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A BATTLE BETWEEN HOST AND PATHOGEN: THE INNATE IMMUNE RESPONSE AND ENTEROVIRUS EVASION STRATEGIES

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Stockholm 2014

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Till min familj

ABSTRACT

Infections with Coxsackieviruses are common and in most cases the infection is asymptomatic and efficiently cleared. On rare occasions, however, an infection can lead to severe diseases including myocarditis, hepatitis and pancreatitis. Infections with Coxsackieviruses have also been implicated in the pathogenesis of type 1 diabetes (T1D). Upon a virus infection, the innate immune response plays an important role in restricting viral replication and further dissemination to susceptible organs. An inability to mount an appropriate immune response may increase virus-induced damage. This thesis focuses on the cross-talk between host and Coxsackievirus. The aims were to identify mechanisms by which the host restricts infection and to unravel strategies used by the virus to evade the host immune response.

We identified that the intracellular virus receptor melanoma differentiation associated factor 5 (MDA5) has an important role in the host response to a Coxsackievirus infection. Absence of this receptor led to a decreased ability to control viral replication, which resulted in severe tissue damage and increased mortality. Polymorphisms in interferon induced with helicase C domain (IFIH1), the gene encoding MDA5, regulate the risk for T1D development, further implicating the involvement of Coxsackieviruses in T1D pathogenesis. Studies in this thesis showed that the Ala946Thr polymorphism in IFIH1 regulates how human pancreatic islets respond to a Coxsackievirus infection. The predisposing allele 946Thr was associated with lower induction of interferons (IFNs) and IFN-inducible genes. It has previously been shown that type I IFNs play an important role in the host defense against Coxsackievirus. Type III IFNs are a recently described group of IFNs that mainly act on epithelial cells and provides protection against virus infection. This thesis established a novel role for the type III IFNs in inducing an antiviral state in infected human pancreatic islets. Moreover, it demonstrated that type III IFNs protect primary human cells, inlcuding pancreatic islets and hepatocytes, from a Coxsackievirus infection. Due to the potent antiviral effect of IFNs, most viruses have developed mechanisms to inhibit their actions. In this thesis, strategies utilized by Coxsackieviruses to inhibit the induction of type III IFN were identified, which further underlines the importance of this group of IFNs in controlling a Coxsackievirus infection.

The findings presented in this thesis further our understanding of how the host recognizes and combats a Coxsackievirus infection, and also describe evasion strategies used by the virus to inhibit these protective mechanisms. The studies also demonstrate that a polymorphism in IFIH1 affects the ability of human pancreatic islets to respond to a Coxsackievirus infection. A better understanding of the host-pathogen interactions may help in the development of therapeutic strategies to reduce the severity of Coxsackievirus infections.

LIST OF SCIENTIFIC PAPERS

I. Michael Hühn, Stephen A. McCartney, **Katharina Lind**, Emma Svedin, Marco Colonna, Malin Flodström-Tullberg.
Melanoma differentiation-associated protein-5 (MDA-5) limits early viral replication but is not essential for the induction of type 1 interferons after Coxsackievirus infection
Virology, 2010, 401, 42-48

II. **Katharina Lind**, Erna Domsgen, Michael Hühn, Olle Korsgren, Malin Flodström-Tullberg
Evaluation of the effect of the type 1 diabetes associated common polymorphism in IFIH1 on the pancreatic islet response to Coxsackievirus
Manuscript

III. **Katharina Lind**, Sarah J Richardson, Pia Leete, Noel G Morgan, Malin Flodström-Tullberg
Induction of an antiviral state and attenuated Coxsackievirus replication in type III interferon-treated primary human pancreatic islets
Journal of Virology, 2013, 87, 7646-7654

IV. **Katharina Lind**, Emma Svedin, Renata Utorova, Virginia M Stone, Malin Flodström-Tullberg
Type III Interferons are Expressed by Coxsackievirus Infected Human Primary Hepatocytes and Regulate Hepatocyte Permissiveness to Infection
Clinical and Experimental Immunology, 2014, 117, 687-695

V. **Katharina Lind**, Emma Svedin, Erna Domsgen, Markus Moll, Malin Flodström-Tullberg
Coxsackievirus counters the host innate immune response by blocking type III interferon expression
Manuscript

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LIST OF ABBREVIATIONS

Ala	Alanine
AP-1	Activator protein-1
CAR	Coxsackie and adenovirus receptor
CARD	Caspase recruitment domain
CVB	Coxsackievirus group B
DAF	Decay accelerating factor
DC	Dendritic cell
ds	Double stranded
eIF4G	Eukaryotic initiation factor 4G
ER	Endoplasmatic reticulum
IFIH1	Interferon induced with helicase C domain
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNλR	Interferon lambda receptor
iNOS	Inducible nitric oxide synthase
IRES	Intracellular replication entry sites
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
JAK1	Janus kinase 1
LGP2	Laboratory of genetics and physiology 2
MDA5	Melanoma differentiation associated factor 5
MEF	Mouse embryonic fibroblast
Mx	Myxovirus resistance protein
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa light chain enhancer of activated B-cells
NK	Natural killer
OAS	Oligoadenylate synthetase
PABP	Poly A binding protein
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell

PKR	Protein kinase R
Poly I:C	Polyinosinic-polycytidylic acid
PP	Pancreatic poly peptide
PRR	Pathogen recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I like receptor
SI	Stimulation index
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducers and activators of transcription
T1D	Type 1 diabetes
Thr	Threonine
TLR	Toll like receptor
TYK	Tyrosine kinase
VP1	Viral protein 1

1 INTRODUCTION

To strive for survival all living organisms have developed mechanisms to defend themselves from predators. The constant pressure from microorganisms has lead to the development of protective mechanisms, collectively known as the immune system. The immune system is typically divided in two parts - the innate immune system which can be found in most organisms and reacts in a non-specific manner, and the adaptive immune system that is only found in vertebrates and provides long lasting protection against microbes. In parallel with the evolution of the immune system, invading microorganisms have also been under selective pressure and have therefore developed ways to evade the immune response. In this thesis, I have studied the interactions between the innate immune system and an infective pathogen namely enterovirus. The aim has been to further our understanding of how the host's innate immune system recognizes enteroviruses and combats the virus infections. In addition, the aim was to decipher evasion strategies used by enteroviruses to circumvent the innate immune responses. A brief introduction to the field is provided to further the understanding of the concepts presented in the five papers (**Paper I-V**) that this thesis is based on.

1.1 THE INNATE IMMUNE RESPONSE

The innate immune system is the first line of defense against invading pathogens. Responses are immediate, within hours, and important for both limitation of viral spread and the orchestration of the adaptive immune response that follows. Several different arms combine to comprise the innate immune system, including physical barriers such as the skin and mucosal surfaces, cellular components like natural killer (NK) cells and phagocytic cells and also inflammatory molecules such as complement and interferons (IFNs) [1]. To enable such an immediate response, the innate immune system responds in a non-specific manner. Induction of the innate immune response relies on the sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). Activation of PRRs results in the production of cytokines and chemokines. Most cells express PRRs and can produce IFNs, which act in both an autocrine and a paracrine manner [2, 3].

1.1.1 Recognition of a viral infection

Recognition of the invading pathogen is imperative to mount a proper immune response. It is therefore not surprising that the immune system has evolved such a diverse array of receptors for efficient pathogen sensing. Two receptor families serve as the main sensors of viral infection, namely the toll like receptors (TLRs) and RIG-I like receptors (RLRs) [4].

Receptor	Localization	Ligands
TLR1	Cell surface	Triacyl lipopeptides
TLR2	Cell surface	Peptidoglycans, hemaglutinin
TLR3	Endosome	ssRNA virus, dsRNA virus
TLR4	Cell surface	Lipopolysaccharide
TLR5	Cell surface	Flagellin
TLR6	Cell surface	Diacyl lipopeptides
TLR7	Endolysosome	ssRNA virus
TLR8	Endolysosome	ssRNA from virus
TLR9	Endolysosome	dsRNA virus
TLR10	?	?
RIG-I	Cytoplasm	ssRNA virus, short poly I:C, short dsRNA with 5’ triphosphate end
MDA5	Cytoplasm	Positive-sense ssRNA virus, long poly I:C

Table 1. TLRs and RLRs, cellular localization and ligands. Adapted from [5-7].

1.1.1.1 Endosomal and extracellular recognition

TLRs are evolutionary conserved and respond to an array of microbial products (Table 1). They are expressed on the cellular surface as well as in intracellular vesicles of both immune- and non-immune cells. Activation of TLRs leads to the secretion of inflammatory cytokines, IFNs and anti-microbial peptides [8, 9]. Recognition by TLRs also leads to maturation of dendritic cells (DCs), which have the ability to initiate and modulate the adaptive immune response [10]. In humans, 10 TLRs have been identified. The functions of TLRs 1-9 have been well characterized, whereas the role of TLR10 remains unclear. A subset of TLRs namely, TLR3, TLR7, TLR8 and TLR9 has been demonstrated to have an involvement in the sensing of viral infections [5, 11]. TLR3 is activated by double stranded (ds)RNA, which is either expressed by viruses carrying a dsRNA genome or produced as an intermediate during replication [12]. It has been shown that viruses including Influenza A virus and West Nile virus are recognized by TLR3 [13, 14]. TLR3 can also serve as sensor for polyinosinic-polycytidylic acid (poly I:C), a synthetic viral mimic [15]. In addition, TLR2 and TLR4 have been implicated in recognition viruses including Cytomegalovirus and Coxsackeivirus [16, 17].

