Master Degree Project

UNIVERSITY OF SKÖVDE

MODULATION OF NLRP3 INFLAMMASOME BY SP110

Regulation and inhibition of NLRP3 inflammasome in Sp110 deficient THP-1 cells

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Abstract:

Upon recognition on Pathogen-associated-molecular-pattern and Danger-associated-with-molecular-pattern of cells in the innate immune system, an intracellular multiprotein complex called inflammasome is formed. Activation of inflammasome lead to recruitment and activation of caspase-1, a cytosolic enzyme responsible for activation of proinflammatory cytokines and excessive activities promote cell death (pyroptosis). This project investigated if the protein Sp110 is involved in the modulation of NLRP3-inflammasome activity. The undifferentiated THP-1 cells with and/or without Sp110 expression were subjected to *Mycobacterium tuberculosis* and proinflammatory response in terms of caspase-1 activation, secretion of the proinflammatory cytokines as well as cell death were quantified. ELISA was used to measure the cytokines and FLUOstar-OPTIMA was used to measure Ac-YVDA-AMC substrate to detect the caspase-1 activity. Furthermore, flow cytometry was used to measure the percentages of cell death by PE-Annexin-V and 7-AAD staining. The study found that the IL-1β, IL-6, and IL-8 were released and significantly increased, but TNF was not significantly increased in cells lacking Sp110 expression. This confirms with our observation that these cells lacking Sp110 expression showed an increased caspase-1 activity upon activation of the bacteria. Additionally, bacterial stimuli induced apoptosis to a higher degree in cells lacking Sp110 expression. The purpose of this study was to assess the potential roles of Sp110 protein in NLRP3-inflammasome activation in undifferentiated THP-1 cells and to know how this protein regulates the NLRP3-inflammasome. In conclusion, the study demonstrated that Sp110 protein regulated NLRP3-inflammasome in an inhibitory fashion, leading to increased secretion of proinflammatory cytokines, caspase-1 activity and apoptotic cell death.

Popular scientific summary:

An inflammation can be initiated by multiple triggers such as an infection, tissue damage, cellular anomalies, and even autoimmune disease. The human body can be infected by different types of bacteria, e.g. *Mycobacterium tuberculosis* (*M. tuberculosis*) bacteria, which is one of the bacteria that causes tuberculosis in human, contributing to 1,5 million casualties in the world 2013. Once the body is infected, the human immune cells go to attack in order to neutralize the infected bacteria.

When there lacks a method to prevent the outbreak of *M. tuberculosis* epidemics, an influence is necessary with the ability to control spreading, to neutralize, and to remove the infected cells. It is crucial to understand how the human body does this and this study has identified a central piece of the puzzle to achieve successful immunity, which is Sp110.

How does this special protein (Sp110) work?

In 2006 *Tosh et al* discovered the gene in mice (*Ipr1*) which adversely controlled the *M. tuberculosis* bacterial infection. However, humans have one gene, which is the same as the mouse gene (*Ipr1*), and this human gene is called SP110. This human gene produces a protein called Sp110 protein and this protein is important for the human immune cells to respond against the bacterial infection. The protein Sp110 may play a major role to control the production of a large, complex protein inside the cells which is called inflammasome. In addition, this complex protein inflammasome, not original in active form, becomes activated by cells infected with different types of bacteria or by a different stimulus. The complex protein produces activated enzymes called caspase-1. The active caspase-1 enzyme come from the activating of inflammasome prosess. This activated enzyme caspase-1 causes the production of proinflammatory molecules (IL-1β, IL-6, IL-8, and TNF-α) and eventually causes the cell death (early apoptosis and late apoptosis/ necrosis cell death) of the healthy cells. This is a good indicator for detection of the condition of the active complex protein inflammasome.

By infecting the monocyte cell line with *M. tuberculosis* without the gene/protein (Sp110) caused the increase of the activated enzyme caspase-1 levels, proinflammatory molecules, and the cell death. By having the protein, the enzyme activity was reduced significantly. The previous researches showed that during autoimmune disease and cells infection the Inflammasome protein highly activated, but this study novel showed that presenting Sp110 in monocyte cell line can reduce inflammasome. Since, the inflammasome is the mechanism of inflammation, the inflammation can be reduced by Sp110 protein. This Sp110 protein can be use in future as a biological drug for controlling inflammation in autoimmune disease patient and also for reducing inflammation during cells infection with different bacteria.

List of Abbreviations:

AIM2	Absent in Melanoma 2
ASC	Associated Speck protein
BIR	Baculovirus inhibitor of apoptosis protein repeat
BMDM	Bone marrow derived macrophages
CARD	Caspase activation recruitment domain
CLR	C-type lectin receptor
DAMP	Danger associated with molecular pattern
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICE	IL-1β converting enzyme
IFN-1	Interferon type-1
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
Ipr1	Intracellular pathogen resistance-1
LRR	Leucine rich repeat
MDA5	Melanoma differentiated-associated gene-5
mROS	Mitochondrial reactive oxygen species
NACHT	Neuronal apoptosis inhibitor protein
NF-$_{\kappa}$B	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NLRC	Nod-like receptor caspase recruitment domain
NLRP	Nod-like receptor pyrin domain
NOD	Nucleotide oligomerization domain
PAMP	Pathogen associated molecular pattern
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PYD	Pyrin domain
SP110	Speckled 110 gene
THP-1	Human monocyte cell line
7-AAD	7-amino-actinomycin D

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1: Introduction:

1:1 Background

The innate immune system is the first line of the host defense against the invading pathogens in a human body. This system plays an essential role in the maintenance of the homeostatic tissue function (Latz et al., 2013). Furthermore, this system has the ability to upregulate inflammatory mediators and to activate the adaptive immune system by inducing adhesion molecules and costimulatory expression (Ciraci et al., 2012). Monocyte and macrophage cells of the innate immune system kill invading pathogens through phagocytosis, and if needed an inflammatory response is triggered leading to the release of proinflammatory cytokines (Auffray et al., 2009). Many extracellular and intracellular molecules are involved in the activation of monocyte and macrophage cells that react against infection and tissue damage (Latz et al., 2013; Auffray et al., 2009).

The early recognition and clearance of the pathogens by innate immune cells depend on germ-line encoded pattern recognition receptors (PRRs) (Newton and Dixit, 2012). The PRRs have the ability to sense the presence of pathogens and to trigger a proinflammatory response against infection by pathogen expressed molecules called pathogen-associated molecular pattern (PAMP) (Akira and Takeuchi, 2010; Mogensen et al., 2009). PAMPs are microorganism-derived molecules important for pathogen survival, such as fungi cell wall component ά-mannan and β-glucan, peptidoglycan components in bacterial cell wall, flagellin protein of bacteria, lipopolysaccharide (LPS) in gram-negative bacteria, and nucleic acids of bacteria and virus (Newton and Dixit, 2012). However, the danger associated with molecular patterns (DAMPs) are endogenous non-microbial molecules which are normally released from damaged cells during necrosis, such as amyloid peptide, uric acid, cytokine interleukin-1α (IL-1α), ATP and calcium binding (Kono and Rock, 2009; Schroder and Tschopp, 2010; Newton and Dixit, 2012). Also, the DAMPs can be recognized by PRRs (Kono and Rock, 2009; Schroder and Tschopp, 2010; Newton and Dixit, 2012).

The PRRs have four different classes, C-type lectin receptors (CLRs) are transmembrane receptors, which recognizes a pathogen carbohydrate by carbohydrate-binding domain (Geijtenbeek and Gringhuis, 2009). The retinoic acid-inducible Gene-I (RIG-I), and melanoma differentiation-associated gene-5 (MDA5), are included in RIC-I-like receptor (RLR) family (Yoneyama et al., 2005; Yoneyama and Fujita, 2008). This type of the PRRs is found in the cytoplasm and RLR is able to recognize viral double strand RNA (Yoneyama and Fujita, 2008; Akira and Takeuchi, 2010). Indeed, the RLRs are essential for inflammation and interferon type-1 (IFN-1) production (Yoneyama et al., 2005; Yoneyama and Fujita, 2008; Akira and Takeuchi, 2010). The first PRRs identified were Toll-Like receptors (TLRs), which were first described in fruit flies (Kawai and Akira, 2006). In humans there are more than 10 membrane bound TLRs that have been identified (Kawai and Akira, 2006). Moreover, the TLRs have the ability to self-ligand and to sense a wide variety of microorganisms from outside the cells or intracellular in endosome and lysosome (Kawai and Akira, 2006; Akira and Takeuchi, 2010). Nucleotide oligomerization domain (Nod)-Like receptor (NLR) is another type of PRRs, but NLRs are strictly cytosolic receptors, which play many essential roles in the innate immune system

regulation and inflammation, by acting as intracellular sensors against invading pathogens (Franchi et al., 2009). There are 23 NLR family members encoded in humans (Ting et al., 2008). Generally, NLRs are divided into several types based on the N-terminal domain:

The first is NOD-like receptor caspase-1 recruitment domain (NLRC) which contains protein nucleotide binding oligomerization domain and the caspase-1 recruitment domain. (Kanneganti et al., 2007; Akira and Takeuchi, 2010). The secound is NOD-like receptors with pyrin domain (NLRP) which is the largest group in the NLRs family, including 14 members. The NLRP contain pyrin domain and nucleotide binding oligomerization domain (Latz, 2010). The third is baculovirus inhibitor of apoptosis protein repeat (BIR) domain which plays a crucial role in cytokines production and apoptotic cell death (Silke and Vaux, 2001; Kanneganti et al., 2007; Akira and Takeuchi, 2010).

