Aus dem Zentrum für Innere Medizin der Universität zu Köln
Klinik und Poliklinik für Innere Medizin I
Direktor: Universitätsprofessor Dr. med. Michael Hallek

Deciphering the role of viral glycoproteins in NLRP3 inflammasome activation and pyroptosis in THP-1 macrophages

Inaugural-Dissertation zur Erlangung der Doktorwürde
der Medizinischen Fakultät
der Universität zu Köln

vorgelegt von
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promoviert am 22. Januar 2026

Gedruckt mit Genehmigung der Medizinischen Fakultät der Universität zu Köln
2026

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Viruses. 2021 Oct 15;13(10):2076. doi: 10.3390/v13102076.

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Danksagung

An dieser Stelle möchte ich allen danken, die mir mein Studium und das Schreiben dieser Promotion ermöglichten:

Prof. Dr. Michael Hallek danke ich für die Möglichkeit die Doktorarbeit in seiner Klinik anzufertigen.

Meinem Doktorvater, Dr. Dr. Jan Rybniker, danke ich für die herzliche Aufnahme in seine Arbeitsgruppe und die stetige Unterstützung während des Projektes.

Meinem Betreuer, Dr. Sebastian Theobald, danke ich für die Einarbeitung in das Projekt und für seine Geduld bei all meinen Fragen. Zudem für die stetige Hilfsbereitschaft während meiner Arbeit im Labor.

Dr. Alexander Simonis, Sandra Winter, Jason Chhen danke ich für die Einführung in die Experimente und die vielen aufmunternden Worte.

Der ganzen Arbeitsgruppe danke ich für die herzliche Aufnahme in das Team, die angenehme Atmosphäre im Labor und die vielen Mittagspausen, die wir zusammen verbracht haben.

Für die Durchsicht meiner Arbeit danke ich Dr. Dr. Jan Rybniker und Dr. Sebastian Theobald.

Ganz besonders danke ich meiner Familie, die mir das Studium ermöglicht hat und mich stets uneingeschränkt unterstützen. Zudem danke ich meinen Freund:innen, die immer motivierende Worte für mich gefunden haben.

Für die finanzielle Unterstützung danke ich dem Köln Fortune Programm sowie der Studienstiftung des deutschen Volkes.

Inhaltsverzeichnis

ABKÜRZUNGSVERZEICHNIS 6

1. ZUSAMMENFASSUNG 7

2. SUMMARY 9

3. INTRODUCTION 11

3.1. Innate immune system 11

3.2. Inflammasomes 11
3.2.1. NLRP1 inflammasome 12
3.2.2. NLRP3 inflammasome 13
3.2.3. NLRC4 inflammasome 14
3.2.4. AIM2 inflammasome 14
3.2.5. Pyrin inflammasome 14

3.3. Pyroptosis 15

3.4. PAMPs and DAMPs – triggers of immune responses 15
3.4.1. Viral glycoproteins 16

3.5. Research question and objectives 17

4. PUBLICATION 18

5. DISCUSSION 34

5.1. Viral glycoproteins and innate immunity 34

5.2. The role of viral glycoproteins and inflammasomes in vaccine-induced immunity 35
5.2.1. Short overview of vaccine development 35
5.2.2. Glycoproteins and inflammasomes as potential targets 36

5.3. Immunological consequences and other potential pathways 36
5.3.1. Receptors for cellular entry and immune response 37
5.3.2. NLRP3 inflammasome activation – signal 1 and signal 2 38
5.3.3. Other inflammasomes 39

5.4. Outlook **40**

6. LITERATURVERZEICHNIS 41

7. ANHANG 44

7.1. Abbildungsverzeichnis **44**

Abkürzungsverzeichnis

AIM2	Absent in melanoma 2
ATP	Adenosin triphosphate
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
COVID-19	Coronavirus disease 2019
DAMP	Danger-associated molecular patterns
DNA	Deoxyribonucleic acid
gB	Glycoprotein B
GSDMD	Gasdermin D
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
IL	Interleukin
mROS	Mitochondrial reactive oxygen species
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRC4	nucleotide-binding oligomerization domain-like receptor family caspase activation and recruitment domain-containing protein 4
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing
N-protein	Nucleocapsid protein
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
S-protein	Spike protein
SARS-CoV	Severe acute respiratory syndrome coronavirus
TLR	Toll-like receptor

1. Zusammenfassung

Infektionen mit viralen Erregern sind weit verbreitet und können eine Vielzahl unterschiedlicher Krankheiten hervorrufen. Die globale Coronavirus disease 2019 (COVID-19) Pandemie hat eindrücklich gezeigt, welch tiefgreifenden Einfluss akute virale Infektionen auf die Gesundheit und die Gesellschaft nehmen können. Ein zentraler Aspekt im Kampf gegen virale Infektionen ist das Verständnis der viralen Pathogenese. Dafür ist ein tiefgreifendes Wissen über die Mechanismen erforderlich, durch die Viren eine Immunreaktion hervorrufen. Die angeborene Immunabwehr, angeführt von Inflammasomen, spielt dabei eine Schlüsselrolle, indem sie Pathogene erkennt und eine Immunantwort initiiert. Zahlreiche Studien zeigten, dass Viren Inflammasome aktivieren können, doch die spezifischen viralen Strukturen, die für diese Aktivierung verantwortlich sind, sind größtenteils noch unbekannt.

Das Ziel der vorliegenden Arbeit war die Untersuchung von vier verschiedenen viralen Glykoproteinen als mögliche Auslöser der Aktivierung des Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (NLRP) 3 Inflammasoms und der Pyroptose in THP-1-Makrophagen. Untersucht wurden das Spike-Protein (S-Protein) des Severe acute respiratory syndrome coronavirus (SARS-CoV) 1 und SARS-CoV-2, das Glykoprotein B (gB) des humanen Cytomegalievirus (HCMV) und E2 des Hepatitis-C-Virus (HCV).

Unter Verwendung verschiedener Methodiken, einschließlich Enzyme-linked Immunosorbent Assays, Western-Blots und Immunofluoreszenz, wurden die Auswirkungen der Stimulation von THP-1-Makrophagen mit den genannten viralen Glykoproteinen untersucht. Im Fokus der Studie stand die Messung der Interleukin (IL)-1β-Sekretion, die Analyse der Expression von NLRP3 und des Apoptosis-associated speck-like proteins containing a caspase-recruitment domain (ASC) sowie die Untersuchung der Bildung von ASC-Specks, einem kritischen Schritt in der Aktivierung des Inflammasoms. Zusätzlich wurden NLRP3- und Gasdermin D (GSDMD) Knockout-Zellen eingesetzt, um die Rolle dieser Proteine in der beobachteten Immunantwort zu validieren. Der GSDMD-vermittelte pyroptotische Zelltod, als Folge der Inflammasom-Aktivierung nach Exposition mit Glykoproteinen, wurde anhand von Laktatdehydrogenase-Freisetzungsassays und Durchflusszytometrie untersucht.

Die Stimulation der THP-1-Makrophagen mit den ausgewählten viralen Glykoproteinen resultierte in einer erhöhten Sekretion von IL-1β, einer Hochregulierung von NLRP3 und der Bildung von ASC-Specks. Die Deletion von NLRP3 oder GSDMD hatten eine signifikante Reduktion der IL-1β-Sekretion zur Folge. Zudem zeigten die Experimente, dass die Exposition gegenüber den viralen Glykoproteinen zu einem verstärkten Zelltod führte.

Die Ergebnisse legen nahe, dass die untersuchten viralen Glykoproteine als Auslöser des NLRP3-Inflammasoms, sowie der Pyroptose, in THP-1-Makrophagen fungieren können. Diese Studie leistet hierdurch einen Beitrag zum Verständnis der Mechanismen in der viralen Pathogenese. In zukünftigen Schritten sollten die Ergebnisse idealerweise *in vivo* bestätigt werden. Offene Fragen betreffen die Rezeptoren, an die die untersuchten Glykoproteine binden, um das NLRP3-Inflammasom in einem ersten Schritt zu aktivieren. Zudem bleibt zu klären, ob andere Inflammasome an der Immunantwort beteiligt sind.

2. Summary

Infections with viral pathogens are a major global health concern, as highlighted by the Coronavirus disease 2019 (COVID-19) pandemic. Understanding the mechanisms of viral pathogenesis, including how viruses modulate the immune response, is a crucial component in the fight against viral infections. Inflammasomes, particularly the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP) 3 inflammasome, play a pivotal role in innate immunity by detecting pathogens and initiating an immune response. Although multiple studies have shown that viruses can activate inflammasomes, the specific viral components responsible for this activation remain largely undefined. This study aimed to investigate the role of four viral glycoproteins, namely the spike protein (S-protein) of the severe acute respiratory syndrome coronavirus (SARS-CoV) 1 and SARS-CoV-2, glycoprotein B (gB) of the human cytomegalovirus (HCMV), and E2 of the hepatitis C virus (HCV), in triggering NLRP3 inflammasome activation and pyroptosis in THP-1 macrophages, thereby offering new insights into viral pathogenesis.

Employing a range of methodologies, including enzyme-linked immunosorbent assays, western blots, and immunofluorescence, this research investigated the stimulatory effects of these viral glycoproteins on THP-1 macrophages. The study focused on measuring interleukin (IL)-1β secretion, on the analysis of the expression levels of NLRP3 and apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) proteins, and on examining the formation of ASC specks, a critical step in inflammasome activation. Furthermore, the research utilized NLRP3 and Gasdermin D (GSDMD) knockout cells to validate the role of these proteins in the observed immune response. GSDMD mediated pyroptotic cell death, as a consequence of inflammasome activation following glycoprotein exposure, was assessed through lactase dehydrogenase release assays and flow cytometry.

The findings revealed that stimulation with the selected viral glycoproteins led to increased IL-1β secretion, upregulation of NLRP3, and formation of ASC specks. Notably, the absence of NLRP3 or GSDMD significantly reduced IL-1β secretion. Additionally, exposure to viral glycoproteins resulted in enhanced cell death.

This study contributes to the field by identifying specific viral glycoproteins that can trigger the NLRP3 inflammasome and pyroptosis in THP-1 macrophages, thereby advancing our understanding of the complex interactions between viral pathogens and the host immune system. The research underscores the need for further investigations into the cellular receptors that these glycoproteins bind to initiate inflammasome activation. Moreover, it highlights the importance of exploring whether other inflammasomes are involved in the immune response to viral infections. Future research should aim to confirm these findings *in vivo* to expand our

knowledge of the molecular mechanisms underlying inflammasome activation by viral pathogens.

3. Introduction

Infectious diseases pose a significant threat to human health, arising from pathogens such as viruses, bacteria, fungi, and parasites. The human body has evolved multiple defense strategies against these pathogens, encompassing mechanical, chemical, and microbial barriers, alongside cellular and humoral immune responses. This study specifically concentrates on viral infections, with a particular focus on the interactions between four viral glycoproteins and the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing (NLRP) 3 inflammasome as part of the innate immune system.

3.1. Innate immune system

The immune system can be divided into the innate and the adaptive immune system. The innate immune system identifies pathogens through conserved molecular patterns known as pathogen-associated molecular patterns (PAMPs) [1]. Recognition of PAMPs by pattern recognition receptors (PRRs) initiates several responses, including phagocytosis, cell movement, pathogen or cell destruction, and cytokine production. Key effector cells within the innate immune system are phagocytes, such as neutrophils, monocytes, macrophages, and natural killer cells. Macrophages play a critical role during infections through the recognition and phagocytosis of pathogens or infected cells, and by producing various cytokines like interleukins (ILs) and tumor necrosis factor [1, 2]. The adaptive immune system, in contrast, targets pathogens with specificity through cellular and humoral responses, involving T-lymphocytes, B-lymphocytes, and antibodies. The formation of long-lived T and B cells creates an immune memory, enabling a faster response upon subsequent exposure to the same pathogen [1].

Historically considered non-specific and incapable of memory, newer research suggests that the innate immune system can exhibit trained innate immunity, characterized by adaptive features and memory-like capabilities. This phenomenon involves temporary, long-term functional reprogramming of cells, including bone marrow progenitors and macrophages, driven by epigenetic and metabolic changes, with lasting effects up to a year. Such reprogramming leads to an enhanced response to repeated stimuli. However, trained innate immunity can also provoke exaggerated immune responses, potentially resulting in tissue damage [2, 3].

3.2. Inflammasomes

Inflammasomes are a crucial component of the innate immune system and play a pivotal role as mediators of the immune response. Although various types of inflammasomes exist, they share common features. These multiprotein complexes assemble in response to activation signals, initiating a tightly regulated process. Through this process, the effector

protein caspase-1 is activated, leading to the activation and release of interleukins. Additionally, caspase-1 induces pyroptotic cell death, resulting in cellular lysis and the release of cytokines and other cellular contents. The proper activation of inflammasomes and the subsequent immune cascade are essential for protecting the body against infections. Conversely, dysregulation of inflammasomes can lead to hyperinflammation and tissue damage, potentially benefiting specific pathogens. Unrestricted inflammasome mediated inflammation is also associated with a range of pathogen independent pathologies, including autoinflammatory diseases, neurodegenerative disorders, and cancer [4, 5].

Inflammasomes can be activated through two main pathways: direct and indirect. Direct activation occurs when immune receptors interact directly with PAMPs or damage-associated molecular patterns (DAMPs). Indirect activation, on the other hand, involves the detection of damage or infection signals through cellular changes, such as alterations in ion fluxes, mitochondrial dysfunction, or the release of endogenous danger signals. Both mechanisms ensure a versatile and responsive defense system, capable of sensing a wide range of microbial threats or cellular injuries [4, 6].

3.2.1. NLRP1 inflammasome

The activation of the NLRP1 inflammasome is associated with several pathogens, including *Bacillus anthracis*, *Shigella flexneri*, *Toxoplasma gondii*, and *Listeria monocytogenes* [7, 8]. Furthermore, a role of the NLRP1 inflammasome has been postulated in monogenetic skin disorders [8].

Unlike other inflammasomes, the NLRP1 inflammasome features a domain with protease activity for self-cleavage, known as the function-to-find domain. However, self-cleavage of NLRP1 alone is not sufficient for its activation, as the carboxy-terminal effector domain remains inhibited by its interaction with the amino-terminal NLRP1b fragment. Studies in murine models have demonstrated that the amino-terminal NLRP1b fragment undergoes proteolytic cleavage, allowing for its ubiquitination and subsequent degradation. The mechanism of this process in human NLRP1 inflammasomes remains incompletely understood. Once the inhibitory domain is degraded, the carboxy-terminal effector domain initiates apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) speck formation, a process where the adaptor protein ASC is recruited and ASC molecules oligomerize. These specks acts as a platform for the recruitment and activation of caspase-1 [4, 5, 8].

3.2.2. NLRP3 inflammasome

The NLRP3 inflammasome, the most extensively investigated inflammasome, plays a critical role in the defense against bacteria, viruses, and fungi. It is also implicated in chronic inflammatory diseases such as diabetes, atherosclerosis, and Alzheimer's disease [9].

