Master's thesis

NTNU
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Exploring the role of the immunoglobulin-like receptor SLAMF1 in infection with human metapneumovirus

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Supervisor: Marit Walbye Anthonsen
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Katja Sæterhaug Bye
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Abstract

Human metapneumovirus (HMPV) is a leading cause of lower respiratory tract infection. Overall, there is little information about how HMPV enters (infects), establishes its viral replication and induces immune responses. Interestingly, recent data obtained by an RNA-sequencing analysis of human monocyte-derived macrophages (MDMs) suggested that the Ig-like receptor, signaling lymphocytic activation molecule family 1 (SLAMF1), was upregulated upon infection with HMPV. The main aim of this thesis was to determine if HMPV regulates SLAMF1 expression and establish if SLAMF1 plays a role in the innate immune responses induced by HMPV.

In this thesis we show that SLAMF1 expression is strongly upregulated upon infection of MDMs by HMPV. Our data suggest that SLAMF1 is involved in HMPV-induced signaling leading to the expression of IFN-β and TNF-α cytokines. Neither silencing of *SLAMF1*, nor the ligation of SLAMF1 on the cell surface by monoclonal antibodies seemed to affect HMPV viral load in infected cells. However, *SLAMF1*-overexpression slightly increased levels of viral mRNA when compared to the infection of control SLAMF1 knockout cells. Therefore, we suggest that SLAMF1 may contribute to the enhanced replication of HMPV, but is not critical for replication to occur. Our results also show that in MDMs TNF-α mRNA expression is induced by HMPV in two waves within 24 h, and that the first wave is TLR4- and SLAMF1-dependent, which could be connected with regulatory role of SLAMF1 in TLR4-mediated intracellular signaling. Silencing of *TLR4* and *SLAMF1* in MDMs reduced the amount of HMPV-mediated secreted TNF-α. Silencing of *TLR4* also reduced the amount of viral mRNA and protein in THP1 cells, suggesting that TLR4 positively regulates HMPV viral entry and replication. Most of the results obtained during the work on this thesis are preliminary, and the role of SLAMF1 in HMPV-infection should be addressed further.

Sammendrag

Humant metapneumovirus (HMPV) er en ledende årsak til nedre luftveisinfeksjon. Generelt finnes det lite informasjon om hvordan HMPV entrer (infiserer), etablerer replikasjon og induserer immunresponser. Nylige funn fra en RNA-sekvenseringsanalyse av humane monocytt-deriverte makrofager (MDMs) avdekket at HMPV-infeksjon førte til økt uttrykk av den Ig-lignende reseptoren, signaliserende lymfocytisk aktiverende molekyl familie 1 (SLAMF1). Hovedmålet med denne oppgaven var å finne ut om HMPV regulerer SLAMF1 uttrykk og om SLAMF1 er involvert i den medfødte immunresponsen indusert av HMPV.

I denne oppgaven viser vi at SLAMF1-uttrykk er sterkt oppregulert ved HMPV-infeksjon i MDMs. Resultatene våre indikerer at SLAMF1 er involvert i HMPV-indusert signalisering som fører til uttrykk av IFN-β og TNF-α cytokiner. Verken hemming av *SLAMF1*, eller ligering av SLAMF1 på celleoverflaten ved bruk av monoklonale antistoff så ut til å påvirke den virale mengden av HMPV i infiserte celler. Derimot førte *SLAMF1*-overuttrykk til en viss økning av viralt mRNA ved sene tidspunkt for infeksjon sammenlignet med SLAMF1-knockout celler. Derfor foreslår vi at SLAMF1 kan bidra til økt virusreplikasjon, men er ikke kritisk for at replikasjonen skal kunne foregå. Resultatene viser også at i MDMs er uttrykk av TNF-α indusert av HMPV i to bølger innen 24 timer, og at den første bølgen er TLR4- og SLAMF1-avhengig. Disse resultatene kan være relatert til den regulatoriske rollen av SLAMF1 i TLR4-mediert signalisering. Hemming av *TLR4* og *SLAMF1* i MDMs reduserte mengden av HMPV-mediert TNF-α sekresjon. Hemming av *TLR4* førte også til en redusert mengde av viralt mRNA og protein i THP1 celler, som kan tyde på at TLR4 positivt regulerer opptaket og replikasjonen av HMPV. De fleste av resultatene er preliminære, og rollen til SLAMF1 i HMPV-infeksjon må utforskes videre.

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Abbreviations

AP-1	Activator protein 1
CD14	Cluster of differentiation 14
cDNA	Complementary DNA
dsRNA	Double-stranded RNA
ERC	Endocytic recycling compartment
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HMPV	Human metapneumovirus
IFN	Interferon
IFNAR	Interferon- α/β receptor
Ig	Immunoglobulin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK	Janus kinase
KD	Knockdown
KO	Knockout
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
MAL	MyD88 adaptor-like protein
MAVS	Mitochondrial antiviral-signaling protein
MHC	Major histocompatibility complex
MDA-5	Melanoma differentiation-associated gene 5
moDC	Monocyte-derived dendritic cell
MDM	Monocyte-derived macrophage
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response protein 88
NFκB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
PAMP	Pattern-associated molecular pattern
pDC	Plasmacytoid dendritic cell
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SD	Standard deviation
SLAMF1	Signaling lymphocytic activation molecule family 1
siRNA	Small interfering RNA
ssRNA	Single-stranded RNA
STAT1	Signal transducer and activator of transcription 1
pSTAT1	Phosphorylated STAT1
TBP	TATA-box binding protein
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β

1 Introduction

1.1 Virus-host interactions

Viruses often rely on host factors for the stages of its life cycle [1]. After entry and release of the viral genome into the host cytoplasm, viruses utilize the host and its own machinery to replicate their genetic material and assemble new viral particles [2]. The requirement of the host in order to carry out these critical functions makes it important to study host-pathogen interactions for the understanding of pathogenicity.

The initial responses of a host cell to virus infection are rapid, initiated by the contact of a virus particle with a receptor or immediately following entry of the virus into the cell [3]. Upon entry, pattern recognition receptors (PRRs) recognize viral components which initiates signal transduction cascades, mobilizing host defenses. Virus infection may lead to alterations in host cell processes that facilitate production and release of progeny virus particles. For instance, infection may modify expression of cellular genes, disrupt and/or subvert trafficking of cellular macromolecules, redirect metabolic pathways or remodel cellular components to promote specific reactions in an infectious cycle [4, 5]. The extent of alterations in host cell processes depend on the properties of the host cell as well as whether the infection is productive. Commonly, differential regulation is seen where expression of some genes is increased while others are decreased. In addition, the defensive responses of the host can be blocked by viral gene products. Together these viral effects promote the growth and survival of progeny viral particles [5].

1.2 Human metapneumovirus (HMPV)

Human metapneumovirus (HMPV) is a common respiratory virus that may cause severe respiratory disease, especially in young children, elderly and immunosuppressed individuals [6-8]. A wide range of symptoms can be caused by HMPV, where difficulty breathing, high fever, wheezing, cough, bronchitis, bronchiolitis and pneumonia may occur [9, 10].

HMPV is a negative single-stranded RNA virus and part of the *Pneumoviridae* family. Like other pneumoviruses, HMPV particles are pleiomorphic and has a diameter that ranges from 150 to 600 nm. HMPV is an enveloped virus, thereby containing a lipid membrane envelope surrounding the matrix (M) protein and three transmembrane surface glycoproteins: the fusion protein (F), attachment glycoprotein (G) and small hydrophobic proteins (SH). Within the envelope resides a helical ribonucleoprotein (RNP) consisting of nucleoprotein (N) that packages genomic RNA, phosphoprotein (P), matrix protein 2 (M2-1 and M2-2) and large RNA-dependent RNA polymerase protein (L) illustrated in **Figure 1** [11]. The genome of HMPV contains eight genes and the

translation of its mRNAs produces nine different polypeptides due to open reading frames (ORF) [12, 13].

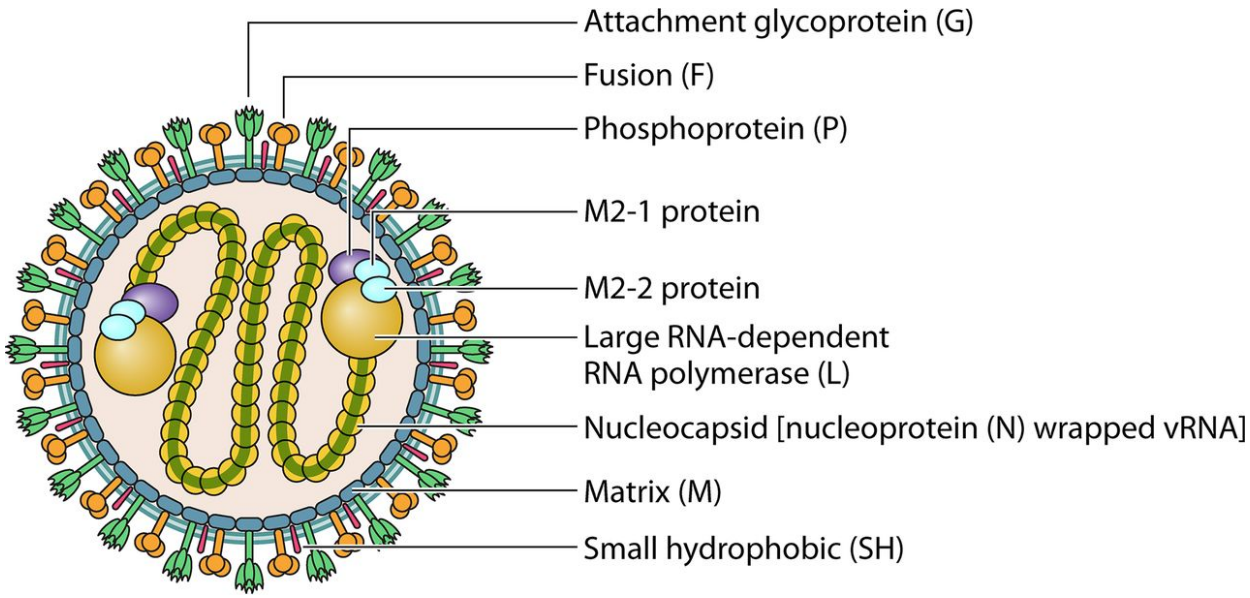

Figure 1: The metapneumovirus is an enveloped, spherical virus consisting of three surface glycoproteins (G, F, SH) surrounding the matrix protein, ribonucleoprotein (containing proteins P, N, L) and the single-stranded negative RNA genome [13].

Like other enveloped viruses, the entry of HMPV into host cells is initiated by attachment to host cell surface receptors followed by membrane fusion (**Figure 2**) [11]. Viral envelopes can be embedded with proteins that the virus use to bind and enter the host cell. The HMPV fusion protein and heparan sulfate have been suggested as the first binding partners upon HMPV binding to the host cell [14]. Helenius et al. showed that viruses have evolved the ability to take advantage of the host cell's endocytic transport mechanism for viral entry [15]. After the virion binds to host cell surface receptors, endocytic vesicles transport the viral particles to the perinuclear area of the host cell where viral replication proceeds [16]. Additionally, a study has shown that HMPV particles are internalized via clathrin-mediated endocytosis in a dynamin-dependent manner and that entry is not triggered by low pH [17].

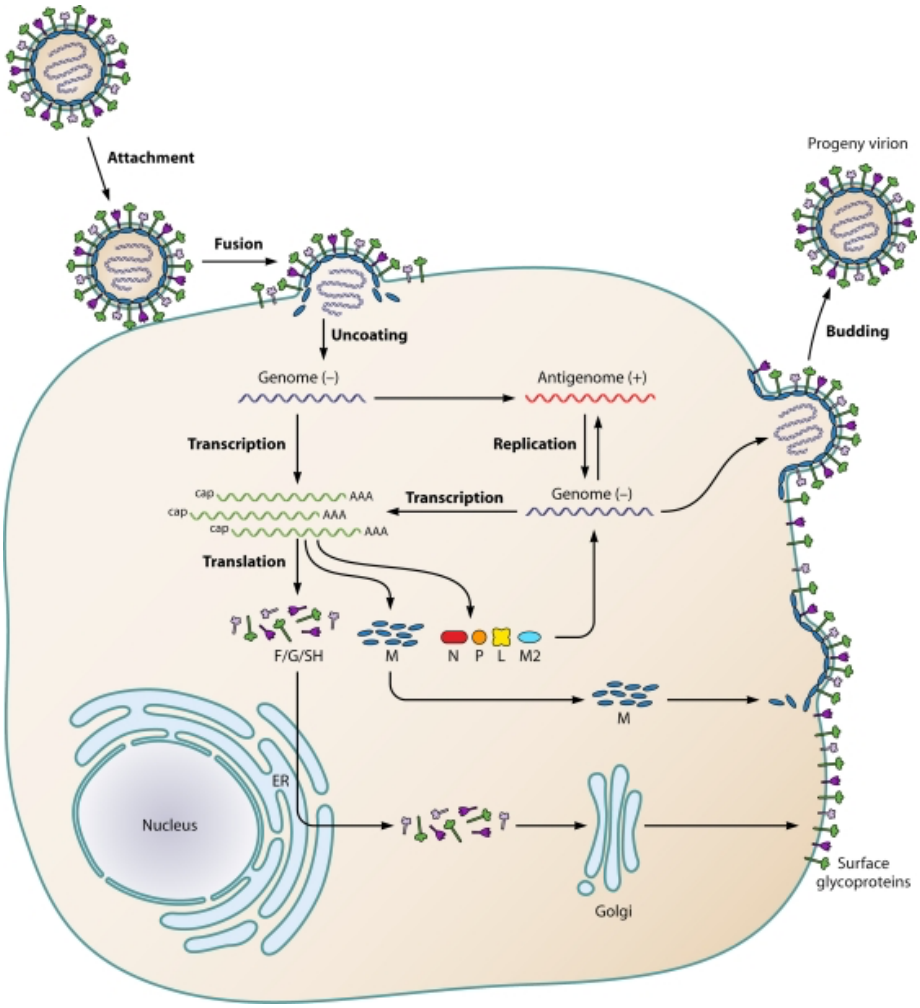

Figure 2: Illustration of the HMPV life cycle. The virion attaches to the plasma membrane and the two membranes fuse followed by uncoating and release of the viral genome into the cytoplasm. The genome is transcribed and replicated to produce the antigenome. Genomic RNA is synthesized from the antigenome which is used to produce additional antigenomes for incorporation into new virions. After translation, RNPs and M proteins are transported to the plasma membrane while the surface glycoproteins go through additional modification in the endoplasmic reticulum (ER) and golgi apparatus before reaching the plasma membrane. Progeny virions are assembled and released through budding. Figure adapted from [13].

1.3 Immune responses induced by virus in the airways

The airway epithelium is crucial in the defense of the lung against viral infection through its role as a physical barrier and its ability to regulate the innate and adaptive immunity [18]. Various cell types present in the lung, including airway epithelial cells, alveolar macrophages and various types of dendritic cells, activate an immune response upon recognition of a pathogen [13].

The initial recognition of pathogens is orchestrated by the innate immunity and occurs through PRRs which are present on the surface or in the cytosol of immune cells. PRRs provide an initial discrimination between self and non-self through detection of pattern associated molecular

patterns (PAMPs). Upon viral invasion, PRRs recognize specific PAMPs present on the viral pathogen and activates innate immune cells to produce various mediators that either act directly to destroy the invading pathogen, or act on other cells to induce an immune response [19]. For example, dendritic cells may produce cytokine mediators that induce proliferation and activation of T lymphocytes that are crucial for initiation of antiviral cytotoxic T-cell responses leading to viral clearance. Alveolar macrophages are major producers of the antiviral interferons, and are also important for viral clearance although this mainly occurs through phagocytosis [20]. The release of cytokines is essential in the immediate defense and in directing the course of the adaptive immune response later in infection [21].

The adaptive immune responses activated by viruses are mediated by a balance of both classes of the adaptive immune system, the humoral and cellular immunity. The humoral immunity is antibody-mediated, and viruses and/or virus-infected cells can stimulate B lymphocytes to produce antibodies specific for viral antigens. The recognition of an antigen by its respective antibody can lead to cell lysis mediated by the complement system or antibody-dependent cytotoxic cells. Antibodies can also neutralize a virus by inhibiting virus-host cell interactions. The cellular immunity occurs inside infected cells and are mediated mostly by T lymphocytes. In this case, antigen-recognition by helper T cells release cytokines that facilitate binding of an activated T cell to the infected cell. The activate T cell is differentiated into a cytotoxic T cell which then kills the infected cell. Together, the innate and adaptive immune system provides a defense against viral infection [19, 22].

The function of interferons and interferon-stimulated genes (ISGs)

Interferons are part of the cytokine family and are central in the innate immune response to virus infections [23]. There are three classes of IFNs: type I (IFN-α/β), type II (IFN-γ) and type III (IFN-λ) [24]. In contrast to type II IFN, type I and III induce a strong antiviral state in responsive cells. Nearly all cell types can produce IFN-α and IFN-β in response to viruses and are therefore the most studied types of interferons [19, 21].

Type I interferons defend against viral infection in several ways. IFN-α and IFN-β bind to the cell surface receptor called interferon-α/β receptor (IFNAR) which uses the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathway, also known as the JAK-STAT-pathway [24, 25]. IFNAR downstream signaling activates the factors STAT1 and STAT2 which eventually leads to production of interferon stimulated genes (ISG) that have direct antiviral effects [24]. Viperin is one of the most highly produced ISGs in response to virus and has shown to have a broad antiviral effect on RNA and DNA viruses through targeting different stages of the

viral life cycle, like genome replication [26]. Release of IFN-α/-β also lead to increased major histocompatibility complex (MHC) class I expression and antigen presentation in all cells, which makes them more susceptible to be killed by cytotoxic T cells. In addition, IFN-α/-β activate natural killer cells which selectively kill virus-infected cells. Expression of IFNs are induced by signaling through PRRs including toll-like receptors (TLRs) and RIG-I-like receptors [19, 27].

RIG-I-like receptors (RLR)

The cytosolic RIG-I-like receptors detect viral RNAs produced within the cell and activate different signaling pathways that lead to expression of type I/III IFNs and proinflammatory cytokines [19, 28]. RLRs bind to viral RNA using an RNA helicase-like domain in their carboxy terminal [29]. Two amino-terminal CARD domains are also present in RLRs which interact with adaptor proteins to activate signaling [19, 29].

The three members of the RLR family are RIG-I, melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2) [30]. RIG-I senses differences at the 5'-end of ssRNA transcripts which allows it to discriminate between host and viral RNA [19]. MDA-5 on the other hand recognizes dsRNA. LGP2 lacs the CARD domains and does not directly sense viral RNA but has been suggested to cooperate with RIG-I and MDA-5 in the recognition of viral RNA [30, 31]. When viral RNA is detected, RIG-I and MDA-5 interact with the downstream adaptor protein mitochondrial antiviral signaling (MAVS), located in the outer mitochondrial membrane and in peroxisomes [31]. MAVS propagate the signal by recruiting various tumor necrosis factor receptor-associated factors (TRAF) that eventually lead to activation of transcription factors including members of the interferon regulatory factor family (IRFs), and a later activation of Nuclear Factor (NF)-κB (NFκB). Activation of IRFs lead to expression of type I and III IFNs, while NFkB lead to expression of proinflammatory cytokines [19, 32].

