FACULTY OF MEDICINE AND HEALTH SCIENCES

# Activation of the NLRP1 inflammasome in epithelial cells

Jade Celis
01507497

Promotor: Prof. Dr. Mohamed Lamkanfi
Copromotor: Dr. Lieselotte Vande Walle
Mentor: Dr. Oonagh Paerewijck

*Laboratory of Medical immunology*

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# 1. PREFACE

Ever since I was in high school, I have looked forward to the moment I would start my career as a biomedical scientist. Throughout the years as a student, this feeling has not diminished, and my enthusiasm for research has grown even more. In June 2022, my thesis comes to an end, and with that, my time as a student finally too. Over the years, I was lucky to be surrounded by many intelligent, kind, and supporting people who helped me reach my goals and made me into the scientist I am today. Now, I can finally, officially, thank them.

First of all, I would like to thank my promotor Prof. Dr. Mohamed Lamkanfi. Throughout the last two years, Prof. Lamkanfi has not only offered me guidance, knowledge, and answers when I had concerns but concomitantly pushed me to be a critical, creative and competent scientist. He gave me the opportunity to work in the fascinating field of inflammasomes which showed me how exciting research could be.

Secondly, I want to thank my co-promotor Dr. Lieselotte Vande Walle, and especially my mentor Dr. Oonagh Paerewijck, who helped and supervised me in the lab during the past two years and have taught me many important lessons. They were always there when I needed them, and even when not everything went to plan, they provided insightful feedback and encouraged me to believe in myself.

Last but not least, I want to thank my friends and family who were always there for me in the past few years:

I especially want to thank Tine Claeys and Margaux Theys, both fantastic researchers in the making, for all these years of friendship. They helped me countless times in my education and on a personal level and encouraged me to be critical and confident.

Next, but equally important, I want to express my gratitude towards my family. First, I want to thank my parents for not only funding my education but for all the love, support, and wisdom they offered me throughout my life and for believing in me no matter what. I want to thank my brother Timen Celis and sister-in-law Sophie Ba for the exciting conversations and critical questions about my thesis. And eventually, I want to thank my significant other, Samuel Huyghe, for all his love and patience when he had to endure my non-stop talking about this thesis.

Lastly, I want to thank Anke Desmet, Stien Lannoy, Aurelie Nollet, Louise Pype, and Astrid Roelens for their unique friendship and support that already started more than ten years ago.

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## 3. SCIENTIFIC SUMMARY

Inflammasomes are indispensable for the innate immune system, protecting the host from various threats. However, when imbalanced, inflammasomes can influence various inflammatory diseases as well. Therefore, it is vital to know what triggers inflammasome activation to determine effective strategies for future therapies.

Even though inflammasomes have increasingly gained interest in recent years, many underlying mechanisms linked to human inflammasomes are still ill-defined. Inflammasomes have been shown to play an equally important role in many cell types, however research is predominantly focused in myeloid cells. This thesis aimed to investigate inflammasome activation in two keratinocyte cell culture models by a suite of stimuli that are known to induce activation of different inflammasomes in myeloid cells and compare our data to other cell types in the literature.

We concluded that Val-boroPro, anisomycin and intracellular Poly (I:C) activate the inflammasome in the N/TERT-2G cell line. This accentuates the essential role of keratinocytes in skin immunity. However, any other stimulus assessed in this project failed to influence inflammasome activation. Furthermore, we did not observe any inflammasome activation in the widely-used HaCaT cell line, revealing its inferiority in inflammasome research.

The results from our experiments advocate that the extrapolation of data from one cell type to the other needs to be contemplated carefully. Moreover, we emphasize that even between cell lines and primary cells many variations are present. Overall, this project has granted crucial insights into the mechanisms of inflammasome activation in keratinocytes, possibly aiding in the discovery of future therapies against epithelial diseases.

## 4. SECTION ON SOCIETAL IMPACT

Inflammasomes play an essential role in human immunity. They are part of the innate immune system, making them the first defense against pathogen- and danger-associated signals and have been proven vital in a balanced immune system to prevent disease. However, excessive activation of the inflammasome can be the cause of many illnesses including cancer, Rheumatoid arthritis, diabetes, atherosclerosis, and crystal arthropathies. An example of the duality of inflammasomes is the NOD-like receptor protein 1 (NLRP1) inflammasome; NLRP1 has been shown to aid tumor destruction in Acute myeloid leukemia cells but simultaneously can be the cause of skin cancer as well due to its role in sunburn after UV irradiation.[1,2] Furthermore, this inflammasome is the cause of many more epithelial diseases such as Psoriasis and Vitiligo.

Since these diseases are ubiquitous in the population and are still not readily cured, insight into these mechanisms is vital to discover therapies to improve the quality of life of patients, lengthen the overall life expectancy of the population and lower the economic costs that accompany these conditions. In addition, since we make use of immortalized cell lines, we also investigate the effectiveness of their use in inflammasome research as well.

# 5. INTRODUCTION

In recent years, the importance of a well-functioning immune system has been proven crucial for a long, healthy life. Our immune system not only protects us from pathogens such as SARS-Cov-2, the cause of the recent pandemic, but plays a role in many inflammatory diseases as well. Multiple metabolic conditions such as atherosclerosis and diabetes, partially caused by an imbalanced immune system, are rising significantly in numbers worldwide. Furthermore, the frequency of allergies and auto-immune diseases increases as well, even in a younger population. This illustrates that a balanced immune system is vital to prevent severe illness or death.

An essential part of our immune system is the innate immune response, which forms a broad and fast defense against threats prior to the deployment of the adaptive immune system. The innate immune system recognizes specific patterns present in pathogens, toxins, or damaged host cells, utilizing pattern recognition receptors (PRR) such as Toll-like receptors (TLR) or nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins. Most PRRs induce changes in gene expression leading to, for instance, increased levels of various inflammatory cytokines and type I interferons. Other PRRs, such as the NLRs, assemble into inflammasomes initiating an immune response through the release of cytokines and the induction of cell death.[3] This project will enlighten fundamental aspects of inflammasome activation in keratinocyte culture models.

Before presenting my results, I will discuss the general mode of action of the investigated inflammasomes in the introduction of the thesis. This will provide the necessary insights into this subject to comprehend the reasoning behind the aim of our project.

## 5.1. Inflammasomes

The innate immune system consists of PRRs that can sense viruses, toxins, bacteria, and danger-associated molecular patterns (DAMPs). As aforementioned, a subset of PRRs can oligomerize and assemble into inflammasomes. This event elicits binding of the PRR to Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) which in its turn binds pro-Caspase-1. [Figure 1] Binding of pro-Caspase-1 to the inflammasome prompts proximity-induced Caspase-1 cleavage leading to its activation. The functions of active Caspase-1 are twofold. Firstly, it leads to the caspase-1-mediated removal of the amino (N)-terminal part of pro-inflammatory cytokines pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL18). Secondly, it cleaves Gasdermin D (GSDMD), which initiates pore formation by the N-terminal part of GSDMD. Due to the pore formation, water can enter the cell leading to lytic cell death called pyroptosis, and matured pro-inflammatory interleukins are released in the extracellular space.[4] These cytokines can bind their receptor on various effector cells, including T cells and neutrophils, eliciting a broader immune response. This immune response can either protect the host from disease or can be the cause of auto-inflammatory conditions.

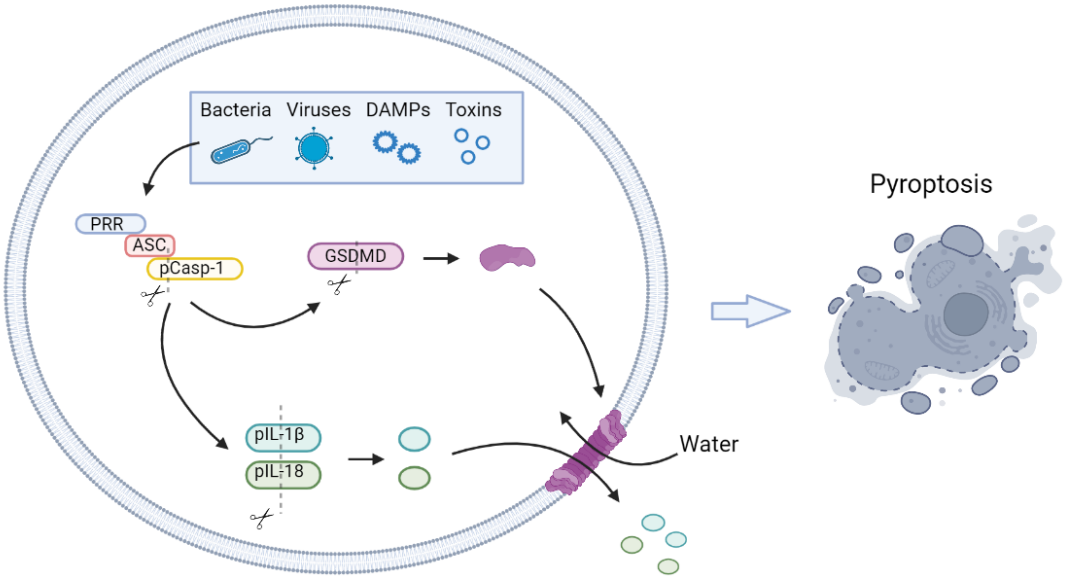


**Figure 1 - General overview of inflammasome activation.**
Inflammasome activation eventually leads to interleukin release and pyroptosis.

5.1.1. The NLRP1 inflammasome

NLRP1, a member of the NLR family, was the first protein to be discovered that assembled into an inflammasome.[5] It was Boyden and Dietrich that spurred the NLRP1b field by showing its role in the response to *Bacillus anthracis* lethal toxin.[6] Ever since, research has revealed the vital influence of this inflammasome on various diseases. The human NLRP1 inflammasome is mainly present in epithelial cells and plays a significant role in skin diseases such as vitiligo, psoriasis, and skin cancer. Moreover, overexpression or overactivation of the NLRP1 inflammasome has been linked to acute lung injury, papillomatosis, and inflammatory bowel disease as well. [7,8]

In general, the NLR family exists out of a Pyrin domain (PYD), a nucleotide-binding and oligomerization (NACHT) domain, and eventually a domain containing leucine-rich repeats (LRR). The PYD domain is thought to be an auto-inhibitory domain that prevents automatic activation of the inflammasome, while the NACHT domain and LRR domain are both sensing domains. In most inflammasomes, the PYD domain can recruit ASC, leading to its activation. In NLRP1, however, the protein contains two additional domains necessary for activation at the carboxy-terminus: The FIIND (function-to-find) domain and the Caspase recruitment domain (CARD) domain. The FIIND domain is autoproteolytically cleaved into a ZU5 and UPA domain; nevertheless, the amino-terminal part degrades only when a particular stimulus arises. Then the carboxy-terminal part binds ASC and pro-Caspase-1 via its CARD domain. [Figure 2] Furthermore, this protein differs between human and mouse species. While humans have only one NLRP1 gene, mice have three. Nlrp1a, nlrp1b, and the pseudogene nlrp1c. The structure of the mouse NLRP1 protein differs from the human NLRP1 since it is missing the amino-terminal PYD domain.[9]

Since the structure of the NLRP1 protein differs significantly from most other inflammasomes in the NLR family, its activation and assembly vary as well. Assembly of the NLRP1 inflammasome is a multiple-step process. Aforementioned, the FIIND domain of NLRP1 undergoes post-transcriptional modification and is autoproteolytically cleaved into subdomains ZU5 and UPA. The N-terminal and C-terminal parts of NLRP1 stay non-covalently bound to each other as long as the inflammasome is not activated. Only when a stimulus is present, will ubiquitination and degradation of the N-terminal part of NLRP1 be triggered, leading to the freeing of the C-terminal UPA-CARD region. Eventually, UPA-CARD subsequently binds ASC and pro-Caspase-1. [Figure 2]

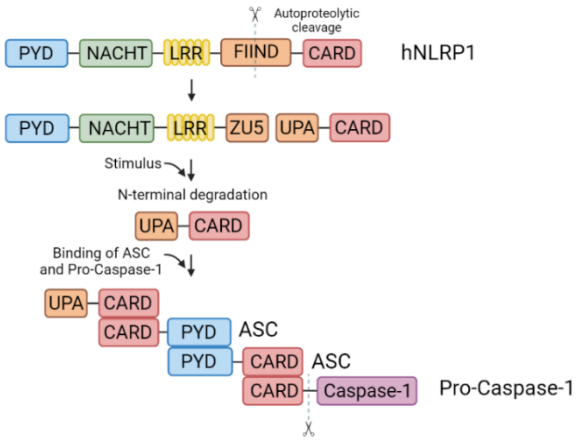


**Figure 2 - Structure and activation of NLRP1.** NLRP1 binds ASC via its CARD domain to cleave Pro-Caspase-1.

As mentioned above, *Bacillus anthracis* is one of the first known activators of NLRP1b in mice and provided essential insights into inflammasomes. However, many more stimuli have been suggested for the NLRP1 inflammasome. I want to discuss the stimuli used in this project more in detail.

**Val-boroPro**

Val-boroPro was first discovered as an anti-tumor drug due to its inhibitory effect on specific protein targets that are often overexpressed in cancer patients and that are known as Fibroblast Activating Proteins (FAP).[10] More recently, Val-boroPro has been shown as an inflammasome activator in mouse and human cells due to its inhibition of DPP8 and DPP9.[11] Last year, Bauernfried et al. clarified how Val-boroPro is able to activate the NLRP1 inflammasome. Evidently, a full-length NLRP1 and a UPA–CARD fragment cooperatively bind to DPP9 to yield a ternary complex that prevents inflammasome formation.[12]

**UVB irradiation/Anisomycin**

In 2018, NLRP1 was shown to play a vital role in UVB irradiation sensing in Normal Human Epidermal Keratinocytes (NHEK) cells.[13] Later on, Robinson et al. showed that NLRP1 inflammasome activation upon UVB irradiation functions through the ZAKα pathway, the same pathway mediated by anisomycin.[2] Activation of the ZAKα pathway initiates phosphorylation of the inter-domain fragment between PYD and NACHT of NLRP1 and is thought to elicit inflammasome activation. This mechanism, however, has not been fully elucidated yet.

Anisomycin, derived from the bacteria *Streptomyces griseolus,* is already used in the clinic as an antibiotic and anti-cancer drug since it functions as a protein synthesis inhibitor in eukaryotic cells.[14] However, the substance has been demonstrated as cytotoxic in high concentrations.[15]

**Poly (I:C)**

Another suggested activator of NLRP1 is cytosolic double-stranded RNA (dsRNA), produced during viral replication of single-stranded RNA (ssRNA) viruses.[16] DsRNA is said to bind the N-terminal part of NLRP1, initiating ubiquitination and degradation of the polypeptide, hence leading to activation of the NLRP1 inflammasome. Polyinosinic:polycytidylic acid (Poly (I:C)), a synthetic analog of dsRNA, has been observed to activate both the NLRP1 and the NLRP3 inflammasomes. In 2017, Poly (I:C) transfection was described to activate the NLRP3 inflammasome in unprimed primary keratinocytes and was upregulated by type I IFN priming. Here, IL-1β levels in supernatants increased upon Poly (I:C) transfection, however, when siRNA for NLRP3 was transfected beforehand, this increase was inhibited.[17] In 2020 however, Bauernfried et al. suggest that dsRNA and Poly (I:C) in the cytosol activate the NLRP1 inflammasome in unprimed N/TERT-1 cells.[16] The inflammasome-dependent cell death could be prevented by a knockout of the NLRP1 gene in these cells.

Untransfected Poly (I:C) can be used as a priming signal since it binds TLR-3 to promote NF-κB activation in the cells.[18] Furthermore, when Poly (I:C) is added without transfection, it is described to activate the NLRP3 inflammasome through Mitochondrial antiviral-signaling protein (MAVS)-induced potassium efflux in murine bone marrow-derived macrophages (BMDM).[19]

### 5.1.2. The NLRP3 inflammasome

The NLRP3 inflammasome, mainly present in myeloid cells, is one of the best-studied inflammasomes. It has been linked to well-known diseases, including Alzheimer's disease, rheumatoid arthritis, gout, atherosclerosis, and even SARS-Cov-2.[20] Furthermore, several gain-of-function mutations have been identified, that cause Cryopyrin-Associated Periodic Syndromes (CAPS) leading to inflammatory symptoms like fever, arthralgia, swelling, and rash.[21]

The structure of NLRP3 is less complicated than NLRP1. It is a tripartite protein with only a PYD-, NACHT- and an LRR domain. Here the PYD domain is essential for inflammasome activation since NLRP3 has no CARD domain and can only interact with ASC through PYD. In contrast to NLRP1, only one ASC is necessary for Caspase-1 cleavage. [22] [Figure 3]

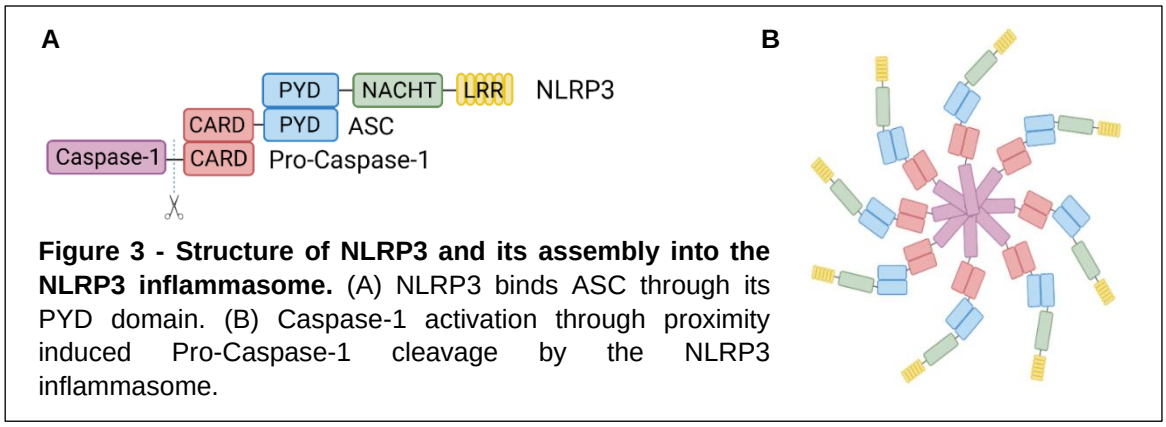


**Figure 3 - Structure of NLRP3 and its assembly into the NLRP3 inflammasome.** (A) NLRP3 binds ASC through its PYD domain. (B) Caspase-1 activation through proximity induced Pro-Caspase-1 cleavage by the NLRP3 inflammasome.

