Thesis for doctoral degree (Ph.D.) 2010

HOST-MICROBE INTERACTIONS IN COXSACKIEVIRUS INFECTIONS FOCUS ON INNATE IMMUNITY

Michael Hühn

Karolinska Institutet

200 1810 – 2010 Years

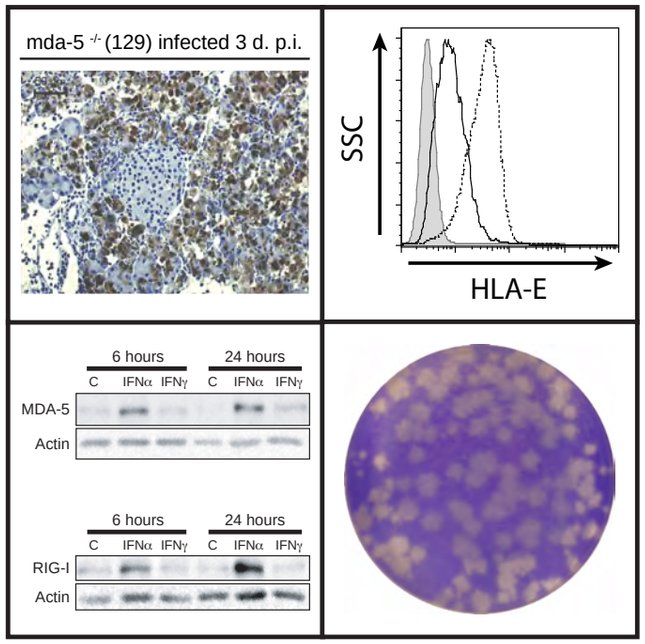
Thesis for doctoral degree (Ph.D.) 2010

HOST-MICROBE INTERACTIONS IN COXSACKIEVIRUS INFECTIONS FOCUS ON INNATE IMMUNITY

Michael Hühn

Thesis for doctoral degree (Ph.D.) 2010

HOST-MICROBE INTERACTIONS IN COXSACKIEVIRUS INFECTIONS FOCUS ON INNATE IMMUNITY



Michael Hühn

Karolinska Institutet

200 1810 – 2010 Years

From
Center for Infectious Medicine
Department of Medicine
Karolinska Institutet, Stockholm, Sweden

HOST-MICROBE INTERACTIONS IN COXSACKIEVIRUS INFECTIONS
FOCUS ON INNATE IMMUNITY

Michael Hühn

Karolinska Institutet

Stockholm 2010

Published by Karolinska Institutet.

ISBN 978-91-7409-955-3

Printed by
REPROPRINT AB
Stockholm 2010
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

ABSTRACT

Coxsackievirus group B (CVB) is a common virus that usually causes only mild symptoms in humans. However, occasionally these infections result in severe diseases like meningitis, myocarditis and pancreatitis. In addition, CVB infections have been suggested to be involved in the etiology of chronic diseases such as dilated cardiomyopathy and type 1 diabetes (T1D). Why the outcome of a CVB infection can vary so greatly is not entirely clear. Besides virus-intrinsic factors it has been hypothesized that the ability to mount an adequate immune response is important in regulating damage after CVB infection. The innate immune response is essential for the control of virus replication early after infection and for initiating the adaptive immune response, which is needed to clear the infection. If the host fails to initiate a suitable immune response, damage may arise as a result of uncontrolled virus replication or excessive inflammation. The aim of this thesis was to increase our understanding of how the innate immune response is initiated after CVB infections and how it may help to control the virus.

In paper I, we showed that the intracellular virus sensor melanoma differentiation-differentiation protein-5 (MDA-5) plays an important role in the immune response to CVB. Lack of MDA-5 weakened the host's ability to limit virus replication during the first days after infection, which lead to increased tissue damage. In addition, we found that the genetic background determines how heavily the host relies on MDA-5 for survival. Mice lacking MDA-5 on a C57/BL6 background rapidly succumbed to CVB infection while 129/SvJ mice survived and eventually cleared the virus even in the absence of MDA-5. Surprisingly, production of type I interferons (IFNs) was not significantly compromised by a lack of MDA-5. Type I IFNs produced by infected cells can stimulate immune cells like natural killer (NK) cells that have been demonstrated to be important in the host response to CVB infections. In paper II, we established that CVB infection modulates the cell surface expression of NK cell receptor ligands. The downregulation of inhibitory class I HLA alleles and the activating NKG2D ligand MICA did not result in increased NK cell mediated killing of the infected cells. However, after encountering infected cells, NK cells responded with enhanced secretion of IFN-γ, a cytokine with well-described antiviral and immuno-modulatory properties. Type I and II IFNs secreted by infected cells and/or immune cells are an indispensable part of the immune response to virus infections. In paper III, it was demonstrated that human pancreatic islet cells respond to IFN stimulation by upregulating the expression of genes involved in virus recognition, limiting virus replication and shaping of the adaptive immune response. This so-called antiviral state reduced the permissiveness of human islet cells to CVB infection.

Patients suffering from cystic fibrosis (CF) have a well-described defect in their antimicrobial immune response, leaving them especially vulnerable to respiratory tract infections. Using a mouse model for CF in paper IV, it was shown that a defective cystic fibrosis transmembrane conductance regulator (*cftr*) gene, the genetic cause for CF, renders mice susceptible to systemic CVB infection. Mice lacking CFTR succumbed to CVB at a dose that was not lethal for their wild type counterparts.

In conclusion, the studies included in this thesis add to our understanding of how the innate immune response recognizes and responds to CVB infections. Interestingly, MDA-5, NK cells and IFNs, while important in the immune response to CVB, have also been implicated to play a role in the development of T1D. This supports the notion that the immune response mounted by the host may influence the outcome of an infection with CVB. Hopefully, a better understanding of the host immune response can help to prevent the severe outcomes sometimes associated with CVB infection.

LIST OF PUBLICATIONS

I. **Hühn, M. H.**, McCartney, S., Lind, K., Svedin, E., Colonna, M., Flodström-Tullberg, M. 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 May 25; 401(1):42-8*

II. **Hühn M.H.**, Hultcrantz M., Lind K., Ljunggren H.-G., Malmberg K.-J., Flodström-Tullberg M. IFN-gamma production dominates the early human natural killer cell response to Coxsackievirus infection. *Cellular Microbiology 2008 Feb;10(2):426-36*

III. Hultcrantz M., **Hühn M.H.**, Wolf M., Olsson A., Jacobson S., Williams B.R., Korsgren O., Flodström-Tullberg M. Interferons induce an antiviral state in human pancreatic islet cells. *Virology 2007 Oct 10; 367(1):92-101*

IV. **Hühn, M.H.**, Lind, K., Svedin, E., Flodström-Tullberg, M. Increased Susceptibility to a Coxsackievirus Infection in a Mouse Model for Cystic Fibrosis. *Manuscript*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
CVB	Coxsackievirus group B
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
eIF2α	Eukaryotic initiation factor 2α
ER	Endoplasmic reticulum
EV	Enterovirus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IFNAR	Interferon α receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	IFN regulatory factor
ISG	Interferon stimulated gene
KIR	Killer cell inhibitory immunoglobulin-like receptor
LPS	Lipopolysaccharide
MCMV	Mouse cytomegalovirus
MDA -5	Melanoma differentiation-associated protein-5
MHC	Major histocompatibility complex
MICA/B	MHC class I chain-related gene A/B
MS	Multiple sclerosis
Mx	Myxovirus resistance protein
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NKR	Natural killer cell receptor

NO	Nitric oxide
OAS	Oligoadenylate synthetase
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cells
PFU	Plaque forming units
pIC	Polyinosinic:polycytidylic acid
PKR	RNA activated protein kinase R
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene-I
RLH	RIG-I like helicases
SOCS-1	Suppressor of cytokine signaling 1
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
Th	T helper cell
TLR	Toll like receptor
TNF	Tumor necrosis factor
ULBP	UL16-binding protein

1 INTRODUCTION

1.1 THE INNATE IMMUNE SYSTEM

All organisms need to protect themselves from the often hostile surrounding environment. The first line of protection is provided by a physical barrier that separates the host from the outside world. In the case of mammals, this function is carried out by the epithelial lining. If this mechanical barrier fails, the host possesses a wide array of specialized cells, small molecules and proteins, collectively referred to as the immune system, that prevents microorganisms from invading and causing damage to the host. The host response to infections can be divided into different stages. The innate arm of the immune system can respond quickly to a large variety of microorganisms in an unspecific manner, and is particular important during the early immune response. It is aimed at detecting invading microbes and limiting their replication and spread until the pathogen-specific adaptive immune response that helps to clear the infection is initiated. Moreover, activation of the adaptive arm of the immune system leads to long-term memory, which helps the host to respond faster to a second infection with the same microorganism. Parts of the evolutionary older innate immune system can be found even in single-cell organisms, while only jawed vertebrates are capable of mounting an adaptive immune response [3].

Most cells are equipped with proteins that enable the detection of intracellular pathogens (see section 1.1.1 of this thesis). This event is crucial for the induction of the immune response. After recognition of an invading pathogen, cells can respond by upregulating the expression of a number of proteins with immunological functions [1]. Cytokines such as interleukins (IL) stimulate and activate other cells [4], while chemokines can recruit immune cells by inducing chemotaxis [5]. Interferons (IFNs) can act in an auto- and paracrine manner to upregulate proteins that can reduce cells' permissiveness to virus infections (see section 1.1.2 of this thesis). By the mechanisms described above, virtually every cell can contribute to the innate immune response. In addition, the host has a set of specialized innate immune cells such as dendritic cells (DCs), monocytes and natural killer (NK) cells. DCs are professional antigen presenting cells (APC). They constantly take up particles from the extracellular space and present peptides in the context of major histocompatibility complex (MHC) molecules to T-lymphocytes (hereafter denoted T-cells) [6], cell types belonging to the adaptive immune system. In addition, DCs are particularly well equipped to recognize intra- and extracellular microorganisms. Plasmacytoid DCs (pDCs) are a subtype of DCs that have the unique ability to rapidly secrete IFN-α upon detection of virus infection [7]. In contrast to most other cell types, even unactivated pDCs express the transcription factor IFN regulatory factor (IRF) 7 needed for the activation of IFN-α transcription [8]. Once DCs become activated, they express co-stimulatory molecules that activate adaptive immune cells that recognize the epitopes presented by DCs [6]. Like DCs, monocytes and macrophages can present antigens to T-cells. In addition, they are highly specialized in engulfing extracellular pathogens. After uptake, macrophages can kill bacteria or parasites by toxic molecules and proteins such as reactive oxygen species and degrading enzymes. Furthermore, monocytes produce cytokines that help to activate and shape the adaptive immune response [9]. NK cells are best known for their ability to recognize and kill malignant and infected cells

without the need for prior sensitization (see section 1.1.3 of this thesis). They are also one of the main producers of IFN-γ, a cytokine with antiviral and immunomodulatory properties [2].

The innate and the adaptive arms of the immune system are highly intertwined and by no means separated parts of the immune response. For example, the activation of the adaptive immune response is highly dependent on the innate response. Antigen presentation by DCs is crucial for its activation, while cytokine production by for example NK cells can determine the quality of a T-cell response [10]. Interestingly, recent studies suggest that NK cells are able to form an immunological memory [11], a function previously attributed only to B- and T-cells, blurring the line between adaptive and innate immunity even more.

1.1.1 Pattern recognition receptors

Mammals possess an intricate system that allows the detection of invading microorganisms. This is facilitated by a set of intracellular and transmembrane proteins, collectively termed pattern recognition receptors (PRR). They are able to recognize conserved structures of bacteria and viruses being either a part of the genome or displayed on the surface, so called pathogen associated molecular-patterns (PAMPs) [1]. Since many microorganisms express the same PAMPs recognized by PRRs, the host can detect a multitude of pathogens with a relatively limited amount of proteins. After ligand binding, PRR initiate a signaling cascade that results in the expression of interferons and/or inflammatory cytokines [1]. This is crucial for the activation and shaping of the innate and adaptive immune responses of the host.

1.1.1.1 Toll like receptors

The toll like receptors (TLRs) were the first mammalian PRR to be discovered. They are homologous to the Toll receptors found in Drosophila [12]. All TLRs are transmembrane proteins, either located at the cell membrane or in the endosomes of the cell (Table 1).

TLR	Location	Typical ligand	Induction of IFN
1	Cell membrane	Triacyllipopeptide	No
2	Cell membrane	Diacyllipopeptide[1] Triacyllipopeptide[2]	No
3	Endosome	dsRNA	Yes
4	Cell membrane	LPS	Yes
5	Cell membrane	Flagellin	No
6	Cell membrane	Diacyllipopeptide	No
7	Endosome	ssRNA	Yes
8	Endosome	ssRNA	Yes
9	Endosome	CpG DNA motives	Yes

Table 1. TLR location and ligands. [1] as a homodimer with TLR6; [2] as a heterodimer with TLR1; after [13].

Known ligands can be divided into two categories. TLRs located at the cell membrane bind to structures on the outside of microorganisms, like LPS, flagellin or lipopeptides, while endosome-restricted TLRs bind to conserved structures of microbial DNA or RNA (Table 1). A subset of TLRs is constitutively expressed in most cell types and can be further upregulated after stimulation with IFNs and/or cytokines. What pathogens can be detected and how cell will respond is determined by the TLRs that the cell expresses. For example, plasmacytoid dendritic cells (pDCs) express TLR7, 8 and 9, and are known to produce large amounts of IFNs after the recognition of viruses [14].

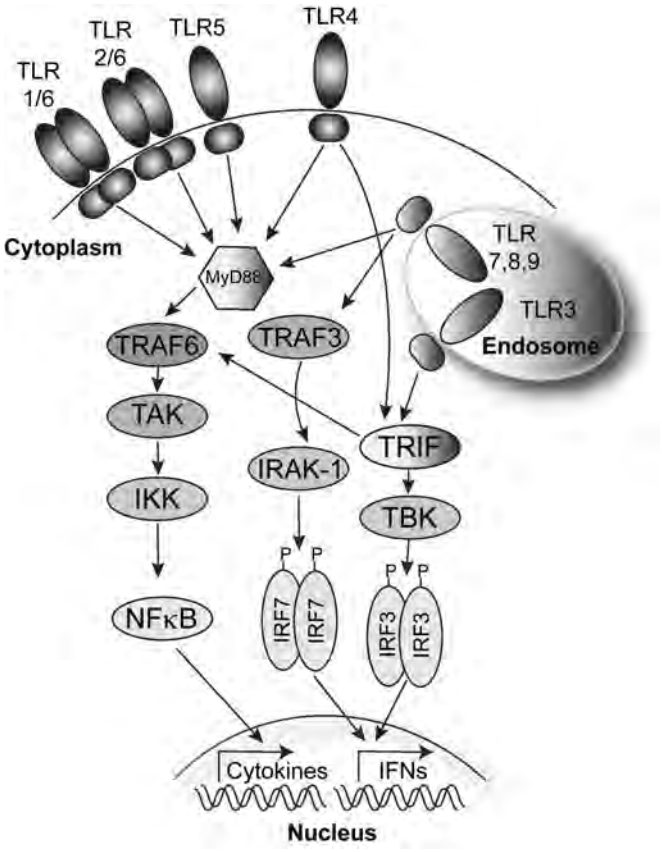


Figure 1. TLR signaling. Adapted after [1].

What cytokines are expressed after TLR stimulation depends on the signaling cascade associated with a given TLR (Figure 1). Inflammatory cytokines are induced by all TLRs, while the ability to induce type I IFNs is restricted to TLR4 and the endosomal TLRs (Table 1). All TLRs except TLR3 interact with the adapter molecule myeloid differentiation factor 88 (MyD88) and activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway. Instead of MyD88, TLR3 utilizes the adaptor TRIF, which can also bind to TLR4 [13].

1.1.1.2 RIG-I like helicases

Retinoic acid-inducible gene I (RIG-I also known as DDX5) and melanoma differentiation associated protein-5 (MDA-5, also known as Helicard or IFIH1) form a group of intracellular PRR called RIG-I like helicases (RLH). RIG-I and MDA-5 consist of two N-terminal caspase recruitment domains (CARD), a helicase domain and a C-terminal repressor domain [15]. A third member of the RLH, LGP-2 lacks the CARD that is necessary for signaling, and has been suggested to be a negative regulator of RIG-I [16]. However, newer results indicate that LPG-2 may instead act as a positive regulator of RLH signaling [17]. Both RIG-I and MDA-5 can recognize viral RNA and the synthetic virus RNA analogue polyinosinic:polycytidylic acid (pIC) [18, 19].After binding of their ligand RIG-I and MDA-5 induce the production of type I IFNs via activation of the adaptor IPS-1 (also called VISA, CARDIF or MAVS) [20], which ultimately results in the activation of the IRF3 and 7 (Figure 2).

