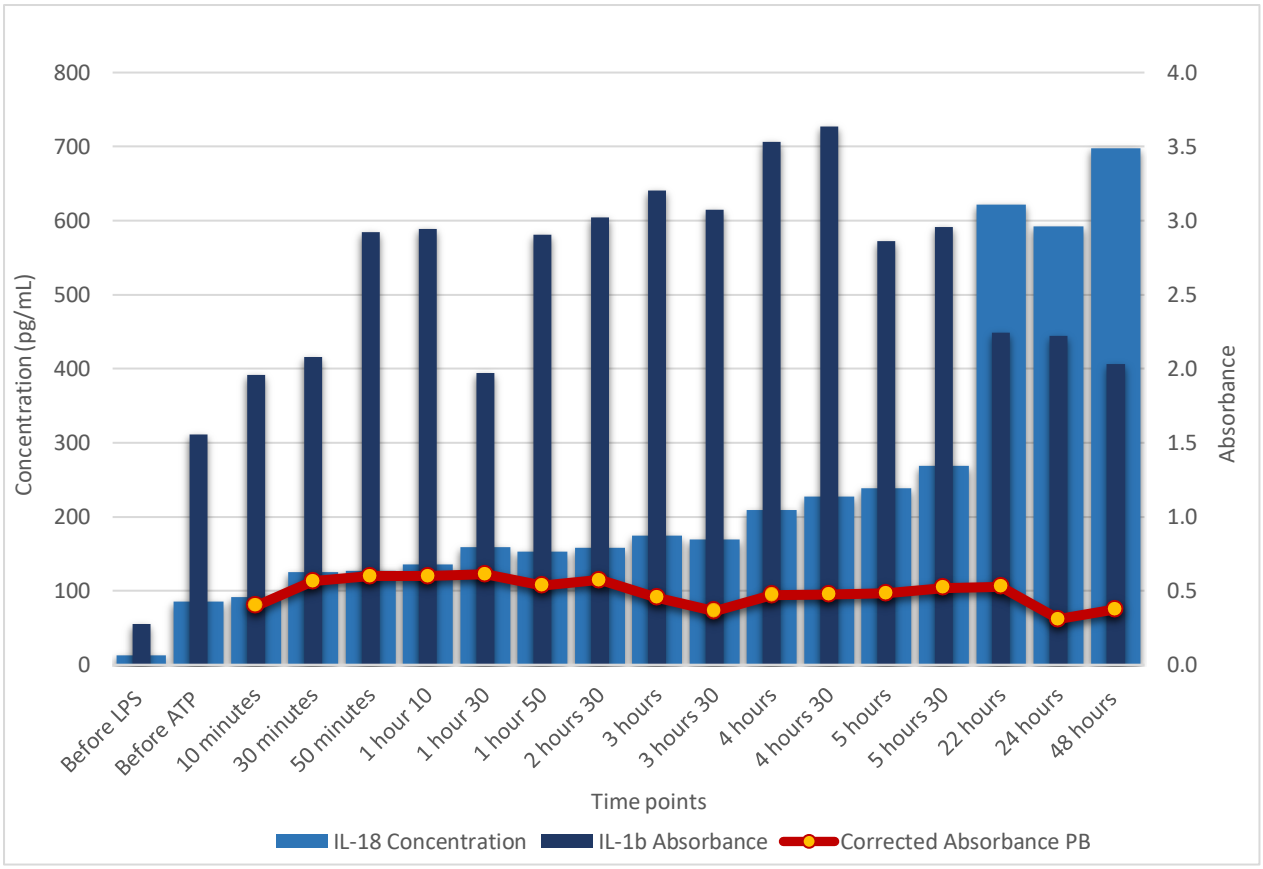

Figure 11. Combined data of IL-1β and IL-18 secretion patterns in superposition with cell viability over time. Time points were averaged using both technical triplicates from both biological replicates. PB values are representative of absorbance values after PrestoBlue incubation.

Discussion

The principles of IL-1β secretion

The exact time and process of NLRP3 inflammasome formation, followed by the secretion of interleukins and their regulation, remain of great interest in the fields of research and in medicine. To better understand the complexity of these mechanisms: detection, assembly and response, the following experiments were performed to obtain some justification on their interrelationship.

In a first step, quantity of secreted interleukin 1β (IL-1β) was measured over differently spaced time points, up until 48 hours after ATP addition. A sandwich ELISA was the method of choice, as it is a rapid and highly accurate tool for protein quantification with high affinity for a single epitope (Chiswick, Duffy, Japp, & Remick, 2011). As seen in Figures 1 and 2, measurement of IL-1β in replicates 1 and 2 were not quantitative due to the inconsistency between very low standards and very high absorbance values for this first replicate (Figure 1 in Appendix 1) and very high standard concentrations for replicate 2 (Figure 2 in Appendix 1). Both standard curves were deemed unreliable for the conversion of absorbance into concentration values (Figures 1 and 2 in Appendix 1). The change in standard concentrations for IL-1β quantification in Replicate 2, were modified based on the parameters used by Li et al. (2015), creating some discrepancies with previous replicates. These modifications were reflective of another limitation of this method as its

dynamic range is narrow, requiring sample concentrations to fit within the linear region of the standard curve and that dilutions might be needed (Leng et al., 2008). Nonetheless, it was decided that throughout this report, change in absorbance can be assumed to reflect the change in concentration over time.