Stimulation of TLRs leads to the induction of IFNs via activation of the transcription factor IFN regulatory transcription factor 3 (IRF3) and nuclear factor kappa light chain enhancer of activated B-cells (NF-κB). According to the use of adaptor protein, TLRs can be divided into two groups. All TLRs, with the exception of TLR3, induce IFN-expression via myeloid differentiation factor 88 (MyD88). Whereas, TLR3 and TLR4 signals via TIR-domain-containing adapter-inducing IFNβ (TRIF) [5, 11].

1.1.1.2 Cytoplasmic recognition

While the TLRs survey the extracellular and the endosomal compartments in the cell the cytoplasm is patrolled by RNA helicases. The RLRs contains three members, retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [18]. RIG-I and MDA5 contain a DExD/H-box helicase domain essential for dsRNA binding and two N-terminal caspase recruitment domains (CARDs) that are important for downstream signaling (Figure 3) [19, 20]. Binding of viral RNA by RIG-I and MDA5 leads to conformational changes in the proteins, which leads to exposure of CARDs and interaction with the adaptor protein interferon-β promoter stimulator 1 (IPS1, also knows as MAVS, CARDIF and VISA) [21-24].

RLRs are important sensors of various RNA viruses, however they have different preferences in the RNA patterns that they bind. RIG-I recognizes RNA bearing a 5' triphosphate end [25-28], as well as short dsRNA stretches [29]. MDA5 on the other hand, cooperatively senses long dsRNA sequences and poly I:C [29, 30]. Because of the different ligand specificities these two receptors recognize different viruses. A wide variety of viruses including members of the paramyxoviridae-, orthomyxoviridae- and rhabdoviridae-family are sensed by RIG-I [18, 25, 30, 31], whereas MDA5 recognizes viruses belonging to the *Picornaviridae* family, such as encephalomyocarditis virus (EMCV) and Coxsackievirus [30, 32-34]. In addition, both RIG-I and MDA5 are required for the detection of some viruses such as West Nile virus and Dengue virus [31].

To date, the role of LGP2 during virus infections is not clear. LGP2 can bind RNA, [18], but it lacks the CARDs that are important for the interaction with downstream molecules and therefore, cannot induce IFNs in a similar manner as RIG-I and MDA5. Initial studies suggested that LGP2 functioned as a negative regulator of RIG-I- and MDA5-signaling [18, 35]. However, more recent studies however, have indicated that LGP2 rather acts as a positive regulator [36, 37]. Further studies are required to elucidate the role of LGP2 in antiviral defense.

1.1.2 Interferons

IFNs were discovered by Isaacs and Lindenmann in 1957 as proteins that, as the name implies, *interfere* with viral replication [38]. Since then, these macromolecules have been extensively studied and besides their inhibitory effects on viral replication, their range of functions also encompasses both anti-proliferative and immunomodulatory properties [39, 40]. IFNs are typically classified into in three groups based on their sequence homology and receptor specificity; type I IFNs, type II IFNs and the type III IFNs. The type I IFNs comprise multiple IFNα subtypes and various single isoforms of IFNβ, -ε, κ, -δ, -ω, -τ and –ζ. IFNα and β have been widely recognized for their importance in combating virus infections, whereas the role of the other type I IFN members is less well known [41, 42]. Type II IFN consists of only one member, IFNγ. IFNγ is mainly produced by immune cells such as activated NK cells and T cells and its main effector function is to modulate of the immune response [43]. The type III IFNs are the most recently added group and consist of IFNλ1 (IL-29), IFNλ2 (IL-28a), IFNλ3 (IL-28b) [44, 45]. A fourth member, IFNλ4, has been added to the family however the expression of this cytokine is only found in a fraction of the population [46]. The type III IFNs have a greater structural similarity to the cytokines that belong to the IL-10-family, however their function and antiviral properties resemble those of IFNα and IFNβ [2, 47].

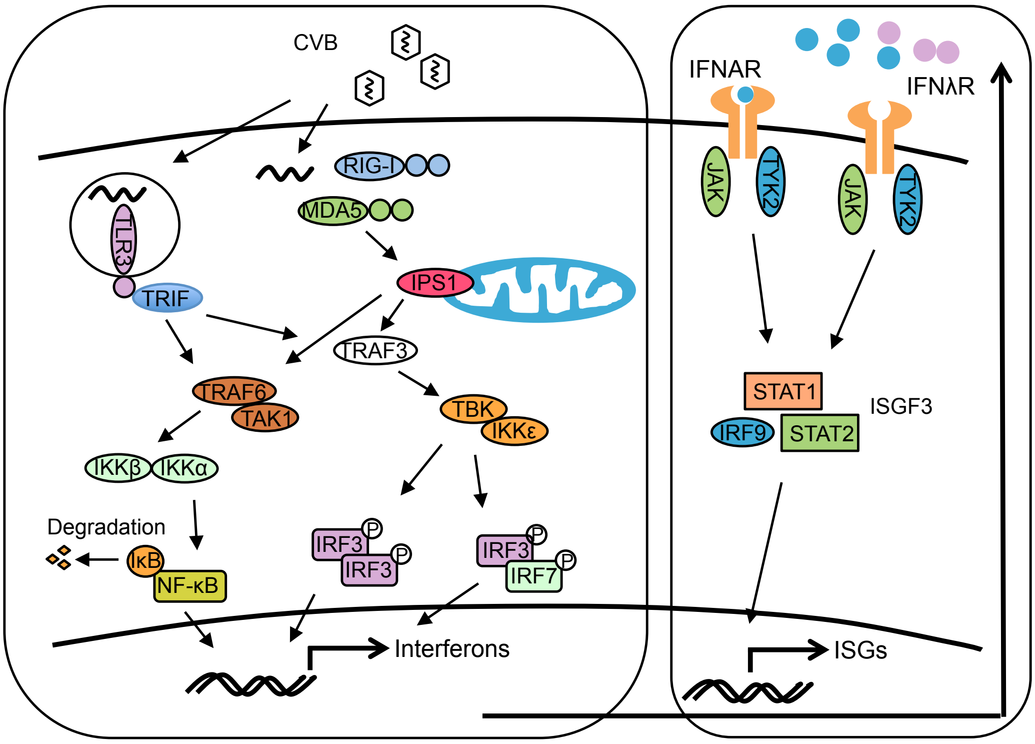

Figure 1. Model for cellular sensing of Coxsackievirus and the induction of IFNs.

1.1.2.1 Induction of type I and III interferons

Virtually all cells have the ability to express IFNs, although the magnitude and timing of expression is dependent on cell type and stimulus [48]. During a viral infection, plasmacytoid DCs (pDCs) are generally considered the major IFN-producing cell and they can express both type I and type III IFNs [48, 49]. In the absence of viral infection or any other pathogen the expression of the IFNs is negligible. However after their induction, the expression is tightly regulated through negative feedback loops to limit tissue damage [50, 51].

Expression of type I and type III IFNs is induced after recognition of pathogens by PRRs, as described in section 1.1.1. The subsequent signaling leads to the activation of transcription factors including IRF3, IRF7, NF-κB and activator protein -1 (AP-1), which bind positive regulatory domains (PRDs) in the promotor region of IFN-genes [52]. After binding to the PRDs, the transcription factors assemble into a complex known as the enhancesome that regulates the transcription of IFNs [52]. Expression of IFNβ requires promotor binding of NF-κB, ATF-2/c-Jun, and IRF3 (or IRF7) while the expression of IFNα is dependent on IRF7. In parenchymal cells, all of these transcription factors are constitutively expressed except IRF7, which is induced in a positive feed back loop by IFNs. Due to its dependency on IRF7, the expression of IFNα upon a virus infection is delayed. This is in contrast to the expression of IFNβ, which is immediate upon a viral infection [53]. In DCs, however, IRF7 is constitutively expressed and they can rapidly express large amounts of IFNs [53, 54]

Regulation of type III IFN expression somewhat differs from that of type I IFNs. Initial studies found that IFNλ1 showed a similar regulation and expression pattern as IFNβ, while IFNλ2 and IFNλ3 were expressed with delayed kinetics and displayed a dependence on IRF7, similar to IFNα [55, 56]. More recent studies have found that IRF3 and/or NF-κB can separately bind to regions outside of the type III IFN promotors [57] and independently induce expression of the type III IFNs [57-60]. In addition, infection with respiratory syncytical virus preferentially induces type III IFNs over type I IFNs [61], further supporting the case that expression is differently regulated.