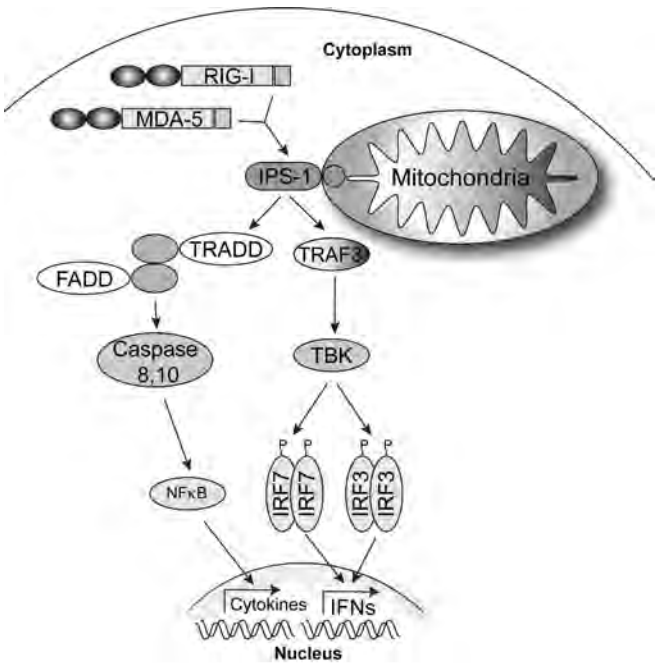


Figure 2. RLH signaling, Adapted after [1].

While RIG-I and MDA-5 have a similar protein structure and share a common signaling pathway, they do not recognize the same features of virus RNA or pIC. It has been demonstrated that pIC shorter than 1000 base paires (bp) is recognized solely by RIG-I, while pIC > 2 kbp is recognized by MDA-5 [21]. How the two proteins distinguish between pIC of different length is not known to date. In addition, it has been shown that RIG-I can be activated by single stranded RNA (ssRNA) carrying a 5´ triphosphate [22, 23] and a polyuridin motive similar to the 3´ UTR region of hepatitis C virus (HCV) [24]. The molecular structure of virus RNA that is recognized by MDA-5 has not been defined.

As it is evident that RIG-I and MDA-5 recognize different patterns in pIC it may not be surprising that they also can distinguish between different viruses [18]. While some viruses can activate both, others are only recognized by RIG-I or MDA-5 (Table 2).

Family	Species	Detected by	
		RIG-I	MDA-5
Paramyxoviridae	Sendai virus	X	
	Newcastle disease virus	X	
Orthomyxoviridae	Influenza virus	X	
Rhabdoviridae	Vesicular stomatitis virus	X	
Picornaviridae	EMCV		X
	Theiler´s virus		X
	Mengo virus		X
Flaviviridae	Hepatitis C virus	X	
	Japanese Encephalitis virus	X	
	Dengue virus	X	X
	West Nile virus	X	X
Reoviridae	Reovirus	X	X

Table 2. Detection of selected viruses by RLH. Adapted after [1].

The preferential detection of certain viruses by one of the two viral sensors can sometimes be associated with a virus family. For example, all picornaviruses studied so far are recognized by MDA-5 but not RIG-I. However, the fact that HCV virus is only recognized by RIG-I [25] but Dengue virus, also belonging to the flaviviridae, can activate both RIG-I and MDA-5 [26] shows that this might be oversimplified. In addition some viruses have evolved mechanisms to impair recognition by RLH. The HCV protease NS3/4 can cleave IPS-1 and the V-proteins of paramyxoviruses can bind to MDA-5 and inhibit its function [27, 28]. This demonstrates that a lack of activation of MDA-5 or RIG-I can be the consequence of a viral immune evasion rather than inability of recognition by RLH.

1.1.2 The interferon system

IFNs were discovered more than 50 years ago by their ability to interfere with virus replication [29]. Today, IFNs are divided into three classes: type I with IFN-α and –β as prototypic members, type II with IFN-γ and type III IFN including IFN-λ 1-3 (also called IL28 a/b and IL29, respectively). The different types of IFNs bind to distinct receptors but all share a similar signaling pathway [30]. After receptor binding the signal is relayed into the cell via the Jak/Tyk and signal transducer and activator of transcription (STAT) pathway. After being phophorylated, STATs form dimers and translocate into the nucleus where they activate gene transcription [31]. Besides their direct antiviral effect, IFNs are also important for the activation and shaping of the immune system. However, an unrestricted IFN response can be detrimental for the host and contribute to immunopathology and autoimmunity [32].

1.1.2.1 Type I interferons

The family of type I IFNs is made up of IFN-α, -β, ε, κ, ω and τ [32]. While IFN-α and -β have been extensively studied little is known about the functions of the other family members. Humans carry at least 13 different functional IFN-α genes and one gene for IFN-β [33]. Most cell types are able to respond to virus infections with the expression of IFN-β (see sections 1.1.1.1 and 1.1.1.2 of this thesis). Secreted IFN-α/β signal via the heterodimeric IFN-α receptor (IFNAR) that leads to the phophorylation of the associated janus kinases JAK1 and Tyk2. Subsequently, JAK1 and Tyk2 will phophorylate STAT-1, 2 and 4, which dimerize and together with IRF9 form the transcription factor ISGF3. ISGF3 induces the transcription of genes that carry the interferon stimulated response element (ISRE) in their promoter region [34]. Among the genes induced by IFN-β is IRF7. IRF7 is needed for the direct induction of IFN-α by PRR (Figure 3) [35]. Since most cell types do not express IRF7 in steady state they depend on IFN-β for the induction of IFN-α. One notable exception are pDCs. The expression of IRF7 by pDC facilitates a rapid production of IFN-α following encounter with virus. In fact, pDCs have been suggested to be the major source of IFN-α after virus infection [8]. Another gene upregulated by type I IFNs is the protein suppressor of cytokine signaling 1 (SOCS-1), a negative regulator of IFN signaling [36].

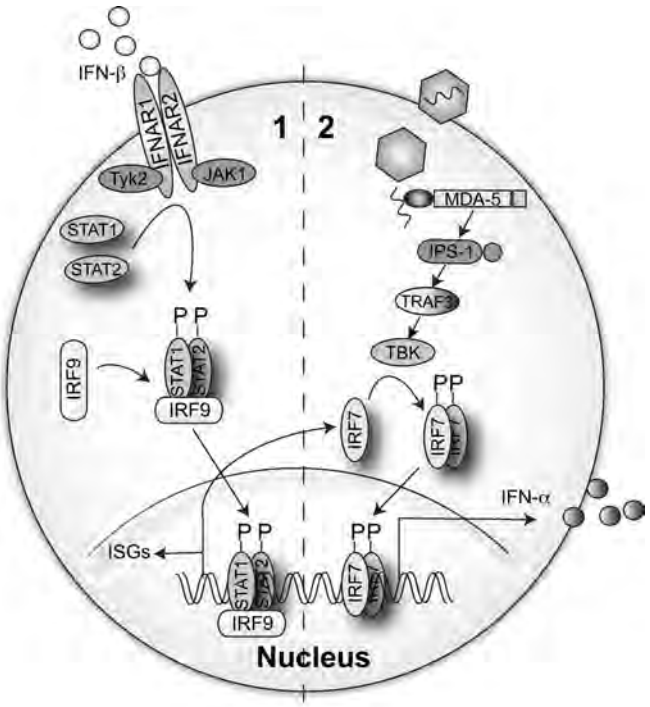


Figure 3. Type I IFN signaling and induction of IFN-α. 1: IFN-β signaling leads to the induction of IRF7 and other ISGs. 2: IFN-α is induced after virus detection by for example MDA-5 via phosphorylation of IRF7. Adapted after [1].

Type I IFNs have the ability to induce an antiviral state (described in more detail in section 1.1.2.3) in host cells and thereby directly limit virus replication and spread [37]. In addition, it has been shown that type I IFNs can activate several immune cells. IFN-α can activate NK cells by enhancing cytokine production and their cytotoxic function [38, 39]. Moreover, IFN-α can stimulate monocyte differentiation and activation [40], as well as induce proliferation of activated CD8+ T-cells. A more indirect way of enhancing the immune response and limiting virus spread is the induction of MHC expression by type I IFNs. This may help to overcome downregulation of MHC, induced by many viruses, and facilitate the efficient presentation of virus-derived peptides to cytotoxic T-cells [33].

The action of type I IFNs can be detrimental to the host if the immune activation is not properly balanced. For example the transgenic expression of type I IFNs in the β-cells of the pancreas induces T1D in normally T1D resistant mice [41]. Furthermore, injections with pIC, a potent inducer of type I IFNs can also induce T1D in diabetes prone rats [42, 43]. Interestingly, the development of T1D has been recognized as a side effect of systemic IFN-α treatment in humans [44].

1.1.2.2 Type II interferons

IFN-γ is the only known type II IFN. In contrast to type I IFNs, it is only expressed by cells of hematopoietic origin, especially by NK and T-cells. IFN-γ is induced in NK cells after stimulation with IL12 and IL18 and/or after encounter with a NK cell susceptible target cell [45, 46]. T-cells can produce IFN-γ after stimulation of the T-cell receptor by its cognate antigen peptide [3]. Binding of IFN-γ to the IFN-γ receptor results in the activation of JAK1 and JAK2 followed by phophorylation of STAT1. STAT1 homodimers can bind to genes possessing the gamma-activated sequence in the promoter region and activate their transcription (Figure 4) [2].

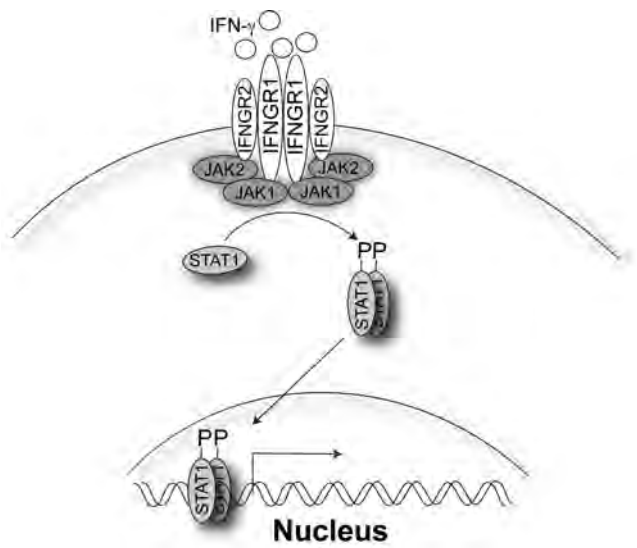


Figure 4. Type II IFN signaling. Adapted after [2].

IFN-γ can, similarly to type I IFNs, induce an antiviral state in host cells, which can inhibit virus replication and spread [37]. In addition, IFN-γ has a profound effect on

immune cells. One of the best-studied effects of IFN-γ is its ability to promote type 1 T helper cell (Th1) differentiation of T-cells, while inhibiting the development of Th2 and Th17 cells [2]. This is achieved by the opposite effect of IFN-γ on the expression of the key transcription factors involved in Th subset differentiation. IFN-γ induces the expression of T-bet [47], needed for Th1 differentiation, and inhibits the expression of GATA-3 [48] and probably RORγt [49], the transcription factors needed for Th2 and Th17 development, respectively.

In addition to skewing the adaptive immune response, IFN-γ also enhances innate immunity. It has been shown that IFN-γ can enhance the TLR signaling in macrophages [50, 51]. The production of inflammatory cytokines is increased in IFN-γ activated macrophages after TLR stimulation. This effect is achieved, at least in part, by inhibition of the anti-inflammatory actions of IL10.

The fact that a lack of SOCS-1, an inhibitor of IFN-γ signaling, is neonatal lethal in mice due to uncontrolled activity of IFN-γ, illustrates the severe effects of unrestricted IFN-γ signaling [36]. Many autoimmune diseases were thought to be exacerbated by IFN-γ. Recently, however, it became clear that in several mouse models of autoimmune diseases, IL17 expressing T-cells are actually responsible for inducing pathology [52, 53]. In fact, IFN-γ seems to be protective in experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis (MS) [54]. Still, it is clear that IFN-γ has a role in the development of some autoimmune conditions. The transfer of autoreactive IFN-γ producing Th1 T-cells can induce EAE in naïve mice [55] and treatment with IFN-γ has been implicated with the exacerbation of MS in humans [56]. Furthermore, it has been shown that IFN-γ can have cytotoxic effects. It is for example been well established that IFN-γ in combination with other inflammatory cytokines like IL1 or tumor necrosis factor (TNF)-α, can induce cell death in pancreatic β-cells. This may contribute to the destruction of insulin producing cells during the development of T1D [57].

1.1.2.3 Interferon stimulated genes and the antiviral state

The antiviral properties of IFNs can be attributed to at least two distinct effects. The immunomodulatory functions of IFNs help the host to clear infected cells and induce an adaptive immune response that confers long-term immunity [32]. Secondly, IFNs can induce an antiviral state in host cells, which can prevent cells from becoming infected and thereby limiting virus replication and spread [58]. The latter effect can be explained by the induction of a set of genes with direct antiviral properties. Genes upregulated by IFNs are collectively called interferon stimulated genes (ISGs). Both type I and type II IFNs induce an antiviral state via the stimulation of an overlapping but not identical set of ISGs [37]. The protective effect of IFNs is most likely due to the combination of genes upregulated and not the result of a single gene product. However, it has been demonstrated that expression of a single ISG or the lack thereof can have a profound effect on the outcome of a virus infection. The function of some ISGs is well established while the mode of action for others is still elusive.

One of the ISGs upregulated following IFN stimulation is the protein kinase R (PKR). Activation by dsRNA results in dimerization of PKR. The eukaryotic initiation factor 2α (eIF2α) is a well-defined substrate for PKR. Phosporylation by PKR prevents the recycling of eIF2α thereby halting host protein synthesis [31]. A recently uncovered consequence of eIF2α phosphorylation is the induction of autophagy, which leads to

the degradation of the cell content [59]. Mice deficient in PKR are more susceptible to infections with coxsackievirus, exemplifying the antiviral activity of PKR [60].

Another well-characterized ISG is the family of 2´-5´ oligoadenylate synthetases (OAS) [61]. After binding of dsRNA, OAS catalyzes the production of 2´-5´ oligoadenylates by cross linking ATP via an unusual 2´5´ phosphodiester binding. OAS or its products do not possess any antiviral activity themselves. Instead, oligoadenylates bind and activate RNase L. RNase L can degrade both cellular and virus RNA, which can prevent virus replication and protein synthesis. The OAS/RNase L pathway has been shown to be important for the induction of the antiviral state by IFNs. Mice lacking RNase L are highly susceptible to coxsackievirus infection [60]. In addition, mice expressing a truncated form of OAS1b are more susceptible to infections with West-Nile virus, a member of the falviviridae [62], and overexpression of RNase L in a human T-cell line reduces HIV replication [63].

Other ISGs with proven antiviral effects include, among others, the myxovirus resistance (Mx) protein family of large GTPases, ISG15 and viperin, although their mode of action is not as well established as for RKR and OAS. ISG15 is an ubiquitin like molecule that can be attached covalently to host proteins [64]. Genetic deletion of ISG15 in mice results in an increased susceptibility to a variety of viruses including herpes and influenza virus [65]. Mx proteins can interact with viral nucleocapsid like proteins of influenza- and Thogotovirus and several members of the *bunyaviridae* family and thereby restrict their cellular location, which limits virus replication [66, 67]. In addition, overexpression of MxA has also been shown to limit CVB replication [68]. An antiviral effect of viperin has been demonstrated for cytomegalovirus (CMV) and HCV, among others [69, 70]. How viperin limits virus replication is not entirely clear but it has been proposed that it can disrupt lipid rafts, which are important for the release of certain viruses from the host cell [71].

1.1.3 Natural killer Cells

NK cells are lymphocytes belonging to the innate arm of the immune system, originally discovered by their ability to lyse tumor cells without the need of prior sensitization. They develop in the bone marrow from the common lymphocyte progenitor and can be defined by their expression of CD56 in the absence of CD3 in humans and by expression of NK1.1 in the absence of CD3 in mice. NK cells make up around 15% of all lymphocytes in peripheral blood. In addition to tumor surveillance, NK cells play a vital role in protecting the host from pathogens by direct killing of microorganism or infected cells. They are also important for shaping the adaptive immune response through their capability to produce cytokines and chemokines, which places them at the intersection of the innate and adaptive immune system. Although NK cells do not rearrange their receptor repertoire like B- and T-cells, recent reports indicate that they may have the ability for memory-like responses [11].

1.1.3.1 NK cell receptors and activation

In contrast to B- and T-cells, NK cells do not rely on a single rearranged receptor for activation. Instead, the integrated signal of a set of activating and inhibitory receptors expressed on NK cells, determines if NK cells become activated (Table 3). Since NK cells do not undergo a selection process in order to delete autoreactive cells, an important question is how NK cells distinguish between self and non-self or normal and

aberrant cells. The observation that NK cells readily kill tumor cells devoid of MHC class I surface expression led to the formulation of the "missing-self"-hypothesis [72]. Normal cells express MHC class I molecules, while tumor cells often lack or downregulate the expression of these molecules. Similarly, many viruses down modulate MHC molecules in order to escape T-cell recognition, which will allows NK cells to recognize infected cells.

The main NK cell receptors (NKR) for the classical human leukocyte antigen (HLA) -A, -B and -C molecules are the killer cell immunoglobulin-like receptors (KIRs). In addition, a family of NKG2/CD94 heterodimeric receptors recognizes the non-classical HLA-E, which presents signal peptides of classical HLA molecules. Interestingly, both the KIR and NKG2 receptor families contain NK cell activating and inhibitory receptors (Table 3). The inhibitory receptors carry an immunoreceptor tyrosine based inhibitory motif (ITIM) in their cytoplasmic tail, while activating receptors bind to adaptor molecules possessing a immunoreceptor tyrosine based activating motif (ITAM) [73].