Not only does the NLR contain the N-terminal domain, NLR contains also leucine rich-repeat domain (LRR) (Martinon et al., 2009). The LRR domain plays an important role in the recognition of PAMPs, which occurs in protein-protein interaction and in the autoregulation of NLRs (Martinon et al., 2009; Franchi et al., 2009). Furthermore, NLR contains the central nucleotide binding oligomerization domain (NACHT) which regulates self-oligomerization (Martinon et al., 2009; Latz, 2010)

In NLRs, the N-terminal mediates signaling through the interaction of downstream factors, and the N-terminal are important for inflammation and apoptosis (Franchi et al., 2009). NLRs initiate the formation of intracellular multi-protein complexes called inflammasomes that were first described in 2002 (Martinon et al., 2002). The inflammasomes are oligomeric molecular platforms, which are more than (700 kDa), and the inflammasome proteins allow the recruitment and the mediation of the activation of caspase-1 enzyme in monocyte and macrophage cells (Martinon et al., 2002, 2009; Kanneganti, 2010). The procaspase-1 is an inactive zymogen present in cytosol, which is recruited by inflammasome to undergo autoactivation, and requires after autoactivation two internal cleavages to become active cysteine proteases (Yamin et al., 1996; Thornbrry et al., 1992; Thornbrry and Molineaux, 1996; Muneta et al., 1999; Lamkanfi et al., 2011). The caspase-1, called interleukin- 1 beta (IL-1β) converting enzyme (ICE), which is responsible for production and the release of the proinflammatory cytokines such as pro-IL-1 β and pro-IL-18 cytosolic precursors (Thornbrry et al., 1992; Agostini et al., 2004; Martinon et al., 2009; Kanneganti, 2010). Pro-IL-1 β and pro-IL-18 are potent cytokines crucial to battle infections (Schroder and Tschopp, 2010; Lamkanfi et al., 2011). Also, the caspase-1 contributes to the host defense through pyroptosis (inflammatory programmed cell death) (Schroder and Tschopp, 2010; Lamkanfi et al., 2011). The pyroptosis term is a Greek word which comes from "(pyro) relating to fire or fever and (ptosis) meaning a falling" (Bergsbaken et al., 2009). And the other name of pyroptosis is caspase-1 dependent cell death, because the caspase-1 activation leads to the rapid rupture of the cell membranes and release of proinflammatory cytokines (Bergsbaken et al., 2009). Additionally, the pyroptosis causes cells lysis to prevent pathogen replication in infected cells (Bergsbaken et al., 2009) and pyroptosis helps to present pathogen antigens and releases DAMPs to be recognized by the immune cells. (Fink and Cookson, 2005; Bergsbaken et al., 2009).

NLRP1, NLRP3, and NLRC4 are the most important of the NLR family that are related to inflammasomes (Martinon et al., 2009). And also absent in melanoma 2 (AIM2) is one of the pyrin HIN-200 family members that is linked to the inflammasome (Martinon et al., 2009). The NLPR1 inflammasome gives a response to a *Bacillus Anthrax* lethal toxin (Boyden and Dietrich, 2006). While,

the NLRC4 has the ability to directly bind itself to flagllin or to basal body rod components of the type III secretion system of bacteria, the NLRC4 becomes activated (Miaoa et al., 2010; Kofoed and Vance, 2011). AIM2 inflammasome can be activated by the dsDNA in cytoplasm (Schroder and Tschopp, 2010). The NLRP3 is characterized by having three domains: NACHT domain, LRR domain, and pyrin domain (PYD) domain and the NLRP3 has been the most reviewed NLR inflammasome (Ciraci et al., 2012).

The NLRP3 inflammasome is not active or even assembled in the normal state, but becomes activated when PYD domain of NLRP3 bind to PYD of apoptosis associated speck protein (ASC) by oligomerization (Ciraci et al., 2012). In the caspase-1 activation, the caspase recruitment domain (CARD) of ASC bind to the CARD domain of procaspase-1, and this binding lead to cleavage of pro-caspase-1 to become functional shown in Figure 1. (Ciraci et al., 2012). The active caspase-1 converts the pro-IL-1β and pro-IL-18 into biological active cytokines, which are released into the extracellular space to provoke and to recruit immune cells. (Mishra et al., 2012; Krishnan et al., 2013).

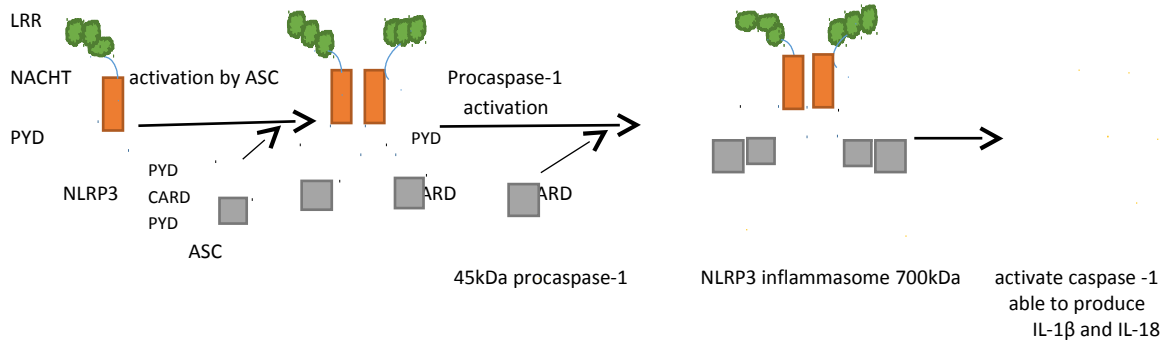

Figure 1. NLRP3 structure is composed of: 1. LRR is important in ligand sense and autoregulation. 2. NACHT is the only domain that contains in all of NLR family, and NACHT is important for regulate self-oligomerization. 3. PYD is important for protein-protein interaction. Several NLRP3 structures bind to each other and then NLRP3 are activated by ASC (PYD-PYD domain binding). Producing this structure, (composed of NLRP3 and ASC), leads to Procaspase-1 autocleveage, when the CARD domain of the caspase-1 bound to the CARD of ASC, activates to form a complex protein inflammasome. The proteolytic active caspase-1 cause production of and release of mature IL-1β and IL-18 cytokines on exterior of cells, which can recruit innate immune cells. The figure adapted from these references. (Stutz et al., 2009; Latz, 2010; Schroder and Tschopp, 2010; Latz et al., 2013)

Activation of NLRP3 inflammasome requires two signals to release mature IL-1β and IL-18: First, the microbial ligands lead to activation of TLRs ligand or NLRs ligand (Vladimer et al., 2013). Actually, microbial ligands upregulate NF-κB, which NF-κB is a protein that regulates DNA transcription (Hoffmann et al., 2006; Vladimer et al., 2013). In addition, the NF-κB protein controls the expression and the transcription of pro-IL-lβ 32 kDa and of pro-IL-18 24 kDa (Robertson et al., 2006; Brough and Rothwell, 2007; Vladimer et al., 2013; Cullen et al., 2015). Shimada *et al.* proposed that mitochondrial DNA oxidized acts as a ligand for activating NLRP3, because during inflammation the oxidized DNA is released from dysfunctional mitochondria into the cytosol (Shimada et al., 2012). The second signal is intracellular signaling, which is required for NLRP3 inflammasome activation of procaspase-1 recruitment that can be triggered by a variety of stimuli to release mature IL-1β and IL-18. (Yamin et al., 1996; Hoffmann et al., 2006; Robertson et al., 2006; Vladimer et al., 2013). The non-pathogenic stimuli are able to activate inflammasome such as heptens, ultraviolet irradiation, protein nonparticles, crystals, and some kinds of antibiotics (Schroder and Tschopp, 2010; Elinav et al., 2011).

Several models have proposed to activated the NLRP3 inflammasome: the first model is extracellular ATP of the pathogens that allow potassium ion (K^+) efflux to the exterior of the cells through active P_2X_7 receptors to trigger NLRP3 inflammasome activation and to trigger the release of mature cytokines (Humphreys and Dubyak, 1998; Davis et al., 2011; Vladimer et al., 2013).

The second model can directly activate NLRP3 inflammasome through a pore forming toxin during the presence of different kinds of bacteria that cause dysregulation of cellular ions (Vladimer et al., 2013). The third model is provided after phagocytosis of the pathogens, the lysosomal destabilized of acidic, which causes cytoplasmic leakage and release cathepsin B to activate NLRP3 inflammasome (Koizumi et al., 2012). Also, during lysosomal disruption the inflammasome can be activated by bacterial RNAs releasing (Koizumi et al., 2012). The NLRP3 inflammasome activation has been shown to be negatively regulated by autophagy, but positively regulated by reactive oxygen species (ROS) (Yazdi et al., 2010). Mitochondria are a source of intracellular ROS production that can be induced by various stress conditions, which include hypoxia, membrane damage, and increase of the metabolic rate (Yazdi et al., 2010). Yazdi *et al* found a relation between mitochondrial ROS (mROS) activity and IL-1β in supernatant from human monocyte cell line (THP-1), and showed, additionally, the removal of mROS by mitophagy that avoids cellular damage by pyroptosis (Yazdi et al., 2010). However, one study argued that increasing mROS instead of activating the NLRP3 inflammasome, promotes the expression as well as the activation of the NLRP3 inflammasome by Ca^{2+} (Wen et al., 2013).

Autophagy is a fundamental mechanism used to regulate cellular homeostasis by removing misfolded protein and dysfunctional organelles. Moreover, autophagy can also be used as a defense against intracellular pathogens (Salminen et al., 2012). In Crohns disease, autophagy deficiency generates inflammatory response and tissue damage (Salminen et al., 2012). The importance of autophagy can directly inhibit the production of IL-1β or indirectly by the autophagy degradation of NLRP3 inflammasome (Chen and Sun, 2013). Mitophagy is a distinct autophagy for mitochondrial degradation, within mitochondria, mitophagy selectively responds to unneeded mitochondria or damaged area. (Zhuet et al., 2013)

Mycobacterium tuberculosis is still one of the biggest public health problems that caused globally 1.5 million mortalities and 9 million morbidities in 2013 (Raviglione, 2015). The *M. tuberculosis* is a complex intracellular pathogen capable of subverting immune response and this pathogen has a long persistent infection in the host (Huynh et al., 2011). The host resistance against Mycobacterium initially dependent on alveolar macrophage after the pathogens evade phagosome lysosome fusion (Zhou et al., 2015; Crevel et al., 2002), further *M. tuberculosis* replicate in macrophages until the cells burst (McDonough et al., 1993). Many antimicrobial mechanisms are involved to limit and to remove *M. tuberculosis* (Placido et al., 1997; Zhou et al., 2015). Furthermore, some proinflammatory cytokines such as IL-18, IL-1β, IL-6, IL-8, and TNF are involved in the regulation of infected macrophage to undergo apoptosis to facilitate bacteria removal (Placido et al., 1997; Zhou et al., 2015). The term “apoptosis”, a Greek word that means to “fall off”, is an immunological silent cell death and avoids inflammation (Kerr et al., 1972; Molloy et al., 1994). Apoptosis controls cell membrane fragmentation until the infected macrophage is engulfed (Kerr et al., 1972; Molloy et al., 1994). In the previous reference to *M. tuberculosis,* showed that the IL-1β production played a major role for the host immune defense to become successful at killing the bacteria. The researchers also had shown that knockout mice lacking IL-1β or the IL-1 receptor were more susceptible to

infection (Briken et al., 2013). In IL-1β double knockout mice, granuloma in the lungs grew significantly more than in the wildtype mice (Zhou et al., 2015). Likewise, the IL-1β deficiency in the mice increased the pulmonary bacteria burden and acute mortality, strongly indicating that IL-1β signaling plays an important role in host defense against *M. tuberculosis* (Zhou et al., 2015). Moreover, IL-1β plays an important role in granuloma and although can mediate in the recruitment of other cytokines such as IL-6. (Kleinnijenhuis et al., 2009; Jayaraman et al., 2013).