In contrast to other inflammasomes, NLRP3 activation typically requires two signals: Priming and activation. For the inflammasome to assemble, the presence of all components necessary for activation is crucial. Priming the cells elicits a change in gene expression, inducing higher expression levels of various components including pro-IL-1β and NLRP3. In some cases, priming can lead to more inflammasome assembly. In others, like the NLRP3 inflammasome, priming is vital for inflammasome activation since the base expression levels of NLRP3 itself in unprimed cells are inadequate. In this instance, posttranslational modifications by adding, for example, lipopolysaccharides (LPS), present in gram-negative bacteria, elicits NF-κB activation and an increase of NLRP3 and pro-IL-1β levels. [Figure 4]

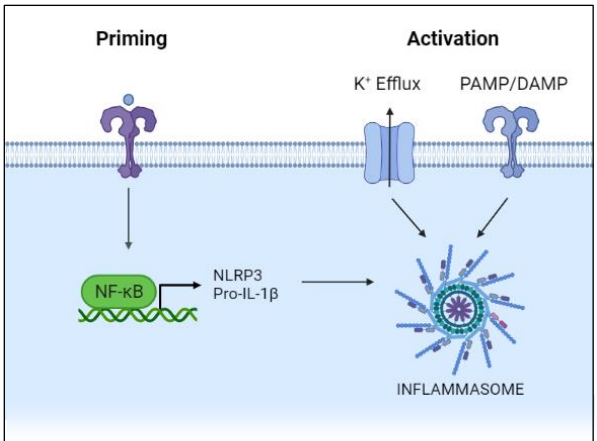


**Figure 4 - Priming and activation of the NLRP3 inflammasome.** Priming induces NLRP3 and Pro-IL-1β expression necessary for inflammasome activation.

After priming the cells, the inflammasome can be activated by different processes. Contrary to most inflammasomes, the NLRP3 inflammasome is believed to be activated not by direct ligand binding but by a general change in the state of the cell, such as potassium efflux or lysosomal damage.[23] However, the NLRP3 inflammasome has two pathways for inflammasome activation. Firstly, a canonical pathway employed by most inflammasome stimuli, causing pyroptosis through Caspase-1. Secondly, the NLRP3 inflammasome can also be activated by a non-canonical pathway triggered by cytosolic LPS independent of TLR4 signaling. Caspase-4/5 in humans and Caspase-11 in mice are activated by LPS and initiate GSDMD cleavage and K+ efflux. This efflux is then sensed, and the NLRP3 inflammasome is assembled.[22]

Eventually, NLRP3 can be an indirect sensor of cytosolic dsDNA using the cGAS-STING-NLRP3 pathway.[24] The cGAS-STING pathway is thought to be a good immunotherapy candidate since it aids in the anti-viral immune response by eliciting interferon and CXCL10 secretion, promoting the maturation and migration of various immune cells.[25] cGAS binds cytosolic dsDNA, producing the second messenger cyclic GMP–AMP (cGAMP). [Figure 5] Thereafter, the STING receptor, present on the endoplasmic reticulum, is activated by binding cGAMP. Activation of STING induces specific lysosomal cell death (LCD), which elicits a $K^+$ efflux activating the NLRP3 inflammasome.[24]

Multiple primers and stimuli have been discovered for the NLRP3 inflammasome. As previously mentioned, LPS is a widely used priming condition for NLRP3 activation. However, seeing that overall NF-κB activation is sufficient for priming, many other priming substances, such as Pam3CSK4, may be used. Here, the stimuli for NLRP3 used in this thesis are discussed more in detail.

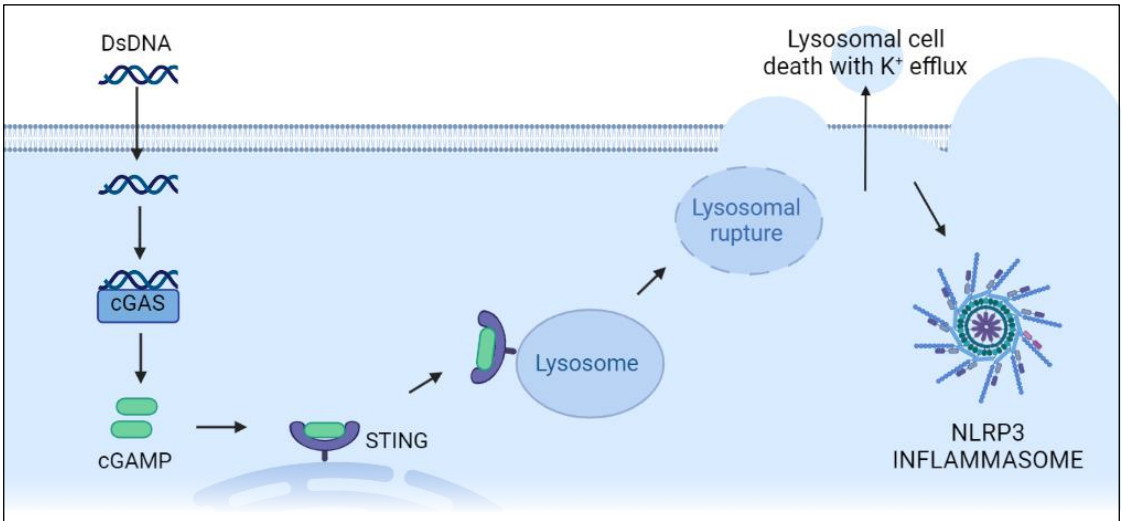


**Figure 5 - NLRP3 inflammasome activation via the cGAS-STING pathway.**
Cytosolic dsDNA binds cGAS eliciting cGAMP production. This induces lysosomal cell death activating the NLRP3 inflammasome due to $K^+$ efflux.

**ATP**

Extracellular ATP is considered a DAMP and binds the P2X7 receptor inducing $K^+$ efflux.[26] In 2006 already, it was discovered to activate the NLRP3 inflammasome in mouse macrophages and has become widely used in human and mouse studies.[27] However, its function in keratinocytes is not fully known yet. Gruber et al. suggested that stimulation with ATP for 20h induces active Caspase-1 in primary keratinocytes suggesting that inflammasome activation occurs.[28] These results are similar to what is described by Ahn et al. which measured increased IL-1β levels in supernatants of primary keratinocytes stimulated for 24h with 1mM ATP in Vitiligo patients.[29]

**Nigericin**

Another well-known NLRP3 inflammasome activator in macrophages, discovered together with ATP, is the potassium ionophore nigericin.[27] Nigericin is a microbial toxin derived from the Gram-positive bacteria *Streptomyces hygroscopicus* that can bind intracellular potassium and transport it through the cell membrane, eventually leading to a significant potassium efflux.[30] Moreover, nigericin has also been a promising anti-cancer drug target since it increases the cytotoxicity of anti-cancer drugs.[31] In keratinocytes, however, the results are controversial. In 2018, Fenini et al. suggested that nigericin activates the NLRP1 inflammasome in primary keratinocytes instead of the NLRP3 inflammasome.[13] Nevertheless, Gruber et al. did not observe inflammasome-dependent Caspase-1 activation upon stimulation with nigericin in primary keratinocytes and Bauernfried et al. 2020 did not see any IL-1β release in supernatants upon stimulation for 10h in N/TERT-1 cells.[16,28]

**HT-DNA**

A recent article suggests an essential role of the cGAS-STING pathway in NLRP3 inflammasome activation in myeloid cells.[24] Allegedly, activation of this pathway leads to a crucial $K^+$ efflux, activating the NLRP3 inflammasome. Thus, activators of this pathway, such as dsDNA, could play a role in activating the NLRP3 inflammasome. More recent data of a study in acute lung injury patients advocates that the NLRP3 inflammasome could play a role in dsDNA sensing via the

cytosolic DNA-STING-NLRP3 axis.[32] Nevertheless, In 2016, Strittmatter et al. observed the influence of both AIM2 and NLRP3 inflammasomes in Herpes simplex virus stimulated primary keratinocytes.[33]

### 5.1.3. The NLRC4 inflammasome

The NLRC4 inflammasome, the last member of the NLR family, can orchestrate a rapid immune response against *Salmonella Typhimurium* and *Pseudomonas aeruginosa*. Additionally, this inflammasome is also involved in autoinflammatory diseases and cancer. For instance, multiple gain-of-function mutations are linked with conditions underlying neonatal enterocolitis and periodic fever.[34]

NLRC4's structure is very similar to NLRP3, except that the PYD domain at the N-terminus is exchanged for a CARD domain. Surprisingly, while ASC is necessary for NLRP1 inflammasome activation, here, ASC is dispensable for pro-Caspase-1 binding. [Figure 6]

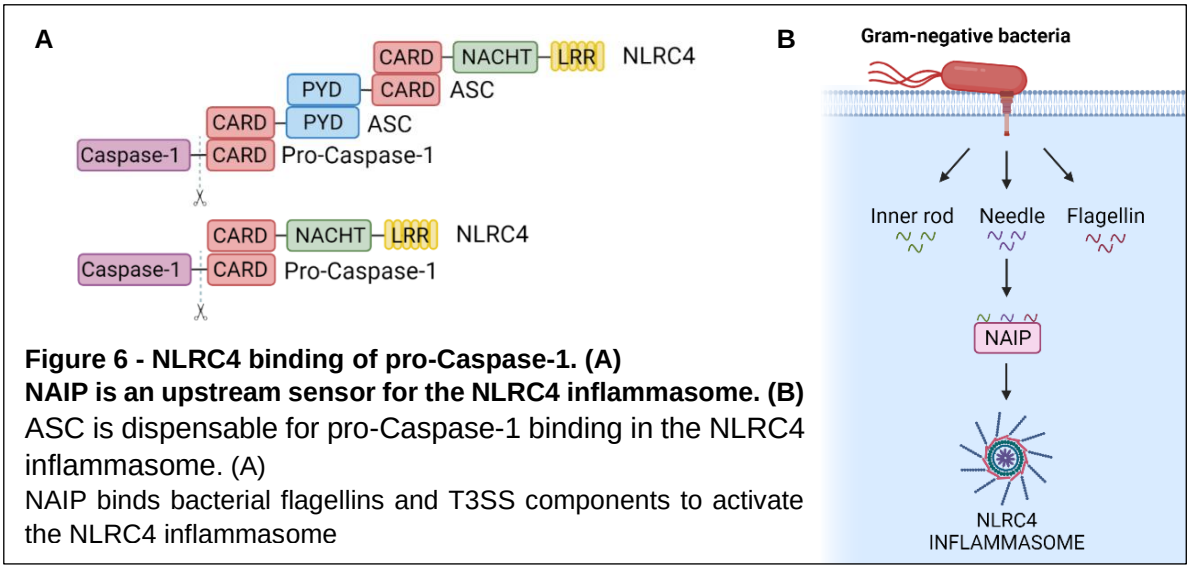


**Figure 6 - NLRC4 binding of pro-Caspase-1. (A) NAIP is an upstream sensor for the NLRC4 inflammasome. (B)** ASC is dispensable for pro-Caspase-1 binding in the NLRC4 inflammasome. (A)
NAIP binds bacterial flagellins and T3SS components to activate the NLRC4 inflammasome

NLRC4 inflammasome activation is distinct from other inflammasomes due to its binding to NLR family apoptosis inhibitory proteins (NAIP). NAIP acts as an upstream sensor for the NLRC4 inflammasome by interacting with bacterial flagellins and type III secretion system (T3SS) components. While multiple NAIPs have been described in mice, a single NAIP operates upstream of NLRC4 in humans and recognizes all activators. [34]

**LFn-Needle**
LFn-Needle is a specific NAIP/NLRC4 inflammasome agonist model. (Invivogen) Needle, a part of the type III secretion systems (T3SS) of intracellular bacteria, binds NAIP, activating the NLRC4 inflammasome. Needle is fused to a second component, the N-terminal domain of *B. anthracis* lethal factor (LFn), to deliver it intracellular.

### 5.1.4. The AIM2 inflammasome

The Absent In Melanoma 2 (AIM2) inflammasome is known for its interaction with cytosolic dsDNA. AIM2 not only protects the host from DNA viruses such as cytomegalovirus and vaccinia virus, but it can also recognize host DNA present in the cytosol due various threats, including cell damage or carcinogenesis.[3]

The AIM2 protein only contains a PYD and a HIN200 domain. the HIN domain primarily mediates DNA interaction and aids oligomerization of the protein into an inflammasome.[35] When HIN interacts with DNA, the PYD domain binds ASC and Pro-Caspase-1. Like the NLRP3 inflammasome, Priming before stimulation is typically necessary for AIM2 inflammasome activation in most cell types. The most abundantly used Priming for AIM2 is type I interferon, which leads to the transcription of interferon-stimulated genes. The primary activator of this inflammasome is cytosolic dsDNA. However, it is worth noting that dsDNA is also able to prime cells for AIM2 via the cGAS-STING pathway.

**HT-DNA**

To assess the susceptibility of the cell culture models to cytosolic dsDNA, we made use of HT-DNA transfection. The AIM2 inflammasome is known to activate through approximately any kind of cytosolic dsDNA and was first discovered in macrophages.[36] However, its function in many other cell types remains ill-defined to this day. AIM2 is determined the essential dsDNA sensor in keratinocytes.[37] Furthermore, Dombrowski et al. showed that AIM2 expression is upregulated in keratinocytes of psoriasis patients.[38]

### 5.1.5. The PYRIN inflammasome

The PYRIN inflammasome protects the host from bacteria, such as *Clostridium difficile,* by detecting bacterial toxin-induced Rho guanosine triphosphatase (Rho GTPase)-inactivation.[3] Rho GTPases normally regulate various signal transduction pathways, including immune responses. When pathogens modulate Rho GTPase activity, the host immune response is repressed, and phagocytosis is impossible, thus leading to the survival of the pathogen.[39] Mutations in the PYRIN inflammasome have been confirmed to play a role in Familial Mediterranean Fever (FMF).[40]

Despite that the Pyrin protein contains a PYD domain, by which it binds ASC and Pro-Caspase-1, similar to many other inflammasome, the structure varies greatly. It has three exclusive domains absent in other inflammasomes; a B-box domain and a coiled-coil (CC) domain, both needed for the oligomerization of Pyrin and at the C-terminus, a B30.2 domain. Although the function of B30.2 is still not fully understood, mutations of this domain are often involved in the cause of FMF.[39]

Known stimuli of this inflammasome are *Clostridium difficile* toxins A (TcdA) and B (TcdB). In this project, we will only make use of TcdA.


**Figure 7 - Structure of Pyrin.**
Pyrin has three exclusive domains. The B-box domain, CC domain and B30.2 domain.

**TcdA**

As previously mentioned, this toxin influences the Rho GTPase activity, which is sensed by Pyrin. TcdA has been proven to activate the Pyrin Inflammasome in mouse macrophages and human monocytes.[40] However, it can also influence NLRP3 inflammasome activation. [41]

## 5.2. Treatment of inflammasome-driven pathologies

Given the apparent influence of inflammasomes on various diseases, the need for treatments explicitly targeting inflammasome-involved pathways is high. As aforementioned, the major consequence of inflammasome activation is the release of pro-inflammatory cytokines, IL-1β and IL-18, leading to auto-inflammatory conditions. Fortunately, certain drugs that aid in these conditions are already in use.

In 2001, the first anti-IL-1 drug, Anakinra, was approved by the Food and Drug Administration (FDA) to treat rheumatoid arthritis.[42] It is a recombinant IL-1 receptor and binds both IL-1α and IL-1β inhibiting their pro-inflammatory function.[3] Later on, a second drug was approved in 2009; Canakinumab is a specific IL-1β monoclonal antibody applied for various inflammasomopathies, including FMF and CAPS. Furthermore, a randomized, double-blind multi-center phase 3 trial studied the effect of Canakinumab on cardiovascular disease and discovered that the risk for cardiovascular events decreased in patients with a history of myocardial infarction with high C-reactive protein levels, a marker for inflammation.[43] Canakinumab's long half-life and high specificity make it an efficient drug with fewer adverse events than Anakinra.[44] However, the adverse events associated with these drugs need to be anticipated since a great share of the innate immune system is compromised upon treatment, increasing the risk for infections.[45]

The high amount of side effects of Anakinra and, to a lesser extent Canakinumab, highlights the need for drugs that specifically target pathways upstream of IL-1. Recently, the NLRP3 inflammasome inhibitor, Inzomelid, has completed its phase I trial and was determined safe and tolerable, and showed rapid clinical improvement in a patient suffering from a CAPS-related flare.[46] Even though these results are promising, drugs inhibiting other inflammasomes are not discovered at the moment including therapeutics for the treatment of NLRP1-related conditions.[47] Although Bortezomib (BTZ), a proteasome inhibitor able to inhibit NLRP1 inflammasome activation, is an FDA-approved compound for the treatment of multiple myeloma, it is highly toxic and unlikely to be a viable drug for inflammasomopathies any time soon.

## 5.3. Aims of the project

Even though inflammasomes have increasingly gained interest in the last couple of years, many underlying mechanisms and pathways linked to human inflammasomes are still ill-defined. Currently, inflammasome research is predominantly performed in rodent species with murine bone marrow-derived macrophages (mBMDMs) as the immune cells of main interest. However, significant variations between human and mouse species are present, demonstrated by the distinctive protein structure and inflammasome assembly of human and mouse NLRP1.[9] Compared to humans, basal expression levels of mouse NLRP1 are deficient in keratinocytes.[48] Furthermore, mouse NLRP1 can be activated by anthrax due to its aberrant N-terminus, which is not seen in human NLRP1. Lastly, While ASC is irreplaceable in human pro-Caspase-1 cleavage, ASC is

dispensable for mouse NLRP1 inflammasome activation.[48] These variations highlight the significance of inflammasome research in human cells.

Another incentive for this project is that inflammasome research is mainly performed in myeloid cells, such as macrophages. Recent research has determined the critical role of inflammasomes in other cell types and has exposed their significant variation in inflammasome activation. Accordingly, many differences have already been proposed between keratinocytes and myeloid cells. For instance, nigericin is proposed to activate the NLRP1 inflammasome in primary keratinocytes, while it's a renowned activator of the NLRP3 inflammasome in macrophages.[13] Consequently, in this project, I aim to investigate inflammasome activation in two keratinocyte cell culture models by a suite of stimuli that are known to induce activation of different inflammasomes in myeloid cells. We will assess the variation between both keratinocyte models and compare our data to other cell types in the literature.

Since no effective therapy exists for NLRP1 inflammasomopathies to this day, this project offers essential information that could aid in the development of future therapies against epithelial inflammatory diseases Furthermore, the use of two keratinocyte cell culture models clarifies how and in what context these different cell types can be applied in inflammasome research.

# 6. MATERIALS AND METHODS

## 6.1. Experimental approach

In general, inflammasome research is multi-faceted. Firstly, it is vital to observe expression of proteins involved in inflammasome pathways to examine if all crucial elements for inflammasome activation are present. Subsequently, analysis of the various downstream signaling events of inflammasome activation, such as lytic cell death and cytokine release in supernatants, offers further necessary information to aid in illuminating essential pathways and mechanisms of the various inflammasomes.