Toll like receptors (TLR)

There are 10 expressed TLR genes in humans that each recognize a distinct set of molecular patterns [33]. Some TLRs are present on the cell surface and detect components of the bacterial cell wall (TLR1, 2, 4, 5, 6), while others are located intracellularly in the membranes of endosomes and are involved in the recognition of viruses (TLR3, 7, 8, 9) [34, 35]. TLR3 recognizes double stranded RNA (dsRNA) whereas TLR7 and TLR8 recognize single stranded RNA (ssRNA). An analog of dsRNA, poly(I:C), is often used in experiments to artificially activate TLR3 or signaling via RIG-I and MDA-5 [19, 36, 37]. Even though TLR4 mainly is known to recognize lipopolysaccharide (LPS) in bacteria, several studies have suggested its involvement in virus

infections [38-40]. TLR4-mediated intracellular signaling has been shown to be regulated by the Ig-like receptor, signaling lymphocytic activation molecule family 1 (SLAMF1) [41], which is further discussed in relation to virus in section 1.6.

Signaling by TLRs induces a wide range of intracellular responses that leads to production of proinflammatory cytokines, chemotactic factors, antimicrobial peptides, and type I interferons [35]. These responses are mediated by four different adaptor molecules: myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like protein (MAL), TIR-domain-containing adapter-inducing interferon-β (TRIF) and thyroid hormone receptor activator molecule (TRAM) [19]. Two major TLR signaling pathways have been described where one pathway is MyD88-dependent resulting in a strong, early activation of NFκB, while the other is TRIF-dependent, activating IRFs and a later activation of NFκB (**Figure 3**) [31, 42]. Signaling from cell surface TLRs primarily leads to production of proinflammatory cytokines via NFκB and activator protein 1 (AP-1). Activation of endosomal TLRs primarily induces expression of type I and III IFNs through IRF3 and IRF7, but can also induce expression of proinflammatory cytokines [43]. Downstream signaling from RLRs and TLRs also involve a wide range of different adaptor proteins and kinases [44], but as this was not a focus in this thesis they will not be further described.

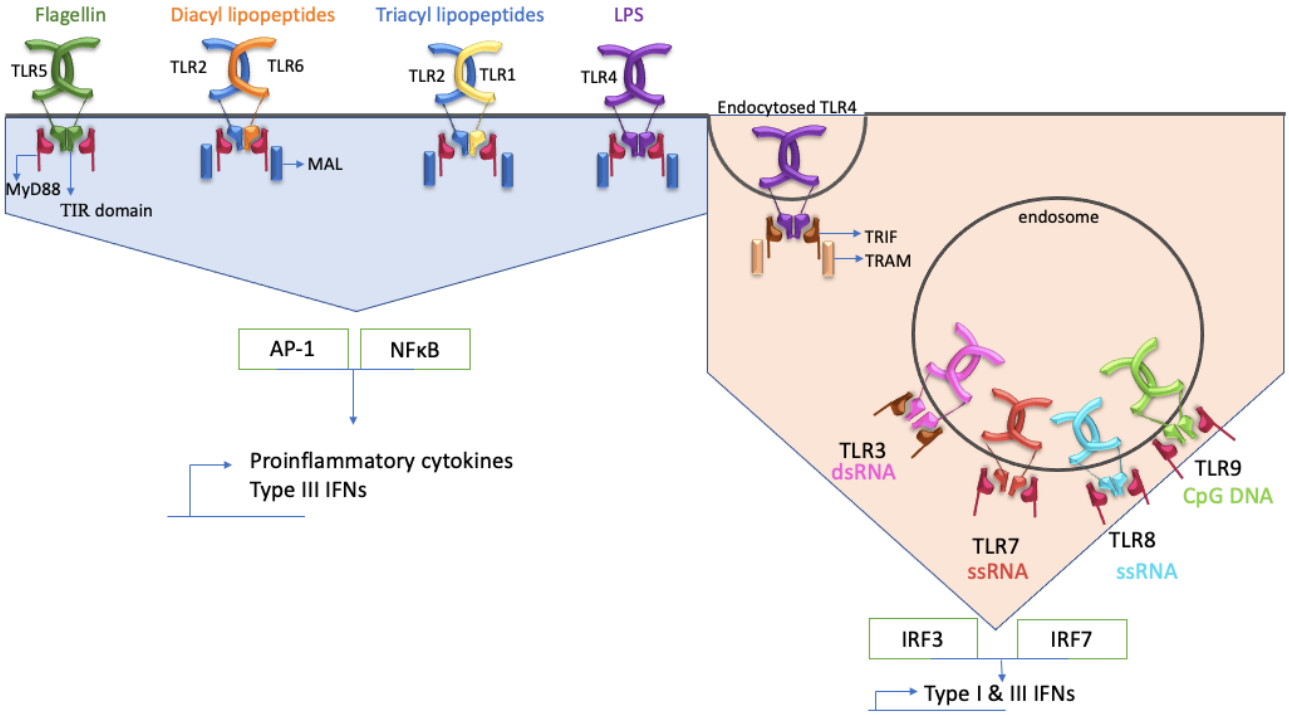

Figure 3: Illustration of TLR localization, ligands and adaptor molecules that activate downstream signaling and induction of proinflammatory cytokines and type I/III IFNs. The ligand for each TLR is shown in color above/below its respective receptor. Binding of a PAMP to a TLR induces TLR dimerization and recruitment of adaptor molecules (MyD88, TRIF) for downstream signaling. TLR1, 2, 4, 5 and 6 are localized on the cell surface and interact with MyD88 to activate the transcription factors AP-1 and NFκB, inducing

expression of proinflammatory cytokines and type III IFNs. TLR3, 7, 8 and 9 reside on the endosomal membrane and interact with MyD88 or TRIF to activate the transcription factors IRF3 and IRF7, resulting in production of type I and III IFNs. Endocytosed TLR4 signal through TRIF/TRAM. Pathways from endosomal TLRs to production of cytokines is not shown.

1.4 TLR4 in virus infections

The last decade, TLR4 has been suggested to play a major role in induction of immune responses by HMPV and the closely related respiratory syncytial virus (RSV) [31, 45]. A previous study found that downregulation of TLR4 expression by siRNA inhibited HMPV-induced production of chemokines and type I IFNs in monocyte-derived dendritic cells (moDCs) [45]. The same was found in bone marrow derived-dendritic cells from TLR4-deficient mice [31]. HMPV was also reported to induce cytokine expression via TLR4 in DCs that was replication-independent at early timepoints of infection which could suggest that components of the viral envelope acts as ligands [45]. However, how HMPV makes use of TLR4 and the type of intracellular signaling that is induced by the HMPV-TLR4-interaction are currently poorly understood. The role of TLR4 in RSV-infection has been more extensively studied and has been reported to mediate inflammatory signaling [46]. Additionally, a study on human monocytes infected by RSV showed that the innate immune response to the fusion protein of RSV was mediated by TLR4 and CD14, an accessory protein that is involved in TLR4 activation. They also found that RSV persisted longer in the lungs of TLR4-deficient mice compared to normal mice, suggesting that TLR4 is important for viral clearance [39].

1.5 Cellular innate immune signaling pathways induced by HMPV

Being a respiratory pathogen, HMPV primarily targets epithelial cells that inhabit the upper and lower respiratory tract and lung-resident leukocytes [13, 17]. However, studies have also shown that HMPV is able to infect dendritic cells and alveolar macrophages although to a less extent [47-49].

Most cell types detect HMPV via TLR3, RIG-I and MDA-5, but in plasmacytoid dendritic cells (pDCs) TLR7 was the preferred receptor [50-52]. Detection of HMPV by TLR3 activates IRF3 via TRIF, whereas RIG-I/MDA5 activates both IRF3/7 and NFκB via MAVS, inducing production of type I/III IFNs and proinflammatory cytokines, respectively [51, 52] (**Figure 4**). In pDCs, HMPV activates IRF7 through TLR7 and MyD88 which mounts a stronger IFN response to HMPV [13]. The current knowledge of which proinflammatory cytokines that are produced upon infection with HMPV are tumor-necrosis factor-α (TNF-α), Il-6, Il-8 and chemokines (RANTES, TSLP) [51, 53]. TNF-α has important functions in homeostasis and disease pathogenesis and is

known to be central for control of viral replication [54, 55]. In epithelial cells, the transcription factors STAT1, STAT2 and IRF9 (called ISGF3 complex), which is necessary for production of ISGs, has been reported to be strongly activated due to infection with HMPV [51]. *In vivo* and *in vitro* studies showed that MDA5 is required for the expression and activation of IRFs, and that HMPV-infection induces activation of IRF3 which regulates expression of IRF7 [52]. The ability to trigger RIG-I and the downstream IFN-α/-β induction has been reported to differ between strains of HMPV [50].

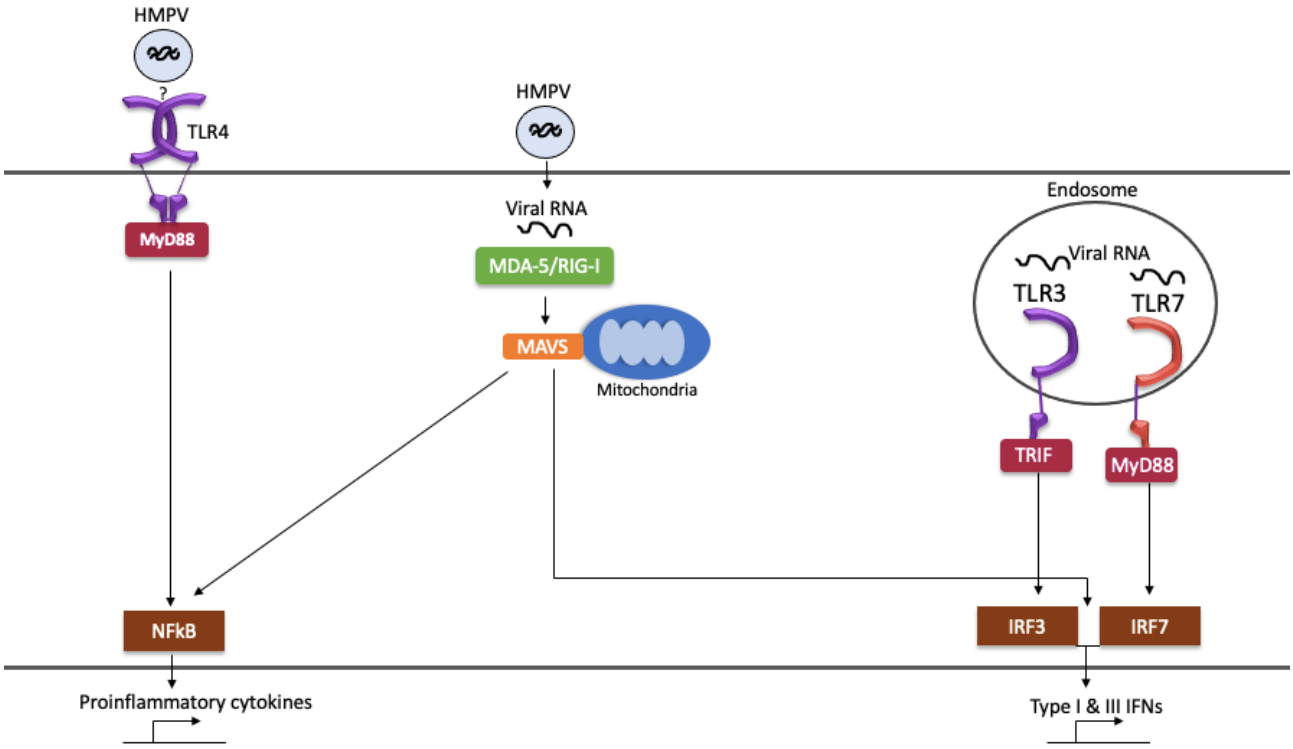

Figure 4: Schematic overview of PRRs and transcription factors leading to production of proinflammatory cytokines and type I/III IFNs by HMPV. An undetermined component of HMPV suggestibly activates TLR4 which through MyD88 and NFκB induces expression of proinflammatory cytokines. Detection of HMPV RNA by MDA-5/RIG-I activates MAVS and induces expression of proinflammatory cytokines and type I/III IFNs via NFκB and IRF3/IRF7, respectively. Expression of IFNs can also be induced by HMPV via TLR3-TRIF-IRF3 and TLR7-MyD88-IRF7 signaling.

1.6 SLAMF1 is required for TLR4-mediated TRAM-TRIF-dependent signaling

SLAMF1 is an Ig-like receptor and a costimulatory molecule that initiates signal transduction networks in a variety of immune cells. In resting macrophages, SLAMF1 is localized to the endocytic recycling compartment (ERC), but upon addition of *Escherichia coli* it is transported together with TRAM from ERC to *E. coli* phagosomes in a Rab-11 dependent manner. Therefore, SLAMF1 is thought to control trafficking of TRAM from ERC to *E. coli* phagosomes. Moreover, a

study showed that SLAMF1 regulates TLR4-mediated TRAM-TRIF dependent signaling by the interaction with TRAM [41]. Interestingly, SLAMF1 has been found to a) be used as a critical entrance receptor for measles virus that was also upregulated by measles virus [14] and b) to mediate vesicular trafficking and signaling from TLR4 during recognition of bacteria or bacterial ligands [41]. In addition, TLR4-mediated induction of IFN-β as well as the destruction of Gram-negative bacteria by human macrophages have been reported to depend on SLAMF1 (**Figure 5**). The role of SLAMF1 in TLR4-mediated induction of IFN-β was studied in macrophages and in THP1 cells, a human leukemia monocytic cell line often used to study monocyte/macrophage functions [41, 56]. Recent results obtained by the research group "Virus in Immunity and Disease" at NTNU suggested that HMPV infection triggered considerable expression of SLAMF1 mRNA in monocyte-derived macrophages (MDMs) [Loevenich, Anthonsen, unpublished]. Hence, it was of interest to explore if SLAMF1 affects HMPV or HMPV-mediated innate immune responses (as IFN-β induction).

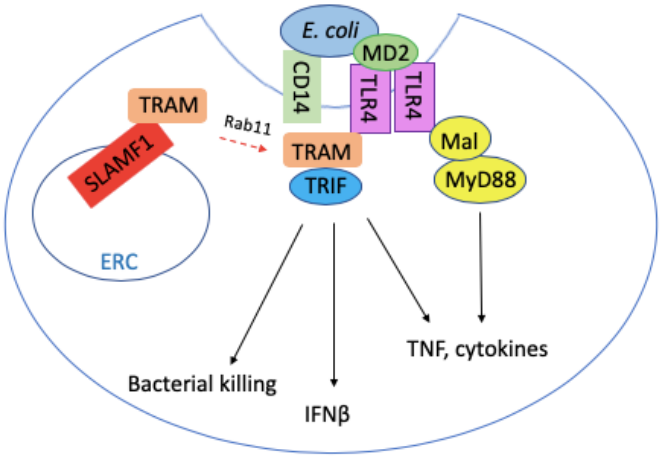

Figure 5: Schematic illustration of how SLAMF1 is thought to act together with TLR4 to induce production of interferon β (IFNβ), TNF and other cytokines as well as bacterial killing in response to an external signal. *E. coli* (LPS) is recognized by the TLR4-MD2 complex and the accessory protein CD14 which through adaptor molecules TRAM-TRIF and MAL-MyD88 activates immune responses [57]. Upon addition of *E. coli,* SLAMF1 is transported together with TRAM from ERC to *E. coli* phagosomes in a Rab-11 dependent manner. The figure is adapted from [41].

1.7 Aim of study

The innate immune responses induced by HMPV have been little described. However, recent results suggested that SLAMF1 mRNA was upregulated in MDMs upon infection with HMPV. Additionally, several studies have suggested the involvement of TLR4 in virus-infection, and SLAMF1 has been suggested to regulate TLR4-mediated intracellular signaling. Hence, the main aim of this thesis was to determine if HMPV regulates SLAMF1 expression and establish if SLAMF1 plays a role in the innate immune responses induced by HMPV. A minor aim was to investigate if silencing of *TLR4* affects HMPV replication and antiviral responses. MDMs and THP1 cells were used as model systems for this purpose. More specifically, the following was pursued:

- Establish if HMPV infects and replicates in THP1 cells, enabling the use of these cells as a model system for HMPV-infection in MDMs
- Establish characteristics of time-dependent expression of SLAMF1 mRNA upon HMPV infection
- Establish if SLAMF1 is involved in virus-triggered cytokine production
- Study how removal (knockdown) of *SLAMF1* affects HMPV replication and antiviral responses
- Study how overexpression of *SLAMF1* affects HMPV replication and antiviral responses

2 Methodology

2.1 Propagation, isolation and titration of HMPV

The clinical isolate HMPV isolate NL/17/00 (A2) was provided by ViroNovative and B. van den Hoogen (Erasmus MC, Rotterdam). LLC-MK2 (ATCC) cells were seeded (2 million) 3 days prior to infection. Monolayers of LLC-MK2 were inoculated with low passage virus at a multiplicity of infection (MOI) at 0.01 in OptiMEM (Gibco, #11058021) containing 2% Fetal Bovine Serum (FBS) and 50 µg/mL trypsin. FBS was added to promote cell growth, while the proteolytic enzyme trypsin was added to facilitate virus infection. The cells were incubated with virus for 2 hrs before the medium was replaced with fresh OptiMEM containing 2% FBS and 50 µg/mL trypsin. After 4 days of incubation, the medium was replaced once more with a fresh batch of OptiMEM containing 2% FBS and 50 µg/mL trypsin. The virus was harvested on the 8th day of incubation through several steps. First, the cells were freeze-thawed at -80 °C, scraped and transferred to tubes for centrifugation (1500 rpm, 5 min, 4 °C). The supernatant was then sterile filtered (0.8 µm) and added carefully to a 20% sucrose cushion followed by centrifugation (26 000 rpm, 120 min, 4 °C). The pellet with virus was resuspended in OptiMEM (2% FBS) and stored at -80 °C.

The virus titer was determined using a cell-based immunoassay. Purified virus was serially diluted (log10) in OptiMEM (2% FBS, 50 ug/ml trypsin) on monolayers of LLC-MK2 (0.1M cells per well) in a 96-well plate. After 4 days of incubation, the cells were fixed with 80% acetone and stained with IMAGEN™ Human Metapneumovirus Kit using Direct Immunofluorescence Assay (Thermo Fisher Scientific, #K612511-2). The focus forming units were determined by manual counting using a fluorescence microscope (Nikon). Determination of number of infectious particles in cell supernatants from virus-infected cells was performed similarly, adding dilutions of the cell supernatant instead of purified virus on monolayers of LLC-MK2 cells.

2.2 Cells and culture conditions

LLC-MK2 (ATCC) cells were cultured in OptiMEM (Gibco) containing 2% FBS, 20 µg/mL gentamicin and 0.7 nM glutamine. Gentamicin was added to prevent contamination while glutamine functioned as an extra source of energy. Three strains of THP1 cells, wildtype, SLAMF1-KO and SLAMF1-Overexpression, were kindly provided by Maria Yurchenko (CEMIR, NTNU Trondheim). All THP1 cells were cultured in RPMI 1640 (Gibco, #A1049101) supplemented by 10% heat-inactivated FBS, 100 nM penicillin/streptomycin (Thermo Fisher Scientific) and 5 µM β-mercaptoethanol (Sigma-Aldrich). Cells that were transfected with siRNA were not cultured with

antibiotics. THP1 cells were differentiated with 60 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 72 hrs followed by 48 hrs in medium without PMA. In a few cases, a shorter differentiation protocol was used where THP1 cells were differentiated with PMA for 24 hrs, and without for 48 hrs. All cells were incubated at 37 ℃ in 5% CO_2.

THP1SLAMF1KO cells were made by Maria Yurchenko using pLentiCRISPRv2 vector (Zhang lab) coding for guiding RNA towards exon 3 in the *SLAMF1* gene. pPax2 and pMD2 packaging and envelope vectors were used to make the virus in HEK293T cells for getting CRISPR/Cas9 and sequence coding guiding RNA to THP1 cells. THP1SLAMF1-Overexpression cells were made using a modified SLAMF1 sequence that was not sensitive to the guiding RNA (the amino acid sequence unchanged, only nucleotide sequence was modified) in lentiviral vector pLVX EF1alpha IRES ZsGreen (Takara Bio Inc.). The same packaging and envelope vector as mentioned earlier was used here as well. Positive cells were selected by sorting in a 488 nm wavelength channel. The control cell line contained an empty vector that also coded for ZsGreen protein for sorting to make sure that there were similar levels of lentiviral vector load. These cell lines were made by Mariya Yurchenko and Kaja Nilsen, and sorting was performed by Unni Nonstad. Cell lines were checked by flow cytometry (by Maria Yurchenko) after unfreezing vials for experimental procedures.