In 2006, Tosh *et al* described many genes that were involved in genetic susceptibility to mycobacterial infections (Tosh et al., 2006). In a mouse model, the *sst1* locus, mapped on the chromosome 1, was essential for *M. tuberculosis* infection control (Kramnik, 2008). Researchers expressed *sst1* locus and identified the gene intracellular pathogen resistance-1 (*Ipr1*) that could control resistance and susceptibility in mice against *M. tuberculosis* infection. The *Ipr1* induces interferon nuclear protein, which is able to control growth of *M. tuberculosis*, and this protein is associated with macrophage nuclear protein. In addition, the macrophage infected cells eventually switched the form of the necrosis programmed cell death to the apoptosis, and apoptosis was beneficial in the removal of the live bacteria. Mice, deficient in *Ipr1,* lack the capacity to control *M. tuberculosis,* which leads to lethal infection hallmarked by extensive loss of macrophages due to chaotic cell death (Kramnik et al., 2000). The human equivalent to murine *Ipr1* is called speckled 110 gene (SP110) which was located on chromosome 2, and Sp110 polymorphisms were linked to *tuberculosis* susceptibility (Kramnik, 2008; Tosh et al., 2006; Lei et al., 2012). The protein was encoded by SP110 gene and Sp110 was described to play an essential role in monocyte differentiation, apoptosis, and activation against pathogens such as inhibition of influenza virus replication, vesicular stomatitis, and implication of the hepatic veno-occlusive disease. (Cliffe et al., 2012; Bloch et al., 2000; Constantoulakis et al., 2010; Nicewonger et al., 2004). The *M. tuberculosis* infection are correlated to Sp110 polymorphism (Kramnik, 2008; Tosh et al., 2006; Fox et al., 2014) However, the effects and functions of Sp110 are not well described. In the recent past a link between Sp110 expression and NLRP3 inflammasome activity in THP-1 cells was identified (unpublished data, Persson *et al*).

1:2 Aim and Objective

There is very little known about NLRP3 inflammasome regulation in general, the aim of this project is to investigate how Sp110 affect the regulation and inhibition of NLRP3 inflammasome activation. In addition, to know the effect of Sp110 on the production caspase-1 activate and the releasing of different kinds of proinflammatory cytokines during NLRP3 inflammasome activation in undifferentiated THP-1 cells by using *M. tuberculosis* strain for cell stimulation.

Since pyroptosis is mediated by NLRP3 inflammasome activity and is believed that Sp110 may act upstream on the actual activity of the NLRP3 inflammasome. The preliminary data indicates that cells lacking Sp110 do indeed have a dramatic increase in NLRP3 inflammasome activity and increasing NLRP3 inflammasome may lead to increasing cell death. The objective is to recognize which mechanisms Sp110-devoid cells are increasingly sensitive to *M. tuberculosis* infection and to observe clearly the role of Sp110 during infection and inflammation.

2: Materials and methods

2:1 Materials

The frozen cell, the three different type of undifferentiated human monocyte cell line THP-1, purchased from American Type Culture Collection (ATCC). Virulent strain, γ-irradiated and dead *M. tuberculosis* (H37Rv) from Colorado State University. TLR ligands, Phorbol 12-myristate 13-acetate (PAM_{SK4}CYS) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Puromycin antibiotic were obtained from InvivoGen (San Diego, California, USA). Ac-YVAP- AMC were obtained from Bachem Feinchemikalien AG (Bachem UK Ltd). Base cell culture media was purchased from RPMI1640 (InvitroGen Life Technologies)

2:2 Undifferentiated THP-1 cell culture

The undifferentiated THP-1 cells were maintained in base cell culture media RPMI1640. In addition, the base cell culture media was supplemented by inactivated FBS 10%, L-glutamine 2mM, Sodium pyruvate 1mM, glucose 4,5g/l, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10mM, and 100unit/ml penicillin streptomycin. The mock transfected and $Sp110^{-/-}$ knockdown of undifferentiated THP-1 cells were kept under selection pressure as the vector also contained resistance gene to puromycin concentration (5unit/ml). Frozen cells, all three types of undifferentiated THP-1 cells, were thawed and resuspended in 10ml of media, centrifuged for 5 mins at 500xg. Then supernatant was discarded and the cells were resuspended in 10ml of fresh media and cultured in a T25 flask at 37°C and 5% CO_2 for 48 hrs. The cells were maintained and were split every 2-3 days to keep the intensity of the cells between $5x10^5$ to $1,5x10^6$ cells/ml. Furthermore, the cells were counted by using a Fast-Read slide 10 chambers (Immune system Ltd) under a Binocular microscope (TOLEXO).

2:3 M. tuberculosis H37Rv strain preparation

In one aliquot a H37Rv strain of *M. tuberculosis,* was comprised of 500µL ($3x10^8$/ml) and stored in -70°C. The H37Rv was defrosted and transferred in one well of a 46-flat bottom tissue culture plate (BD Biosciences) and was syringed more than 10 times with a 27g. Afterwards the H37Rv strain was transferred into a sterile centrifuge tube for centrifuging 3000xg in 10 mins. The supernatant was discarded and the pellet was resuspended in fresh cell media for preparing different multiplicity of infection MOI (20, 10, 5 and 2).

2:4 Undifferentiated THP-1 cell infection assays and supernatant collection

The undifferentiated THP-1 cells (5 x 10^5cells/ml), which include wild type, mock transfected and knockdown $Sp110^{-/-}$ cells, in fresh media were seeded in sterile 24-well flat bottom 100-mm diameter tissue culture plates (BD Biosciences). The cells were stimulated and infected with the H37Rv virulent strain of *M. tuberculosis* MOI (10 and 2) bacteria per undifferentiated THP-1 cell and untreated cells (cultured in complete medium only) were used as unstimulated cells. The prepared 24-wells plates were incubated at 37°C in a humidified, 5% CO_2 atmosphere (Alexis Biochemicals) for 24hrs. The

samples were transferred to sterile centrifuge tubes (each well's sample in one tube) and centrifuged for 5 mins at 5000xg. Furthermore, the supernatant was collected and stored in -20°C for analyzing cytokine concentration.

2:5 Enzyme-linked immunosorbent ELISA assays for cytokine analysis

The undifferentiated THP-1 cells ($1x10^6$ cells/ml) were cultivated for 24hrs in 24-well flat bottom tissue culture plate for supernatants collection. The plate included unstimulated and stimulated H37Rv *M. tuberculosis* strain MOI (10 and 2) bacteria/cell. The levels of cytokines IL-1β, TNF-α, IL-8, and IL-6 were measured in supernatant by using a commercial ELISA kit Diaclone SAS (BioSite) according to the manufacturer's instructions. In the ELISA kit methods, the samples, standards, and 50µl of the detection antibody were incubated together for 3hrs and then washed two times. Minor changes were performed in methods: in the first step was to add samples and standards in each well and to incubate for 3hrs, and afterward to wash four times. In the second step 100µL of biotinylated detection antibody was used with the same manufacture's concentration in each well. Additionally, incubation times were two hrs. longer and the washing step was four times. The linear standard curve for each cytokine was generated by using standard cytokine supplied by manufacture. In addition, the cytokines absorbance was measured on an ELISA plate reader set at 450nm and 550nm and raw data calculation was made using Excel files to find cytokines concentration value.

2:6 Fluorescent peptide substrate essay for measuring caspase-1 activity

Caspase-1 activity was measured by using fluorescent peptide substrate Ac-YVDA-AMC (7-amino-4-methylcoumarin) 1µM final concentration in 96-wells tissue culture flat bottom plate (BD Biosciences). All the three types of undifferentiated THP-1 cells $1.5x10^5$ cells/ml were transferred to each wells of the plate and the plate was incubated at 37°C under 5% CO_2 for one hr. protected from light. After incubation for one hr., the ATP (Sigma-Aldrich) (final concentration 5µM) and LPS (final concentration 5µg/ml) were added in four wells as a positive control. In order to compare each type of undifferentiated THP-1 cells to each other, a H37Rv strain MOI (20 and 10)/ cell were used to stimulate the cells and a base cell culture media was used as a standard. Later, within a 24hrs period every two hrs., the Ac-YVDA-AMC substrate was attached to the caspase-1 active site and the Ac-YVDA-AMC was measured by using a FLUOstar OPTIMA system (BMG Labtech) software version 1, 30 R3, to record *(excitation-$_{max}$ 480-nm ex. and; emission-$_{max}$ 520-nm)* which is the raw data calculated by using an Excel File.