To assess these facets, we utilized two human keratinocyte cell culture models. Firstly, expression levels of multiple inflammasome components of both cell lines were determined. Secondly, to investigate inflammasome activation upon a variety of priming conditions and stimuli, we performed LDH activity assays and Luminex assays to respectively measure cell death and cytokine release in the supernatants. In further experiments, we established if the obtained results were inflammasome-specific utilizing specific inflammasome or Caspase inhibitors. Furthermore,

### 6.1.1. Cell culture

As aforementioned, two human keratinocyte cell culture models were used to investigate inflammasome activation: the widely used HaCaT cell line and the more recent N/TERT-2G cell line. The HaCaT cell line originated from a cancer patient and has been proven to be a good model for inflammatory studies in general.[49] However, since this line is derived from cancer tissue, many modifications in the genotype are present.[50] On the contrary, the N/TERT-2G keratinocyte cell line was deliberately immortalized by altering only a few genes.[51] Although this cell line is still in its infancy, it has been proven useful in inflammasome research.[2,13,16] Finally, we also performed some preliminary experiments using NHEK cells. Since these cells have a normal genotype and phenotype, experiments with this cell type will generally be more comparable to experiments conducted *in vivo*.

### 6.1.2. Priming

Since priming is often an essential step for inflammasome activation, we utilized two priming conditions; Pam3CSK4 and interferon-γ (IFNγ).

Pam3CSK4 is a TLR1/TLR2 ligand that potently activates the NF-κB pathway and is widely used in the literature.[18,52] It binds TLR2 by mimicking lipopeptides present on gram-positive and -negative bacteria, instigating the heterodimerization of the receptor with TLR1.[53] Subsequently, MYD88 is activated and induces NF-κB activation. Aforementioned, NF-κB is an important pro-inflammatory transcription factor influencing gene expression of, for instance, NLRP3. [Figure 8] Another beneficial NF-κB activator is lipopolysaccharides (LPS). In this project, however, we did not apply this priming due to its own role in inflammasome activation in transfection experiments. As aforementioned, LPS is a powerful activator of the non-canonical NLRP3 inflammasome in various cell types when present in the cytosol of the cell. We do not want LPS to influence our results when using stimuli that need to be transfected.

The second priming condition we utilized is IFNγ. IFNγ influences gene expression by interacting with the IFNγ receptor, activating the Janus kinase (JAK)-signal transducer and activator of transcription 1 (STAT1) pathway. This increases the expression of multiple interferon-stimulated genes (ISG), which play a major role in inflammation. [Figure 8] priming of macrophages with IFNγ has been shown to elicit 'Super-activation', increasing the cells' responsiveness to pro-inflammatory stimuli.[54] Furthermore, priming of keratinocytes with IFNγ aids in the response to Herpes simplex virus via the AIM2 inflammasome and is demonstrated to prime keratinocytes and increase their susceptibility to CD8+ T cell-mediated cytotoxic responses through MHC class I induction in a coculture model.[33,55]

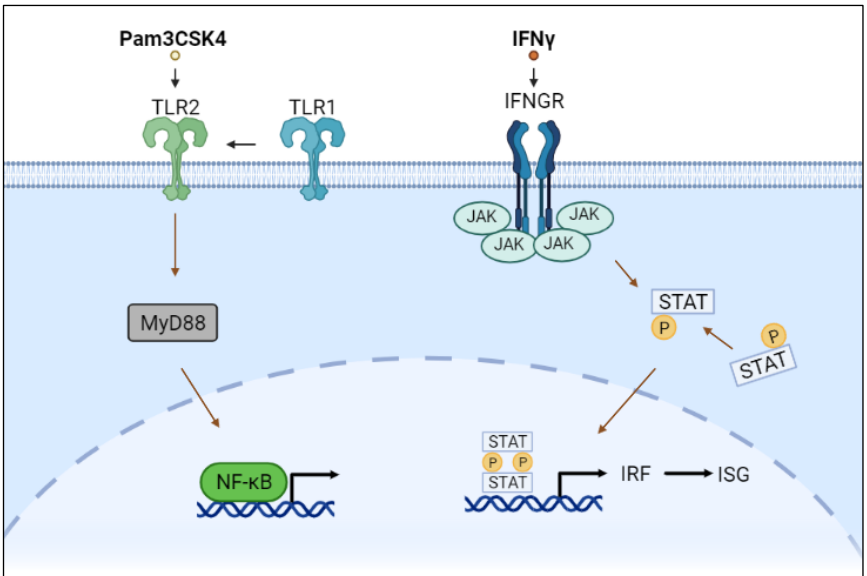


**Figure 8 - Overview of the Pam3CSK4 and IFNγ pathways.** Pam3CSK4 induces transcription via NF-κB activation while IFNγ induces phosphorylation of STAT to induce the transcription of interferon stimulated genes.

### 6.1.3. Stimuli

Table 1 lists the stimuli used in this thesis together with the inflammasome they are generally known to activate according to the literature. However, as mentioned before, many differences exist between cells.

| NLRP1 | NLRP3 | NLRC4 | AIM2 | PYRIN |
|---|---|---|---|---|
| Val-boroPro<br>Anisomycin<br>Poly (I:C) | ATP<br>Nigericin<br>Porphyrins<br>Imiquimod/Resiquimod<br>DiABZI | LFn-Needle | HT-DNA | TcdA |

**Table 1 - Overview of the stimuli used in this project.**

Concentrations of the used stimuli are in accordance with the literature. Each stimulus was added for 24h unless stated otherwise. Poly (I:C) and HT-DNA were transfected and LFn-needle was added together with the protective antigen (PA) of Bacillus anthracis to ensure cytosolic entry.

### 6.1.4. Inhibitors

Since all inflammasomes have overlapping downstream signaling events such as cell death and IL-1β release, it is impossible to know which inflammasome is activated solely based on these occurrences. Therefore, we made use of specific inflammasome inhibitors BTZ and MCC950 to establish if the observed cell death and cytokine release are inflammasome specific. BTZ is a proteasome inhibitor that specifically inhibits the NLRP1 inflammasome while MCC950 is a direct NLRP3 inflammasome inhibitor. In this project, we mainly focus on the NLRP1 and NLRP3 inflammasomes since there is a great overlap between NLRP1 and NLRP3 activators in various cell types. For instance, nigericin is an NLRP3 inflammasome activator in macrophages but recently has been suggested to activate the NLRP1 inflammasome in keratinocytes.[13]

### 6.1.5. LDH activity assays

For this project, we made use of LDH activity assays, a widely-used method to measure lytic cell death.[56] LDH is a large protein present in nearly all cells and is only released when the cell dies. This enzyme catalyzes the conversion of lactate into pyruvate, concomitantly producing NADH. (Promega) Released LDH activity can be measured by providing lactate and NAD+. The produced NADH can then be used to convert a tetrazolium salt (INT) into red formazan. The total absorbance can then be measured to know the amount of cell death. [Figure 9]

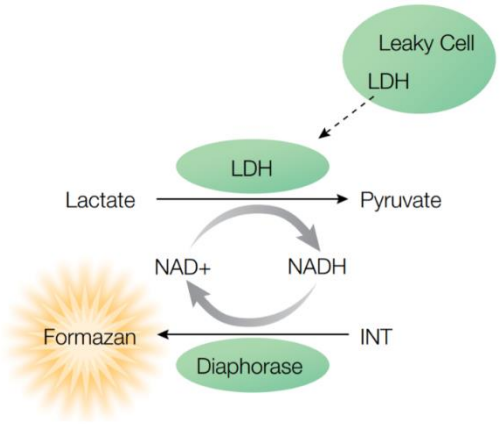


**Figure 9 - Mechanism of LDH activity assays.** LDH activity leads to the formation of a red formazan. This can then be measured by spectrometry. Image by Promega.

### 6.1.6. Luminex assays

Luminex assays permit the measurement of various cytokines simultaneously. In this project, we measured IL-1β, interleukin-1alpha (IL-1α), Interferon-gamma-inducible protein 10 (IP-10; also known as CXCL10), Tumor Necrosis Factor (TNF), and interleukin-6 (IL-6) release in the supernatants.

As aforementioned, IL-1β is generally released upon inflammasome-induced pyroptosis. IL-1α, on the contrary, cannot be cleaved by Caspase-1 and is only indirectly influenced by the inflammasome.[57] Caspase-1 can only cleave the cytosolic “decoy receptor” that binds IL-1α, IL-1R2, which normally inhibits the proteolytic activation of IL-1α. However, IL-1R2 can be cleaved by Caspase-1, freeing IL-1α and permitting its release in the supernatants.[58] IL-1α itself is cleaved by various proteases including granzyme B and Caspase-4/5 in human and Caspase-11 in mouse. This suggests a role of the non-canonical NLRP3 inflammasome in IL-1α maturation. Pro-IL-1α is cleaved by Caspase-5 upon activation of the non-canonical inflammasome and is then released in the supernatants.

IP-10, TNF, and IL-6 are measured to assess if any inflammatory pathways are influenced by, for instance, the use of priming, stimuli or inhibitors. IL-6 and TNF are hallmarks of NF-κB activation

and are influenced in different priming conditions. IL-6 release increases when the TLR-1/2 inflammatory pathway is activated after Pam3CSK4 priming while TNF release can be increased by various TLR's including TLR2, TLR3, TLR4.[59,60] IP-10 is secreted upon stimulation with type I and II interferons and is recorded to assess the influence of IFNγ priming and the activation of the cGAS-STING pathway.[24]

## 6.2. Materials

### 6.2.1. Cell culture

HaCaT cells (kind gift of Prof. Jo Lambert; dermatology department of Ghent University) were grown in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose; w/L-Gln; w/ sodium pyruvate; #LO BE12-604F; Westburg) supplemented with 10% Fetal bovine serum (FBS; #34090719FBS; Serana), 1mM sodium pyruvate (#LO BE13-115E; Westburg), 1mM penicillin-streptomycin (#LO DE17-602E; Westburg) and 1mM non-essential amino acids (#LO BE13-114E; Westburg). Low Calcium medium for HaCaTs was made in a similar manner using 3:1 Gibco™ DMEM, high glucose, no glutamine, no calcium (#115305560; Fisher Scientific) and 1:3 Dulbecco modified Eagle medium (DMEM; 4.5 g/L glucose; w/L-Gln; w/ sodium pyruvate; #LO BE12-604F; Westburg) instead of 100% DMEM with calcium. The N/TERT-2G cell line (kind gift of J. Rheinwald, Harvard University) and NHEK cells from healthy patients (kind gift of Prof. Jo Lambert; dermatology department of Ghent University) were grown in Epilife medium (#11684842; Fisher Scientific) supplemented with 1% Gibco™ Human Keratinocyte Growth Supplement (HKGS; #10761364; Fisher Scientific), 6% DMEM (#LO BE12-604F; Westburg), 2% Gibco™ Ham's F-12 Nutrient Mix (#15172529, Fisher Scientific), 1% FBS, and 0,1mM penicillin-streptomycin. Other media were tested as well: supplemented K-SFM medium with Gibco™ Keratinocyte SFM (0,2ng/ml epidermal growth factor (EGF) and 25µg/ml bovine pituitary extract (BPE) were added in accordance with the manufacturer's instructions; #12539079; Fisher Scientific) and 0,3mM of $CaCl_2$. Supplemented DF-K medium containing Gibco™ DMEM, high glucose, no glutamine, no calcium (#115305560; Fisher Scientific), 2ng/ml EGF and 25µg/ml BPE, 0,3mM $CaCl_2$ and 1,5mM L-Glutamine (#LO BE17-605E; Westburg) was used to make a 1:1 K-SFM/DF-K medium. Lastly, due to delivery problems of HKGS growth factors, we made use of a Epilife medium supplemented with the equal concentrations of EGF and BPE used in the K-SFM medium. The culture flasks for NHEK and N/TERT-2G cells were coated with 1% human plasma fibronectin (#F0895-2MG; Sigma) in calcium-free Dulbecco's Phosphate Buffered Saline (dPBS; #LO BE17-512F; Westburg) for at least 45 minutes and eventually washed with dPBS before use. All cells were incubated at 37°C in a 5% $CO_2$ atmosphere and medium was renewed every two days.

### 6.2.2. Priming, stimulation, and inhibition

Cells were seeded in 96-well plates and cultured at 37°C in a humidified 5% $CO_2$ atmosphere at a confluency of approximately 80% at the point of stimulation. HaCaT cells were seeded at a concentration of 7.000 cells per well while the N/TERT-2G cells were seeded at a concentration of 15.000 cells per well. Concentrations for priming and stimulation were decided in accordance with the literature. Cells were left untreated or were exposed to 1µg/ml Pam3CSK4 (#TLRL-PMS; Invivogen) or 200ng/ml IFNγ (#300-02; PeproTech) overnight (17 hours) before stimulation (24 hours). After stimulation, supernatants was collected and centrifuged at 1200RPM for 5 min to

remove cellular debris. Eventually, it was used for LDH activity assays or frozen at -80°C for future use in Luminex assays. Stimuli and inhibitors were used at the following concentrations: 10µM of VbP (Val-boroPro - CAS 150080-09-4 – Calbiochem; #5314650001; Sigma Aldrich), 5µM of anisomycin (#S7409; Sellekchem), 10µg/ml of Poly (I:C) or 6,7µg/ml (1µg/well) of Poly (I:C) transfection (#TLRL-PIC; Invivogen), 5mM of ATP (#10519987001; Sigma Aldrich), 10µM of nigericin (#N7143-10MG; Sigma Aldrich), 10µg/ml of Imiquimod (#S1211; Sellekchem); 2µg/ml of Resiquimod (R848; #TLRL-R848; Invivogen); 100µM of Mesoporphyrin IX dihydrochloride (#258806; Sigma (Merck), 1µM of diABZI (Cmdp3 Mouse/human sting agonist; #S8796; Bio-Connect), 100ng/ml of LFn-Needle (#TLRL-NDL; Invivogen), 6,7µg/ml (1µg/well) HT-DNA (#10164142; Bio-Techne),1µg/ml of C. difficile Toxin A (TcdA; #BML-G140-0050; Enzo Life Sciences). Inhibitors were added 30 min. before stimulation; 10nM of BTZ (#S1013; Sellekchem), 10µM MCC950 (#5381200001; Sigma), 50µM of VX-765 (#273404-37-8; Selleck Chemicals). For transfections, cells were maintained in the original medium utilized in cell culture and were transfected in 96-well plates with Lipofectamine 2000 ((#10696153; Thermo Fisher Scientific) according to the manufacturer's instructions. Mocks for each stimulus were made according to the substance the stimulus was solved in.

### 6.2.3. Cytokine analysis

Cytokine levels in supernatants were determined by a magnetic bead-based multiplex assay using Luminex technology according to the manufacturers' instructions. Two kits were used: Bio-Plex Pro Human Cytokine Screening Panel (LUMINEX); 1. Bio-Plex Pro Human Cytokine IL-1β Set (#171B5001M; Bio-rad), Bio-Plex Pro Human Cytokine lL-6 Set (#171B5006M; Bio-rad) Bio-Plex Pro Human Cytokine TNF-α Set (#171B5026M; Bio-rad) Bio-Plex Pro™ Human Cytokine IP-10 Set (#171B5020M; Bio-rad) Bio-Plex Pro Human Cytokine IL-1α Set (#171B6001M; Bio-rad) Bio-Plex Pro Reagent Kit III with Flat Bottom Plate, 1 x 96-well (#171304090M; Bio-rad) Bio-Plex Pro Human Cytokine Screening Panel Standards, 96 assays (#12007919; Bio-rad) 2. Human Discovery Luminex Assay (#LXSAHM-03; R&D Bio-Techne)

### 6.2.4. Cell death assays

The supernatant of cells was collected after 24h stimulation (or earlier when using BTZ) and centrifuged at 250 × g for 5 min to remove cellular debris. LDH measurement was performed with the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit according to the manufacturers' instructions. Absorbance per well was measured using SpectraMax i3x Multi-Mode Microplate Reader.

### 6.2.5. Light microscopy

Cell viability and differentiation levels during cultivation and stimulation was assessed by bright-field microscopy prior to supernatants collection.

### 6.2.6. Western blotting

Cells were cultured at 250.000 cells per well in a 6-well plate. Concentrated cell lysates of untreated cells were prepared in Caspase Lysis Buffer (20 mM Tris pH 7.4, 200mM NaCl, 1% NP-40). Supernatant samples or lysates were denatured using 50 mM dithiothreitol (DTT) and samples were boiled at 95C° for 10 minutes. SDS-PAGE-separated proteins were transferred to PVDF nitrocellulose membranes. Immunoblots were then blocked with 3% milk and incubated with primary antibodies against human Caspase-1 (p20) (#AG-20B-0048-C100; Adipogen), IL-1β (#GTX74034; Genetex), GSDMD (#HPA044487; Atlas Antibodies), ASC (#AG-25B-0006-C100; Adipogen), NLRP1 (#679802; Biolegend) or NLRP3 (#AG-20B-0014-C100; Adipogen). Subsequently, Horseradish-peroxidase–conjugated goat anti-mouse (115-035-146; Jackson Immunoresearch Laboratories, West Grove, PA, USA) or anti-rabbit secondary antibody (111-035-144; Jackson Immunoresearch Laboratories) was used to detect proteins by enhanced chemiluminescence (Thermo Fisher Scientific). The Bio-rad ChemiDoc Touch Imaging System was used to visualize the results.

### 6.2.7. Figures and graphs

Figures were created with Biorender.com. Graphs were made using GraphPad Prism 9.

### 6.2.8. Statistical analysis

Experiments were performed in triplicate unless stated otherwise. Gathered data were analyzed using one-way ANOVA or unpaired simple t-test, and results are presented as mean ± SEM. A p-value lower than 0.05 was considered significant. Asterisks on graphs represent the p-value: $p \leq 0.05$: *, $p \leq 0.01$: **, $p \leq 0.001$: ***, $p \leq 0.0001$: ****. Statistical analysis was performed using GraphPad Prism 9 and Microsoft Excel.

# 7. RESULTS

## 7.1. Establishment and optimization of keratinocyte culture protocols

We set up an optimization process to find competent culture conditions for each cell line.

### 7.1.1. N/TERT-2G immortalized cell line

N/TERT-2G cells were a kind gift of Prof. J. Rheinwald (Harvard University). To define the most optimal culture conditions for this immortalized cell line, we assessed a number of different N/TERT culture media such as Epilife medium, Keratinocyte serum-free medium (K-SFM), and DF-K/K-SFM (1:1) medium. Furthermore, we utilized varying flask brands (VWR, Bioswisstec, Thermofisher) and varying passaging protocols. However, these conditions failed to provide an efficient culture environment for the N/TERT-2G cells in our hands.

However, we succeeded in identifying proper growth conditions for N/TERT-2G cells by using protocols for primary keratinocyte cultures. Two well-known techniques for keratinocyte cell culture were evaluated; the use of a fibroblast feeder layer (3T3) and fibronectin-coating of the flasks prior to the addition of the cells.[61,62,63] Both tested conditions provided good N/TERT-2G cell growth with various media in our hands. Since fibronectin-coating is time-efficient, this technique eventually was selected as our standard N/TERT-2G cell culture protocol.