IL-1β is one of the most important cytokines to play a role in protection against infection. It is a very potent protein with a wide scope of organ targets. Its potency is both an advantage and a disadvantage, as it is also known to cause tissue damage (Sahoo, Ceballos-Olvera, Barrio, & Re, 2011). Thus, IL-1β is only expressed under stringent and "urgent" conditions. Upon LPS addition, the inflammasome is primed and the transcription factor NFκB is induced to synthesize pro-IL-1β and pro-IL-18 (Kelley et al., 2019; Murphy & Weaver, 2017). The importance of priming macrophages is reflected by the upregulation of pro-IL-1β expression, which was initially absent from the cell's cytosol. As can be seen in Figures 1, 2 and summarized in Figure 3, there was a noticeable and unexpected increase in secreted IL-1β after LPS addition. According to literature, IL-1β is expected to be secreted upon NLRP3 inflammasome activation, following a second DAMP-induced activation signal (Kelley et al., 2019; Bauernfeind et al., 2009). However, LPS also plays a distinct role within the non-canonical pathway of inflammasome formation. Caspase-11, which is found in mice, and its orthologues caspases 4 and 5 in humans were found capable of initiating an inflammatory response by direct contact with intracellular LPS (He, Hara, & Nuñez, 2016; Yi, 2017). The activation of the non-canonical pathway could explain the rise in secreted IL-1β after LPS addition, yet its occurrence is unlikely as it is induced by intracellular lipopolysaccharide (Kayagaki et al., 2013). As Stoffels et al. (2015) stated, priming of human Peripheral Blood Mononuclear Cells (PBMCs) for three hours with LPS, led to the secretion of IL-1β, preceded by a large increase in mRNA expression.

Following this step, THP-1 cells were further stimulated with ATP to provoke NLRP3 activation, interleukin maturation and secretion. ATP is classified as a DAMP, usually secreted by damaged cells in the surrounding and responsible for conducting the "second signal" of inflammasome activation (Groslambert & Py, 2018). IL-1β release was further intensified after ATP addition and its increase was maintained up until four hours of incubation (Figure 3). The rapid release of IL-1β into the medium is mediated by the actions of ATP on P2X7 Receptors (P2X7R). Upon interaction between ATP and P2X7R, a potassium and sodium efflux and calcium influx is created, allowing the activation of caspase-1 (Franchi, Eigenbrod, Planillo & Nuñez, 2009; Solle et al., 2000). Based on the observations by Mehta, Hart and Wewers (2000), ATP greatly enhances the release of IL-1β in LPS-primed monocytes and that the combination of both LPS and ATP is necessary for inflammasome activation. The rapid induction of IL-1β secretion is explained by the formation of P2X7 gated ion channels and are believed to transform into pores after prolonged ATP exposure. The formation of these pores, allows molecules of 900 Da in size to dissipate from the cell into the exterior (Solle et al., 2000; Ferrari et al., 1999). The disruption of cellular integrity is followed by cell death creating a passage for interleukins to be released (Solle et al., 2000; Gandelman, Peluffo, Beckman, Cassina, & Barbeito, 2010; Virginio, MacKenzie, North, & Surprenant, 1999). This assumption is one amongst many others attempting to explain how IL-1β and its "relative", IL-18 are secreted. Opposing opinions consider cell death "dispensable" as a variety of secretory pathways exist allowing the secretion of such cytokines (Stoffels et al., 2015). Nevertheless, cytokine secretion is expected to increase after being exposed to foreign particles, explaining the functionality of these messenger molecules: to promote defenses and eventually eradicate pathogens.

Ultimately, IL-1β secretion decreased after approximately four hours of ATP addition. This decrease could be due to cell death via P2X7 pores or pyroptosis, ending the production of *de-novo* protein synthesis. Also, according to Stoffels et al. (2015), ATP did not influence IL-1β mRNA expression in PBMCs. Initially, pro-IL-1β mRNA is highly induced by LPS priming and remains unchanged. Thus, only the initial amount of mRNA will be translated into functional proteins,

limiting the overproduction of such cytokines even upon inflammasome activation. In addition, Zhu and Kanneganti (2017), discussed that the decline in IL-1β is explained firstly by the reduction of levels of *Il1b* gene transcription with increased toll-like receptor activation. In other words: less mRNA is produced with increased LPS activation, thus, causing a decrease in pro- IL-1β protein and its secretion. Other than these changes, pro-IL-1β is believed to be an unstable protein, with a relatively short half-life and a susceptibility for degradation by microRNA action (at the mRNA level) or via ubiquination at the protein level (Dinarello, 2009). Supporting these observations, Schott et al. (2014) demonstrated that "triggered" immune cells (*in vivo*) synthesize large amounts of different mRNAs that compete for translation into functional proteins and that for LPS-activated macrophages, negative feedback regulators genes for inflammation were given priority for translation. This showed the importance of a negative-feedback loop for such effective and sometimes devastating messenger molecules such as IL-1β for both *in vitro* and *in vivo* conditions.

The differential time kinetics of IL-18 secretion

Another important interleukin secreted by macrophages is interleukin-18. IL-18 shares structural homology with IL-1β making it also subject to unconventional secretory pathways (Dubyak, 2012). IL-18 is known to be constitutively expressed in macrophages, where the protein is constantly synthesized previously to any inflammatory stimulus (Dinarello, Novick, Kim, & Kaplanski, 2013). This cytokine is a pleiotropic protein with a broad spectrum of targets and functions, linking innate and adaptive immune systems (Mehta et al., 2000; Ghose et al., 2011). A sandwich ELISA was applied to quantify IL-18, as it could differentiate both, IL-1β and IL-18 from each other within "crude" samples.

The secretion of this cytokine was firstly marked after exposure to LPS (Figures 6 and 7), as the cytosolic protein was already expressed at low levels. This pattern is also observed in monocytes where TLR4 stimulation is sufficient to partially induce interleukin secretion (Yang et al., 2019; Netea et al., 2009; Puren, Fantuzzi, & Dinarello, 1999). Following this first step, IL-18 was in a constant state of increase (Figures 4,5and 6). Contrary to IL-1β, IL-18 had quite a different progression of concentration over time (Figure 4,5 and 6). There was an observed increase in IL-18 upon LPS addition followed by maintained increase up until 22 hours after ATP addition.

The time kinetics of IL-18 were simplified by plotting data in an hourly manner (Figure 7). The significance of the changes in IL-18 concentration before, during and after inflammasome activation, were determine by repeated measures ANOVA followed by a Bonferroni post-hoc test. As samples were taken consecutively from the same culture, making them paired data, the Bonferroni post-hoc test was deemed adequate for the type of data collected. The number of time points selected were ten (see Figure 7), which is the recommended maximum number of comparable variables. It is also a more rigorous test compared to its counterpart Least Significant Difference (LSD) test as it accounts for type I errors (Lee & Lee, 2018; Chen, Feng, & Yi, 2017). Nonetheless, there are also restrictions with this type of test as it has a risk of type II error, and the adjusted α is smaller than required which diminishes the power of the test (Chen et al., 2017). As the avoidance of type I error was prioritized, as there were ten samples to be compared and as the SPSS software only offered few options, the Bonferroni post-hoc test was the opted choice for significant difference (see table 3 Appendix 2).