2:7 Determination of Apoptosis and late Apoptosis/Necrosis cell death in undifferentiated THP-1 cells by flow cytometry

The undifferentiated THP-1 cells (wild type, mock transfected and knockdown $Sp110^{-/-}$ protein) ($1x10^5$cells/ml) were cultured in 24-wells flat bottom tissue culture plate. Cells were infected by using the H37Rv strain MOI (10 and 5) per cell, by using PamCys (1ng/ml) for cell stimulation and by using a cell culture media for cell control. While the prepared plate incubated for 24hrs, the harvested three types of undifferentiated THP-1 cells were transferred to sterile centrifuge tubes (each well's sample in one tube). For measuring the cell death (early apoptotic or late apoptotic/necrotic cell death), the undifferentiated THP-1 cells the detached cells were stained in accordance with the manufacturer's instructions in a PE-Annexin V Apoptosis Detection Kit (BioLegend). After 15 mins' incubation in a

dark place the stained cells were analyzed on the BD Accuri™ C6 flow cytometer (BD Biosciences) with the BD Accuri™ C6 software to detect phosphatidylserine PS *(excitation-$_{max}$ 488-nm and; emission-$_{max}$ 575±20-nm)* and 7-amino-actinomycin D 7-AAD detected *(650 nm long-pass filter).*

2:8 Statistical analysis

The statistics analyzing of all the variances were done by using the IV version of GraphPad Prism software (GraphPad software). The cytokines data and the cell death data results were illustrated by mean value ± 1 standard error (SEM) from triplicated or quadruplicated independent experiments, which were performed in all parallel manner. The differences between all the groups were evaluated by using One-way ANOVA followed by Bonferroni post-test to compare differences between each type of undifferentiated THP-1 cells. The caspase-1 activity data were also represented by means ± 1 SEM of three independent experiments. The comparison of the variances of caspase-1 activity between the different hours of the different groups of each type of undifferentiated THP-1 cell was evaluated by Two-way ANOVA followed by Bonferroni post-test in order to compare the differences of each group with the control at different hours. Additionally, the p-value less 0.05 was considered statistically significant.

3: Results

3:1 Sp110$^{-/-}$ upregulate IL-1β cytokine secretion in undifferentiated THP-1 cells

In order to study the regulation and inhibition of NLRP3 inflammasome by Sp110 the levels of IL-1β cytokines were measured in all three types of undifferentiated THP-1 cells. The IL-1β cytokine secretion was induced in all three types of undifferentiated THP-1 cells following stimulation with H37Rv strain MOI (10 and 2). However, the Sp110$^{-/-}$ knockdown cells responded with significantly greater amounts of releasing IL-1β cytokine production compared to wild type and mock transfected cells (***P<0,001) Figure 2A. The Sp110$^{-/-}$ knockdown cells during stimulation with MOI 2 secreted 2500-3000 pg/ml, whereas wild type and mock transfected cells secreted 900-1100 pg/ml. In addition, no differences in the cytokine release were detected between the wild type and the mock transfected cells stimulated MOI 2 Figure 2A. Furthermore, the Sp110$^{-/-}$ knockdown cells stimulation MOI 10 increased IL-1β secretion to 12500 pg/ml compared to Sp110$^{-/-}$ knockdown cells stimulated MOI 2 Figure 2A. In addition, the results of unstimulated of undifferentiated THP-1 cells did not release IL-1β cytokines after 24hrs incubation Figure 2A.

3:2 Sp110$^{-/-}$ induced more IL-8 cytokine secretion in undifferentiated THP-1 cells

With the purpose of knowing the roles of Sp110 in NLRP3 inflammasome regulation the IL-8 cytokines were detected in all different types of undifferentiated THP-1 cells. The results of mock transfected cells could not release IL-8 cytokines by the cell simulating of H37Rv MOI (10 and 2) after incubation for 24hrs at 37°C under 5% CO_2 Figure 2B. In contrast, the Sp110$^{-/-}$ knockdown cells stimulated MOI (10 and 2) of H37Rv strain released significantly higher of IL-8 cytokines secretion compared to the wild type (***P<0,001). In addition, the Sp110$^{-/-}$ knockdown cells stimulated MOI 2 were (400,000 pg/ml) and MOI 10 were (2100,000 pg/ml) whereas wild type cells stimulated MOI 2 secreted (100,000 pg/ml) and wild type cells stimulated MOI 10 secreted (900,000 pg/ml) respectively Figure 2B. In addition, the results of unstimulated of undifferentiated THP-1 cells did not release IL-8 cytokines after 24hrs incubation Figure 2A.

3:3 Sp110$^{-/-}$ did not induce more TNF-α cytokine secretion in undifferentiated THP-1 cells

TNF-α cytokine secretion was studied in different type of undifferentiated THP-1 cells to detect the effect of Sp110 in the production of this cytokines. The TNF-α cytokine secretion was induced in all of the cell types following stimulation by H37Rv and incubation for 24hrs Figure 2C. In the Sp110$^{-/-}$ knockdown cells stimulated MOI 10 the concentration of TNF-α were (650± 30 pg/ml), in the wild type MOI 10 stimulated cells the concentration of TNF-α were (600- 630 pg/ml), while in the mock transfected cells concentration were (600pg/ml) Figure 2C. Moreover, the Sp110$^{-/-}$ did not significantly increase the secretion of TNF-α cytokines in undifferentiated THP-1 cells, (P>0,05) Figure 2C. However, the Sp110$^{-/-}$ knockdown cells stimulated MOI 2 (460± 20 pg/ml) were significantly more than the wild type (400± 20 pg/ml), (*P<0,05), but were not significantly more than the mock transfected cells (420± 20 pg/ml) Figure 2C. Furthermore, the TNF-α cytokine was not secreted in unstimulated of all types undifferentiated THP-1 cells Figure 2C.

Generally, Figure 2C showed no significant difference between the different kinds of undifferentiated THP-1 cells in the releasing of TNF-α cytokine (P>0,05).

3:4 Sp110$^{-/-}$ induced more IL-6 cytokine secretion in undifferentiated THP-1 cells

The results detected the effect of Sp110 in the secretion of IL-6 cytokines during NLRP3 inflammasome activation in different types of undifferentiated THP-1 cells. All types of the undifferentiated THP-1 cells released IL-6 cytokines by using the H37Rv strain of *M. tuberculosis*, for cells stimulation Figure 2D. The Sp110$^{-/-}$ knockdown stimulated MOI 2 cells (7000 pg/ml of IL-6 concentration) induced significantly more IL-6 secretion compared to the wild type and the mock transfected cells stimulated MOI 2, (***P<0,001) Figure 2D. Additionally, the Sp110$^{-/-}$ knockdown MOI 10 cells (15000 pg/ml), which were secreted significantly more IL-6 cytokines compared to the wild type stimulated MOI 10 cells (2500± 100 pg/ml concentration) and the mock transfected stimulated MOI 10 cells (1000± 100 pg/ml), (***P<0, 001) Figure 2D. At the same time the wild type cells did not induce significantly more than the mock transfected cells (P>0,05). The deficiency of Sp110 affected significantly increase the transcription and release of IL-6 cytokines in undifferentiated THP-1 cells Figure 2D. Additionally, the IL-6 cytokines did not induce in unstimulated of undifferentiated THP-1cells after 24hrs incubation

In summary, the Sp110$^{-/-}$ knockdown cells increased significantly the secretion of the potent proinflammatory IL-1β, IL-8, and IL-6 cytokines, contrary to TNF-α cytokines secretion. Also, the concentration of each cytokine in Sp110$^{-/-}$ in knockdown Sp110$^{-/-}$ knockdown undifferentiated THP-1 cells were different Figure 2.

3:5 Sp110$^{-/-}$ protein increased the caspase-1 activity in undifferentiated THP-1 cells

In order to detect the effect of Sp110 in the caspase-1 activity production during NLRP3 inflammasome in undifferentiated THP-1 cells, the cells were stimulated with *M. tuberculosis* MOI 20 and as positive control with LPS+ ATP. The cells were then incubated at 37°C and 5% CO_2 in the presence of 1μM Ac-YVDA-AMC for 24hrs. Fluorescence was measured the caspase-1 activity in every 2hrs to in the undifferentiated THP-1 cell population Figure 3.

At 4hrs all types of undifferentiated THP-1 cells showed similar levels of caspase-1 activity to all stimuli and this time point can be considered the base line. However, at 24hrs of incubation the level of caspase-1 activity production in all cell types of undifferentiated THP-1 were increased Figure 3. Upon stimulation MOI 20, the Sp110$^{-/-}$ knockdown cells increased the caspase-1 activity to 7300 RFI whereas the caspase-1 activity in the control was 2100 RFI (0,001), and the caspase-1 activity was significantly increased in the Sp110$^{-/-}$ knockdown cells stimulation MOI 20 compared to control Figure 3C. Furthermore, the Sp110$^{-/-}$ knockdown cells in stimulated LPS+ATP did not significantly increase the amount of caspase-1 activity. In contrary, the wild type and mock transfected cell stimuli LPS+ ATP produced significantly more caspase-1 activity compared to the control (P>0,001) Figure 3A, B. However, the caspase-1 activity in the MOI 20 stimuli cell wild type were 4500 RFI and mock transfected cells were 5600 RF. Additionally, the wild type and mock transfected cells did not produce significantly more caspase-1 activity compared to control Figure 3A, B.

In summary, the caspase-1 activity were increased in the knockdown Sp110$^{-/-}$ cells compare to the wild type and mock transfected cells after 24hrs incubation, by using the H37Rv strain of *M.*

tuberculosis MOI (20 and 10) to cells stimulate, but the results were not significantly different between all types of undifferentiated THP-1 cells (P>0,05) *Figure in Appendices I*. Additionally, at 24hrs the wild type and mock transfected cells appeared to have produced the same amount of cleaved substrate indicating similar caspase-1 activity in the wild type and the mock transfected cells when stimulated with the MOI 10 *Figure in Appendices I.*