Ultimately, we made use of the Epilife medium supplemented with 1% Human Keratinocyte Growth Supplement (HKGS), 6% low calcium Dulbecco's modified Eagle medium, and 2% Ham's F12 nutrient mix. We worked in low serum concentrations; 1% Fetal Bovine Serum (FBS) and added 0,1mM penicillin-streptomycin to lower the risk of contamination. The culture flasks were coated with 1% human plasma fibronectin in calcium-free Dulbecco's Phosphate Buffered Saline (dPBS) for at least 45 minutes and eventually washed with dPBS before use. Cells were incubated at 37°C in a 5% $CO_2$ atmosphere and medium was renewed every two days. Cells were passaged when a confluency of 80% was reached and cells were cultured at 1/5 of the original concentration.

Unfortunately, because the supplier of the growth factors used in our supplemented Epilife medium was not able to deliver in timely manner at the end of this project, we switched to using Epilife medium supplemented with the K-SFM growth factors, 0,2ng/ml epidermal growth factor (EGF) and 25µg/ml bovine pituitary extract (BPE), instead of the HKGS. We determined that this supplemented medium also provided an effective environment for our cells since we could not observe changes in the morphology, differentiation and growth rate of the cells.

### 7.1.2. Normal human epidermal keratinocytes

We performed preliminary experiments in NHEK cells (Kind gift of Prof. Jo Lambert; Dermatology department of Ghent University). We confirmed that the fibronectin-coating protocols and culture conditions we established in the N/TERT-2G cells provided a suitable environment for growth of the NHEK cells. Although the slower growth and greater susceptibility of the primary cells prevented us from extensively experimenting with NHEK cells in the context of this project, a

number of key preliminary experiments where confirmed in NHEK to validate some of our findings in the immortalized cell lines described above.

#### 7.1.3. HaCaT immortalized cell line

Cultivation of the HaCaT cell line (Kind gift of Prof. Jo Lambert; Dermatology department of Ghent University) was already established in the previous year. This cell line is a widely-used keratinocyte model, and efficient protocols and media have already been described in the literature.[64]

We confirmed that a DMEM medium supplemented with 10% FBS, 1mM sodium pyruvate, 1mM non-essential amino acids, and 1mM penicillin-streptomycin provided an excellent environment for the HaCaT cell line. Cells were incubated at 37°C in a 5% $CO_2$ atmosphere and medium was renewed every two days. Passaging of the cells occurred when a confluency of 80% was reached. However, we witnessed that the growth rate of the HaCaT cell line is significantly higher than the N/TERT-2G and NHEK cells. Therefore we applied a 20 times dilution upon passaging the cells.

Calcium levels in the medium can trigger signals for growth, survival, and differentiation and could influence the susceptibility of the HaCaT cells to our stimuli.[65] Therefore, in some experiments we also assessed the effect of a lower calcium concentration in culture media of the HaCaT cell line. (i.e. ±0,36mM instead of ±1,6mM). In other experiments, we compared growth of HaCaT cells in the selected culture conditions of the N/TERT-2G cells. This analysis indicated that HaCaTs can be grown efficiently in a variety of media without complications.

### 7.2. Optimization of stimulation experiments

As aforementioned, we assessed various priming conditions and stimuli on the N/TERT-2G and HaCaT cells. Therefore, we had to optimize important factors in our experiments to reach a good reproducibility.

First of all, we defined competent plating conditions for all three cell culture models. A suitable cell confluency per well is required for stimulation experiments; limited confluency would not optimally determine the effect of our stimulus, while excessive confluency could diminish the impact of the stimulus due to the decreased cell-stimulus contact. Eventually, confluency of 80% at the point of stimulation was determined to be ideal for all three cells. (data not shown) Since the HaCaT cell line is a fast- and easy-growing cell culture model, cells were seeded at a concentration of 7.000 cells per well in a 96-well plate. NHEK and N/TERT-2G cells were plated at a concentration of 15.000 cells per well.

Secondly, we decided on the duration of priming and stimulation times. Although each priming and stimulation takes effect at different time points, generally cytokine levels in supernatants do not deteriorate quickly. Since we want to capture both early and late inflammasome-induced cell death and cytokine release, we made use of the 17-hour time point for all priming conditions and the 24-hour time point for all stimulations.

Thirdly, we assessed the cytotoxicity of the NLRP1 inhibitor, BTZ. As aforementioned, inhibition of inflammasomes is an essential part of inflammasome research. However, off-target mechanisms of inhibitors is a well-known cause of cytotoxicity. This is true for BTZ since it inhibits the proteasome to prevent NLRP1 activation.[66]

Since BTZ's cytotoxicity has insufficiently been described in keratinocytes, we assessed various concentrations of BTZ in N/TERT-2G cells. [Figure 10] Our data suggest that BTZ alone is not toxic until at least the 9h time point at concentrations as high as 200nM.

The well-established NLRP3 inhibitor, MCC950, and the Caspase inhibitor VX-765 have repeatedly been proven not to significantly affect cell viability *in vitro* at least up to 100µM concentrations.[28,66,67] Therefore, we did not perform experiments to assess their cytotoxicity in this project.

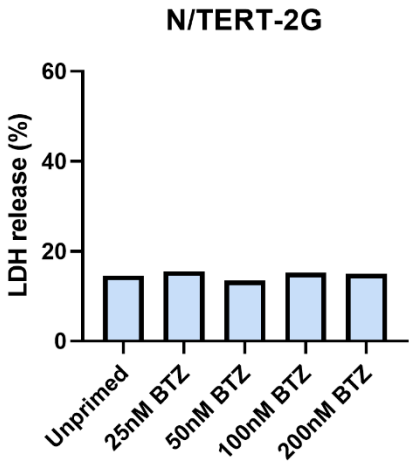


**Figure 10 – Assessment of cytotoxicity of BTZ.**
There was no significant cytotoxicity due to BTZ after 9h with concentrations as high as 200nM. The graphs present one experiment in singleton.

## 7.3. Influence of priming conditions

Transcriptional priming can influence inflammasome pathways by upregulating components of the pathways that are insufficiently present in untreated cells. As aforementioned, we assessed the impact of Pam3CSK4 and IFNγ priming on NF-κB and interferon stimulated genes such as IL-6, TNF, and CXCL10. To examine whether Pam3CSK4 stimulation effectively activated the NF-κB pathway, we measured two hallmark cytokines, IL-6 and TNF, in both cell lines and NHEK cells using Luminex assays. Surprisingly, we could not measure significant changes in IL-6 [Figure 11A] and TNF [Figure 11B] levels upon Pam3CSK4 priming in the two cell lines and the NHEK cells. This hints that TLR2 may be inoperative in keratinocytes or that NF-κB activation is compromised at another step downstream of TLR1/2. Notably, IFNγ treatment modestly induced IL-6 levels in HaCaT, but not in N-TERT-2G and NHEK. However, IL-6 levels were basally higher in N/TERT-2G cells than in HaCaT and NHEK.

Unfortunately, due to time constraints and repeated delays in delivery of Luminex kits, we could not assess other TLR agonists in the timeframe of the project.

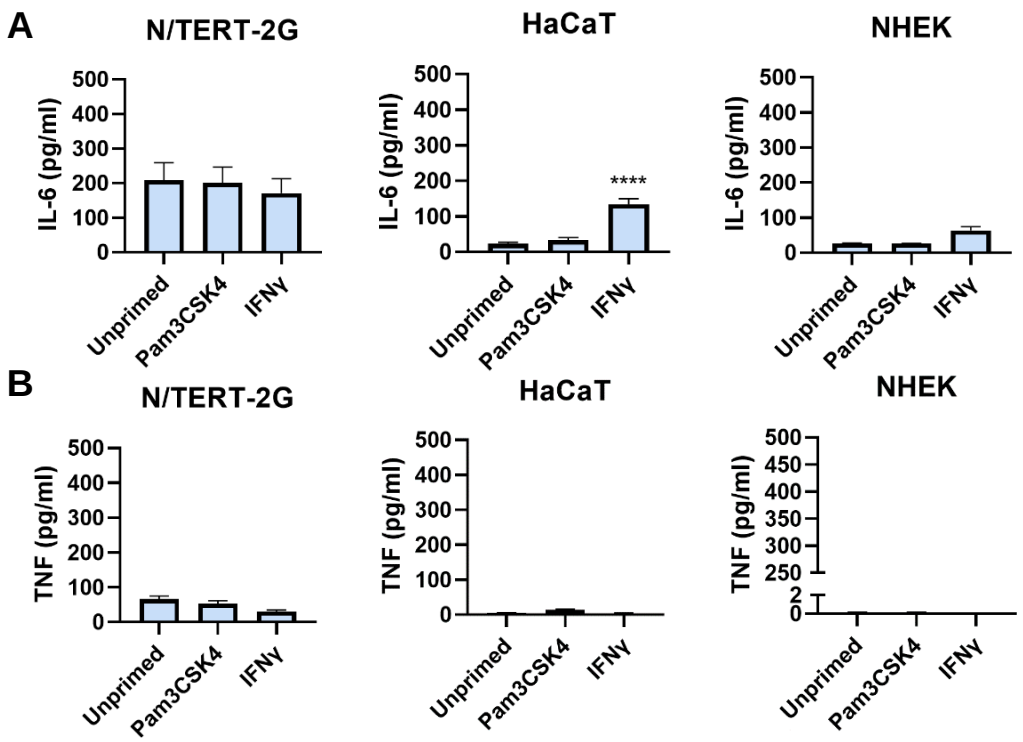


**Figure 11 – Impact of priming on IL-6 (A) and TNF (B) levels in supernatants of N/TERT-2G, HaCaT, and NHEK cells.**

There are no significant changes in IL-6 and TNF upon Pam3CSK4 priming in all cell types. However, IFNγ priming increased IL-6 levels in HaCaT cells. Graphs of N/TERT-2G and HaCaT cells visualize the mean of three experiments with error bars presenting the Standard Error of the Mean (SEM). The graphs of NHEK cells show one experiment in technical duplicates with error bars presenting the standard deviation (SD).

Activity of IFNγ signaling was assessed by measuring IP-10 using Luminex assays. We witnessed that IFNγ elicited a tremendous IP-10 response in the N/TERT-2G cell line, confirming that this pathway is functional and gene expression of interferon-stimulated genes will be upregulated. [Figure 12] Unfortunately, our data exceeded the expected outcome and was higher than the used standards in this experiment.

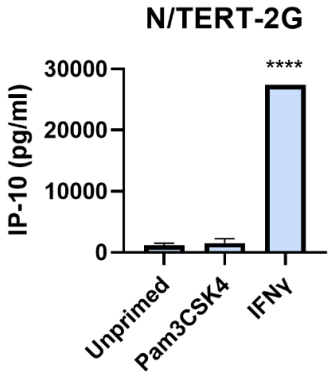


**Figure 12 – Impact of priming on IP-10 release in N/TERT-2G cells.**

IP-10 surged upon IFNγ treatment in N/TERT-2G cells. The graph presents the mean of three experiments with error bars presenting the SEM.

## 7.4. Inflammasome-involved protein expression of N/TERT-2G, HaCaT, and NHEK cells

As a complementary approach to the transcriptional priming studies described above, we assessed the protein expression of key proteins that are vital in inflammasome activation and assembly.

In preliminary experiments from the previous year, we already provided evidence that untreated HaCaT cells contain all necessary components for inflammasome activation except for NLRP3. [Figure 13] This observation is not surprising as, in contrast with monocytes, the majority of cell types do not express adequate NLRP3 levels for inflammasome activation in an unprimed state.[22] The fact that low levels of pro-IL-1β are present in the HaCaT cells is notable. Expression of Pro-IL-1β is habitually only elicited by priming in monocytes and macrophages.[69,70,71] However, Watanabe et al. have already shown that this is not the case in primary keratinocytes.[72]

We also assessed the NLRP1 and NLRP3 expression levels in untreated NHEK, N/TERT-2G, and HaCaT cells. [Figure 13B] We detected varying NLRP1 protein expression levels in all three cell types. The NHEK and N/TERT-2G cells showed a clear band at the 165kDa mark indicating that these cells both constitutively express NLRP1, albeit at different levels. The HaCaT sample showed only a minor band, suggesting that NLRP1 may not be expressed in these cells. When we assessed the NLRP3 expression levels we obtained unexpected results. As discussed in the paragraph above and visualized in the NHEK and HaCaT samples, NLRP3 expression is often not present in unprimed cells. However, the N/TERT-2G cells showed a clear band just below 130kDa and NLRP3 is expressed in a basal state.

It is important to mention that the HaCaT cell samples were not lysed in a complete identical manner as the NHEK and N/TERT-2G cells. However, the number of cells per sample was roughly the same. This is confirmed by the thickness of the β-actin band.

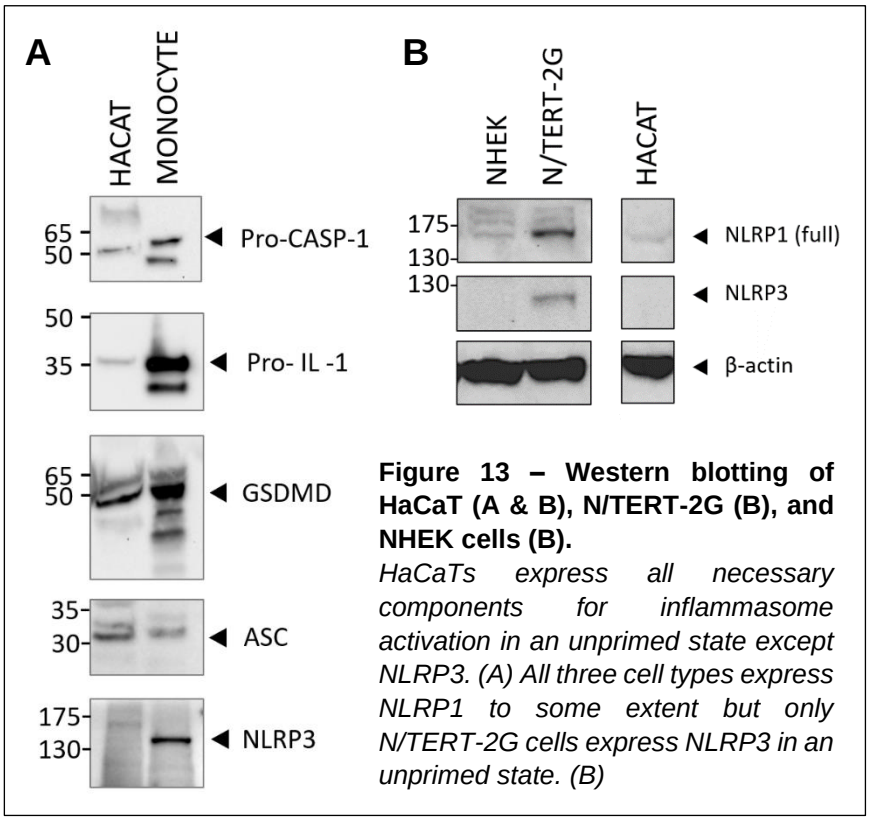


**Figure 13 – Western blotting of HaCaT (A & B), N/TERT-2G (B), and NHEK cells (B).**
*HaCaTs express all necessary components for inflammasome activation in an unprimed state except NLRP3. (A) All three cell types express NLRP1 to some extent but only N/TERT-2G cells express NLRP3 in an unprimed state. (B)*

## 7.5. Stimulation

As aforementioned, we also studied the downstream signaling events, lytic cell death and cytokine release, to assess inflammasome activation upon stimulation.

### 7.4.1. NLRP1 inflammasome stimuli

#### *7.4.1.1. Val-boroPro*

Recent publications suggest an evident influence of Val-boroPro (VbP) on NLRP1 inflammasome activation in unprimed keratinocytes.[11,12] To confirm this in our N/TERT-2G and HaCaT cells, we performed stimulation experiments using 10µM VbP stimulation for 24h.

We first obtained the LDH activity data and detected that the amount of cell death rose considerably in the N/TERT-2G cells in unprimed, Pam3CSK4-, and IFNγ-primed conditions but not in the HaCaT cells. [Figure 14A] Surprisingly, upon cytokine analysis in supernatants, we observed a significant increase in both IL-1β and IL-1α levels in the N/TERT-2G cells in all conditions. Thus, upregulation of pro-IL-1β expression levels is redundant in this cell line. These data confirm that VbP induces inflammasome activation in the N/TERT-2G cells independent of priming and that all the components for VbP-induced inflammasome activation are present without priming.

However, similar to the LDH activity data, all signs of inflammasome activation were absent in the HaCaT cell line. [Figure 14B] Indeed, there was no rise in IL-1β nor IL-1α levels in the supernatants after stimulation with VbP. This indicates that specific steps in inflammasome pathways are impaired in this cell line, hampering inflammasome activation upon stimulation with VbP.

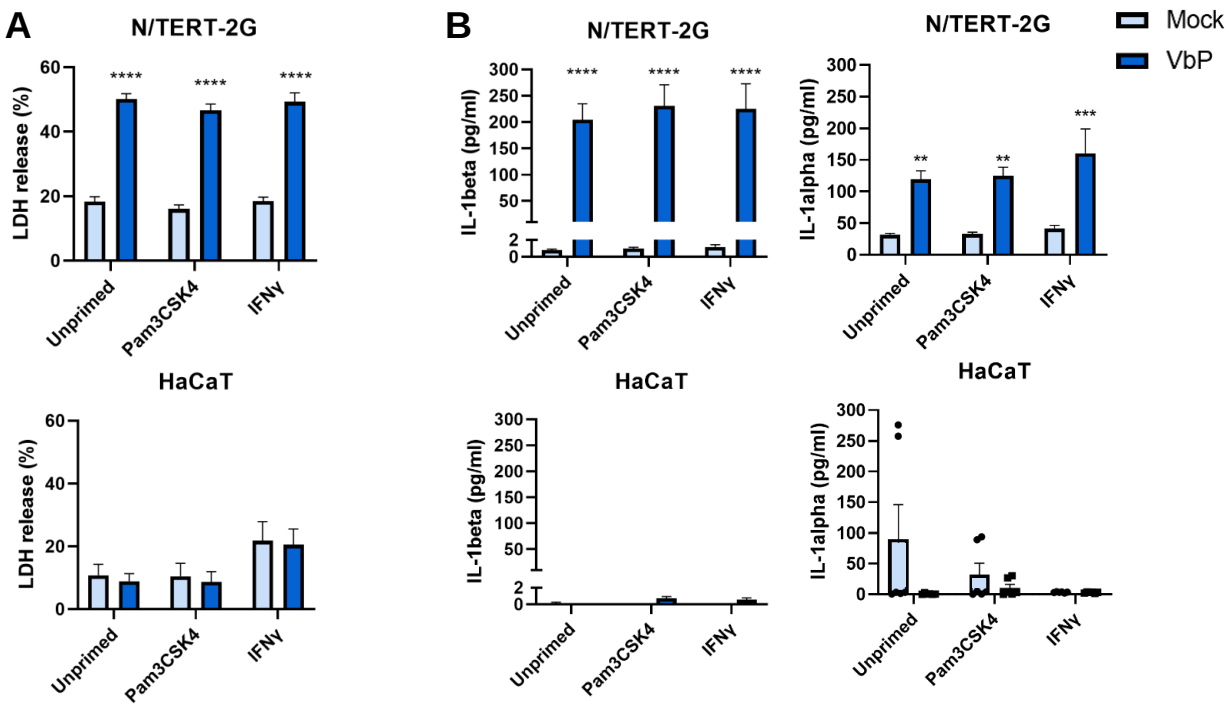


**Figure 14 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h VbP stimulation (10µM) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.** *VbP induces cell death and IL-1β release in N/TERT-2G cells but not in HaCaT cells. The graphs present the mean of three experiments. Error bars visualize the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001*

Since the outcome of the HaCaT cells was unexpected, we wanted to ensure that the culture conditions did not influence our results. As aforementioned, cell characteristics can be influenced by the culture conditions. Therefore, we cultured the HaCaTs in three different media and compared the amount of VbP-induced cell death of the HaCaTs in each medium. We made use of the normal supplemented DMEM medium as a control (±1,36mM), a similar DMEM medium with low calcium concentration (±0,36mM), and the supplemented Epilife medium we use for the N/TERT-2G cells. [Figure 15]

We did not detect any changes upon use of the different media. Therefore we continue experiments in the original DMEM medium and conclude that VbP does not induce inflammasome activation in the HaCaT cells.