Interestingly, Mehta et al. (2000) noticed that the combination of LPS and ATP induced a strong release of both IL-1β and IL-18 as soon as 30 minutes after incubation. These observations were further developed by comparing the effects of LPS on the intracellular expression of both interleukins. By comparing the regulation of protein expression, pro-IL-1β was dramatically produced after addition of LPS however, pro-IL-18 did not show any significant change in its expression after LPS priming (Stoffels et al., 2015). Regardless of the unchanged expression of

pro-IL-18 mRNA, LPS is required for the maturation of caspase-1 and ATP-induced interleukin secretion (Mehta et al., 2000). The continuous expression of IL-18 in immune cells, coupled with the actions of ATP, explain the enhanced secretion of IL-18 after ATP addition and its maintained increase over time (figure 6). Another particularity of IL-18 secretion over time, is the absence of a decrease in later hours after ATP stimulation. In accordance with the results obtained by Zhu and Kanneganti (2017), chronic TLR4 stimulation (using LPS), created an increase in IL-18 expression and allowed its maintenance 24 hours later. Also, IL-18 lacks gene destabilizing-elements, making it a stably expressed cytokine (Kaplanski, 2017).

The abundant production of IL-18 in immune cells would naturally require regulations before and after its release, as excessive amounts of this cytokine are the cause for many autoimmune diseases (Volin & Koch, 2011). However, *in vitro* conditions such as those applied here, were characterized by the accumulation of IL-18 over time without any sign of "return to normal" levels. The progression of IL-18 *in vivo* would be expected to decline after a certain amount of time as it is tightly regulated by the IL-18 Binding Protein (IL-18BP) and by its own by-products such as interferon gamma (IFN-γ) (Kaplanski, 2017; Volin & Koch, 2011). IL-18BP is also constitutively expressed, has high affinity for IL-18 and circulates in the plasma to regulate any excess interleukin secretion (Bachmann, Paulukat, Pfeilschifter, & Mühl, 2008). IFN-γ is released by Natural Killer cells (NK), T helper lymphocyte cells type 1 (Th1), $CD8^+$ cytotoxic lymphocytes, with the purpose of optimising pathogen recognition, eliminating any viral infections by recruiting other immune cells to the site and by controlling cell growth to avoid cancer development. The production of IFN-γ is induced by IL-18, while the latter is simultaneously regulated by IFN-γ, creating an effective negative feedback loop (Schroder, Hertzog, Ravasi, & Hume, 2003). Thus, *in vivo* conditions differ from those *in vitro*, however, it could be expected that in live organisms, IL-18 might increase after exposure to pathogenic substances (such as LPS) but the prolonged exposure would activate regulatory mechanisms to counteract and decrease levels of secreted IL-18. Essentially, the trend of IL-18 secretion *in vivo* would resemble that of IL-1β *in vitro* where IL-18 secretion is regulated and decreases after prolonged ATP exposure.

The link between interleukin secretion and pyroptosis

Although IL-1β and IL-18 are structurally related proteins, they have different functions and regulatory mechanisms representative of their roles in leading an adequate immune response. These differences were visible throughout the research with diversities in the trends of secretion, the stability of each protein as well as the constant availability of IL-18 as opposed to the promptness of IL-1β production in stimulated THP-1 cells. Both cytokines are products of the activation of the NLRP3 inflammasome with the aim of collaborating and creating a fitting immune response. IL-1β and IL-18 are both synthesized without any signal peptide and requires cleavage by caspase-1 for maturation and subsequent release. The release mechanism of these particular molecules is still in active research, however, a form of cell death known as pyroptosis has been proposed as one of the methods of secretion (Dubyak, 2012). Pyroptosis, also translated as "fiery death", is a proinflammatory form of cell death that requires caspase-1 activation. It is characterized by the rapid formation of cell membrane pores, maturation of interleukins IL-1β/IL-18 via caspase-1, morphological changes such as swelling, followed by membrane rupture and discharge of intracellular constituents (Jorgensen & Miao, 2015). Pyroptosis is thought to be the defence mechanism used by macrophages (ATP-stimulated) to expose pathogens from intracellular niches while simultaneously building an immune response by releasing the proinflammatory cytokines IL-1β and IL-18 (Bergsbaken et al., 2009).

Cell death is known to precede interleukin secretion, the link between both mechanisms and the time line of all occurring events was put to test by measuring the change in cell viability over time using PrestoBlue. PrestoBlue, is an assay composed of a blue-colored compound known as: resazurin which is reduced by metabolically active cells into a pink product named resorufin

(Erikstein et al., 2010). The conversion of PrestoBlue into resorufin can be measured visually, using absorbance or even fluorescence. It also yields rapid results that might be visible after only 10 minutes of incubation and is not toxic to cells, making it a great method for measuring live cell activity. First, the assay was tested to determine ideal cell number and incubation time for cultured THP-1 cells. Based on the experiments with suspension cells (Figure 8) and adherent cells (Figure 9), the smallest cell density per well (10000 cells/100 μL) and the shortest incubation time (30 minutes) that yielded the best results were chosen as the ideal conditions to determine cell viability in THP-1 cells.