Activation of the NLRP3 inflammasome involves a two-step process. Initially, a priming signal, comprising either PAMPs or DAMPs recognized by PRRs, is necessary. This step activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), which then upregulates the expression of NLRP3 and pro-IL-1β. Subsequently, a second activation signal is required. Known activators include adenosine triphosphate (ATP), pore-forming toxins, lysosomal damage, mitochondrial reactive oxygen species (mROS), nucleic acids, and invading pathogens. Exposure to these signals triggers the oligomerization of the adaptor protein ASC, leading to the assembly of the inflammasome. This assembly activates the protease caspase-1, which processes IL-1β for release into the extracellular environment. Additionally, Gasdermin D (GSDMD) and IL-18 are cleaved. GSDMD is activated to its N-terminal form (GSDMD-N), forming pores in the cell membrane and inducing pyroptotic cell death [4, 9, 10].

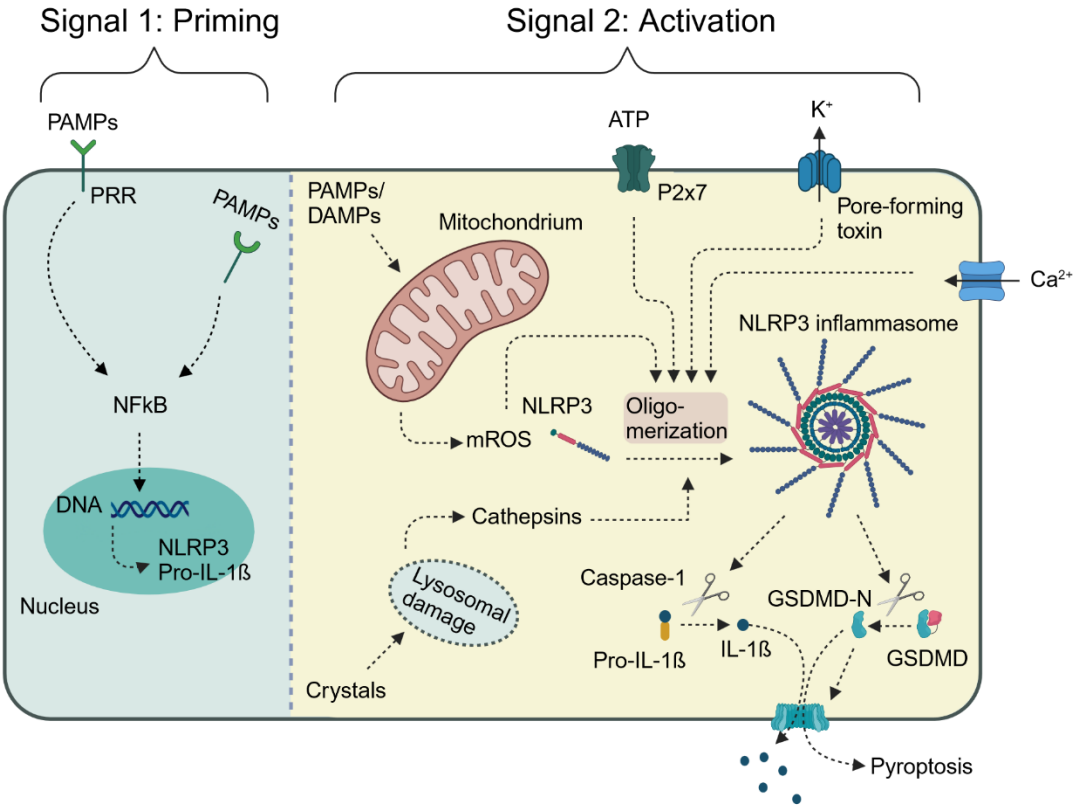

Figure 1: NLRP3 inflammasome priming and activation. The first signal, priming, consists of PAMPs that are recognized by PRRs. Through this interaction, the transcription factor NFkB is activated and upregulates the transcription of NLRP3 and pro-IL-1ß. A variety of second signals for NLRP3 inflammasome activation are known. PAMPs and DAMPs can lead to the production of mROS. Crystalline structures, such as uric acid, can lead to lysosomal damage and the release of cathepsins. Extracellular adenosin triphosphate (ATP) and consequent potassium efflux can trigger inflammasome formation through a specific receptor. Furthermore, aberrant ion concentrations through potassium efflux or calcium influx are known activation signals. All these signals lead to the

recruitment of ASC and the oligomerization of NLRP3. Once the inflammasome is assembled, activated caspase-1 proteolytically cleaves pro-IL-1ß and GSDMD into their active forms. GSDMD-N form pores in the cellular membrane, which leads to pyroptosis and the release of the pro-inflammatory cytokine IL-1ß. (Created with BioRender.com)

3.2.3. NLRC4 inflammasome

Research has demonstrated that the nucleotide-binding oligomerization domain-like receptor family caspase activation and recruitment domain-containing protein 4 (NLRC4) inflammasome plays an important role in the defense against enteric pathogens, such as *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. It also contributes to protection against pathogens like *Listeria monocytogenes* and *Pseudomonas aeruginosa*. Additionally, the NLRC4 inflammasome has been implicated in certain human pathologies, with two NLRC4-associated disorders identified: infantile enterocolitis and recurrent macrophage activation syndrome both of which are characterized by excessive inflammation and dysregulated immune responses [4, 11].

Activation of the NLRC4 inflammasome occurs through ligand binding of PAMPs. It employs specific proteins to detect pathogenic components. These proteins, upon recognizing bacterial flagellin or proteins from the Type III secretion system, recruit NLRC4 to assemble the inflammasome complex. This complex directly interacts with caspase-1, triggering its activation and the subsequent inflammatory response [4, 11].

3.2.4. AIM2 inflammasome

The Absent in melanoma 2 (AIM2) inflammasome plays a vital role in the defense against bacterial, fungal, protozoan, and viral infections. It has also been implicated in a range of autoinflammatory diseases, including arthritis, psoriasis, atopic dermatitis, colitis, Sjögren's syndrome, and systemic lupus erythematosus [12].

Activation of the AIM2 inflammasome occurs through the direct recognition of cytoplasmic double-stranded deoxyribonucleic acid (DNA), originating either from dying host cells or invading pathogens. This interaction prompts the activation of caspase-1, which in turn proteolytically processes IL-1β, IL-18, GSDMD, leading to inflammatory responses and pyroptosis [5, 12].

3.2.5. Pyrin inflammasome

The pyrin inflammasome serves as an innate immune sensor, detecting bacterial toxins that downregulate Rho guanosine triphosphatases. Mutations in the *MEFV* gene, which encodes pyrin, result in the overactivation of the pyrin inflammasome and contribute to the development of the autoinflammatory disorder known as familial mediterranean fever [13].

In its resting state, the pyrin inflammasome is kept inactive through phosphorylation. However, bacterial toxins that inactivate RhoA lead to dephosphorylation and subsequent

activation of the inflammasome. This activation induces caspase-1, which then cleaves IL-1β, IL-18, and GSDMD, driving the inflammatory response [4, 13].

3.3. Pyroptosis

Pyroptosis is a pro-inflammatory form of programmed cell death. Although it shares characteristics with apoptosis, such as chromatin fragmentation and nuclear condensation, pyroptosis is distinct from apoptosis due to pore formation in the plasma membrane and the release of inflammatory intracellular contents [14]. Unlike accidental necrosis, which involves the uncontrolled release of cell contents due to cell rupture, pyroptosis is a regulated form of necrotic cell death [15].

Pyroptosis is induced through the cleavage of GSDMD by caspases. GSDMD consists of two domains. The C-terminal domain acts as a repressor while the N-terminal domain has the ability to oligomerize and create membrane pores. These pores allow pro-inflammatory cytokines such as IL-1β and IL-18 to exit the cell into the extracellular space, while the influx of water leads to cell swelling, membrane rupture, and lysis [14, 16].

There are multiple molecular pathways leading to pyroptosis. The canonical pathway begins with inflammasome activation via PRRs, leading to caspase-1-mediated cleavage of GSDMD. In the non-canonical pathway, caspases 4 and 5 are directly activated by intracellular lipopolysaccharide, cleaving GSDMD to form pores. Additionally, research indicates that certain chemotherapeutic drugs can prompt caspase-3-mediated pyroptosis [14, 16].

Pyroptosis plays a role in the host defense against various pathogens, including *Salmonella* and *Shigella*. Its involvement in numerous diseases, such as cardiovascular diseases and certain cancers, has also been documented [15].

3.4. PAMPs and DAMPs – triggers of immune responses

PAMPs are conserved molecules specific to certain pathogens and absent in the host. Examples include microbial ribonucleic acid (RNA) and DNA, surface glycoproteins, lipoproteins, and membrane components [10, 17]. On the other hand, DAMPs are endogenous signals that indicate cellular distress or tissue damage, arising from changes in cellular homeostasis. DAMPs can originate from the extracellular matrix, such as biglycan or fibrinogen, or from intracellular sources including the cytosol, nucleus, mitochondria, endoplasmic reticulum, or the plasma membrane. Molecules such as ATP, uric acid, RNA, DNA, mitochondrial DNA and defensins can function as DAMPs [17, 18].

Both PAMPs and DAMPs can trigger an immune response upon recognition by PRRs expressed by cells of the innate immune system. These receptors are located on the cell surface, in the cytosol, or in the nucleus, with membrane-bound PRRs detecting extracellular pathogens and intracellular PRRs sensing internal cellular distress [19]. PRRs are generally

categorized into five main groups: toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors, C-type lectin receptors, and AIM2-like receptors [17]. The binding of PAMPs or DAMPs to these receptors can stimulate the release of pro-inflammatory molecules such as interferons, chemokines, and cytokines, and may also lead to the activation of the adaptive immune response [10, 17, 20].

3.4.1. Viral glycoproteins

This study specifically examines viral glycoproteins due to their key role in both cellular entry and in the recognition of pathogenic patterns. Viral glycoproteins, present on the surface of enveloped viruses such as the human cytomegalovirus (HCMV), the hepatitis C virus (HCV), the severe acute respiratory syndrome coronavirus (SARS-CoV) 1, and SARS-CoV-2 facilitate the initial contact with host cells by binding to specific receptors. Following receptor binding, some viral glycoproteins trigger the fusion of the viral envelope with the host cell membrane, allowing the viral genome to enter the host cell and enabling viral replication [21].

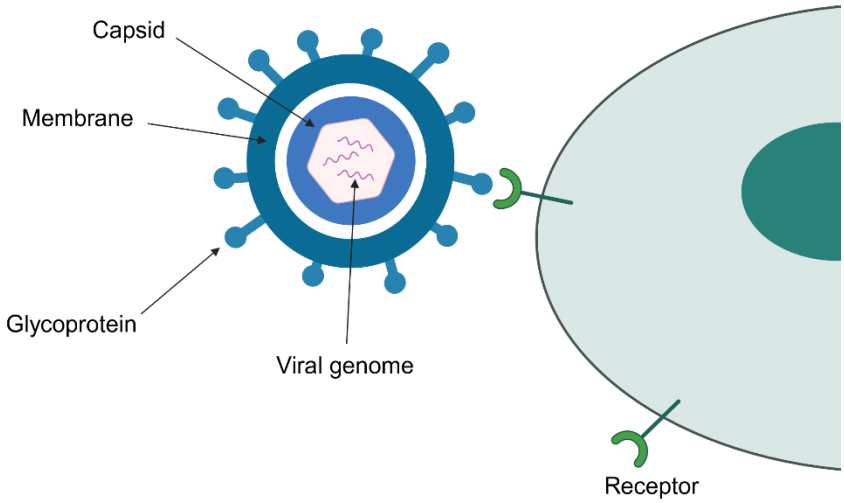

Figure 2: Simplified scheme of virus and host cell interaction. The enveloped virus is made up of a viral genome, the capsid, and glycoproteins on the outer membrane. Glycoproteins are responsible for the interaction with host cell receptors. (Created with BioRender.com)

In HCMV infections, viral glycoproteins are crucial for cellular entry. The glycoprotein M/N complex is needed for attachment to host cells, while the glycoprotein H/L complex, along with glycoprotein B (gB), is essential for membrane fusion. Potential receptors for gB include the epidermal growth factor receptor, platelet-derived growth factor receptor alpha, and integrins, though gB may also mediate fusion without a receptor [22]. For HCV, viral entry occurs through the interaction between viral glycoproteins E1/E2 and host cell receptors. Glycosaminoglycans and low-density lipoprotein receptors are required for the initial

attachment. Four specific receptors have been identified to play a role in cellular entry: scavenger receptor class B type 1, CD81, claudin-1, and occludin [23]. During infections with SARS-CoV-1 and SARS-CoV-2, the spike protein mediates viral entry through its interaction with the angiotensin-converting enzyme 2 receptor. The spike protein consists of two subunits: S1, the receptor-binding subunit, and S2, the membrane-fusing subunit. The transmembrane serine protease 2 is necessary for cellular entry by cleaving the spike protein into the S1 and S2 subunits [24].

In addition to facilitating cellular entry, viral glycoproteins play a critical role in immune evasion by altering glycosylation patterns, thereby masking viral polypeptide epitopes to evade antibody detection. Furthermore, viral glycoproteins contribute to cell-to-cell transmission, allowing viruses to spread directly between adjacent cells, thereby avoiding extracellular immune defenses, such as neutralizing antibodies [25, 26].

3.5. Research question and objectives

Viruses pose a significant threat to global health, a fact highlighted by the recent Coronavirus disease 2019 (COVID-19) pandemic. There is a critical need for a deeper understanding of the immune response mechanisms against viral pathogens to facilitate the development of new medications and vaccines. This research aimed to investigate the interactions between various viral glycoproteins and the NLRP3 inflammasome in macrophages. Specifically, the study focused on four glycoproteins, namely the spike protein (S-proteins) of SARS-CoV-2 and SARS-CoV-1, gB of HCMV, and E2 of HCV, encompassing a wide spectrum of viral infections. The research not only concentrated on the activation of the NLRP3 inflammasome but also examined the induction of cell death, particularly pyroptosis.

To test the hypothesis that viral glycoproteins trigger the NLRP3 inflammasome in macrophages, we conducted *in vitro* experiments using THP1 monocytes, a human monocytic cell line.

4. Publication

Underlying reference of this dissertation (see below):

Eisfeld, HS; Simonis, A; Winter, S; Chhen, J; Ströh, LJ; Krey, T; Koch, M; Theobald, SJ; Rybniker, J. Viral Glycoproteins Induce NLRP3 Inflammasome Activation and Pyroptosis in Macrophages. Viruses. 2021 Oct 15;13(10):2076, doi: 10.3390/v13102076.