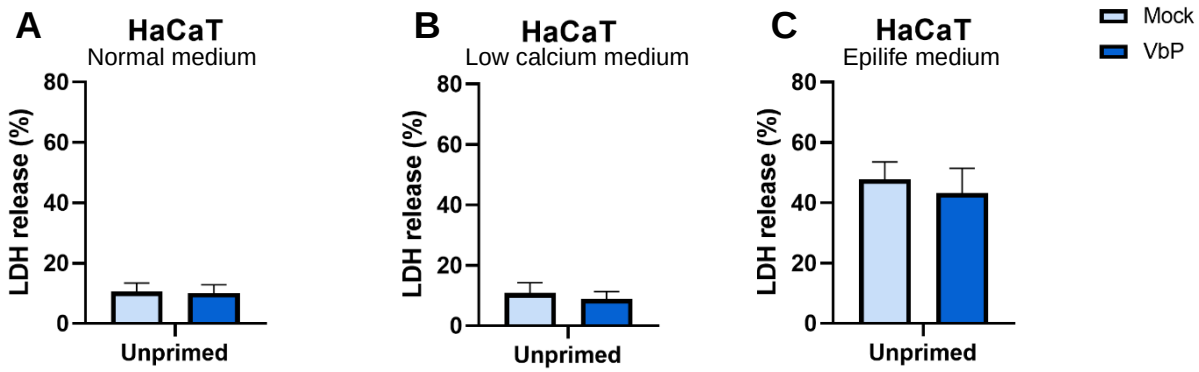


**Figure 15 – 24h VbP (10µM) stimulation in normal calcium HaCaT (A), low calcium HaCaT (B), and N/TERT-2G Epilife medium (C).**
The normal medium graph shows the mean of three independent experiments with error bars presenting the SEM. (A) The low calcium medium (B) and Epilife medium (C) graphs show the mean of one experiment in technical duplicates. Error bars represent the SD.

Since LDH activity data as well as the Luminex data suggest inflammasome activation in the N/TERT-2G cells, we examined the possibility of NLRP1 inflammasome activation by pharmacological inhibition of NLRP1 utilizing BTZ. Although we already assessed its cytotoxicity after 8h, the minimum effective dose of BTZ was not yet described in keratinocytes. Therefore, we assessed various concentrations of BTZ to inhibit VbP-induced inflammasome activation upon 8h of stimulation in the N/TERT-2G cells. [Figure 16]

We observed that VbP-induced cell death was completely inhibited [Figure 16A] and cytokine release diminished [Figure 16B] with BTZ concentrations as low as 8,33nM. We decided to use a 10nM concentration of BTZ for further experiments.

Overall, we can conclude that VbP explicitly activates the NLRP1 inflammasome in N/TERT-2G cells but not in HaCaT cells.

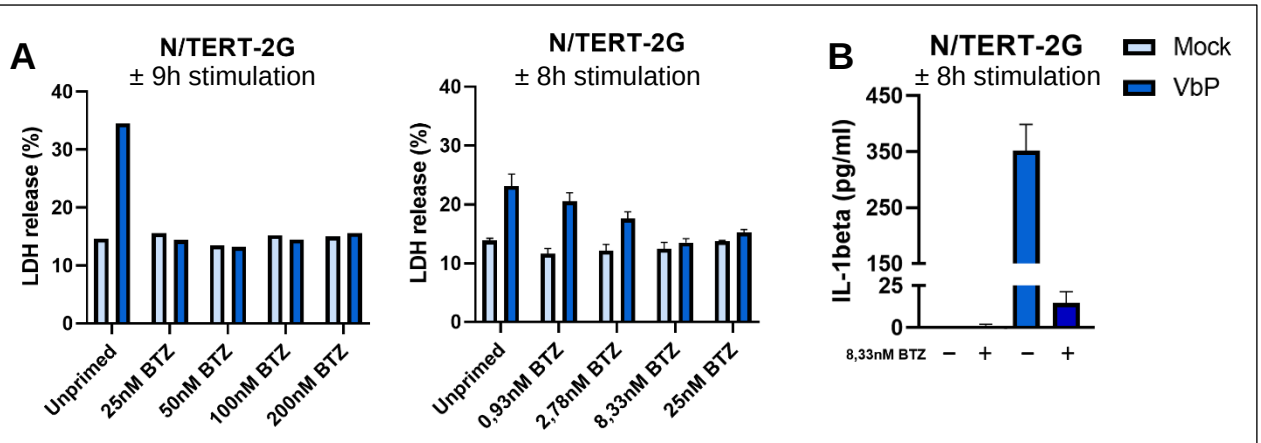


**Figure 16 – Effectivity of BTZ in unprimed N/TERT-2G cells.**
VbP-induced cell death [Figure 16A] and cytokine release [Figure 16B] was almost completely inhibited with BTZ concentrations as low as 8,33nM.. The graphs present one experiment in singleton or technical duplicates, error bars represent the SD.

*7.5.1.2. Anisomycin*

Anisomycin was recently discovered to activate NLRP1 in various cell types, including N/TERT cells and primary keratinocytes.[2] To confirm the impact of anisomycin on NLRP1 inflammasome activation in N/TERT-2G and HaCaT cells, we performed stimulation experiments using 5µM anisomycin for 24h.

Similar to VbP stimulation, we detected a significant increase in cell death in unprimed Pam3CSK4, and IFNγ primed conditions of the N/TERT-2G cells but not the HaCaT cells. [Figure 17A] This trend continued in the Luminex data; we measured a surge in IL-1β and IL-1α release irrespective of priming in the N/TERT-2G cells but not in the HaCaT cells. [Figure 17B] Even though we observe a statistically significant elevation in IL-1β levels in the Pam3CSK4 Priming in HaCaTs, we do not consider this biologically substantial due to the diminutive change in values. In HaCaTs, IL-1α levels showed a statistically significant increase in the unprimed stimulation condition. However, we deem this result to be the cause of the outliers present in one of the three experiments. Furthermore, IL-1α can be present in the supernatants without caspase-1 activation. As aforementioned, IL-1α is cleaved by various proteins and can be released in the supernatants due to general lytic cell death.

To assess whether anisomycin-induced inflammasome activation is NRLP1 dependent, we performed anisomycin stimulation in the presence of 10nM BTZ. [Figure 18] After 8h of anisomycin stimulation, we detected a significant increase in cell death percentages and IL-1β levels in supernatants, which were diminished in all conditions upon the use of BTZ. However, unsurprisingly, complete inhibition of cell death induction was not present. Anisomycin influences a broad range of pathways and is therefore linked with cytotoxicity.[15] Curiously, the IL-1β levels upon anisomycin stimulation have surged in comparison with our previous experiments. These varying results can be explained by 2 possibilities; firstly, cells were now only stimulated for 8h instead of 24h. Additionally, this experiment was performed in the other culture medium we had to implement at the end of this project. This experiment was the only experiment with anisomycin in this medium.

All together, we can conclude that anisomycin explicitly activates the NLRP1 inflammasome in N/TERT-2G cells but not in HaCaT cells.

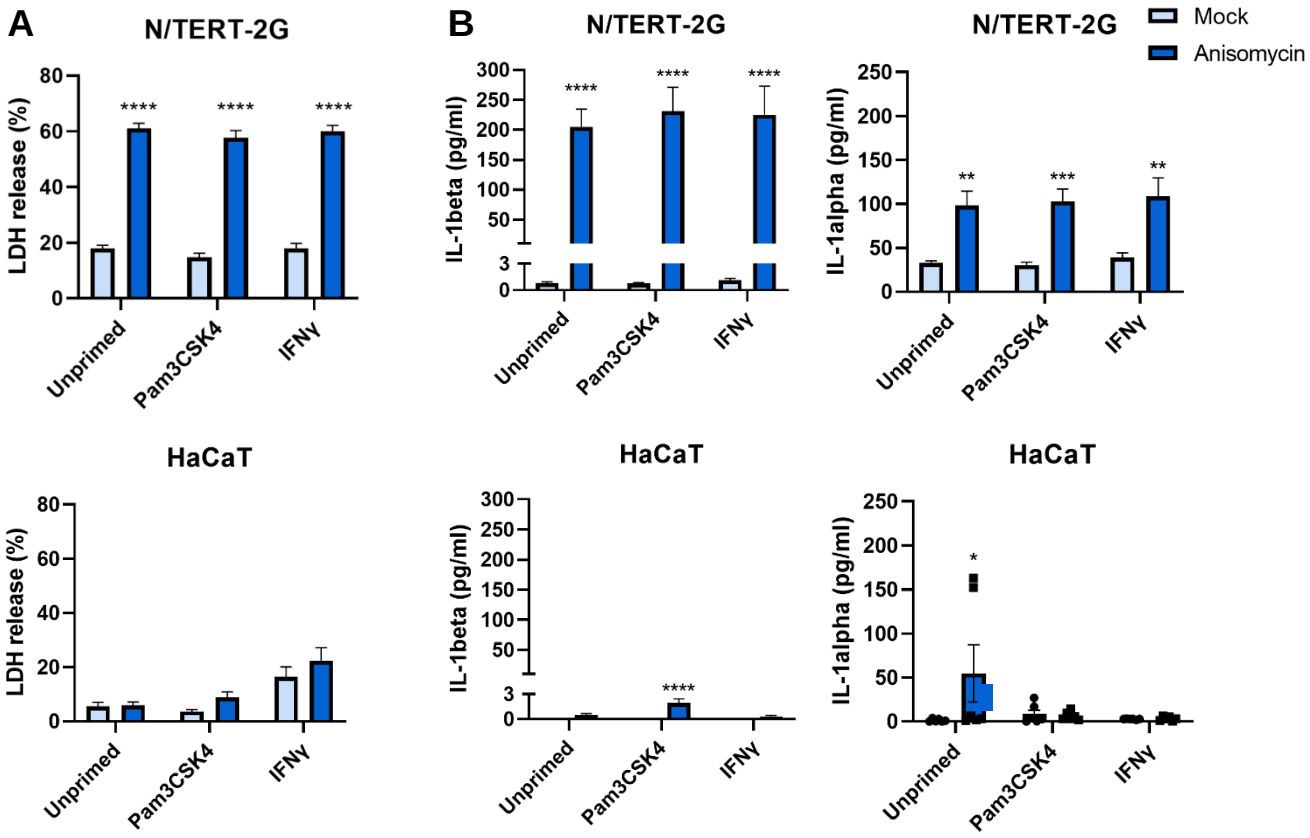


**Figure 17 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h anisomycin stimulation (5µM) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.**
*Anisomycin elicits cell death and IL-1β release in N/TERT-2G cells but not in HaCaT cells. The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001*

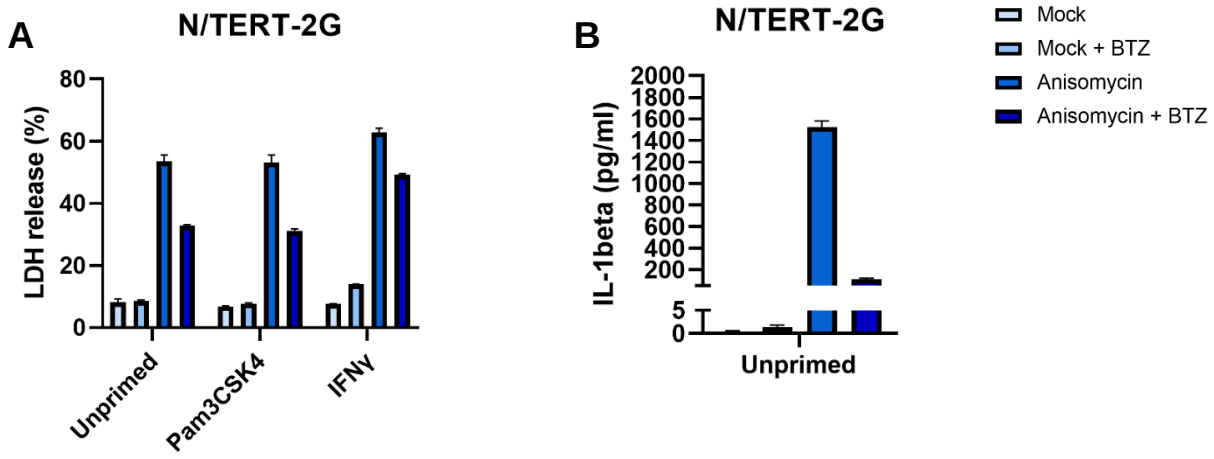


**Figure 18 – Inhibition of cell death (A) and cytokine release (B) upon 8h anisomycin stimulation in N/TERT-2G cells using 10nM BTZ.**
10nM of BTZ inhibits anisomycin-induced cell death and IL-1β release. Graphs visualize one experiment with technical duplicates. Error bars present the SD.

*7.5.1.3. Poly (I:C)*

DsRNA has been shown to induce inflammasome activation in various cell types.[17,19] We assessed the possible influence of Poly (I:C) transfection utilizing Lipofectamine 2000 to transfect cells with 6,7µg/ml Poly (I:C) (1µg/well). Poly (I:C) stimulation alone was used as an additional mock to confirm that the transfection of Poly (I:C) is essential for activation.

Using LDH activity assays, we observed significant cell death in both cell lines in all priming conditions. [Figure 19A] In HaCaTs we saw that untransfected Poly (I:C) stimulation also greatly induced cell death percentages in the IFNγ priming. Luminex experiments revealed no significant changes in the HaCaTs. However, a significant increase in IL-1β and IL-1α levels was observed in the NTERT-2G cells upon Poly (I:C) transfection in all conditions but not upon untransfected Poly (I:C) stimulation. [Figure 19B] We conclude that Poly (I:C) transfection can activate the inflammasome. However, the fact that N/TERT-2G cells express both NLRP1 and NLRP3 basally, the contribution of both inflammasomes needs to be assessed.

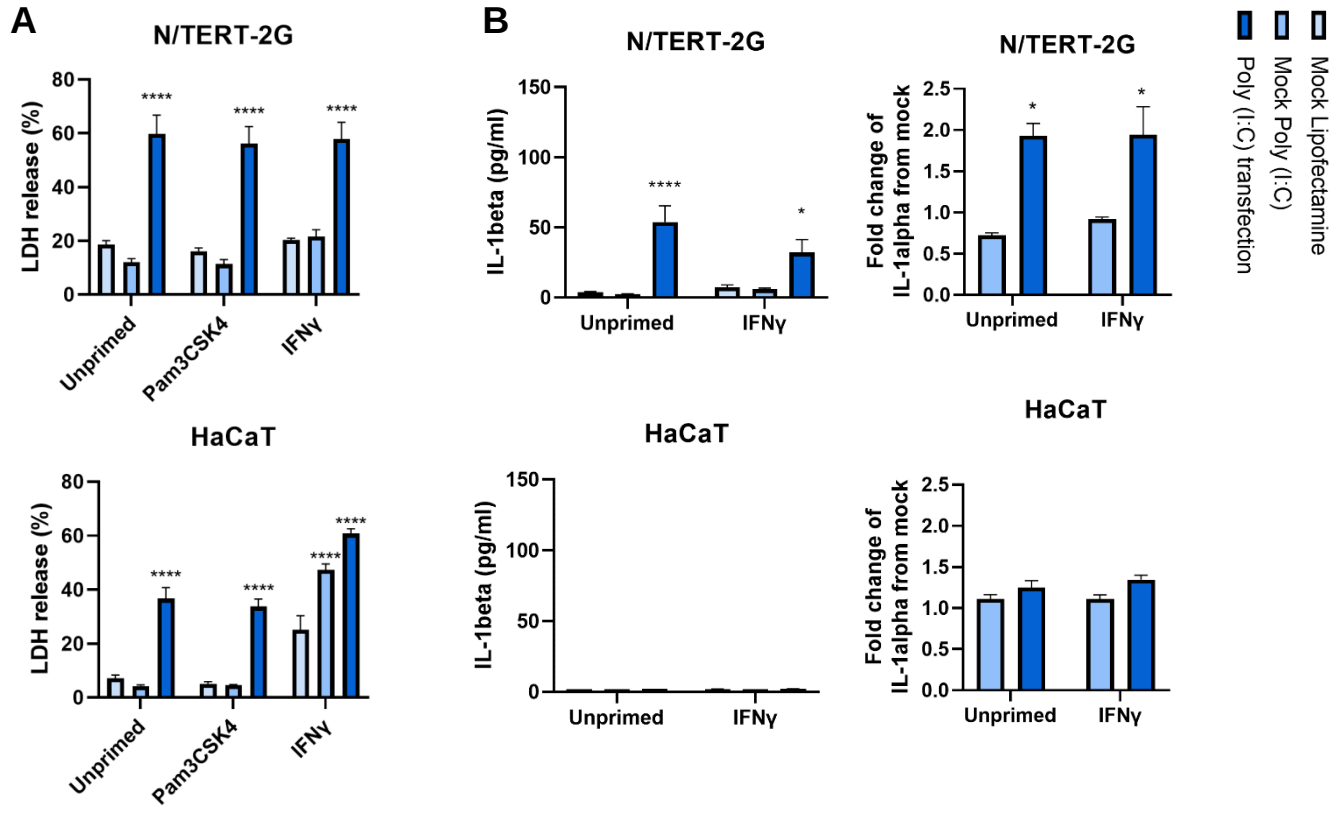


**Figure 19 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h Poly (I:C) transfection (6,7µM) in unprimed, Pam3CSK4-primed) and IFNγ-primed N/TERT-2G and HaCaT cells.**
*The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001.* Unfortunately, due to the slow delivery of Luminex assays, we did not have the right standard to measure IL-1α levels in supernatants. Therefore, graphs are visualized using the fold change from the mock with only Lipofectamine.