1.1.2.2 Interferon signaling and induction of an antiviral state

Upon release, IFNs bind to distinct cell surface receptors. Type I IFNs bind to the IFNα receptor (IFNAR), a ubiquitously expressed receptor composed of IFNAR1 and IFNAR2 subunits [3]. Type III IFNs exert their biological effect through the IFNλ-receptor (IFNλR), which consists of two subunits: IFNλR2 and IL-10R [44, 45]. In contrast to IFNAR, the IFNλR shows a restricted tissue distribution and is mainly expressed on specific immune cells and epithelial cells [62, 63]. Despite the use of different receptors, type I and type III IFNs induces the same signalling pathways and consequently induce similar types of biological activities [64-66] and **Paper III**. Binding of the IFNs to their respective receptor activates Janus activated kinases 1 (Jak1) and tyrosin kinase 2 (Tyk2). Activation of these kinases leads to the phosphorylation and activation of signal transducers and activators of transcription 1 (STAT1) and STAT2. STAT1/STAT2 dimers associate with IRF9 to form the IFN stimulated gene factor 3 (ISGF3) complex, which initiates the transcription of interferon stimulated genes (ISGs) [67].

IFNs display antiviral properties that can be attributed to at least two distinct effects. The immunomodulatory function of IFNs is important for inducing the adaptive immune system and helps to clear the virus infection [68]. In addition, IFNs can induce an antiviral state, which prevent the cells from becoming infected and is therefore an important event for controlling a virus infection. The antiviral state includes the upregulated expression of over hundreds of genes [69], however, the function of many of the expressed proteins is not clear. One of these genes with a proven antiviral effect is 2´5 oligodenylate synthetase (OAS). Upon binding to dsRNA, OAS activates RNaseL, which leads to degradation of both viral and cellular RNA resulting in inhibition of viral replication [70]. OAS/RNaseL is important in the antiviral defense against Coxsackievirus as mice lacking the expression of RNase L fails to restrain virus replication and succumb early to the infection [71]. OAS/RNaseL has also been shown to have a protective effect upon infection with West Nile virus [72]. The family of Myxovirus resistance protein (Mx), consisting of MxA and MxB, has also been demonstrated to have important antiviral properties [73, 74]. Studies have shown that MxA restricts influenza virus and La Crosse virus replication [75, 76]. Moreover, over-expression of MxA reduces Coxsackievirus replication [77]. One of the earliest expressed ISGs is IRF7, which act as transcription factor potentiating IFN signaling [53]. IRF7 is also important for the induction of IFNα as described in section *1.1.2.1*. In addition to these ISGs, PRRs such as RIG-I and MDA5 are also induced in response to IFN [66, 78] and **Paper III**.

1.1.2.3 Antiviral activity of type III interferons

The type III IFNs are to a large extend similar to the type I IFNs. As discussed above, the signaling cascades induced by type I IFNs and type III IFNs are the same. Furthermore, type I IFNs and type III IFNs are often co-expressed and the protective effect is to a large extent overlapping [44, 45, 79]. Studies have shown that type III IFNs have been shown to protect against a wide array of viruses including encephalomyocarditis virus (EMCV) and vesicular stomatitis virus [44, 45]. Moreover, type III IFNs block infection with hepatitis B virus, hepatitis C virus and influenza A virus [62, 80-83].

The most striking difference between type I IFNs and type III IFNs is the selective expression of the IFNλR on epithelial cells, which suggests that the type III IFNs are important for viral defense at the mucosal surface [62, 63]. Indeed, several studies have shown that type III IFNs protect epithelial cells originating in the intestine, lung and vagina from virus infection [82, 84-86]. Interestingly, type III IFNs play a non-redundant role in protecting the epithelial cells in the small intestine from Rotavirus infection [85].

In humans, type III IFNs also play a role in protecting hepatocytes against virus infection. Hepatocytes are epithelial like cells and respond well to type III IFNs [80, 86-88] and **Paper IV**. Pretreatment with type III IFNs protect hepatic cell lines and hepatocytes against HCV [83, 88] and Coxsackievirus infection [86] and **Paper IV**. Moreover, the type III IFNs are currently being clinically tested as a treatment against HCV infection [89, 90].

1.2 ENTEROVIRUS

Enteroviruses belong to the *Picornaviridae* family and they are currently divided into 12 species named Enterovirus A-H and -J, as well as Rhinovirus A-C [91]. All enteroviruses are non-enveloped single-stranded RNA viruses that contain a small genome of approximately 7500 bases [92].

1.2.1 Coxsackievirus group B

The first Coxsackievirus were isolated from two paralyzed children more than 60 years ago in the town of Coxsackie [93]. Later, several serotypes were identified and the Coxsackieviruses were divided into groups A and B based on pathogenicity [94]. Nowadays, Coxsackievirus group B (CVB) consists of six serotypes (CVB1-6) that belong to the enterovirus B genus [91]. Infections with CVBs are commonly encountered, especially during the neonatal period and childhood. The primary mode of infection is via the fecal oral route and to a lesser extent, via the respiratory route. Most infections with CVB are asymptomatic or associated with mild flu-like symptoms, however they can on rare occasions disseminate to other organs and cause severe conditions such as myocarditis, meningitis and hepatitis [92]. In addition, infections with CVB have also been associated with the development of type 1 diabetes (T1D) [95].

1.2.2 Coxsackievirus lifecycle

The main receptor required for cellular entry of the CVBs is the coxsackie and adenovirus receptor (CAR) [96, 97]. However, decay accelerating factor (DAF) has been proposed as attachment receptor for some serotypes [98-100]. After entry, the virus is uncoated and the positive sense RNA is immediately translated. Translation of cellular RNA involves the recognition of a 5'cap, however the Coxsackievirus genome lacks this structure. Instead, a viral protein known as VpG is attached to the 5'end, which has been shown to be important for initiation of RNA-synthesis. Translation of the viral RNA therefore occurs in a cap-independent manner via intracellular replication entry sites (IRES). Upon translation, the genome is made into a single polyprotein, which is cleaved into structural proteins (viral protein (VP), 1-4) and non-structural proteins (2A-C, 3A-D) by the virally encoded proteases 2A and 3C (Figure 2). The structural proteins make up the viral capsid whereas the non-structural proteins are mainly involved in synthesizing new viral RNA [101]. In addition, the non-structural proteins can also interfere with cellular processes and are known for shutting off cellular translation as well as inhibiting the induction of an immune response [102-106] and **Paper V**.

Viral replication takes place in the cytoplasm on the outer surface of membraneous vesicles [101]. The first step in viral replication involves the synthesis of a negative complementary strand, which serves as a template for the production of positive RNA-strands. After assembly of new viral particles, the virus is released from the cell through cellular lysis, however non-lytic mechanisms have been suggested [107, 108].

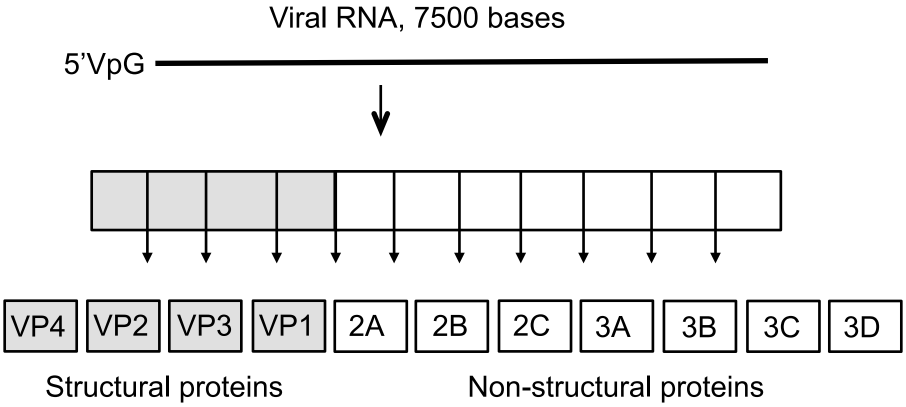

Figure 2. Coxsackievirus genome and polyprotein. Adapted from [109].

1.3 HOST-ENTEROVIRUS INTERACTIONS

1.3.1 Detection of Coxsackievirus and the role of interferons

Both TLRs and RLRs have been shown to play a role in recognizing CVB. Among the TLRs, TLR3 seem to play the most essential role as demonstrated that mice who lack TLR3 expression show a heightened mortality after infection with CVB compared to wild type mice [110, 111]. Abston *et al* have also demonstrated that expression of TLR3 is protective against CVB-induced myocarditis [112]. In addition, the expression of TRIF (the adaptor protein for TLR3 signaling, Figure 1) is important in controlling CVB3 replication in the heart [113]. Further supporting an important role for TLR3 is the finding that two SNPs in TLR3 are associated with the development of enterovirus-induced myocarditis [114]. Additional TLRs with a suggested involvement in Coxsackievirus recognition are TLR4, TLR7 and TLR8. TLR4 has been proposed to sense CVB in human pancreatic cell lines [17], whereas TLR7 and TLR8 have been implicated in CVB recognition in pDCs and cardiac cells respectively [115, 116].

RLRs are also involved in the sensing of Coxsackievirus. Since Coxsackieviruses do not carry a 5' triphosphate (the ligand for RIG-I) the focus has mainly been on the role of MDA5. Early studies showing that the sensing of EMCV (belongs to the family of *Picornaviridae*) was dependent on MDA5 suggested that Coxsackieviruses may also be recognized by this receptor [30]. This hypothesis was further supported by a study performed by Wang *et al* and our results presented in **Paper I** [33, 34]. Both studies showed that in the absence of MDA5, mice have an increased mortality upon CVB infection. Further supporting a role for MDA5 in sensing CVB, is the finding that mouse embryonic fibroblasts (MEFs) lacking MDA5 or IPS1 (the adaptor protein used by MDA5, Figure 1) are unable to produce IFNβ in response to transfection with CVB [32]. Of additional interest to the studies performed in this thesis is the finding that several SNPs in IFIH1 have been associated with the development of T1D, which is further described in section *1.4.1.3* of this thesis.