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viruses

Article

Viral Glycoproteins Induce NLRP3 Inflammasome Activation and Pyroptosis in Macrophages

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Abstract: Infections with viral pathogens are widespread and can cause a variety of different diseases. In-depth knowledge about viral triggers initiating an immune response is necessary to decipher viral pathogenesis. Inflammasomes, as part of the innate immune system, can be activated by viral pathogens. However, viral structural components responsible for inflammasome activation remain largely unknown. Here we analyzed glycoproteins derived from SARS-CoV-1/2, HCMV and HCV, required for viral entry and fusion, as potential triggers of NLRP3 inflammasome activation and pyroptosis in THP-1 macrophages. All tested glycoproteins were able to potently induce NLRP3 inflammasome activation, indicated by ASC-SPECK formation and secretion of cleaved IL-1β. Lytic cell death via gasdermin D (GSDMD), pore formation, and pyroptosis are required for IL-1β release. As a hallmark of pyroptosis, we were able to detect cleavage of GSDMD and, correspondingly, cell death in THP-1 macrophages. CRISPR-Cas9 knockout of NLRP3 and GSDMD in THP-1 macrophages confirmed and strongly support the evidence that viral glycoproteins can act as innate immunity triggers. With our study, we decipher key mechanisms of viral pathogenesis by showing that viral glycoproteins potently induce innate immune responses. These insights could be beneficial in vaccine development and provide new impulses for the investigation of vaccine-induced innate immunity.

Keywords: NLRP3; IL-1β; pyroptosis; viral glycoproteins; innate immunity; macrophages

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Citation: Eisfeld, H.S.; Simonis, A.; Winter, S.; Chhen, J.; Ströh, L.J.; Krey, T.; Koch, M.; Theobald, S.J.; Rybniker, J. Viral Glycoproteins Induce NLRP3 Inflammasome Activation and Pyroptosis in Macrophages. *Viruses* **2021**, *13*, 2076. https://doi.org/10.3390/v13102076

Academic Editors: Parikshit Bagchi and Anupam Mukherjee

Received: 12 September 2021
Accepted: 11 October 2021
Published: 15 October 2021

1. Introduction

Viral infections and infection-associated complications represent a major global health problem. The diversity of viruses and viral biology is reflected by the broad range of clinical symptoms ranging from asymptomatic, to chronic to acute and fulminant courses. In addition to the spectrum of severity, viral diseases differentiate highly in contagiosity, including a low infectivity to highly pathogenic viruses, which can cause global pandemics affecting millions of people. In addition to the ongoing HIV pandemic, several outbreaks

have aroused public attention in the last years as the SARS epidemic in 2003 or several Ebola outbreaks in West Africa between 2014 to 2016. Furthermore, the world is facing an ongoing coronavirus disease 19 (COVID-19) pandemic with over 220 million confirmed cases and over 4 million deaths worldwide [1].

COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is a single-stranded positive-sense RNA virus and belongs to the ß-coronavirus subfamily [2]. The most common symptoms in patients are fever, cough, and fatigue [3]. As some patients fail to control viral infection, SARS-CoV-2 can cause severe viral pneumonia leading to respiratory failure [4]. The genome of SARS-CoV-2 shows nearly 80% sequence identity to SARS-CoV, which caused an epidemic in 2003 affecting approximately 8000 people with 774 deaths [2,5]. Similar to COVID-19, patients presented fever, cough, myalgia, and dyspnea and developed in some cases atypical pneumonia [5].

Hepatitis C virus (HCV) and human Cytomegalovirus (HCMV) are examples of viruses causing mostly chronic infections. HCV is an enveloped virus with a positive-sense single-stranded RNA genome [6]. The virus is transmitted through exchange of body fluids. In 20% of infected individuals, HCV causes an acute infection with subsequent viral clearance. However, in 80% of patients the infection progresses to a chronic state and patients are at risk for liver cirrhosis and other complications [6]. HCMV is an enveloped virus with a double-stranded DNA genome. HCMV infections are mainly asymptomatic but can also cause severe infections in immune compromised hosts or congenital infections [7,8].

In the defense against viral pathogens, the immune system of the host has developed several strategies to avert viral entry and viral reproduction. In the last years, the adaptive immune system, including the cellular response of lymphocyte to viral infections has been the focus in the research of host–pathogen interactions and viral pathogenesis. However, the innate immunity plays an underestimated role in viral defense [9]. Components of the innate immune system represent the first line of defense and are essential for the establishment of full antiviral immunity. Here, inflammasomes play a crucial role for host immune responses. They are intracellular multi-protein complexes essential for the secretion of the inflammatory cytokines Interleukin-1ß (IL-1ß) and IL-18, required for the recruitment of immune cells [10]. The inflammasome activation process is initiated through the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PAMPs are molecular motifs conserved within a class of microbes, e.g., lipopolysaccharides (LPS) [10]. This first step leads to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), a transcription factor which upregulates the expression of pro-IL-1ß and NLRP3 [10]. In a second step, an activation signal is needed to induce complete inflammasome activation. Activation signals are PAMPs or danger-associated molecular patterns (DAMPs), such as aberrant ionic fluxes through viroporins [10]. The most investigated inflammasome is the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome, a multi-protein complex, which upon activation triggers the oligomerization of the adaptor protein ASC. After inflammasome assembly, pro-caspase-1 is auto-cleaved and subsequently processes pro-Interleukin-1ß (pro-IL-1ß) proteolytically [10]. Further, the NLRP3 inflammasome induces pyroptosis, a form of pro-inflammatory and regulated necrotic cell death. Here, gasdermin D (GSDMD) is cleaved by caspase-1 and forms pores in the cellular membrane leading to cell lysis [11]. Inflammasomes are highly important for a successful antiviral response; however, they can also contribute to the immunopathology and hyperinflammatory states [9,12,13]. Therefore, a better understanding of the underlying mechanisms leading to inflammasome activation in viral diseases is of particular importance. In fact, there remain several open questions on how viral pathogens induce an inflammasome response in cells of the innate immune system and which virus derived structural components function as triggers.

Activation of immune cells by viral pathogens is mainly mediated by surface proteins including glycoproteins, which interfere with the host cell. Glycoproteins play a crucial role

in cellular entry and are known as important antigens for host immune responses [14–16]. In SARS-CoV and SARS-CoV-2 the spike protein mediates cellular entry by binding to the angiotensin-converting enzyme 2 (ACE2) [14]. The spike protein of SARS-CoV-2 (SP-CoV-2) has generated particular interest as the main target in vaccine development [17]. In hepatitis C infections viral entry is mediated through the interactions of the surface proteins E1 and E2 (E2 HCV) with host cell receptors. These glycoproteins are crucial factors of HCV pathogenicity [16]. In HCMV infections, several structures contributing to cellular entry are known. One important antigen is the glycoprotein B (gB HCMV), which is responsible for membrane fusion during viral entry [15]. Commonly, all proteins represent potent viral antigens, which are targeted by the cellular and humoral immune system and represent important vaccine antigens [18–21]. However, mechanisms of activation of cells associated with innate immunity by these glycoproteins is poorly understood. Recently, we were able to show that SP-CoV-2 potently triggers inflammasome formation and consequently IL-1ß secretion in macrophages derived from COVID-19 patients, but not healthy individuals. Epigenetic changes and gene expression profiles from COVID-19 macrophages suggest an innate memory response of in vivo primed and SARS-CoV-2 experienced macrophages [22]. Still, the question remains if other viral glycoproteins can have similar effects.

Within this manuscript, we analyze the role of several viral glycoproteins as innate immunity triggers in vitro. All studied viral glycoproteins potently induced the NLRP3 inflammasome and GSDMD-dependent pyroptosis with subsequent IL-1β secretion in THP-1 macrophages. Our findings are of particular importance to understand viral pathogenesis and surface exposed glycoproteins, which seem to have a dual function as mediators of viral entry and inflammasome activation. Furthermore, our results can foster vaccine development, since most antigens used so far also impact innate immunity.

2. Materials and Methods

2.1. Cell Lines and Cell Culture

THP-1 cells, a human monocytic cell line, were cultured in RPMI medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% FBS (PAN Biotech, Aidenbach, Germany). For differentiation into macrophages cells were incubated with 20 nMol Phorbol 12-myristate 13-acetate (PMA) (Merck, Darmstadt, Germany) for 24 h. After 24 h, medium was exchanged, and cells were cultured for another 24 h without PMA. All cells were cultured at 37 °C with 5% CO_2.

2.2. Stimulation of THP-1 Macrophages

The medium was exchanged, and cells were incubated with DMSO (Merck; solvent of inhibitors) or MCC950 (Merck; 10 µM) for 2 h. LPS (Merck; 5 µg/mL) and viral glycoproteins (10 µg/mL) were added, and cells were incubated for another 4 h for inflammasome priming. Nigericin (Merck; 5 µM) or adenosine triphosphate (ATP) (Thermo Fisher Scientific; 5 mM) were added and incubated for 2 h for inflammasome activation. For ELISA, the supernatant was transferred to a new plate and stored at −80 °C. For cell death analysis cells were incubated another 24 h with nigericin.

2.3. CRISPR-Cas9 Knockout Cell Line Generation

THP-1 macrophages expressing Cas9 and eGFP (kindly provided by Prof. Michael Hallek, University Hospital of Cologne) were resuspended at a concentration of 10^6 cells/mL in RPMI with 10% FBS and 1% penicillin streptomycin (Thermo Fisher Scientific). An amount of 20 mM HEPES buffer solution (Thermo Fisher Scientific) and lentiviral vectors with guide RNAs (LentiArray CRISPR Guide RNA; Thermo Fisher Scientific) for GSDMD and NLRP3 were added (MOI = 5). Tubes were centrifuged (800 rpm, 2 h, 37 °C) for spin infection. Transduced cells were seeded into 6-well plates and incubated for 24 h. After 24 h the medium was changed. Cells were expanded and 4 days after transduction puromycin (ROTH, Karlsruhe, Germany; 1 µg/mL) was added. Efficacy of knockout was confirmed by Western Blot analysis.

2.4. IL-1β ELISA

Human IL-1 beta Uncoated ELISA Kit (Thermo Fisher Scientific) was used following manufacturer's instructions. All supernatants were diluted 1:50 in ELISA diluent. The samples were measured using a Hidex sense microplate reader (Hidex, Turku, Finland) at OD 450 nm–570 nm. Sample concentrations were determined using the provided standard curve (range: 2–150 pg/mL).

2.5. Immunoblot Analysis

2.5.1. Preparation of Supernatants

Cells were stimulated as stated above. The supernatant was transferred and prepared using methanol (ChemSolute, Renningen, Germany) and chloroform (AppliChem, Darmstadt, Germany). Pellet was suspended in loading buffer (100 mM Tris-HCL/4% sodium dodecyl sulfate/0.2% bromophenol blue/20% glycerol/ 200 mM β-mercaptoethanol) and incubated at 95 °C for 5 min.

2.5.2. Preparation of Cell Lysates

After stimulation, cell culture plates were stored on ice and RIPA buffer (Thermo Fisher Scientific) was used to lyse the cells. Samples were centrifuged (16,000× *g*, 4 °C, 20 min) and the supernatant was used for analysis. Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was performed to determine protein concentrations. Samples were diluted in loading buffer and incubated at 95 °C for 5 min.

2.5.3. Immunoblot

Samples and markers were applied to gels, which were run at 120 V. Subsequently, gels were blotted using the Trans-Blot Turbo Transfer System (BioRad, Berkely, CA, USA). Membranes were blocked for 1 h and primary antibodies ASC (B-3) (Santa Cruz Biotechnology, INC., Dallas, TX, USA; 1:500 in 5% dried milk), NLRP3 (Cell Signaling Technology, Danvers, MA, USA; 1:1000 in 5% BSA in TBST buffer), P-NFkB (Cell Signaling Technology; 1:1000 in 5% dried milk), cleaved Interleukin-1β (Cell Signaling Technology; 1:1000 in 5% dried milk), IL-1β (Cell Signaling Technology; 1:1000 in 5% BSA in TBST buffer), cleaved GSDMD (Cell Signaling Technology; 1:500 in 5% dried milk) or GSDMD (Merck; 1:500 in 5% dried milk) were added and incubated at 4 °C overnight. After washing, anti-mouse IgG or anti-rabbit IgG (both Cell Signaling Technology; 1:5000 in 5% dried milk) were added for 1 h. Membranes were developed using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized using the FUSION SOLO S imaging system (Vilber Lourmat, Eberhardzell, Germany).

2.6. Flow Cytometry

Cells were stimulated and incubated as described before. FITC Annexin V Apoptosis Detection Kit with PI (BioLegend, San Diego, CA, USA) were used according to manufacturer's instructions. Stained cells were analyzed using the MACS Quant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7. Immunofluorescence Staining

Cells were plated into 8-well chamber culture slides at 6×10^4 cells/well, differentiated with PMA and stimulated as described before. Cells were washed with PBS (Thermo Fisher Scientific), fixed with 4% PFA for 15 min, washed and incubated with 5% FBS/0.1% Tween/0.1% Triton/PBS for 1 h. For ASC speck staining, cells were incubated overnight with the ASC Antibody (B-3) Alexa Fluor 488 (Santa Cruz Biotechnology, INC.; 1:100) in 3% FBS/0.1% Tween/0.1% Triton/PBS at 4 °C. After washing with PBS, DAPI (Thermo Fisher Scientific; 1:1000) was added and cells were incubated for 10 min. Cells were washed with PBS and slides were mounted with ProLong Gold antifade reagent (Thermo Fisher Scientific). Slides were scanned using the Olympus FV1000 Microscope (Olympus, Shinjuku, Japan). Images were taken with the same microscope settings for all conditions.

On average we acquired the following number of cells per condition per biological replicate: Ø: 126, LPS: 178, SP-CoV-2: 174, gB HCMV: 178, E2 HCV: 140. Three biological replicates were performed per condition.

2.8. LDH Assay

THP-1 macrophages were plated at 5×10^3 cells/well, differentiated with PMA and stimulated. After 24 h CyQUANT LDH Cytotoxicity Assay (Thermo Fisher Scientific) was performed following the manufacturer's instructions. Plates were measured with Hidex sense microplate reader at 490 nm–680 nm.

2.9. Protein Production

The plasmid for the SARS-CoV-2 spike protein (MN908947; AA: 1-1208; RRAR to GSAS; F817P; A892P; A899P; A942P; K986P; V987P) was kindly provided by Jason S. McLellan [23] and the SARS-CoV-1 plasmid (AAP13567; AA: 18-1190) was purchased from Sino Biological (Codon Optimized). The two coronavirus ectodomains (SARS-CoV-1 AA: 18-1190; SARS-CoV-2 AA: 1-1208) as well as the S2 region from SARS-CoV-2 (AA: 686-1208) were amplified from the synthetic gene plasmids by PCR and subsequently cloned into a modified sleeping beauty transposon expression vector containing a C-terminal Twin-Strep-Tag and verified by sequencing. In the case of the two ectodomains, a T4 foldon was added at the C-terminus. For the recombinant protein productions, stable HEK293 EBNA cell lines were generated employing the sleeping beauty transposon system [24]. Briefly, expression constructs were transfected into the HEK293 EBNA cells and after selection with puromycin, cells were expanded into triple flask and induced with doxycycline. Cell supernatants were filtered, passed through Strep-Tactin XT (IBA Lifesciences, Göttingen, Germany) resin, washed with 1 M NaCL/TBS, and eluted with biotin-containing TBS-buffer (IBA Lifesciences). After dialysis against TBS-buffer, the recombinant proteins were validated by SDS-PAGE followed by Coomassie staining. All reusable materials were treated with 1 M NaOH for 2 h and rinsed with ddH_2O water to remove putative LPS contamination.