To establish the role of the NLRP3 inflammasome in transfected Poly (I:C)-induced cytokine release, we repeated aforementioned experiments in the presence or absence of NLRP3 inhibitor, MCC950. Overall, we did not observe any significant difference in cell death percentages and IL-1β release in the presence or absence of MCC950, suggesting that NLRP3 is not the cause of the visible inflammasome activation markers. [Figure 20] This suggests a role for NLRP1 in cytokine release upon Poly (I:C) transfection in N/TERT-2G cells.

Therefore, we assessed inflammasome activation upon Poly (I:C) transfection in the presence of 10nM BTZ to inhibit NLRP1 signaling. We had already tested various BTZ concentrations after 17h Poly (I:C) transfection and did not see significant cytotoxicity of BTZ alone at this concentration. [Figure 21] However, we did not observe any changes in transfected Poly(I:C)-induced cell death percentages in the presence or absence of BTZ. Unfortunately, we could not obtain any Luminex data to measure IL-1β release levels and LDH activity assay alone is inadequate to conclude if the inflammasome is inhibited or not.

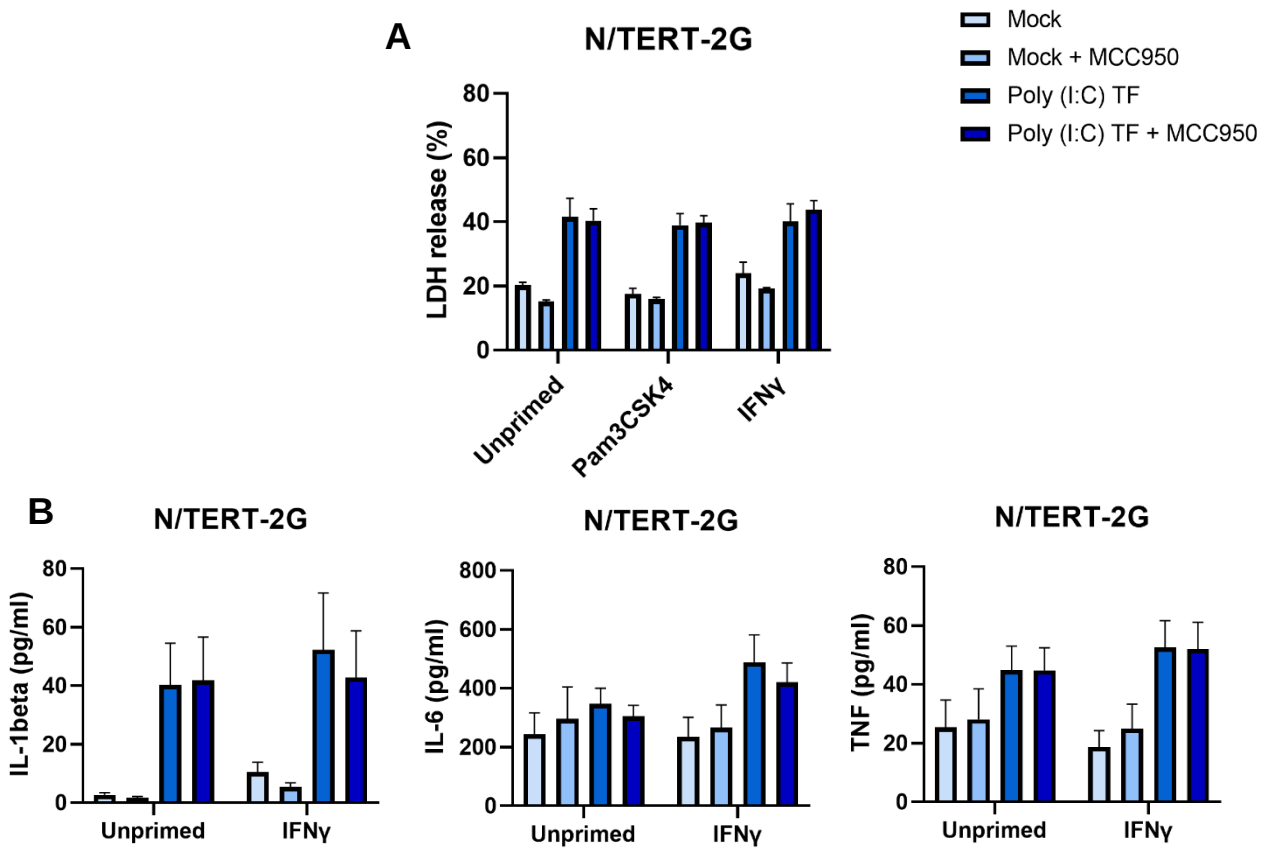


**Figure 20 – Inhibition of the NLRP3 inflammasome upon 24h Poly (I:C) transfection in N/TERT-2G and HaCaT cells using 10µM MCC950.**
There is no inhibition of cell death and cytokine release using MCC950. LDH activity graphs visualize one experiment with technical duplicates with error bars presenting the SD. Luminex data visualize the mean of three experiments with error bars presenting the SEM.

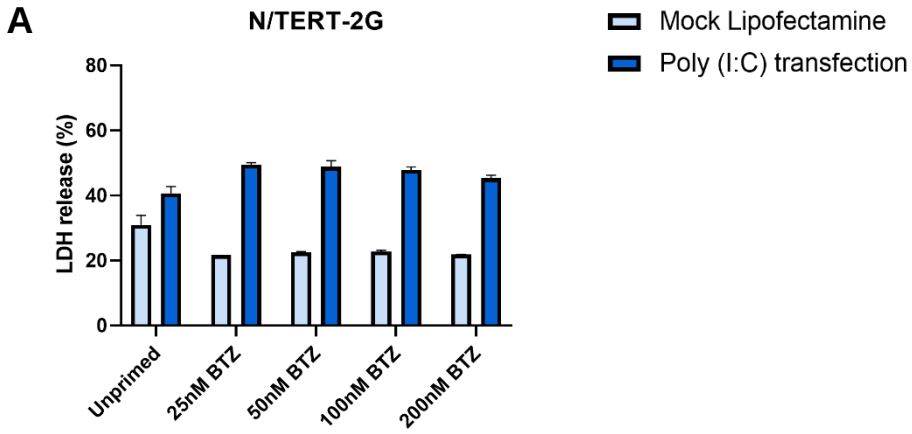


**Figure 21 – Inhibition of LDH release percentages (A) upon 17h Poly (I:C) transfection (6,7µM) using BTZ in unprimed N/TERT-2G cells.**
*We did not see inhibition of cell death upon the use of BTZ. The graph visualizes one experiment in technical duplicates. Error bars represent the SD.*

### 7.5.2. NLRP3 inflammasome stimuli

#### *7.5.2.1. ATP*

Extracellular ATP is a well-known activator of the NLRP3 inflammasome and has recently been suggested to activate the inflammasome in healthy primary keratinocytes as well as primary cells derived from Vitiligo patients.[28,29] To confirm these publications and to specify which inflammasome is assembled, we performed stimulation experiments using 5mM of ATP.

Although ATP was determined as an NLRP3 inflammasome activator in various cell types, we cannot conclude the same in our experimental setups. We detected a significant amount of cell death in the N/TERT-2G cells in unprimed and IFNγ primed cells following ATP stimulation [Figure 22A] and a statistically significant increase in ATP-induced IL-1β secretion with prior IFNγ priming. [Figure 22B] However, since the IL-1β values were extremely low (around detection limit of the Luminex assay), we do not consider this increase biologically relevant. Unsurprisingly, in the HaCaT cells, stimulation with ATP did not show any increase in cell death, and the Luminex data revealed no changes in IL-1β and IL-1α levels in the supernatants (except for an outlier in IL-1α levels). Hence, we conclude that ATP cannot activate any inflammasome in both cell lines.

Since two publications propose ATP as an inflammasome activator in primary cells, we performed preliminary experiments with ATP stimulation in NHEK cells as well. [Figure 23] Nevertheless, we obtained similar results as the HaCaT and N/TERT-2G cells. Indeed, cell death nor IL-1β levels rose upon ATP stimulation in our experimental setups, establishing that no inflammasome is activated in ATP-stimulated primary keratinocytes.

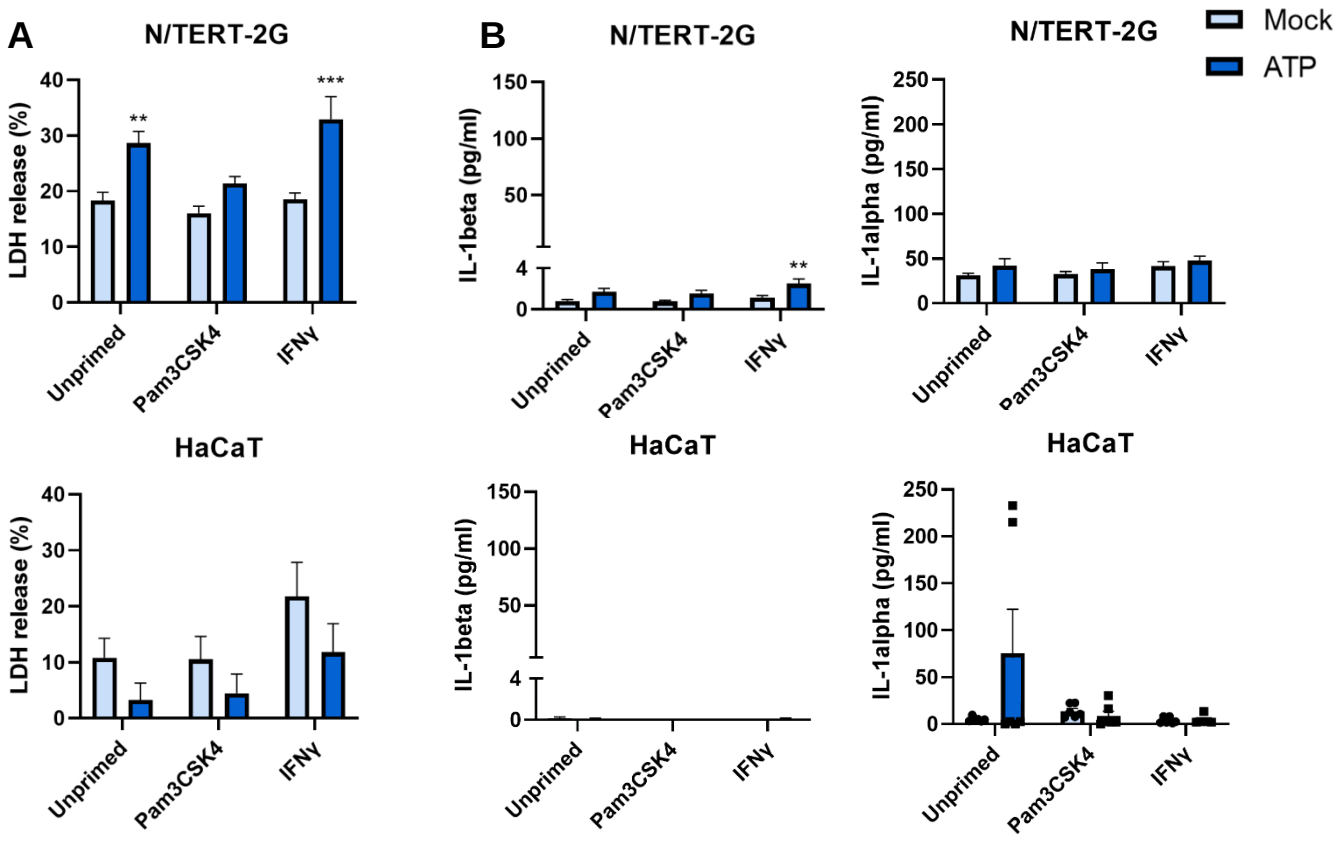


**Figure 22 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h ATP stimulation (5mM) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.**
*We did not observe any inflammasome activation markers upon stimulation with ATP. The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001*

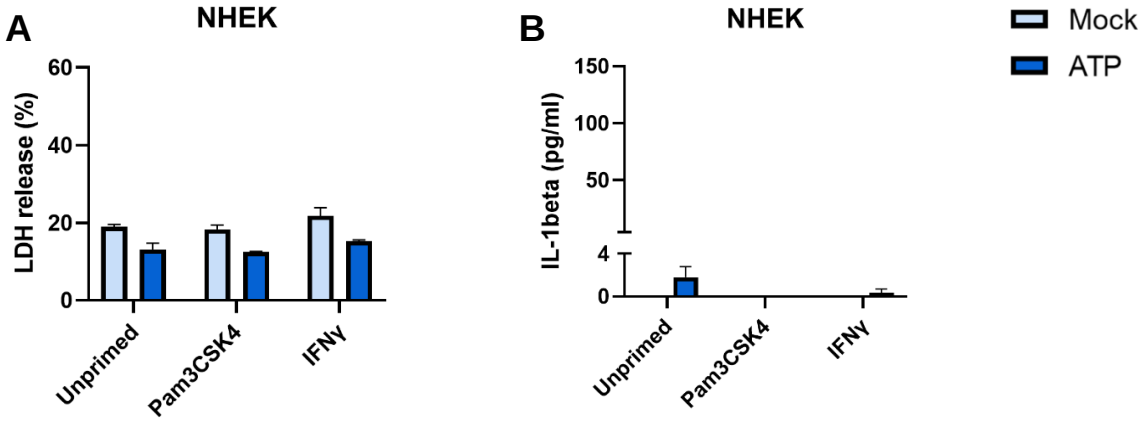


**Figure 23 - LDH release percentages (A) and IL-1β release (B) upon 24h ATP stimulation (5mM) in unprimed, Pam3CSK4-primed and IFNγ-primed NHEK cells.**
*We did not observe any inflammasome activation markers upon stimulation with ATP. The graphs visualize one experiment in technical duplicates. Error bars represent the SD.*

### *7.5.2.2. Nigericin*

Nigericin is a well-known NLRP3 inflammasome activator and has recently been revealed to activate NLRP1 in keratinocytes.[13] We wanted to confirm these data and stimulated the two keratinocyte cell models with 10µM of nigericin.

LDH activity assays revealed extreme amounts of cell death (>80%) in N/TERT-2G cells in unprimed, Pam3CSK4 and IFNγ primed conditions but not in HaCaT cells. [Figure 24A] However, nigericin has already been proven to be cytotoxic and could have influenced the amount of cell death independent of inflammasome activation.[73] Therefore, we performed Luminex assays to assess the cytokine release. Nevertheless, we did not observe any augmentation of IL-1β and IL-1α levels in the supernatants upon stimulation with nigericin in both cell lines. [Figure 24B] We conclude that nigericin does not influence inflammasome activation in N/TERT-2G and HaCaT cells in our experimental setup.

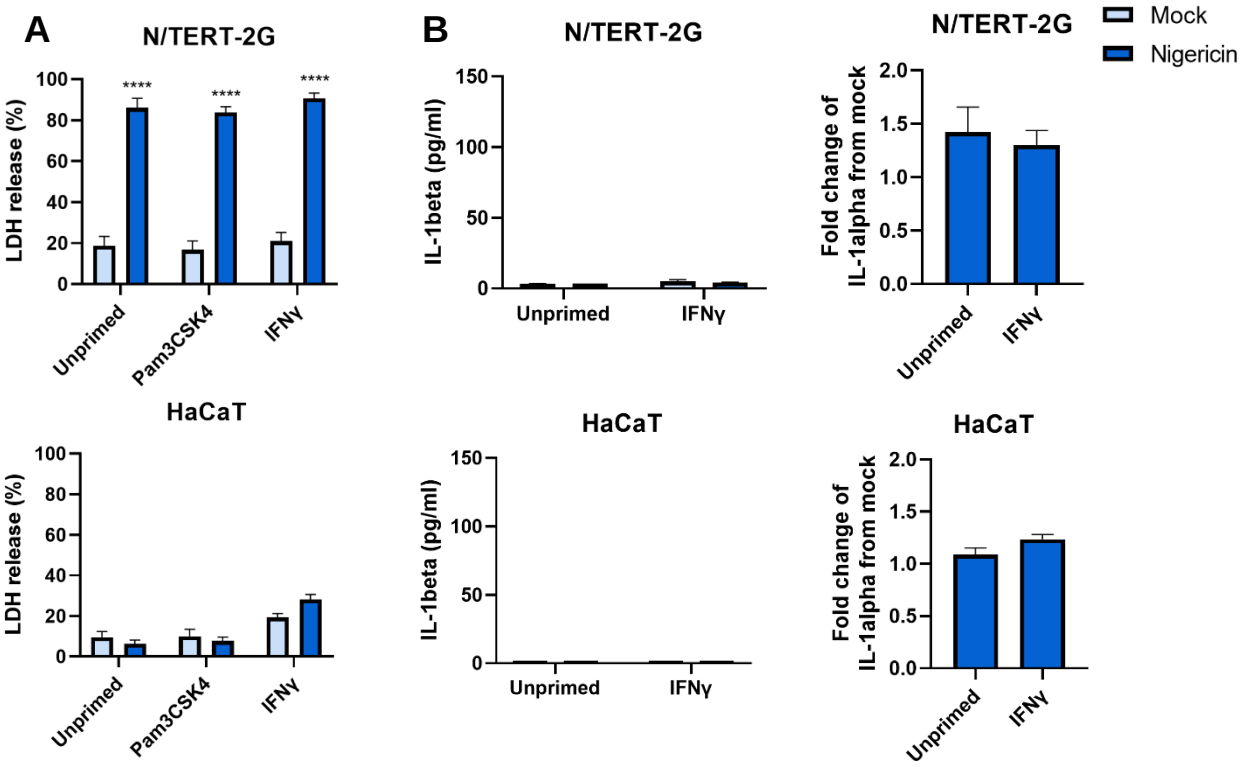


**Figure 24 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h nigericin stimulation (10µM) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.** *No signs of inflammasome activation were present upon nigericin stimulation.* IL-1α levels graphs are visualized using the fold change from the mock with only Lipofectamine. *The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001.*

### *7.5.2.5. DiABZI*

Gaidt et al. discovered that dsDNA could activate the NLRP3 inflammasome via the cGAS-STING pathway in monocytes.[24] However, analogous experiments have not yet been performed in keratinocytes. In this project we assessed possible inflammasome activation via the cGAS-STING pathway in keratinocytes using 24h stimulation with 1µM of the direct STING-agonist, DiABZI.