An intact innate immune response is critical for restricting viral replication and promoting host survival after a Coxsackievirus infection. The protective role of the type I IFNs has been demonstrated *in vivo* using mice that are unable to respond to type I IFNs [117, 118] or are deficient in IFNβ [119]. These mice showed an increased susceptibility to Coxsackievirus infection and the severity of disease was much higher compared to wild type animals. *In vitro* studies also support a protective role for type I IFNs in Coxsackievirus infections [78, 120]. The protective effect of IFNs is suggested to be mediated largely by proteins with antiviral activity such as protein kinase-R (PKR), RNaseL and inducible nitric oxid synthase (iNOS). Indeed, mice lacking these genes succumb to an infection with Coxsackievirus [71, 121, 122]. In addition to the type I IFNs, type III IFNs also induce the expression of antiviral proteins and can mediate protection against virus infections [44, 66, 86, 123]. In **Papers III and IV**, we describe a novel finding that type III IFNs also has a protective effect during Coxsackievirus infections.

1.3.2 Coxsackievirus evasion strategies to evade the innate immune response

Coxsackieviruses have developed multiple mechanisms to counteract the innate immune response. By targeting several pathways, the virus can ensure that detection and the subsequent induction of IFNs is avoided or limited. Some of these alterations do not just affect the production of IFNs, but have a more global effect on the host's cellular machinery. The Coxsackievirus proteases 2A and 3C have been shown to cleave eukaryotic initiation factor 4G (eIF4G) and poly A-binding protein (PABP) [103-105], two proteins important for translation initiation [124]. In addition, it has been demonstrated that the non-structural proteins 2B, 2BC and 3A interfere with endoplasmatic reticulum (ER) to Golgi transport leading to inhibition in protein secretion [125, 126]. Coxsackieviruses also restrict the induction of IFNs by targeting events that occur before translation. By targeting MDA5 and IPS1, the virus inhibits IFN-induction via the RLR pathway [102, 106] and **Paper V**. In a similar manner, induction of type I IFNs via TLR3 is blocked by reducing the expression of TRIF [106] and **Paper V**. Coxsackieviruses have also been found to interfere with the activation of the transcription factor NF-κB [127, 128], although it remains to be established whether this affects the induction of IFNs.

The fact that Coxsackieviruses have developed multiple evasion strategies to interfere with the production of type I IFNs emphasizes the importance of this group of cytokines in combating Coxsackievirus infection. Our findings in **Paper III and IV** show that type III IFNs are also important in suppressing Coxsackievirus replication [66, 86], however whether the type III IFNs were also inhibited during infection with Coxsackievirus has not been investigated and was the focus of **Paper V**.

1.4 TYPE 1 DIABETES

T1D is a chronic disease characterized by a loss of functional insulin producing beta cells resulting in an inability to correctly regulate blood glucose levels. There is currently no cure for the disease and it is only controlled by life long administration of exogenous insulin. Manifestation of disease usually occurs during childhood or adolescence but can also develop in adults [129]. The incidence varies around the world ranging from 0.1 cases in 100 000 individuals in low incidence countries such as Venezuela and up to 60 cases per 100 000 individuals in countries with a high incidence including Finland and Sweden [130-133]. It is particularly important to note that there has been a rapid increase in the incidence during the past decades, especially among the young [131, 133]. T1D is a multifactorial disease resulting from a complex network of genetic and environmental factors [134]. It is considered to be an autoimmune disease based on the presence of predisposing HLA class II haplotype genes in the majority of cases together with the presence of autoreactive B- and T-cells [129]. However, how immunological tolerance to beta cell is broken and how beta cells are destroyed remains elusive.

1.4.1.1 The islets of Langerhans

The pancreas is composed of exocrine and endocrine cells, which have two main functions, namely the digestion of ingested food (exocrine) and the regulation of glucose metabolism (endocrine). The exocrine cells produce digestive enzymes and make up the major volume of the organ. The endocrine cells produce hormones and are gathered in small clusters called the islets of Langerhans, which are scattered throughout the exocrine tissue. In total, the islets make up 1-2% of the total pancreas mass, each containing around 1000 cells. The islets mainly consist of five different cell types: alpha cells, beta cells, delta cells, epsilon cells and pancreatic polypeptide (PP) cells. Beta cells are the most abundant cell type and respond to elevated glucose concentrations through the release of insulin. Alpha cells are responsible for producing glucagon, which as oppose to insulin increase the glucose concentrations in the blood. Delta cells and epsilon cells produce somatostatin and ghrelin respectively and have regulatory functions. PP cells produce pancreatic polypeptide [135].

1.4.1.2 Genetic factors

Susceptibility to T1D is largely inherited and more than 50 genes have been linked to the development of the disease [134]. A common factor among many of these genes is that they play a role in modulating the immune response in one way or another and also, many of them are expressed by the beta cells themselves. The predominant genetic risk factors are the HLA class II genes [136]. However, genome wide association studies have, during the past decade, identified several additional genes, which contribute to disease susceptibility [134]. Presence of a susceptibility gene does not automatically lead to the development of T1D, however having multiple predisposing genes can increase risk [137].

IFIH1

The function of MDA5 as a viral sensor is important for the induction of IFNs and is described in section *1.1.1.2*. Interestingly, several polymorphisms in the gene encoding IFIH1 have been associated with T1D [138, 139]. These findings have been confirmed in several other populations [140-144], however are not valid in all [142, 145, 146]. Moreover, an association between polymorphisms in IFIH1 and other diseases with an autoimmune background such as systemic lupus erythematosus (SLE) [147, 148], psoriasis [149] and autoimmune thyroid disease [150] has also been found. The most common single nucleotide polymorphism (SNP) is the Ala946Thr (rs1990760). Among Europeans, threonine (Thr) is most common allele and the frequency in the British population is approximately 65% [138, 139, 141, 151]. Presence of Thr at codon 946 in IFIH1 is associated with an increased risk of developing T1D, whereas alanine (Ala), the ancestral amino acid is linked to protection against the disease [139]. In addition, two polymorphisms have been identified, Glu627X (rs3574460) and Ile923Val (rs35667974) which are associated with protection against T1D [151]. However the presence of the protective alleles among the population is rare, less than 3 %. Exactly how polymorphisms in IFIH1 alter the risk of developing T1D remains to be established in detail. Studies performed so far have suggested that the SNPs in IFIH1 alter expression levels and function of the protein [142, 151, 152]. How polymorphisms in IFIH1 affect the response to enterovirus is however, not yet known and we aim to address this question in **Paper II**.

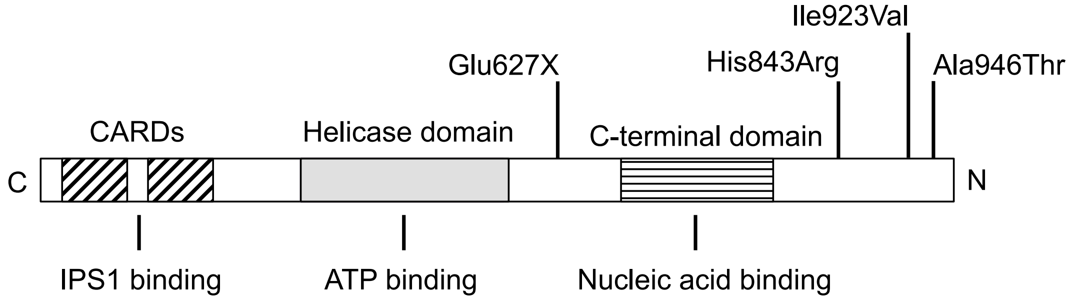

Figure 3. Structure of the IFIH1 gene, positions of polymorphisms associated with T1D are indicated. Adapted from [153]

1.4.1.3 Environmental factors -a role for enteroviruses?

The rapid increase in the number of T1D cases during recent years coupled with a relatively low concordance rate between monozygotic twins [154, 155] suggests that environmental factors also play a role in disease development. Several factors have been proposed such as dietary components [156], vitamin D status [157] and a lack of exposure to microbes (the "hygiene hypothesis") [158]. In addition, numerous studies have linked virus infections to the development of T1D and several viruses have been proposed, including rotavirus [159], Rubella virus [160] and enterovirus [161]. The strongest associations are found with the enteroviruses and especially with Coxsackieviruses [161-163]. This link is supported by several epidemiological studies and clinical observations [158, 164-167]. An association has also been found between the appearance of autoantibodies and enterovirus infections [165, 168]. Moreover, enterovirus has been found more often in the pancreas and intestine of patients with T1D compared with healthy control group [163, 167, 169-171]. *In vitro*, enteroviruses have a tropism for the pancreatic beta cells and can cause severe damage to the cells [172]. Although many studies point towards an association between enteroviruses and development of T1D, there are some studies that fail to find a relationship [173, 174].