Function	NK cell receptor	Endogenous ligand
Activating/ Co-activating	KIR	HLA-class I
	NKp30	B7-H6
	NKp44	?
	NKp46	?
	NKG2C/CD94	HLA-E
	NKG2D	MIC A/B, ULBP 1-6
	DNAM-1	PVR, Nectin-2
	FcγRIIIa (CD16)	IgG
Inhibitory	KIR	HLA-class I
	NKG2A/CD94	HLA-E
	LIR-1	HLA-class I
	KLRG1	E-cadherin
	NKR-P1	LLT1
	LAIR-1	Collagen
	Siglec-7	Sialic acid
	Siglec-9	Sialic acid
	IRp60	?

Table 3. Selected NK cell receptors and their cellular ligands. Adapted after [74].

In addition to KIRs and NKG2/CD94 receptors, NK cell activation is governed by several activating NKRs. Maybe the best-studied is the activating receptor NKG2D. The endogenous proteins MHC class I chain related gene (MIC) A/B and UL 16 binding proteins (ULBPs) 1-6 are known to bind NKG2D [75, 76]. NKG2D ligand-expression is induced in cells after DNA damage and/or virus infection. It has been demonstrated that MICA and ULBP 1-3 are upregulated in non-tumor cells after the induction of DNA brakes and DNA replication arrest [77].

Tumor cells are genetically instable and often display DNA aberrations that may trigger the expression of NKG2D ligands. Similarly viruses can cause cellular stress that induces MIC and/or ULBP protein expression. Both CMV and HIV have been demonstrated to induce the expression of NKG2D ligands [78, 79].

CD16 (FcRγIIIa) is an activating NKR that can trigger antibody dependent cytotoxicity by binding of the Fc part of immunoglobulins present on opsonised pathogens or antibody coated host cells [80]. Other activating NKR include DNAM-1 and NKp30, 44 and 46. PVR (CD155) and nectin-2 (CD112) have been demonstrated to bind DNAM-1 [81] but the ligands for the NKp receptors remain elusive.

In addition to activation by direct interaction with tumor or infected host cells, NK cells can also be activated in an indirect fashion by cytokines. DCs and monocytes secrete a variety of cytokines like IL1, -12, -15 and -18 (hereafter collectively referred to as monokines) as well as type I IFN. In fact, these cytokines have the ability to induce the expression and secretion of cytokines by NK cells and increase NK cell cytotoxicity [45]. This suggests that for optimal activation NK cells need both indirect signals supplied by cytokines and direct interaction with the target cell.

1.1.3.2 NK cell subsets and their immunomodulatory function

In humans, NK cells can be divided into at least two distinct subsets based on their expression of CD56. In peripheral blood roughly 90% of the NK cells express low levels of CD56 (CD56dim) while the remaining 10% have high expression of CD56 (CD56bright) [80]. The rational behind the division into two distinct subsets is derived from various studies providing evidence for differences in the phenotype and function of CD56dim and CD56bright NK cells. CD56dim NK cells express high levels of CD16 while CD56bright cells have no or only low levels of this activating NKR [82]. In addition, CD56bright cells have low or no expression of KIRs but a high expression of the inhibitory NKR CD94/NKG2A, while the opposite is the case for CD56dim NK cells [83, 84]. This phenotypical difference has implications for the functions of the two NK cell subsets. The almost exclusive expression of activating NKRs on CD56dim cells suggests a role for those cells in killing. This notion is substantiated by the fact that CD56dim NK cells are more granular and have a higher resting cytotoxicity than CD56bright cells [85]. On the other hand CD56bright cells have been suggested to be more potent in the production of cytokines and chemokines following cytokine stimulation [10, 45]. This might in part be attributed to the higher expression of some monokine receptors on CD56bright cells. Several hypotheses attempts to explain the developmental relationship between the two NK cell subsets (reviewed in [80]). It has been proposed that CD56bright cells represent a more immature NK cell that can develop into CD56dim NK cells in the periphery. Today, most data support the notion that CD56dim/bright NK cells might develop from a common NK cell precursor or develop from a NK cell progenitor via completely separate pathways.

NK cells have a unique ability to shape the immune response. Although NK cells maybe best known for their ability to produce great amounts of the prototypical Th1 cytokine IFN-γ they can produce several other cytokines including the Th2 priming cytokines IL10 and IL13. Interestingly, the induction of the different cytokines can be manipulated by monokines. The presence of IL15 seems to be required for optimal induction of Th2 cytokines, while a combination of IL12 and IL18 gives rise to high production of IFN-γ [45]. Lymph nodes are the major site for the activation of the

adaptive immune response. NK cells have been demonstrated to be present in relative large quantities in secondary lymphoid organs. At such sites, NK cell derived cytokines could efficiently influence T-cell activation. Interestingly, NK cells found in lymph nodes phenotypically and functionally resemble peripheral blood CD56bright NK cells [86]. Furthermore, NK cells can produce TNF-α and GM-CSF and thereby directly influence DC and macrophage maturation [45]. Another way of shaping the immune response is the direct killing of resting DCs and activated macrophages by NK cells [87, 88].

1.1.3.3 NK cells in virus infection and virus evasion

The importance of NK cells during virus infections has been demonstrated in mice for several viruses. Cell culture experiments with human NK cells have shown that they can recognize and respond to cells infected with different viruses. Moreover, specific HLA/KIR haplotype combinations seem to be beneficial during certain virus infections, suggesting that NK cells are involved in the immune response to these viruses. The fact that many viruses have evolved intricate mechanisms to evade NK cell recognition further strengthens the notion that NK cells are an important part of the immune response during virus infections.

Maybe the most extensively studied virus with regards to NK cells is CMV. Studies in mice have shown that resistance to mouse CMV (MCMV) is conferred by NK cells. In C57/BL6 mice, binding of the virus protein m157 to the activating NKR Ly49H is important for MCVM clearance [89]. Viruses with a mutated m157 protein, unable to bind Ly49H, are more virulent then their wild type counterparts [90]. In humans, CMV infections drive the preferential expansion of NK cells expressing the activating NKR NKG2C/CD94 [91]. Importantly, humans specifically lacking functional NK cells can suffer from life threatening CMV infections [92]. Evidence point towards a role of NK cells in controlling other herpesviruses like Ebstein-Barr virus and herpes simplex virus (reviewed in [93]). In cell culture experiments it has been shown that human NK cells can recognize and kill cells infected with vaccinia virus, a member of the *poxviridae* family, in a NKp30, -40 and, -46 depended manner [94]. Since HLA-E is selectively downregulated after infection with vaccinia virus, NK cells expressing the inhibitory receptor NKG2A/CD94 can preferentially kill infected cells [95]. Moreover, influenza virus infected DCs can be lysed by autologous human NK cells. Inhibition of NKG2D and NKp46 signaling by neutralizing antibodies can block NK cell recognition in this system [96]. Epidemiological studies have shown that individuals carrying distinct HLA/KIR allele combinations have a higher likelihood of clearing infection with HCV or show a slower progression to AIDS after HIV infections, respectively [97, 98]. This suggests that NK cells may confer at least some protection from HIV and HCV infections. Several studies show that NK cells are important in the immune response to coxsackievirus infections (discussed in more detail in section 1.2.1.2.2 of this thesis).

The fact that many viruses try to interfere with NK cell responses indicates that evasion of NK cell recognition is important for these viruses to successfully establish a productive infection and ensure virus spread. Viral evasion of NK cell recognition is again most carefully studied for CVM and MCMV. As many other viruses, CMV has the ability to downregulate MHC surface expression, probably as a mean to avoid recognition by T-cell. However, this should render cells susceptible to NK cell mediated lysis. To escape NK cell recognition, the virus has evolved mechanisms to

selectively downregulate certain HLA alleles but spare others [99]. The leader peptide of UL40 can bind to HLA-E and thereby retain HLA-E on the cell surface [100]. This could potentially protect infected cells from recognition by NK cells expressing the inhibitory receptor NKG2A/CD94. LIR-1 is an inhibitory NKR that binds a conserved structure on most HLA alleles. CMV expresses the LIR-1 binding protein UL18, which could potentially inhibit LIR-1 expressing NK cells [101].

CMV also expresses several proteins that prevent infected host cells from displaying activating NK cell ligands. For example, the CMV encoded protein UL16 can down regulate the surface expression of MICB and ULBP 1 and 2 [102]. Infection of human fibroblasts with a strain of CVM lacking UL16 makes them more susceptible to NK cell mediated killing compared to cells infected with wild type CMV [78]. In addition, the CMV protein UL142 has been shown to interfere with the host cell expression of MICA [103]. Moreover, a novel way of downmodulating host protein expression has recently been demonstrated for CVM. Besides the interference with MICB expression by virus proteins, CMV expresses the miRNA UL112 that binds to the mRNA of MICB and thereby reduces expression of the protein [104].

As CMV, HIV has evolved a mechanism to selectively downregulate HLA molecules. The HIV protein Nef can down regulate HLA-A and B while leaving HLA-C and HLA-E surface expression unaltered [105, 106]. This selective effect on HLA expression may represent the best compromise for HIV infected cells to avoid both T- and NK cell recognition. Recently, Nef has also been shown to interfere with the expression of MICA and ULBP1 and 2 [107].

1.2 THE MAJOR HISTOCOMPATIBILITY COMPLEX

In order to determine if a cell has become aberrant or infected by microorganisms the immune system needs to be able to survey the content of any given cell. This is achieved by the presentation of the cell content to specialized immune cells. NKT cells can recognize lipid antigens presented by CD1 molecules, while T cells bind to MHC molecules presenting degraded protein antigens [3].

The MHC constitutes a family of highly polymorphic molecules, also termed HLA in humans. Based on their structure and function HLA molecules can be divided into two classes. All cell types can express class I HLA molecules. They are made up of a polymorphic 44 kDa α-chain that pairs with the constant 12 kDa β2-microglobulin (β2M) chain. The α–chain determines if a molecule belongs to the HLA-A, -B or -C family. The function of class I HLA molecules is to present peptides derived, mainly but not exclusively, from intracellular proteins. After protein degradation by the proteasome, peptides will be transported to the endoplasmic reticulum (ER) in a transporter associated with antigen presentation (TAP) dependent manner. With the help of tapasin and the calcium-binding chaperone protein calreticulin, peptides are loaded into the binding groove of class I HLA molecules. Finally, the HLA-peptide complex binds to β2M and is transported to the plasma membrane, were it can be recognized by cytolytic CD8+ T-cells. Besides the classical class I HLA molecules cells can also express the non-classical HLA class I molecules HLA-E and –G [108].

Class II HLA molecules are expressed mainly by APCs like B-cells, DCs and macrophages. They are composed of two polymorphic α- and β-chains. Class II HLA molecules are divided into HLA-DQ, -DR and -DP molecules made up of different α- and β-chains. Peptides presented on these molecules are derived from proteins taken up

by APCs from the cell surrounding. Microbes or dying cells will be endocytosed by the cell, transported into the lysosome and than proteolytically degraded. Class II HLA molecules synthesized in the ER are transported to the lysosome where they are loaded with the exogenous peptides. After translocation to the plasma membrane CD4+ T-cells can bind to the class II HLA-peptide complex [109].

Certain class I and II HLA alleles predispose for autoimmune diseases. For example 30 – 50% of all children with T1D carry the HLA DR3/4 genotype [110].

1.3 ENTEROVIRUSES

Enteroviruses (EVs) are small non-enveloped positive single stranded RNA (+ssRNA) viruses belonging to the family of *picornaviridae.* So far over 60 serotypes have been isolated from man and grouped into the 5 species of Poliovirus and human enteroviruses A - D. EVs are spread by the fecal-oral route, however, transmission via the respiratory route has also been proposed. Their +ssRNA genome serves as mRNA and is translated into a single polyprotein, which is subsequently cleaved into the individual proteins by virus-encoded proteases. EVs have a small genome of approximately 7500 kb that encodes 4 structural and 7 non-structural proteins. Cleavage intermediates have been reported to carry out functions distinct from the mature proteins. The replication of the virus occurs in the cytoplasm of the host cell. In a first step the positive RNA strand is transcribed into a negative RNA strand. This serves as a template for the production of new positive RNA strands, which are incorporated into newly formed virus particles [111].

1.3.1 Coxsackievirus group B

Coxsackieviruses of the group B (CVB) belong to the species human enterovirus B and comprise 6 different serotypes [112]. Infections with CVB are often subclinical or present with only mild, common cold-like symptoms [111]. In rare cases CVB infections cause more severe manifestations. In addition to respiratory symptoms, CVB is a major cause of aseptic meningitis, myocarditis and pancreatitis. CVB has also been associated with chronic conditions such as dilated cardiomyopathy (DCM) and type 1 diabetes (T1D). This is supported by studies detecting Enterovirus RNA and/or protein in the heart muscle of patients with DCM [68, 113] and in the pancreatic β-cells of T1D patients [114, 115], respectively. The broad spectrum of diseases associated with CVB reflects their wide tissue tropism. After infection of mice, CVB can be recovered from most organs including pancreas, heart, lung and small intestine [116]. In humans CVB and/or EV RNA or protein has been detected among others in the pancreas, heart muscle, duodenum and periferal blood mononuclear cells [113, 115, 117, 118].

1.3.1.1 Interaction of coxsackievirus group B with the host cell

The main receptor for CVB entry into the host cell is the coxsackievirus and adenovirus receptor (CAR) [119]. CAR is a member of the immunoglobulin superfamily implicated in cell-cell adhesion and is expressed for example by epithelial cells of the intestine, pancreas, and heart as well as cardiomyocytes [120]. Many CVB serotypes can also utilize decay accelerating factor (DAF) as a co-receptor [121]. Even though CAR is used for cell entry by most CVB strains, it has also be shown that CVB can adapt to infect cells deficient in CAR [122].

After the initial infection of the cell, CVB has to manipulate the host cell machinery in order to initiate protein translation, RNA replication and to release virus progeny from the cell. Major alterations of host proteins have been described after CVB infection (reviewed in [123]). Viral proteases have been reported to cleave host proteins such as the eukaryotic initiation factor 4G and the inhibitor of κBα [124, 125], which could result in the shut-off of host protein synthesis and an impairment of cytokine production, respectively. The non-structural proteins 2B, its precursor 2BC and 3A have been shown to interfere with the host secretory pathways by disrupting protein transport trough the ER and Golgi network [126, 127]. This may profoundly impede the cell's ability to initiate a proper immune response due to diminished cytokine secretion and decreased upregulation of membrane proteins such as MHC molecules. In addition, CVB can manipulate the induction of apoptosis and necrosis in the host cell by several mechanisms [124, 128, 129]. Ultimately, most CVB infections result in the lysis of the host cell, which facilitates the release of newly formed virus particles. However, it is becoming increasingly clear that CVB can establish a persistent or latent infection under certain conditions. Exactly how and when CVB may establish persistent/latent infections is not well understood. It has been suggested that the cell cycle state of the host cell can influence the outcome of a CVB infection. In actively dividing cells infection will progress to cell lysis, while infection of resting or quiescent cells may result in persistency/latency of CVB [130]. However, it is not clear how CVB particles are released from cells persistently infected with CVB without an obvious deleterious effect.

1.3.1.2 The host immune response to coxsackievirus infection

Many studies using mouse models have addressed the immune response to CVB. Only few attempts have been made to address the human immune response elicited by these viruses. Some studies have been performed using cell culture systems, while additional information has been provided from epidemiological studies and the examination of biopsies from CVB infected tissues. Together with the studies in mouse models, this information clearly indicates that both the innate and the adaptive immune response are indispensable for the control of CVB. However, increasing evidence suggest that the immune system also contributes to the pathology seen after CVB infection [131].

1.3.1.2.1 Detection of coxsackievirus and the interferon response

The host possesses several receptors that facilitate the detection of invading viruses as described in section 1.1.1 of this thesis. All RNA sensing TLRs have been implicated in the detection of CVB. TLR7 and 8 have been shown to recognize CVB in human cardiac cells and pDCs [132, 133]. In monocytes, TLR3 has been suggested to be important for CVB detection [134, 135]. Maybe somewhat surprising, TLR4 has been shown to detect a structural motif of CVB [136]. The role of intracellular RNA sensors has not been extensively studied. However, the 5´ end of the CVB RNA is covalently linked to the virus protein VPg [137], suggesting that RIG-I cannot recognize CVB because of its requirement for a 5´ phosphate in the 5´ end [23].

One of the most immediate host cell responses to virus infection is the production of type I IFNs. Several cell types have been shown to produce type I IFNs after CVB infection, including pancreatic islets, cardiac cells and hematopoietic cells [132, 138,

139]. The host's ability to mount an IFN response is crucial, since a lack of IFN-β or the type I IFN receptor results in rampant virus replication and early mortality [140, 141] in mice. The presence of IFN-α and type I IFN signaling is also important for the protection of host cells from CVB induced cell death [138, 142]. The protective effect of type I IFNs is probably mediated by the upregulation of antiviral proteins. In fact, mice lacking the known ISGs PKR or inducible nitrix oxide synthase (iNOS) succumb to an infection with CVB at a dose that is not lethal for wild type mice [60, 143].

In the context of CVB infection, IFN-γ has been shown to protect the host from lethal infection, virus induced tissue damage and myocarditis [144, 145].