We observed a significant increase in cell death in the IFNγ priming in both cell lines. [Figure 25A] Nonetheless, although we observed a surge of IP-10 levels in the supernatants of N/TERT-2G cells confirming that the cGAS-STING pathway is activated [Figure 25C], overall no changes in IL-1β release in the N/TERT-2G nor the HaCaT cells could be detected. [Figure 25B] We did determine a significant rise in IL-1α alone but this cytokine is also released upon lytic cell death independent of inflammasomes. We conclude that the cGAS-STING pathway is activated upon the use of diABZI in N/TERT-2G cells, however no inflammasome activation is present in both N/TERT-2G and HaCaT cells.

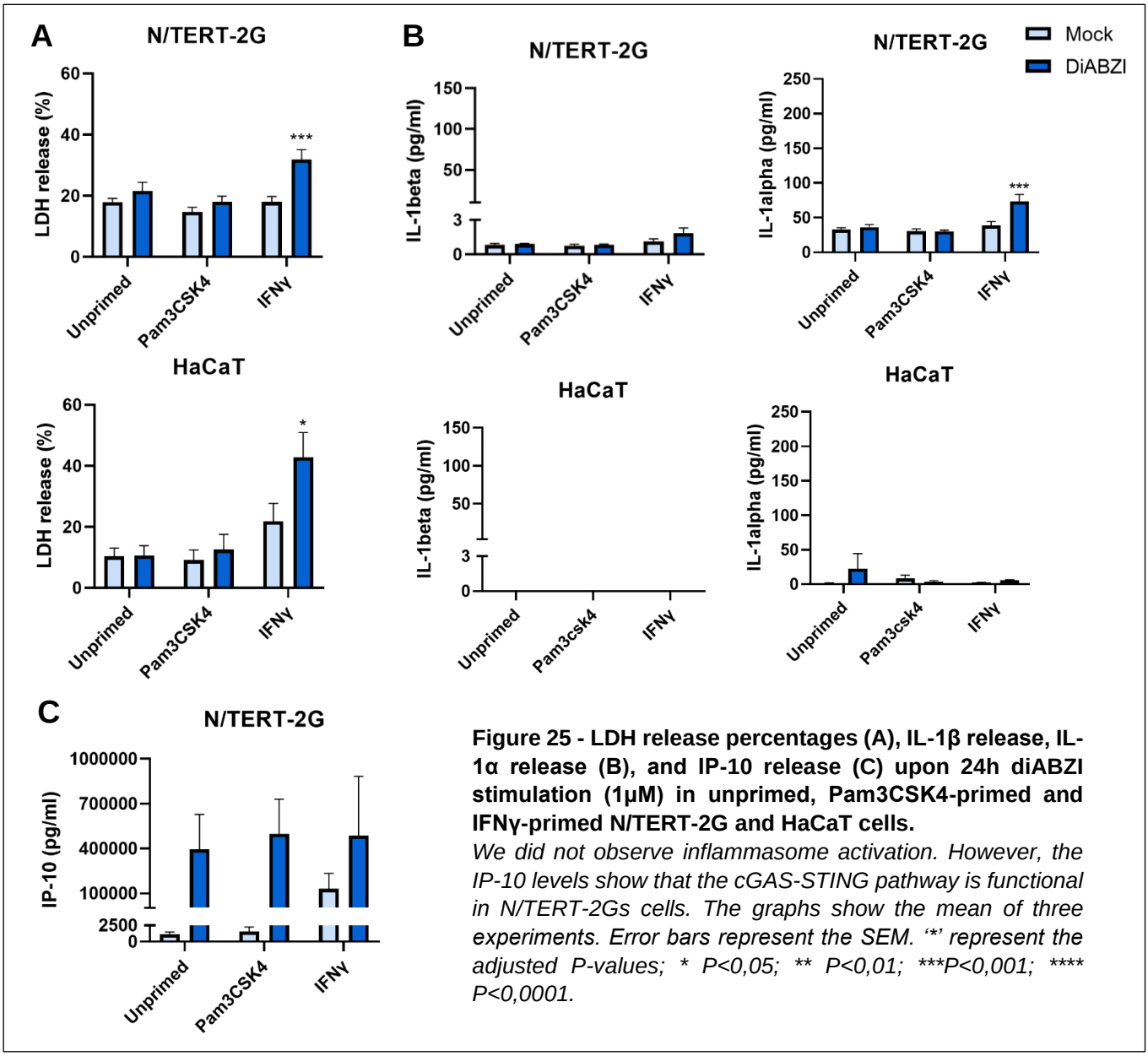


**Figure 25 - LDH release percentages (A), IL-1β release, IL-1α release (B), and IP-10 release (C) upon 24h diABZI stimulation (1µM) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.**
*We did not observe inflammasome activation. However, the IP-10 levels show that the cGAS-STING pathway is functional in N/TERT-2Gs cells. The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001.*

### 7.5.3. NLRC4/NAIP inflammasome stimuli

#### *7.5.3.1. LFn-Needle*

We did not observe any significant changes upon 100µg/ml LFn-Needle stimulation in both cell lines during preliminary experiments. Therefore, we discontinued research on this stimulus.

### 7.5.4. AIM2 inflammasome stimuli

#### *7.5.4.1. Herring testes DNA*

In this project, we transfected HT-DNA into the cells, to assess the role of both NLRP3 and AIM2 in N/TERT-2G and HaCaT cells. Similar to our previous experiments with Poly (I:C) transfection, we transfected 6,7µg/ml HT-DNA (1µg/well) using Lipofectamine 2000. HT-DNA stimulation alone was used as an extra mock control to confirm that the transfection of HT-DNA is essential for activation.

Using LDH activity assays, we witnessed cell death in both N/TERT-2G and HaCaT cells, with the most significant change being observed with IFNγ priming. [Figure 26A] However, Luminex assays detected no rise in IL-1β levels [Figure 26B], refuting that inflammasome activation is promoted by HT-DNA. Notably, this is markedly contrasting to transfected Poly (I:C) that did induce inflammasome activation.

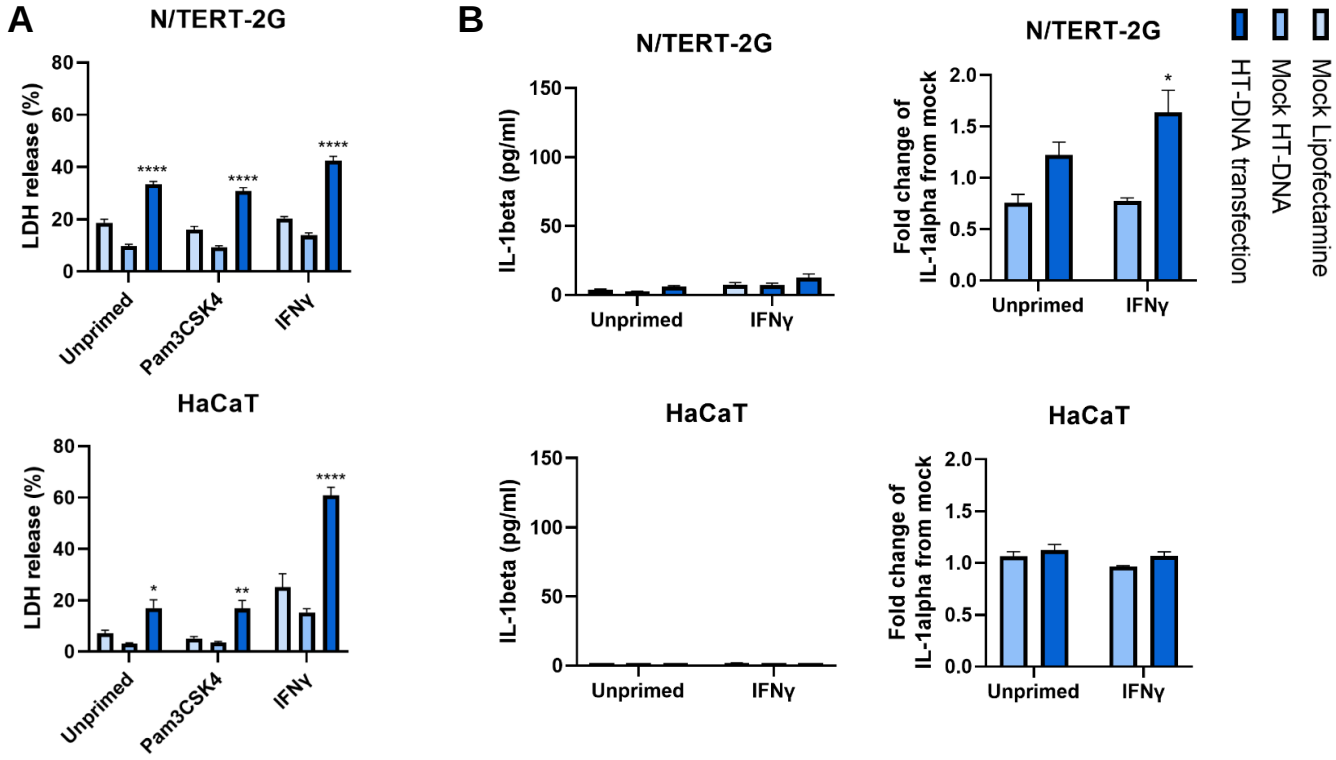


**Figure 26 - LDH release percentages (A), IL-1β release, IL-1α release (B), and upon 24h HDNA stimulation (1µg/well) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.**
*There were no markers for inflammasome activation present. The graphs present the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001.*

### 7.5.5. Pyrin inflammasome stimuli

#### *7.5.5.1. Clostridium difficile toxin A*

Although the Pyrin inflammasome has been described in dept in BMDM and mutations in the gene encoding this inflammasome sensor causes the autoinflammatory disease FMF, knowledge of its function in keratinocytes is scarce.[31] We assessed the possibility of Pyrin activation in N/TERT-2G and HaCaT cells by stimulation with 1µg/ml TcdA in both cell culture models.

LDH activity assays revealed a substantial increase of cell death in some priming conditions. [Figure 27A] In the N/TERT-2G cells we could distinguish a significant change in cell death in the IFNγ priming conditions, while the HaCaTs showed a trend towards overall increase in unprimed, Pam3CSK, and IFNγ primed conditions (although not significant). Nevertheless, IL-1β release remained absent in both cell lines, suggesting there is no activation of any inflammasome in both cell lines. [Figure 27B]

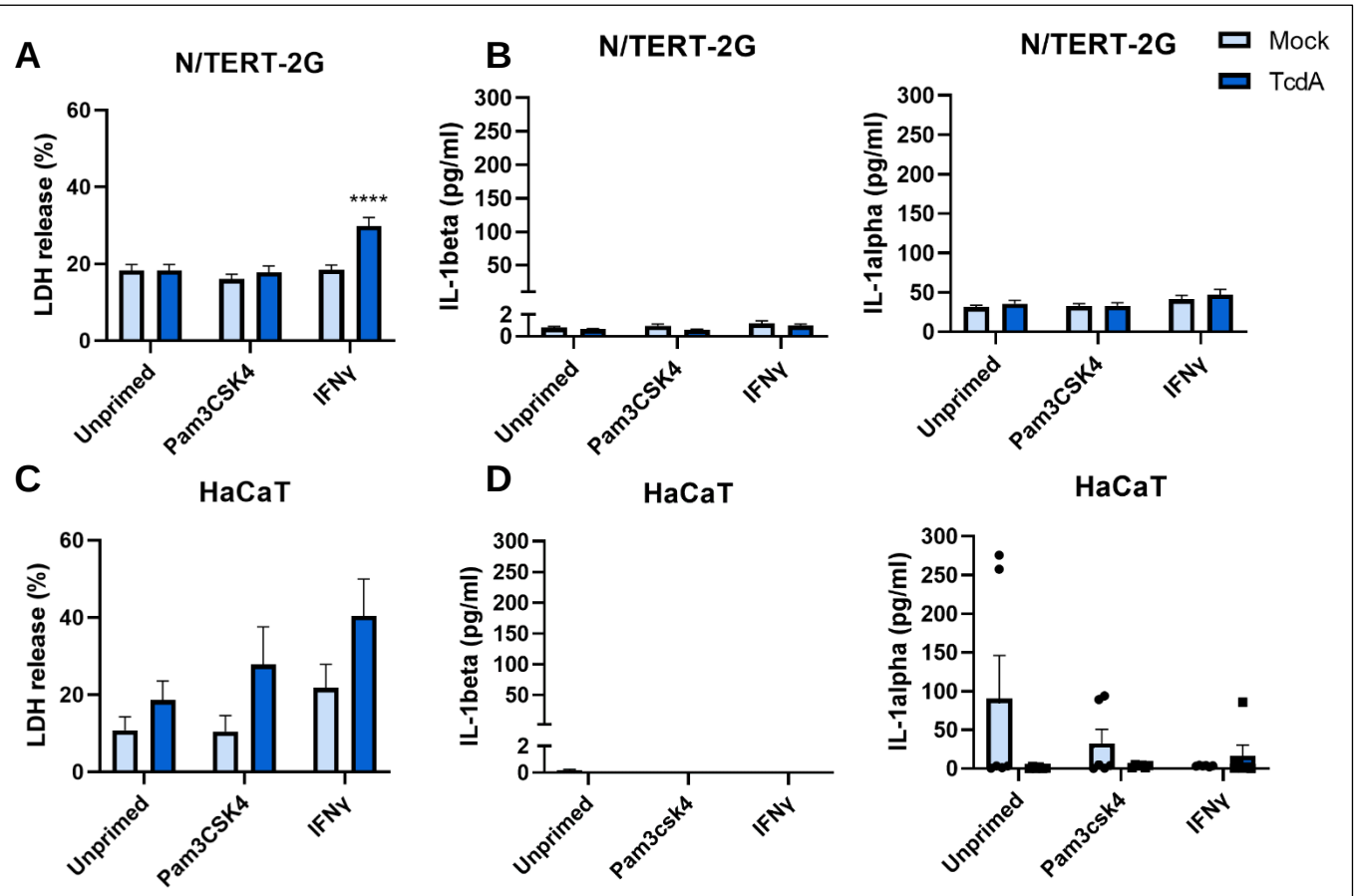


**Figure 27 - LDH release percentages (A), IL-1β release, and IL-1α release (B) upon 24h TcdA stimulation (1µg/ml) in unprimed, Pam3CSK4-primed and IFNγ-primed N/TERT-2G and HaCaT cells.**
*There were no signs of inflammasome activation present. The graphs show the mean of three experiments. Error bars represent the SEM. '*' represent the adjusted P-values; * P<0,05; ** P<0,01; ***P<0,001; **** P<0,0001.*

# 8. DISCUSSION

Although inflammasomes have already been well-documented in mouse myeloid cells, insight into the role of inflammasomes in human keratinocytes remains relatively limited. This work sought to elucidate the inflammasome repertoire and characterize their activation mechanisms in two keratinocyte cell lines and compare the results obtained with the scientific literature.

Firstly, we explored various cultivation conditions of N/TERT-2G cells and the HaCaT cell lines. Whereas HaCaT grew robustly in a number of cell media, N/TERT-2G cells were more difficult to maintain into culture. Eventually, we succeeded in growing them in supplemented Epilife medium under culturing conditions also used for primary keratinocyte cultures. We cannot fully define which factors restrict their growth in other conditions, but our detailed documentation of the successful culture optimization process will provide an interesting resource for future use of this cell line in the laboratory. Furthermore, the complications we encountered highlight the complexity of reaching reproducibility between laboratories.

Using our optimized growth conditions, we next systematically examined inflammasome activation in our keratinocyte cell lines using a list of stimuli that are well-established to induce activation of specific inflammasomes in macrophages and monocytes. In comparing our results with those of the scarce literature, we identified a number of commonalities as well as inconsistencies that I will discuss here.

The first unexpected results emerged from Luminex analysis of unprimed, Pam3CSK4-primed and IFNγ-primed cells. Unlike IFNγ, which induced immense IP-10 secretion levels in culture supernatants, Pam3CSK4 proved inadequate for priming of keratinocyte cell lines. Indeed, Pam3CSK4 was incapable to activate the NF-κB pathway and could not induce IL-6 and TNF after 17h of priming in N/TERT-2G, NHEK and HaCaT cells. Even when N/TERT-2G cells were stimulated with Poly (I:C) transfection for 24h, we did not observe a significant increase in abovementioned cytokines. This outcome was unforeseen since primary keratinocytes have been proven to functionally express TLR2 and TLR3, and NF-κB activation is already described upon priming with 5µg/ml Pam3CSK4 or Poly (I:C) transfection for less than 6h.[52,74] Furthermore, we did observe basal levels of IL-6 and TNF in N/TERT-2G cells and a slight increase in IL-6 and TNF in the HaCaTs, showing that the NF-κB pathway is intact in these cells.

From our results, we have no evidence that NLRP3 activation can occur in keratinocytes. According to our Western blot results, N/TERT-2G cells express NLRP3, however it is still unknown if these levels are sufficient to activate the NLRP3 inflammasome. Strittmatter et al. observed NLRP3 inflammasome activation in primary keratinocytes upon stimulation with cytosolic DNA and independent of priming.[33] This is in contradiction with our experiments in HaCaT and N/TERT-2G cells using transfection of Herring Testes-DNA (HT-DNA). Nevertheless, in line with what is observed in bone marrow derived macrophages (BMDMs) or monocytes, priming conditions could augment NLRP3 expression to stimulate its activation.[75] As we mentioned before, LPS priming is a widely-used priming condition for NLRP3 expression upregulation. However transfection experiments are not feasible with this priming as cytosolic LPS activates the non-canonical NLRP3 inflammasome in BMDMs and could lead to IL-1β in keratinocytes.[76] However, LPS priming in the presence or absence of transfection agents is not assessed in the cell lines in this thesis. Other

priming agents, such as TNF, could be assessed to substitute priming conditions for future transfection experiments.[77]

More unforeseen results transpired from SDS-PAGE experiments. As aforementioned, we revealed that N/TERT-2G cells express NLRP3 in an unprimed state. This is in great contrast to our results in the NHEK cells. Although Dai et al. and Watanabe et al. have suggested that neonatal primary keratinocytes express NLRP3 in an unprimed state, we could not conclude the same because we observed no NLRP3 expression via Western blotting in NHEK cells.[72,77] Moreover, our Western blot analysis showed that N/TERT-2G cells expressed significantly higher NLRP1 levels than NHEK cells and definitely much more than the HaCaT cell line. The NLRP1 expression levels in HaCaTs and NHEKs were already discussed by Watanabe et al. and are in accordance with our results. In contrast, Dai et al. suggested that primary keratinocytes do not express NLRP1 basally.[77] Nevertheless, NLRP1 expression levels in N/TERT-2G cells have not been reported in the literature yet. Although we don't have a clear explanation, the contradictory results between research groups regarding NLRP1 and NLRP3 expression in keratinocytes could be caused by various underlying differences such as the difference in culture conditions, variation in lysis conditions and loading of the samples, differences in specificity and sensitivity of the antibodies etc.