The use of buffycoats from blood donors was approved by REK (Regional Etisk Komitè). Human monocytes from blood donors (Blodbanken, St. Olavs Hospital) were isolated from buffycoat by adherence on the same day as the blood was donated. The buffycoat was diluted in Phosphate buffered saline (PBS, Sigma Aldrich, #D8537), applied on top of Lymphoprep™ (Axis Shield Poc AS™, #11508545) and centrifuged (690 x *g*, no deceleration, 25 min, RT) in order to separate the different cell types. After centrifugation, the layer of peripheral PBMC was retained in the interphase between the top layer with plasma and the layer with ficoll and granulocytes below. PBMCs were collected and centrifuged (840 x *g*, 10 min, 20 ℃), and the supernatant was discarded. The pellet was resuspended in Hank's balanced salt solution (HBSS, Sigma-Aldrich, #55021C) and centrifuged (200 x *g*, 8 min, 20 ℃). This step was repeated two times (in total 3 times). The cells were counted with a CoulterCounter (Beckman Coulter) on program B, using ZAP-OGLOBIN II lytic reagent (Beckman Coulter, #7546138) to exclude possible presence of erythrocytes from the count. The cells were resuspended in RPMI 1640 (Gibco) containing 5% human serum and seeded in 24-well cell culture dishes (0.5 ml per well). After 1 h incubation, allowing surface adherence of monocytes, the dishes were washed three times by HBSS to remove nonadherent cells. Monocyte-derived macrophages (MDMs) were obtained by differentiating cells for 3 days in RPMI 1640

(Gibco), 10% human serum, penicillin/streptomycin (1:100) and 50 ng/ml recombinant human macrophage colony-stimulating factor (rhMCSF, R&D Systems, #216-MC-025), followed by 4 days without penicillin/streptomycin. Antibiotics were removed from the medium on day 3 as the cells were treated with siRNA on the 4th day after seeding (see section 2.7). Human A+ serum was used as this was the blood type of the donors. MCSF was added to initiate differentiation of monocytes into MDMs. Two days after seeding, an additional amount of RPMI 1640 medium (Gibco) supplemented with 10% human serum and 50 ng/ml MCSF was added. Before stimulation, the medium was changed to OptiMEM serum free medium (Gibco).

2.3 *In vitro* HMPV infection and stimulation with LPS/poly(I:C)

All THP1 cell lines and MDMs were infected with HMPV (A2 strain) at a MOI of 1 in OptiMEM (Gibco) supplemented with 2% FBS and 0.7 nM glutamine. Only 10% of the seeded primary cells was assumed to be MDMs, and the amount of virus was calculated followingly. Cells were incubated with HMPV for different time points up to 48 hrs as indicated in the results section. The THP1 cell line was transfected with 10 µg/ml high molecular weight poly(I:C) using RNAiMAX (Invitrogen, #13778500) as described by the manufacturer. All cell lines were stimulated with 200 ng/ml LPS diluted in OptiMEM (2% FBS).

2.4 Quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) is a technique that is used to amplify, detect and quantify DNA/RNA sequences. Reverse transcription qPCR (RT-qPCR) makes it possible to use RNA as a template where RNA transcripts are reverse transcribed into complementary DNA (cDNA) before proceeding with qPCR. DNA/cDNA is amplified by repeating 3 steps: denaturation, annealing and elongation. Each step of the cycle has a specific temperature that is optimal for the primer set and template that is used, and the cycle is repeated 20-40 times. By using a florescent dye that binds to dsDNA or a fluorescent-labeled target-specific probe, the amount of DNA amplicons in the sample is reflected in the intensity of fluorescent signal that is measured after each cycle. In the initial cycles, the fluorescence is usually too low to be distinguished from the background. The number of cycles required for the fluorescent signal to exceed the background level is referred to as the cycle threshold (Ct). The Ct-value corresponds proportionally to the initial number of template DNA in the sample. In this thesis, RNA was used as template.

Total RNA was isolated from the cells using QIAzol lysis reagent (Qiagen, #79306), and chloroform extraction followed by purification using RNeasy Mini Kit (Qiagen, #74104) including the DNase digestion step according to the manufacturers protocol. Quantification of isolated RNA was carried out using a NanoDrop ND-1000 Spectrophotometer (Saveen Werner).

cDNA was synthesized using qScript cDNA synthesis kit (QuantaBio, #95047-025) for RT-qPCR as suggested by the manufacturer. qPCR was performed in replicates using PerfeCTa SYBR Green Fast mix (QuantaBio, #733-1386) for analysis of the majority of genes. Primer sequences are listed in Supplementary section 7.1, **Table S1**. For analysis of *TNF-α* and *TLR4*, the following TaqMan gene expression assays were used: *TLR4*: (Hs00152939), *TNF* (Hs000174128), *TATA-Box Binding Protein* (*TBP,* used as the endogenous control) (Hs00427620) and PerfeCTa qPCR FastMix (Quanta Biosciences, #733-1394). qRT-PCR was performed using a StepOnePlus real-time PCR cycler (Applied Biosystems) and was run with a holding state of 95 °C (20 sec), a cycling state (40x) at 95 °C (3 sec) and 60 °C (30 sec) for a duration of 45 min. Gene expression was calculated as fold change (ΔΔCt) normalized against the expression of *TBP* in the same sample and presented as the relative expression to the untreated (medium) control. The only exception was the expression of *HMPV-N* which was presented as the relative expression compared to the earliest time point of infection with HMPV.

2.5 Gel electrophoresis and western blot

Western blot is a technique used to identify and analyze proteins in a sample and involves separation by electrophoresis followed by staining with antibodies. After separation of the proteins on a polyacrylamide gel, the proteins are transferred to a blotting membrane. The membrane is first incubated with a primary antibody specific to the protein of interest, then a second antibody is used that binds to the primary antibody. The secondary antibody is linked to a fluorescent dye or an enzyme that produces color or light which allows it to be detected.

Cell lysates were prepared by simultaneous extraction of proteins and total RNA using QIAzol reagent (Qiagen, #79306) as described by the manufacturer. Protein pellets were dissolved in a buffer containing 2% Sodium dodecyl sulfate (SDS) and 4M Urea (Sigma-Aldrich) in order to break up the secondary and tertiary structure of the protein, leaving the proteins linearized and with an overall negative charge. The samples were treated with 4X LDS sample buffer containing Dithiothreitol (DTT, 1:10) and heated for 10 minutes at 95 ℃ before analysis or storage at -20 ℃. DTT was important to add as it has the ability to reduce disulfide bonds in the proteins.

Protein extracts were separated by size by using 4-12% or 10% NuPAGE® Bis-Tris gels (Invitrogen, #NP0322BOX, #WG1402BOX). SeeBlue Prestained Protein Standard (Invitrogen, #LC5925) and Magic Mark™ XP Western Protein Standard (Invitrogen, #LC5602) were used as ladders. The gel was run in 1XMOPS buffer (Invitrogen, #NP0001) for 30 min at 90V and 45 min at 180V.

The proteins were transferred from the gel to a nitrocellulose membrane using iBlot transfer stacks and iBlot™ 2 Gel Transfer device (Invitrogen, #IB23001, #IB23002) as described by the manufacturer. The P0 program on the iBlot transfer device was used for the protein transfer and included three steps: 20 V for 1 min, 23 V for 4 min and 25 V for 2 min. The membrane was rinsed in Tris buffered saline with 0.1% Tween-20 (TBST, 5 min), and to avoid unspecific binding of antibodies, the membrane was blocked for 1h with Bovine serum albumin in TBST (BSA, 5%). After blocking, the membrane was washed 3 times with TBST (5 min) and incubated with the primary antibody (**Table S2**) overnight. Then the membrane was washed again, 3 times with TBST (5 min), and incubated with the secondary antibody for 1 h (**Table S3**). The washing step with TBST was then repeated twice (5 min) followed by a last wash with Tris buffered saline (TBS, 10 min) before drying the membrane. The secondary antibodies were conjugated to fluorescent dyes that, upon excitation by light, emitted light at an emission wavelength which was detected via LI-COR Odyssey imager (LI-COR Biosciences), allowing visualization of the protein of interest. The relative intensity of protein bands was quantified using Image Studio™ Software.

2.6 Sandwich ELISA

Enzyme-linked immunosorbent assay (ELISA) is a technique that uses antibodies to detect the presence of antigens in biological samples. A modification of ELISA known as sandwich ELISA uses two antibodies that are specific for different epitopes of the antigen. One of the antibodies is used to capture the antigen, while the other is conjugated to a reporter-enzyme which upon exposure to the appropriate substrate produces a measurable product. Generally, the detection antibody binds to horseradish peroxidase (HRP) conjugated to streptavidin. Substrate oxidation can then be measured by absorbance using a spectrophotometer.

In this thesis, levels of secreted TNF-α protein in the supernatant from cell cultures of monocyte-derived macrophages were determined using a TNF-α DuoSet ELISA (DY210-05; R&D Systems)

following the manufacturer's protocol. The concentration of TNF-α in the samples was determined based on a standard curve.

2.7 siRNA-mediated knockdown

Small interfering RNAs (siRNA) were used to silence *SLAMF1* and *TLR4* in THP1 cells and MDMs. Oligonucleotides used for silencing were AllStars negative control siRNA (SI03650318), FlexiTube siRNA Hs_SLAMF1_2 (SI00047250) and Hs_TLR4_2 (SI00151011; Qiagen). siRNA duplexes were reverse transfected into THP1 cells (two days after seeding) using Lipofectamine RNAiMAX (Invitrogen, #13778500) according to the manufacturer's instructions. Transfected THP1 cells were allowed to grow for another 48 hrs before replacing the medium with fresh RPMI 1640 (Gibco) containing 10% FBS and 5 µM β-mercaptoethanol. Another 48 hrs later, THP1 cells were infected with HMPV or treated with LPS or poly(I:C).

MDMs were transfected with siRNA the 4th day after seeding by using Lipofectamine^{TM} 3000 Transfection Reagent (Invitrogen, #L3000001) according to the manufacturer's instructions. 24 hrs after transfection of MDMs (day 5), the medium was replaced with antibiotic free RPMI 1640 (Gibco) containing 10% human serum and 25 ng/ml of MCSF. On day 6, the transfection with siRNA was repeated. The medium was replaced 24 hrs later (day 7) with RPMI 1640 (10% human serum, 25 ng/ml MCSF). On day 8, MDMs were infected with HMPV or treated with LPS/poly(I:C).

2.8 Ligation of surface SLAMF1

Anti-SLAMF1 antibodies were added to THP1 cells to induce surface SLAMF1 ligation. For this experiment, THP1SLAMF1-KO and THP1SLAMF1-Overexpression cells provided by Mariya Yurchenko were seeded in 12-well plates and differentiated with PMA for 24 hrs, followed by 48 hrs without PMA. The cells were infected on the third day after seeding. Anti-SLAMF1 antibody (Invitrogen, #11-1509-42) and an isotype control (Invitrogen, #MA1-10406) were diluted in OptiMEM (2% FBS, 10 µg/ml) and added to the respective wells 30 min prior to infection with HMPV or stimulation with LPS.

3 Results

Recent data obtained by RNA-sequencing of human MDMs showed that SLAMF1 mRNA was upregulated by HMPV (Loevenich, Anthonsen, unpublished]. The main aim of this thesis was to determine if HMPV regulates SLAMF1 expression and establish if SLAMF1 plays a role in the innate immune responses induced by HMPV. This was studied in MDMs and THP1 cells, which is frequently used as a model system of human macrophages [55].

3.1 HMPV infection of THP1 cells and the induced innate immune responses

The current knowledge on how HMPV acts in THP1 cells is rather limited, but due to their several advantages these cells were chosen to investigate the aim of study. Unlike MDMs, THP1 cells are easily genetically manipulated, are not affected by donor variation and are considered easy to work with *in vitro* [56]. In order to test if THP1 cells could be used as a model of HMPV-infection in MDMs, we studied the ability of HMPV to establish an infection and induce an immune response in THP1 cells. THP1 cells were differentiated to macrophage-like cells and infected with HMPV ranging from 1 – 48 hrs or stimulated with LPS or poly(I:C) as described in section 2.3.

3.1.1 HMPV is able to enter and replicate in THP1 cells

As the viral infectivity in THP1 cells was unknown, the first issue we wanted to address was if HMPV was able to enter and replicate in these cells. This was determined through assessing the number of infectious particles in the supernatant by titration and measuring levels of viral RNA using RT-qPCR as described in section 2.1 and 2.4, respectively. Viral replication was analyzed by quantifying the amounts of RNA transcripts of the viral nucleocapsid, HMPV-N.

Quantification by RT-qPCR showed a continuous increase of HMPV-N mRNA over time with a peak at 24 hrs followed by a decrease (**Figure 6 and S1**). This is in accordance with earlier findings in MDMs and MDDMs [58]. Titration of the infected cell supernatants also revealed the presence of infectious particles (not shown), suggesting that progeny virus particles have been produced. Unfortunately, due to technical difficulties with the procedure we were not able to determine the exact number of focus forming units. In summary, the results suggested that HMPV was able to enter and replicate in THP1 cells.

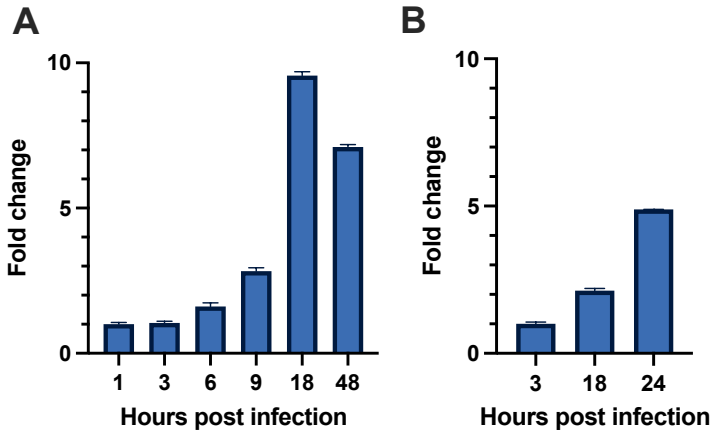

Figure 6: HMPV is able to enter and replicate in THP1 cells. Quantification of HMPV-N mRNA carried out by RT-qPCR in THP1 cells infected with HMPV (m.o.i =1) for the indicated time points. Results from two independent experiments are shown (A, B) where Figure B is representative of two independent experiments showing the same trends for the time points used in B. The bars represent three technical replicates per sample where their average C_T values have been normalized against the earliest time point of infection as well as the reference gene, TBP. The results are presented as means with SD.

3.1.2 The kinetics of IFN-β and TNFα expression differs in response to HMPV

Since the virus was able to replicate in THP1 cells, we wanted to investigate HMPV-induced expression of IFNs and proinflammatory cytokines and how this correlated to viral replication. We chose to focus on IFN-β and TNF-α expression as they are important cytokines in the antiviral immune response and to determine which signaling pathways had been activated. IFN-β is mostly regulated by IRFs (less by NFκB and AP-1), while TNF-α is regulated by NFκB [19]. Levels of IFN-β and TNF-α mRNA in response to HMPV, LPS and poly(I:C) were measured by RT-qPCR. LPS and poly(I:C) functioned as positive controls for cytokine expression.

IFN-β mRNA increased along with viral replication with a maximal peak at 24 hrs (**Figure 7**). TNF-α mRNA on the other hand was induced at an early time point (3 hrs) and decreased thereafter. IFN-β expression was also much stronger induced by HMPV compared to TNFα. A repetition of this experiment showing the same trends of expression is located in Supplementary section 7.2, **Figure S1**. Based on these results, the kinetics of IFN-β and TNFα expression in THP1 cells seem to differ from one another.

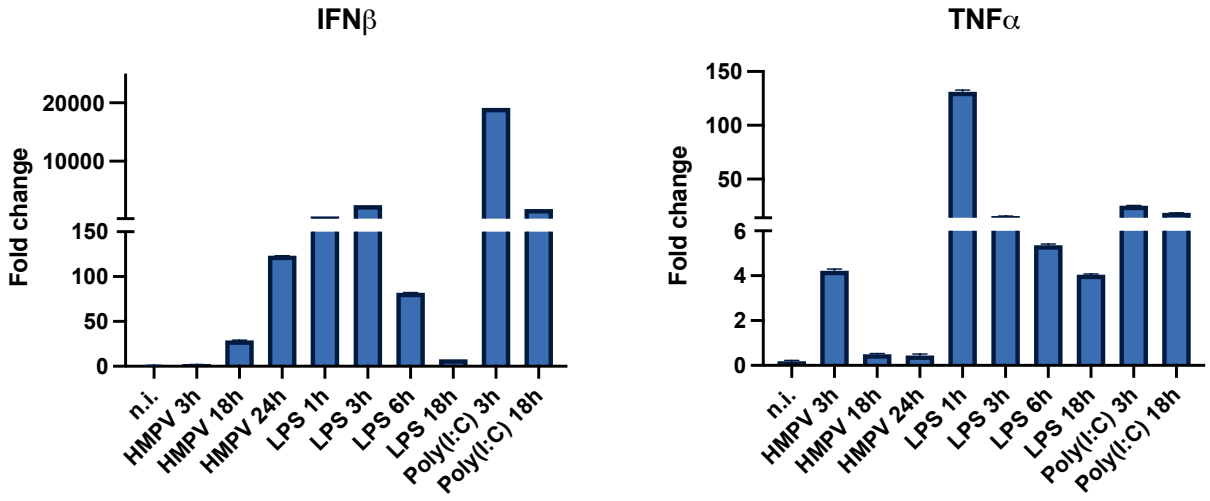

Figure 7: The kinetics of IFN-β and TNF-α mRNA expression differs in response to HMPV in THP1 cells. Quantification of IFN-β and TNF-α mRNA expression by RT-qPCR in THP1 cells infected with HMPV (m.o.i = 1) or stimulated with LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points. The bars represent three technical replicates per sample where their average C_T values have been normalized against the non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD and are representative for two independent experiments.

3.1.3 Knockout of *TLR4* does not affect HMPV-induced IFN-β expression

Earlier studies done on moDCs and mice showed that TLR4 was important for chemokine and type I IFN production [45]. However, how HMPV suggestibly makes use of TLR4 and the type of intracellular signaling that is induced by the HMPV-TLR4-interaction is not well known. In this regard, we wanted to explore how knockout of *TLR4* would affect HMPV-induced IFN-β expression. RNA samples from a THP1 cell line where *TLR4* had been knocked out was kindly provided by Kristin Rian (IKOM, NTNU). IFN-β mRNA expression was measured by RT-qPCR. The results showed that LPS-induced IFN-β expression was dependent on TLR4, while HMPV-induced IFN-β expression was not (**Figure 8**).