1.3.1.2.2 The role of NK cells in coxsackievirus infection

Given their central role at the intersection between the innate and adaptive immunity, surprisingly little is known about the contribution of NK cells to the human immune response to CVB infection. In mouse models of CVB-induced pancreatitis and myocarditis, it has been shown that NK cells are important in limiting virus replication and thus tissue damage [146, 147]. In addition, the susceptibility of different mouse strains to CVB-induced pancreatitis is inversely correlated with their endogenous NK cell activity [148]. The protective effect of NK cells might be independent of their ability to kill infected cells since mice deficient in perforin do not show increased virus replication [149].

In humans only indirect evidence for a contribution of NK cells in the host response to CVB is available. In seven patients with acute myocarditis, NK cells were found in the lesions of the heart [150]. However, since a viral cause for the myocarditis was not confirmed, one can only speculate that at least in some of the 7 cases myocarditis might have been caused by a CVB infection. The presence of NK cells in the heart after CVB infection has also been observed in mice [151, 152]. More conclusive results came from a study of six patients with recent onset T1D. In three patients, which all showed signs of enterovirus infection of the β-cells, the lymphocyte infiltration around the pancreatic islets consisted mainly of NK cells. Interestingly, no NK cells could be detected in the pancreas of the remaining patients having no detectable signs of virus infections [153]. However, the presence of NK cells around the islets could not be confirmed by another study investigating 29 pancreata from recent onset T1D patients [154].

1.3.1.2.3 Adaptive immune response to coxsackievirus infection

During the early phase of infection the innate arm of the immune system limits virus replication and spread, thereby preventing excessive tissue damage. In order to achieve clearance of the infecting agent and long-term immunity, the host has to mount a strong adaptive immune response to the invading pathogen. The importance of neutralizing antibodies for the control of CVB is supported by the observation that patients with agammaglobulinaemia are highly susceptible to enterovirus infection [155]. Likewise, mice lacking B-cells are unable to clear CVB and show persistent CVB infection [156].

The contribution of T-cells in CVB infections is not as clear as for B-cells. On one hand it has been demonstrated that T-cells are part of the infiltrating lymphocytes in virus-induced pancreatitis and myocarditis, and are able to limit virus replication [157, 158]. On the other hand several studies failed to isolate virus-specific T-cells from mice

after infection. In fact, CVB seems to be able to suppress T-cell activation, since mice infected with a recombinant virus carrying known immunodominant CD4+ and CD8+ T-cell epitopes, fail to mount a T-cell response towards these epitopes [159, 160].

1.3.1.2.4 Pathology caused by the immune system after Coxsackievirus infection

As described above the ability to mount an immune response is important for the host in order to limit virus replication and eventually clear CVB. However, under certain conditions immune cells may also help in spreading the virus. In addition, the immune response, if not balanced properly, can also be detrimental and contribute to CVB associated disease.

Several studies have suggested that T-cells can exacerbate CVB-induced pathology. In a mouse model for pancreatitis caused by CVB, CD8+ T-cells were important in the early phase of the infection but contributed to tissue damage at later time points. Mice lacking CD4+ T-cells had lower virus titers and presented with milder pathology [158]. In an experimental model for CVB-induced myocarditis the antiviral function of CD8+ T-cells could be uncoupled from their pathological effect by genetic deletion of perforin [149]. Mice lacking perforin cleared CVB with similar efficacy as wild type mice, but suffered less damage to their heart muscle. Similarly, B-cells are needed to clear CVB but at the same time help to spread the virus at the early phase of infection [156]. B-cell may also facilitate increased virus infectivity by a more indirect mechanism. It has been shown that CVB specific antibodies, especially non-neutralizing antibodies, can enhance virus infection of cells previously not permissive to CVB [161]. As for B- and T-cells, NK cells are important for controlling CVB infection but have also been shown to contribute to CVB induced disease. In a mouse model for virus-induced diabetes, the depletion of NK cells almost completely prevented diabetes development [142].

The pathological effect of immune cells after virus infection can be mediated by several mechanisms. In CVB-induced myocarditis and possibly diabetes, the attempt to clear virus infected cells through direct killing by T- or NK cells may result in extensive irreversible tissue damage. A more indirect mechanism for immunopathology could be the inflammation caused by the virus. In contrast to the immune response directed against infected cells, inflammation may also damage surrounding cells and tissues. This mechanism has been proposed to be important in CVB-induced pancreatitis [162]. Interestingly, mice lacking MyD88, the adaptor protein utilized by most TLRs, show increased survival and less tissue damage after CVB infection [163].

Taken together, it is becoming increasingly evident that the immune response, while being important in controlling CVB infections, also contributes to the pathology associated with these infections. In fact, it may be hard to delineate to what extent damage is induced directly by the virus and how much is caused by either an imbalanced, misdirected and/or excessive immune response.

1.4 TYPE 1 DIABETES

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of the insulin producing β-cells within the pancreatic islets of Langerhans. This results in insulin deficiency, which requires life long treatment [164]. The early events causing the initiation of the autoimmune process remain mostly elusive, however, over the last

decades many studies have added to our understanding of the pathogenesis of T1D. It has been demonstrated that most patients develop both autoreactive T- and B-cells recognizing β-cell antigens [165, 166]. Studies in mouse models have clearly shown that T-cells are involved in the destruction of the beta cells, while the role of B-cells is more controversial and has been mostly linked to their function as APCs [167, 168]. A phase II clinical trial with a B-cell depleting anti-CD20 antibody showed promising results in preserving residual β-cells functions in recent onset T1D patients [169], indicating a pathogenic role for B-cells in human T1D. Differences in the function and/or activation in other immune cells such as NKT-cells [170, 171], macrophages [172] and DCs [173] have been described in patients and mouse models of T1D. The role of NK cells in the development of T1D is discussed in more detail in section 1.4.3 of this thesis.

Advances in technology have made it possible to define gene variants that predispose individuals to T1D. Most of the genes found are involved in regulating immune functions. The major genetic risk factors for T1D are HLA class II genes, with HLA DR3 and DR4 conferring the highest risk. Other gene variants that increase the likelihood of T1D development have been mapped to genes encoding insulin, PTPN22, IL-2, IL-2Ra and CTLA-4 among others [174]. Interestingly, recently polymorphisms in the gene for the intracellular virus sensor MDA-5 has been linked to T1D [175, 176].

The discordance for the disease in monozygotic twins and the increase in T1D incidence in many countries [177, 178], show that disease development can only partially be explained by genetic factors. Several environmental factors have been studied for their potential involvement in the etiology of T1D. Breast-feeding has been shown to be protective, while exposure to cow milk proteins early in life is suggested to be a risk factor for the development of the disease [179]. Vitamin D has been proposed to protect from T1D [180], which could potentially explain the lower T1D incidence in countries closer to the equator.

1.4.1 The islet of Langerhans in health and type 1 diabetes

Pancreatic cell types responsible for the production of endocrine hormones are clustered in the islet of Langerhans (called islets hereafter). Islets contain four different cell types each specialized in the production of one hormone. β-cells make up most of the cells within the islets [181] and secrete insulin after stimulation with for example glucose. Insulin induces the translocation of glucose transporter 4 (Glut4) to the plasma membrane of muscle and adipose cells, enabling the up take of glucose from the blood stream [182]. Low blood glucose levels trigger the release of glucagon from α-cells, which stimulates gluconeogenesis in the liver and glucose release. Somatostatin and pancreatic polypeptide, two hormones with a broad regulatory function of the endocrine system, are produced by the δ- and PP-cells, respectively [183]. The pancreas of a healthy individual contains approximately 1 million islets that constitute around 1 to 1.5% of the total pancreas mass [184].

The nature of the immune-mediated destruction of β-cells has been studied extensively in the non-obese diabetic (NOD) mouse model of T1D. Like humans, NOD mice develop autoreactive T-cells specific for β-cell antigens. Such T-cells can migrate to the pancreas after the autoimmune process is initiated. In the beginning, T-cells are localized around the islets and at later stages of disease development they invade the islets. Besides T-cells other cell types such as B-cells, macrophages and NK cells, are

found in the immune cell infiltrate around and within the islets. The cellular composition of the islet infiltrating cells is less well studied for humans. A recent study by Willcox et al. attempted to address this question by identifying the infiltrating cells around the islets in a set of pancreata from recent onset patients. They found that at early time points CD8+ T-cells and macrophages dominated the infiltrate, while the percentage of CD20+ B-cells increased with progressing β-cell destruction. CD4+ T-cells were found at all stages but less frequently than CD8+ T-cells and macrophages [154]. Although NK cells were only rarely detected in this study, another report suggested that, at least in a group of patients, NK cells may represent the main cell type found around the islets after T1D onset [153]. Interestingly, the islet architecture was different in the patients of the latter study compared to the classical pathological picture normally seen in T1D. Within the islets a large amount of insulin producing cells were still present. In addition, β-cells were positive for staining with an antibody detecting EVs. Intriguingly, it has been shown that infection of human islet cells with some CVB strains impairs insulin secretion [185].

It was long believed, that at the time of overt T1D onset, almost all β-cells are destroyed, a notion that was supported by immunohistological studies of pancreata from patients. However, it has now been reported by several studies that a considerable percentage of patients still have a significant number of insulin positive cells within the islets at the time of onset [153, 186]. These results are puzzling because patients still have no or very low serum insulin levels and require treatment with insulin. This suggests that the cause of insulin deficiency, at least in a group of patients, cannot entirely by explained by β–cell destruction and that our current hypothesis of the etiology of T1D needs to be revised.

1.4.2 Virus infection as environmental factor in development of type 1 diabetes

Virus infections have for a long time been suggested to be important environmental factors in the etiology of T1D. Several viruses have been studied for their possible association with T1D, with the most convincing evidences pointing towards congenital rubella infections and enteroviruses [187]. The viruses most often implicated in the development of T1D are group B coxsackieviruses. Already 50 years ago it was shown that antibodies against CV were more prevalent in T1D patients than in control individuals [188]. A strain of CVB serotype 4, isolated from a recent onset T1D patient in 1979 by Yoon et al., could also induce the disease in mice [189]. Since then, many studies have addressed the potential role of CVB or enterovirus infections in T1D. In a prospective study, EV infections were associated with the appearance of autoantibodies [190]. Furthermore, several studies confirmed the initial findings that recent onset T1D patients more often than healthy conrols show signs of CVB or enterovirus infections [191, 192].

Several mechanisms have been proposed by which CVB infections could induce T1D. It has been demonstrated both *in vivo* and *in vitro* that CVB can directly infect mouse and human beta cells [60, 138, 193, 194]. The presence of EVs and/or CVB in the islets of T1D patients, but rarely in controls has been confirmed by several studies [114, 115, 153]. An inability of the host to control virus replication in the beta cells, for example due to a lack of type 1 IFN production or signaling, can result in direct virus induced lysis of the β-cells [138, 142]. In addition, cytokines that are produced by

immune cells after CVB infection may contribute to β-cell death. It has been shown that treatment of isolated human and rodent islets cells with either TNF-α and IFN-γ or IL1β and IFN-γ can have a deleterious effect on β-cells by the induction of apoptosis [57].

Another possible process that could give rise to CVB-induced T1D is the so-called bystander activation of autoreactive T-cells. The inflammatory milieu created after infection can result in the migration of potentially β-cell specific T-cells to the pancreas. In addition, previously sequestered autoantigens may be released by virus and/or cytokine induced damage to the β-cells [195]. In combination this may lead to the recruitment and priming of autoreactive T-cells.

A third proposed mechanism is a cross-reactivity between CVB and endogenous antigens, a mechanism termed molecular mimicry. It has been demonstrated that some autoantigens have a sequence homology to CVB peptides [196, 197]. In fact, cross reactivity of antibodies raised against CVB peptides with autoantigens has been reported [197].

The time point at which the host encounters a virus infection might be important in determining its effect on the autoimmune process. Studies in diabetes prone mice suggest that infections are protective before the autoimmune response is established, while they exacerbate the disease at a later stage [198].

1.4.3 NK cells in type 1 diabetes

NK cells have not been studied as extensively for their involvement in the development of T1D as other cell types. However, their ability to directly kill for example infected cells and to produce cytokines that can shape the adaptive immune response could make them potential players in the autoimmune process. The finding that a HLA KIR haplotype favoring the activation of NK cells is found more often in patients with T1D than in healthy individuals could suggest a scenario in which NK cells contribute to the development of T1D [199]. Reduced numbers of peripheral NK cells with an increased activation state have been reported for recent onset T1D patients [200]. A study by Dotta et al. further supports an involvement of NK cells in the diabetes process [153]. Of note, recently it has been shown that NK cells are present in the pancreas of several mouse strains and are part of the infiltrating lymphocytes around the islet of pre-diabetic NOD mice [201]. In several mouse models for T1D the depletion of NK cells results in an attenuation or prevention of disease development [202-204]. In a mouse model of virus induced T1D the ability of CVB4 to induce the disease was shown to depend on the presence of NK cells [142].

Taken together, a growing body of evidence suggests that NK cells could contribute to the development of T1D. However, the exact mechanism by which NK cells might promote the development and/or progression of the disease, and at what stage of the autoimmune process is still not delineated. In addition several factors have to be considered when interpreting available studies; 1) Both in humans and mice the identification of NK cells relies mostly on a combination of surface markers, which makes it difficult to identify NK cells *in vivo*. 2) Functional surface receptors such as NKG2D are also expressed on a subset of T- and/or NKT-cells. 3) The function and/or phenotype of NK cells in the blood might not reflect their state at the site of inflammation.

1.5 CYSTIC FIBROSIS

Cystic fibrosis (CF) is an inherited autosomal recessive disease caused by a defect in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein [205]. The prevalence of CF varies greatly between different populations ranging from 1:1350 in Ireland to 1:25000 in Finland [206]. Several hundred *cftr* mutations have been described but a deletion of the phenylalanine residue at position 508 (F508del) is by far the most common. This mutation is found in approximately 70% of all defective *cftr* alleles in the Caucasian population [207].

CFTR is expressed by many cell types and functions as a cAMP-regulated chloride channel [208, 209]. The absence or a reduced function of CFTR results in an imbalance of ion transport across epithelial membrans that causes the production of a thick viscous mucus and disruption of proper secretion in organs such as the lung, intestine and pancreas. As a consequence of this, patients suffer from many different symptoms. Common problems include intestinal obstruction, meconium ileus, pancreas insufficiency and chronic infections of the lungs, with the latter being the greatest contributing factor for morbidity and mortality [207]. Improved clinical care and treatment of patients has resulted in a dramatic increase in life expectancy over the last decades. This has led to the appearance of other clinical manifestations presenting later in life. In patients above 20 years of age, up to 40-50% suffer from impaired glucose tolerance or overt diabetes. Cystic fibrosis related diabetes (CFRD) is now recognized as a major co-morbidity factor in CF [210].

1.5.1 Immuno-dysregulation in cystic fibrosis

CF patients often fail to clear bacterial infections resulting in chronic bacterial colonization of the lungs. Recent research also suggests an increased susceptibility to respiratory virus infections in both patients and mouse models for CF [211-213]. This indicates that an impaired anti-microbial defense may be a direct consequence of CF. In fact, several defects in the innate immune response have been found. CF patients show lower levels of exhaled nitric oxide (NO), a second messenger molecule with well-described immune function [214]. The enzyme inducible NO synthase (iNOS), which catalyzes the production of NO, is decreased in airway epithelial cells of CF patients and a mouse model for CF [215, 216]. This has been attributed to an increased expression of the protein inhibitor of activated Stat1 (PIAS) leading to a reduction of the IFN regulating factor 1 (IRF1) [217]. Another study found a decrease in the basal expression level of the anti-viral protein OAS1 in CF lung epithelial cells compared to normal cells [215].

Given that CF patients are more susceptible to infections with bacteria and viruses, the finding that CF is associated with a proinflammatory phenotype, might be counterintuitive. However, several studies have confirmed that CF patients show increased expression of proinflammatory cytokines [218-220]. This phenotype is, at least partially, a consequence of the defect in CFTR and not only due to chronic infections, as the same phenotypes have been observed in mouse models of CF and in cell cultures of CF lung epithelial cells [215, 221]. The underlying mechanism that link *cftr* mutations to the increased expression of proinflammatory cytokines has not been delineated, however recently a defect in the acid sphingomyelinase pathway has been suggested to be a contributing factor [221].

It is of interest to note that most studies addressing the immune functions in CF have been focused on the airways. Since other organs are also affected by a defective CFTR, it can be assumed that similar defects are present outside the lung. Indeed, for both the pancreas and intestine a proinflammatory phenotype has been reported [220, 222, 223].

1.5.2 Mouse models for cystic fibrosis

Mouse models are helpful tools for studies on human diseases. Several murine models for CF are available today [224]. The first CF mice were created by disruption of the *cftr* gene, resulting in complete absence of cftr mRNA and protein [225]. Most of those mice displayed a very severe phenotype, often with high mortality early after birth or weaning. Providing mice with a liquid high nutrient diet increased survival [226]. Later on, mouse models carrying specific human *cftr* mutations were created. Three mouse strains harboring the F508del mutation and one carrying the G551D and G480C mutations, respectively are available today. Because in these mice CFTR mRNA is produced, which allows the expression of the aberrant protein and mirroring the situation in human CF, they show milder symptoms and improved viability [227-231].