Interestingly, we observed important differences between the tested cell lines and primary cells in our stimulation experiments. [Table 2] We found that VbP activated the NLRP1 inflammasome in unprimed N/TERT-2G cells. This is in accordance with other recent publications in NHEK and N/TERT-2G cells and the mechanism of activation by VbP is already described in these reports.[12,79] However, our data in the HaCaT cell line showed that HaCaT cells do not display any signs of inflammasome activation by VbP. This result, together with the Western blot results for NLRP1 suggest that NLRP1 expression levels in HaCaT are inadequate for effective NLRP1 inflammasome activation in this cell line. More proof in support of this concept was obtained through stimulation experiments using anisomycin. Robinson et al. recently described anisomycin as an NLRP1 inflammasome activator in both primary and N/TERT cells and we obtained confirmed this in the N/TERT-2G cells.[2] Nevertheless, similar to VbP stimulation, we could not detect any markers for inflammasome activation upon 24h anisomycin stimulation in the HaCaT cells. Together, these results indicate that NLRP1 inflammasome signaling can be studied in N/TERT and NHEK cells, but not in the HaCaT cell line. Further research, using gene sequencing, qPCR, and Western blotting could clarify why NLRP1 activation is defective in these cells.

Although we did not have sufficient time during the thesis to broadly examine expression of many inflammasome components by Western blotting in the N/TERT-2G cells, the fact that inflammasomes can be activated in unprimed NTERG-2G cells by VbP and anisomycin is a point of proof that an adequate amount of pro-IL-1, GSDMD, ASC, pro-Caspase-1 and NLRP1 is present in keratinocytes.

We next focused on stimulation experiments using Poly (I:C) transfection. These experiments allowed us to conclude that, once more, inflammasome activation by this stimulus varies between keratinocyte cell lines. We observed inflammasome activation in all priming conditions of the N/TERT-2G cells, but not in HaCaT cells. Since we propose that N/TERT-2G cells express both NLRP1 and NLRP3 in a basal state, both inflammasomes could be driving the observed inflammasome responses by transfected Poly (I:C). In support of this hypothesis, intracellular Poly

(I:C) is known to only activate the NLRP3 inflammasome in macrophages.[19,80] In contrast, Poly (I:C) transfection was described to activate the NLRP3 inflammasome in unprimed primary human keratinocytes but has been shown to activate the NLRP1 inflammasome in unprimed N/TERT cells.[16,17] We therefore assessed the impact of both inflammasomes via the pharmacological inhibition of NLRP1 and NLRP3. Through the use of MCC950, we revealed that the NLRP3 inflammasome did not affect IL-1β release in supernatants and cell death percentages remained unchanged in MCC950-pretreated N/TERT-2G cells. This outcome suggests that NLRP3 is dispensable and that NLRP1 may have a central role in this cell line. However, information on the impact of the NLRP1 inflammasome could not be completely obtained. In preliminary experiments, we could not detect a decrease in cell death levels using various concentrations of bortezomib (BTZ), a proteasome inhibitor that indirectly inhibits NLRP1 activation. However, Luminex assays need to be performed to assess cytokine levels in the supernatants for a more sensitive readout of its potential impact. As depicted in literature as well as this thesis, differences obtained in different cell lines and primary cells are ubiquitous. A possible reason could be the use of different media for growing different cells in reported experiments. In agreement, in this thesis we encountered great variations in inflammasome activation with two different media as well. Equal concentrations of anisomycin showed greater IL-1β secretion levels in the Epilife medium supplemented with only EGF and BPE than in the original Epilife medium for our N/TERT-2G cells. VbP did not induce cell death in our N/TERT-2G cells in the second mentioned growth medium (data not shown), showing the critical impact the medium can have on results in *in vitro* experiments. However, these data stem from preliminary experiments and results should be further validated to achieve robust conclusions.

Using other well-established inflammasome stimuli, we showed that extracellular ATP, nigericin, diABZI, HT-DNA and TcdA all fail to trigger inflammasome activation in the N/TERT-2G and HaCaT cells. Indeed, none of these stimuli promoted IL-1β release in culture supernatants as measured by Luminex assay. This suggests that the AIM2, NLRP3 and Pyrin inflammasomes may not be functional. Our data from ATP stimulation in HaCaTs are in accordance with findings from Zheng et al.[81] This group described that LPS priming and stimulation with extracellular ATP did not induce IL-1β levels in HaCaT cells. However, experiments in primary keratinocytes from healthy donors reported by Gruber et al. suggested inflammasome activation since caspase-1 activation upon 20h stimulation with 5mM ATP was observed.[28] A possible cause for these differences could be due to the fact that our NHEK data were preliminary (transpired from one experiment performed in technical duplicates) and should be further confirmed. The data obtained with nigericin stimulation are in accordance with reports from two other research groups that observed no caspase-1 activation and IL-1β release upon nigericin stimulation.[16,28] However, Fenini et al. detected IL-1β release upon stimulation with nigericin in primary keratinocytes that could be inhibited with NLRP1 knockouts.[13] Data from stimulation experiments using diABZI and HT-DNA, both stimuli that influence the cGAS-STING pathway, did not show any promising results in our hands. Although this pathway is described to activate the NLRP3 inflammasome in myeloid cells, not much is known about their effects in keratinocytes.[24] Our analyses showed that the cGAS-STING pathway is functional in N/TERT-2G cells, however, we failed to detect inflammasome activation with these stimuli in both N/TERT-2G and HaCaT cells. IP-10 was not measured in HaCaT cells in this project, however, Li et al. described that the cGAS-STING pathway is functional in this cell line and induces apoptosis.[82] This is in accordance with our experiments that prove that the inflammasome is not activated by these cGAS-STING agonists. Although we determined that cytosolic dsDNA did not

induce inflammasome activation in our cell systems, the literature does describe an important role of this stimulus in promoting inflammasome activation in myeloid cells. In macrophages it is a well-established NLRP3 activator.[36] However, its ability to promote inflammasome activation in keratinocytes remains open for discussion. Dombrowski et al. observed inflammasome activation in IFNγ primed primary keratinocytes derived from healthy people but not in unprimed cells. Only when primary keratinocytes were derived from psoriasis patients, priming of the cells was redundant since AIM2 was already upregulated.[38] However, Kopfnagel et al. observed AIM2 expression and IL-1β release in unprimed healthy keratinocytes.[37] Moreover, Bauernfried et al. did not observe any IL-1β release in the N/TERT cells upon 10h dsDNA transfection.[16] Now, we reveal that even with the addition of IFNγ, there is no inflammasome activation to be observed in neither the N/TERT-2G and HaCaT cells.

In a final set of experiments, we assessed the ability of TcdA to promote inflammasome activation. TcdA has been shown to activate the pyrin inflammasome in macrophages.[40] However, no experiments with this stimulus had been described in keratinocytes yet. In this project, we revealed that TcdA does not induce inflammasome activation in both N/TERT-2G and HaCaT cells, using concentrations that induce robust pyrin activation in macrophages. This prompts the question if the pyrin inflammasome plays a role in keratinocytes or not.

Although we uncovered many important novelties concerning inflammasomes in keratinocytes, research into this subject is far from finished yet. For instance, the further use of primary cells, derived from different donors, could reveal more important variations between keratinocyte cell lines, primary keratinocytes, and other cell types. Furthermore, the exact timeline of when the studied stimuli influence certain pathways could clarify variations between publications. In this project we observed cell death induction and cytokine release after 24 hour stimulation to capture all possible inflammasome-linked events. However, many articles describe shorter stimulation times, which could cause that inflammasome activation by certain stimuli is missed. Furthermore, longer stimulation times to assess inflammasome activation could influence the results negatively as well. Proteins involved in our experiments, including LDH and various cytokines, have varying half-lives. The possibility exists that some protein levels could be underestimated. Therefore, we suggest the use of kinetic live-cell analysis to observe the cell death induction by the stimuli or even inhibitors at various timepoints. Even though we assessed the expression levels of various proteins via Western blotting in unprimed cells, the use of SDS-PAGE or qPCR could help clarify the different results we often observed in the various cells. Moreover, genetic studies could uncover mutations that are the cause of these differences. Eventually, since the use of inhibitors is often not fully feasible due to cytotoxicity or aspecificity, we could make use of knockouts via Crispr-CAS9 or small interfering RNAs (siRNA) to genetically confirm the key involvement of specific inflammasome pathways.

| Stimulus | Literature research | Results |
|---|---|---|
| **VbP** | Keratinocytes<br>NLRP1 inflammasome activator in N/TERT-1 and primary keratinocytes.[79]<br>Myeloid cells<br>Activates CARD8 in monocytes.[1] | NLRP1 activator in N/TERT-2G cells but not in HaCaT cells. |
| **UVB/ Anisomycin** | Keratinocytes<br>UVB is a NLRP1 inflammasome activator in primary keratinocytes.[13]<br>Anisomycin is a NLRP1 inflammasome activator in N/TERT-1 and primary keratinocytes.[2]<br>UVB triggers apoptosis via cGAS-STING pathway in HaCaT cells.[82]<br>Myeloid cells<br>/ | Anisomycin is an NLRP1 activator in N/TERT-2G cells but not in HaCaT cells. |
| **DsRNA (Poly (I:C) transfection)** | Keratinocytes<br>NLRP1 inflammasome activator in unprimed primary cells, 10h stimulation.[16]<br>NLRP3 inflammasome activator in unprimed primary cells, 24h stimulation.[17]<br>Myeloid cells<br>NLRP3 inflammasome activation in macrophages.[19,80] | We observed NLRP3-independent inflammasome activation in N/TERT-2G cells. HaCaTs did not show inflammasome activation markers. |
| **ATP** | Keratinocytes<br>Caspase-1 activation upon ATP stimulation in unprimed primary cells.[28]<br>Inflammasome activation in Vitiligo patient-derived primary cells.[29]<br>No inflammasome activation upon LPS+ATP stimulation in HaCaTs.[81]<br>Myeloid cells<br>Widely-used NLRP3 inflammasome activator.[27] | No inflammasome activation in N/TERT-2G, NHEK and HaCaT cells. |
| **Nigericin** | Keratinocytes<br>NLRP1 inflammasome activator in unprimed primary cells.[13]<br>No inflammasome activation upon nigericin stimulation in unprimed NTERT-1 cells.[16]<br>No caspase-1 activation after 20h nigericin stimulation in primary cells. [28]<br>Myeloid cells<br>Widely-used NLRP3 inflammasome activator.[27] | No inflammasome activation in N/TERT-2G and HaCaT cells. |
| **DiABZI** | The use of diABZI has not been described in inflammasome-linked studies keratinocytes. | The cGAS-STING pathway is activated in N/TERT-2G cells. No inflammasome activation was observed. |

| | | |
|---|---|---|
| **DsDNA (HT-DNA)** | Keratinocytes<br>No inflammasome activation upon dsDNA stimulation in unprimed NTERT-1 cells.[16]<br>AIM2 activator in unprimed primary keratinocytes.[37]<br>AIM2 activator in IFNγ primed primary keratinocytes.[38]<br>Myeloid cells<br>NLRP3 activator in unprimed monocytes.[24] | No inflammasome activation in N/TERT-2G and HaCaT cells. |
| **TcdA** | Keratinocytes<br>/<br>Myeloid cells<br>NLRP3 inflammasome activator in macrophages.[40] | No inflammasome activation in N/TERT-2G and HaCaT cells. |

**Table 2 – Summary of inflammasome activation for each stimulus in human cells according to the literature and our experiments.**

# 9. GENERAL CONCLUSION

We can conclude that Val-boroPro, anisomycin and intracellular Poly (I:C) do activate the inflammasome in N/TERT-2G cells, accentuating the essential role of keratinocytes in skin immunity. However, any other stimulus assessed in this project failed to influence inflammasome activation in general. Furthermore, we did not observe any inflammasome activation in the widely-used HaCaT cells, revealing the inferiority of this cell line for inflammasome research, especially for the NLRP1 inflammasome.

The results from our experiments and literature research advocate that the extrapolation of data from one cell type to the other needs to be contemplated carefully. Moreover, we emphasize that even between cell lines and primary cells of the same cell type the outcome of experiments can deviate.

Overall, this project has granted crucial insights into the mechanisms of inflammasome activation in keratinocytes, possibly aiding in the discovery of future therapies against epithelial diseases.

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## 11. POSTER

FACULTY OF MEDICINE AND HEALTH SCIENCES

LABORATORY OF MEDICAL IMMUNOLOGY

# ACTIVATION OF THE NLRP1 INFLAMMASOME IN EPITHELIAL CELLS

**Jade Celis, Oonagh Paerewijck, Lieselotte Vande Walle and Mohamed Lamkanfi**

## 1. Introduction

Pathogen recognition receptors (**PRR**) are an essential component of the innate immune system, protecting the host from various threats. Several PRRs **initiate an immune response by assembling into inflammasomes**. These multi-protein complexes elicit the **release of** intracellular proteins such as **IL-1β and lactate dehydrogenase (LDH)**, eventually causing an inflammatory cell death called **pyroptosis**.[1] [Fig. 1]

Imbalance of inflammasome activation has been associated with various serious diseases including **Alzheimer's disease, atherosclerosis, vitiligo, crystal arthropathies and cancer**.[2,3]

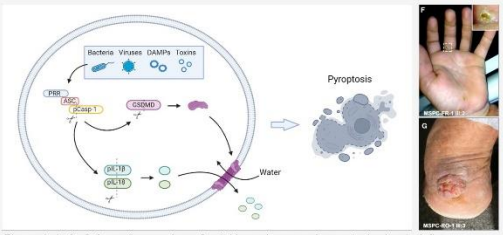


*Figure 1: Left: Schematic overview of cytokine release and pyroptosis due to inflammasome activation. Right: multiple self-healing palmoplantar carcinoma (MSPC) due to mutations in NLRP1.*[4]

**Hypothesis**

At present, differences in inflammasome activation between various epithelial cell types are still insufficiently identified. Insight into this subject would not only lead to better strategies for future therapies in epithelial tissue diseases but would also help to clarify how these different cell types can be used in inflammasome research. In this project, **we are investigating inflammasome activation upon specific stimulation in three different keratinocyte cell culture models.**

## 2. Experimental approach

Primary keratinocytes (NHEK) and two immortalized keratinocyte cell lines, HaCaT and N/TERT-2G (N/TERT-2Gs were kindly gifted by J. Rheinwald from Harvard University), are first plated and cultured. Next, they are **primed** with the TLR1/2 agonist Pam3csk4 or with interferon-γ (IFNγ) for 17 hours followed by **stimulation** with specific inflammasome activators as indicated in Table 1. Subsequently, cell culture media are collected and used to **measure levels of pyroptosis by LDH activity assay** and to investigate levels of the inflammasome-dependent cytokine IL-1β by **Luminex assay**. An overview of our experimental approach is visualized in Figure 2. We will repeat certain experiments with inflammasome **inhibitors** to establish **if the observed cell death and cytokine release is inflammasome specific**.

| **Stimuli:** | |
|---|---|
| NLRP1 | Val-boroPro (VbP)<br>Poly (I:C)<br>Anisomycin |
| NLRP3 | ATP |
| AIM2 | DsDNA |
| Pyrin | *C. difficile* toxin A |
| **Inhibitors:** | |
| NLRP3 | MCC950/CRID3 |
| NLRP1 | Bortezomib (proteasome) |
| CASP1 | VX-765 |

Table 1: Top: overview of the stimuli already tested in this project. Bottom: the inhibitors we will use in future experiments.

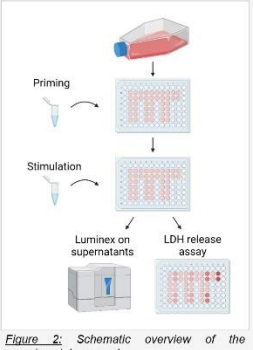


*Figure 2: Schematic overview of the experimental approach.*

## 3. Results

Preliminary experiments in NHEK and HaCaT cells show **different outcomes between both cell types upon inflammasome stimulation**. For instance, stimulation with the anti-tumor drug Val-boroPro (VbP), a well established NLRP1 inflammasome activator[4] displayed clear cell death and IL-1β release in NHEK cells. However, in HaCaT cells these changes were absent. [Fig. 3]

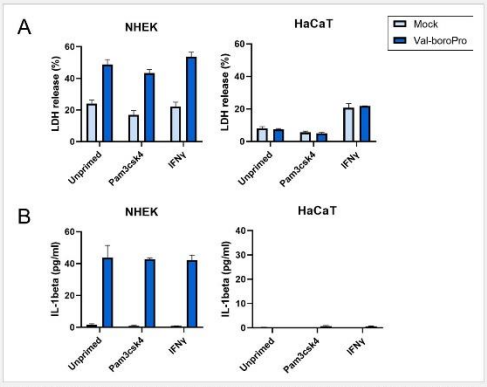


*Figure 3: LDH release **(A)** and IL-1β release **(B)** after stimulation of unprimed, Pam3csk4- and IFNγ-primed NHEK and HaCaT cells with 10μM VbP. NHEKs were primed for 4h and stimulated with VbP for 16h. HaCaTs were primed for 17h and stimulated with VbP for 24h. NHEK graphs show the mean of preliminary results derived from one experiment in technical duplicates. HaCaT graphs show the mean of one experiment representative of three independent experiments. Error bars represent the standard deviation.*

Moreover, we did not observe a significant change in LDH and IL-1β release in HaCaT cells upon stimulation with anisomycin while in NHEKs the cell death increased greatly upon stimulation. [Fig. 5] Anisomycin was recently reported to activate the NLRP1 inflammasome in NHEKs.[6] However, Luminex assays still need to be performed in NHEKS since anisomycin is an eukaryotic protein synthesis inhibitor and can be toxic by itself.[7]

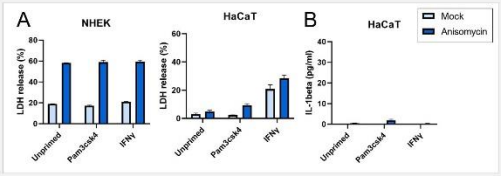


*Figure 5: LDH **(A)** and IL-1β release **(B)** in supernatants of HaCaT cells and LDH release in NHEK cells **(A)** after stimulation with anisomycin (5 μM) in unprimed, Pam3csk4- and IFNγ-primed cells. HaCaTs were primed for 17h and stimulated with anisomycin for 24h. NHEKS were primed for 4h and stimulated for 20h. NHEK graphs show the mean of preliminary results derived from one experiment in technical duplicates. HaCaT graphs show the mean of one experiment representative of three independent experiments. Error bars represent the standard deviation.*

After completion of these studies, we will next use the various inhibitors in Table 1 to address whether these responses are inflammasome-mediated. In addition, we will test various additional stimuli on the NHEK and HaCaT cells and we will perform similar experiments in N/TERT-2G cells.

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GHENT UNIVERSITY

**Contact**
Jade.Celis@ugent.be
Jade Celis