Following the initial experiments, differentiated THP-1 cells were incubated with ATP for different times, with the expectation that prolonged ATP exposure would induce cell death. By means of caspase-1 activation through NLRP3 formation, interleukin secretion and ultimately loss of membrane integrity, a decrease in absorbance over time would be observed. However, absorbance or cell metabolism did not show any considerable variation representative of a decrease in cell viability (Figure 10). Evidently, there were no indications of pyroptosis or cell death over time, contradicting the findings of Feuvre et al. (2001) on effects of LPS, ATP and caspase-1 on cell viability in macrophages. In the study, cultured mouse macrophages, were treated with LPS and ATP (with an ATP concentration of 5mM, same as in this study) for 30 minutes, and cell viability was measured by change in Lactate Dehydrogenase (LDH) using Trypan Blue dye (Feuvre et al., 2001). As stated by the article, ATP-induced pores formed (through P2X7 receptors), accompanied with an increase in IL-1β transcription, translation and maturation via caspase-1. Subsequently, cell death seemed inevitable (Feuvre et al., 2001). Pyroptosis was observed as independent of interleukin secretion but cell viability was both dependent and independent of caspase-1, possibly referring to two different variants of cell death. Again, in another study, macrophages from mice were exposed to the same quantities of ATP, showed that P2X7 receptors formed in response to ATP, interleukin secretion was augmented and imminent cell death preceded these processes (Brough, & Rothwell, 2007). Yet, according to Stoffels et al. (2015), cell death was not always a consequence of ATP addition. By adding ATP at lower concentrations (1mM and 3mM) than those used and cited previously, Peripheral Blood Mononuclear Cells (PBMCs) did not die. Similar patterns of interleukin secretion were found in chronically stimulated macrophages with no relation to pyroptosis (Zhu & Kanneganti, 2017). Regardless of the contrast between data, all could agree that cell death is indispensable to cytokine secretion, subsequently, IL-1β and IL-18 are not a direct cause of pyroptosis but merely a product of it.

The discrepancies found between these findings and the results obtained in this thesis can be generally explained by the differences between cell lines, culture conditions, concentrations of stimulants used (LPS and ATP), incubation times and methods used to measure cell viability. Also, the use of the PrestoBlue assay presents limitations in its capacity to measure cell viability efficiently over time. It is light sensitive and is processed differently between cell types as it is dependent on their metabolic activity, making the color development vary from a few minutes to a couple of hours (Lall, Henley-Smith, Canha, Oosthuizen, & Berrington, 2013). These variations were observed throughout the experiment (Figures 8, 9 and 10) with absorbance values differing depending on cell culturing and incubation time. In addition, Stoffels et al. (2015), highlighted the importance of ATP preparation just before its addition to cell cultures. ATP dissolved for longer than two minutes was found to lose its effects, this was not taken into account when performing the cell viability assay as ATP was prepared immediately before its addition to the first sample and was left at room temperature throughout the experiment (about 5 hours and 30 minutes). This could potentially explain the lack of change in cell viability over time.

Conclusion and Future perspectives

The data obtained in this thesis was supposed to illustrate the relationship between interleukin secretion and cell viability as it is commonly thought that both phenomena are synchronized. IL-1β and IL-18 are structurally related proteins, their lack of signal peptide makes them subject to unconventional secretory pathways. It was clear that both interleukins are differentially expressed and regulated in THP-1 cells (macrophage-like cell line). It could be agreed that both cytokines were partially released upon priming and that release was further increased after ATP addition. Both interleukins were regulated differently. IL-1β was spontaneously expressed and downregulated over time, whilst IL-18 was constitutively expressed with a maintained increase over time. The common or differential mode of secretion adopted by these cytokines has yet to be elucidated.

One form of release frequently observed in macrophages is pyroptosis, a proinflammatory form of cell death that is dependent on caspase-1 activation. The correlation found between caspase-1 activation, cell death and interleukin release was not evident, which could be explained by the insufficient incubation of PrestoBlue with THP-1 cells, but also, the possibility of interleukin secretion through unconventional pathways without requiring cell death. These secretory pathways are described as vesicular transportation methods such as: exosomal, lysosomal and microvesicular pathways (Dubyak, 2012). Further studies should encompass all possible modes of secretion in relation to different cell lines and culturing conditions. Nonetheless, the mechanisms of IL-1β, IL-18 secretion and pyroptosis function together with the aim of developing an adequate immune response to eliminate potential threat. However, the inflammatory response is susceptible to the intensity of the stimulus making it diverse in its process of pathogen elimination.

The understanding of how these mechanisms intertwine, the limits of pyroptosis, the potential unconventional pathways adopted by interleukins give a global understanding about inflammation. The foundations of their regulatory mechanisms give insight on the potential treatment of certain, commonly found diseases such as: Alzheimer's, Parkinson's, CAPS and even COVID-19.

Ethical considerations and the impacts on society

THP-1 cells are immortalized monocyte-like cells that have been extensively modified, cultured, and transformed into multiple derivatives called cell lines. Their sole purpose is to mimic the behavior of monocytes in an *in-vitro* fashion. All advances on the functions and behaviors of THP-1 cells can aid in the understanding of immune-related diseases, such as autoinflammatory diseases, cancers and many more. The development of such understandings is reflected on multiple scales: Firstly, at a cellular level with the grasp of molecular events within cells and the processes involved with these. Secondly, these findings can be adapted to investigate diseases and their treatment through *in-vivo* studies using animal models, most commonly mice. The practice of animal testing requires ethical approval by animal welfare associations within nations, which assess the purpose of the study, the damage and discomfort inflicted on the animals in question. These restrictions have been in place since 1964 as stated by the Declaration of Helsinki where well-fare of the animals is at utmost priority. Ultimately, the study of cellular events, the use of animal models for disease/treatment, leads to their "customization" unto human subjects calling for stricter ethical approval. As stated by World Medical Association through the Declaration of Helsinki (2008): "A physician shall act in the patient's best interest when providing medical care" and must protect their subject's dignity, integrity, privacy, confidentiality and health. Patients are to be fully aware of the procedure, its repercussion and all that entails; as well as consent is necessary for the completion of human studies. Studies on THP-1 cells does not have any ethical constraints, however, the intent behind the analysis of these cells is firstly to determine how they

behave normally, but also how their functionality is affected in altered or abnormal states. The further progression of this study can be seen as the resulting improvement of human quality of life and the ethical limitations that accompany it.