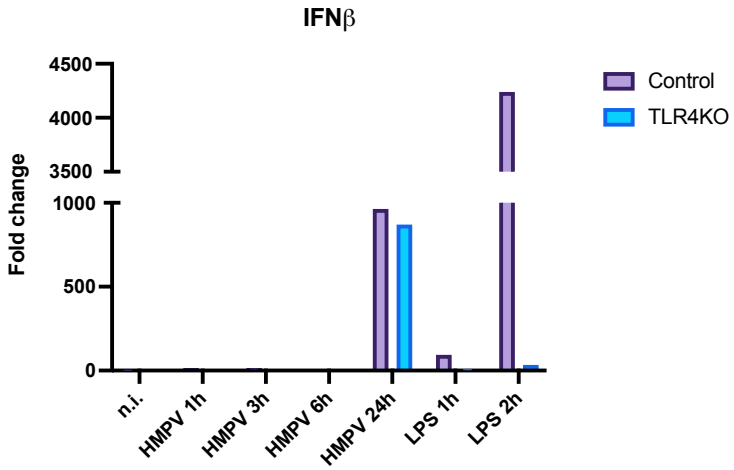

Figure 8: Knockout of TLR4 strongly reduces IFN-β mRNA expression in response to LPS, but not in response to HMPV. Quantification of IFN-β mRNA expression by RT-qPCR in THP1 cells and THP1-TLR4KO cells treated with HMPV (m.o.i. = 1) or LPS (200 ng/ml) for the indicated time points. The bars represent three technical replicates per sample where their average C_T values have been normalized against the noninfected sample as well as the reference gene, GAPDH. The results are presented as means with SD.

3.1.4 Infection with HMPV induces phosphorylation of STAT1 and production of viperin

Measuring levels of mRNA revealed a strong induction of IFN-β by HMPV and a weak induction of TNF-α, but this does not give information about the amount of secreted protein. By determining levels of phosphorylated STAT1 (pSTAT1), this would indicate signaling via IFN-α/-β that has bound to the IFNAR receptor, which leads to phosphorylation of STAT1. As mentioned in the introduction, this will in turn lead to production of ISGs, like viperin [24]. TNF-α expression is induced by activation of NFκB which occurs through phosphorylation of one of its subunits, p65 [59]. Activation of NFκB and the JAK-STAT pathway by HMPV in THP1 cells was investigated by quantifying levels of phosphorylated p65 (pp65)/total p65, pSTAT1/total STAT1 and viperin by western blot analysis as described in section 2.5.

Western blot analysis showed that the amount of HMPV-N protein increased with infection and, as with HMPV-N mRNA, peaked at 24 hrs and decreased thereafter (**Figure 9**). The same pattern of expression as IFN-β mRNA was observed with pSTAT1 and viperin, increasing with viral replication. Nevertheless, viperin expression peaked at 48 hrs while pSTAT1 and the viral protein, HMPV-N, peaked at 24 hrs. Phosphorylation of p65 was weakly induced by HMPV. Results from two repetitions of the experiment showing the same trends can be found in Supplementary section 7.2, **Figure S2** and **S3**. In summary, the protein analysis were in agreement with the gene expression analysis.

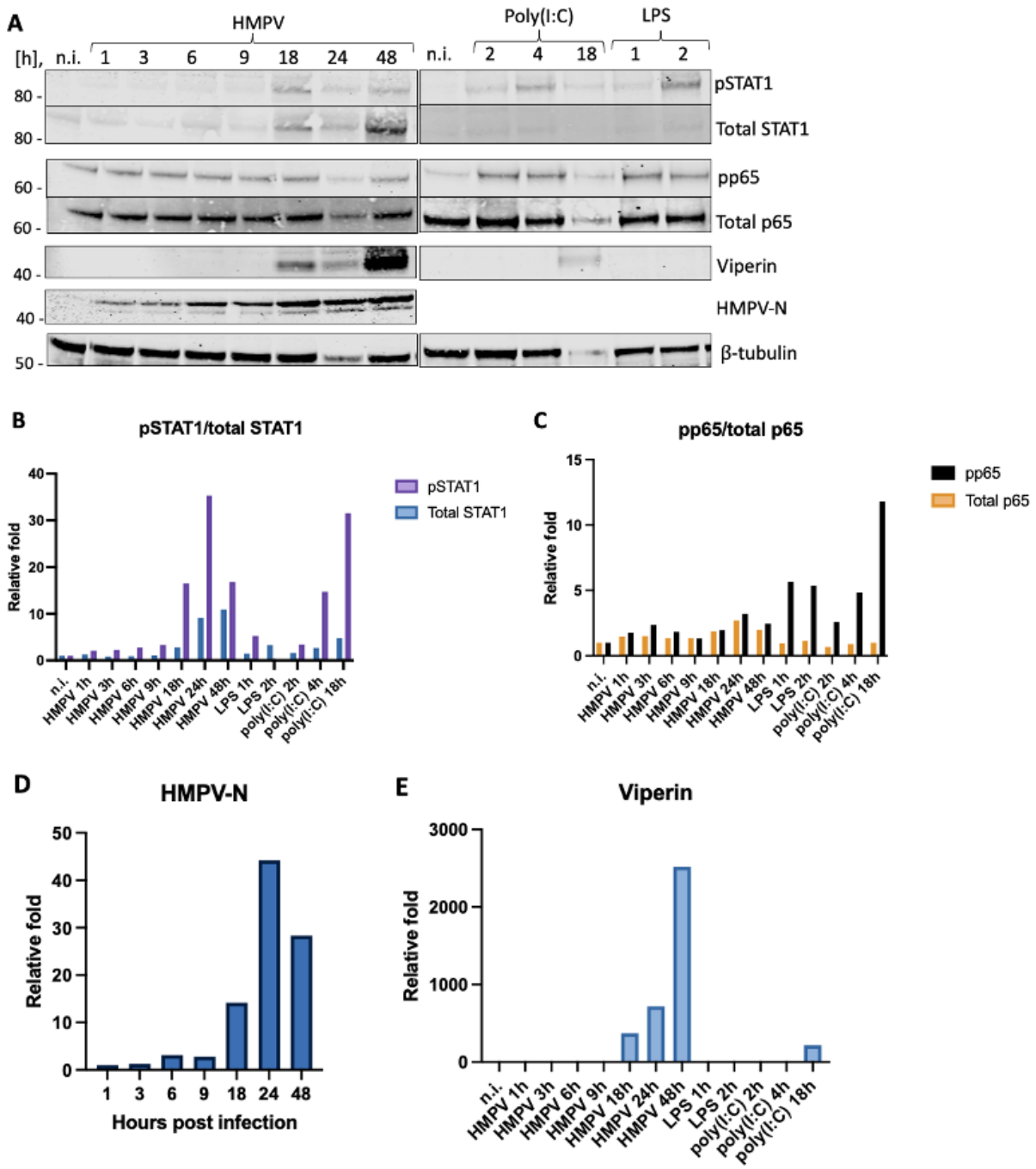

Figure 9: **HMPV infection in THP1 cells induces phosphorylation of STAT1 and production of viperin.** Western blotting of THP1 cells infected with HMPV (m.o.i. = 1) or stimulated with LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points (A). The antibodies used are indicated in the figure. β-tubulin was used as a loading control. Molecular weight is given in kilodaltons. Graphs show quantification of pSTAT1/total STAT1 (B), pp65/total p65 (C), viral protein (D) and viperin (E) levels relative to β-tubulin obtained with ImageStudio software.

3.1.5 SLAMF1 expression is weakly induced by HMPV in THP1 cells

As mentioned earlier, a previous study showed that SLAMF1 was strongly induced in response to HMPV in MDMs. These results are shown in **Figure 10** (left panel). In order to establish if HMPV regulate SLAMF1 levels and to determine if THP1 cells could be used as a model for HMPV-infection in MDMs, we measured SLAMF1 expression in THP1 cells. SLAMF1 mRNA was measured by RT-qPCR in THP1 cells stimulated with HMPV, LPS or poly(I:C). Stimulation with LPS for 6 hrs was considered as a positive control for SLAMF1 expression.

Unlike MDMs, only a weak induction of SLAMF1 mRNA at 3 hrs post infection was observed in THP1 cells (Fig. 10, right panel). HMPV-induced expression of SLAMF1 mRNA therefore seem to differ between THP1 cells and MDMs. Results from two additional experiments showing the same trend as the right panel in Fig. 10 are shown in Supplementary section 7.2, **Figure S4**.

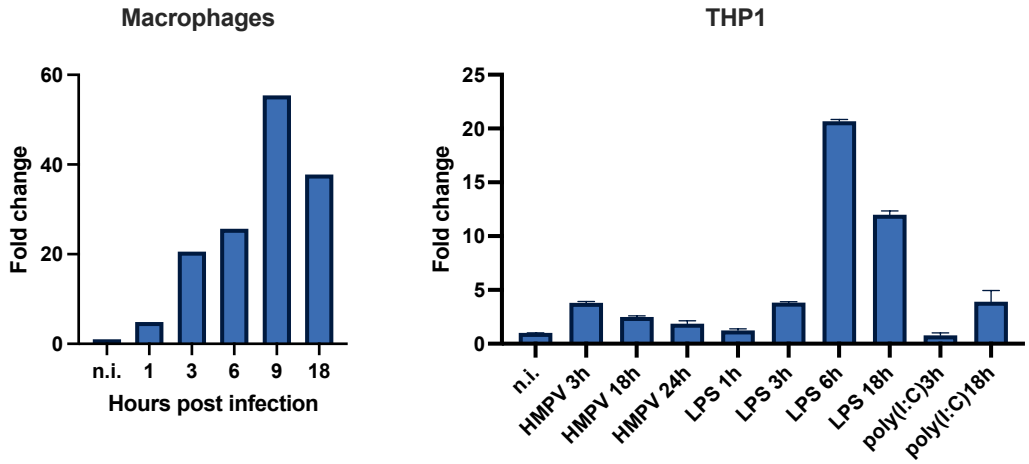

Figure 10: SLAMF1 mRNA is weakly induced by HMPV in THP1 cells compared to MDMs.
Quantification of SLAMF1 mRNA expression by RT-qPCR in human macrophages or THP1 cells infected with HMPV (m.o.i = 1) or stimulated with LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points. The results from macrophages have been previously obtained by the "Virus in Immunity and Disease"-group and is only representative for one experiment. The bars represent three technical replicates per sample where their average C_T values have been normalized against the noninfected-sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD. The data on THP1 cells is one representative for three experiments.

3.2 HMPV-induced innate immune responses in MDMs

Considering that SLAMF1-induction by HMPV in THP1 cells was very weak compared to MDMs, we wanted to confirm the previously obtained results on HMPV-induced SLAMF1-expression in MDMs (Fig. 10, left panel). To evaluate the innate response of HMPV-infection it was also of interest to compare the expression kinetics of IFN-β and TNF-α with SLAMF1. Therefore, we proceeded with MDMs and measured levels of HMPV-N, SLAMF1, IFN-β and TNF-α mRNA expression by RT-qPCR.

The amount of viral RNA was measured first as this would have an impact on levels of SLAMF1 and cytokines. As with HMPV-infection in THP1 cells (Fig. 6), qPCR analysis showed that the level of HMPV-N mRNA in MDMs increased with time (**Figure 11,** top panel). We also confirmed that SLAMF1 was induced by HMPV in MDMs (Fig. 11, second panel); A weak induction of SLAMF1 mRNA was detected at 3 hrs post infection followed by a plateau until a rapid increase was detected at 18 hrs post infection. Similarly, levels of IFN-β mRNA increased with infection with a rapid increase of expression observed at 18 hrs post infection (Fig. 11, third panel). In contrast, TNF-α mRNA seemed to be induced in two waves in MDMs (Fig. 12, lower panel). A weak expression of TNF-α was detected at 1 h post infection followed by a decrease until a stronger induction occurred at 18 hrs. This was observed in two of three donors, while a continuous TNF-α expression was observed in the third donor. In summary, HMPV-mediated TNF-α expression kinetics seem to differ from that of IFN-β and SLAMF1 in MDMs.

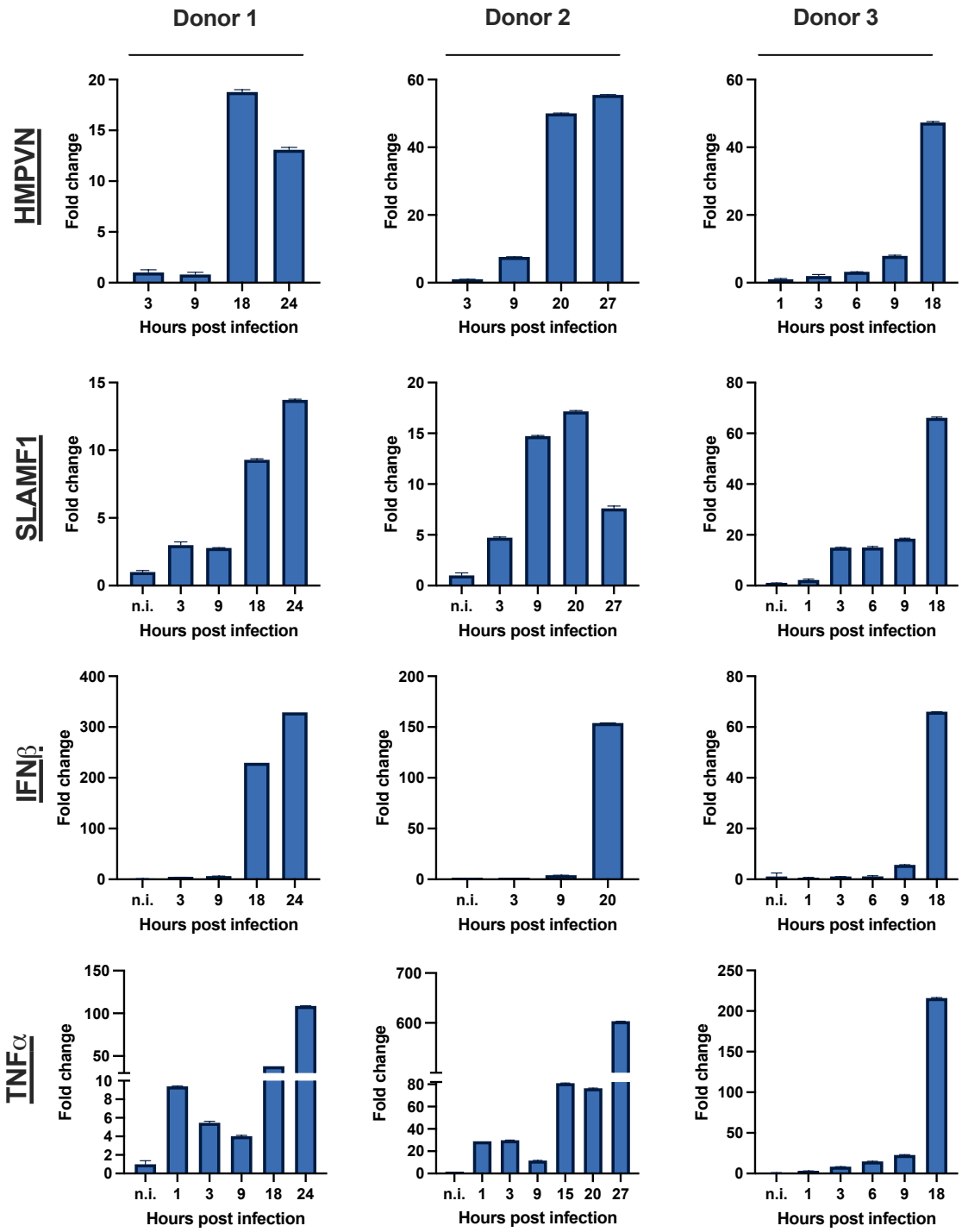

Figure 11: HMPV-infection in MDMs induces expression of SLAMF1, IFN-β and TNF-α mRNA. Quantification of HMPV-N, SLAMF1, IFN-β and TNF-α mRNA expression by RT-qPCR in MDMs from three donors infected with HMPV (m.o.i = 1) for the indicated time points. The bars represent two technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

3.3 The effects of silencing *TLR4* and *SLAMF1* on viral replication and innate responses to HMPV-infection in THP1 cells

In the previous section we found that SLAMF1 was strongly expressed in response to HMPV in MDMs and were able to map the expression kinetics of IFN-β and TNF-α mRNA in HMPV-infection. Next, we wanted to explore whether knockdown (KD) of specific genes would affect viral replication and/or innate immune signaling. THP1 cells were chosen to investigate the effects of silencing as these cells are considered more easily genetically modified than MDMs. Despite that only a weak expression of SLAMF1 mRNA was detected in THP1 cells (section 3.1.5) there was still a possibility that HMPV could be affected by silencing of *SLAMF1* in these cells.

TLR4 has earlier been proposed to be implicated in HMPV-induced expression of proinflammatory cytokines and IFNs and may be activated by components of the viral envelope [45]. However, it is not known if SLAMF1 expression affects HMPV-responses and if TLR4 mediates SLAMF1-induction by HMPV. We therefore performed siRNA-mediated knockdown of *TLR4* or *SLAMF1* to study the effects on viral replication and downstream signaling. Silencing was performed in differentiated THP1 cells as described in section 2.7 followed by infection with HMPV or stimulation with LPS.

3.3.1 Silencing of *TLR4* reduces levels of HMPV-N mRNA

The effects of *TLR4* and *SLAMF1* knockdown on HMPV-induced immune signaling and replication were first studied by measuring levels of HMPV-N, TNF-α and IFN-β mRNA by RT-qPCR. The silencing efficiency of *TLR4* and *SLAMF1* was determined by qPCR and are shown in **Table 1, Figure 12**. The silencing was considered overall successful.

Table 1: Overview of KD efficiencies of *SLAMF1* and *TLR4* in THP1 cells. Silencing efficiencies (%) of *TLR4* and *SLAMF1* in THP1 cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Cells were infected with HMPV (m.o.i = 1) or stimulated with LPS (200 ng/ml) for the indicated time points. *TLR4*-silencing in Exp 1 did not include HMPV 3 hrs or the non-infected (n.i) sample. The KD efficiencies for each sample were calculated as the percentage of SLAMF1/TLR4 mRNA expression in relative to the respective non-silencing control subtracted from 100%.

Gene	Sample	**Exp 1** Silencing efficiency (%)	**Exp 2** Silencing efficiency (%)
SLAMF1	n.i.	94	76
	HMPV 3h	91	90
	HMPV 24h	80	77
	LPS 2h	85	66
TLR4	n.i.	-	74
	HMPV 3h	-	74
	HMPV 24h	78	56
	LPS 2h	0	66

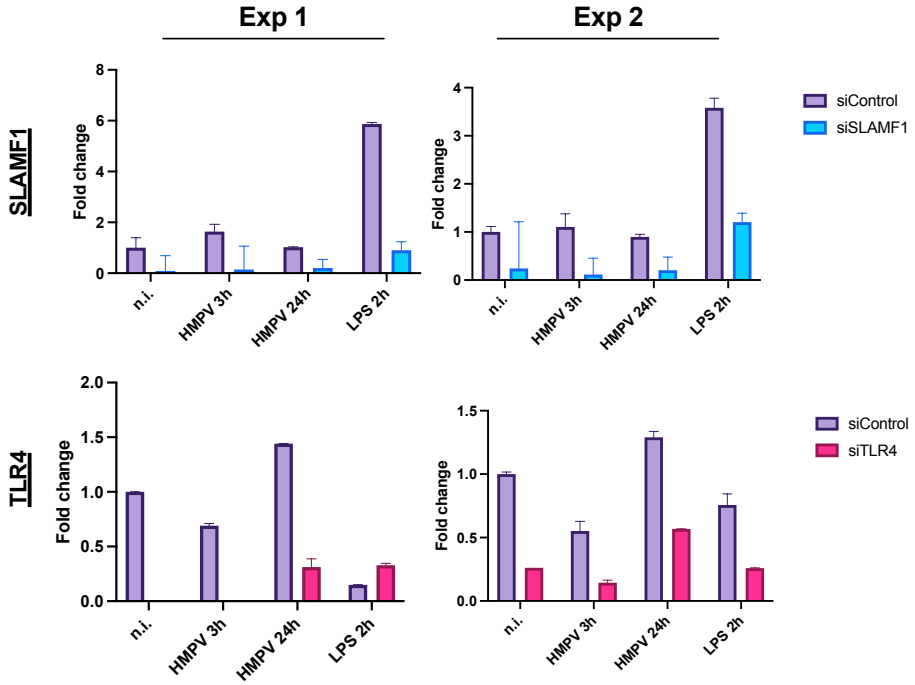

Figure 12: **KD efficiency of *SLAMF1* and *TLR4* in THP1 cells.** Quantification of SLAMF1 and TLR4 mRNA expression by RT-qPCR in THP1 cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Results from two independent experiments infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points are shown. The bars represent two technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

Silencing of *TLR4* led to a reduced amount of HMPV-N mRNA compared to the control, which could suggest that TLR4 is important for viral entry and/or replication (**Figure 13**). Unfortunately, the effects of silencing *SLAMF1* on HMPV-N mRNA were not consistent. LPS-mediated expression of IFN-β mRNA at 2 hrs was reduced by silencing of *SLAMF1* and *TLR4*. HMPV-mediated IFN-β mRNA expression seemed to be reduced by siSLAMF1 in one of the experiments, while siTLR4 did not seem to have an effect at all.