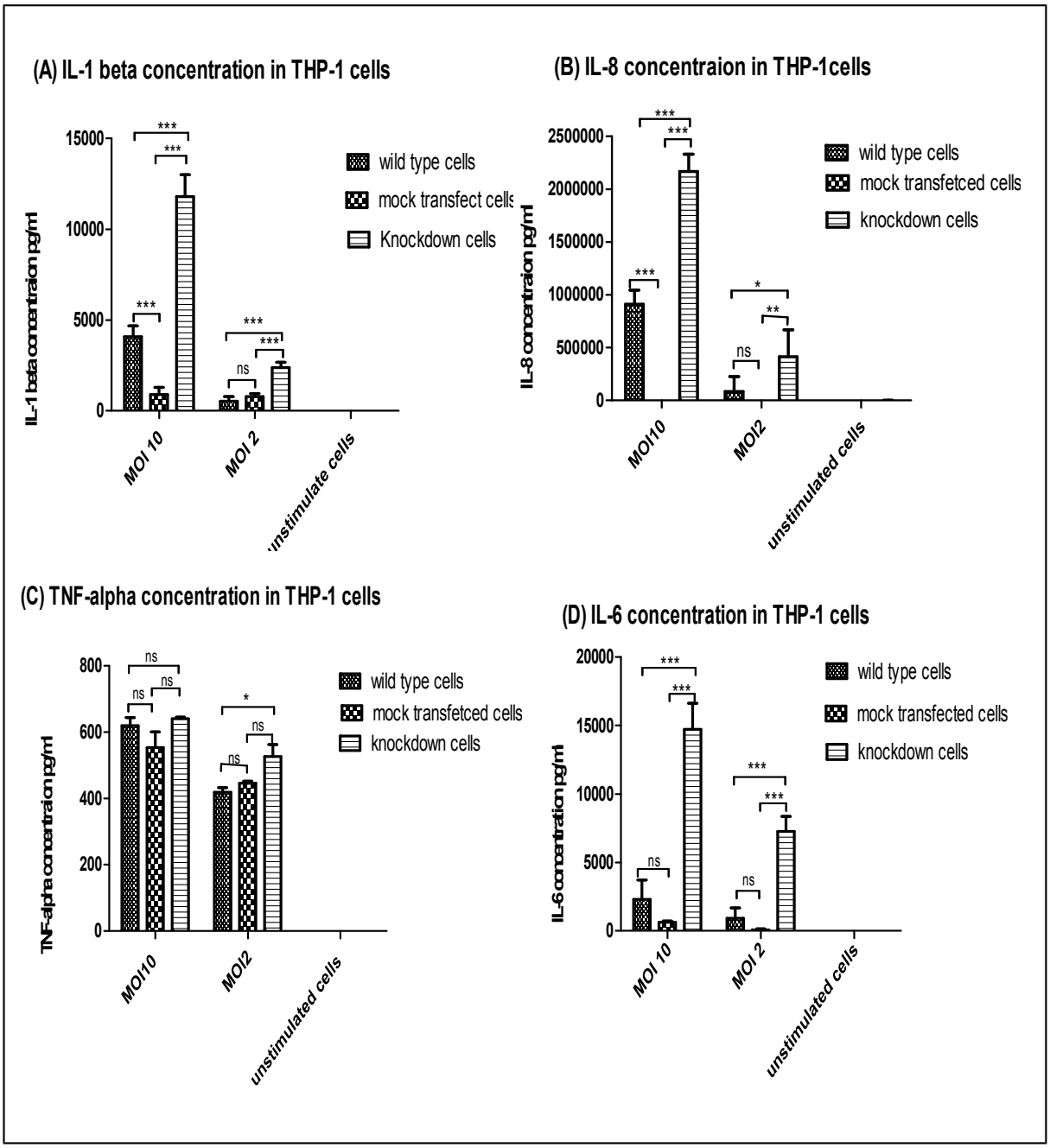

Figure 2. Secretion of cytokines IL-1β, IL-8, TNF-α and IL-6 from wild type, mock transfected and knockdown Sp110$^{-/-}$undifferentiated THP-1 cells. The cells (stimulated by H37Rv MOI (10 and 2) and unstimulated) were incubated for 24hrs in 37°C and 5% CO_2, the levels of cytokine secretion were evaluated by ELISA. The data were analyzed by one-way ANOVA followed Bonferroni post-test and expressed as a mean ± 1 SEM. All three types of undifferentiated THP-1, unstimulated cells had not released cytokines IL-1β, IL-8, TNF-α and IL-6. (A) H37Rv induced IL-1β in all types of undifferentiated THP-1 cells and Sp110$^{-/-}$ knockdown cells secreted

significantly more IL-1β than wild type and mock transfected (***P<0,001, n=4) (B) IL-8 in knockdown $Sp110^{-/-}$ knockdown cells were significantly induced more than wild type cells (***P<0,001, n=3), but IL-8 cytokines did not secret in mock transfected cells. (C) TNF-α cytokines induced in all types of undifferentiated THP-1 cells and TNF-α was stimulated by H37Rv MOI (10 and 2), but there were not significant different between undifferentiated THP-1 cells, (P>0,05, n=3). (D) IL-6 induced in all types of undifferentiated THP-1 cells and $Sp110^{-/-}$ knockdown cells secreted significantly more IL-6, (***P<0,001, n=4).

3:6 Lacking expression of Sp110 induced the undifferentiated THP-1 cells to undergo early apoptosis and late apoptosis/necrosis cell death

In order to know the effect of the Lacking expression Sp110 on cell death was evaluated by PE-Annexin V and 7-AAD staining and flow cytometry. In early apoptosis the PE-Annexin V stain bind to the PS located in the lower right quadrant of scatter of region A-2, *Figure in Appendices II*. The 7-AAD bind with DNA, was used for late apoptotic/necrotic cell death and 7-AAD was detected in the upper right quadrant of the plot of region A-3, *Figure in Appendices II*. The live cells did not stain with Annexin V and 7-AAD, in the lower left quadrant of scatter region A-1, *Figure in Appendices II.*

After 24hrs incubation at 37°C and 5% CO_2 the percentages of the early apoptotic cell death in the negative wild type cells were 17%, in the negative mock transfected cells were 23%, and in the negative knockdown $Sp110^{-/-}$ protein cells were 15,5% Figure 4A. Apoptosis cell death in the negative control of all three types undifferentiated THP-1 cells after 24hrs were not statistically significantly different between Figure 4A. While the cells stimulated with PAM (PamCys) 1ng/ml and then incubated for 24hrs, the early apoptosis cell death percentages were not significantly different in any type of undifferentiated THP-1 cells Figure 4A.

When the undifferentiated THP-1 cells were stimulated by H37Rv strain MOI 10 and MOI 5 and incubated for 24hrs, then the percentages of the early apoptotic cell death were detected in each cell. In addition, the apoptotic percentages in the wild type infected MOI 5 were 21% and in the mock transfected cells were 26%, but the results were not significantly different in compare to the wild type and mock transfected cell controls respectively (P>0,05) Figure 4A. However, the early apoptotic cell death in the $Sp110^{-/-}$ knockdown stimulated MOI 5 cells were 40% and the result was increased significantly compared to the negative control the $Sp110^{-/-}$ knockdown (**P<0,01) and to the wild type infected MOI 5 (*P<0,05) Figure 4A. Likewise, the results of the cell death in $Sp110^{-/-}$ knockdown stimulated MOI 10 were 44% which were significantly more than the wild type and mock transfected stimulated MOI 10 cells (21%) (*P<0,05) Figure 4A.

In general, the removed Sp110 in knockdown undifferentiated THP-1 cells increased early apoptotic cell death by cell stimulating with H37Rv strain MOI 5 and MOI 10 after 24hrs incubation Figure 4A.

The 7-AAD stain detected both late apoptotic and late necrotic cell death, which was stained cell's nucleus and bind to the DNA. The percentages of late apoptotic/necrotic cell death after 24hrs incubation in the negative wild type cell were 8,5%, in the negative mock transfected cells were 10% and in the negative $Sp110^{-/-}$ knockdown were 13,5% Figure 4B. The results were not significantly different between all of the types of undifferentiated THP-1 cells negative control Figure 4B. While, in cells stimulated with PAM (PamCys) 1ng/ml and following incubation for 24hrs, the late apoptosis/necrosis cell death percentages were not significantly different in any type of undifferentiated THP-1 cells Figure 4B.

The cell stimulation with H37Rv MOI 5 were not only increased in early apoptotic cell death, but also increased in the positive staining of late apoptotic/necrotic cell death compared to the negative controls. Following 24hrs incubation the wild type cells were 20%, the mock transfected cells were 14%, and the $Sp110^{-/-}$ knockdown cells were 30% of the cell population were positively stained the 7-AAD Figure 4B. Infection by MOI 10 increased the number of late apoptotic/necrotic cell death in wild type was by 24%, in mock transfected was by 20%, and in $Sp110^{-/-}$ knockdown was by 39% Figure 4B. $Sp110^{-/-}$ knockdown cells were more likely to become more late apoptotic/necrotic cell death compared to wild type and to mock transfected.

Generally, Figure 4 showed the Sp110 deficient in knockdown undifferentiated THP-1 cells induced more cell death during 24hrs incubation in 37°C and 5% CO_2 when the cells infected with H37Rv strain of *M. tuberculosis*.