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References

Aderem, A., & Ulevitch, R. J. (2000). Toll-like receptors in the induction of the innate immune response. *Nature*, *406*(6797), 782–787. doi: 10.1038/35021228

Bachmann, M., Paulukat, J., Pfeilschifter, J., & Mühl, H. (2008). Molecular mechanisms of IL-18BP regulation in DLD-1 cells: pivotal direct action of the STAT1/GAS axis on the promoter level. *Journal of Cellular and Molecular Medicine*, *13*(8b), 1987–1994. doi: 10.1111/j.1582-4934.2008.00604.x

Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., Macdonald, K., Speert, D., … Latz, E. (2009). Cutting Edge: NF-κB Activating Pattern Recognition and Cytokine Receptors License NLRP3 Inflammasome Activation by Regulating NLRP3 Expression. *The Journal of Immunology*, *183*(2), 787–791. doi: 10.4049/jimmunol.0901363

Bergsbaken, T., Fink, S. L., & Cookson, B. T. (2009). Pyroptosis: host cell death and inflammation. *Nature Reviews Microbiology*, *7*(2), 99–109. doi: 10.1038/nrmicro2070

Brough, D., & Rothwell, N. J. (2007). Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *Journal of Cell Science*, *120*(5), 772–781. doi: 10.1242/jcs.03377

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., … Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6). doi: 10.18632/oncotarget.23208

Chiswick, E. L., Duffy, E., Japp, B., & Remick, D. (2011). Detection and Quantification of Cytokines and Other Biomarkers. *Methods in Molecular Biology Leucocytes*, 15–30. doi: 10.1007/978-1-61779-527-5_2

Clark, R., & Kupper, T. (2005). Old Meets New: The Interaction Between Innate and Adaptive Immunity. *Journal of Investigative Dermatology*, *125*(4), 629–637. doi: 10.1111/j.0022-202x.2005.23856.x

Cullen, S. P., Kearney, C. J., Clancy, D. M., & Martin, S. J. (2015). Diverse Activators of the NLRP3 Inflammasome Promote IL-1β Secretion by Triggering Necrosis. *Cell Reports*, *11*(10), 1535–1548. doi: 10.1016/j.celrep.2015.05.003

Dagenais, M., Skeldon, A., & Saleh, M. (2011). The inflammasome: in memory of Dr. Jurg Tschopp. *Cell Death & Differentiation*, *19*(1), 5–12. doi: 10.1038/cdd.2011.159

Dinarello, C. A. (2006). Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. *The American Journal of Clinical Nutrition*, *83*(2). doi: 10.1093/ajcn/83.2.447s

Dinarello, C. A. (2009). Immunological and Inflammatory Functions of the Interleukin-1 Family. *Annual Review of Immunology*, *27*(1), 519–550. doi: 10.1146/annurev.immunol.021908.132612

Dinarello, C. A., Novick, D., Kim, S., & Kaplanski, G. (2013). Interleukin-18 and IL-18 Binding Protein. *Frontiers in Immunology*, *4*. doi: 10.3389/fimmu.2013.00289

Dubyak, G. R. (2012). P2X7 receptor regulation of non-classical secretion from immune effector cells. *Cellular Microbiology*, *14*(11), 1697–1706. doi: 10.1111/cmi.12001

Ferrari, D., Los, M., Bauer, M. K., Vandenabeele, P., Wesselborg, S., & Schulze-Osthoff, K. (1999). P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. *FEBS Letters*, *447*(1), 71–75. doi: 10.1016/s0014-5793(99)00270-7

Feuvre, R. A. L., Brough, D., Iwakura, Y., Takeda, K., & Rothwell, N. J. (2001). Priming of Macrophages with Lipopolysaccharide Potentiates P2X7-mediated Cell Death via a Caspase-1-dependent Mechanism, Independently of Cytokine Production. *Journal of Biological Chemistry*, *277*(5), 3210–3218. doi: 10.1074/jbc.m104388200

Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., & Nuñez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nature Immunology*, *10*(3), 241–247. doi: 10.1038/ni.1703

Franchi, L., Warner, N., Viani, K., & Nuñez, G. (2009). Function of Nod-like receptors in microbial recognition and host defense. *Immunological Reviews*, *227*(1), 106–128. doi: 10.1111/j.1600-065x.2008.00734.x

Gandelman, M., Peluffo, H., Beckman, J. S., Cassina, P., & Barbeito, L. (2010). Extracellular ATP and the P2X7 receptor in astrocyte-mediated motor neuron death: implications for amyotrophic lateral sclerosis. *Journal of Neuroinflammation*, *7*(1), 33. doi: 10.1186/1742-2094-7-33

Ghose, P., Ali, A. Q., Fang, R., Forbes, D., Ballard, B., & Ismail, N. (2011). The Interaction between IL-18 and IL-18 Receptor Limits the Magnitude of Protective Immunity and Enhances Pathogenic Responses following Infection with Intracellular Bacteria. *The Journal of Immunology*, *187*(3), 1333–1346. doi: 10.4049/jimmunol.1100092

Groslambert, M., & Py, B. (2018). Spotlight on the NLRP3 inflammasome pathway. *Journal of Inflammation research, 11*, 359-374. doi: 10.2147/JIR.S141220

Gurung, P., Malireddi, R. K. S., Anand, P. K., Demon, D., Walle, L. V., Liu, Z., … Kanneganti, T.-D. (2012). Toll or Interleukin-1 Receptor (TIR) Domain-containing Adaptor Inducing Interferon-β (TRIF)-mediated Caspase-11 Protease Production Integrates Toll-like Receptor 4 (TLR4) Protein- and Nlrp3 Inflammasome-mediated Host Defense against Enteropathogens. *Journal of Biological Chemistry*, *287*(41), 34474–34483. doi: 10.1074/jbc.m112.401406

Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., … Golenbock, D. T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-β. *Nature Immunology*, *9*(8), 857–865. doi: 10.1038/ni.1636

Harijith, A., Ebenezer, D. L., & Natarajan, V. (2014). Reactive oxygen species at the crossroads of inflammasome and inflammation. *Frontiers in Physiology*, *5*. doi: 10.3389/fphys.2014.00352

He, W.-T., Wan, H., Hu, L., Chen, P., Wang, X., Huang, Z., … Han, J. (2015). Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. *Cell Research*, *25*(12), 1285–1298. doi: 10.1038/cr.2015.139