Silencing of *TLR4* reduced levels of TNF-α mRNA in response to LPS and HMPV, but only at only 3 hrs post infection. *SLAMF1*-silencing did not have consistent results on HMPV- or LPS-induced TNF-α mRNA expression. The experiment has to be repeated to provide enough data for statistical analysis in order to determine whether the observed differences are significant or not. Therefore, these results are preliminary and merely show the trends of expression.

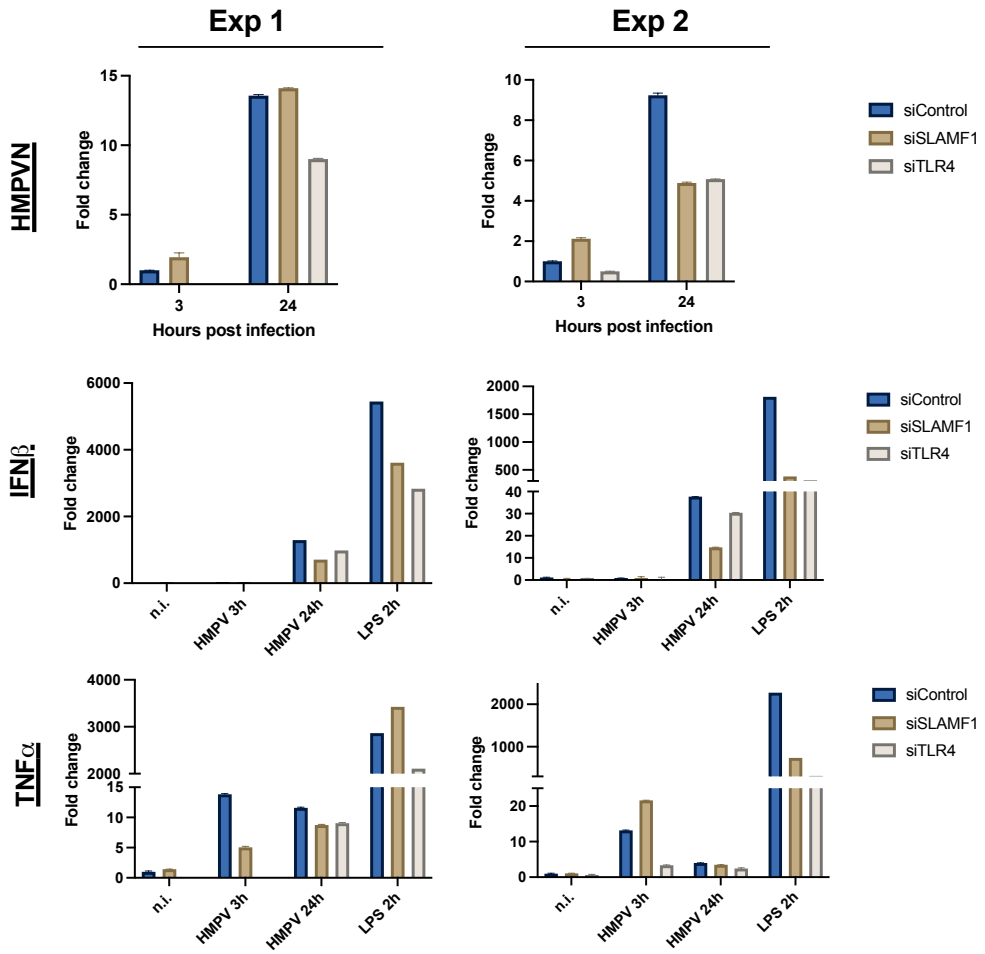

Figure 13: Silencing of *TLR4* reduces levels of HMPV-N mRNA in THP1 cells. Quantification of HMPV-N, IFN-β, TNF-α mRNA expression by RT-qPCR in THP1 cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Results from two independent experiments infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points are shown. The bars represent two technical replicates per sample where their average C_T values have been normalized

against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

3.3.2 Silencing of *SLAMF1* reduces levels of phosphorylated STAT1

Next, we determined the effects of *TLR4*- and *SLAMF1*-silencing on protein expression induced by HMPV. This was studied by measuring levels of HMPV-N protein, pSTAT1 and total STAT1 by western blot analysis as described in section 2.5.

In agreement with the mRNA expression results, *TLR4*-silencing reduced the amount of viral protein while *SLAMF1*-silencing did not (**Figure 14**). Phosphorylation of STAT1 in response to HMPV and LPS was strongly reduced by siSLAMF1, which could indicate that SLAMF1 is important for activation of the JAK-STAT pathway. The effects of siTLR4 on levels of pSTAT1 were unfortunately not consistent between the experiments.

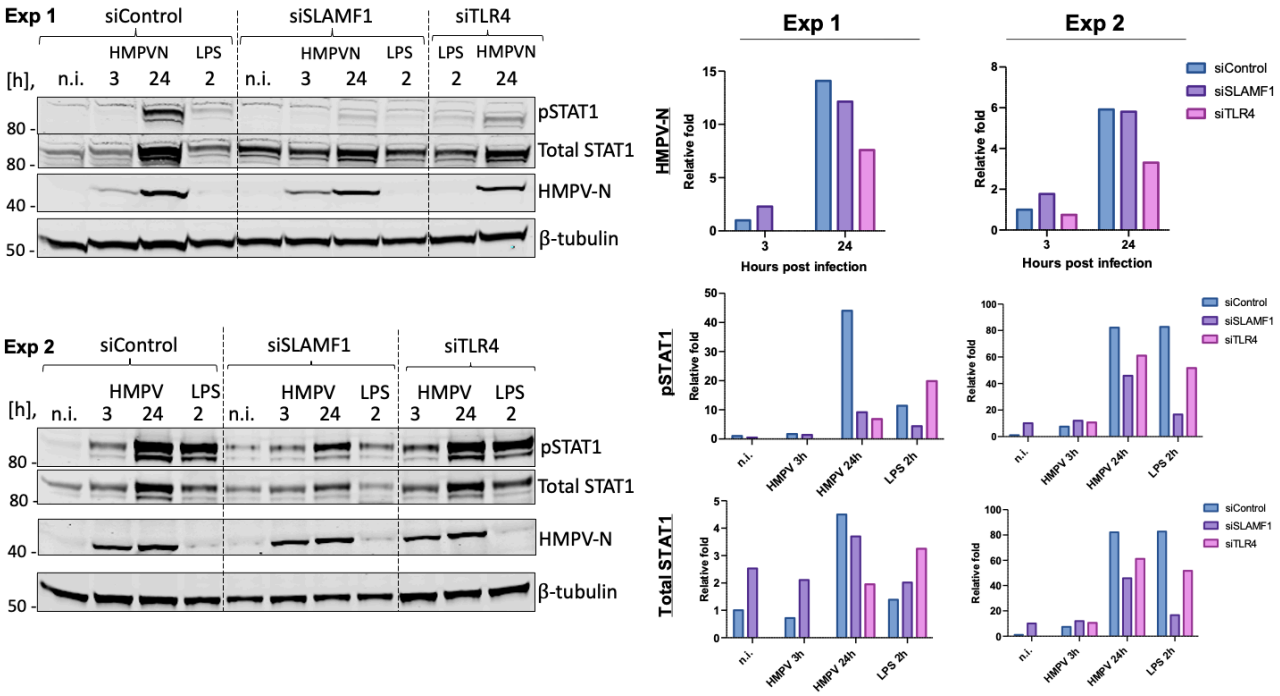

Figure 14: Silencing of *TLR4* and *SLAMF1* in HMPV-infected THP1 cells reduces levels of HMPV-N protein and pSTAT1, respectively. Western blotting of THP1 cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides and infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. The antibodies used are indicated in the figure and β-tubulin was used as a loading control. Molecular weight is given in kilodaltons. Results from two independent experiments are shown. Graphs to the right show the quantitative analysis of the protein levels from the blots to the left. Quantification of viral protein, pSTAT1 and total STAT1 levels relative to β-tubulin were obtained with ImageStudio software.

3.4 The effects of silencing *TLR4* and *SLAMF1* on viral replication and innate responses to HMPV-infection in MDMs

Considering that SLAMF1 was much stronger expressed by HMPV in MDMs than in THP1 cells, we wanted to investigate the effect of silencing *SLAMF1* on HMPV-infection in MDMs. A stronger expression of SLAMF1 could possibly also lead to a stronger effect of silencing on viral replication and downstream signaling. *TLR4* was also silenced for comparison. Human monocytes were obtained from blood donors as described in section 2.2. *TLR4* and *SLAMF1* were silenced by siRNA-mediated knockdown as described in section 2.7 before infection with HMPV or stimulation with LPS.

3.4.1 Silencing of *TLR4* and *SLAMF1* in MDMs reduces early induction of TNFα mRNA by HMPV

The effects of siTLR4 and siSLAMF1 on viral replication and the induced cytokine expression was studied by measuring levels of HMPV-N, IFN-β and TNF-α mRNA in MDMs by RT-qPCR (see section 2.4). The silencing efficiency of *TLR4* and *SLAMF1* in MDMs were determined by qPCR and are shown in **Table 2, Figure 15**.

Table 2: Overview of KD efficiencies of *SLAMF1* and *TLR4* in MDMs. Silencing efficiencies (%) of *TLR4* and *SLAMF1* in MDMs from two donors treated with a control non-silencing oligonucleotide (n.i.) or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Cells were infected with HMPV (m.o.i = 1) or stimulated with LPS (200 ng/ml) for the indicated time points. The KD efficiencies for each sample were calculated as the percentage of SLAMF1/TLR4 mRNA expression in relative to the respective non-silencing control subtracted from 100%.

Gene	Sample	**Donor 1** Silencing efficiency (%)	**Donor 2** Silencing efficiency (%)
SLAMF1	n.i.	60	68
	HMPV 3h	77	57
	HMPV 6h	82	89
	HMPV 24h	70	67
	LPS 2h	35	45
TLR4	n.i.	53	21
	HMPV 3h	54	27
	HMPV 6h	42	63
	HMPV 24h	33	79
	LPS 2h	55	52

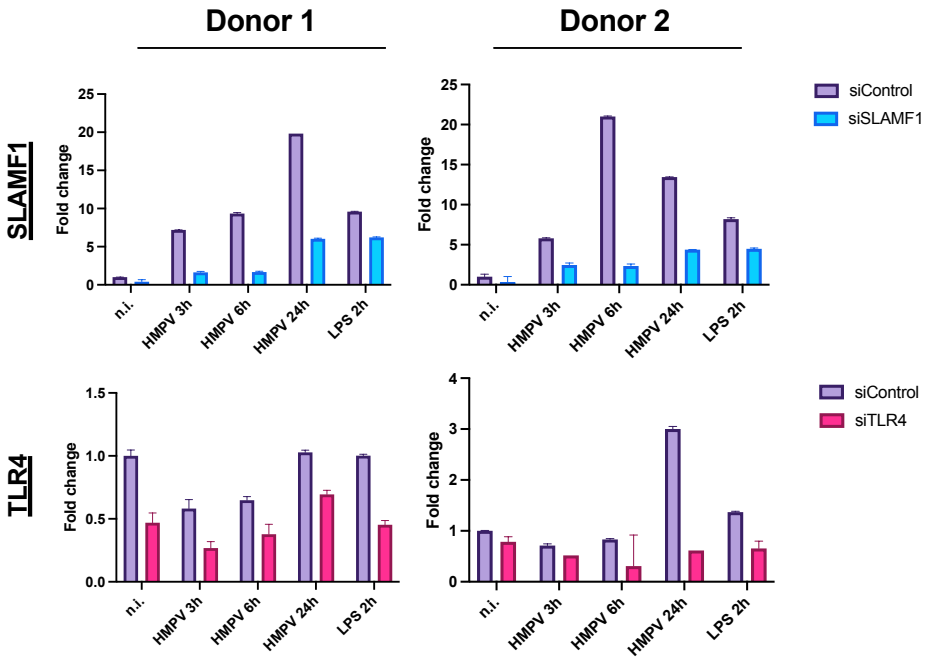

Figure 15: KD efficiency of *SLAMF1* and *TLR4* in MDMs. Quantification of SLAMF1 and TLR4 mRNA expression by RT-qPCR in MDMs cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Results from MDMs infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points in two donors are shown. The bars represent two technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

The fold change of HMPV-N mRNA was unfortunately too low to make any conclusions from the effect of silencing on viral replication (**Figure 16**). Additionally, the donor variation was too high in order to determine the effects of *TLR4*- and *SLAMF1*-silencing on IFN-β expression in MDMs. However, the effects of silencing on TNF-α expression was quite consistent between donors, suggesting a donor-dependent effect on HMPV-stimulated IFN-β expression. siSLAMF1 and siTLR4 reduced levels of TNF-α mRNA in response to HMPV at an early time point (3 hrs) but did not seem to have an effect at 24 hrs post infection. These results suggest that only the early wave of TNF-α mRNA expression in MDMs is TLR4- and SLAMF1-dependent. Similar to another study, our results showed that LPS-induced expression of TNF-α mRNA at 2 hrs post stimulation was not significantly reduced by silencing of *SLAMF1* [41]. Unexpectedly, silencing of *TLR4* also did not lead to reduced levels of LPS-induced TNF-α expression. Data from several donors is needed in order to conclude on the effects of silencing.

Protein expression of pSTAT1/total STAT1 and HMPV-N were analyzed by western blot analysis, but due to high donor variation we were not able to draw any conclusions. These results are located in Supplementary section 7.3, **Figure S5**.

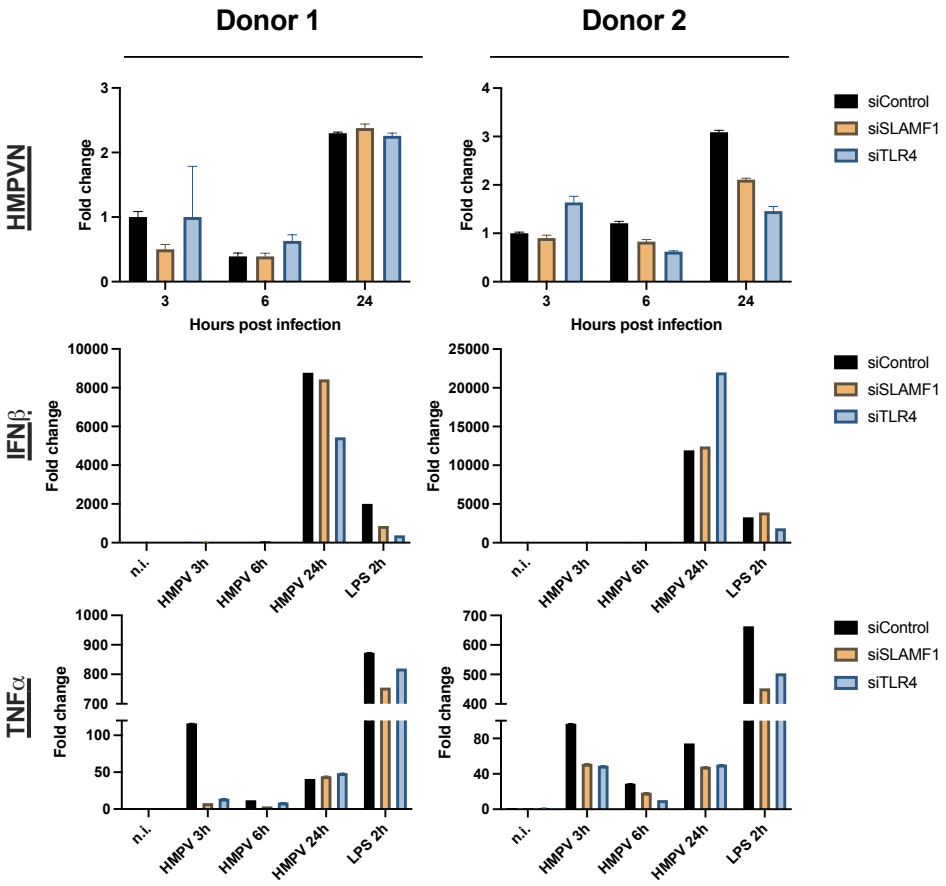

Figure 16: Silencing of *SLAMF1* and *TLR4* reduces early induction of TNF-α mRNA by HMPV in MDMs. Quantification of HMPV-N, IFN-β, TNF-α mRNA expression by RT-qPCR in MDMs treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides. Results from MDMs infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points in two donors are shown. The bars represent two technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

3.4.2 *TLR4*- and *SLAMF1*-silencing in MDMs reduces early TNF-α-secretion by HMPV

Gene expression analysis showed that HMPV-induced TNF-α mRNA expression was strongly reduced by silencing of *SLAMF1* and *TLR4* (Fig. 16), but this does not reveal the amount of secreted TNF-α protein. Therefore, ELISA was performed as described in section 2.6 to measure TNF-α protein secretion in the same samples.

ELISA revealed a marked reduction of TNF-α protein secretion in response to HMPV by *TLR4*- and *SLAMF1*-silencing at 3 hrs in one donor, and at 6 hrs in the second donor (**Figure 17**). At 24 hrs post infection, silencing did not seem to have any effect on the levels of TNF-α secretion. In response to LPS there was no difference in TNF-α secretion as a consequence of silencing. Hence, these results are in accordance with the results from the mRNA expression analysis, suggesting that early induction of TNF-α expression in MDMs is dependent on TLR4 and SLAMF1 upon infection with HMPV.

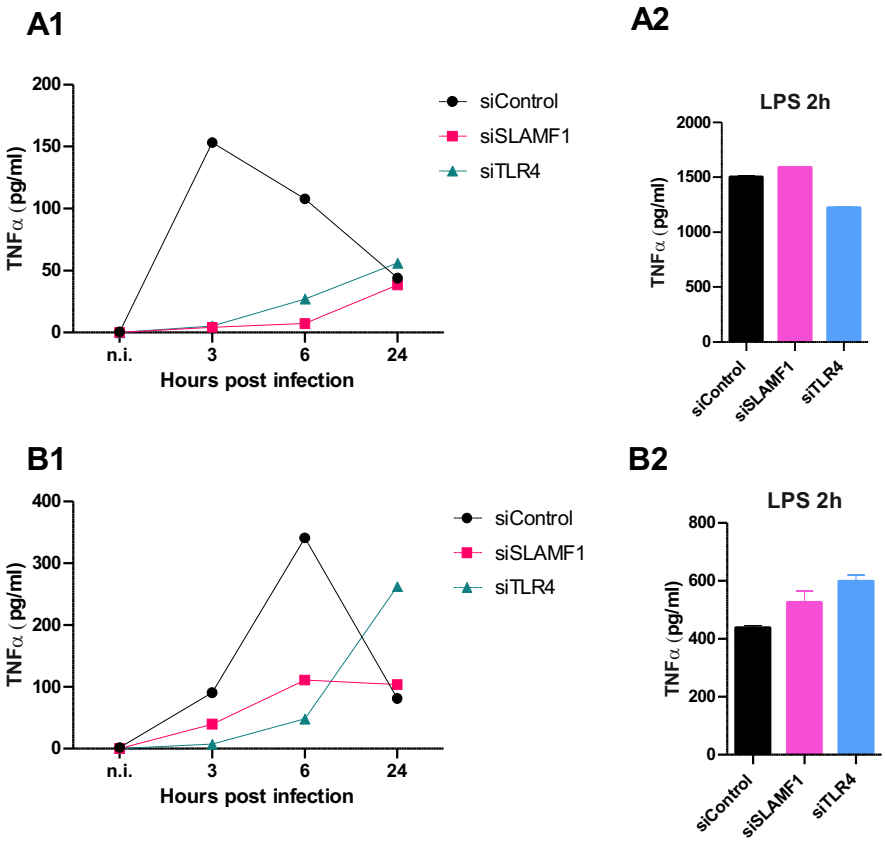

Figure 17: Silencing of *SLAMF1* and *TLR4* in MDMs reduces secretion of TNF-α protein in response to HMPV. TNF-α secretion levels in MDMs treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides and infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. Levels of secreted TNF-α protein in response to HMPV (A1, B1) and LPS (A2, B2) were assessed by ELISA. Data are presented as means with SD for two technical replicates from two donors (A, B).