For gB HCMV and E2 HCV, codon-optimized synthetic cDNA corresponding to soluble C-terminally truncated versions of HCMV gB comprising residues [18] or HCV E2 isolate UKN2b_2.8 comprising residues 384–715 were cloned into a modified Drosophila S2 expression vector described previously and transfection was performed as reported earlier [25]. For large-scale production, cells were induced with 4 µM CdCl2 at a density of approximately 7×10^6 cells/mL for 8 days, pelleted, and the soluble ectodomain was purified by affinity chromatography from the supernatant using a StrepTactin Superflow column followed by size exclusion chromatography using a Superdex200 column in 10 mM TRIS pH 8.0, 150 mM NaCl.

3. Results

3.1. Viral Glycoproteins Activate the NLRP3 Inflammasome

Viral host cell innate immune response varies broadly and it is known that several cellular inflammation-associated pathways can be activated by different viruses [9,10,26]. Therefore, we aimed to decipher the effect of purified viral glycoproteins of various viruses (SARS-CoV-2, SARS-CoV-1, HCMV, and HCV) on the activation of the NLRP3 inflammasome. We used glycoproteins of four different viruses to cover different forms of infections, such as acute vs. chronic.

As an in vitro model, we used human THP-1 monocytes, which were differentiated to macrophages using PMA. We utilized a chemical genetics approach to analyze NLRP3 activation. MCC950, a well-known NLRP3 inhibitor [27], was used to pre-incubate THP-1 macrophages. Subsequently, THP-1 macrophages were stimulated with each viral glycoprotein as the first stimulus for inflammasome priming. In a second step, we incubated the cells with nigericin as an activation signal (Figure 1a).

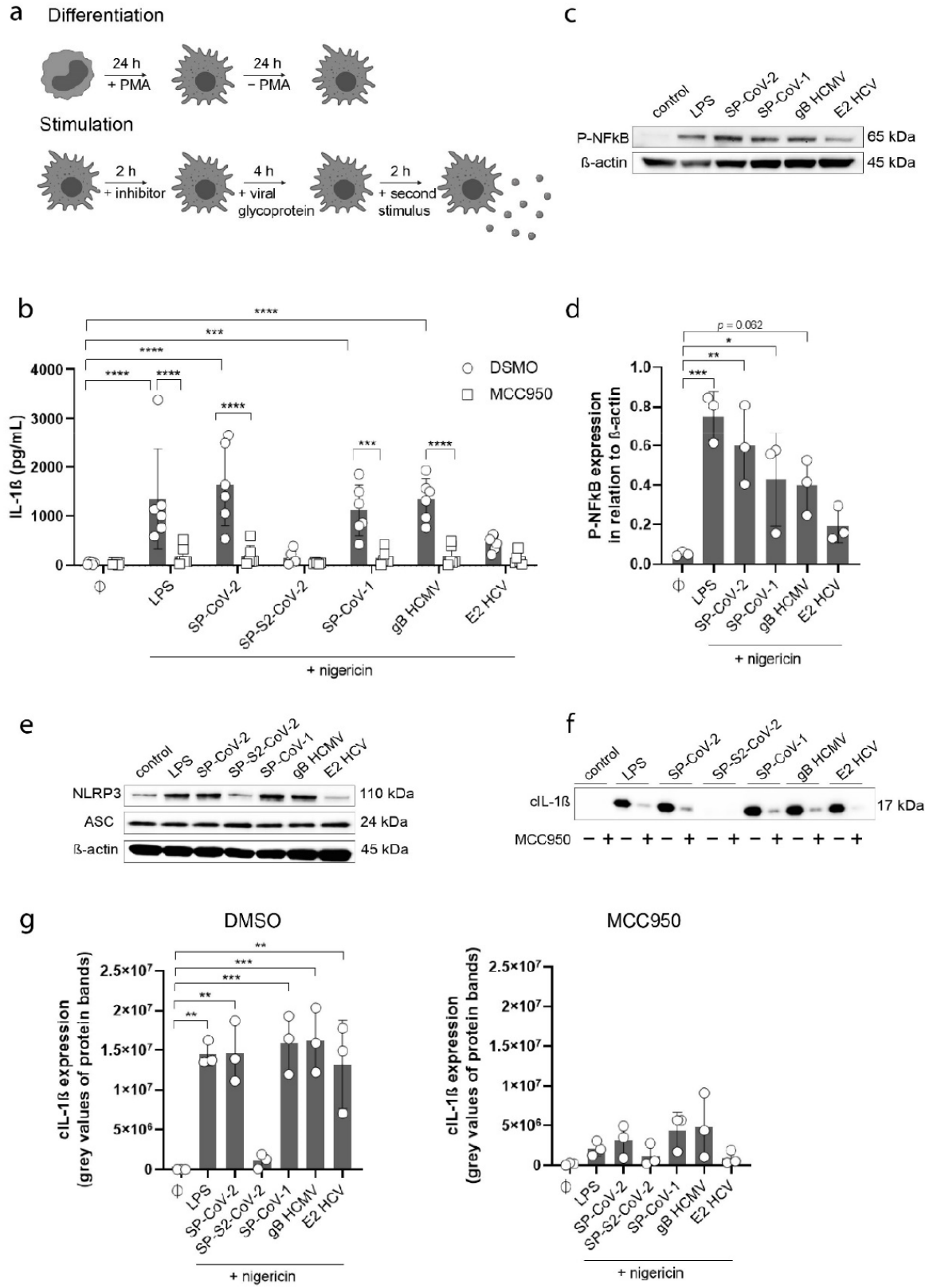

Figure 1. (**a**) Scheme of the experimental setup. THP-1 monocytes were differentiated using PMA (20 nMol) for 24 h and additionally incubated for 24 h without PMA. THP-1 macrophages were exposed to inhibitors for 2 h, subsequently stimulated with viral glycoproteins as priming signal for 4 h and as activation signal with nigericin for an additional 2 h. (**b**) Quantification of IL-1β concentration (pg/mL) in the supernatant of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) in the presence (square) or absence (circle) of MCC950 (10 µM) ($n = 6$). All cells were stimulated with nigericin for 2 h (5 µM). Results are expressed as mean ± SD. Statistical analysis was done using two-way ANOVA with Tukey's and Sidak's multiple comparisons test (*** = $p \leq 0.001$; **** = $p \leq 0.0001$). (**c**) Detection of P-NFkB (1:1000 for P-NFkB antibody) in total cell lysates of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral

antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). ß-actin (1:1000 dilution) was used as a loading control. (**d**) Quantified results of Western Blot in Figure 1c are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. Results were compared to a control (Ø) (* = $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). (**e**) Detection of NLRP3 and ASC (1:1000 dilution for NLRP3 antibody, 1:500 dilution for ASC antibody; n = 3) in total cell lysates of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). ß-actin (1:1000 dilution) was used as a loading control. (**f**) Detection of cleaved IL-1ß (cIL-1ß) (n = 3) in the supernatant of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) in the presence (+) or absence (-) of MCC950 (10 µM) (top). All cells were stimulated with nigericin (5 µM). (**g**) Quantified results of Western Blot in Figure 1d are expressed as mean ± SD. Statistical analysis was done using one-way ANOVA with Tukey's multiple comparisons test (** $p \leq 0.01$; *** $p \leq 0.001$).

As viral glycoproteins, SP-CoV-2, the spike protein of SARS-CoV-1 (SP-CoV-1), gB HCMV and the viral structure protein E2 HCV were used. The S2 subunit of SP-CoV-2 (SP-S2-CoV-2) which fails to bind plasma membranes was used as a negative control. SP-S2-CoV-2 is the membrane-fusion domain and does not prime the NLRP3 inflammasome in macrophages [22]. LPS, a well-known inflammasome activator, was used as positive control. To determine NLRP3 inflammasome activation, we measured IL-1β in the supernatants of THP-1 macrophages upon stimulation. LPS, SP-CoV-2, SP-CoV-1 and gB HCMV were able to significantly increase the concentration of IL-1β in the cell supernatant (Figure 1b). Increased IL-1β levels (11.2-fold increase) were also detected upon stimulation with E2 HCV. Chemical inhibition with MCC950 abrogated IL-1β secretion upon stimulation with viral glycoproteins (Figure 1b). As verification and to exclude unspecific effects by high protein concentrations, we stimulated THP-1 macrophages with a 10-fold lower protein concentration (1 µg/mL) and were able to see similar results, which occurred in a dose-dependent manner (Supplementary Figure S1a). NLRP3 inflammasome activation could also be induced by using ATP instead of nigericin after as a second signal for IL-1ß release (Supplementary Figure S1b). After the stimulation with viral glycoproteins without a second signal such as nigericin or ATP, no increase in IL-1ß levels could be detected (Supplementary Figure S1c).

To investigate the upregulation of an immune response, we first quantified phospho-NFkB (P-NFkB) in total cell lysates. We were able to detect an increase in P-NFkB after the exposure to LPS, SP-CoV-2, SP-CoV-1, and gB HCMV (Figure 1c,d). Then, we analyzed the molecular mechanism of NLRP3 activation by quantifying NLRP3 and ASC protein levels in total cell lysates of stimulated THP-1 macrophages. Here, we could detect increased NLRP3 levels after stimulation with LPS, SP-CoV-2, SP-CoV-1, and gB HCMV (Figure 1e and Supplementary Figure S1d) indicating increased transcription of NLRP3 upon viral glycoprotein stimulation. In contrast, ASC protein levels were not affected by the stimulation (Figure 1e and Supplementary Figure S1e). We then analyzed IL-1ß levels in total cell lysates and could detect an increase in proteins levels after stimulation with LPS, SP-CoV-2, SP-CoV-1, and gB HCMV (Supplementary Figure S1f,g). As a hallmark of inflammasome activation, we analyzed the presence of cleaved IL-1β (cIL-1β) in the supernatant of stimulated THP-1 macrophages using Western blot analysis (Figure 1f). Strikingly, a significant increase in cIL-1β could be detected after stimulation with LPS and all viral glycoproteins (Figure 1f,g), whereas MCC950 was able to effectively block the secretion of cIL-1β (Figure 1f,g). SP-S2-CoV-2, however, did not increase cIL-1β (Figure 1f,g).

Next, we visualized ASC-SPECK formation, which is an important step in NLRP3-inflammasome activation via immunofluorescence microscopy of THP-1 macrophages stimulated with viral glycoproteins. ASC-SPECK formation indicates complete assembly of the inflammasome. The amount of ASC-SPECKS per cell ($DAPI^+$) increased upon stimulation with LPS and the tested glycoproteins (Figure 2a,b).

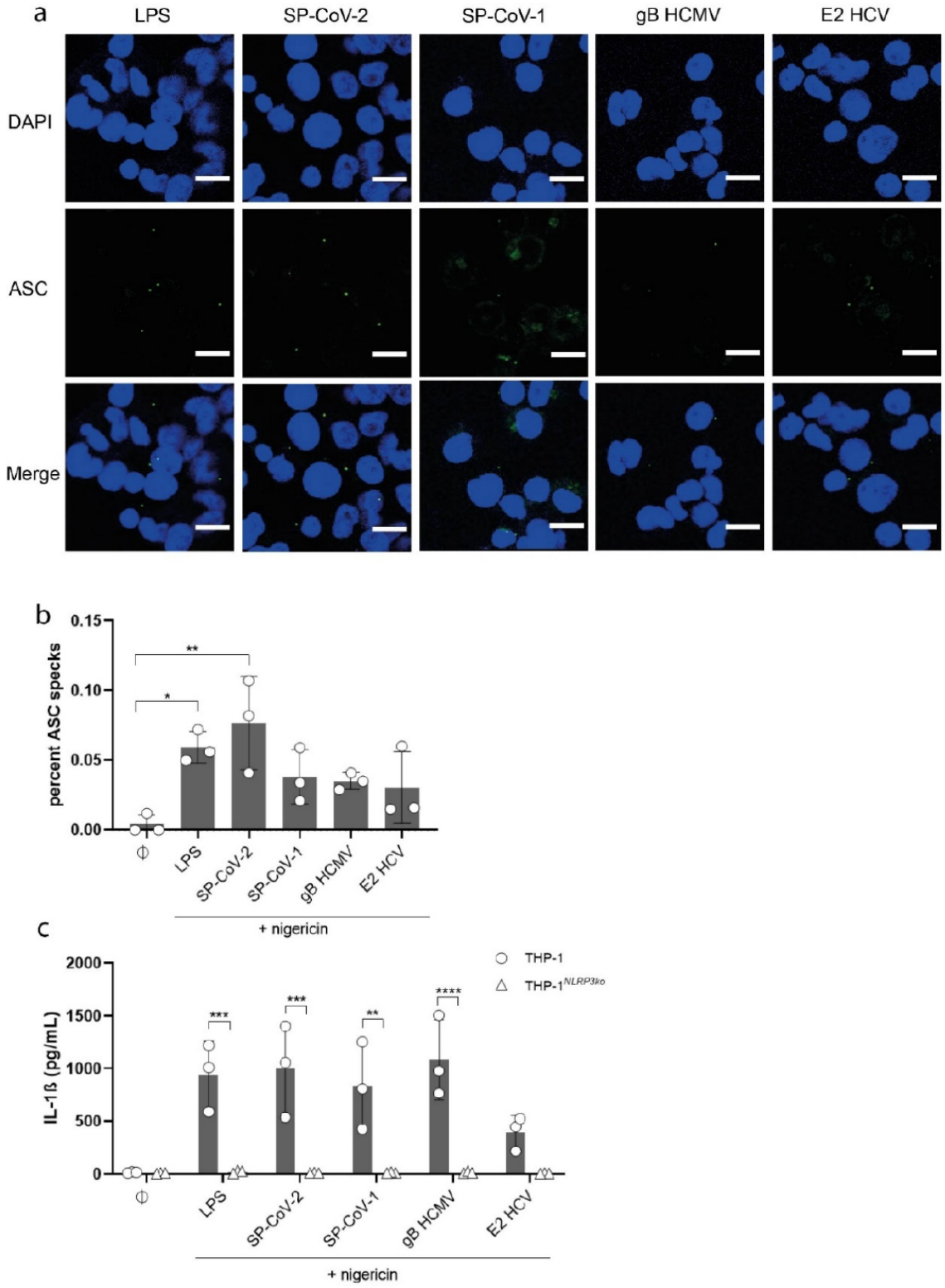

Figure 2. (**a**) Confocal fluorescence microscopy images of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). Cells were stained with ASC Antibody (B-3) Alexa Fluor 488 (1:100 dilution) and DAPI and analyzed with the same microscope settings (n = 3). Representative images are shown (scale bar = 15 µm). (**b**) Quantified results of fluorescence microscopy shown in Figure 2a are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test (* = $p \leq 0.05$; ** = $p \leq 0.01$). (**c**) Quantification of IL-1β concentration (pg/mL) in the supernatant of THP-1 macrophages (circle) and THP-1^{NLRP3ko} cells (triangle) stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) (n = 3). All cells were stimulated with nigericin (5 µM). Results are expressed as mean ± SD. Statistical analysis was done using two-way ANOVA with Sidak's multiple comparisons test (** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$).