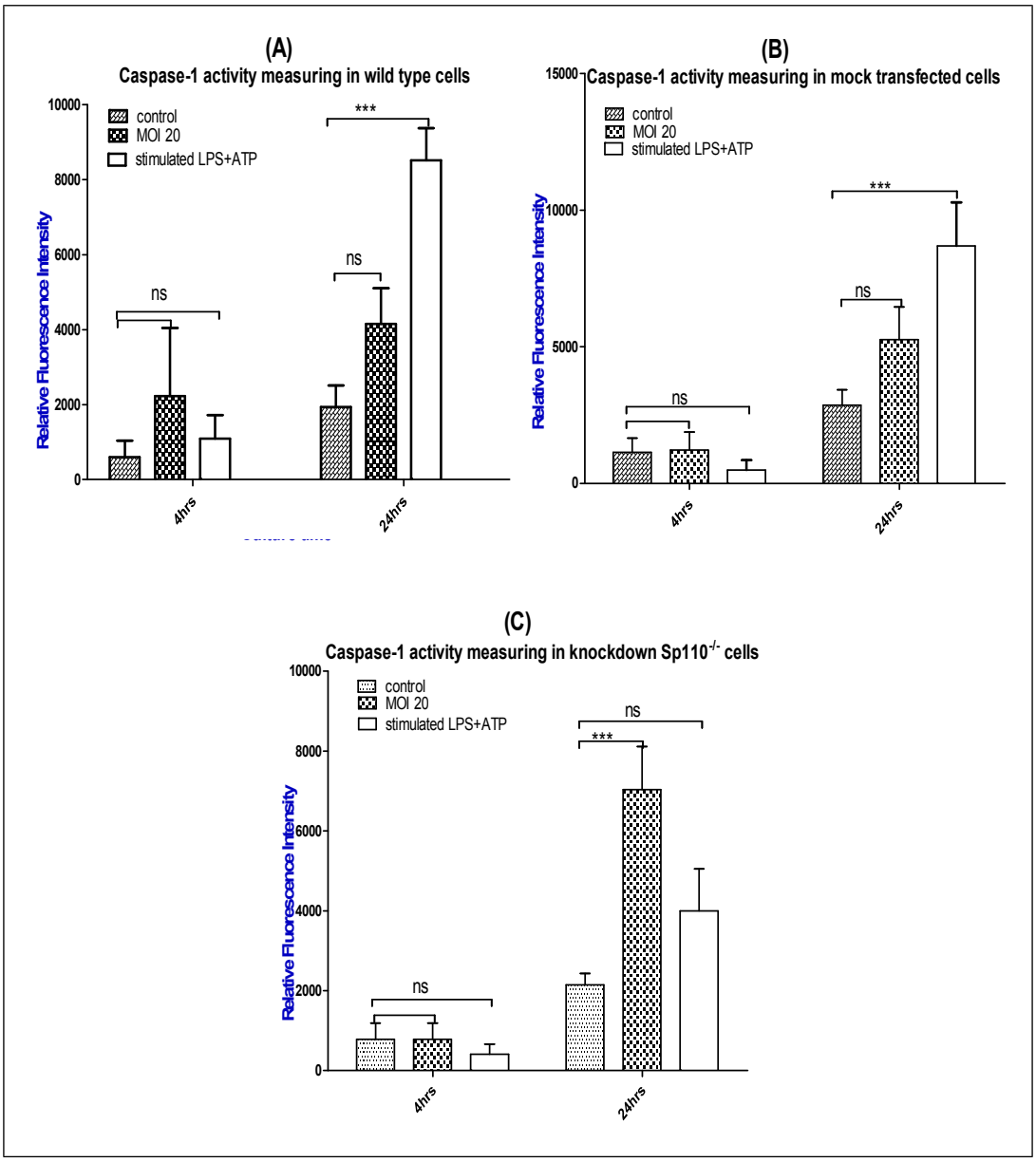

Figure. 3 Caspase-1 activities were measured in wild type, mock transfected and knockdown Sp110$^{-/-}$, which were incubated at 37°C and 5%CO_2 in the presence of 1µM Ac-YVDA-AMC for 24hrs. Fluorescence was measured every 2hrs to quantify caspase-1 activity and the data were analyzed by Two-way ANOVA followed by Bonferroni post-test means ± 1 SEM. At 4hrs all types of undifferentiated THP-1 cells had similar levels of caspase-1 activity to all stimuli. (A) In wild type cells stimulated MOI 20 at the 24hrs incubation induced more caspase-1 activity, but was not significantly more than the control (p>0,05, n=3). (B) The amount of caspase-1 activity in mock transfected cells stimulated MOI 20 at 24hrs incubation had the same results as a wild type cells, which were not significantly more than control (p>0,05, n=3). (C) The knockdown Sp110$^{-/-}$ stimulated MOI 20 produced significantly more caspase-1 activity after 24hrs incubation compared to control (***P<0,001, n=4). But the ATP+LPS did not induce more caspase-1 activity in the Sp110$^{-/-}$ knockdown cells.

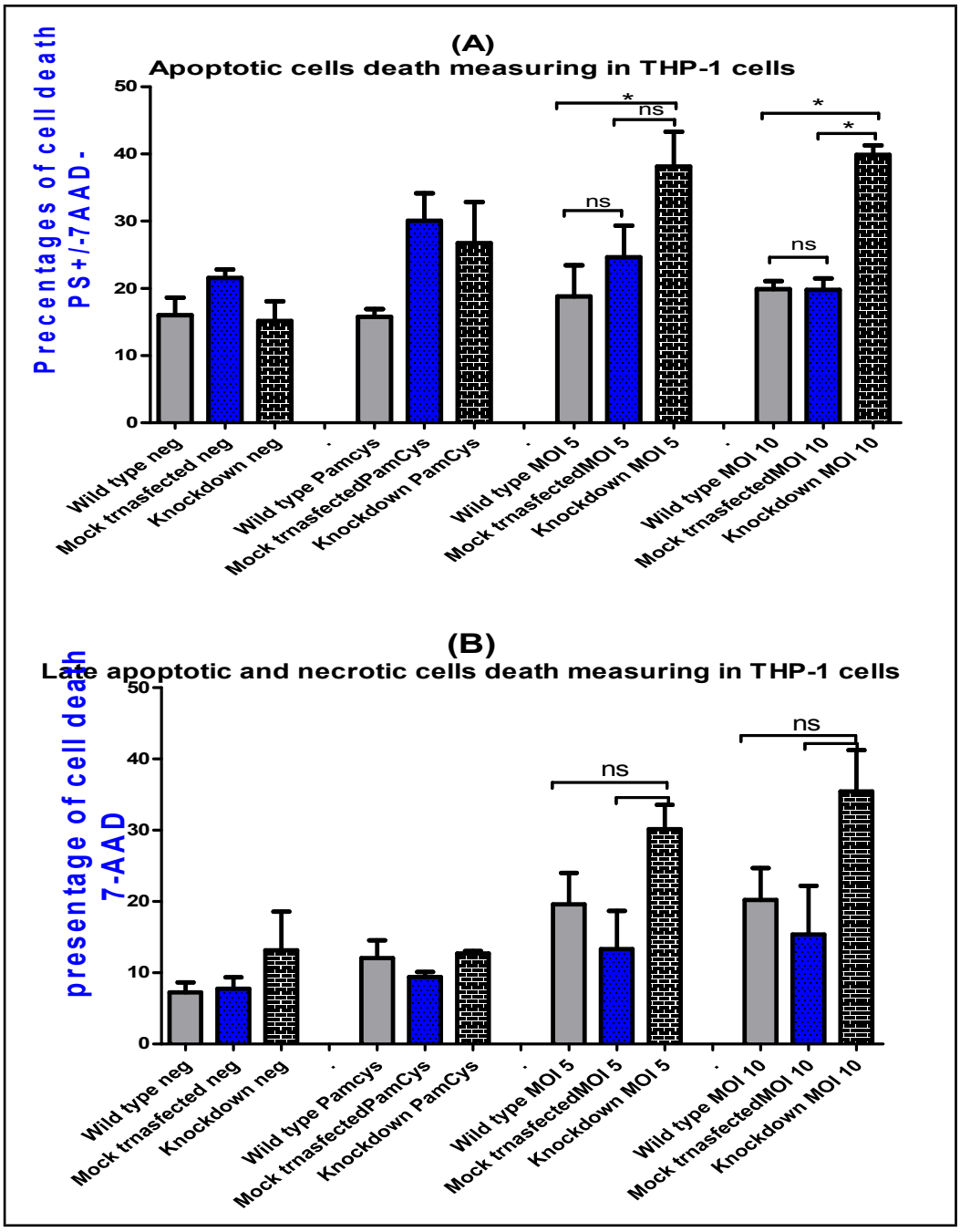

Figure 4. Undifferentiated THP-1 cells (wild type, mock transfected and knockdown Sp110$^{-/-}$) (1x10^5) were infected by H37Rv MOI (10 and 5), stimulated by PAMCys and negative control following an incubation for 24hrs. Cells analyzed by flow cytometry with PE-Annexin V and data analyzed by One-way ANOVA followed by Bonferroni post-test representing means ±1 SEM. (A) Percentages of early apoptotic cell death, which was PS positive and 7-AAD negative, were significantly increased in Sp110$^{-/-}$ knockdown infected MOI 10 and 5 (*P<0,05, n=3). (B) Percentages of late apoptotic/necrotic cell death, which was PE and 7-AAD positive, increased late apoptotic/necrotic cell death in Sp110$^{-/-}$ knockdown cells, but there was no significant difference between all types of undifferentiated THP-1 cells by the cells infected MOI 10 and 5 (P>0,05, n=3).

4: Discussion

This study used the H37Rv dead strain of *M. tuberculosis* bacteria to activate the NLRP3 inflammasome in undifferentiated human monocyte cell line THP-1, because the H37Rv dead strain had the ability to promote inflammasome activation and to recruit procaspase-1 (Latz, 2010; Schroder and Tschopp, 2010; Latz et al., 2013). The procaspase-1 has been shown an inactive form of cysteine protease in the cytoplasm of the cells and the caspase-1 become activated through ASC adaptor protein in inflammasome (Yamin et al., 1996; Thornbrry et al., 1992; Schroder and Tschopp, 2010; Latz et al., 2013). However, the inflammasome was shown to be a multi-protein complex and was a critical component in the host response against microbial infection in the innate immune system (Martinon et al., 2002; Walle and Lamkanfi, 2011; Greaney et al., 2015). This experimental model investigated the role of the Sp110 in the NLRP3 inflammasome activation in undifferentiated THP-1 cells because the resent studies linked Sp110 protein to NLRP3 inflammasome in THP-1 cells (unpublished data, Persson *et al*). The previous researchers identified the SP110 gene that can control the *M. tuberculosis* infection in human (Kramnik, 2008; Tosh et al., 2006; Lei et al., 2012). The Sp110 was encoded by the SP110 gene and the Sp110 is described to play an essential role in monocyte cells differentiation, apoptosis, and the activation against pathogens (Cliffe et al., 2012; Lei et al., 2012). The Sp110 polymorphisms was linked to *M. tuberculosis* susceptibility (Kramnik, 2008; Tosh et al., 2006; Lei et al., 2012), but the role and the function of the human Sp110 during inflammation was not described very well.

In order to know the role of Sp110 in inflammasome activation in undifferentiated THP-1 cells. The cells lacking Sp110 through shRNA expression against Sp110 (short hairpin and complimentary of Sp110 mRNA). This cell had been prepared and verified by RT-qPCR and Western blot showing that neither Sp110 mRNA nor protein is present in the cells. This novel study measured the caspase-1 activity in all types of undifferentiated THP-1 cells by Fluorescence, which detected the Ac-YVDA-AMC substrate. The Ac-YVDA-AMC substrates had the ability to bind to the active site of cleavage caspase-1. This study result identified that the Sp110 deficient knockdown cells induced significantly higher caspase-1 activity in response to laboratory H37Rv strains stimulated MOI 20 after 24hrs incubation in 37°C and 5% CO_2 compared to the control Figure 3C. Additionally, the amount of caspase-1 activity in $Sp110^{-/-}$ knockdown cells were higher than the wild type cells and mock transfected cells *Figure in Appendices II*. However, the results were not statistically significantly different between all types of undifferentiated THP-1 cells, that may be due to unspecified Ac-YVDA-AMC substrate *Figure in Appendices II. Bai et al* showed that inhibition of caspase-1 and cathepsin B increased the THP-1 cells' become more susceptible during *M. tuberculosis* H37Rv strain infection (Bai et al., 2015). Moreover, the other studies of experimental models of infection *in vivo* demonstrated that the susceptibility of infection by a variety of pathogens were increased by the lacking of caspase-1 enzyme in knockout mice (Netea et al., 2010). Definitely, the previous studies showed that the caspase-1 was activated by the activation of the multiprotein complex inflammasome (Schroder and Tschopp, 2010; Latz et al., 2013). This thesis study detected the caspase-1 activity, while the caspase-1 was used as a hallmark for detecting NLRP3 inflammasome activation. Moreover, the data of the present study demonstrated that the amount of caspase-1 activity was induced more in the knockdown $Sp110^{-/-}$ cells Figure 3C. The caspase-1 activity results

implied the increased levels of NLRP3 inflammasome activation by removing the Sp110 in the undifferentiated THP-1 cells and the cells infected by *M. tuberculosis* H37Rv strain.