3.5 The impact of knockout or overexpression of *SLAMF1* on HMPV replication and HMPV-induced innate immune responses

Measles virus, which is related to HMPV, seem to rely on SLAMF1 as an entrance receptor [14]. In addition, since infection with HMPV induces expression of SLAMF1 it was interesting to

investigate whether overexpressing or knockout (KO) of *SLAMF1* would affect HMPV replication or the induced signaling pathways. THP1 cell lines where *SLAMF1* was knocked out or overexpressed were provided by Mariya Yurchenko (CEMIR, NTNU) for this purpose (see section 2.2). The cells were infected with HMPV or stimulated with LPS .

3.5.1 Overexpression of *SLAMF1* leads to slightly increased levels of HMPV-N mRNA

To investigate the effects of *SLAMF1*-overexpression or KO on viral replication and cytokine expression, levels of HMPV-N, IFN-β and TNF-α mRNA were measured by RT-qPCR. We focused on IFN-β and TNF-α expression in order to determine which transcription factors, IRF or NFκB respectively, were activated by HMPV. By performing western blot analysis, we confirmed that the *SLAMF1*-overexpressing cell line indeed overexpressed *SLAMF1* compared to the KO cell line (**Figure 18**).

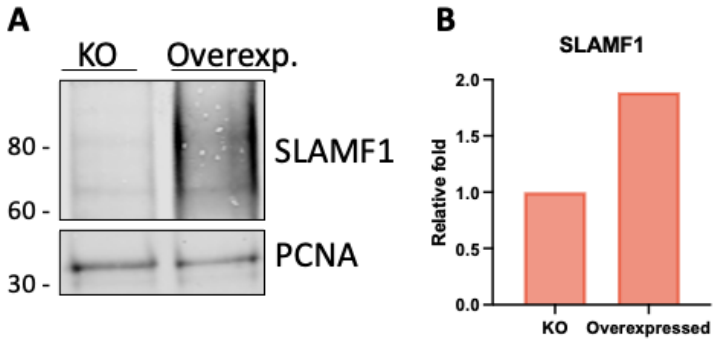

Figure 18: Protein expression analysis confirmed a higher amount of SLAMF1 protein in *SLAMF1*-overexpression cell line compared to *SLAMF1*-KO. Western blot analysis of THP1-SLAMF1KO and THP1SLAMF1-Overexpression cell lines where non-infected samples (medium 24 hrs) of the two cell lines were blotted against SLAMF1 with PCNA as the loading control (A). Molecular weight is given in kilodaltons. Quantification of levels of SLAMF1 protein were obtained with ImageStudio software (B).

The levels of HMPV-N mRNA were slightly increased at late time points of infection by overexpressing *SLAMF1* compared to the knockout, although the differences were not significant (**Figure 19**). However, these results were observed repeatedly in all 3 repetitions of the experiment and might suggest a minor positive impact of *SLAMF1*-overexpression on viral replication. The same small increase was observed by overexpressing *SLAMF1* on HMPV-mediated IFN-β mRNA expression at 24 hrs for all three experiments. Because of variable results, we were unable to determine the effects of *SLAMF1*-overexpression on TNF-α mRNA expression and on LPS-mediated IFN-β mRNA expression.

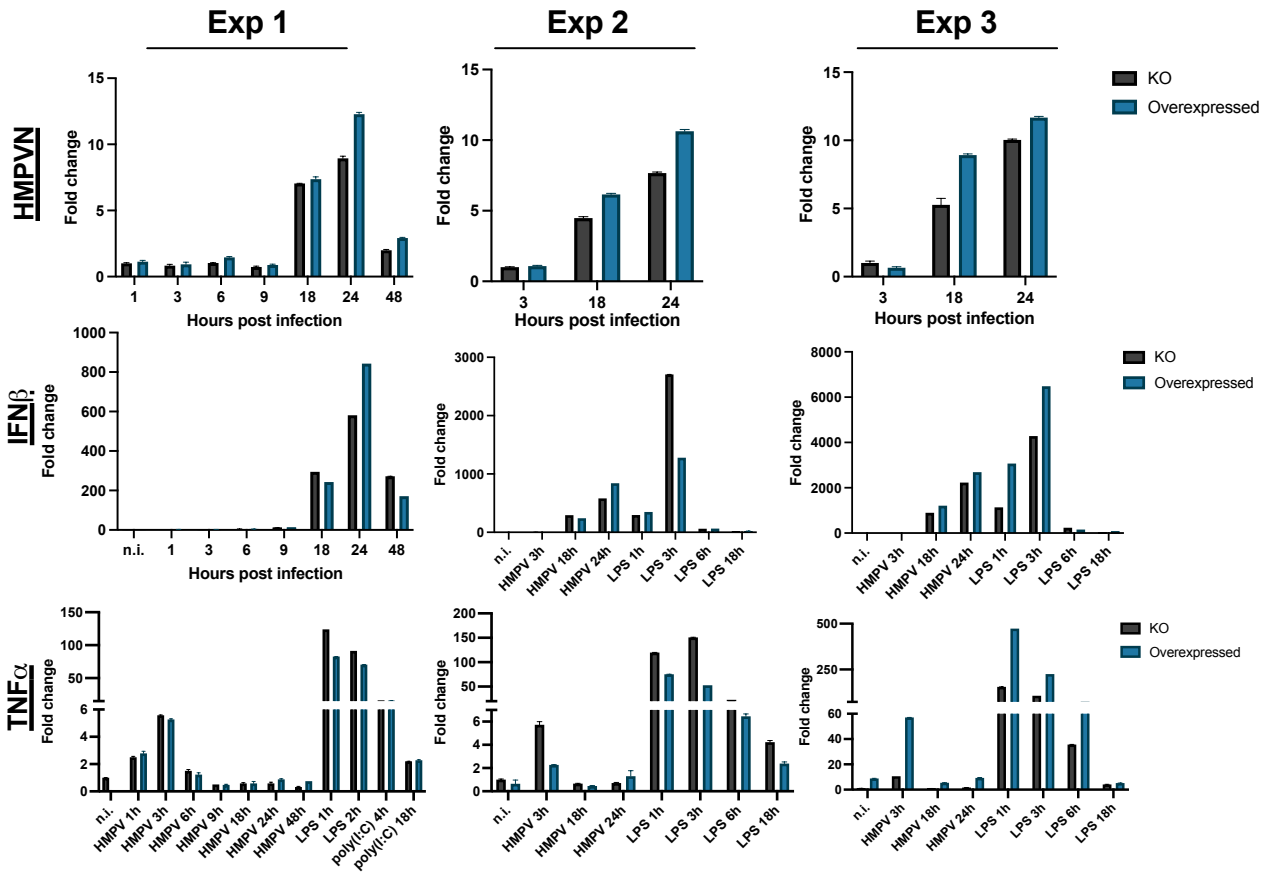

Figure 19: Overexpression of *SLAMF1* slightly increases levels of HMPV-N mRNA in THP1 cells. Quantification of HMPV-N, IFN-β, TNF-α mRNA expression by RT-qPCR in THP1SLAMF1-Overexpression and THP1SLAMF1-KO cell lines infected with HMPV (m.o.i = 1), LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points. The bars represent three technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD from three independent experiments. A two-sided student t-test (p-value < 0.05) was performed to determine if the difference between HMPV-N mRNA levels in the two cell lines were significant. The t-test did not reveal any statistically significant differences and is therefore not shown.

3.5.2 Overexpression of *SLAMF1* may increase levels of pSTAT1 and viperin

In order to follow up the results observed by mRNA expression analysis, we measured levels of viral protein, pp65/total p65, pSTAT1/total STAT1, and viperin in THP1SLAMF1-KO and THP1SLAMF1-overexpression cell lines by western blot analysis. Only 2/3 immunoblots were successful.

Unlike HMPV-N mRNA (Fig. 19), levels of HMPV-N protein did not seem to be affected by KO or overexpression of *SLAMF1* (**Figure 20**). On the other hand, levels of viperin seemed to be slightly

increased by overexpression of *SLAMF1* in response to HMPV, and even stronger increased in response to LPS. The same effect was observed in one of the two experiments on levels of pSTAT1. These findings support the results observed in section 3.3.2 where siSLAMF1 reduced levels of pSTAT1, suggesting that SLAMF1 is important for activation of the JAK-STAT pathway.

As with the unmodified THP1 cells (Fig. 9), HMPV-induced expression of pp65/total p65 was almost non-existent and did not seem to be affected by KO or overexpressing *SLAMF1*. These results suggest that a) HMPV infection weakly induces the signaling pathway that activates NFκB and b) that SLAMF1 does not appear to regulate this signaling pathway. However, these observations were only based on two experiments which did not provide enough data to perform statistical analysis. Several repetitions of the experiment are necessary to conclude on these results.

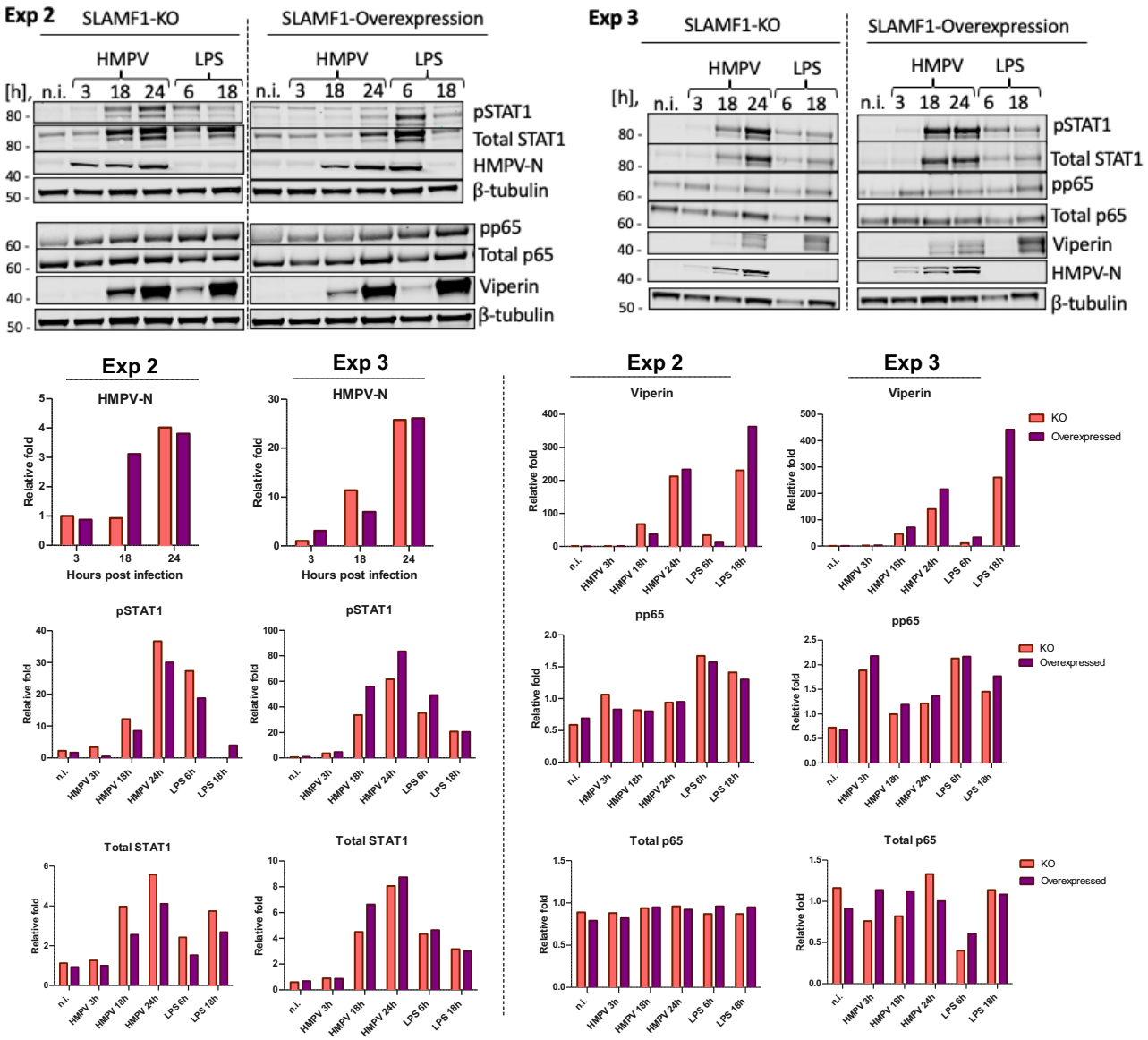

Figure 20: Overexpression of *SLAMF1* may increase levels of pSTAT1 and viperin in THP1 cells. Western blot analysis of HMPV-N protein, pSTAT1/total STAT1 and pp65/total p65 levels in

THP1SLAMF1KO (orange) and THP1SLAMF1-Overexpression (purple) cell lines. Two of three experiments are shown where the immunoblot of Exp 1 was not successful. The cells were lines infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. Molecular weight is given in kilodaltons. Graphs show quantification of protein levels relative to β-tubulin and were obtained with ImageStudio software.

3.6 The effects of surface SLAMF1 ligation on HMPV-infection in THP1 *SLAMF1*-KO and -overexpression cells

Combining the results from silencing and overexpressing *SLAMF1*, it seems like SLAMF1 might have an effect on replication of HMPV and downstream signaling. However, we still did not know where in the infectious cycle SLAMF1 encountered the virus. It could be on the surface upon early entry or perhaps in the cytosol during replication. A recent study on the receptors involved in HMPV entry suggested that the fusion protein (F) of HMPV interacts with multiple binding partners during attachment [60], and we speculated that surface SLAMF1 could be of importance. We therefore wanted to upregulate SLAMF1 on the surface of the cells in order to test if this was beneficial for HMPV. To do this, we mimicked a situation that occurs when HMPV infects a cell. Upon infection, macrophages that have not yet been encountered by HMPV are activated by cytokines (TNF-α or other proinflammatory cytokines) released from neighboring infected cells. This induces upregulation of SLAMF1 on the cell surface of the non-infected macrophages. Infected cells and activated cells would also release soluble SLAMF1 which serves as a ligand for surface SLAMF1 (homotypic receptor) [61]. By incubating cells with anti-SLAMF1 antibodies it has been found that this leads to upregulation of SLAMF1 on the cell surface [M. Yurchenko, CEMIR, unpublished]. The use of monoclonal antibodies to ligate specific molecules have also been performed in other studies [62, 63]. Hence, we treated THP1SLAMF1-KO and -overexpression cells with anti-SLAMF1 antibodies as described in section 2.8 before infection with HMPV or stimulation with LPS.

3.6.1 Ligation of surface SLAMF1 does not seem to affect HMPV replication or HMPV-induced IFN-β and TNFα expression

The effects of surface SLAMF1 ligation in HMPV-infection was first investigated by measuring the amount of HMPV-N, IFN-β and TNF-α mRNA by RT-qPCR. The THP1SLAMF1-KO cell line was used as a negative control in this experiment where ligation of surface SLAMF1 expectantly would not occur. LPS functioned as a positive control for cytokine expression.

qPCR analysis showed that the amount of HMPV-N mRNA was higher in the SLAMF1-overexpressing cells when compared to the infection of control *SLAMF1*-knockout cells regardless of SLAMF1-ligation (**Figure 22**). These results suggest that surface SLAMF1 does not have an effect on the entry or replication of HMPV, but that overall overexpressed SLAMF1 does. This corresponds to the results that were observed with *SLAMF1*-overexpression described in section 3.5.1. The same effect was observed with levels of IFN-β; A stronger IFN-β mRNA expression was detected by *SLAMF1*-overexpression when compared to the knockout, and ligation of surface-SLAMF1 did not have any effect. Even though LPS only was included as a positive control, we observed that ligation of SLAMF1 led to a strong increase of LPS-mediated levels of IFN-β mRNA. These results indicate that non-surface SLAMF1 is important for entry/replication of HMPV and HMPV-mediated expression of IFN-β mRNA, while surface SLAMF1 is important for LPS-mediated expression of IFN-β mRNA. TNF-α mRNA expression was not affected by ligation of surface SLAMF1 in response to either virus or LPS, but the amount of TNF-α was higher in the SLAMF1-overexpression cell line compared to the knockout. The experiment should be repeated in order to state these findings with confidence.

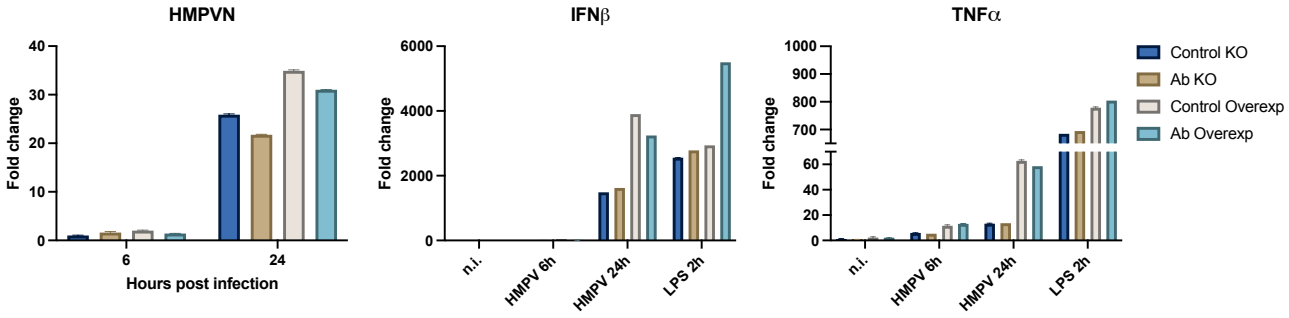

Figure 22: Surface SLAMF1 ligation does not affect levels of HMPV-N mRNA or HMPV-induced IFN-β, or TNF-α mRNA in THP1 cells. Quantification of HMPV-N, IFN-β, TNF-α mRNA by RT-qPCR in THP1SLAMF1-Overexpression and THP1SLAMF1-KO cell lines treated with anti-SLAMF1 antibodies or an isotype control. The cells were infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. The bars represent three technical replicates per sample where their average C_T values have been normalized against a non-infected sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

3.6.2 Overexpression of *SLAMF1* increases levels of pSTAT1 regardless of SLAMF1 ligation

To follow up the results from the previous section, levels of pSTAT1, total STAT1 and viral protein were measured by western blot analysis.