Almost no specks were detected in unstimulated cells (Supplementary Figure S2a and Figure 2b). To confirm NLRP3 activation as the major driver of IL-1β secretion, we generated a THP-1 NLRP3 knockout cell line (THP-$1^{NLRP3ko}$) by CRISPR-Cas9 technology (Supplementary Figure S2b). A stable Cas9 expressing THP-1 line was infected with lentiviral vectors encoding gRNAs against NLRP3. Wild-type THP-1 macrophages and THP-$1^{NLRP3ko}$ cells were stimulated as previously described. While an increase in IL-1β secretion could be detected in wild-type cells, IL-1β levels in the supernatants of THP-$1^{NLRP3ko}$ cells stimulated with LPS, SP-CoV-2, SP-CoV-1, gB HCMV or E2 HCV remained at baseline (Figure 2c). These results clearly confirm that viral glycoprotein stimulation of macrophages induces NLRP3 inflammasome activation and IL-1ß secretion.

3.2. Viral Glycoprotein Exposure Induces Necrotic Cell Death

Necrotic cell death, in particular pyroptosis, is closely linked to inflammasome activation. To draw that connection, we performed an Annexin V and PI cell death staining on macrophages to analyze if viral glycoproteins can induce cell death. THP-1 macrophages were stimulated as stated above and incubated for an additional 24 h. Cells were then stained with Annexin V and PI and analyzed using flow cytometry (representative images and gating strategy can be found in Figure 3a and Supplementary Figure S2c).

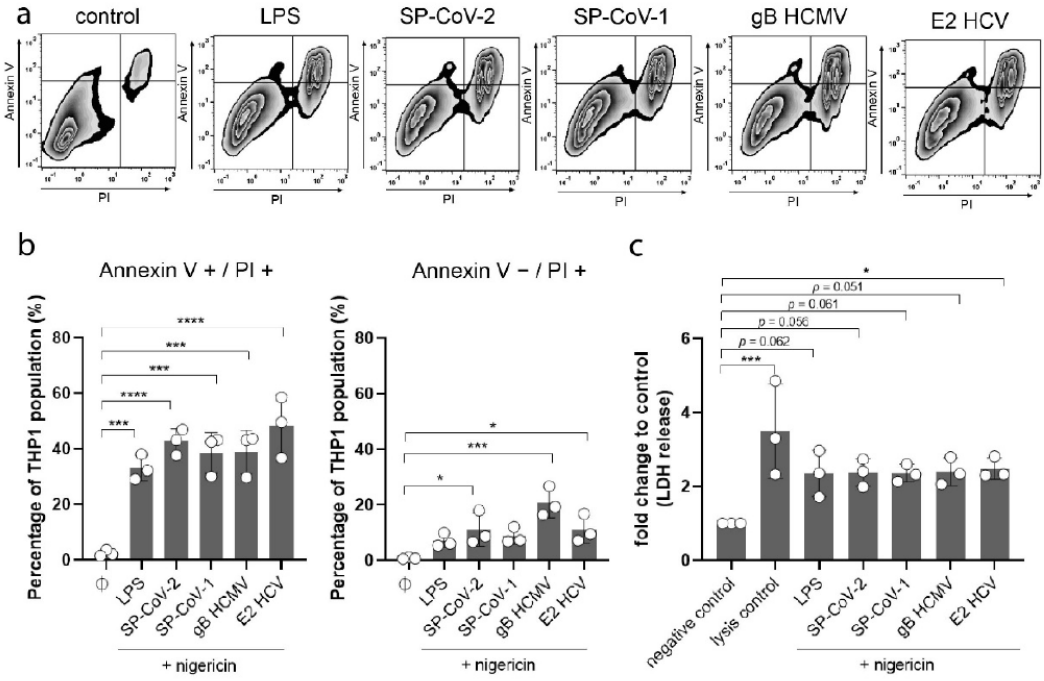

Figure 3. (**a**) THP-1 macrophages were stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). After 24 h cells were stained with Annexin V and PI. Cells were analyzed using flow cytometry (n = 3). Representative images are shown. (**b**) Quantified results of flow cytometry analysis shown in Figure 3a are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. Results were compared to a control (Ø) (* = $p \leq 0.05$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$). (**c**) Quantification of lactate dehydrogenase (LDH) in the supernatant of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) was performed 24 h after stimulation (n = 3). All cells were stimulated with nigericin (5 µM). Results are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons test. Results were compared to a control (negative control). (* $p \leq 0.05$; *** $p \leq 0.001$).

We could detect a significant increase in cells undergoing late apoptosis/necrosis (Annexin V+/PI+) after the stimulation with LPS or with the respective viral glycoproteins (Figure 3b). Interestingly, we could also detect a significant increase in necrosis (Annexin V-/PI+) in cells exposed to SP-CoV-2, gB HCMV, and E2 HCV (Figure 3b). To further investigate necrotic cell death, we performed an LDH releasing assay on stimulated THP-1

macrophages after 24 h of incubation. Compared to the negative control we detected an increase in LDH levels in the supernatant of cells exposed to LPS, SP-CoV-2, SP-CoV-1, gB HCMV, and E2 HCV (Figure 3c). These results indicate that viral glycoproteins could induce necrotic cell death.

3.3. Viral Glycoproteins Induce GSDMD Dependent Pyroptosis

NLRP3 inflammasome activation and GSDMD dependent pyroptosis are closely interconnected and known as a cause of cell death of macrophages upon exposure to viral or bacterial pathogens [28,29]. Cleavage of GSDMD by caspase-1 represents an important step in pyroptotic cell death, thus we performed immunoblots of cleaved GSDMD (GSDMD-N) in total cell lysates of stimulated THP-1 macrophages (Figure 4a).

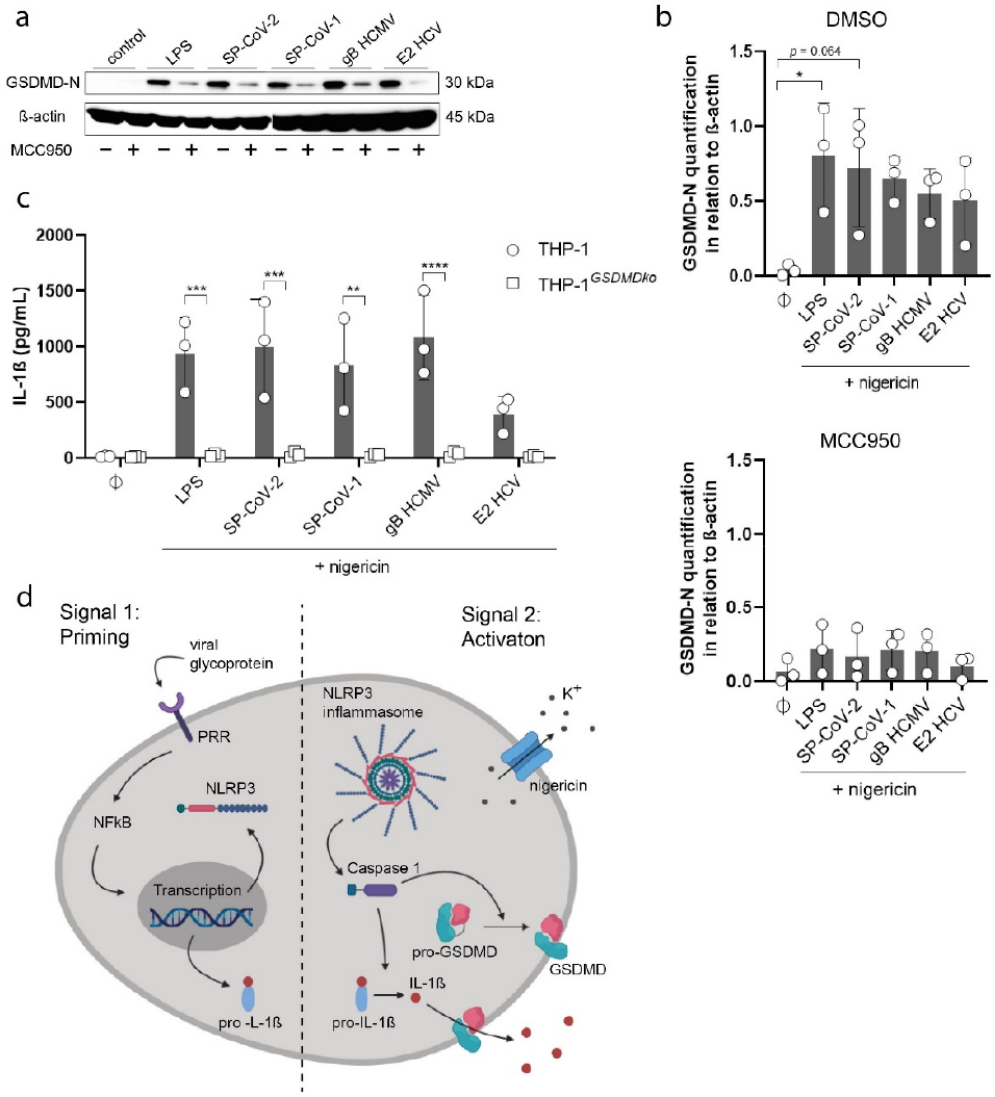

Figure 4. (**a**) Detection of cleaved GSDMD (GSDMD-N) in cell lysates of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) in the presence (+) or absence (-) of MCC950 (10 µM). All cells were stimulated with nigericin (5 µM). (**b**) Quantified results of Western Blot shown in Figure 4a are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test (* $p \leq 0.05$). (**c**) Quantification of IL-1β concentration (pg/mL) in the supernatant of THP-1 macrophages (circle) and THP-1^{GSDMDko} cells (square) stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL) (n = 3). All cells were stimulated with nigericin (5 µM). Bars indicate mean ± SD. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test (** = $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). (**d**) Scheme of NLRP3 activation and induction of pyroptosis by viral antigens.

Here, we could detect a strong increase of GSDMD-N levels in cell lysates of cells stimulated with LPS and with all viral glycoproteins (Figure 4b). As expected, MCC950 diminished GSDMD-N levels efficiently (Figure 4b). We also detected GSDMD in total cell lysates and could not detect an increase in protein expression after stimulation (Supplementary Figure S2d,e). To confirm the induction of pyroptosis via GSDMD cleavage, we generated THP-1 GSDMD knockout cells (THP-1^{GSDMDko}) (Supplementary Figure S2b). In comparison to wild-type THP-1 macrophages, THP-1^{GSDMDko} stimulated with LPS or viral glycoproteins failed to secrete IL-1β (Figure 4c).

In summary, we show that several viral glycoproteins act as viral PAMPs that potently induce NLRP3 inflammasome activation and consequently pyroptotic cells death in THP-1 macrophages (Figure 4d).

4. Discussion

Inflammasomes, as an essential part of the innate immune response, are of particular importance for the host defense against various pathogens. While the role of inflammasomes in the antiviral response is well described, in-depth knowledge on exact triggers and structural components activating associated signaling pathways is scarce [9,10,30,31]. In this paper, we were able to show that surface exposed glycoproteins of several viruses can function as activators of the NLRP3 inflammasome and pyroptotic cell death.

Several viruses have been connected with inflammasome activation before. Huang et al. show that the AIM2 inflammasome is activated in THP-1 macrophages when exposed to CMV [32]. When exposed to HCV genotype 2a, THP-1 macrophages, primary macrophages and Kupffer cells show an increase in the secretion of the inflammatory cytokine IL-1β [33]. Most recently, the inflammasome has been discussed as part of the inflammation process in COVID-19 and is associated with severe outcomes in patients [13]. While these studies confirm an interaction between whole viruses and inflammasomes, especially the AIM2 inflammasome, the influence of surface-associated glycoproteins as triggers of the innate immune system is not well studied.

Detection of invading viruses involves PRRs. These receptors can bind to several viral components such as viral RNA, viral DNA, virus coat proteins, or viral glycosylated proteins [9]. In this paper, we show that glycoproteins of several important viruses are capable of priming the NLRP3 inflammasome. All proteins were able to induce the secretion of cleaved IL-1β, which represents the hallmark for inflammasome activation. For further evidence, we showed that the glycoproteins are able to trigger the formation of ASC-SPECKS as the proof of NLRP3 inflammasome formation. Furthermore, we could also demonstrate increased NLRP3 protein levels after stimulation with the viral glycoproteins. Why E2 HCV was able to induce secretion of cleaved IL-1β without strong upregulation of NLRP3 requires further analysis. In fact, our data strongly links virus-related inflammasome activation, as discussed above, to the corresponding viral glycoproteins, which activate host inflammasome pathways and therefore act as viral PAMP for the innate immune system. Further research is required to understand which receptors and signaling events leads to NFkB and NLRP3 activation. While we have focused on the most studied and well-described inflammasome complex (NLRP3), we cannot rule out that stimulation with viral glycoproteins can also activate other inflammasome complexes.

Studies surrounding viral glycoproteins and their effects on innate immunity are even more important, as they are increasingly exploited as key components of vaccine constructs. A prominent example is the COVID-19 vaccine. So far, all approved vaccines use the SARS-CoV-2 spike protein as a target antigen [17]. For HCMV infections, the most promising vaccine candidate in development is a soluble gB subunit vaccine [34]. For HCV infections several vaccine candidates are currently examined and potential targets are nonstructural proteins, HCV E1, E2, and core proteins [35]. Several studies have investigated the immune response to vaccines. As a link between innate and adaptive immunity, they show an interaction between vaccine components and PRRs leading to chemokine secretion and subsequent attraction of innate immune cells [36,37]. This highlights the importance of

our findings, which show that all studied proteins are potent triggers of innate immune signaling via the inflammasome. It is highly conceivable that the observed NLRP3 inflammasome activation and subsequent IL-1β secretion is important for a vaccine-induced immunity, which requires further confirmation in additional in vivo and ex vivo studies, or clinical trials. Our analyses are based on in vitro experiments with a monocytic cell line which allows for genetic manipulation. At least for SARS-CoV-2 we were able to show that primary human macrophages derived from COVID-19 patients also activate the NLRP3 inflammasome upon spike protein exposure [22]. We will now analyze NLRP3 activation in primary macrophages derived from seropositive or seronegative individuals for the other viruses.

Currently, we cannot rule out that the glycosylation pattern of the viral glycoproteins could have an impact on inflammasome formation and activation. However, crystal structures for all glycoproteins have been published and all affinity purified proteins have been shown to induce immune responses in vitro and in vivo [18,38]. Usage of different producer cells such as HEK293 cells and drosophila S2 insect cells as performed in our study rules out expression system dependent artefacts with regard to glycosylation pattern.

Furthermore, we analyzed the inflammatory cell death pyroptosis, as one key component in the defense against viral pathogens. Pyroptosis was first described in bacterial infections, e.g., with *Shigella*, *Salmonella*, and *Yersinia* [39]. However, in more recent studies pyroptosis has also been linked to viral pathogens [29,30]. An example of pyroptosis in viral infections is the inflammatory necrosis in cultured liver cells following the infection with HCV [40]. Here we provide evidence that pyroptosis can be induced by viral glycoproteins in THP-1 macrophages. All proteins investigated in this study induce necrotic cell death in THP-1 macrophages, which could be abrogated by a GSDMD knockout.