Several mechanisms have been proposed to explain how an enterovirus infection would cause T1D, including direct infection of the beta cell, molecular mimicry and bystander activation [175]. A direct infection of beta cells is the mechanism that has been most extensively studied and is supported by the studies showing that enterovirus can replicate in islets *in vitro* [66, 78] and **Paper III,** which result in severe cellular damage [172]. Direct infection of beta cells has been proposed to be involved in the progression to fulminant diabetes. This subset of T1D is characterized by a rapid onset and a near complete loss of beta cells. In contrast to T1D, autoimmunity is believed to play a less prominent role in fulminant diabetes [176, 177]. Direct infection of beta cells can also lead to a persistent infection, which is supported by *in vitro* studies showing that Coxsackieviruses can persist in beta cells for several months [120].

A persistent infection can also lead to functional impairment of the beta cell such as suppression of insulin release [167, 171, 172].

Molecular mimicry has also been suggested. This theory proposes that similarities in the sequence between virus epitopes and beta cell antigens leads to a cross-reactive T cell response that eliminates not only virus but also beta cells [178]. Resemblance has been found between enterovirus protein 2C and host protein GAD65 that supports this theory [179, 180].

Bystander activation is yet another proposed mechanism. In this scenario, auto-reactive T cells specific for beta cell antigens are activated and initiate an autoimmune attack on the pancreatic beta cells. An infectious agent may be involved in this scenario. A persistent infection may lead to a low-grade inflammatory response thus creating a cytokine milieu, which attracts T cells resulting in beta cell destruction [178].

2 AIMS

The overall aim with this thesis was to gain a better understanding of the immune response against enterovirus infections. Moreover, the aim was to unravel the mechanisms used by the virus to evade the immune system.

Specific aims:

- To study the role of the intracellular receptor MDA5 in host survival and regulation of CVB replication (**Paper I**)
- To assess the human pancreatic islet response to CVB infection (**Paper II**)
- To study whether a polymorphism in IFIH1 dictates the antiviral response to CVB (**Paper II**)
- To determine if type III IFNs are expressed upon a CVB infection (**Paper III and IV**)
- To study if type III IFNs elicit a biological response and reduce CVB replication in human pancreatic islets (**Paper III**)
- To study whether type III IFNs regulates permissiveness to CVB infection in primary human hepatocytes (**Paper IV**)
- To study if CVB has evolved mechanisms to evade the type III IFN response (**Paper V)**

3 MATERIAL AND METHODS

3.1.1.1 Animals

In **Paper I**, mda5-/- and wild-type animals on two different backgrounds, C57BL/6 and 129/SvJ were used. The animals were bred and housed under specific pathogen free conditions at Washington University School of Medicine, St. Louis, USA. All animal experiments were conducted in accordance with the institutional guidelines for animal care and use and with appropriate ethical permission.

3.1.1.2 Primary cells

Pancreatic islets isolated from human organ donors were used in **Paper II and III**. The islets were isolated at Uppsala University within the Nordic Network for islet transplantation. Primary human hepatocytes were isolated at Karolinska University Hospital and used in **Paper IV**. Informed consent was obtained from all donors or their relatives. A benefit of using primary cells isolated from human donors is that they display a diverse genetic variation and may provide a better representation of the population compared to cell lines and animal models. However, there are also limitations associated with the use of primary cells. Due to the varying genetic backgrounds and characteristics of the donors (such as age and previous infections) the results are likely to have a larger variation. Also, it is important to remember that the time from isolation to experimental use and the isolation process itself can affect the quality of the cells. The quality of the pancreatic islets was tested by measuring the insulin release (stimulation index (SI)) in response to glucose. Only islets with a SI above 4 were used to assess the innate immune response to Coxsackievirus infection in **Paper II**.

3.1.1.3 Cell lines

HeLa, HepG2 and Huh7.5 cells have been used throughout the studies presented in this thesis. HeLa cells were chosen since it is a cell line that is widely used and well characterized. HepG2 and Huh7.5 cells are derived from the liver and were used in **Paper IV** to determine whether type III IFNs protects against a Coxsackievirus infection.

3.1.1.4 Virus strain

The CVB3-Nancy strain was used in all studies described in this thesis. This virus strain was chosen since it is well characterized and widely used in the field. Also, the CVB3 strain has been associated with several diseases such as T1D and myocarditis. HeLa cells were used for propagation and titration of the virus.

3.1.1.5 Virus detection

Throughout this thesis, several methods were used for detection of CVB3 replication. During viral replication the protein VP1 is expressed and the presence of this protein in infected cell were detected using Western blot and/or flow cytometry. To measure the amounts of infectious virus particles in tissues (**Paper I**) or culture supernatants (**Paper II – V**), plaque assays were performed.

3.1.1.6 Real time RT-PCR

Real time RT-PCR provides a sensitive and powerful tool to analyze changes in mRNA expression. Compared to conventional PCR, real-time RT-PCR is an efficient method used to quantify gene expression. Real-time RT-PCR was used in all of the papers included in this thesis, and in addition it was also used to perform the PCR array described in **Paper III**. Throughout this thesis, relative RT-PCR was used to compare the expression levels between sample. To normalize the sample, the expression levels of the gene of interest were always compared with an internal control. Samples that displayed cycles above 35 cycels was considered below detection limit. Real-time RT-PCR was also used to genotype the donors used in **Paper II**. This assay is based on fluorescent probes specific for the polymorphism in IFIH1 (Ala946Thr).

3.1.1.7 Western blot

Western blot was used in **Paper II, II and V** to measure the protein expression levels following infection with Coxsackievirus or IFN-treatment. Western blot was used as a complement to the mRNA analysis to confirm the upregulation of a gene also on protein level in **Paper III**. In addition, Western blot was used to identify proteins that are targeted by CVB3 **Paper V**. This technique was also used to measure the phosphorylation status of IRF3 in **Paper V**.

3.1.1.8 Immunohistochemistry and immunofluorescence

Immunohistochemistry was used in **Paper I** to detect tissue damage and inflammation in organs. Immunohistochemistry together with immunofluorescence was used to detect the cellular localization of the IFNλR in **Paper III**, which was carried in collaboration with Dr. Sarah Richardson and Prof. Noel Morgan at the University of Exeter, United Kingdom.

3.1.1.9 ELISA

ELISA was used to detect the amounts of secreted IFNα in **Paper I** and secreted CXCL10 in **Paper III**. ELISA and a sensitive bioassay were also used to measure accumulation of type I and type I IFNs and III IFNs in culture supernatants from CVB3 infected human pancreatic islets, but these analyses failed to detect IFNs (data not shown).

3.1.1.10 Measurement of cytopathic effects

In **Paper IV** we performed a 96-well assay to measure the cytopathic effect after a Coxsackievirus infection. This assay was modified from a previously described protocol to examine the protective effect of type III IFNs [181].

4 RESULTS AND DISCUSSION

4.1 RECOGNITION OF COXSACKIEVIRUS

4.1.1 The role of MDA5 in the host response to a Coxsackievirus infection

The ability to recognize a virus infection is crucial for the initiation of a proper immune response. PRRs such as TLRs and RLRs play an important role in sensing viral structures and inducing the expression of IFNs. Previous studies have identified a role for several TLRs in sensing Coxsackieviruses [17, 110, 111, 115, 116]. In 2006 Smyth *et al* reported that polymorphisms in the gene encoding the viral sensor MDA5 were associated with an increased risk for T1D development [139], a finding that has provided a potential explanation for the association seen between enterovirus infections and susceptibility to T1D. However, whether MDA5 was of importance in recognizing Coxsackieviruses was not known. The aim of **Paper I** was, therefore, to address the role of MDA5 during a CVB3 infection.

To determine the role of MDA5 in CVB infections, mice that were deficient in MDA5 (*mda5*-/-) and wild type (wt) controls were challenged with CVB3. We found that mice lacking *mda5* that were on a C57BL/6 (B6) background succumbed early to CVB3 infection while most wt mice survived (**Paper I**, Figure 1A). Mice deficient in MDA5 also displayed a marked disability in their control of virus replication early after infection (**Paper I**, Figure 2A), suggesting that MDA5 controls viral replication early during infection. Binding of viral RNA to MDA5 triggers the induction of IFNs. Since a rapid expression of IFNs is important for controlling a Coxsackievirus infection [118, 119] we wished to examine the expression of IFN and IFN-inducible genes in the animals. We found that wt mice had an increased production of IFNα in serum at 48 h post infection (**Paper I**, Figure 3A). At this time point, CVB3-infected *mda5-/-* mice also had an increased production of IFNα, although the induction was lower compared to infected wt animals (**Paper I**, Figure 3A). An increased induction of IFNβ mRNA was detected in the liver and pancreas of both *mda5-/-* and wt mice post CVB3 infection (**Paper I**, Figure 3B). There was also an increased expression of OAS1a and CXCL10 in these organs (Paper I, Figure 3C-D). Finally, the inability to control early viral replication led to severe tissue damage in the liver and exocrine pancreas in the *mda5* deficient mice (**Paper I**, Figure 4 A-C).

The findings in **Paper I** show that MDA5 is important for controlling early CVB3 replication and that an inability to appropriately regulate the virus has devastating effects on the host. Our results showing that MDA5 is important for survival upon a CVB3 infection are similar to those reported by Wang *et al* [34]. There are however, some differences between the studies. Wang *et al* report that MDA5 is important for survival after a CVB infection in mice on 129/SvJ background, whereas our results show no difference in survival rates post CVB3 infection on this genetic background (**Paper I**, Figure 1B). This difference may be explained by the higher dose of virus used in the study performed by Wang *et al*. Our results do, however show that a lack of MDA5 in 129/SvJ mice leads to tissue damage long after the virus is cleared (**Paper I**, Figure E).