During *M. tuberculosis* infection the different kinds of potent proinflammatory cytokines has been shown to be induced by caspase-1 activity during NLRP3 inflammasome activation (Cooper et al., 2012). The cytokines played an important role in determining the outcome of intracellular *M. tuberculosis* pathogen infection (Cooper et al., 2012). In addition, the previous observation studies in the patient and experimental models demonstrated that the drugs or genetic factors induced the deficiency in cytokine secretion and in induced signaling pathways (Cooper et al., 2012). In order to investigate the role of Sp110 in NLRP3 inflammasome activation in undifferentiated THP-1 cells and the effect of caspase-1 activity on the production of IL-1β, the cells were stimulated by H37Rv strain MOI (2 and 10) and then used ELISA to detect the levels of IL-1β cytokine secretion. Notably, the data of this novel study showed that IL-1β cytokine secretion induced significantly more in knockdown $Sp110^{-/-}$ cells compared to wild type and mock transfected cells Figure 2A. In addition, the IL-1β cytokine secretion in the mock transfected cell was significantly less than the wild type cells Figure 2A. This thesis result demonstrated, that the deficiency of Sp110 in undifferentiated THP-1 cells induced more IL-1β secretion, and revealed the IL-1β concentration in undifferentiated THP-1 cells were strictly correlated to the MOI of H37Rv strain Figure 2A. *In vivo* Walter group revealed that the NLRP3 inflammasome was essential for the host resistance against *M. tuberculosis* infection and proved also that the IL-1β production were strongly dependent on the NLRP3 inflammasome activation (Walter et al., 2010). According to *Walter et al* the IL-1β secretion was strongly depend on NLRP3 inflammasome, when the macrophage cells infected by H37Rv strain MOI=3 (Walter et al., 2010). Certainly, here in this context, the Sp110 affected the caspase-1 activity and the levels of IL-1β cytokine secretion in undifferentiated THP-1 cell. The results meant that the Sp110 had the ability to downregulate the activation of NLRP3 inflammasome. Indeed, the previous experimental model revealed that IL-1β cytokines were not detected in the bone marrow derived macrophages (BMDM) $ACS^{-/-}$ and $caspase\text{-}1^{-/-}$ cells, when the cells were infected by *M. tuberculosis,* whereby both ACS and caspase-1 were involved in NLRP3 inflammasome activation (Abdalla et al., 2012; Dorhoi et al., 2012). Moreover, the IL-1β cytokines were reduced in the BMDM generated from $NLRP3^{-/-}$ knockout mouse cells in comparison to the BMDM generated from the wild type cell by using *M. tuberculosis* for cell infection (Abdalla et al., 2012; Dorhoi, et al., 2012). The NLRP3 inflammasome, but not the NLRC4 inflammasome, had impact on IL-1β cytokine secretion (Abdalla et al., 2012; Dorhoi et al., 2012).

Friedland at al demonstrated that IL-8 was produced in THP-1 cells after phagocytosis of *M. tuberculosis* (Friedland et al., 1992). In addition, the IL-8 cytokines were important for granuloma formation by acting as a chemoattract molecules for attracting the T cells (Friedland et al., 1992). The data of this novel proved that the IL-8 cytokines were induced significantly more in knockdown $Sp110^{-/-}$ knockdown cells compared to wild type and mock transfected cells Figure 2B. While according to this study data, IL-1β cytokine had the likelihood to promote IL-8 in undifferentiated THP-1 cells k by H37Rv. *Kim* and colleagues reported that the proinflammatory IL-1β cytokines had the ability to induce IL-8 secretion in mast cells (Kim et al., 2010), while IL-1β cytokine were secreted through the activation of NLRP3 inflammasome (Abdalla et al., 2012; Dorhoi et al., 2012). Here in this context the result of IL-1β and IL-8 cytokines implied that the NLRP3 inflammasome activation can be downregulated by Sp110 in undifferentiated THP-1 cells. The IL-6 was a potent proinflammatroy cytokine that responded against *M. tuberculosis* infection, while these cytokines had no direct role in the inflammasome mediated mouse models, but IL-6 was used as a hallmark for inflammation

(McGeough et al., 2012; Cooper et al., 2012; Tang et al., 2012). The IL-6 cytokine activation depended on the proinflammatory IL-1β cytokines in murine mouse models (McGeough et al., 2012). In this study ELISA were used for analyzing IL-6 secretion in knockdown $Sp110^{-/-}$ stimulated by H37Rv strain MOI (10 and 2) pre cell, while the $Sp110^{-/-}$ cells were induced significantly more IL-6 compared to wild type and mock transfected cells Figure 2D. In addition, the IL-6 concentration in undifferentiated THP-1 cells was strictly correlated to the MOI of H37Rv strain Figure 2D. In contrast, the wild type cells did not induce significantly more IL-6 secretion than the mock transfected cells Figure 2D. These study results showed that the secretion of IL-6 cytokines in undifferentiated THP-1 cells had the same statistically results as the IL-1β, also. While the IL-6 and IL-1β promoted various processes, IL-6 was essential for fully elaboration of the IL-1β cytokines in the NLRP3 inflammasome dependent (McGeough et al., 2012; Tang et al., 2012). The present experimental models resembled that the IL-6 were strongly induced by the $Sp110^{-/-}$ in knockdown THP-1 cells, and the results of IL-1β, IL-8, and IL-6 implied the Sp110 had a role to downregulate the NLRP3 inflammasome activation in THP-1 cells.

Also, this study investigated additional proinflammatory cytokines and detected if Sp110 removed effect on the TNF-α cytokines secretion in undifferentiated THP-1 cells. However, the previous observation demonstrated that the absence proinflammatory TNF-α cytokines in the host cells triggered rapid susceptibility of infection and cell death by *M. tuberculosis* bacteria (Cooper et al., 2012; Cavalcanti et al., 2012). In contrast, the other studies data revealed that the TNF-α had no role in susceptibility rather than that the TNF increased bacterial burden in late infection (Cooper et al., 2011; Harris and Keane, 2010). The TNF-α cytokine secretion were strongly increased in the stimulated monocyte and macrophages $IL\text{-}1\beta^{-/-}$ cells after 5hrs incubation (Rojas et al., 2000). While here in this experimental model the TNF-α cytokines were strongly induced by *M. tuberculosis* stimulation MOI (10 and 2) per cell, the results of TNF-α were not significantly different between all types of undifferentiated THP-1 cell Figure 2C. All the previous results of proinflammatory cytokines (IL-1β, IL-6, and IL-8) of this study, which were correlated with NLRP3 inflammasome (Walter et al., 2010; McGeough et al., 2012; Tang et al., 2012), were changed by the Sp110 removing in the undifferentiated THP-1 cells Figure 2. This novel study suggested that the Sp110 had no effect on TNF-α secretion in undifferentiated THP-1 cells Figure 2C. The TNF-α had not increased by $Sp110^{-/-}$ protein in undifferentiated THP-1 cells due to two findings. The first finding was that the TNF-α cytokines secretion was not activated through NLRP3 inflammasome activation in THP-1 cells by *M.tuberculosis* stimulation and the another finding was that Sp110 had no role in the TNF-α cytokines secretion in undifferentiated THP-1 cells.

The previous papers showed that inflammasome activation did not only produce caspase-1 activation and proinflammatory cytokine secretion, but caused also cell death (Schroder and Tschopp, 2010; Lamkanfi et al., 2011). Since, the previous study showed that the pathogens infection induced cell death, the apoptotic cell death was induced directly by some gram-negative bacteria through caspase-1 activation (Hilbi et al., 1998; Hersh et al., 1999). In addition, both inflammatory and cell death signaling were induced by the bacterial cell-well component (lipoprotein) binding to TLR-2 (Aliprantis et al., 1999; Aliprantis et al., 2000). The dendritic cell stimulation by *M. tuberculosis* induced more apoptotic cell death instead of pyroptosis (Abdalla et al., 2012). In this context, the result showed that the early apoptotic cell death, which was PS positive stain, was induced significantly more by removing the Sp110 in undifferentiated THP-1 cells upon stimulation MOI (5 and 10) followed incubation for 24hrs Figure 4A. The previous evidence had reported that the *M.tuberculosis* bacteria in monocyte and macrophage cells in murine models bacterial lipoprotein

induced apoptosis, and the bacterial lipoprotein was interacted with TLR2 (Ciaramella et al., 2002; Rojas et al., 2000). Furthermore, the apoptotic cell death in monocyte and macrophages depended on caspase-1, but did not depend on caspase-3 and caspase-4 (Ciaramella et al., 2002; Rojas et al., 2000). Actually, this novel study results revealed that Sp110 had effect on the early apoptotic cell death, which implied that Sp110 had role in the active NLRP3 inflammasome to reduce apoptosis cell death in undifferentiated THP-1 cells during infection. However, the results showed increased late apoptotic/necrotic cell death in $Sp110^{-/-}$ knockdown cells, but that was not significantly more than wild type and mock transfected Figure 4B. Additionally, the results were more likely to induce significantly late apoptotic/necrotic cell death, possibly due to the few experiments Figure 4B.