Even though none of the CF mice resemble human CF in every aspect, many of the phenotypes seen in patients are also found in the CF mouse models. Intestinal pathology is apparent at varying degrees in all CF mice [232]. Interestingly, this seems to correlate with the remaining expression of CFTR with milder pathology in mice that do express at least some protein. When challenged with bacteria or bacterial lipopolysaccharide all mouse strains tested respond with hyperinflammation and show impaired bacterial clearance. Defects in the antiviral response have also been demonstrated for some CF mice [211, 233]. Pancreatic abnormalities are absent or only very mild in all mouse models available [232].

Interestingly, while the proinflammatory phenotype, seen in CF patients, is also present in the lung, pancreas and intestine of CF mice, more severe pathology is often only seen after challenging the mice with microorganisms or after other manipulations [211, 223]. This, together with the observation that housing conditions influence the phenotype and viability of CF mice [231], suggest that environmental factors, especially infections, influence CF pathology.

2 AIMS OF THIS THESIS

Coxsackievirus group B (CVB), a member of the *picornaviridae* family, is a common virus that in most instances only causes mild symptoms. However, under certain conditions it can cause severe disease such as myocarditis or pancreatitis. How infections with CVB can result in such different outcomes is puzzling. One possible explanation is that more pathogenic virus variants cause the more severe diseases. However, it is very likely that the host itself plays an important role in determining the outcome of an infection with CVB. In particular the immune response mounted by the host after infection may influence the severity of a CVB infection.

The overall aim of this thesis was to extend our knowledge on the contribution of the innate immune system in the response to CVB infection, in the hope that a better understanding for the virus-host interactions can help to determine the underlining mechanisms that lead to severe CVB induced pathology.

Specific aims

To determine if the intracellular virus sensor MDA-5 is important for the recognition of CVB by studying the consequences of a CVB infection in the absence of MDA-5. (**paper I**)

To evaluate how CVB influences the expression of ligands for NK cell receptors on the surface of infected cells. (**paper II**)

To study if CVB infection alters susceptibility to NK cell mediated killing and if NK cells become activated after encountering CVB infected cells. (**paper II**)

To analyze the expression of antiviral proteins in human islets of Langerhans after IFN stimulation. (**paper III**)

To investigate if the expression of IFN stimulated genes prevent CVB replication in human islets of Langerhans. (**paper III**)

To study the antiviral response towards CVB in a mouse model for cystic fibrosis. (**paper IV**)

3 RESULTS AND DISCUSSION

3.1 RECOGNITION OF CVB INFECTION BY THE HOST

3.1.1 Intracellular recognition of CVB by MDA-5

In order to initiate an immune response to an infection it is crucial for the host to recognize the invading pathogen. As described in section 1.1.1 of this thesis, PRR receptors are one of the major means by which the host detects potentially dangerous microorganisms and induces the production of inflammatory cytokines and IFNs. Type I IFNs are particularly important in the defense against CVB infection [140, 141]. Interestingly, polymorphisms in the gene for the intracellular virus sensor MDA-5 have been linked to an increased risk for the development of T1D [175, 176], offering a potential explanation to how genetic variations could influence susceptibility to virus-induced diabetes.

Since it was not known if MDA-5 is important for the recognition of CVB we wished to address the role of MDA-5 in CVB infection (**paper I**).

We infected mice deficient in MDA-5 and wild type (wt) controls with CVB serotype 3 (CVB3) and studied virus replication, the induction of IFNs and ISGs and the consequences of CVB3 infection for the host with regards to tissue damage and host survival. We found that mice lacking MDA-5 on a C57/BL6 background (hereafter denoted mda-$5^{-/-}$ (B6)) were strikingly more susceptible to CVB3 infections that their wt counter parts (**paper I, Figure 1A**). Surprisingly, mice deficient in MDA-5 on a SvJ/129 background (denoted mda-$5^{-/-}$ (129)) did not succumb to the infection and showed no difference in survival compared to wt (129) mice (**paper I, Figure 1B**). Even though mda-$5^{-/-}$ (129) mice did not show increased mortality after infection with CVB3, the lack of MDA-5 clearly impaired their ability to control virus replication (**paper I, Figure 2C**). Three days after infection CVB titers in pancreata were several orders of magnitude higher in mda-$5^{-/-}$ (129) mice compared to wt (129) mice. The difference was even more pronounced in mda-$5^{-/-}$ (B6) animals, which exhibited higher virus titers in most organs compared to wt (B6) mice three days after infection. (**paper I, Figure 2A**). Unexpectedly, virus titers did not differ between mda-$5^{-/-}$ (B6) and wt (B6) mice at day 4 after infection (**paper I, Figure 2B**). MDA-5 is important for the induction of type I IFNs after infection with different viruses [18], therefore we wanted to study the increased susceptibility of mda-$5^{-/-}$ mice to CVB3 infection could be explained by a lack of type I IFN production. This however, did not turn out to be the case. We observed a partial but not significant decrease of serum IFN-α levels 48 hours after infection in C57/BL6 animals lacking MDA-5 (**paper I, Figure 3A**). On the tissue level we observed a robust induction of IFN-β and the two ISGs OAS1a and CXCL10 even in the absence of MDA-5 (**paper I, Figure 3B-C**). The induction of IFN-β in the liver and pancreas appeared to correlate well with virus titers, as mice with low or undetectable levels of CVB3 did not upregulate IFN-β mRNA (**paper I, Supplementary Figure 3**). The increased virus replication early after infection had severe ramifications for the host. Tissue damage in the liver and pancreas was dramatically increased in mda-$5^{-/-}$ (B6) mice three days after infection (**paper I, Figure**

4A and C). Moreover, as all mice on the SvJ/129 background survived infection, we could show that damage to the exocrine pancreas had long-term consequences. Even 28 days after infection immune cell infiltration was more pronounced in mice lacking mda-5 and the tissue damage appeared to be irreversible (**paper I, Figure 4D**).

In summary, we showed that mice deficient in MDA-5 cannot control CVB3 replication early after infection, and that this had profound effects for the host. Increased virus replication lead to exacerbated tissue damage, and increased mortality of mda-$5^{-/-}$ mice on a C57/BL6 background.

These results are, at least in parts, in line with a recent study by Wang et al. [234] that also demonstrated increased susceptibility to CVB3 infection in *mda-5* knock-out mice. However, our results add to the understanding of the role of MDA-5 during CVB infection in several ways. Firstly, we report increased virus replication three days after infection in the pancreas of mda-$5^{-/-}$ mice, while this was not seen in the other study. A possible explanation for this obvious discrepancy could be a difference in the infectious dose used in the two studies. In fact, in our hands the majority of C57/BL6 mice survived an infection with 10^3 plaque forming units (PFU) of CVB3, while in the study by Wang et al. [234] all mice succumbed to the infection with the higher dose. This difference is also reflected by the amount of virus recovered from wt animals three days after infection. It is feasible that at a higher dose of virus the host is overwhelmed and the innate immune system is simply unable to control virus replication regardless of the presence or absence of MDA-5. Secondly, we showed that the increase in tissue damage early after infection, also seen by Wang et al., had long-term consequences. Inflammation of the exocrine pancreas was still present 28 days after infection indicating that the tissue damage was irreversible. Finally, our study suggests that other factors besides MDA-5 determine the severity of CVB3 infection, as mice on a SvJ/129 background eventually clear CVB, while mice on a B57/BL6 background succumb in the absence of MDA-5.

Our finding that mice lacking MDA-5 are still able to induce IFNs implies that other PRR can detect CVB *in vivo* and induce robust IFN production. In fact, studies have suggested that TLR3, -4, -7 and -8 can recognize CVB [132, 134, 135]. It is possible that one of these PRR is responsible for IFN induction in mda-$5^{-/-}$ mice. If RIG-I can also respond to CVB has not been studied. The fact that recognition by RIG-I depends on uncapped RNA carrying 5´ phosphates [23], a structure that CVB RNA lacks, makes it at least questionable. In addition, it is possible that CVB has mechanisms that could interfere with RIG-I detection. It has been shown that a protease of poliovirus, a virus closely related to CVB, can cleave RIG-I [235].

The result that mda-$5^{-/-}$ mice are unable to restrict early virus replication although they can mount an IFN response raises the question on how MDA-5 may help the host to control virus replication. It could in fact be that mda-$5^{-/-}$ mice do have a reduced IFN response. We did see a reduction in serum IFN-α levels 48 hours after infection but this difference failed to reach statistical significance. Considering the importance of IFNs for the host during CVB infection, this slight reduction might be enough to impair the host's ability to restrict CVB replication. Another possibility is that the lack of MDA-5 has a larger impact on the IFN production at even earlier time points at the local site of infection. As we have not addressed this we cannot rule out this scenario. A different way by which MDA-5 could help to restrict virus replication is by its often overlooked

ability to induce apoptosis. It has been shown that, after treatment with the viral RNA analogue pIC, MDA-5 can induce apoptosis in melanoma and non-malignant cells by upregulating pro-apoptotic proteins and the activation of caspases [236]. It is possible that MDA-5 helps the host to restrict virus spread by inducing apoptosis in infected cells.

In the light of the recently established link between MDA-5 and T1D [175, 176] it is tempting to speculate on the role of MDA-5 in virus-induced diabetes. Even though our results indicate that absence of MDA-5 *per se* does not predispose mice to virus-induced diabetes, our results might still have implications for how MDA-5 could contribute or protect the host from virus-induced diabetes. However, as the impact of the *mda-5* SNPs associated with T1D on the function of the protein is not firmly established, one can envision two rather different scenarios. One is based on the observation that two rare SNPs associated with protection from T1D abolish the ability of MDA-5 to induce IFN after stimulation with pIC [237]. This would favor the hypothesis that production of IFN after CVB infection can be detrimental and contribute to the development of T1D. However, the facts that the two SNPs of *mda-5* are very rare, with allele frequencies of around 2% and 0,5%, respectively, and that the much more common protective SNP A964T displayed no defect in pIC-induced IFN production, suggests that results linking protective *mda-5* variants to reduced IFN production, cannot be generalized. Our results cannot confirm or disproof the hypothesis that a defect in MDA-5 function may protect from CVB induced diabetes.

As we studied the outcome of CVB3 infection in the absence of MDA-5, it is easier to address the hypothesis that a reduced function of MDA-5 may contribute to CVB induced diabetes. The observation that mice deficient in MDA-5 do not develop diabetes after CVB3 infection might speak against this hypothesis. However, this result is not surprising. Pancreatic β-cells seem to be specifically well equipped to handle CVB infection. Unless IFN signaling is abrogated specifically in the β-cells [142], β-cells are spared by CVB even if the exocrine pancreas is completely destroyed by the virus [116]. Our finding that mda-$5^{-/-}$ mice are unable to control early virus replication could suggest that individuals having a less functional MDA-5 may suffer more often and/or from more severe CVB infections. This could increase the likelihood that the virus reaches the pancreas and infects the β-cells. This could have detrimental effects in persons that are genetically predisposed to developing T1D. The infection of the pancreas itself could lead to an inflammatory milieu that can attract potentially autoreactive T-cells [238]. This would fit with our observations that mice lacking MDA-5 suffer from more severe infection and show increased lymphocyte infiltration in infected organs. In addition, damage to the β-cell by CVB infection could increase the release of sequestered autoantigens and prime autoreactive T-cells, previously attracted to the side of infection [195]. Interestingly, prospective studies have suggested that individuals later progressing to T1D suffer more often from enterovirus infection [190]. The same study suggests that enterovirus infections are associated with the induction of autoimmunity as assessed by the appearance of autoantibodies shortly after infection. In this context, it should be note that mice used in our study are non-autoimmune prone mouse strains. It would be interesting to study the effect of MDA-5 deficiency in mice genetically susceptible to autoimmune diseases, such as the non-obese diabetes mouse strain.

In conclusion, the results presented in **paper I** show that MDA-5 plays an important role in the host immune defense against CVB infection by limiting early virus replication and preventing CVB induced tissue damage. These results warrant further studies about the link of MDA-5 with virus-induced diabetes.

3.1.2 NK cell recognition of CVB infected host cells

NK cells play an important role in the innate immune response to virus infection. They can limit virus replication by direct killing of infected cells, through the production of IFN-γ, a cytokine with known antiviral effects, and by activating the adaptive immune response [93]. Studies in mice suggest that NK cells are important for the host immune response after infection with CVB [147, 152]. However, NK cells have also been reported to exacerbate virus-induced pathology. In a mouse model for virus-induced T1D, NK cells were essential for the induction of disease [142].

Since little was known about the role of NK cells in the human immune response to CVB infections, we wanted to investigate if CVB has the ability to alter the expression of NK cell receptor ligands and how NK cells respond to cells infected with CVB (**paper II**).

First, we studied the direct effect of CVB infection on the expression of NK cell-ligands. Therefore, we established a flow cytometry based method that enabled us to discriminate infected from uninfected cells by staining with an antibody specific for the enterovirus coat protein VP1 (**paper II, Figure I**). The balance of activating and inhibitory signals presented on a cell determines if a NK cell will become activated [73]. The main inhibitory ligands for NK cells are HLA class I molecules [239]. Infection of the two tumor cell lines HeLa and HepG2, and primary human umbilical vein endothelia cells (HUVECs) with CVB3 resulted in a approximately 40% reduction of cell surface HLA class expression (**paper II, Figure 2A**), as assessed by staining with a pan-HLA antibody. Several viruses have evolved mechanisms to selectively downregulate specific HLA alleles while leaving others unaltered. To see if this was the reason for the incomplete downregulation of HLA molecules, we stained with antibodies specific for HLA-A, -B or -C (**paper II, Figure 2B**). All HLA alleles were affected equally by CVB3 infection, suggesting that a selective downregulation of HLA molecules cannot explain the partial reduction of HLA surface expression. The non-classical HLA class I protein HLA-E presents leader peptides from other HLA class I proteins and plays a dual role during NK cell activation. Binding of HLA-E to NKG2A/CD94 or NKG2C/CD94 inhibits or activates NK cells, respectively. Surprisingly, CVB3 infection had a much stronger effect on HLA-E surface expression compared to the classical HLA alleles. Only 15% of HLA-E remained on the cell surface after CVB3 infection (**paper II, Figure 2B**).

It has been shown that cellular stress (e.g. virus infection) may induce the expression of MICA/B and/or ULBPs, which can engage the activating NK cell receptor NKG2D. However, after infection of HeLa cells with CVB3 we did not detect an upregulation of either protein. Instead we saw a downregulation of MICA surface expression (**paper II, Figure 2B**).

As both activating and inhibitory NK cell ligands were downregulated after CVB3 infection the effect on NK cell activation was unpredictable. After becoming activated,

NK cells can respond in different ways. They can actively kill aberrant cells in a perforin/granzyme-, TRAIL- or FAS-L-dependent manner and/or by the production of cytokines such as IFN-γ [240, 241]. To study if NK cells were able to kill CVB3 infected HeLa cells, we co-cultured infected or control cells with either resting or activated peripheral blood mononuclear cells (PBMCs). Three complementary methods were used to assess killing: 1) mobilization of CD107a on the cell surface of CD56+/CD3- PBMCs, 2) the activation of caspase 3 in HeLa cells, and 3) uptake of propidium iodide by HeLa cells (**paper II, Figure 3**). All experiments indicated that NK cells are unable to kill HeLa cells infected with CVB3. As mentioned above, in addition to killing of infected cells, NK cells can also respond with the production of cytokines [46]. To investigate if NK cells become activated after encountering CVB-infected HeLa cells we measured the intracellular production of IFN-γ in CD56+/CD3- PBMCs. We observed some IFN-γ production by NK cells after co-culture with uninfected HeLa cells. However, this was dramatically increased after co-culture of PBMC with CVB3 infected HeLa cells (**paper II, Figure 4A and B**). In addition, we measured IFN-γ in the culture supernatants. Again infection of HeLa cells with CVB3 increased the amount of IFN-γ released by PBMC, which was primarily depended on NK cells (**paper II, Figure 4C**).

Taken together the results presented in **paper II** indicate that infection with CVB3 leads to the downregulation of classical and non-classical HLA molecules. Stress-inducible ligands of the activating NK cell receptor NKG2D are not induced by CVB3. In fact, infection resulted in a downregulation of MICA. The alteration of NK cell ligands did not induce NK cell mediated killing of infected HeLa cells, but stimulated the production of IFN-γ.