Our results also show that MDA5 is not absolutely required for induction of type I IFNs. Furthermore, the finding that mice lacking MDA5 are still able to induce the expression of type I IFNs upon CVB3 infection suggests that additional receptors may also recognize Coxsackievirus. So far, a role for RIG-I in Coxsackievirus infection has not been described, however TLR3, TLR4, TLR7 and TLR8 are potential receptors that may also recognize Coxsackieviruses [14, 17, 110, 115]. It is likely that the induction of IFNα seen in the *mda5-/-* mice is mediated by one or several of these TLRs. Furthermore, although we detect IFNα in the serum of *mda5*-/- at 48 h post infection, it is interesting to note that the levels are lower compared to wt mice. This suggests that MDA5 is important for the early induction of type I IFNs but other receptors may have a role later. Since type I IFNs are critical in limiting CVB3 replication, it is possible that the slight reduction in IFN levels lead to increased virus titers and as a consequence of this, severe tissue damage. In **Paper III and IV**, we identified a protective role for type III IFNs in CVB3 infection and it would be interesting to see whether the lack of MDA5 also influence the induction of type III IFNs.

Several polymorphisms in IFIH1 that regulates susceptibility to T1D have been identified [138, 139]. Two studies recently reported that overexpression of MDA5 leads to the development of autoimmune symptoms [182, 183]. This suggests that a reduced function of MDA5 is protective in autoimmune diseases. In our study, we saw no signs of T1D development in the *mda5*-/- animals. However, we found that MDA5 plays an important role in the host defense against a Coxsackievirus infection, which can have implications for how MDA5 regulates virus-induced T1D. Based on our result that a lack of MDA5 leads to uncontrolled viral replication, one can speculate that a strong immune response will protect against a virus infection. Coxsackievirus replication initially takes place in the intestine, and an inability to block virus here may increase the risk for the infection to spread to other organs, such as the pancreas. A reduced capacity to control the virus infection may also lead increased number infections and also more severe infections. Interestingly, enterovirus infections are more commonly found among persons with T1D than healthy controls [184]. A strong immune response can however also lead to the activation of auto reactive T cells that initiate an autoimmune attact on the host [179]. On the other hand, a weak early response may lead to that the virus can replicate and spread within the body. This can result in an increased expression of antiviral genes in the target organs and an increased activation of the immune response, which may contribute to the activation of self-reactive T-cells and subsequent induction of autoimmunity. This scenario is supported by the results in (**Paper I**, Figure 3), which show that the *mda5-/-* animals display increased levels of ISGs in target organs.

In summary, in **Paper I** we show that MDA5 play an important role in controlling CVB3 infection, especially early in the infection. Moreover, we show that MDA5 is not absolutely required for induction of type I IFNs.

4.1.2 A polymorphism in IFIH1 modulates the antiviral response

Infections with enteroviruses, especially those with Coxsackieviruses have been linked to T1D in several studies [161, 163]. In **Paper I** we found that MDA5 plays an important role in recognizing and controlling an infection with CVB3. Several polymorphisms in IFIH1 have been associated with T1D, however how these SNPs affect the ability of MDA5 to recognize Coxsackieviruses and induce an innate immune response is not currently known. The aim with **Paper II** was to study the human islet response to a Coxsackievirus infection and determine if a polymorphism in IFIH1 affects the antiviral response.

We started by examining the islets antiviral response against a Coxsackievirus infection. We found that the islets responded to a CVB3 infection by inducing the expression of type I and type III IFNs (**Paper II**, Figure 1A and 2B). We made an attempt to measure secreted IFNs both using ELISA and a bioassay, however the levels were below detection limit (data not shown). Secreted IFNs act in an autocrine and a paracrine manner to induce the expression of IFN-inducible genes [185] and measuring the expression of these genes can be used as an indirect measurement of IFN production. In response to CVB3 infection, the islets strongly induced the expression of genes involved in viral recognition (MDA5, RIG-I and TLR3) and antiviral defense mechanisms (CXCL10 and MxA) (**Paper II**, Figure 1B-C).

The polymorphism Ala946Thr (rs1990760) in IFIH1 shows the strongest association with T1D. The risk allele of this polymorphism encodes threonine (Thr, here denoted “TT”) in codon 946, whereas the non-predisposing allele encodes alanine (Ala, here denoted “CC”) in this position [139]. To determine if this polymorphism affects the islet’s ability to induce an antiviral response after Coxsackievirus infection, we genotyped the donors included in the study. Surprisingly, our cohort did not contain any donors with the protective CC-genotype. Instead, the cohort consisted of similar numbers of donors carrying the TT- or TC-genotype (**Paper II**, Table 1). After dividing the donors according to genotype, we found no difference in the induction of either type I and type III IFNs between the two genotypes. However, donors carrying the TT-genotype showed a trend towards lower amounts of type III IFNs compared to the TC-genotype (**Paper II**, Figure 2B). Next, we analyzed the induction of IFN-stimulated genes with respect to the polymorphism in IFIH1. Intriguingly, after infection with CVB3 a lower induction and/or expression level of MDA5, RIG-I and TLR3 was seen in donors carrying the risk TT-genotype compared to the TC-genotype (**Paper II**, Figure 3).

It is important to highlight that there are, however, some limitations in **Paper II**. The first is that our cohort is rather small due to the scarcity of human islets donors. Secondly, we lack donors with the protective CC-genotype. Examples of polymorphisms exist where the heterozygous mutation is associated with a higher or lower effect than the homozygous mutations, a phenomena known as molecular heterosis [186]. In fact, a study by Cinek *et al* found that the presence of enterovirus in blood from T1D patients was more frequent in patients carrying the TC-genotype of the Ala946Thr polymorphism [187]. Therefore, one should be careful in drawing conclusions regarding how donors with a CC-genotype would respond to infection and furthermore how this relate to the TT-donors. Thirdly, we only study the time point 48 h after infection. Assessing gene induction following infection at other time points may increase the chance of finding an association between the Ala946Thr polymorphism and the human islets response upon infection.

A large number of studies support the association between polymorphisms in IFIH1 and autoimmune diseases such as T1D [139-144], SLE [147, 148], autoimmune thyroid disease [150] and Aicardi-Goutiers syndrome [183, 188]. Interestingly, a recent study found an association between the 946Thr polymorphism in IFIH1 and the development of dilated cardiomyopathy [189]. In a similar manner to T1D, infections with enteroviruses have been implicated in the pathogenesis dilated cardiomyopathy [190].

Since the discovery of the association between polymorphisms in IFIH1 and the development of T1D, several studies have attempted to elucidate the mechanism by which the polymorphisms affect risk for disease development. Nejentsev *et al* identified four rare variants of IFIH1 that are associated with protection against T1D [138]. These variants correlated with lower expression levels of MDA5 and they displayed a reduced ability to bind dsRNA, implying that diminished expression and function is protective against T1D [151, 152]. In line with this, Liu *et al* reported that the predisposing 946Thr polymorphism was associated with increased basal expression of MDA5 [142]. However, others failed to reproduce this finding [191, 192] and in addition, we did not find an association between the Ala946Thr polymorphism and basal expression levels of MDA5 in human islets (**Paper II**, Figure 3). A recent study found that the 946Thr variant of MDA5 was constitutively active [182]. In **Paper II**, we measured basal expression (i.e. uninfected islets) levels of IFNs and IFN-stimulated genes in human islets carrying the TT and TC genotypes and found no difference in their expression in the absence of a CVB3 infection (**Paper II**, Figure 2B). In parenchymal cells such as human islets, the basal expression of MDA5 is very low [66, 78] and **Paper II**, Figure 1D) which may explain why we fail to detect a difference in basal expression of IFNs between the two genotypes. Immune cells such as DCs, however, may express higher levels of MDA5 and it is possible that a constitutively active variant might be detected with greater ease in these cell types. Immune mediated diseases such as SLE and T1D have been associated with an IFN-signature [182, 193, 194] and a polymorphism in IFIH1 that leads to a constitutively active protein in combination with other predisposing genes may contribute to this IFN-signature. Interestingly, Funabiki *et al* also showed that the 946Thr variant of IFIH1 is unable to respond to an infection with EMCV with the induced expression of IFNs [182]. We show that the donors expressing the TT-genotype respond with lower expression of type III IFNs and IFN-stimulated genes after infection with CVB3 compared to donors carrying the TC-genotype (**Paper II**, Figure 2B, 3 and 4), which may indicating that the TT-donors are less responsive.

To summarize our findings in **Paper II**, we show that the Ala946Thr polymorphism affects the ability of human islets to respond to a CVB3 infection. We found that the risk genotype (TT) is associated with a lower induction of viral sensors after infection compared to the TC-genotype.

4.2 PROTECTIVE EFFECT OF TYPE III INTERFERONS

4.2.1 Protective effect in human islets

Restriction of early viral replication is heavily dependent on the induction of IFNs. The type III IFNs are the most recently identified IFN-family and they have been shown to protect against infection with various viruses [84, 85, 195]. This protective effect however, is limited to a narrow subset of cell types [62, 63]. In **Paper III**, we wished to investigate whether human islets were able to respond to type III IFNs and if such stimuli render islets resistant to a Coxsackievirus infection.