He, Y., Hara, H., & Núñez, G. (2016). Mechanism and Regulation of NLRP3 Inflammasome Activation. *Trends in Biochemical Sciences*, *41*(12), 1012–1021. doi: 10.1016/j.tibs.2016.09.002

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E. O., Kono, H., Rock, K. L., … Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology*, *9*(8), 847–856. doi: 10.1038/ni.1631

Iyer, S. S., He, Q., Janczy, J. R., Elliott, E. I., Zhong, Z., Olivier, A. K., … Sutterwala, F. S. (2013). Mitochondrial Cardiolipin Is Required for Nlrp3 Inflammasome Activation. *Immunity*, *39*(2), 311–323. doi: 10.1016/j.immuni.2013.08.001

Jorgensen, I., & Miao, E. A. (2015). Pyroptotic cell death defends against intracellular pathogens. *Immunological Reviews*, *265*(1), 130–142. doi: 10.1111/imr.12287

Kaplanski, G. (2017). Interleukin-18: Biological properties and role in disease pathogenesis. *Immunological Reviews*, *281*(1), 138–153. doi: 10.1111/imr.12616

Kayagaki, N., Wong, M. T., Stowe, I. B., Ramani, S. R., Gonzalez, L. C., Akashi-Takamura, S., … Dixit, V. M. (2013). Noncanonical Inflammasome Activation by Intracellular LPS Independent of TLR4. *Science*, *341*(6151), 1246–1249. doi: 10.1126/science.1240248

Kelley, N., Jeltema, D., Duan, Y., & He, Y. (2019). The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *International Journal of Molecular Sciences*, *20*(13), 3328. doi: 10.3390/ijms20133328

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., … Melino, G. (2008). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death & Differentiation*, *16*(1), 3–11. doi: 10.1038/cdd.2008.150

Lall, N., Henley-Smith, C. J., Canha, M. N. D., Oosthuizen, C. B., & Berrington, D. (2013). Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays. *International Journal of Microbiology*, *2013*, 1–5. doi: 10.1155/2013/420601

Lamkanfi, M., & Dixit, V. M. (2012). Inflammasomes and Their Roles in Health and Disease. *Annual Review of Cell and Developmental Biology*, *28*(1), 137–161. doi: 10.1146/annurev-cellbio-101011-155745

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

Latz, E., Xiao, T. S., & Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nature Reviews Immunology*, *13*(6), 397–411. doi: 10.1038/nri3452

Leng, S. X., Mcelhaney, J. E., Walston, J. D., Xie, D., Fedarko, N. S., & Kuchel, G. A. (2008). ELISA and Multiplex Technologies for Cytokine Measurement in Inflammation and Aging Research. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *63*(8), 879–884. doi: 10.1093/gerona/63.8.879

Li, X., Liu, S., Luo, J., Liu, A., Tang, S., Liu, S., … Zhang, Y. (2015). Helicobacter pylori induces IL-1β and IL-18 production in human monocytic cell line through activation of NLRP3 inflammasome via ROS signaling pathway. *Pathogens and Disease*, *73*(4). doi: 10.1093/femspd/ftu024

Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H. I., Spinas, G. A., … Donath, M. Y. (2002). Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets. *Journal of Clinical Investigation*, *110*(6), 851–860. doi: 10.1172/jci200215318

Medzhitov, R., Preston-Hurlburt, P., & Janeway, C. A. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, *388*(6640), 394–397. doi: 10.1038/41131

Mehta, V. B., Hart, J., & Wewers, M. D. (2000). ATP-stimulated Release of Interleukin (IL)-1β and IL-18 Requires Priming by Lipopolysaccharide and Is Independent of Caspase-1 Cleavage. *Journal of Biological Chemistry*, *276*(6), 3820–3826. doi: 10.1074/jbc.m006814200

Menu, P., & Vince, J. E. (2011). The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. *Clinical & Experimental Immunology*, *166*(1), 1–15. doi: 10.1111/j.1365-2249.2011.04440.x

Misawa, T., Takahama, M., Kozaki, T., Lee, H., Zou, J., Saitoh, T., & Akira, S. (2013). Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome. *Nature Immunology*, *14*(5), 454–460. doi: 10.1038/ni.2550

Muñoz-Planillo, R., Kuffa, P., Martínez-Colón, G., Smith, B. L., Rajendiran, T. M., & Núñez, G. (2013). K Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity*, *38*(6), 1142–1153. doi: 10.1016/j.immuni.2013.05.016

Murphy, J.-E., Morales, R. E., Scott, J., & Kupper, T. S. (2003). IL-1α, Innate Immunity, and Skin Carcinogenesis: The Effect of Constitutive Expression of IL-1α in Epidermis on Chemical Carcinogenesis. *The Journal of Immunology*, *170*(11), 5697–5703. doi: 10.4049/jimmunol.170.11.5697

Murphy, K., & Weaver, C. (2017). *Janeway's Immunobiology* (9. Ed.). New York: Garland Science.

Netea, M. G., Nold-Petry, C. A., Nold, M. F., Joosten, L. A. B., Opitz, B., Jonathan H. M. Van Der Meer, … Dinarello, C. A. (2009). Differential requirement for the activation of the inflammasome for processing and release of IL-1β in monocytes and macrophages. *Blood*, *113*(10), 2324–2335. doi: 10.1182/blood-2008-03-146720

Nirmala, J. G., & Lopus, M. (2019). Cell death mechanisms in eukaryotes. *Cell Biology and Toxicology*, *36*(2), 145–164. doi: 10.1007/s10565-019-09496-2

Pellegrini, C., Antonioli, L., Lopez-Castejon, G., Blandizzi, C., & Fornai, M. (2017). Canonical and Non-Canonical Activation of NLRP3 Inflammasome at the Crossroad between Immune Tolerance and Intestinal Inflammation. *Frontiers in Immunology*, *8*. doi: 10.3389/fimmu.2017.00036

Puren, A. J., Fantuzzi, G., & Dinarello, C. A. (1999). Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1 are differentially regulated in human blood mononuclear cells and mouse spleen cells. *Proceedings of the National Academy of Sciences*, *96*(5), 2256–2261. doi: 10.1073/pnas.96.5.2256