These results raise some interesting questions. One is how CVB can interfere with the surface expression of HLA molecules. Several not mutual exclusive mechanisms could play a role. A general host protein shut off could be responsible for the observed effects. However, prevention of protein synthesis by treatment of cells with cyclohexamide could not induce HLA downregulation to the same degree as CVB infection (**paper II, Figure 2C**). This suggests that additional factors are involved in the downregulation of HLA. It has been shown that CVB expresses several proteins that interfere with intracellular protein transport, thereby impairing protein translocation to the cell surface [126]. Another possibility is that CVB actively increases the removal of HLA from the cell surface. A study published by Cornell el al. offers some insight into this question and complements our results [242]. In this study it was confirmed that CVB is able to reduce the surface expression of HLA molecules. In addition, the authors addressed the mechanisms behind this phenomenon. It was shown that three different proteins encoded by CVB, namely 2B and its precursor 2BC as well as 3A, have the ability to interfere with the surface transport of HLA. In addition, 2B and 2BC can increase endocytosis, which may further decrease HLA surface expression. Interestingly, both the study by Cornell et al. and our study (**paper II**) suggest that CVB only inhibits HLA expression to a certain degree, leaving around 40-60% of the HLA molecules on the surface. It might be that evolutionary pressure to avoid detection by both T-cells and NK cells has resulted in this “compromise”. Partial downregulation might be enough to impair T-cell responses without leaving cells vulnerable to NK cell mediated killing. The observation that HLA downregulation by

CVB seems insufficient to induce NK cell mediated killing of infected HeLa cell, together with poor T-cell responses to CVB infection demonstrated by others [159], might be interpreted in support of this notion. Other viruses achieve similar results by selectively downregulating HLA-A and –B, but not HLA-C [243]. The limited number of proteins expressed by CVB could be the explanation for the different approach taken by CVB. However, it has to be mentioned that the system employed in our study may not resemble the *in vivo* situation in all aspects. HeLa cells are tumor cells with high surface levels of HLA. Other cell types with lower HLA expression might well become susceptible to NK cell mediated killing after CVB infection. Moreover, a study with fetal thymocyte cultures has shown that CVB leads to the upregulation rather than downregulation of HLA on immature T-cells [244]. Collectively, these observations suggest that the effect of CVB infection might be cell type dependent. However, the study on fetal thymocyte cultures did not discriminate between infected and uninfected cells, which make it hard to establish if HLA upregulation was caused directly by the virus in infected cells. To confirm our results that NK cells respond with the production of IFN-γ to CVB infected cells rather than with direct killing, further studies with preferably primary cells should be conducted. Analysis of NK cell responses to CVB infected pancreatic islet cells are of particular interest. Results from several mouse models suggest that NK cell might be involved in the development of T1D after virus infection or other interventions [142, 202, 203, 245]. In addition, it has been demonstrated in a subset of recent onset T1D patients that NK cells are present in the vicinity of enterovirus-infected islets [153], but it is not clear if NK cells were involved in the development of the disease.

Another question is what the consequence the NK cell produced IFN-γ might have. Like type I IFNs, IFN-γ is able to stimulate the expression of ISGs and thereby induce an antiviral state in host cells [37]. This would help to limit virus replication and spread. For example, the transgenic expression of IFN-γ limits virus replication and protects mice from CVB-induced myocarditis and ameliorates pancreatitis [144, 145].

On the other hand, NK cell activation during CVB infection could potentially be detrimental for the host, as they have been implicated in diabetes exacerbation [142, 202, 203, 245]. For example, blocking of the activating receptor NKG2D prevents diabetes development in NOD mice [204]. However, NKG2D is also expressed on some effector T-cells, which makes it difficult to attribute the effect solely on NK cells. Interestingly, IFN-γ in combination with other inflammatory cytokines has been shown to induce cell death in pancreatic β-cells and impair their function, which could explain how NK cells can contribute to diabetes development [57, 246, 247]. Direct killing of infected cells could also induce immunopathology after CVB infection. In fact, it has been demonstrated that CVB-induced myocarditis is at least in part due to tissue destruction by immune cells [149]. Mice lacking perforin show no impairment of virus clearance in the heart but have considerably less tissue damage. Similarly, the same mice do not show increased CVB replication in the pancreas [116]. These results suggest that direct killing of infected cells by NK cells is not important for control of the virus and can in fact be detrimental to the host.

In conclusion in **paper II** we showed that CVB can downregulate surface expression of several NK cell ligands and that human NK cells respond with the production of IFN-γ rather than direct killing of infected cells.

3.2 THE ANTIVIRAL EFFECT OF TYPE I AND II INTERFERONS IN HUMAN β-CELLS

Recognition of virus infections by PRR receptors results in the rapid production of type I IFNs. Furthermore, activation of NK and/or T-cells by virus-infected cells can induce the production of IFN-γ. Both type I and II IFNs upregulate the expression of proteins that induce an antiviral state in host cells [37]. The production of IFN is crucial for the host immune response to virus infections. In the absence of IFN or IFN signaling mice are exceptionally susceptible to CVB infections [140, 141]. Specific abrogation of IFN signaling in β-cells results in the rapid induction of T1D after CVB infection in mice [142]. In human islets the neutralization of IFN-α after CVB infection *in vitro* leads to complete cell destruction [138]. This illustrates that pancreatic β-cells relay heavily on IFNs in order to prevent virus replication and cell death. However, how human islets respond to IFN stimulation and if IFNs can induce an antiviral state in human islets had not been investigated.

In **paper III** we studied how human pancreatic islet cells respond to stimulation with IFN-α or IFN-γ. Specifically, we wanted to know if IFNs upregulate the expression of proteins with antiviral properties, and if this results in the induction of an antiviral state that prevents CVB replication. In addition, we examined the induction of PRR, suggested to be important for CVB recognition.

To gain insight into what genes are upregulated after IFN stimulation, we stimulated islets from four donors with either IFN-α or IFN-γ. Six hours after stimulation we isolated RNA and evaluated gene expression using a custom-made microarray comprising 2178 probes for genes known to be stimulated by IFNs and/or involved in the immune response. We found that 23 and 6 genes were consistently upregulated in islets from all four donors after IFN-α and IFN-γ treatment, respectively (**paper III, Table 1 and 2**). For example, CXCL10, viperin and IFIT1 were upregulated by both IFNs, while OAS was only stimulated by IFN-α. To confirm the results obtained by the microarray we studied the gene expression of selected genes by Western blot and/or Real-Time (RT) PCR analysis. We observed a robust induction of CXCL10, viperin and OAS mRNA after IFN-α treatment. As seen in the microarray experiments, IFN-γ was less potent in inducing those genes (**paper III, Figure 1**). Western blot analysis of Viperin protein expression verified results obtained by other methods (**paper III, Figure 3**).

The production of IFNs can be induced after recognition of virus RNA by PRR. In fact, it has been shown that human islets express IFN-α after CVB infection [138]. However, the expression of PRR by human islets that could induce IFN production after CVB infection had not been studied. Therefore we decided to investigate the expression of TLR3, MDA-5 and RIG-I. Treatment with IFN-α led to a substantial upregulation of mRNA expression of all three PRR. Again, IFN-γ had only a minor effect on the gene expression of all PRR studied (**paper III, Figure 2**). Analysis of RIG-I and MDA-5 protein expression confirmed the results obtained by the RT-PCR analysis (**paper III, Figure 3**).

As we observed a robust upregulation of proteins known to be involved in the host antiviral defense, we asked if IFN treatment could prevent CVB replication in human islets. Treatment with IFN-α resulted in powerful protection from CVB infection of

human islets. Mirroring the reduced effect on gene expression, pre-treatment with IFN-γ showed only an intermediated effect but could still reduce CVB replication by around 90% (**paper III, Figure 4**).

Taken together, the results from **paper III** showed that human islets respond to IFN treatment with a robust upregulation of proteins associated with the host antiviral response. This induced an antiviral state, which potently suppressed CVB replication. The effect of IFN-α was more powerful than IFN-γ.

Several genes were induced in the IFN-treated human islets. We can only speculate which proteins are responsible for the protection from CVB infection. It has been shown that the OAS/RNase L pathway is important for the host immune response to CVB infection in mice [60]. As the production of oligoadenylates by OAS is the rate-limiting step in the activation of RNase L, it is feasible to assume that an upregulation of OAS after IFN treatment has the potential to inhibit CVB replication in human islets. Interestingly, *in vitro* infection of mouse islets has confirmed that the protective effect of IFN-α is considerably dependent on an intact RNase L pathway [60]. The effect of Viperin on CVB replication has not been addressed. However it has been shown that Viperin has a protective role in CMV and HCV infection [69, 70]. How Viperin can support the host antiviral response is not entirely clear. For Influenza A, it has been suggested that Viperin prevent virus budding from the cell membrane by disrupting lipid raft formation [71]. As CVB is normally released after the infected cells are destroyed by the cytopathic effect induced by the virus, it seems unlikely that Viperin can inhibit CVB release by the same mechanism. However, it has been shown that human islets can be infected persistently with CVB [138]. It is not evident how virus is released during persistent infection without an obvious deleterious effect on beta cells, therefore a role for Viperin during CVB infection cannot been ruled out.

A protein upregulated by both IFN-α and IFN-γ was the chemokine CXCL10. The role for CXCL10 is to attract NK and T-cells expressing the chemokine receptor CXCR3 to the site of infection, rather than contributing directly to the induction of an antiviral state. The upregulation of CXCL10 is important in the host immune response to CVB infection, as illustrated by the fact that mice lacking CXCL10 are more susceptible to CVB-induced myocarditis, while overexpression of CXCL10 leads to a reduction of virus titers and tissue damage in the heart [152]. In contrast to the protective effect on virus replication and virus induced tissue damage, CXCL10 can also attract potentially autoreactive T-cells and thereby exacerbate autoimmune diseases. It has been shown that transgenic expression of CXCL10 in β-cells accelerates T1D development in mice [248]. This suggests an interesting link between CVB infections and the development of T1D, a notion that is supported by two recently published studies that investigated CXCL10 expression in the islets of patients with recent onset diabetes. It was shown that CXCR3 expressing T-cells infiltrated the islets of those patients [238, 249]. Of note, enterovirus proteins were detected in many of the islets studied.

After detection of a virus by PRR, infected cells can respond themselves by the production of type I IFNs. This has also been shown for human pancreatic islets [138]. We demonstrated that human islets express several PRR receptors potentially able to detect RNA viruses such as CVB. Interestingly, MDA-5 was also expressed and upregulated after IFN treatment by human islets. Several polymorphisms in MDA-5

have recently been suggested to predispose to T1D [175, 176]. As MDA-5 is expressed by human β-cells one could speculate that the β-cells themselves can determine the response to CVB infection and the immune response initiated. As a consequence, β-cells may play an active role in the initiation or exacerbation of T1D. In this regard it is interesting to note that we did observe a considerable variation in the response to IFN treatment in islets from different donors. One reason for the relative low numbers of genes found to be upregulated after IFN stimulation, were the stringent criteria we used to define altered gene expression with only genes upregulated ≥ 1,5 fold in all four donors included. For example IL-15 was not studied further as it was only upregulated ≥ 1,5 fold in tree out of the four donors. Similarly IRF1 was upregulated in some but not all islet preparations (**paper III, Supplementary Table 1**). Even genes significantly upregulated in the islet from all donors showed variation. This illustrates that the IFN response can differ substantially between individuals. Hence, the ability to control virus infection and the immune response initiated may vary accordingly. It would be interesting to correlate the antiviral response of β-cells with the presence or absence of gene polymorphisms implicated in T1D development.

In summary, in **paper III** we showed that human islets respond to IFN treatment by entering an antiviral state that can inhibit CVB replication. This supports the notion that islets cells can regulate their own permissiveness to CVB infection. In the light of the suggested link between CVB infections and T1D development [250], studies addressing the differences of the antiviral response between individuals are clearly needed.

3.3 ANTIVIRAL RESPONSE AGAINST COXSACKIEVIRUS IN A MOUSE MODEL FOR CYSTIC FIBROSIS

Cystic fibrosis (CF), the most common monogenetic recessive disease in the Caucasian population, presents with a well-established defect in antimicrobial defense (see section 1.5.1 of this thesis). Chronic bacterial infections of the airways represent the major determinant of morbidity and mortality in CF patients [251]. It has also been shown that respiratory virus infections cause more severe disease in CF patients compared with healthy controls and that this can exacerbate lower respiratory tract symptoms [212]. Enteroviruses have been found among the viruses shown to infect the airways of CF patients [212]. In addition, infection with respiratory syncytial virus can increase the susceptibility for bacterial infection in a mouse model of CF [211]. Even though an increased vulnerability to virus infection in the lungs has been demonstrated both for CF patients and in mouse models of CF, the general effect of a defective *cftr* on antiviral immunity had not been studied.

In **paper IV** we utilized a mouse model of CF, harboring the most common human *cftr* mutation F508del ($CFTR^{tm1EUR}$ [230]). We investigated the effect of an aberrant CFTR protein on the systemic antiviral defense against coxsackievirus infection, a virus with broad tissue tropism (see section 1.3.1). We studied if the F508del *cftr* alters general susceptibility to infections with CVB3 “Nancy” by measuring survival, virus titers found in several organs after infection and tissue damage induced by CVB. In addition, as CVB infection have been linked to the development of T1D, and cystic fibrosis

related diabetes (CFRD) is often observed in patients with CF, we wanted to know if the F508del mutation of *cftr* predispose mice to CVB induced diabetes.

To establish if a defective CFTR leads to an increased susceptibility to CVB infection, we infected $CFTR^{tm1EUR}$ and wild type littermate controls mice (hereafter denoted as ΔF508 and wt, respectively) with either 10^2 or 10^4 PFU CVB3. All wt mice survived infection with the lower dose of CVB3, while approximately half of the ΔF508 succumbed to infection with this virus dose (**paper IV, Figure 1A**). Even though most of the wt and ΔF508 animals died after an infection with the higher infectious dose, transgenic mice showed a significant decrease in median survival compared to wt controls (**paper IV, Figure 1B**). To establish if the decreased survival of ΔF508 mice was due to uncontrolled virus replication, virus titers were measured in different organs three days after infection. Only few animals showed detectable virus in the lung, intestine and pancreas after infection with a low dose of CVB (1/5 and 2/5 for ΔF508 and wt mice respectively; **paper IV, Figure 2A**). Infection with 10^4 PFU/mouse resulted in vigorous viral replication in all organs tested, with no significant difference between ΔF508 and wt mice (**paper IV, Figure 2B**). However, for some organs we observed a trend towards higher virus replication in ΔF508 mice compared to littermate controls. Despite the similarity in virus replication we wanted to compare tissue damage three days after low dose CVB infection. Histological evaluation of liver section did not reveal any abnormalities. Mild lymphocyte infiltration and exocrine tissue damage was apparent in most pancreas sections with no difference between ΔF508 and wt control mice (data not shown). A wt animal with high virus replication in the pancreas (**paper IV, Figure 2A**) exhibited extensive tissue damage and lymphocyte infiltration (data not shown).

In order to evaluate if ΔF508 mice are prone to CVB induced diabetes, we measured blood glucose levels after infection. None of the animals developed hyperglycemia at any time point. In contrast, most animals infected with a high dose of CVB3 showed a decrease in blood glucose levels early after infection (**paper IV, Supplementary Figure 1A**). Even after infection with 10^2 PFU CVB3 some ΔF508 mice became hypoglycemic, while most wt controls remained normoglycemic (**paper IV, Supplementary Figure 1B**). Almost all animals that showed severe hypoglycemia eventually succumbed to the infection.

In conclusion, the results presented in **paper IV** suggest that mice carrying the *cftr* F508del mutation have an increased susceptibility to CVB3 infections but can control early virus replication comparable to wt littermates. We did not observe any difference in virus-induced tissue damage between ΔF508 and wt mice early after infection. In addition, the lack of a functional CFTR did not render mice susceptible to CVB3 induced diabetes.

It has been shown that CF patients and mouse models for CF have an impaired defense against different viruses in their airways [212, 215, 233]. However, to our knowledge our study is the first attempt to address the impact of a defective CFTR on antiviral immune response to a virus with a broad tissue tropism. We demonstrated that ΔF508 mice have a dramatically increased susceptibility to CVB3 infection. Infections with CVB, and enteroviruses in general, are common and have been identified in the respiratory tract of CF patients [212]. This is of special interest as it has been suggested

that virus infections can exacerbate lung pathology in patients, and increase bacterial colonization in mouse models for CF [211]. It would therefore be interesting to study if enterovirus infections are common in CF patients around the time point when initial bacterial colonization of the lung is established or at times of exacerbated respiratory tract symptoms. One study showed that enterovirus infections are not more common in CF patients following lung transplantation compared to individuals without CF [213]. However, our results show that ΔF508 mice are more susceptible to CVB3. Therefore it would be of interest to evaluate the prevalence of CVB infections in a larger group of CF patients to see if infections are more common alternatively, more severe than in healthy individuals.

Surprisingly, despite the striking increase in CVB susceptibility we did not observe an increase in virus replication in ΔF508 mice three days after infection. This suggests that ΔF508 mice do not have a defect in their initial ability to control virus replication. A similar phenotype has been observed in mice lacking expression of iNOS. Mice deficient in iNOS show an increased susceptibility to CVB infection but have no alteration in early virus replication [252]. However, absence of iNOS leads to impaired virus clearance. By measuring virus replication at later time points we will be able to establish if this is also the case in ΔF508 mice. Of note, it has been shown that CF patients and mouse models for CF display an impaired iNOS expression in lung epithelial cells with a lower production of NO [215, 253]. As we did not observe an increased virus replication it might not be surprising that we did not see more tissue damage in ΔF508 mice after infection.