We started by determining whether human islets express type III IFNs following a Coxsackievirus infection. We found that the islets induced expression of IFNλ1 and IFNλ2 (**Paper III**, Figure 1A). The ability of a cell to respond to type III IFNs is dependent on the expression of IFNλR [44, 45]. To determine if human islets are able to respond to type III IFNs, we first examined whether islets express the IFNλR. Human islets were shown to express both receptor subunits at mRNA level, although the levels varied amongst the donors (**Paper III**, Figure 2A). To further evaluate the cellular localization of the subunits, we studied pancreas sections from healthy subjects using immunofluorescence. The expression IL-10R2 was detected in alpha- and beta-cells but not in delta cells, whereas expression of IFNλR1 was found only in alpha cells (**Paper III**, Figure 2B). Although our studies cannot exclude a low expression level (i.e. below the detection limit of our assay), the finding that beta cells do not express IFNλR, suggests that beta cells are unable to respond or have a low response to type III IFNs. Interestingly, it has been shown that enteroviruses preferentially replicate in beta cells and not alpha cells [163, 196]. However, whether beta cells have the ability to induce the expression of the IFNλR upon Coxsackievirus infection or other stress inducing stimuli, as has been shown for Hepatitis C virus [197], remains to be established.

Expression of both IFNλR subunits in at least some of the cells in the islets indicated that the islets had the ability to respond to type III IFNs. To gain an insight into what genes are upregulated upon type III IFN stimulation, we treated islets with IFNλ1, IFNλ2 or IFNα. After six hours the upregulation of 88 genes that are known to be modulated by IFNα and -β was evaluated using a PCR array. In agreement with other studies [64, 65] we found that IFNλ1 and IFNλ2 induced expression of a similar set of genes as IFNα (**Paper III**, Figure 3). However, IFNα induced a greater number of genes compared to the type III IFNs (**Paper III**, Figure 3). In addition, we confirmed the upregulation of some of the genes at the protein level such as MDA5 and RIG-I (**Paper III**, Figure 4).

Due to our observation that a number of genes with antiviral activity are upregulated after type III IFN treatment in human islets, we next tested whether the type III IFNs could prevent CVB replication in human islets. Viral replication was measured using two techniques: by determining the protein expression of VP1 using Western blot and by measuring accumulated virus titers in the supernatant by plaque assay. Infected islets had a high expression of VP1 (**Paper III**, Figure 5A). However, treatment with IFNλ1 and IFNλ2 before infection with

CVB3 reduced the expression of VP1 to levels below the detection limit (**Paper III**, Figure 5A). In addition, lower titers of replicating virus were detected in the supernatants of islet pretreated with IFNλ1 or IFNλ2 before infection (**Paper III**, Figure 5B).

IFNs are important for protecting pancreatic islets against virus infection [66, 78, 119]. Mice harboring beta cells that cannot respond to type I IFNs rapidly develop diabetes following a CVB infection [117]. Our findings in **Paper III** suggest that in addition to the type I IFNs, type III IFNs also contribute to this protection. Our study, however does not provide a mechanistic insight into the proteins which are important in protecting islets against a CVB3 infection. Nonetheless, the gene array results can give some hints. For example, PKR and OAS were induced by type III IFNs. Activation of PKR leads to the attenuation of protein translation, while OAS generates 2’5’ oligoadenylate, which activates RNase L as a means to enhance degradation of viral RNA. The involvement of both proteins in protection against CVB infection has been demonstrated [71]. Another gene that was upregulated by type III IFNs was the chemokine CXCL10. CXCL10 has a key role in the immune system were it can manipulate NK and T cell trafficking and cause effector functions [198]. Using a mouse model for virus induced myocarditis, it was shown that CXCL10 played an important role in limiting Coxsackievirus infection in the heart [199]. However, CXCL10 can also have a negative effect via contributing to autoimmune diseases by attracting autoreactive T cells to the site. In line with this, it has been shown that expression of CXCL10 in pancreatic beta cells accelerates the development of T1D [200].

To summarize, in **Paper III** we show that human islets respond to a Coxsackievirus infection by inducing the expression of type III IFNs. Moreover, islets stimulated with type III IFNs upregulate genes with antiviral activity and render the cells resistant to Coxsackievirus infection.

4.2.2 Protective effect in hepatocytes

In **Paper III** we found that type III IFNs have a protective effect against Coxsackievirus infection in pancreatic islets. Coxsackieviruses however, have a broad tissue tropism and infection is not limited to just the pancreas but other organs such as the heart and liver are also targeted [92]. Especially neonates and young children are at risk of developing complications following a Coxsackievirus infection. On rare occasions, the infection reaches the liver and can cause hepatitis, which may progress to acute hepatic necrosis associated with liver failure and coagulopathy [201, 202]. Factors regulating susceptibility to Coxsackievirus infections of the liver remain poorly understood and whether type III IFNs play a role was currently unknown. Based on our observations made in **Paper III** we hypothesized that type III IFNs could have an important role in protecting hepatocytes from infection. In **Paper IV** we aimed to address this hypothesis by testing whether type III IFNs protect human hepatocytes against a Coxsackievirus infection.

To our knowledge no studies had been conducted whereby primary human hepatocytes were infected with Coxsackievirus *in vitro*, so the first question we asked was whether hepatocytes were permissive to CVB infection. We found that hepatocytes were susceptible to infection with CVB as replicating virus was detected, as shown by the presence of intracellular VP-1 in the hepatocytes (Figure 4). Moreover, infectious viral particles were released from the cells into the supernatant and this together with an increased cell death indicated that the virus underwent a complete replication cycle and resulted in cell lysis (Figure 4, and data not shown). Infecting the cells with a higher dose of virus led to increasing titers in the supernatant (Figure 4), and maximum titers were found at 24 h post infection (**Paper IV**, Figure 1).

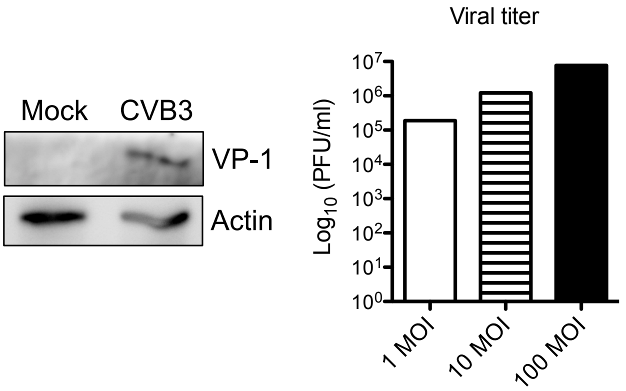

Figure 4. CVB3 infects and replicates in primary human hepatocytes. A. Primary hepatocytes were infected with CVB3 and at 24 h post infection, total protein was isolated and the expression of VP1 was measured using Western blot. Actin was used as loading control. B. Primary human hepatocytes were infected with CVB3 at an MOI of 1, 10 or 100. At 48 h post infection, viral titers were measured in the supernatant using plaque assay. (Lind K and Flodström-Tullberg M, unpublished results).

Hepatocytes have previously been shown to express type III IFNs upon infection with Hepatitis C virus [203, 204]. Together with our findings in **Paper III**, showing that CVB induce type III IFNs in human islets, we hypothesized that CVB infection of primary hepatocytes would induce the expression of type III IFNs. Indeed, hepatocytes responded to the infection by inducing the mRNA expression of IFNλ1 and IFNλ2 (**Paper IV**, Figure 1B). In agreement with other studies [87, 205], we found that hepatocytes express the IFNλR and induce the expression of ISGs in response to stimulation with IFNλ1 and IFNλ2 (**Paper IV**, Figure S1 and 3A). Importantly, we found that pretreatment of the cells with IFNλ1 or IFNλ2 resulted in protection against infection with CVB3 (**Paper IV**, Figure 3B).

In **Paper III and IV** we found that IFNλ1 and IFNλ2 protect cells from a Coxsackievirus infection. One limitation with these studies is that they did not include IFNλ3. The sequence homology between IFNλ2 and IFNλ3 is around 96% suggesting that the two would have similar potency [45]. However, IFNλ3 has been shown to have a more potent protective effect against EMCV (belongs to the enterovirus genus) than IFNλ1 and IFNλ2 [206]. In addition, a recent study found that IFNλ3 induced a greater induction of IFN-inducible genes compared two IFNλ1 and IFNλ2 [207]. Extending the studies to also include IFNλ3 would therefore be of interest.

The results presented in **Paper III and IV** demonstrate that infected cells express type III IFNs upon a Coxsackievirus infection. Our findings also indicate that this group of IFNs induce an antiviral state in cells and participates in the defense against CVB by limiting viral replication. These results support the notion that pancreatic beta cells and hepatocytes can regulate their own permissiveness to Coxsackievirus infection. An increasing number of reports suggest that the role of type III IFNs is to protect mucosal surfaces from pathogenic viruses [85, 195]. Interestingly, a recent study identified a non-redundant role of the type III IFNs in protecting intestinal epithelial cells from rotavirus infection [85]. Coxsackievirus normally infects the host via the fecal oral route and initial replication occurs in the epithelial cells of the intestine. One can speculate that type III IFNs would play an important role in limiting Coxsackievirus entry into the host by preventing infection of the epithelial cells lining the intestine. Studies examine the role of type III IFNs in Coxsackievirus infections of the intestine are warranted.