It is not clear whether the described inflammasome activation and the induction of pyroptosis will be beneficial to the host, e.g., by promoting viral clearance, or whether detrimental consequences such as tissue damage predominate. These questions are crucial when analyzing potential therapeutics interfering with innate immunity. As an example, in COVID-19, IL-1β has been linked to hyperinflammation with respiratory failure and blocking IL-1 has been studied as a therapeutic approach [41,42]. In HCV infections, IL-1 is discussed to have antiviral activity and an important role in viral clearance in acute infections [43]. However, IL-1ß is also discussed as a potential stimulus for inflammation and possibly consequent liver fibrosis in chronic HCV infections [33]. This demonstrates that there are still a lot of uncertainties associated with IL-1β and its role in viral infections and innate immune responses. Confirmation of our findings in in vivo models of infection or vaccination will help to answer these open questions. We provide new impulses for further investigation of viral glycoproteins as innate immunity triggers as a starting point for these studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13102076/s1, Supplementary Figure S1. (a) Quantification of IL-1ß concentration (pg/mL) in the supernatant of THP-1 macrophages stimulated with LPS (5 µg/mL; n = 6) or viral antigens (as indicated, 1 µg/mL; n = 3). All cells were stimulated with nigericin (5 µM). Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. (* = $p \leq 0.05$). (b) THP-1 macrophages were stimulated with LPS (5 µg/mL) or viral glycoproteins (as indicated, 10 µg/mL) and subsequently exposed to ATP (5 mM) (n = 3). IL-1ß concentration (pg/mL) was quantified in supernatant via IL-1ß ELISA. (c) THP-1 macrophages were stimulated with viral glycoproteins (as indicated, 10 µg/mL). IL-1ß concentration (pg/mL) was quantified in supernatant via IL-1ß ELISA. (d) Quantification of NLRP3 Western Blot shown in Figure 1e. NLRP3 expression was quantified in relation to ß-actin expression. (e) Quantification of ASC Western Blot shown in Figure 1e. ASC expression was quantified in relation to ß-actin expression. (f) Detection of IL-1ß (1:1000 for IL-1ß antibody) in total cell lysates of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). ß-actin (1:1000 dilution) was used as a loading control. (g) Quantified results of Western Blot in Figure S1f are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple

comparisons test. Results were compared to a control (Ø) (**, $p \leq 0.01$). Supplementary Figure S2. (a) Fluorescence microscopy of unstimulated THP-1 macrophages (control). Cells were stained with ASC Antibody (B-3) Alexa Fluor 488 (1:100 dilution) and with DAPI. Representative images are shown (scale bar: 15 µm). (b) Scheme of the generation of knockout (KO) cell lines. THP-1 macrophages were first transduced with Cas9-GFP. Cells were then transduced with guide RNAs (gRNA). (c) Representative gating strategy showing flow cytometry analysis for THP-1 cell death staining with Annexin V and PI. (d) Detection of GSDMD (1:500 for GSDMD antibody) in total cell lysates of THP-1 macrophages stimulated with LPS (5 µg/mL) or viral antigens (as indicated, 10 µg/mL). All cells were stimulated with nigericin (5 µM). ß-actin (1:1000 dilution) was used as a loading control. (e) Quantified results of Western Blot in Figure S2d are expressed as mean ± SD.

Author Contributions: Conceptualization, S.J.T. and J.R.; Data curation, H.S.E. and S.J.T.; Formal analysis, H.S.E., A.S. and S.J.T.; Funding acquisition, J.R.; Investigation, H.S.E., A.S., S.W., J.C., L.J.S., T.K., M.K. and S.J.T.; Methodology, H.S.E., A.S., S.W., J.C., L.J.S., T.K., M.K. and S.J.T.; Resources, T.K., M.K. and J.R.; Software, H.S.E., A.S. and S.J.T.; Supervision, J.R.; Visualization, H.S.E. and S.J.T.; Writing—original draft, H.S.E., A.S., S.J.T. and J.R.; Writing—review and editing, H.S.E., A.S., S.J.T. and J.R. All authors have read and agreed to the published version of the manuscript.

Funding: H.S.E.: S.J.T. and A.S. receive funding from the Cologne Fortune Program/Faculty of Medicine, University of Cologne. A.S. is supported by the Cologne Clinician Scientist Program (CCSP), funded by the German Research Council (FI 773/15-1) J.R. receives funding from the Thematic Translational Unit Tuberculosis (TTU TB, Grant number TTU 02.806 and 02.905) of the German Center of Infection Research (DZIF). L.J.S. is funded by the Deutsche Forschungsgemeinschaft (DFG)—Projektnummer 158989968—SFB 900, project B10. Financial support was also received from the German Research Foundation (DFG RY 159/3-1, SFB1403 (project number 414786233), and FOR2722 to M.K.) and the Center for Molecular Medicine Cologne (ZMMK-CAP8). Funding was received from the COVIM project of NaFoUniMedCovid19 network (FKZ: 01KX2021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. All generated data is represented within this manuscript.

Acknowledgments: We thank all members from the Translational Research Unit—Infectious Diseases (TRU-ID) for their help. We kindly thank A.G. Hallek (Michael Hallek, Cologne) for providing THP-1-Cas9-eGFP cells. We kindly thank Marija Backovic (Structural Virology Unit, Institut Pasteur) for her participation and help in this project. We acknowledge Jakob J. Malin for illustrations made with biorender.com.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. World Health Organization. WHO Coronavirus (COVID-19) Dashboard/. Available online: https://covid19.who.int/ (accessed on 13 October 2021).
2. Wang, M.Y.; Zhao, R.; Gao, L.J.; Gao, X.F.; Wang, D.P.; Cao, J.M. SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development. *Front. Cell Infect. Microbiol.* **2020**, *10*, 587269. [CrossRef]
3. Huang, C.; Wang, Y.; Li, X.; Ren, L.; Zhao, J.; Hu, Y.; Zhang, L.; Fan, G.; Xu, J.; Gu, X.; et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* **2020**, *395*, 497–506. [CrossRef]
4. Giamarellos-Bourboulis, E.J.; Netea, M.G.; Rovina, N.; Akinosoglou, K.; Antoniadou, A.; Antonakos, N.; Damoraki, G.; Gkavogianni, T.; Adami, M.-E.; Katsaounou, P.; et al. Complex Immune Dysregulation in COVID-19 Patients with Severe Respiratory Failure. *Cell Host. Microbe* **2020**, *27*, 992–1000.e3. [CrossRef] [PubMed]
5. Xu, J.; Zhao, S.; Teng, T.; Abdalla, A.E.; Zhu, W.; Xie, L.; Wang, Y.; Guo, X. Systematic Comparison of Two Animal-to-Human Transmitted Human Coronaviruses: SARS-CoV-2 and SARS-CoV. *Viruses* **2020**, *12*, 244. [CrossRef]
6. Wilkins, T.; Akhtar, M.; Gititu, E.; Jalluri, C.; Ramirez, J. Diagnosis and Management of Hepatitis C. *Am. Fam. Physician* **2015**, *91*, 835–842.
7. Emery, V.C. Investigation of CMV disease in immunocompromised patients. *J. Clin. Pathol.* **2001**, *54*, 84–88. [CrossRef]
8. Fowler, K.B.; Boppana, S.B. Congenital cytomegalovirus infection. *Semin. Perinatol.* **2018**, *42*, 149–154. [CrossRef] [PubMed]
9. Carty, M.; Guy, C.; Bowie, A.G. Detection of Viral Infections by Innate Immunity. *Biochem. Pharmacol.* **2021**, *183*, 114316. [CrossRef]
10. Zhao, C.; Zhao, W. NLRP3 Inflammasome-A Key Player in Antiviral Responses. *Front. Immunol.* **2020**, *11*, 211. [CrossRef] [PubMed]

11. D'Arcy, M.S. Cell death: A review of the major forms of apoptosis, necrosis and autophagy. *Cell Biol. Int.* **2019**, *43*, 582–592. [CrossRef]
12. He, Z.; Chen, J.; Zhu, X.; An, S.; Dong, X.; Yu, J.; Zhang, S.; Wu, Y.; Li, G.; Zhang, Y.; et al. NLRP3 Inflammasome Activation Mediates Zika Virus-Associated Inflammation. *J. Infect. Dis.* **2018**, *217*, 1942–1951. [CrossRef] [PubMed]
13. Rodrigues, T.S.; de Sá, K.S.G.; Ishimoto, A.Y.; Becerra, A.; Oliveira, S.; Almeida, L.; Gonçalves, A.V.; Perucello, D.B.; Andrade, W.A.; Castro, R.; et al. Inflammasomes are activated in response to SARS-CoV-2 infection and are associated with COVID-19 severity in patients. *J. Exp. Med.* **2021**, *218*, e20201707. [CrossRef] [PubMed]
14. Shang, J.; Wan, Y.; Luo, C.; Ye, G.; Geng, Q.; Auerbach, A.; Li, F. Cell entry mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 11727–11734. [CrossRef]
15. Nguyen, C.C.; Kamil, J.P. Pathogen at the Gates: Human Cytomegalovirus Entry and Cell Tropism. *Viruses* **2018**, *10*, 704. [CrossRef]
16. Khan, A.G.; Miller, M.T.; Marcotrigiano, J. HCV glycoprotein structures: What to expect from the unexpected. *Curr. Opin. Virol.* **2015**, *12*, 53–58. [CrossRef] [PubMed]
17. Samrat, S.K.; Tharappel, A.M.; Li, Z.; Li, H. Prospect of SARS-CoV-2 spike protein: Potential role in vaccine and therapeutic development. *Virus Res.* **2020**, *288*, 198141. [CrossRef]
18. Theobald, S.J.; Kreer, C.; Khailaie, S.; Bonifacius, A.; Eiz-Vesper, B.; Figueiredo, C.; Mach, M.; Backovic, M.; Ballmaier, M.; Koenig, J.; et al. Repertoire characterization and validation of gB-specific human IgGs directly cloned from humanized mice vaccinated with dendritic cells and protected against HCMV. *PLoS Pathog.* **2020**, *16*, e1008560. [CrossRef]
19. Gerna, G.; Lilleri, D. Human cytomegalovirus (HCMV) infection/re-infection: Development of a protective HCMV vaccine. *New Microbiol.* **2019**, *42*, 1–20.
20. Ströh, L.J.; Krey, T. HCV Glycoprotein Structure and Implications for B-Cell Vaccine Development. *Int. J. Mol. Sci.* **2020**, *21*, 6781. [CrossRef]
21. Kumar, A.; Dowling, W.E.; Román, R.G.; Chaudhari, A.; Gurry, C.; Le, T.T.; Tollefson, S.; Clark, C.E.; Bernasconi, V.; Kristiansen, P.A. Status Report on COVID-19 Vaccines Development. *Curr. Infect. Dis. Rep.* **2021**, *23*, 9. [CrossRef] [PubMed]
22. Theobald, S.J.; Simonis, A.; Georgomanolis, T.; Kreer, C.; Zehner, M.; Eisfeld, H.S.; Albert, M.C.; Chhen, J.; Motameny, S.; Erger, F.; et al. Long-lived macrophage reprogramming drives spike protein-mediated inflammasome activation in COVID-19. *EMBO Mol. Med.* **2021**, *13*, e14150. [CrossRef]
23. Hsieh, C.L.; Goldsmith, J.A.; Schaub, J.M.; DiVenere, A.M.; Kuo, H.C.; Javanmardi, K.; Le, K.C.; Wrapp, D.; Lee, A.G.; Liu, Y.; et al. Structure-based design of prefusion-stabilized SARS-CoV-2 spikes. *Science* **2020**, *369*, 1501–1505. [CrossRef]
24. Kowarz, E.; Löscher, D.; Marschalek, R. Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines. *Biotechnol. J.* **2015**, *10*, 647–653. [CrossRef] [PubMed]
25. Krey, T.; d'Alayer, J.; Kikuti, C.M.; Saulnier, A.; Damier-Piolle, L.; Petitpas, I.; Johansson, D.X.; Tawar, R.G.; Baron, B.; Robert, B.; et al. The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule. *PLoS Pathog.* **2010**, *6*, e1000762. [CrossRef] [PubMed]
26. Newton, A.H.; Cardani, A.; Braciale, T.J. The host immune response in respiratory virus infection: Balancing virus clearance and immunopathology. *Semin. Immunopathol.* **2016**, *38*, 471–482. [CrossRef]
27. Tapia-Abellán, A.; Angosto-Bazarra, D.; Martínez-Banaclocha, H.; de Torre-Minguela, C.; Cerón-Carrasco, J.P.; Pérez-Sánchez, H.; Arostegui, J.I.; Pelegrin, P. MCC950 closes the active conformation of NLRP3 to an inactive state. *Nat. Chem. Biol.* **2019**, *15*, 560–564. [CrossRef]
28. Beckwith, K.S.; Beckwith, M.S.; Ullmann, S.; Sætra, R.S.; Kim, H.; Marstad, A.; Åsberg, S.E.; Strand, T.A.; Haug, M.; Niederweis, M.; et al. Plasma membrane damage causes NLRP3 activation and pyroptosis during Mycobacterium tuberculosis infection. *Nat. Commun.* **2020**, *11*, 2270. [CrossRef] [PubMed]
29. Dubois, H.; Sorgeloos, F.; Sarvestani, S.T.; Martens, L.; Saeys, Y.; Mackenzie, J.M.; Lamkanfi, M.; van Loo, G.; Goodfellow, I.; Wullaert, A. Nlrp3 inflammasome activation and Gasdermin D-driven pyroptosis are immunopathogenic upon gastrointestinal norovirus infection. *PLoS Pathog.* **2019**, *15*, e1007709. [CrossRef] [PubMed]
30. Man, S.M.; Karki, R.; Kanneganti, T.D. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol. Rev.* **2017**, *277*, 61–75. [CrossRef] [PubMed]
31. Thomas, P.G.; Dash, P.; Aldridge, J.R., Jr.; Ellebedy, A.H.; Reynolds, C.; Funk, A.J.; Martin, W.J.; Lamkanfi, M.; Webby, R.J.; Boyd, K.L.; et al. The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* **2009**, *30*, 566–575. [CrossRef]
32. Huang, Y.; Liu, L.; Ma, D.; Liao, Y.; Lu, Y.; Huang, H.; Qin, W.; Liu, X.; Fang, F. Human cytomegalovirus triggers the assembly of AIM2 inflammasome in THP-1-derived macrophages. *J. Med. Virol.* **2017**, *89*, 2188–2195. [CrossRef] [PubMed]
33. Shrivastava, S.; Mukherjee, A.; Ray, R.; Ray, R.B. Hepatitis C virus induces interleukin-1β (IL-1β)/IL-18 in circulatory and resident liver macrophages. *J. Virol.* **2013**, *87*, 12284–12290. [CrossRef] [PubMed]
34. Jenks, J.A.; Nelson, C.S.; Roark, H.K.; Goodwin, M.L.; Pass, R.F.; Bernstein, D.I.; Walter, E.B.; Edwards, K.M.; Wang, D.; Fu, T.M.; et al. Antibody binding to native cytomegalovirus glycoprotein B predicts efficacy of the gB/MF59 vaccine in humans. *Sci. Transl. Med.* **2020**, *12*, 3611. [CrossRef] [PubMed]
35. Bailey, J.R.; Barnes, E.; Cox, A.L. Approaches, Progress, and Challenges to Hepatitis C Vaccine Development. *Gastroenterology* **2019**, *156*, 418–430. [CrossRef] [PubMed]