The heightened susceptibility to CVB3 infections in ΔF508 mice may have additional implications for CF associated pathology. It is evident that environmental factors, especially bacterial infections, contribute to the development of severe respiratory symptoms in CF patients [207]. However, if and how virus infections may affect other CF related complications has not been studied. Pancreatitis and/or pancreas insufficiency are often observed in CF patients [207]. It has been suggested that due to the aberrant composition of secretory contents, digestive enzymes secreted by the exocrine pancreas may precipitate and lead to obstruction and tissue damage. Most mouse models for CF show no or only mild pancreas abnormalities [232]. This discrepancy has been attributed to a complementary Cl- channel in the mouse pancreas and/or residual CFTR activity. However, it is possible that environmental factors, which CF mice are not exposed to, may contribute to the development of pancreas abnormalities. Interestingly, CVB is known to cause acute pancreatitis in humans [111]. One could speculate that an increased susceptibility to CVB infections may result in an increase of CVB-induced exocrine pancreas tissue damage and thereby exacerbate the development of pancreas insufficiency. We did not observe more pancreas damage or pancreatitis in ΔF508 mice after infection compared to wt controls. However, it is possible that such an effect will only be apparent at later time points and/or after repeated insults to the exocrine pancreas. Interestingly, mutations in *cftr* have also been linked to chronic idiopathic pancreatitis [254, 255]. If enterovirus infections are involved in the etiology of idiopathic pancreatitis has not been studied.

CFRD is another common complication of CF, often observed only later in life [210]. As CVB infections have been implicated with the development of T1D it is tempting to speculate on a role of CVB infection in the CFRD. Our observations that ΔF508 mice do not develop diabetes after infection might speak against this notion. However, this does not rule out the possibility that β-cells of ΔF508 mice have an

impaired antiviral response to CVB infections. In fact, mice lacking RNase L or PKR are highly susceptible to CVB infections but do not develop hyperglycemia after infection. Still, treatment with IFN-α or IFN-γ fails to protect islets of those mice from infection with CVB in vitro [60]. Interestingly, it has been shown that IFN-γ failed to upregulate iNOS and OAS, two proteins known to be important in the antiviral defense against CVB, in human alveolar epithelial cells derived from CF patients [215]. This lead to an increased replication of parainfluenza virus in cells from CF patients compared to control cells. Moreover, a recent study found enterovirus protein in pancreatic islets of 2 out of 11 CF patients [114]. The fact that the two patients with positive enterovirus staining had CFRD, while the remaining patients did not, may support the hypothesis that CVB could be involved in the development of CFRD.

In summary in **paper IV** we demonstrated that the F508del mutation of *cftr* results in an increased susceptibility to CVB3 infections in a mouse model of CF. This may have implications for the development of several CF associated complications, as CVB infections can cause pathology in many organs also affected by CF.

4 CONCLUDING REMARKS

The aim of this thesis was to gain new insight into the contribution of the innate immune response during CVB infection. We focused on different stages either important for the initiation of the immune response and/or control of virus replication. Moreover, we used a mouse model of CF to evaluate the outcome of CVB infection in a disease with an underlying defect in innate immune functions.

We showed that several mechanisms of the innate immune response are important for detecting and limiting CVB. The first event, that has to occur during every infection, is the recognition of the invading pathogen. Results from **paper I** clearly demonstrated that MDA-5 is an important component in the initial host response to CVB. After the virus has been detected, it is crucial for the host to rapidly suppress virus replication in order to limit damage caused directly by the virus. We demonstrated in **paper II** that NK cells might be important effector cells during CVB infection, especially due to their ability to produce IFN-γ after encountering CVB infected cells. The immune response is aimed at restricting the production of new virus progeny and ultimately clearing the virus infection. For this to happen, it is pivotal to prevent infection of virus permissive cells. Our experiments presented in **paper III** assign an important role to the target cell itself in successfully preventing virus spread. The response of CVB-permissive human islets to IFNs, produced by different cells during the infections, is critical for a successful immune response. Taken together, these results indicate that the host relies on a complex chain of events in order to mount a favorable immune response towards CVB. How fragile the highly coordinated immune response to CVB infection might be, is illustrated by the results presented in **paper IV**. Mice having a single mutation in the chloride channel CFTR, which has no known direct immunological functions, have an impaired antimicrobial defense and are highly susceptible to CVB infections.

Most infections with CVB are asymptomatic or present with only mild symptoms. This suggests that in most cases the immune system can fend of CVB infections successfully. However, why CVB infections occasionally cause severe diseases and how the immune response differs in such instances is not clear. Furthermore, CVB has been detected in and isolated from the pancreas of T1D patients, and thereby linked to the development of the disease. So far it has been impossible to define a specific structure that would make a certain CVB isolate more diabetogenic than others. This fact could be used to argue against a causative link between CVB infections and the development of T1D, with the reasoning being that CVB is a common virus and that most infections obviously do not result in T1D. However, this assumes that only virus intrinsic factors determine the outcome of an infection. Given the dynamic interaction between the host immune system and the virus, this is a gross oversimplification. A more likely scenario would be that a combination of virus- and host factors determines the outcome of a CVB infection. This is especially likely for a complex disease as T1D. The recent observation that some *mda-5* alleles increase the risk for T1D development has put the spotlight on the innate immune response and could offer some insight into how virus infections could cause T1D in genetically predisposed individuals. We showed that MDA-5 is indeed important for the host immune response to CVB infections. However, until the functional consequences of predisposing *mda-5* alleles for the immune response against CVB have been clarified, one can only speculate

about the events that might lead to CVB-induced T1D. One scenario could be an unbalanced IFN production. On one hand it has been shown that β-cells rely on IFNs for protection from CVB-induced damage, on the other hand overexpression of IFN-β, specifically in the β-cells, can break the immunological tolerance and induce T1D in non-diabetes prone mice. This highlights the delicate balance that the immune response has to achieve in order to prevent damage to the host.

By further increasing our knowledge about how CVB and the immune system interact, we will be able to delineate the events that lead to severe infections with CVB. This will hopefully help to prevent some of the serious diseases and late sequels associated with CVB infections.

5 ACKNOWLEDGEMENTS

During the past five and a half years I have spent here at CIM, many great people helped me to sail smoothly through the rough sea that is research in general and especially doing a PhD. I don't know what I would have done without all of you, but I'm quite certain I would not be were I am right now (sitting here and try not to forget someone):

First, I want to thank my supervisor **Malin Flodström-Tullberg** for accepting me as a student in her group. You not only introduced me to this exciting field of research, but also taught me that science truly is fun. Thank you for keeping me on track, while at the same time giving me the freedom to explore all the interesting things I found off the road. I really appreciate that you always had time to discuss science with me and never lost your patience (it must have been hard sometimes).

I would like to thank my co-supervisor **Hans-Gustaf Ljunggren** for sending my application to Malin, you are a fantastic match maker. Also, thank you for all the energy you invested to make CIM a great place to work at. I hope many more students will enjoy the same privilege as I did.

During my time, I also had the opportunity to work with people outside KI. I would especially like to thank **Marco Colonna** for inviting me to work in his lab at the Washington University, St. Louis, USA. Without **Steve McCartney**, my stay in St. Louis would not have been so successful and definitely not as much fun. Also, thanks to everybody in Marcos lab for making me feel welcome and helping with my experiments. I want to thank all the people working for the Nordic Network for Clinical Islet transplantation particularly **Olle Korsgren** and **Margareta Engkvist** (Uppsala Universitet), without whom much of my work would not have been possible.

Work would have been boring without all the past and present members of the MFT group. Thank you **Monica** for being my mentor when I joined the group and for many interesting discussions, both science related and not. **Stella**, I hope some of your organization skills have rubbed off on me. Thank you **Katharina**, it was really fun working with you. **Emma**, thank you for spending the summer in the lab with me and helping me to get things done. **Terezia** thank you for teaching me so many thinks about cystic fibrosis and vitamin D. Thank you **Pär** for adding some additional Y to the group. I also would like to thank **Mark** for teaching me how to work with CVB and for great concert memories.

I would also like to thank the staff at the animal facilities, especially **Maria Ottoson** and **Anette Bergman**.

There are so many people at **CIM** it would be impossible to thank each and everyone of you individually without forgetting someone. Therefore I want to thank all of you for being around and making CIM such a nice and stimulating work environment. However, without **Lena**, **Anette**, **Elisabeth** and **Hernan** none of us could do any work. Thank you for keeping CIM running. Also a big thanks to **Ann** and **Carina** for help

with all the administrative work throughout the years. Thanks to **Yenan**, **Stephanie**, **Sofia**, **Kim**, **Carlotta**, **Lidija** and **Axana** for sharing an office and all the nice conversations we had. Also a big thank you to **Sandra**, **Julius**, **Linda**, **Sayma**, **Erika**, **Ulrika**, **Anh Thu**, **Annette**, **Sanna**, **Niklas**, **Jakob**, **Veronica**, **Kalle** and **Robban** for nice times both in the lab and outside. **Steve** at the end you finally became my official mentor. Thank you for all the science talk and helping me save money on overpriced gadgets

I want to thank all my friends that reminded me that there is life outside the lab: **Markus** and **Kirsten**, I hope we will be neighbors for some time to come. **Claudia** and **Samuel**, Öregrundsgatan was never the same after you left. **Sven**, thank you for helping us move and all the great movie nights! **Bettina**, I really enjoyed having you around in- and outside the lab (will we ever dance Salsa again?). **Annette,** thank you for your great sense of humor and always laughing when I tried to be funny. My oldest and best friend **Jens**: You are an amazing person and the best godfather Malte could have! Our hour-long skype calls always gave me energy when I most needed it.

I would like to thank my first supervisor **Jens Hellwage**. I will always be grateful for the chance you gave me!

I want to thank the whole **Öhlund family** for making me feel welcome and inviting me into their lives. **Bengt** and **Marie-Louise,** thank you for the wonderful and relaxing times I had in Boden and Kusträsk.

Meine Eltern **Doris** und **Harald**: Ich kann euch nicht genug danken für alles was ihr für mich getan habt. Die Gewissheit, dass ihr immer da sein werdet wenn ich euch brauche, hat mir die Kraft gegeben all die Dinge zu verwirklichen die ich mir vorgenommen habe. Ich weiß das es nicht leicht gefallen sein muss mich ziehen zu lassen. Aber ich möchte das ihr wisst das mein zu Hause immer da sein wird wo ihr seid!

Pia you are the most important person in my life! I can only hope that you know how much you mean to me. You showed me the world, made me appreciate the little things and were always there to catch me. Now we are a family and I cannot imagine anything else that would make me happier than spending time with you and **Malte**!

6 REFERENCES

1. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
2. Hu, X. and L.B. Ivashkiv, *Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases.* Immunity, 2009. **31**(4): p. 539-50.
3. Chaplin, D.D., *Overview of the immune response.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
4. Renauld, J.-C., *Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators.* Nat Rev Immunol, 2003. **3**(8): p. 667-76.
5. Commins, S.P., L. Borish, and J.W. Steinke, *Immunologic messenger molecules: cytokines, interferons, and chemokines.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S53-72.
6. McCullough, K.C., N. Ruggli, and A. Summerfield, *Dendritic cells--at the front-line of pathogen attack.* Vet Immunol Immunopathol, 2009. **128**(1-3): p. 7-15.
7. Honda, K., et al., *Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction.* Nature, 2005. **434**(7036): p. 1035-40.
8. Izaguirre, A., et al., *Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells.* Journal of Leukocyte Biology, 2003. **74**(6): p. 1125-38.
9. Gordon, S., *The macrophage: past, present and future.* European Journal of Immunology, 2007. **37 Suppl 1**: p. S9-17.
10. Fehniger, T.A., et al., *Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response.* J Immunol, 1999. **162**(8): p. 4511-20.
11. Sun, J.C., J.N. Beilke, and L.L. Lanier, *Adaptive immune features of natural killer cells.* Nature, 2009. **457**(7229): p. 557-61.
12. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.
13. Kawai, T. and S. Akira, *TLR signaling.* Cell Death Differ, 2006. **13**(5): p. 816-25.
14. Kadowaki, N., et al., *Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.* The Journal of Experimental Medicine, 2001. **194**(6): p. 863-9.
15. Wilkins, C. and M. Gale, *Recognition of viruses by cytoplasmic sensors.* Curr Opin Immunol, 2010. **22**(1): p. 41-7.
16. Yoneyama, M., et al., *Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity.* J Immunol, 2005. **175**(5): p. 2851-8.
17. Satoh, T., et al., *LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses.* Proc Natl Acad Sci USA, 2010. **107**(4): p. 1512-7.
18. Kato, H., et al., *Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.* Nature, 2006. **441**(7089): p. 101-5.
19. Gitlin, L., et al., *Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus.* Proc Natl Acad Sci USA, 2006. **103**(22): p. 8459-64.

20. Kumar, H., et al., *Essential role of IPS-1 in innate immune responses against RNA viruses.* J Exp Med, 2006. **203**(7): p. 1795-803.
21. Kato, H., et al., *Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5.* The Journal of Experimental Medicine, 2008. **205**(7): p. 1601-10.
22. Hornung, V., et al., *5'-Triphosphate RNA is the ligand for RIG-I.* Science, 2006. **314**(5801): p. 994-7.
23. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(5801): p. 997-1001.
24. Saito, T., et al., *Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA.* Nature, 2008. **454**(7203): p. 523-7.
25. Sumpter, R., et al., *Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I.* Journal of Virology, 2005. **79**(5): p. 2689-99.
26. Loo, Y.-M., et al., *Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity.* J Virol, 2008. **82**(1): p. 335-45.
27. Meylan, E., et al., *Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus.* Nature, 2005. **437**(7062): p. 1167-72.
28. Andrejeva, J., et al., *The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter.* Proc Natl Acad Sci USA, 2004. **101**(49): p. 17264-9.
29. ISAACS, A. and J. LINDENMANN, *Virus interference. I. The interferon.* Proc R Soc Lond, B, Biol Sci, 1957. **147**(927): p. 258-67.
30. Stark, G.R., *How cells respond to interferons revisited: from early history to current complexity.* Cytokine Growth Factor Rev, 2007. **18**(5-6): p. 419-23.
31. Sadler, A.J. and B.R.G. Williams, *Interferon-inducible antiviral effectors.* Nat Rev Immunol, 2008. **8**(7): p. 559-68.
32. Theofilopoulos, A.N., et al., *Type I interferons (alpha/beta) in immunity and autoimmunity.* Annu Rev Immunol, 2005. **23**: p. 307-36.
33. Randall, R.E. and S. Goodbourn, *Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures.* J Gen Virol, 2008. **89**(Pt 1): p. 1-47.
34. Smith, P.L., G. Lombardi, and G.R. Foster, *Type I interferons and the innate immune response--more than just antiviral cytokines.* Mol Immunol, 2005. **42**(8): p. 869-77.
35. Honda, K., et al., *IRF-7 is the master regulator of type-I interferon-dependent immune responses.* Nature, 2005. **434**(7034): p. 772-7.
36. Alexander, W.S., et al., *SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine.* Cell, 1999. **98**(5): p. 597-608.
37. Der, S.D., et al., *Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays.* Proc Natl Acad Sci USA, 1998. **95**(26): p. 15623-8.
38. Della Chiesa, M., et al., *Multidirectional interactions are bridging human NK cells with plasmacytoid and monocyte-derived dendritic cells during innate immune responses.* Blood, 2006. **108**(12): p. 3851-8.
39. Gerosa, F., et al., *The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions.* J Immunol, 2005. **174**(2): p. 727-34.

40. Mohty, M., et al., *IFN-alpha skews monocyte differentiation into Toll-like receptor 7-expressing dendritic cells with potent functional activities.* J Immunol, 2003. **171**(7): p. 3385-93.
41. Alba, A., et al., *IFN beta accelerates autoimmune type 1 diabetes in nonobese diabetic mice and breaks the tolerance to beta cells in nondiabetes-prone mice.* J Immunol, 2004. **173**(11): p. 6667-75.
42. Ewel, C.H., et al., *Poly I:C accelerates development of diabetes mellitus in diabetes-prone BB rat.* Diabetes, 1992. **41**(8): p. 1016-21.
43. Sobel, D.O., et al., *Poly I:C induces development of diabetes mellitus in BB rat.* Diabetes, 1992. **41**(4): p. 515-20.
44. Fabris, P., et al., *Insulin-dependent diabetes mellitus during alpha-interferon therapy for chronic viral hepatitis.* J Hepatol, 1998. **28**(3): p. 514-7.
45. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.
46. Dokun, A.O., et al., *Specific and nonspecific NK cell activation during virus infection.* Nat Immunol, 2001. **2**(10): p. 951-6.
47. Lighvani, A.A., et al., *T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells.* Proc Natl Acad Sci USA, 2001. **98**(26): p. 15137-42.
48. Hwang, E.S., et al., *T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3.* Science, 2005. **307**(5708): p. 430-3.
49. Tanaka, K., et al., *Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads.* J Immunol, 2008. **180**(6): p. 3746-56.
50. Hu, X., et al., *IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins.* Immunity, 2006. **24**(5): p. 563-74.
51. Hu, X., et al., *Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation.* Nature Immunology, 2002. **3**(9): p. 859-66.
52. Kleinschek, M.A., et al., *IL-25 regulates Th17 function in autoimmune inflammation.* J Exp Med, 2007. **204**(1): p. 161-70.
53. Nakae, S., et al., *Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice.* J Immunol, 2003. **171**(11): p. 6173-7.
54. Ferber, I.A., et al., *Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE).* J Immunol, 1996. **156**(1): p. 5-7.
55. Axtell, R.C., et al., *T helper type 1 and 17 cells determine efficacy of interferon-beta in multiple sclerosis and experimental encephalomyelitis.* Nature medicine, 2010. **16**(4): p. 406-12.
56. Panitch, H.S., et al., *Exacerbations of multiple sclerosis in patients treated with gamma interferon.* Lancet, 1987. **1**(8538): p. 893-5.
57. Cetkovic-Cvrlje, M. and D.L. Eizirik, *TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide.* Cytokine, 1994. **6**(4): p. 399-406.
58. Haller, O., G. Kochs, and F. Weber, *The interferon response circuit: induction and suppression by pathogenic viruses.* Virology, 2006. **344**(1): p. 119-30.
59. Tallóczy, Z., H.W. Virgin, and B. Levine, *PKR-dependent autophagic degradation of herpes simplex virus type 1.* Autophagy, 2006. **2**(1): p. 24-9.
60. Flodström-Tullberg, M., et al., *RNase L and double-stranded RNA-dependent protein kinase exert complementary roles in islet cell defense during coxsackievirus infection.* J Immunol, 2005. **174**(3): p. 1171-7.