The amount of HMPV-N protein was differently affected than levels of HMPV-N mRNA and the results makes us unable to interpret the effect of surface SLAMF1 ligation on expression of viral protein without repeating the experiment (**Figure 23**). However, the results did reveal that *SLAMF1*-overexpression led to an increase of pSTAT1/total STAT1 in response to HMPV compared to the knockout control regardless of SLAMF1-ligation. This is in agreement with the results in section 3.5. Unlike levels of IFN-β mRNA, LPS-induced pSTAT1 expression did not seem to be affected by surface SLAMF1 ligation. Several repetitions of the experiment are necessary to follow up and confirm these results.

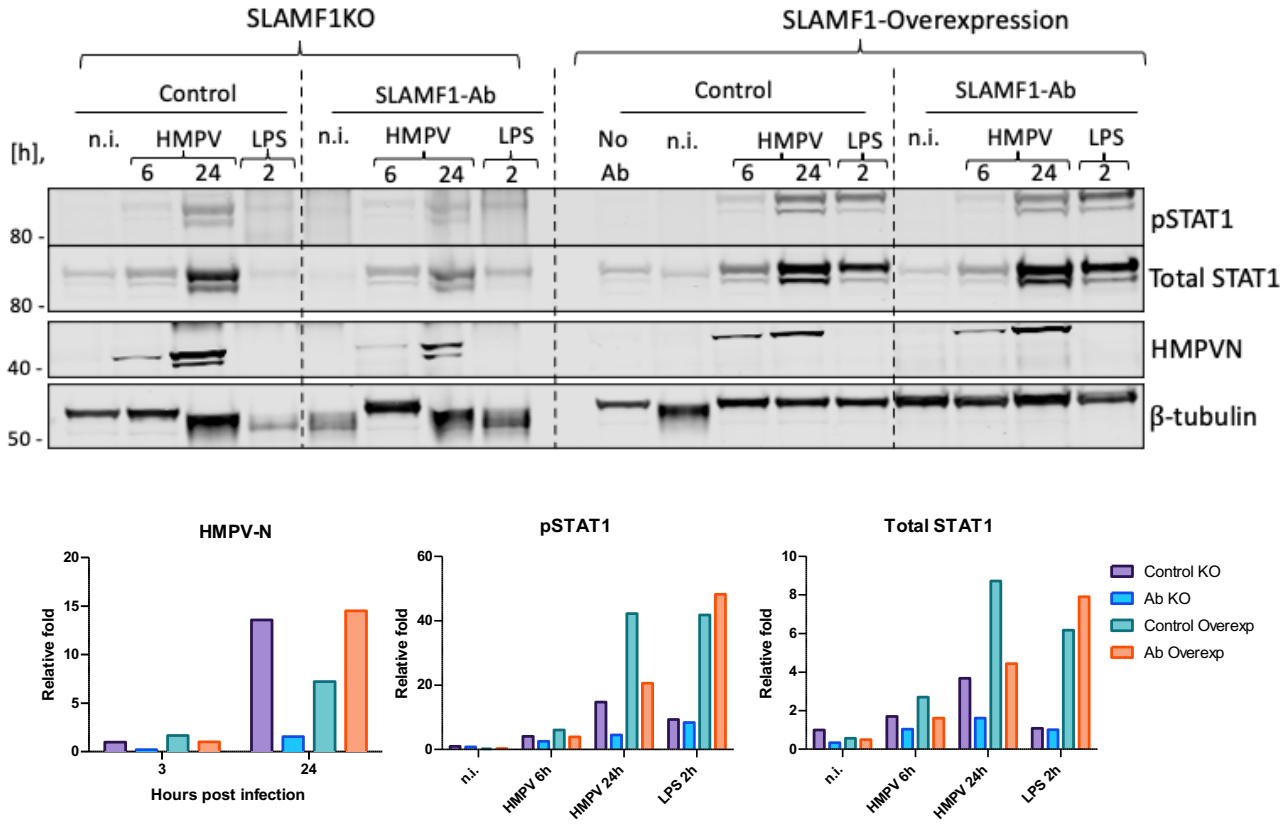

Figure 23: Overexpression of SLAMF1 increases levels of pSTAT1/total STAT1 compared to the KO control regardless of SLAMF1 ligation. Western blot analysis of protein levels in THP1-SLAMF1KO and THP1SLAMF1-Overexpression cell lines treated with anti-SLAMF1 antibodies or an isotype control (A). The cells were lines infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. Molecular weight is given in kilodaltons. Levels of pSTAT1, total STAT1 and viral protein (HMPV-N) were calculated relative to β-tubulin and quantified using ImageStudio software.

4 Discussion

The aim of this thesis was to determine if HMPV regulates SLAMF1 and establish if SLAMF1 plays a role in the innate immune responses induced by HMPV. This was done by studying the immune response induced by HMPV and viral replication when *SLAMF1* was overexpressed, silenced, unmodified or when SLAMF1 was ligated on the cell surface. In addition, we investigated whether silencing of *TLR4* had an effect on viral replication and signaling.

4.1 HMPV-induced SLAMF1 expression differs in THP1 cells and MDMs

We observed a clear difference of the expression kinetics of SLAMF1 mRNA by HMPV in THP1 cells and MDMs. While SLAMF1 mRNA was weakly induced at 3 hrs post infection in THP1 cells followed by a decrease, the expression was much stronger in MDMs and increased along with viral replication (Fig. 10). Loevenich et al. found that HMPV-stimulated expression of different immune mediators was highly dependent on the cell type [58], and cell type specificities could be an explanation to why SLAMF1 is differentially expressed in the two cell lines. Nevertheless, we were able to conclude that HMPV induces expression of SLAMF1 upon infection.

When comparing HMPV-mediated TNF-α and SLAMF1 mRNA expression patterns, there is a possibility that there could be a connection between the signaling pathways leading to their expression. Two waves of TNF-α mRNA expression were observed in response to HMPV in MDMs and the occurrence of these two waves could be explained by separate molecular events (Fig. 11). The first wave might be induced when the virus is first sensed upon entry, while the second could be induced by viral replication. Moreover, as we speculated that induction of SLAMF1 could be a secondary effect of cytokine release, it is possible that the first wave of TNF-α induces expression of SLAMF1. Yurchenko et al. showed that SLAMF1 mRNA expression was not driven by IFN-β-mediated signaling, but whether TNF-α -mediated signaling does has not been studied [41]. If our hypothesis on TNF-α-mediated SLAMF1 expression is correct, the low expression of TNF-α mRNA observed in THP1 cells could be a reason why SLAMF1 is weakly expressed in these cells. On the other hand, TNF-α and SLAMF1 mRNA levels both peaked at 3 hrs in THP1 cells (Fig. 7 and 10) which could mean that they are activated by the same factor, like NFκB. To further explore the cause of SLAMF1-induction it could be interesting to test whether it is driven by TNF-α-mediated signaling or if it perhaps is due to activation of NFκB. This could be done by exploring if addition of TNF- or TNFR-blocking antibodies or treatment with TNF-α affect SLAMF1 expression in HMPV-infected cells.

4.2 SLAMF1 expression affects HMPV-induced innate immune signaling

During investigation of the role of SLAMF1 in HMPV-infection, we found that silencing of *SLAMF1* led to a reduction of HMPV-induced IFN-β mRNA expression in THP1 cells while overexpression of *SLAMF1* led to a an increase (Fig. 13, 19 and 22). These results suggest that SLAMF1 is involved in HMPV-mediated expression of IFN-β. This was supported by analyzing the levels of pSTAT1 and the production of viperin as a read-out of IFN-β-mediated signaling. The amount of pSTAT1 and viperin were affected similarly to IFN-β by silencing and upregulation of *SLAMF1* in response to HMPV (Fig. 14, 20 and 23). These results are in agreement with a previous study on THP1 cells and MDMs where silencing of *SLAMF1* reduced expression of IFN-β in response to LPS [41]. Therefore, SLAMF1 seems to be involved in the signaling pathways that drives both LPS- and HMPV-induced IFN-β expression. However, several repetitions of the experiment and additional research approaches are needed in order to confirm this.

Regarding HMPV entry and replication, neither silencing of *SLAMF1* nor the ligation of SLAMF1 on the cell surface by monoclonal antibodies seemed to affect HMPV viral load in infected cells (Fig 13, 14 and 22). Our speculation that surface SLAMF1 acts in HMPV-entry (similar to measles virus) as one of the binding partners upon attachment [60] may therefore not be valid. However, we did not confirm that the ligation of surface SLAMF1 had been successful which means there is a possibility that ligation may not have occurred. Additionally, the entry of HMPV was only indirectly studied through measuring the levels of viral RNA and protein; If silencing of *SLAMF1* inhibited HMPV entry, consequently we would expect a detection of lower levels of viral RNA and protein. Nevertheless, we did observe a slight increase of viral mRNA at late time points of infection by overexpressing *SLAMF1* when compared to the infection of control SLAMF1 knockout cells in four independent experiments (Fig. 19 and 22). Therefore, we suggest that SLAMF1 may contribute to enhanced replication, but is not critical for replication to occur. The approaches used are not suitable to reveal at which stage in the infectious cycle HMPV encounters SLAMF1 and this is yet to be discovered. Investigating if HMPV affects SLAMF1 localization would be interesting to assess in future studies.

4.3 TLR4 levels impacted on HMPV levels

Kolli et al. showed that HMPV-induced cytokine expression occurred via TLR4 in dendritic cells and that it was replication-independent, suggesting that TLR4 is activated by components of the viral envelope [45]. We found that silencing of *TLR4* in THP1 cells reduced the amount of viral mRNA and protein as well as cytokine expression (Fig. 13 and 14), thereby supporting these results. As the reduction of viral mRNA and protein in THP1 cells was present at an early time point of

infection, this could indicate that HMPV was affected by *TLR4*-silencing before replication began. In other words, TLR4 might be involved in the entry of HMPV. Indeed it has been suggested that TLR4 mediates entry and proinflammatory signaling of several viruses, such as RSV, HMPV, dengue virus and Ebola virus [46]. Moreover, Kurt Jones et al. hypothesized that TLR4 was involved in the innate immune recognition of the RSV fusion protein and showed that TLR4 was important for viral clearance [39]. As the RSV fusion protein is known to mediate fusion of the viral envelope with the target cell membrane during virus entry, it is possible TLR4 recognizes the fusion protein of RSV and affects entry of the virus [64]. Considering that RSV and HMPV are closely related, this hypothesis might also be applied for HMPV.

Even though our data suggest the involvement of TLR4 in HMPV-infection in THP1 cells, we do not know if the same results would be found *in vivo*. An earlier published study investigating the role of TLR4 in HMPV-infected mice showed that viral replication and clearance of HMPV was unaffected in *TLR4*-deficient mice, contradicting the results obtained by Kurt Jones et al. [38]. Although, the study on TLR4-deficient mice was only performed in females which makes it questionable whether the results were female-dependent. In order to further validate our own results of the effect of *TLR4*-silencing in HMPV-infection, the number of infectious particles could be measured by an infectious viral titer assay. Additionally, confocal microscopy could be performed to directly study the role of TLR4 in the entry of HMPV. It is important to have in mind that these suggestions are based on our results observed from two repetitions of the experiment which did not provide enough data for statistical analysis. In order to state whether the amount of viral mRNA and protein as a consequence of silencing were significantly different from the controls, several repetitions of the experiment should be performed.

Another interesting perspective for future experiments could be to investigate the involvement of the co-receptor of TLR4, CD14, in HMPV-infection [19]. Bao et al. downregulated CD14 to study HMPV-induced moDC responses and showed a significant reduction in cytokine, chemokine and type I IFN gene expression and protein secretion [65]. CD14 has also been reported to be critical in sensing the RSV F protein in macrophages [39]. Based on this information it would be interesting to investigate if the entry of HMPV not only involves TLR4, but also its co-receptor CD14.

A limitation of the work performed in this thesis is that most findings are based on experiments performed in THP1 cells, and we do not know whether the same results can be reproduced in other cell lines. Several attempts were made to characterize the effect of *TLR4*- and *SLAMF1*-silencing in MDMs to confirm what was observed in THP1 cells in response to HMPV, but the donor variation

was too high to be able to make any conclusions with the number of repetitions performed. Donor variation is one of the challenges when using human primary cells. Factors like genetic background and health status causes variation between biological replicates [66]. Hence, a large sample size is necessary to produce consistent results. Kinetics of cytokine expression is also a factor that varies between donors and should be accounted for when creating experiments [67]. For example, one donor could induce two waves of TNF-α within 24 hrs, while another donor might not have induced the second wave within the same time span. A difference in expression kinetics was observed when measuring the secreted amount of TNF-α which peaked at different time points in the two donors. The fact that only 2-3 time points were included in this experiment limits the amount of information we can use to interpret the result and should be modified in future studies. Mapping each donor's expression kinetics for a specific cytokine is therefore important in order to get an informative overview of the cytokine expression pattern. In order to follow up and test if the results observed by HMPV-infection in THP1 cells can be reproduced in MDMs, experiments should be carried out in several donors. Additionally, mouse-studies could be performed to test if the effects of silencing *TLR4* and *SLAMF1* in HMPV-infection is conserved *in vivo.*

4.4 The first wave of TNF-α expression in response to HMPV is TLR4- and SLAMF1-dependent

In this thesis we also found that in MDMs TNF-α mRNA expression is induced by HMPV in two waves, and that the first wave is TLR4- and SLAMF1-dependent. Earlier we suggested that the two waves of TNF-α mRNA expression in MDMs are caused by different events of the infectious cycle, but the results also indicate that each wave involves different receptors. One explanation could be that TLR4 is activated upon entry of HMPV, and this could be the cause of the first wave of TNF-α mRNA expression. This theory can be backed up by the results from *TLR4*- and *SLAMF1*-silencing in MDMs where only the first wave of TNF-α mRNA expression at 3 hrs post infection was reduced by silencing (Fig. 16). Analysis of secreted TNF-α protein from the same experiment revealed the same effect (Fig 17). These results suggest that the first wave of TNF-α expression is TLR4- and SLAMF1-dependent while the second is not. This could be connected with the regulatory role of SLAMF1 in TLR4-mediated intracellular signaling [41].

Levels of TNF-α secretion in response to LPS in MDMs were unaffected by silencing of *TLR4* and *SLAMF1*. One reason could be due to poor silencing in these samples and/or because the length of stimulation with LPS was too short. Yurchenko et al. found that LPS-induced TNF-α mRNA expression only was affected by *SLAMF1*-silencing at 3-6 hrs post stimulation, while secreted TNF-

α was not affected by silencing until 4 hrs post stimulation [41]. Considering that we only treated MDMs for 2 hrs with LPS, it is likely that the induction of TNF-α was not optimal at this time point. Adding several time points for LPS-stimulation could give a more informative overview on the effects of silencing on TNF-α expression.

It is relevant to mention that the role of TLR4 in HMPV-induced IFN-β expression could be questioned from the results obtained in this thesis. Two studies have suggested that HMPV-induced cytokine, chemokine and type I IFN mRNA levels and secretion occurs via TLR4 in various types of dendritic cells [38, 65]. Unfortunately, we were not able to conclude on the effects of *TLR4*-silencing in macrophages due to donor variation, but in THP1 cells TLR4 did not seem to mediate HMPV-induced IFN-β expression. Neither knockdown nor knockout of *TLR4* seemed to reduce IFN-β mRNA in response to HMPV in THP1 cells (Fig. 8 and 13) and could be explained by several reasons. First of all, the silencing may not have been efficient enough to block the IFN-β expression induced by HMPV. Second, as explained in section 4.1, expression of HMPV-stimulated immune mediators was found to be cell-type specific and could be an explanation why our results on IFN-β expression in THP1 cells are not in agreement with the previous studies on DCs. Thus, it is possible that HMPV-induced IFN-β expression in DCs and THP1 cells rely on different receptors. Additionally, siRNA has been reported to induce an immune response, originating from siRNA sequence-dependent delivery vehicles or from the RNA-interference process [68]. Therefore, the reason why a reduction of IFN-β is not observed after silencing of *TLR4* could be due to siRNA-stimulated IFN-β. On the other hand, levels of pSTAT1 protein, being an indicator of IFN-β signaling, was reduced by silencing of *TLR4*. Western blot analysis is a semi-quantitative method and the effects of silencing varied between experimental repetitions, which could question the quality of these results. It is important to keep in mind that these suggestions are based on only two repetitions of the experiment and did not provide enough data for statistical analysis. These results are therefore preliminary and should be repeated.

5 Conclusion

In this thesis we showed that HMPV was able to infect THP1 cells and that SLAMF1 was weakly induced by HMPV at an early time point of infection. SLAMF1 expression in MDMs on the other hand was strongly upregulated upon infection with HMPV and correlated with viral replication. Our results also suggest that SLAMF1 is involved in HMPV-triggered IFN-β and TNF-α expression, and that the expression kinetics of SLAMF1 and TNF-α mRNA seemed to differ in THP1 cells and MDMs. Moreover, our studies demonstrated that TNF-α expression was induced in two waves within 24 hrs in response to HMPV in MDMs, and that the first wave was TLR4- and SLAMF1-dependent.

Here we also suggest that TLR4 positively regulates the entry and replication of HMPV as *TLR4*-silencing reduced the amount of HMPV-N mRNA and protein in THP1 cells. However, the role of TLR4 in the entry of HMPV should be further investigated by other research approaches. While knockdown and knockout of *SLAMF1* did not seem to affect HMPV viral load in infected cells, *SLAMF1*-overexpression slightly increased the amount of viral mRNA at late time points of infection when compared to the infection of control SLAMF1 knockout cells. Therefore, we suggest that SLAMF1 may contribute to enhanced replication, but is not critical for replication to occur. Most of the results obtained during this thesis are preliminary and further studies are necessary to confirm these results.

6 References

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7 Supplementary

7.1 Primer sequences and antibodies

The primer sequences used when performing RT-qPCR are listed in Table S1 while primary and secondary antibodies used in western blot analysis are listed in Table S2 and S3, respectively.

Table S1: Primer sequences used to quantify mRNA expression of different genes by RT-qPCR.

Gene	Forward	Reverse
IFN-β	5′-GCCGCATTGACCATCTATGAGA-3′	5′-GAGATCTTCAGTTTCGGAGGTAAC-3′
HMPV-N	5′-CATATAAGCATGCTATATTAAAAGAGTCTC-3′	5′-CCTATTTCTGCAGCATATTTGTAATCAG-3′
Cyt SLAMF1	5’-TGAGAAGAAGAGGTAAAACGAACC-3’	5’- ATATGGTGGTGCAAGGGTCC-3’
Ext SLAMF1	5’-AGGCCCTCCACGTTATCTA-3’	5’- GCAAAAGCGCTGAACTGA-3’
TBP	5’- GAGCCAAGAGTGAAGAACAGTC-3’	5’-GCTCCCCACCATATTCTGAATCT-3’

Table S2: **Primary antibodies used in western blot staining.**

Antibody	Species	Molecular Weight (kDa)'	Dilution in TBST	Manufacturer
HMPV-N	Mouse	42	1:500	Abcam (#ab94802)
STAT1, phospho (Tyr701)	Rabbit IgG	84, 91	1:1000	Cell Signaling Technology (#9167)
STAT1	Rabbit IgG	84, 91	1:1000	Cell Signaling Technology (#9172)
P65, phospho (Ser536)	Rabbit IgG	65	1:1000	Cell Signaling Technology (#3033)
P65	Rabbit IgG	65	1:1000	Cell Signaling Technology (#8242)
Viperin	Rabbit IgG	45	1:1000	Cell Signaling Technology (#13996)
SLAMF1	Rabbit IgG	60-80	1:1000	Sino Biological (#10837-R008)
Beta-tubulin	Rabbit IgG	55	1:1000	Abcam (#ab6046)
Proliferating cell nuclear antigen (PCNA)	Rabbit IgG	30	1:1000	Santa Cruz Biotechnology Inc. (FL-261; sc-7907)

Table S3: Secondary antibodies used in western blot staining.