Sahoo, M., Ceballos-Olvera, I., Barrio, L. D., & Re, F. (2011). Role of the Inflammasome, IL-1β, and IL-18 in Bacterial Infections. *The Scientific World JOURNAL*, *11*, 2037–2050. doi: 10.1100/2011/212680

Schott, J., Reitter, S., Philipp, J., Haneke, K., Schäfer, H., & Stoecklin, G. (2014). Translational Regulation of Specific mRNAs Controls Feedback Inhibition and Survival during Macrophage Activation. *PLoS Genetics*, *10*(6). doi: 10.1371/journal.pgen.1004368

Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2003). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of Leukocyte Biology*, *75*(2), 163–189. doi: 10.1189/jlb.0603252

Schroder, K., & Tschopp, J. (2010). The Inflammasomes. *Cell*, *140*(6), 821–832. doi: 10.1016/j.cell.2010.01.040

Shimada, K., Crother, T. R., Karlin, J., Dagvadorj, J., Chiba, N., Chen, S., … Arditi, M. (2012). Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis. *Immunity*, *36*(3), 401–414. doi: 10.1016/j.immuni.2012.01.009

Silverman, N. (2001). NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes & Development*, *15*(18), 2321–2342. doi: 10.1101/gad.909001

So, A. (2008). Developments in the scientific and clinical understanding of gout. *Arthritis Research & Therapy*, *10*(5), 221. doi: 10.1186/ar2509

Solle, M., Labasi, J., Perregaux, D. G., Stam, E., Petrushova, N., Koller, B. H., … Gabel, C. A. (2000). Altered Cytokine Production in Mice Lacking P2X7Receptors. *Journal of Biological Chemistry*, *276*(1), 125–132. doi: 10.1074/jbc.m006781200

Sonnaert, M., Papantoniou, I., Luyten, F. P., & Schrooten, J. (2015). Quantitative Validation of the Presto Blue™ Metabolic Assay for Online Monitoring of Cell Proliferation in a 3D Perfusion Bioreactor System. *Tissue Engineering Part C: Methods*, *21*(6), 519–529. doi: 10.1089/ten.tec.2014.0255

Stoffels, M., Zaal, R., Kok, N., Jos W. M. Van Der Meer, Dinarello, C. A., & Simon, A. (2015). ATP-Induced IL-1Î² Specific Secretion: True Under Stringent Conditions. *Frontiers in Immunology*, *6*. doi: 10.3389/fimmu.2015.00054

Subramanian, N., Natarajan, K., Clatworthy, M. R., Wang, Z., & Germain, R. N. (2013). The Adaptor MAVS Promotes NLRP3 Mitochondrial Localization and Inflammasome Activation. *Cell*, *153*(2), 348–361. doi: 10.1016/j.cell.2013.02.054

Takeda, K., & Akira, S. (2005). Toll-like receptors in innate immunity. *International Immunolog*, *17*(1), 1-14. doi: https://doi.org/10.1093/intimm/dxh186

Tsutsumi, N., Yokota, A., Kimura, T., Kato, Z., Fukao, T., Shirakawa, M., Ohnishi, H. & Tochio, H. (2019). An innate interaction between IL-18 and the propeptide that inactivates its precursor form. *Scientific Reports*, *9*(1). doi: 10.1038/s41598-019-42661-5

Vanaja, S. K., Rathinam, V. A., & Fitzgerald, K. A. (2015). Mechanisms of inflammasome activation: recent advances and novel insights. *Trends in Cell Biology*, *25*(5), 308–315. doi: 10.1016/j.tcb.2014.12.009

Virginio, C., Mackenzie, A., North, R. A., & Surprenant, A. (1999). Kinetics of cell lysis, dye uptake and permeability changes in cells expressing the rat P2X7receptor. *The Journal of Physiology*, *519*(2), 335–346. doi: 10.1111/j.1469-7793.1999.0335m.x

Volin, M. V., & Koch, A. E. (2011). Interleukin-18: A Mediator of Inflammation and Angiogenesis in Rheumatoid Arthritis. *Journal of Interferon & Cytokine Research*, *31*(10), 745–751. doi: 10.1089/jir.2011.0050

Wilson, S. P., & Cassel, S. L. (2010). Inflammasome-Mediated Autoinflammatory Disorders. *Postgraduate Medicine*, *122*(5), 125–133. doi: 10.3810/pgm.2010.09.2209

Xie, Q., Shen, W.-W., Zhong, J., Huang, C., Zhang, L., & Li, J. (2014). Lipopolysaccharide/adenosine triphosphate induces IL-1β and IL-18 secretion through the NLRP3 inflammasome in RAW264.7 murine macrophage cells. *International Journal of Molecular Medicine*, *34*(1), 341–349. doi: 10.3892/ijmm.2014.1755

Yang, Y., Wang, H., Kouadir, M., Song, H., & Shi, F. (2019). Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. *Cell Death & Disease*, *10*(2). doi: 10.1038/s41419-019-1413-8

Yi, Y.-S. (2017). Caspase-11 non-canonical inflammasome: a critical sensor of intracellular lipopolysaccharide in macrophage-mediated inflammatory responses. *Immunology*, *152*(2), 207–217. doi: 10.1111/imm.12787

Yi, Y.-S. (2018). Regulatory Roles of the Caspase-11 Non-Canonical Inflammasome in Inflammatory Diseases. *Immune Network*, *18*(6). doi: 10.4110/in.2018.18.e41

Yolken, R. (1980). Enzyme-linked Immunosorbent Assay (ELISA): a practical tool for rapid diagnosis of viruses and other infectious agents. *The Yale Journal of biology and medicine, 53*(1), 85-92.