36. Iavarone, C.; O'Hagan, D.T.; Yu, D.; Delahaye, N.F.; Ulmer, J.B. Mechanism of action of mRNA-based vaccines. *Expert Rev. Vaccines* **2017**, *16*, 871–881. [CrossRef]
37. Edwards, D.K.; Jasny, E.; Yoon, H.; Horscroft, N.; Schanen, B.; Geter, T.; Fotin-Mleczek, M.; Petsch, B.; Wittman, V. Adjuvant effects of a sequence-engineered mRNA vaccine: Translational profiling demonstrates similar human and murine innate response. *J. Transl. Med.* **2017**, *15*, 1. [CrossRef] [PubMed]
38. Vasiliauskaite, I.; Owsianka, A.; England, P.; Khan, A.G.; Cole, S.; Bankwitz, D.; Foung, S.K.H.; Pietschmann, T.; Marcotrigiano, J.; Rey, F.A.; et al. Conformational Flexibility in the Immunoglobulin-Like Domain of the Hepatitis C Virus Glycoprotein E2. *mBio* **2017**, *8*, e00382-17. [CrossRef] [PubMed]
39. Xia, X.; Wang, X.; Zheng, Y.; Jiang, J.; Hu, J. What role does pyroptosis play in microbial infection? *J. Cell Physiol.* **2019**, *234*, 7885–7892. [CrossRef]
40. Kofahi, H.M.; Taylor, N.G.; Hirasawa, K.; Grant, M.D.; Russell, R.S. Hepatitis C Virus Infection of Cultured Human Hepatoma Cells Causes Apoptosis and Pyroptosis in Both Infected and Bystander Cells. *Sci. Rep.* **2016**, *6*, 37433. [CrossRef]
41. Huet, T.; Beaussier, H.; Voisin, O.; Jouveshomme, S.; Dauriat, G.; Lazareth, I.; Sacco, E.; Naccache, J.M.; Bézie, Y.; Laplanche, S.; et al. Anakinra for severe forms of COVID-19: A cohort study. *Lancet Rheumatol.* **2020**, *2*, e393–e400. [CrossRef]
42. van de Veerdonk, F.L.; Netea, M.G. Blocking IL-1 to prevent respiratory failure in COVID-19. *Crit. Care* **2020**, *24*, 445. [CrossRef] [PubMed]
43. Zhu, H.; Liu, C. Interleukin-1 inhibits hepatitis C virus subgenomic RNA replication by activation of extracellular regulated kinase pathway. *J. Virol.* **2003**, *77*, 5493–5498. [CrossRef] [PubMed]

5. Discussion

Viral infections pose a significant global health threat. Pathogenic viruses can cause a variety of diseases, ranging from acute respiratory infections to chronic hepatitis. The course of an infection is largely determined by the interaction between the virus and the immune system. However, viral pathogenesis and the associated immune responses are often poorly understood. The global COVID-19 pandemic has highlighted the importance of in-depth research for adequate patient care and prevention strategies, such as vaccine design. In this study, four different viral glycoproteins were analyzed to determine if they function as PAMPs and thus induce an inflammasome-dependent innate immune response in macrophages.

5.1. Viral glycoproteins and innate immunity

This study shows that the viral glycoproteins gB of HCMV, E2 of HCV, and the S-protein of SARS-CoV-1 and SARS-CoV-2 can prime the NLRP3 inflammasome in THP-1 macrophages. Consequently, the pro-inflammatory cytokine IL-1ß is released and the cell undergoes pyroptotic cell death. To contextualize these results within viral pathogenesis, the following chapter addresses previous research on the studied viruses and innate immune responses.

Negash et al. show that patients chronically infected with HCV have elevated IL-1ß serum levels, suggesting HCV dependent inflammasome activation *in vivo*. Furthermore, they show that the NLRP3 inflammasome is activated in macrophages after exposure to the virus *in vitro* [27]. Although studies suggest that human hepatocytes are not the primary source of IL-1β secretion, one *in vitro* study indicates that the NLRP3 inflammasome is still activated after HCV infection and may contribute to liver inflammation. However, the mechanisms behind this activation remain poorly understood [27, 28]. While previous studies show NLRP3 inflammasome activation after exposure to the whole virus, this study indicates that the E2 protein functions as a priming signal in this process, which could be important for further research.

Inflammasome activation has been discussed in the context of COVID-19 associated inflammation. Early studies have shown elevated lactase dehydrogenase serum levels in COVID-19 patients, as a marker for cell destruction and pyroptosis. In addition to lactase dehydrogenase, pro-inflammatory cytokines were elevated in patient serums [29]. A more recent study detected inflammasome associated specks in blood monocytes from COVID-19 patients. Interestingly, NLRP3 inflammasome activation is associated with the severity of COVID-19 [30]. Theobald et al. demonstrate that the spike protein primes NLRP3 inflammasome activation in peripheral blood mononuclear cells (PBMCs) derived from COVID-

19 patients, but not in healthy individuals, suggesting a role of trained innate immunity in this process [31].

Several studies have shown that SARS-CoV-1 activates the NLRP3 inflammasome *in vitro*. The ORF3a protein activates the inflammasome by changing the potassium ion permeability of the plasma membrane and by the production of mROS. The envelop protein causes changes in calcium permeability, leading to inflammasome activation [30, 32]. In contrast, we found that the S-protein functions as a PAMP, inducing NLRP3 inflammasome activation via caspase-1.

Studies on HCMV and the NLRP3 inflammasome are limited. One study reports elevated levels of NLRP3 mRNA in blood samples from critically ill patients with CMV viremia and sepsis. However, the authors note that the small sample size means these results should be considered preliminary [33].

Taken together, these results support our study and highlight the role of viral glycoproteins in innate immune responses. As our work included only THP-1 macrophages, the next step could be to perform experiments with patient-derived material. Stimulating patient-derived PBMCs with viral glycoproteins would allow us to analyze inflammasome activation more accurately. Additionally, *in vivo* experiments are necessary to strengthen our findings. Developing a mouse model could help analyze the effects of viral glycoproteins or whole viruses on NLRP3 inflammasome expression, activation, and cytokine release. Using NLRP3 knockout mice could further elucidate the role of the inflammasome in innate immune responses to these viruses.

5.2. The role of viral glycoproteins and inflammasomes in vaccine-induced immunity

5.2.1. Short overview of vaccine development

The goal of vaccines is to protect the body from infections by creating specific antimicrobial immunity in the host. Vaccines designed against viral infections usually induce the production of antibodies that target immunogenic viral proteins, preventing the virus from entering and infecting host cells [34]. Besides antibodies, T cells are crucial for vaccine efficacy, by forming memory T cells that respond rapidly to subsequent encounters with the vaccine or pathogen [35].

Traditionally vaccine development focused on whole viruses, either live-attenuated or inactivated, or virus-like particles. However, this approach is not effective for all viral infections or might be too time-consuming. In recent decades, structure-based vaccine design has become a research focus. This approach studies viral proteins with immunogenic properties as potential vaccine constructs, identifying vulnerable sites for neutralizing antibody

attachment. A deep mechanistic understanding of the respective proteins is crucial for this approach to vaccine development [34]. To enhance immunogenicity in structure-based vaccines, adjuvants, such as aluminum salts, are often used to modulate the immune response to specific antigens [36]. Most recently, during the COVID-19 pandemic, the first mRNA vaccines were approved. These vaccines deliver a small, non-infectious segment of the virus mRNA into human cells, which then produce and express a viral antigen, in this case the S-protein, triggering an immune response [37].

5.2.2. Glycoproteins and inflammasomes as potential targets

In our research, we analyzed the impact of four different viral glycoproteins on the innate immune system. All four glycoproteins induced NLRP3 inflammasome activation and pyroptosis, demonstrating their immunogenic properties and potential as vaccine targets. Previous studies have highlighted the importance of B and T cell responses against these viral glycoproteins, underscoring their suitability for vaccine research [38-42]. Notably, the S-proteins of SARS-CoV-2 has been pivotal in COVID-19 vaccine development, with all approved vaccines targeting the S-protein to elicit neutralizing antibodies that inhibit viral entry [43]. Our research extends the understanding of the immune response to the S-protein, emphasizing its role in inducing a robust immune response.

Inflammasomes, particularly the NLRP3 inflammasome, play a crucial role in mediating immune responses, which can influence vaccine efficacy. Studies show that aluminum hydroxide, a common vaccine adjuvant, activates the NLRP3 inflammasome to induce the secretion of IL-1β and IL-18 [36, 44]. While NLRP3 is critical for aluminum-mediated IL-1β secretion, research on its importance for adjuvant activity *in vivo* shows mixed results. Li et al. report an impaired antibody response to aluminum-containing vaccines in NLRP3 knockout mice, while other studies present contradictory data [36, 44]. Other adjuvants, such as QuilA and chitosan, also induce NLRP3 inflammasome activation, implying a broader significance of the NLRP3 inflammasome in vaccine efficacy [36]. However, the immune response to the Pfizer-BioNTech BNT162b2 mRNA vaccine appears independent of NLRP3 inflammasome activation and various TLR pathways, suggesting that different vaccine platforms may utilize distinct innate immune mechanisms [37]. These findings underscore the complexity and variability of viral glycoproteins and inflammasomes in vaccine-induced immunity, emphasizing the need for further research into their roles in vaccine efficacy.

5.3. Immunological consequences and other potential pathways

An immune response to pathogens involves a complex interplay of various immune signals and responses. This complexity poses a challenge in understanding viral pathogenesis. This study demonstrates that viral glycoproteins can activate the NLRP3

inflammasome in macrophages, leading to the release of pro-inflammatory cytokines and pyroptotic cell death. While these findings are a step toward deciphering virus-host immune interactions, they represent only a small part of a complex system. The following chapter will address knowledge gaps and explore additional inflammatory pathways that may be activated beyond the NLRP3 inflammasome.

5.3.1. Receptors for cellular entry and immune response

Cells express a variety of different receptors. Some are used by viruses for cellular entry, others can detect pathogens and trigger an immune response. The interaction between viral pathogens and cellular receptors remains largely unknown. Understanding how host cell receptors recognize viral PAMPs to trigger innate immune responses is crucial for developing antiviral or host-directed immune modulatory treatments and vaccines.

To detect pathogens, cells use a variety of different PRRs. NLRs, such as NLRP1, NLRP3, and NLRC4, are the sensors for inflammasome complexes [19]. While this study shows the activation of the NLRP3 inflammasome through viral glycoproteins, the receptors involved in the priming and activation process remain largely unknown. TLRs have an important role in sensing pathogens and activating the immune system. Cell-surface TLRs mainly bind to proteins, lipids, and lipoproteins, while intracellular TLRs detect nucleic acids or microbial components derived from endolysosomal degradation. TLR2, expressed on the cell surface, has been described to detect gB of HCMV and consequently activate NFkB, leading to pro-inflammatory cytokine production [45]. Studies suggest that HCV RNA binds to TLR7, triggering an immune response in various cell types [46]. Knockdown of TLR7 significantly reduces IL-1ß messenger RNA expression in HCV-exposed cells, suggesting that TLR7 mediates one of the priming signals for the NLRP3 inflammasome [27]. TLR4 binds lipopolysaccharide, a major component of the outer membrane of gram-negative bacteria. Research however also describes a role in the detection of viral glycoproteins, such as, the fusion protein of the respiratory syncytial virus and the S1 subunit of the spike protein of SARS-CoV-2 [45]. Interestingly, Theobald et al. show that TLR2 is upregulated in PBMCs isolated from COVID-19 patients compared to healthy controls, suggesting a role for TLR2 in COVID-19 pathogenesis [31]. When considering the entire virus, other TLRs might come into play. Sartorius et al. suggest that TLR7 and TLR8 could be involved in sensing the single-stranded RNA of SARS-CoV-2 [45].

The interaction between TLRs and viral PAMPs often activates interferon signaling cascades [45]. In HCMV infections, the NFkB/interferon-mediated signaling pathway is a well-described innate immune response [47]. Further analysis of inflammasome-independent pathways in viral pathogenesis could enhance the understanding of viral-host interactions.

Analyzing PRRs in experiments could uncover specific mechanisms of viral recognition and immune activation, potentially revealing new targets for antiviral therapies and vaccine adjuvants. These experiments might involve gene knockout or knockdown techniques to assess the impact of individual receptors on immune responses and cytokine release. Techniques such as CRISPR-Cas9 or RNA interference, through small interfering RNA, could be used to precisely manipulate receptor expression and function, allowing for detailed dissection of their roles in innate immunity.

5.3.2. NLRP3 inflammasome activation – signal 1 and signal 2

NLRP3 inflammasome activation requires two signals: a priming signal and an activation signal. This study analyzes four viral glycoproteins as priming signals, using nigericin to provide the second signal. Nigericin induces potassium efflux, resulting in abnormal ion concentrations within the cell. Identifying the physiological second signal that triggers NLRP3 assembly during infections with SARS-CoV-1, SARS-CoV-2, HCV, and HCMV requires further research.

For SARS-CoV-2 infections, the nucleocapsid protein (N-protein), which is involved in viral RNA packaging, has been suggested as a possible second signal. Pan et al. demonstrate that the N-protein directly interacts with NLRP3 and promotes ASC binding, facilitating inflammasome assembly. This interaction induces IL-1β secretion and triggers an immune response [48].

Chen et al. show that the viroporin 3a of SARS-CoV-1 activates the NLRP3 inflammasome through potassium efflux [32]. Viroporins, which form ion channels in host cell membranes, disrupt cellular homeostasis and facilitate viral replication and pathogenesis [49]. Changes in membrane ion permeability are a well-known second signal for inflammasome activation [32].

Negash et al. analyze the effect of HCV infection on liver macrophages, showing that the intact virus is required for processing pro-IL-1β. The study demonstrates that HCV-induced potassium efflux serves as a second signal for NLRP3 inflammasome activation. However, although the HCV p7 protein, a transmembrane cation channel, was discussed as a potential cause, the specific protein responsible for this potassium efflux was not identified [27].

Next steps could be to employ danger signals, such as ATP or uric acid, as second signals to further enhance the experimental design, replacing nigericin in future studies. Additionally, the analysis of other viral proteins, particularly viroporins, could provide valuable insights.

5.3.3. Other inflammasomes

In our research, we focused on the most studied inflammasome, the NLRP3 inflammasome. However, it is conceivable that the viruses investigated in our study trigger other inflammasomes as well.