Antibody	Species	Dilution in TBST	Manufacturer
IRDye® GAR 800CW	Rabbit IgG	1:5000	LI-COR Biosciences (#926-32211)
IRDye® GAR 680RD	Rabbit IgG	1:20 000	LI-COR Biosciences (#926-68071)
IRDye® GAM 800CW	Mouse IgG	1:5000	LI-COR Biosciences (#926-32210)

7.2 Supplementary material for experiments performed in THP1 cells

This section includes material from the experimental repetitions done on THP1 cells described in section 3.1. Results from qPCR-analysis on HMPV-N, IFN-β and TNF-α mRNA are shown in Figure S1, while western blot analysis from two independent experiments are shown in Figure S2 and S3. Figure S4 shows quantification of SLAMF1 mRNA expression.

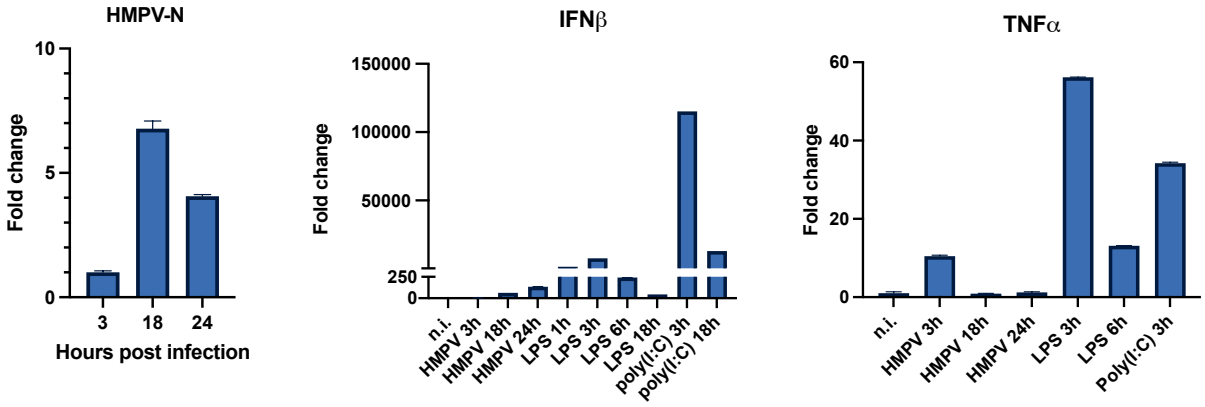


Figure S1: Quantification of HMPV-N, IFN-β and TNF-α mRNA expression by RT-qPCR in THP1 cells infected with HMPV (m.o.i = 1) or stimulated with LPS/poly(I:C) for the indicated time points. The bars represent three replicates per sample where their average C_T values have been normalized against the 1h-sample as well as the reference gene, TBP. The results are presented as means with SD.

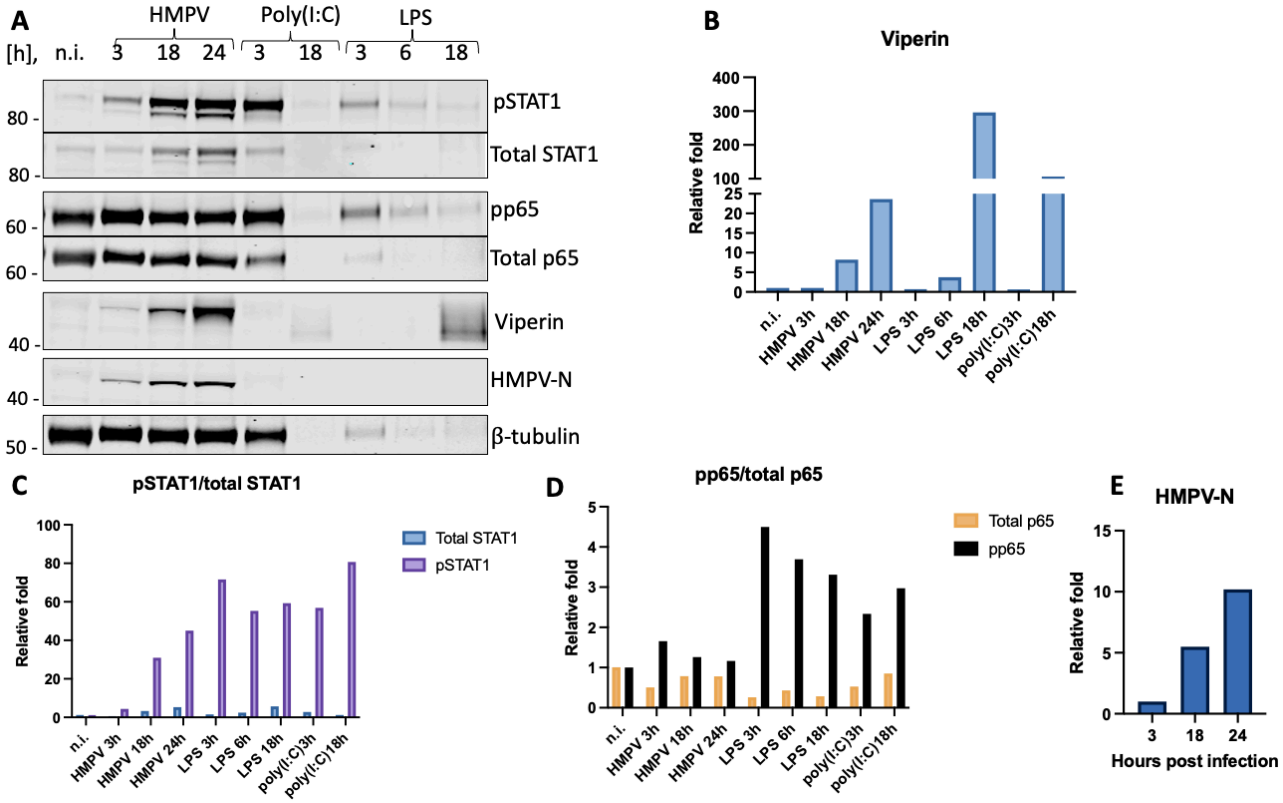


Figure S2: HMPV infection in THP1 cells induces phosphorylation of STAT1 and production of viperin. Western blotting of THP1 cells infected with HMPV (m.o.i. =1) or stimulated with LPS (200 ng/ml)

or poly(I:C) (10 µg/ml) for the indicated time points (A). The antibodies used are indicated in the figure. β-tubulin was used as a loading control. Molecular weight is given in kilodaltons. Graphs show quantification of viperin (B), pSTAT1/total STAT1 (C), pp65/total p65 (D) and viral protein (E) levels relative to β-tubulin obtained with ImageStudio software.

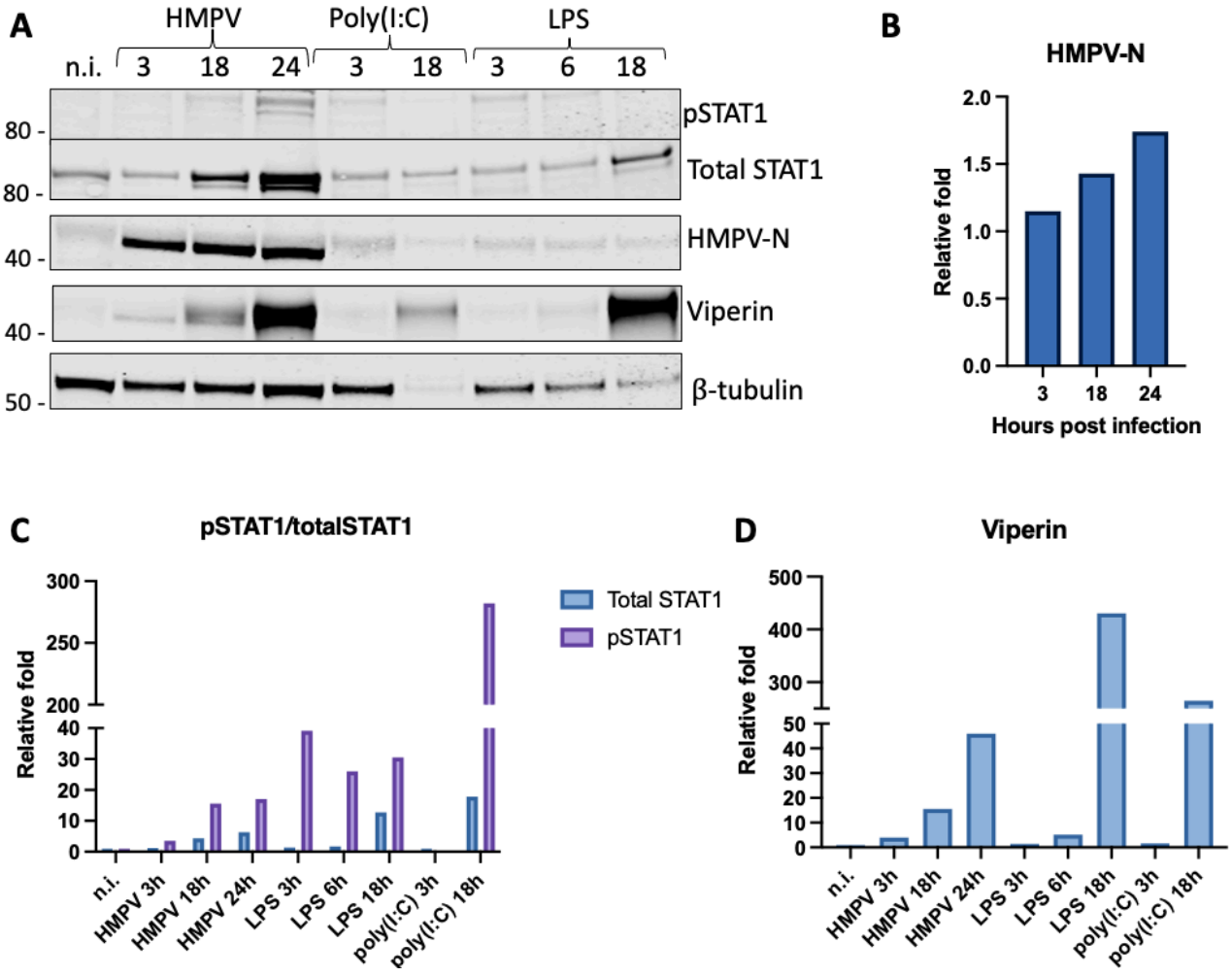


Figure S3: **HMPV infection in THP1 cells induces phosphorylation of STAT1 and production of viperin.** Western blotting of THP1 cells infected with HMPV (m.o.i. =1) or stimulated with LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points (A). The antibodies used are indicated in the figure. β-tubulin was used as a loading control. Molecular weight is given in kilodaltons. Graphs show quantification of viral protein (B), pSTAT1/total STAT1 (C) and viperin (D) levels relative to β-tubulin obtained with ImageStudio software. This membrane was also blotted against pp65 but did not show any signal.

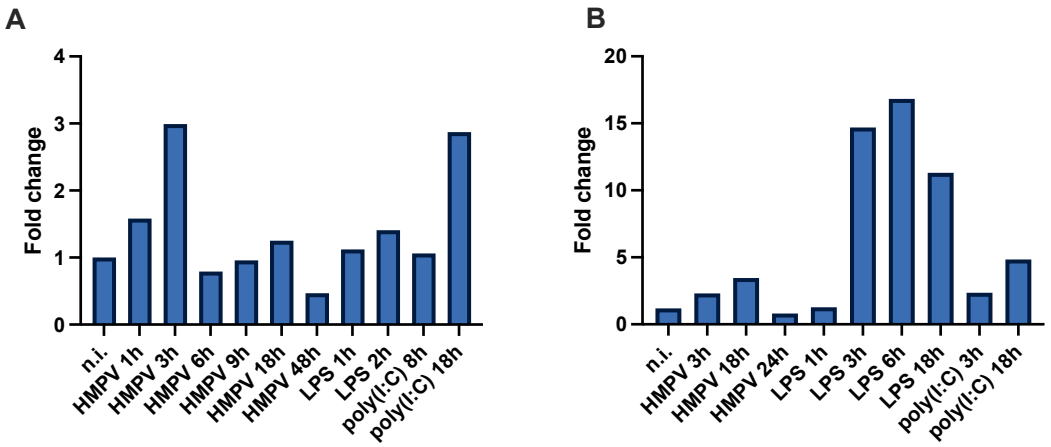


Figure S4: SLAMF1 is weakly induced by HMPV in THP1 cells. Quantification of SLAMF1 mRNA expression in two independent experiments (A, B) by RT-qPCR in THP1 cells infected with HMPV (m.o.i = 1) or stimulated with LPS (200 ng/ml) or poly(I:C) (10 µg/ml) for the indicated time points. The bars represent three technical replicates per sample where their average C_T values have been normalized against the noninfected-sample (n.i.) as well as the reference gene, TBP. The results are presented as means with SD.

7.3 Supplementary material from silencing *SLAMF1* and *TLR4* in MDMs

This section includes the western blot analysis of pSTAT1/total STAT1 and HMPV-N protein levels in MDMs from section 3.4 where *TLR4* and *SLAMF1* has been silenced (Fig. S5).

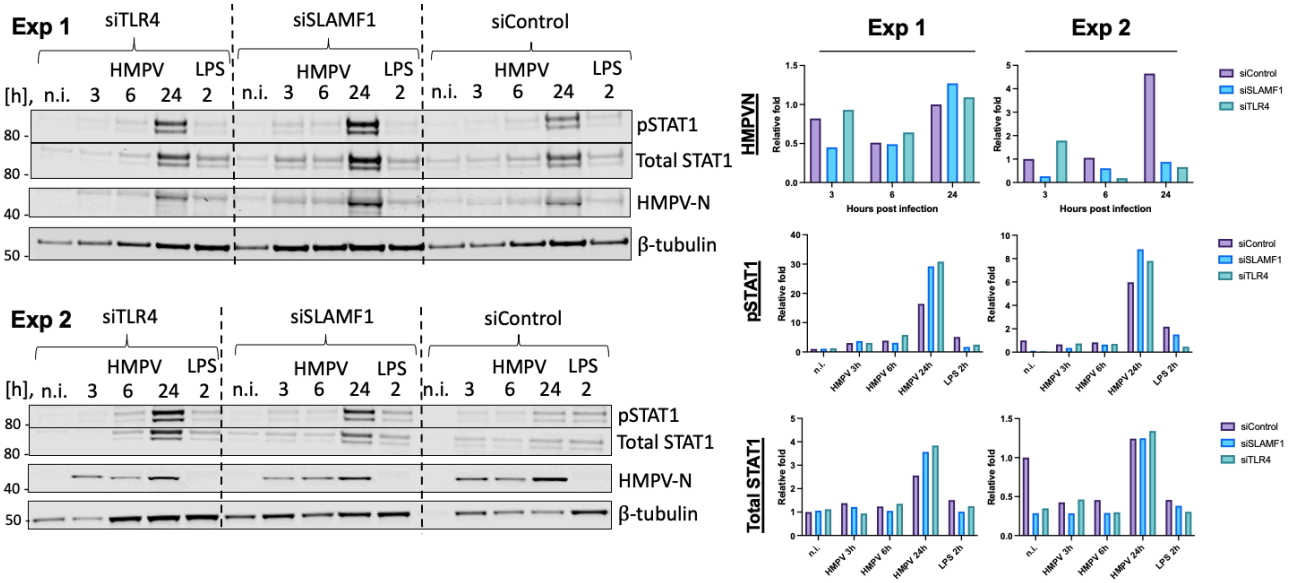
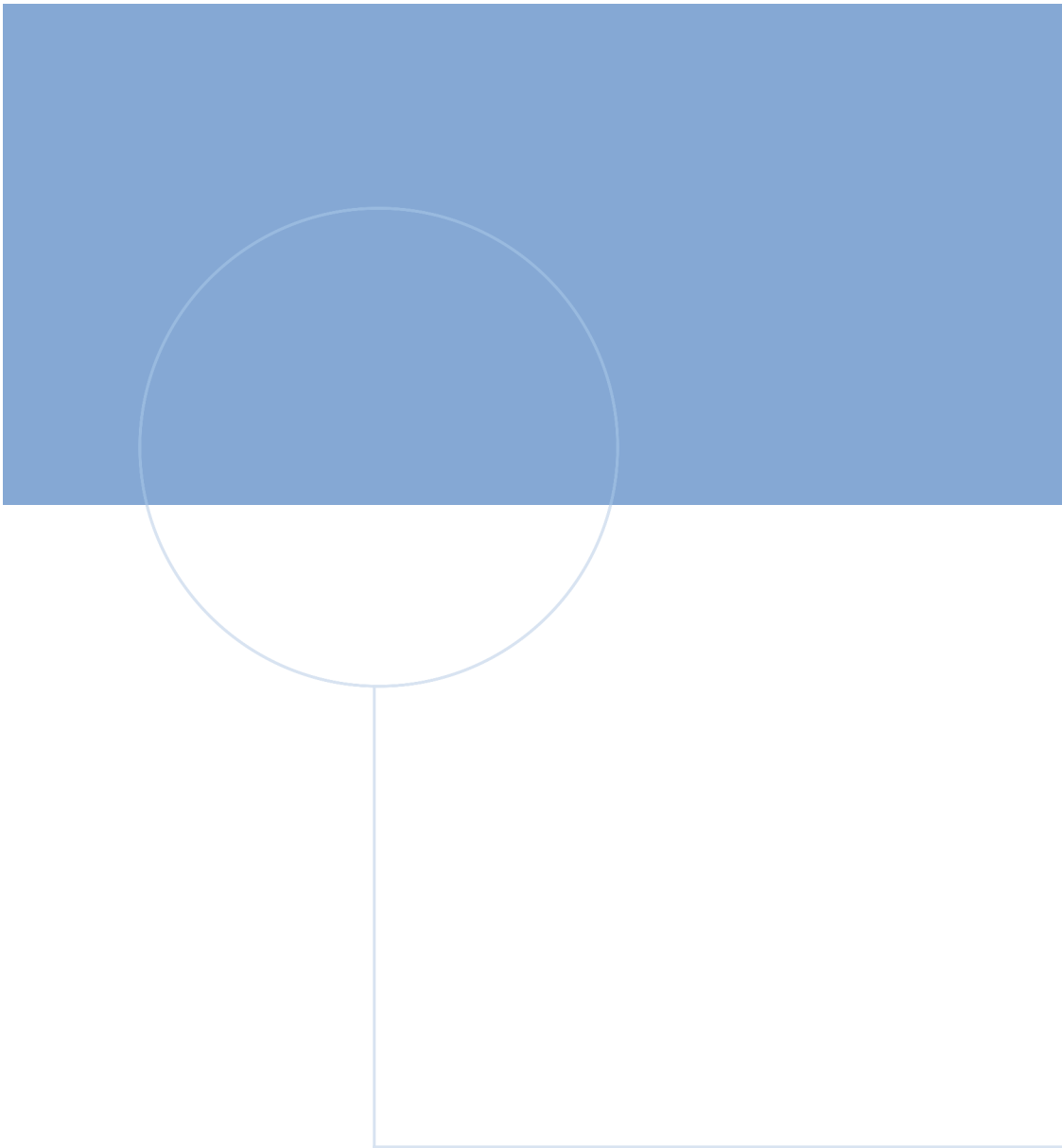


Figure S5: The effects of *SLAMF1*- and *TLR4*-silencing in MDMs on protein expression varied between donors. Western blotting of MDMs cells treated with a control non-silencing oligonucleotide or *SLAMF1*-/*TLR4*-specific siRNA oligonucleotides and infected with HMPV (m.o.i = 1) or LPS (200 ng/ml) for the indicated time points. The antibodies used are indicated in the figure and β-tubulin was used as a loading control. Molecular weight is given in kilodaltons. Graphs to the right show the quantitative analysis of the protein levels from the blots to the left. Quantification of viral protein, pSTAT1 and total STAT1 levels relative to β-tubulin obtained with ImageStudio software.



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