To summarize, the findings in **Paper** IV shows that type III IFNs protect primary human hepatocytes from a Coxsackievirus infection.

4.3 COXSACKIEVIRAL IMMUNE EVASION STRATEGIES

The importance of type I IFNs in the defense against a Coxsackievirus infection is underlined by the existence of evasion strategies developed by the virus to impede the immune response [208]. In **Paper III and IV**, we found that type III IFNs limit Coxsackievirus replication in pancreatic islets and also in primary hepatocytes. These observations led us to hypothesize that Coxsackieviruses have evolved mechanisms to also evade the type III IFN response. Our aim in **Paper V** was therefore to determine whether Coxsackievirus has developed mechanisms to inhibit the expression of type III IFNs.

We started by determining whether HeLa cells induced the expression of type III IFNs upon infection with CVB3. As a positive control, the cells were treated with the viral mimic poly I:C. Treatment with poly I:C, led to an increased in the expression of type III IFNs, demonstrating that the cells responded to an virus infection (**Paper V**, Figure 1A). Interestingly, we did not detect any IFNλ1 or IFNλ2 mRNA expression following infection with CVB3 (**Paper V**, Figure 1A). In order to establish whether the virus is able to inhibit the induction of type III IFNs, we infected cells with CVB3 prior to treatment with poly I:C. Recognition of Coxsackievirus infection has been shown to be dependent on both MDA5 and TLR3 [33, 34, 110, 111] and **Paper I**. To determine if the virus were able to inhibit both these pathways we delivered poly I:C by two different routes: via transfection (where it is sensed by TLR3 [15, 209]) or exogenously (as sensed by MDA5 [209, 210]. We found that infection with CVB3 strongly inhibited the induction of type III IFNs following both the exogenous application and transfection of poly I:C (**Paper V**, Figure 2), indicating that the virus blocks both MDA5- and TLR3-signalling.

Induction of type III IFNs is dependent on the activation of the transcription factor IRF3 [57, 58, 60]. Activation of IRF3 is regulated via hyperphosphorylation of the protein [211]. We found that transfection of poly I:C led to hyperphosphorylation of IRF3. In contrast, this was not detected after infection with CVB3 (**Paper V**, Figure 3A). The finding that IRF3 was not fully activated following CVB3 infection indicated that signaling molecule(s) upstream of IRF3 were targeted. Indeed, we found that the expression of TRIF (the adaptor protein utilized by TLR3, Figure 1) declined in CVB3-infected cells (**Paper V**, Figure 2B-C). Moreover, we found that the MDA5-pathway was inhibited during CVB3 infection, as indicated by the reduction of IPS1-expression (the adaptor protein utilized by MDA5, Figure 1) during the course of infection (**Paper V**, Figure 2B-C). In addition, the expression of MDA5 and RIG-I were lower following CVB3 infection (**Paper V**, Figure 2B).

At least two strategies have been described for how enteroviruses cleave signaling molecules important for the induction of IFNs. Either, the infection can activate caspases that cleaves the protein. Subsequently, the protein is degraded by the proteasome [212] or proteins can be targeted and degraded by virally encoded proteases [103, 213]. We started by investigating the role of capsases and the proteasome during a Coxsackievirus infection, by infecting HeLa cells in the presence of caspase or proteasome inhibitors. As seen previously, infection with CVB3 led to reduced expression of both TRIF and IPS1 and this occurred in the presence of inhibitors (**Paper V**, Figure 4). Thus, we concluded that the decreased expression of TRIF and IPS1 was independent of both caspases and proteasomal degradation. We continued to investigate the second possible strategy: namely the targeting of host proteins by virally encoded proteases. Coxsackieviruses encode two proteases, 2A and 3C. By constructing vectors containing 2A or 3C and expressing these in HeLa cells, we were able to study their possible role in blocking the expression of IFNs. We found that, expression of 2A inhibited the poly I:C-induced expression of IFNλ1 and IFNλ2 (**Paper V**, Figure 5). Moreover, expression of 2A led to decreased the levels of TRIF and IPS1 (**Paper V**, Figure 6). We found no inhibitory effect on type III IFN induction after expressing 3C nor did we see a decrease in the expression of TRIF and IPS1 (**Paper V**, Figure 6).

Type III IFNs were identified in 2003 [44, 45] and so far, only a few reports have been published that describe the viral evasion strategies which target type III IFNs [58, 214-216]. The results in **Paper V** demonstrate that Coxsackieviruses have also evolved inhibitory mechanisms to block the induction of type III IFNs. Furthermore, our study provides insight into the strategy by which the virus evades this host antiviral immune response. We found that the activation of IRF3 was severely inhibited during a Coxsackievirus infection, which is in agreement with other reports [102, 106]. Furthermore, we found that the expression of both TRIF and IPS1 were reduced following a CVB3 infection. TRIF and IPS1 act as adaptor molecules important for the induction of IFNs, and targeting these molecules may be a strategy used by the virus to block type III IFNs. However, coxsackieviruses are known for inhibiting several host functions such as shutting of host transcription and translation [103-105]. Most likely all these inhibitory actions are acting together to efficiently block IFN induction. Furthermore, the exact mechanism that the virus uses to inhibit the induction of type III IFNs remains to be determined.

Moreover, we also show that the expression of MDA5 and RIG-I were reduced after infection with CVB3. The fact that CVB3 also targets RIG-I was somewhat surprising since Coxsackieviruses do not bear a 5' triphosphate and therefore, it would be unexpected for them to be recognized by RIG-I. However, the finding that CVB3 reduces the expression of RIG-I, suggests that it might play a so far unidentified role during Coxsackievirus infection.

During the preparation of **Paper V**, two studies reported that CVB-encoded proteases target MDA5- and TLR3-pathways [102, 106]. Our findings support the results described by Feng *et al*, which show that 2A targets IPS1, and similar observations have been made with the 2A protease from poliovirus and Enterovirus 71 (both belonging to the enterovirus genus) [102, 217]. In addition, our results demonstrate that TRIF is targeted by 2A (**Paper V**, Figure 6). This is in contrast to the findings seen by Mukherjee *et al,* which show that TRIF is cleaved by 3C [106].

In **Paper III and IV** we found that a Coxsackievirus infection induces the mRNA expression of type III IFNs in primary cells, which may seem contradictive to the results presented in **Paper V**. It is however difficult to estimate the level of type III IFNs detected in **Paper III and IV** as we do not have a suitable positive control to compare with, such as poly I:C or a modified virus that lacks the ability to inhibit the induction low levels of IFNs. In addition, in **Paper II** we measured the levels of secreted IFNs in the supernatant using two sensitive methods namely, ELISA and a bioassay. However, with both methods the levels of IFNs were below detection limit, indicating that Coxsackievirus induce very low levels of IFNs in primary cells. We can nonetheless conclude from **Paper I** that a Coxsackievirus infection *in vivo* leads to the induction of IFNs. Although the levels were not sufficient to efficiently block viral replication (**Paper I**, Figure 2B).

In summary, we show in **Paper V** that Coxsackieviruses have developed mechanisms through which they can interfere with the induction of type III IFNs.

5 CONCLUDING REMARKS

The aim of this thesis was to expand our understanding of the interplay between host and virus during an enterovirus infection. Our focus has been on the innate immune system and the proteins that are important for recognizing and controlling a Coxsackievirus infection.

Our findings in **Paper I** highlight the role of MDA5 as an important protein that is involved in controlling the outcome of Coxsackievirus infections. Infections with Coxsackieviruses have been associated with severe diseases and have in addition been implicated in the development of T1D. Several polymorphisms in IFIH1, the gene encoding MDA5, have been associated with the development of T1D. In **Paper II,** we show that the Ala946Thr polymorphism in IFIH1 affects the ability of human islets to induce the expression of type III IFNs and IFN-inducible genes after a Coxsackievirus infection. Induction of IFNs is important both for protecting target cells from a virus infection and for limiting the replication of virus in cells that are already infected. In **Paper III and IV** we found that type III IFNs contribute to the antiviral defense initiated against Coxsackieviruses. Our results in **Paper III** show that human islets respond to type III IFNs by inducing genes with known antiviral effects. In addition, type III IFNs render human islets resistant to Coxsackievirus infection. Furthermore, in **Paper IV** we show that type III IFNs also protect primary human hepatocytes against an infection with Coxsackievirus. Due to the potent protective effect of IFNs, viruses have evolved multiple strategies to circumvent the immune response and in **Paper V**, we describe a mechanism utilized by Coxsackieviruses to prevent the induction of type III IFNs. This identifies a new strategy, through which Coxsackieviruses evade the immune response and at the same time provides insight into the proteins, which are important in combating a Coxsackievirus infection.

The studies of which this thesis is comprised, contribute to a deeper understanding of the interplay between host and pathogen during an enterovirus infection. Our findings emphasize the importance of the innate immune system in controlling Coxsackievirus infections. Increasing our knowledge of the interactions between Coxsackieviruses and the immune system will hopefully contribute to the development of therapeutic agents that could prevent diseases associated with such infections.

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