All in all, all of this data indicated that the Sp110 may modulate the activity of NLRP3 inflammasome in undifferentiated THP-1. The Sp110 reduced cell death during inflammation in undifferentiated THP-1 cells. In the novel data shows that the Sp110 deficient in undifferentiated THP-1 cells, made the cells become more susceptible to *M. tuberculosis* infection and the cells infection increased percentages of early apoptotic cell death while the percentages of late apoptotic/necrotic cell death did not increase significantly by lacking the Sp110. Additionally, the Sp110 deficiency in undifferentiated THP-1 cells triggered a cascade of potent proinflammatory cytokines and were upregulated when the cells were stimulated. Furthermore, the caspase-1 activity in $Sp110^{-/-}$ undifferentiated THP-1 cells was induced significantly more than the control by H37Rv strain. While $Sp110^{-/-}$ could not upregulate the caspase-1 activity in undifferentiated THP-1 cells, the results of $Sp110^{-/-}$ knockdown cells were not significantly more than wild type and mock transfected cells. The Sp110 had the ability to reduce NLRP3 inflammasome activation and by detected the caspase-1 activity, proinflammatory cytokines, and cell death which were used as a hallmark. These findings indicate that SP110 played a role in the signaling that was leading up to caspase-1 activation. Since cells depleted of SP110 showed greater activation, one finding is that SP110 may act in an inhibitory fashion by keeping the caspase-1 activity down. This interaction may be directed by acting upon the caspase itself, but it may also be that the presence of Sp110 inhibited the NLRP3 inflammasome from forming and thus could caspase-1 not be activated.

In conclusion:

The H37Rv strain cause an inflammatory response in undifferentiated THP-1, promote NLRP3 inflammasome activation, caspase-1 activation, cytokine secretion, and cell death. Absence of Sp110 protein in undifferentiated THP-1 cells makes the cells more susceptible to apoptosis and possibly late apoptosis/necrosis when the cells stimulated by H37Rv strain. Upon stimulation of the cells by *M. tuberculosis* H37Rv strain, cells without Sp110 protein expression produce and release significantly more Il-1β, Il-6, and IL-8 than control cells. Cell deficiency in Sp110 expression showed greater caspase-1 activity upon cells stimulation. In addition, SP110 may act as an inhibitory fashion by keeping the caspase-1 activity down. Since activated caspase-1 was responsible for Il-1β production, these findings explained the detected increase in Il-1β production as well as in Il-6 and IL-8 whereby Il-1β triggered the production of IL-6 and IL-8 cytokines.

The SP110 play an essential role in the regulation of NLRP3 inflammasome activation in undifferentiated THP-1 cells and Sp110 also can inhibit inflammation because inflammasome is the mechanisms of inflammation. The Sp110 protein can be used as biological drug for inhibition inflammation in autoimmune disease patient and this research is continued at Orebro university.

5: Ethical statement

THP-1 cells are a human monocytic cell line which has been isolated from the blood of one-year-old boy with acute monocytic leukemia. The THP-1 cells had C3b and Fc receptors, but lack surface and cytoplasmic immunologlublins (Bowdish, 2011). The boy is no longer alive, but his cells are and are still widely used to investigate various inflammatory mediators and the cells can be differentiated into monocyte or macrophage providing researchers with an invaluable tool for studying innate immune responses.

H37Rv of *M.tuberculosis* stains were the most used strain in the laboratory, but this strain was originally isolated from a patient with *M.tuberculosis* infection in 1905. The H37Rv strain was multidrug resistance, high virulence and had the ability to produce granuloma. Although has high capacity to induce cytokines, and is valuable for inflammasome detection (Krishnan et al, 2013).

6: Future Perspectives

Inflammasome activation is like a double edge sword which have the ability to control the pathogen infection and the cell death. However, dysregulation and dysfunction of the inflammasome may lead to human inflammatory disease. Hence, the different types of proinflammatory cytokines tightly regulated by the complex protein inflammasome, the inflammation must be strongly regulated for guarantee avoiding lateral damage of the host. I like to direct studies for further investigation about the inflammasome activation mechanism and about how the inflammasome regulate and inhibited by different factors, and also hopefully to discover a targets drug that can be used for both anti-inflammatory and antimicrobial pathogenicity. The challenge for the future will be to investigate more about the different kinds of the genes to that correlated to the inflammasome activation and control the inflammation.

7: Acknowledgments

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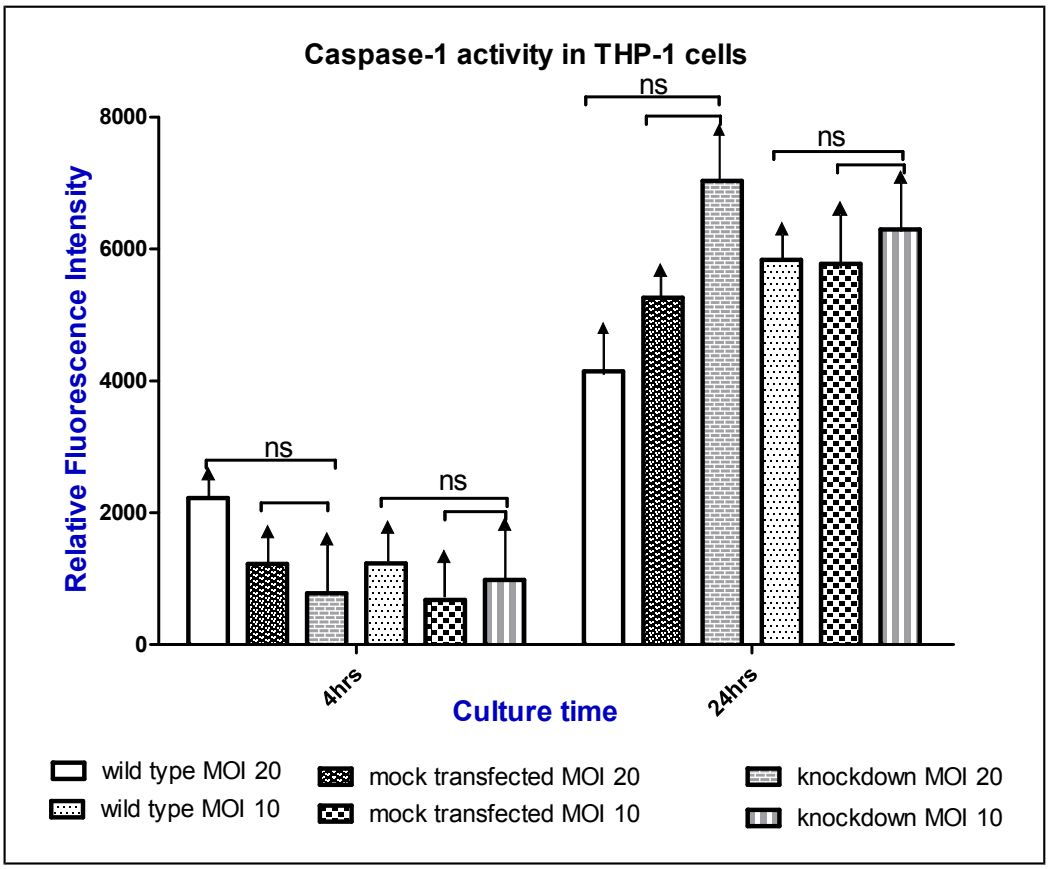
References:

1. Abdalla H., Srinivasan L., Shah S., Mayer-Barber K., Sher A., Sutterwala F. and Briken V. (2012) Mycobacterium tuberculosis infection of dendritic cells leads to partially caspase-1/11-independent IL-1β and IL-18 secretion but not to pyroptosis. *PloS One, 7,* e40722.

Appendices

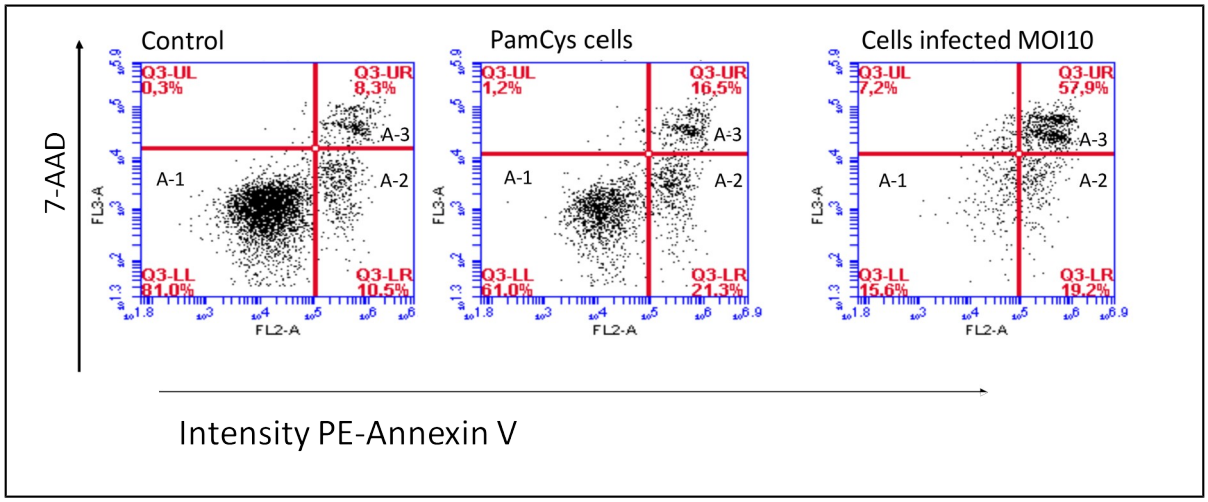
Appendices I



Caspase-1 activity measured in undifferentiated THP-1 cells (wild type, mock transfected and knockdown Sp110$^{-/-}$). 1.5x10^5 cells/ml with 1µM of Ac-YVDA-AMC substrate incubated for 1hr at 37°C under 5% CO2. Later, cells infected by H37Rv MOI (20 and 10) and caspase-1 activity was measured in 4 and 24hrs by

FLUOstar OPTIMA. Data were analyzed by Two-way ANOVA followed by Bonferroni post-test, which represented means ± 1 SEM. The caspase-1 activity after 24hrs incubation were increased in knockdown $Sp110^{-/-}$ cells, but not significantly more than wild type and mock transfected cells ($P<0,05$, n=3).

Appendices II



The undifferentiated THP-1 ($1x10^5$cells/ml) cells stimulated by H37Rv MOI 10, and PAMCys, then followed incubated for 24hrs at 37°C of 5% CO2. Afterward, cell death was stained PE-Annexin V n=3 and was analyzed by flow cytometry. Percentages of early apoptotic cell death, which was PE positive (X-axis), and percentages of late apoptotic/necrotic cell death was PE and 7-AAD positive (Y-axis). Living cell percentages in control were 80% (A-1), and cell stimulated PAMCys which induced early apoptotic cell death were 21,3% (A-2). Late apoptotic/necrotic percentages in cells stimulated MOI 10 increased to 57,9% (A-3).