Zhu, Q., & Kanneganti, T.-D. (2017). Cutting Edge: Distinct Regulatory Mechanisms Control Proinflammatory Cytokines IL-18 and IL-1β. *The Journal of Immunology*, *198*(11), 4210–4215. doi: 10.4049/jimmunol.1700352

Appendices:

Appendix 1: Standard curves for IL-1β measurements

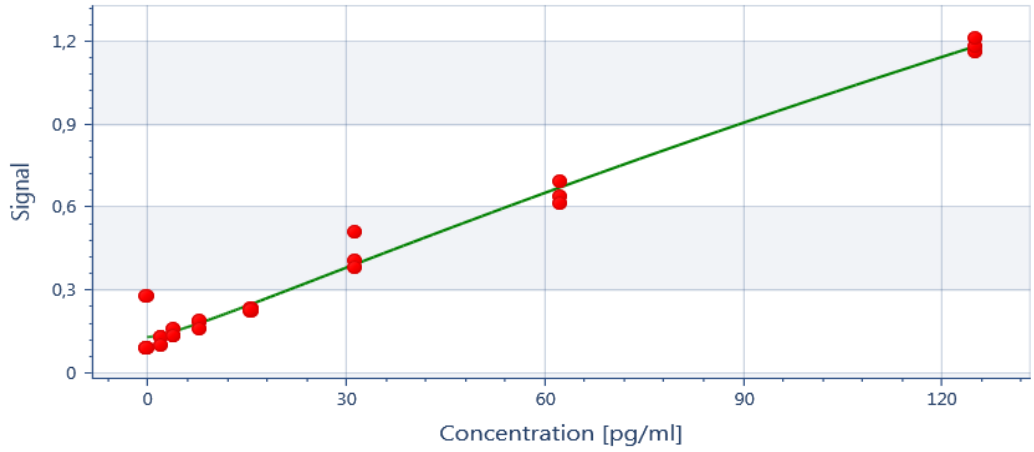

Figure 1. Standard curve plot for ELISA IL-1β Replicate 1. Standard concentrations used (in triplicates): 125 pg/mL; 62.5 pg/mL; 31.3 pg/mL; 15.6 pg/mL; 7.8 pg/mL and 3.9 pg/mL. The coefficient of determination R^2= 0.996 and the equation of the curve is: $y = 0.133247 + (\frac{121.288-0.133247}{\left(\left(1+\left(\frac{x}{15.6829}\right)^{-0.239844}\right)\right)^{10}})$

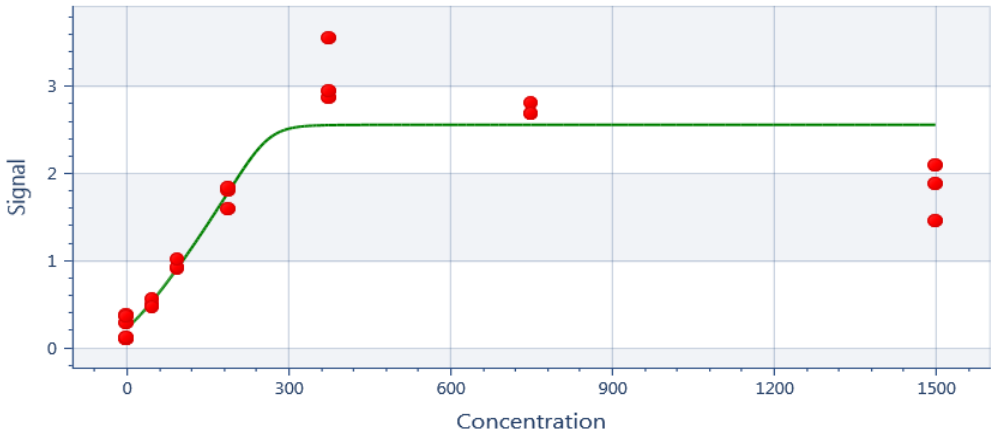

Figure 2. Standard curve plot for ELISA IL-1β Replicate 2. Standard concentrations used (in triplicates): 1500 pg/mL; 750 pg/mL; 375 pg/mL; 187.5 pg/mL; 93.75 pg/mL; 46.875 pg/mL and 23.438 pg/mL. The coefficient of determination R^2= 0.873 and the equation of the curve is: $y = 0.243178 + \left(\frac{2.56639-0.243178}{(1+\left(\frac{x}{264.224}\right)^{-11.9435}))^{0.1}}\right)$

Appendix 2: Collected data for IL-18 secretion and statistical analysis results

Table 1. Concentration of secreted IL-18 (combined data average)

Time points	Concentration in pg/mL
Before LPS	31,18133099
Before ATP	218,1088057
10 mins	285,4170402
20 mins	302,0671862
30 mins	417,8884472
40 mins	479,566012
50 mins	506,7299296
1 hour	534,1269971
1h10	524,4008302
1h20	552,344649
1h30	
1h40	520,8970832
1h50	588,6800627
2 hours	586,5227351
2h30	605,0338336
3 hours	569,0593524
3h30	
4 hours	578,6061066
4h30	518,0672004
5 hours	414,2585131
5hours 30	354,490513
22 hours	261,8926083
24 hours	256,5162855

Table 2. Shapiro-Wilk test of Normality results for each time point within both replicates 1 and 2.

Time points	Significance (p-value) combined replicates
Before LPS	0.391
Before ATP	0.489
10 minutes	0.148
20 minutes	0.079
30 minutes	0.332
40 minutes	0.602
50 minutes	0.442
1 hour	0.547
1 hour 10	0.780
1 hour 20	0.834
1 hour 30	0.081
1 hour 40	0.740
1 hour 50	0.762
2 hours	0.267
2 hours 30	0.046
3 hours	0.590
3 hours 30	0.491
4 hours	0.161
4 hours 30	0.073
5 hours	0.859
5 hours 30	0.908
22 hours	0.711
24 hours	0.341
48 hours	0.911

Table 3. Results of Repeated-Measures ANOVA for the combined hourly data from the ELISA IL-18 replicates:

Concentration A	Concentration B	Significance
Before LPS	After LPS/Before ATP	0.001
After LPS/Before ATP	10 minutes	0.311
	1 hours	<0.001
10 minutes	1 hour	0.002
1 hour	2 hours	0.064
2 hours	3 hours	0.003
3 hours	4 hours	0.003
4 hours	5 hours	0.619
5 hours	22 hours	0.003
22 hours	24 hours	1.00
	48 hours	0.018
24 hours	48 hours	0.027

The repeated measures ANOVA was corrected using Bonferroni post-hoc test.