Research shows that HCMV infections activate the AIM2 inflammasome in THP1 macrophages, leading to the release of IL-1β and induction of cell death. AIM2-deficient cells exhibit significantly lower levels of cleaved caspase-1 and mature IL-1β in the initial hours post-infection, suggesting a critical role of the AIM2 inflammasome. Interestingly, the secretion of mature IL-1β in AIM2-deficient cells increased after 24 hours, suggesting the involvement of other inflammasomes at this later stage [47]. *In vivo* studies are needed to understand the relevance of these findings.

Rossi et al. studied liver biopsies from HCV-positive patients and compared them to those from patients with metabolic chronic liver diseases, such as non-alcoholic fatty liver disease and non-alcoholic steatohepatitis. The NLRP3 inflammasome was expressed in all biopsies but to a greater extent in HCV-positive patients, where it was localized in migrating macrophages. The AIM2 inflammasome was almost exclusively expressed in HCV-positive biopsies and was localized in Kupffer cells, suggesting a role for AIM2 in HCV infections and indicating that inflammasome activation may be cell-type dependent. However, as mentioned by the authors, these results were not compared to biopsies from healthy controls, and the cohort size was relatively small, necessitating confirmation in future research [50]. Negash et al. demonstrate that an NLRP3 knockdown in THP-1 macrophages resulted in the loss of IL-1β maturation after HCV exposure, suggesting that the NLRP3 inflammasome is a primary driver of IL-1β release, potentially ruling out a prominent role for other inflammasomes [27].

Pan et al show that the N-protein of SARS-CoV-2 failed to activate the NLRP1, NLRC4, or AIM2 inflammasome, suggesting that the N-protein specifically triggers NLRP3 [48].

This study utilizes NLRP3 knockout cells to highlight the crucial role of the NLRP3 inflammasome in response to viral glycoproteins. Stimulated knockout cells exhibited minimal IL-1β secretion after 6 hours. These findings suggest that, in this specific cell type and time frame, the NLRP3 inflammasome is the primary driver of IL-1β maturation and secretion. Notably, the AIM2 inflammasome is activated by direct binding of DNA, and in some cases RNA, rather than glycoproteins, which were the focus of our experiments. Additionally, the activation of other inflammasomes may be triggered by different viral structures and could vary based on time or cell type. Therefore, it could be valuable to stimulate NLRP3 knockout cells with whole viruses to determine if IL-1β is released, potentially indicating the involvement of other inflammasomes or other viral glycoproteins.

5.4. Outlook

This study demonstrates the immunogenic properties of the viral glycoproteins E2 of HCV, gB of HCMV, and the S-protein of SARS-CoV-1 and SARS-CoV-2, as they prime the NLRP3 inflammasome for activation in THP-1 macrophages. These findings underscore the importance of these glycoproteins in viral pathogenesis and the host immune response. However, further research is needed to elucidate the underlying mechanisms. Future studies could aim to identify macrophage-derived receptors that bind these viral glycoproteins to trigger the observed innate immune response. Furthermore, the second physiological signal required for NLRP3 inflammasome activation remains to be determined. This signal could originate from the virus itself or be an intrinsic molecule released upon viral infection.

This study was conducted with cell lines *in vitro*. While inflammasome activation can aid the host by facilitating viral clearance, it can also cause harm through hyperinflammation and tissue destruction. Delineating these inflammasome mediated effects in viral infections requires *in vivo* or *ex vivo* studies. However, these studies are often limited due to the lack of suitable animal models for the respective viral disease [51].

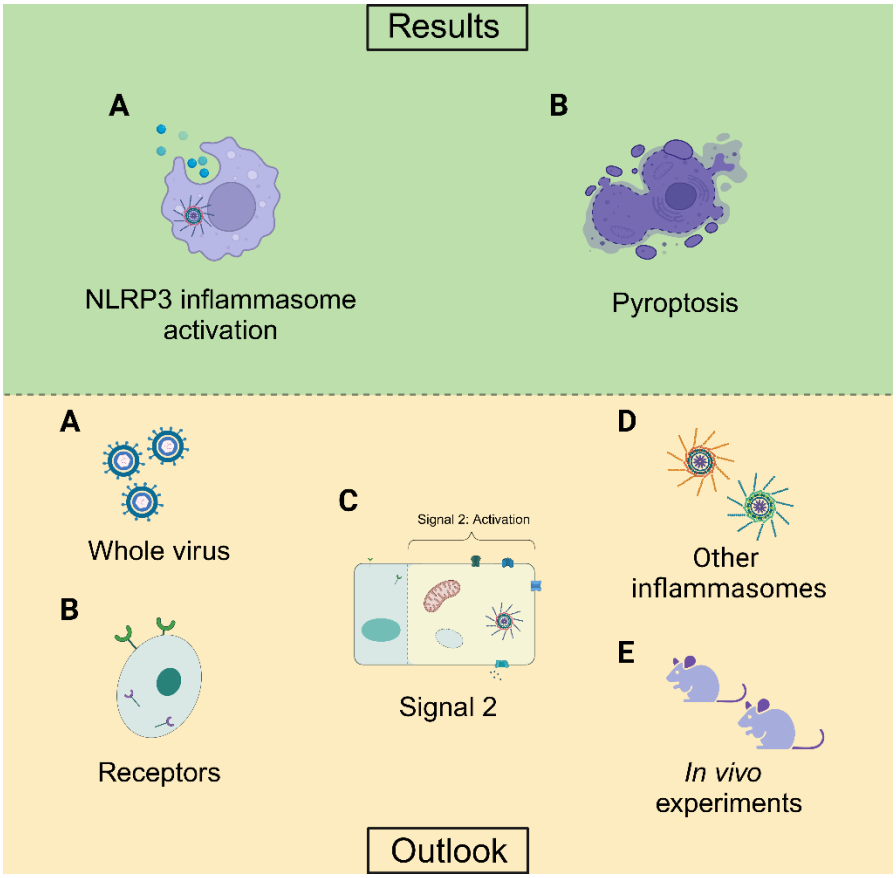

*Figure 3: Summary of results and outlook. On top the results of this study are shown, being NLRP3 inflammasome activation and IL-1ß release (**A**), as well as pyroptotic cell death (**B**). The bottom part shows the outlook with important aspects being the stimulation with whole viruses (**A**), the identification of involved receptors (**B**), the detection of second signals for inflammasome activation (**C**), the role of other inflammasomes in the activation of innate immune responses concerning viral infections with HCMV, HCV, SARS-CoV-1 and SARS-CoV-2 (**D**), and in vivo experiments (**E**). (Created with BioRender.com)*

6. Literaturverzeichnis

1. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
2. Netea, M.G., et al., *Defining trained immunity and its role in health and disease.* Nat Rev Immunol, 2020. **20**(6): p. 375-388.
3. Hajishengallis, G., et al., *Trained Innate Immunity and Its Implications for Mucosal Immunity and Inflammation.* Adv Exp Med Biol, 2019. **1197**: p. 11-26.
4. Christgen, S. and T.D. Kanneganti, *Inflammasomes and the fine line between defense and disease.* Curr Opin Immunol, 2020. **62**: p. 39-44.
5. Fenini, G., et al., *The NLRP1 Inflammasome in Human Skin and Beyond.* Int J Mol Sci, 2020. **21**(13).
6. Fu, J. and H. Wu, *Structural Mechanisms of NLRP3 Inflammasome Assembly and Activation.* Annu Rev Immunol, 2023. **41**: p. 301-316.
7. Bauernfried, S. and V. Hornung, *Human NLRP1: From the shadows to center stage.* J Exp Med, 2022. **219**(1).
8. Mitchell, P.S., A. Sandstrom, and R.E. Vance, *The NLRP1 inflammasome: new mechanistic insights and unresolved mysteries.* Curr Opin Immunol, 2019. **60**: p. 37-45.
9. Kelley, N., et al., *The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation.* Int J Mol Sci, 2019. **20**(13).
10. Zhao, C. and W. Zhao, *NLRP3 Inflammasome-A Key Player in Antiviral Responses.* Front Immunol, 2020. **11**: p. 211.
11. Duncan, J.A. and S.W. Canna, *The NLRC4 Inflammasome.* Immunol Rev, 2018. **281**(1): p. 115-123.
12. Kumari, P., et al., *AIM2 in health and disease: Inflammasome and beyond.* Immunol Rev, 2020. **297**(1): p. 83-95.
13. Schnappauf, O., et al., *The Pyrin Inflammasome in Health and Disease.* Front Immunol, 2019. **10**: p. 1745.
14. Yu, P., et al., *Pyroptosis: mechanisms and diseases.* Signal Transduct Target Ther, 2021. **6**(1): p. 128.
15. Zhaolin, Z., et al., *Role of pyroptosis in cardiovascular disease.* Cell Prolif, 2019. **52**(2): p. e12563.
16. Fang, Y., et al., *Pyroptosis: A new frontier in cancer.* Biomed Pharmacother, 2020. **121**: p. 109595.
17. Li, D. and M. Wu, *Pattern recognition receptors in health and diseases.* Signal Transduct Target Ther, 2021. **6**(1): p. 291.
18. Roh, J.S. and D.H. Sohn, *Damage-Associated Molecular Patterns in Inflammatory Diseases.* Immune Netw, 2018. **18**(4): p. e27.
19. Kanneganti, T.D., *Intracellular innate immune receptors: Life inside the cell.* Immunol Rev, 2020. **297**(1): p. 5-12.
20. Carty, M., C. Guy, and A.G. Bowie, *Detection of Viral Infections by Innate Immunity.* Biochem Pharmacol, 2021. **183**: p. 114316.
21. Banerjee, N. and S. Mukhopadhyay, *Viral glycoproteins: biological role and application in diagnosis.* Virusdisease, 2016. **27**(1): p. 1-11.
22. Nguyen, C.C. and J.P. Kamil, *Pathogen at the Gates: Human Cytomegalovirus Entry and Cell Tropism.* Viruses, 2018. **10**(12).
23. Khan, A.G., M.T. Miller, and J. Marcotrigiano, *HCV glycoprotein structures: what to expect from the unexpected.* Curr Opin Virol, 2015. **12**: p. 53-8.
24. Hoffmann, M., et al., *SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor.* Cell, 2020. **181**(2): p. 271-280 e8.
25. Reis, C.A., R. Tauber, and V. Blanchard, *Glycosylation is a key in SARS-CoV-2 infection.* J Mol Med (Berl), 2021. **99**(8): p. 1023-1031.

26. Gardner, T.J. and D. Tortorella, *Virion Glycoprotein-Mediated Immune Evasion by Human Cytomegalovirus: a Sticky Virus Makes a Slick Getaway.* Microbiol Mol Biol Rev, 2016. **80**(3): p. 663-77.
27. Negash, A.A., et al., *IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease.* PLoS Pathog, 2013. **9**(4): p. e1003330.
28. Ramachandran, A., et al., *Deubiquitination and Activation of the NLRP3 Inflammasome by UCHL5 in HCV-Infected Cells.* Microbiol Spectr, 2021. **9**(1): p. e0075521.
29. Huang, C., et al., *Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China.* Lancet, 2020. **395**(10223): p. 497-506.
30. Vora, S.M., J. Lieberman, and H. Wu, *Inflammasome activation at the crux of severe COVID-19.* Nat Rev Immunol, 2021. **21**(11): p. 694-703.
31. Theobald, S.J., et al., *Long-lived macrophage reprogramming drives spike protein-mediated inflammasome activation in COVID-19.* EMBO Mol Med, 2021. **13**(8): p. e14150.
32. Chen, I.Y., et al., *Severe Acute Respiratory Syndrome Coronavirus Viroporin 3a Activates the NLRP3 Inflammasome.* Front Microbiol, 2019. **10**: p. 50.
33. Singh, N., et al., *Inflammasome expression and cytomegalovirus viremia in critically ill patients with sepsis.* J Clin Virol, 2017. **93**: p. 8-14.
34. Graham, B.S., M.S.A. Gilman, and J.S. McLellan, *Structure-Based Vaccine Antigen Design.* Annu Rev Med, 2019. **70**: p. 91-104.
35. Palgen, J.L., et al., *Optimize Prime/Boost Vaccine Strategies: Trained Immunity as a New Player in the Game.* Front Immunol, 2021. **12**: p. 612747.
36. Li, H., et al., *Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3.* J Immunol, 2008. **181**(1): p. 17-21.
37. Li, C., et al., *Mechanisms of innate and adaptive immunity to the Pfizer-BioNTech BNT162b2 vaccine.* Nat Immunol, 2022. **23**(4): p. 543-555.
38. Luxenburger, H., et al., *HCV-Specific T Cell Responses During and After Chronic HCV Infection.* Viruses, 2018. **10**(11).
39. Smith, C.J., M. Quinn, and C.M. Snyder, *CMV-Specific CD8 T Cell Differentiation and Localization: Implications for Adoptive Therapies.* Front Immunol, 2016. **7**: p. 352.
40. Moss, P., *The T cell immune response against SARS-CoV-2.* Nat Immunol, 2022. **23**(2): p. 186-193.
41. Stuart, J.D., E. Salinas, and A. Grakoui, *Immune system control of hepatitis C virus infection.* Curr Opin Virol, 2021. **46**: p. 36-44.
42. Dauby, N., et al., *Limited Effector Memory B-Cell Response to Envelope Glycoprotein B During Primary Human Cytomegalovirus Infection.* J Infect Dis, 2016. **213**(10): p. 1642-50.
43. Martinez-Flores, D., et al., *SARS-CoV-2 Vaccines Based on the Spike Glycoprotein and Implications of New Viral Variants.* Front Immunol, 2021. **12**: p. 701501.
44. Franchi, L. and G. Núñez, *The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity.* Eur J Immunol, 2008. **38**(8): p. 2085-9.
45. Sartorius, R., et al., *Exploiting viral sensing mediated by Toll-like receptors to design innovative vaccines.* NPJ Vaccines, 2021. **6**(1): p. 127.
46. Xu, C., J. Chen, and X. Chen, *Host Innate Immunity Against Hepatitis Viruses and Viral Immune Evasion.* Front Microbiol, 2021. **12**: p. 740464.
47. Huang, Y., et al., *Human cytomegalovirus triggers the assembly of AIM2 inflammasome in THP-1-derived macrophages.* J Med Virol, 2017. **89**(12): p. 2188-2195.
48. Pan, P., et al., *SARS-CoV-2 N protein promotes NLRP3 inflammasome activation to induce hyperinflammation.* Nat Commun, 2021. **12**(1): p. 4664.
49. Breitinger, U., et al., *Viroporins: Structure, function, and their role in the life cycle of SARS-CoV-2.* Int J Biochem Cell Biol, 2022. **145**: p. 106185.

50. Rossi, C., et al., *The P2X7R-NLRP3 and AIM2 Inflammasome Platforms Mark the Complexity/Severity of Viral or Metabolic Liver Damage.* Int J Mol Sci, 2022. **23**(13).
51. Rong, N. and J. Liu, *Development of animal models for emerging infectious diseases by breaking the barrier of species susceptibility to human pathogens.* Emerg Microbes Infect, 2023. **12**(1): p. 2178242.

7. Anhang

7.1. Abbildungsverzeichnis

Figure 1: NLRP3 inflammasome priming and activation 13
Figure 2: Simplified scheme of virus and host cell interaction..... 16
Figure 3: Summary of results and outlook..... 40