61. Silverman, R.H., *Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response.* J Virol, 2007. **81**(23): p. 12720-9.
62. Perelygin, A.A., et al., *Positional cloning of the murine flavivirus resistance gene.* Proc Natl Acad Sci USA, 2002. **99**(14): p. 9322-7.
63. Maitra, R.K. and R.H. Silverman, *Regulation of human immunodeficiency virus replication by 2',5'-oligoadenylate-dependent RNase L.* Journal of Virology, 1998. **72**(2): p. 1146-52.
64. Loeb, K.R. and A.L. Haas, *The interferon-inducible 15-kDa ubiquitin homolog conjugates to intracellular proteins.* J Biol Chem, 1992. **267**(11): p. 7806-13.
65. Lenschow, D.J., et al., *IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses.* Proc Natl Acad Sci USA, 2007. **104**(4): p. 1371-6.
66. Haller, O., P. Staeheli, and G. Kochs, *Interferon-induced Mx proteins in antiviral host defense.* Biochimie, 2007. **89**(6-7): p. 812-8.
67. Andersson, I., et al., *Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus.* Journal of Virology, 2004. **78**(8): p. 4323-9.
68. Andreoletti, L., et al., *Detection of enteroviral RNA by polymerase chain reaction in endomyocardial tissue of patients with chronic cardiac diseases.* J Med Virol, 1996. **48**(1): p. 53-9.
69. Helbig, K.J., et al., *Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector.* Hepatology, 2005. **42**(3): p. 702-10.
70. Chin, K.C. and P. Cresswell, *Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus.* Proc Natl Acad Sci USA, 2001. **98**(26): p. 15125-30.
71. Wang, X., E.R. Hinson, and P. Cresswell, *The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts.* Cell Host Microbe, 2007. **2**(2): p. 96-105.
72. Ljunggren, H.G. and K. Kärre, *In search of the 'missing self': MHC molecules and NK cell recognition.* Immunol Today, 1990. **11**(7): p. 237-44.
73. Biassoni, R., *Human natural killer receptors, co-receptors, and their ligands.* Curr Protoc Immunol, 2009. **Chapter 14**: p. Unit 14.10.
74. Flodström-Tullberg, M., et al., *Natural killer cells in human autoimmunity.* Curr Opin Immunol, 2009. **21**(6): p. 634-40.
75. Eagle, R.A. and J. Trowsdale, *Promiscuity and the single receptor: NKG2D.* Nat Rev Immunol, 2007. **7**(9): p. 737-44.
76. Eagle, R.A., et al., *ULBP6/RAET1L is an additional human NKG2D ligand.* Eur J Immunol, 2009. **39**(11): p. 3207-16.
77. Gasser, S., et al., *The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor.* Nature, 2005. **436**(7054): p. 1186-90.
78. Rölle, A., et al., *Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein.* J Immunol, 2003. **171**(2): p. 902-8.
79. Ward, J., et al., *HIV-1 Vpr triggers natural killer cell-mediated lysis of infected cells through activation of the ATR-mediated DNA damage response.* PLoS Pathog, 2009. **5**(10): p. e1000613.
80. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends Immunol, 2001. **22**(11): p. 633-40.
81. Bottino, C., et al., *Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule.* The Journal of Experimental Medicine, 2003. **198**(4): p. 557-67.

82. Lanier, L.L., et al., *The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes.* J Immunol, 1986. **136**(12): p. 4480-6.
83. Voss, S.D., et al., *Participation of the CD94 receptor complex in costimulation of human natural killer cells.* J Immunol, 1998. **160**(4): p. 1618-26.
84. André, P., et al., *Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin.* Proc Natl Acad Sci USA, 2000. **97**(7): p. 3400-5.
85. Nagler, A., et al., *Comparative studies of human FcRIII-positive and negative natural killer cells.* J Immunol, 1989. **143**(10): p. 3183-91.
86. Fehniger, T.A., et al., *CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity.* Blood, 2003. **101**(8): p. 3052-7.
87. Carbone, E., et al., *Recognition of autologous dendritic cells by human NK cells.* Eur J Immunol, 1999. **29**(12): p. 4022-9.
88. Nedvetzki, S., et al., *Reciprocal regulation of human natural killer cells and macrophages associated with distinct immune synapses.* Blood, 2007. **109**(9): p. 3776-85.
89. Lee, S.H., et al., *Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily.* Nat Genet, 2001. **28**(1): p. 42-5.
90. Voigt, V., et al., *Murine cytomegalovirus m157 mutation and variation leads to immune evasion of natural killer cells.* Proc Natl Acad Sci USA, 2003. **100**(23): p. 13483-8.
91. Gumá, M., et al., *Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts.* Blood, 2006. **107**(9): p. 3624-31.
92. Biron, C.A., K.S. Byron, and J.L. Sullivan, *Severe herpesvirus infections in an adolescent without natural killer cells.* N Engl J Med, 1989. **320**(26): p. 1731-5.
93. Lanier, L.L., *Evolutionary struggles between NK cells and viruses.* Nat Rev Immunol, 2008. **8**(4): p. 259-68.
94. Chisholm, S.E. and H.T. Reyburn, *Recognition of vaccinia virus-infected cells by human natural killer cells depends on natural cytotoxicity receptors.* J Virol, 2006. **80**(5): p. 2225-33.
95. Brooks, C.R., et al., *The inhibitory receptor NKG2A determines lysis of vaccinia virus-infected autologous targets by NK cells.* J Immunol, 2006. **176**(2): p. 1141-7.
96. Draghi, M., et al., *NKp46 and NKG2D recognition of infected dendritic cells is necessary for NK cell activation in the human response to influenza infection.* J Immunol, 2007. **178**(5): p. 2688-98.
97. Martin, M.P., et al., *Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS.* Nat Genet, 2002. **31**(4): p. 429-34.
98. Khakoo, S.I., et al., *HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection.* Science, 2004. **305**(5685): p. 872-4.
99. Wagner, M., et al., *Major histocompatibility complex class I allele-specific cooperative and competitive interactions between immune evasion proteins of cytomegalovirus.* The Journal of Experimental Medicine, 2002. **196**(6): p. 805-16.
100. Ulbrecht, M., et al., *Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis.* J Immunol, 2000. **164**(10): p. 5019-22.

101. Prod'homme, V., et al., *The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells.* J Immunol, 2007. **178**(7): p. 4473-81.
102. Dunn, C., et al., *Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity.* J Exp Med, 2003. **197**(11): p. 1427-39.
103. Chalupny, N.J., et al., *Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142.* Biochem Biophys Res Commun, 2006. **346**(1): p. 175-81.
104. Stern-Ginossar, N., et al., *Host immune system gene targeting by a viral miRNA.* Science, 2007. **317**(5836): p. 376-81.
105. Yamada, T., et al., *Proline 78 is crucial for human immunodeficiency virus type 1 Nef to down-regulate class I human leukocyte antigen.* J Virol, 2003. **77**(2): p. 1589-94.
106. Cohen, G.B., et al., *The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells.* Immunity, 1999. **10**(6): p. 661-71.
107. Cerboni, C., et al., *Human immunodeficiency virus 1 Nef protein downmodulates the ligands of the activating receptor NKG2D and inhibits natural killer cell-mediated cytotoxicity.* J Gen Virol, 2007. **88**(Pt 1): p. 242-50.
108. Dick, T.P., *Assembly of MHC class I peptide complexes from the perspective of disulfide bond formation.* Cell Mol Life Sci, 2004. **61**(5): p. 547-56.
109. Cresswell, P., *Assembly, transport, and function of MHC class II molecules.* Annu Rev Immunol, 1994. **12**: p. 259-93.
110. Eisenbarth, G.S., *Update in type 1 diabetes.* J Clin Endocrinol Metab, 2007. **92**(7): p. 2403-7.
111. Pallansch, M., R. Roos, and D.M. Knipe, *Enteroviruses: Polioviruses, Coxsackieviruses, Echoviruses, and Newer Enteroviruses,* in *Fields Virology*. 2007. p. 839-894.
112. Oberste, M.S., *Comparative genomics of the coxsackie B viruses and related enteroviruses.* Current topics in microbiology and immunology, 2008. **323**: p. 33-47.
113. Bowles, N.E., et al., *Detection of Coxsackie-B-virus-specific RNA sequences in myocardial biopsy samples from patients with myocarditis and dilated cardiomyopathy.* Lancet, 1986. **1**(8490): p. 1120-3.
114. Richardson, S.J., et al., *The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes.* Diabetologia, 2009. **52**(6): p. 1143-51.
115. Ylipaasto, P., et al., *Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells.* Diabetologia, 2004. **47**(2): p. 225-39.
116. Mena, I., et al., *Coxsackievirus infection of the pancreas: evaluation of receptor expression, pathogenesis, and immunopathology.* Virology, 2000. **271**(2): p. 276-88.
117. Oikarinen, M., et al., *Detection of enteroviruses in the intestine of type 1 diabetic patients.* Clin Exp Immunol, 2008. **151**(1): p. 71-5.
118. Schulte, B.M., et al., *Detection of enterovirus RNA in peripheral blood mononuclear cells of type 1 diabetic patients beyond the stage of acute infection.* Viral Immunol, 2010. **23**(1): p. 99-104.
119. Bergelson, J.M., et al., *Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5.* Science, 1997. **275**(5304): p. 1320-3.

120. Freimuth, P., L. Philipson, and S.D. Carson, *The coxsackievirus and adenovirus receptor.* Current topics in microbiology and immunology, 2008. **323**: p. 67-87.
121. Bergelson, J.M., et al., *Coxsackievirus B3 adapted to growth in RD cells binds to decay-accelerating factor (CD55).* Journal of Virology, 1995. **69**(3): p. 1903-6.
122. Polacek, C., et al., *Cytolytic replication of coxsackievirus B2 in CAR-deficient rhabdomyosarcoma cells.* Virus Res, 2005. **113**(2): p. 107-15.
123. Marchant, D., et al., *The impact of CVB3 infection on host cell biology.* Current topics in microbiology and immunology, 2008. **323**: p. 177-98.
124. Zaragoza, C., et al., *Viral protease cleavage of inhibitor of kappaBalpha triggers host cell apoptosis.* Proc Natl Acad Sci USA, 2006. **103**(50): p. 19051-6.
125. Lamphear, B.J., et al., *Mapping the cleavage site in protein synthesis initiation factor eIF-4 gamma of the 2A proteases from human Coxsackievirus and rhinovirus.* J Biol Chem, 1993. **268**(26): p. 19200-3.
126. Cornell, C.T., et al., *Inhibition of protein trafficking by coxsackievirus b3: multiple viral proteins target a single organelle.* J Virol, 2006. **80**(13): p. 6637-47.
127. de Jong, A.S., et al., *Determinants for membrane association and permeabilization of the coxsackievirus 2B protein and the identification of the Golgi complex as the target organelle.* J Biol Chem, 2003. **278**(2): p. 1012-21.
128. Chau, D.H.W., et al., *Coxsackievirus B3 proteases 2A and 3C induce apoptotic cell death through mitochondrial injury and cleavage of eIF4GI but not DAP5/p97/NAT1.* Apoptosis, 2007. **12**(3): p. 513-24.
129. Esfandiarei, M., et al., *Coxsackievirus B3 activates nuclear factor kappa B transcription factor via a phosphatidylinositol-3 kinase/protein kinase B-dependent pathway to improve host cell viability.* Cell Microbiol, 2007. **9**(10): p. 2358-71.
130. Feuer, R., et al., *Cell cycle status affects coxsackievirus replication, persistence, and reactivation in vitro.* J Virol, 2002. **76**(9): p. 4430-40.
131. Huber, S., *Host immune responses to coxsackievirus B3.* Current topics in microbiology and immunology, 2008. **323**: p. 199-221.
132. Triantafilou, K., et al., *Human cardiac inflammatory responses triggered by Coxsackie B viruses are mainly Toll-like receptor (TLR) 8-dependent.* Cell Microbiol, 2005. **7**(8): p. 1117-26.
133. Wang, J.P., et al., *Cutting Edge: Antibody-mediated TLR7-dependent recognition of viral RNA.* J Immunol, 2007. **178**(6): p. 3363-7.
134. Richer, M.J., et al., *Toll-like receptor 3 signaling on macrophages is required for survival following coxsackievirus B4 infection.* PLoS ONE, 2009. **4**(1): p. e4127.
135. Negishi, H., et al., *A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity.* Proc Natl Acad Sci USA, 2008. **105**(51): p. 20446-51.
136. Triantafilou, K. and M. Triantafilou, *Coxsackievirus B4-induced cytokine production in pancreatic cells is mediated through toll-like receptor 4.* J Virol, 2004. **78**(20): p. 11313-20.
137. Sean, P. and B.L. Semler, *Coxsackievirus B RNA replication: lessons from poliovirus.* Current topics in microbiology and immunology, 2008. **323**: p. 89-121.
138. Chehadeh, W., et al., *Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells.* J Virol, 2000. **74**(21): p. 10153-64.

139. Chehadeh, W., et al., *Human antibodies isolated from plasma by affinity chromatography increase the coxsackievirus B4-induced synthesis of interferon-alpha by human peripheral blood mononuclear cells in vitro.* J Gen Virol, 2001. **82**(Pt 8): p. 1899-907.
140. Deonarain, R., et al., *Protective role for interferon-beta in coxsackievirus B3 infection.* Circulation, 2004. **110**(23): p. 3540-3.
141. Wessely, R., et al., *Cardioselective infection with coxsackievirus B3 requires intact type I interferon signaling: implications for mortality and early viral replication.* Circulation, 2001. **103**(5): p. 756-61.
142. Flodström, M., et al., *Target cell defense prevents the development of diabetes after viral infection.* Nat Immunol, 2002. **3**(4): p. 373-82.
143. Zaragoza, C., et al., *Inducible nitric oxide synthase protection against coxsackievirus pancreatitis.* J Immunol, 1999. **163**(10): p. 5497-504.
144. Horwitz, M.S., et al., *Pancreatic expression of interferon-gamma protects mice from lethal coxsackievirus B3 infection and subsequent myocarditis.* Nat Med, 2000. **6**(6): p. 693-7.
145. Horwitz, M.S., et al., *Protection from lethal coxsackievirus-induced pancreatitis by expression of gamma interferon.* J Virol, 1999. **73**(3): p. 1756-66.
146. Godeny, E.K. and C.J. Gauntt, *Involvement of natural killer cells in coxsackievirus B3-induced murine myocarditis.* J Immunol, 1986. **137**(5): p. 1695-702.
147. Godeny, E.K. and C.J. Gauntt, *Murine natural killer cells limit coxsackievirus B3 replication.* J Immunol, 1987. **139**(3): p. 913-8.
148. Vella, C. and H. Festenstein, *Coxsackievirus B4 infection of the mouse pancreas: the role of natural killer cells in the control of virus replication and resistance to infection.* J Gen Virol, 1992. **73 (Pt 6)**: p. 1379-86.
149. Gebhard, J.R., et al., *Coxsackievirus B3-induced myocarditis: perforin exacerbates disease, but plays no detectable role in virus clearance.* Am J Pathol, 1998. **153**(2): p. 417-28.
150. Young, L.H., et al., *Perforin-mediated myocardial damage in acute myocarditis.* Lancet, 1990. **336**(8722): p. 1019-21.
151. Godeny, E.K. and C.J. Gauntt, *In situ immune autoradiographic identification of cells in heart tissues of mice with coxsackievirus B3-induced myocarditis.* Am J Pathol, 1987. **129**(2): p. 267-76.
152. Yuan, J., et al., *CXCL10 inhibits viral replication through recruitment of natural killer cells in coxsackievirus B3-induced myocarditis.* Circulation Research, 2009. **104**(5): p. 628-38.
153. Dotta, F., et al., *Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients.* Proc Natl Acad Sci USA, 2007. **104**(12): p. 5115-20.
154. Willcox, A., et al., *Analysis of islet inflammation in human type 1 diabetes.* Clin Exp Immunol, 2009. **155**(2): p. 173-81.
155. Rudge, P., et al., *Encephalomyelitis in primary hypogammaglobulinaemia.* Brain, 1996. **119 (Pt 1)**: p. 1-15.
156. Mena, I., et al., *The role of B lymphocytes in coxsackievirus B3 infection.* Am J Pathol, 1999. **155**(4): p. 1205-15.
157. Henke, A., et al., *The role of CD8+ T lymphocytes in coxsackievirus B3-induced myocarditis.* Journal of Virology, 1995. **69**(11): p. 6720-8.
158. Ramsingh, A.I., et al., *T cells contribute to disease severity during coxsackievirus B4 infection.* J Virol, 1999. **73**(4): p. 3080-6.

159. Kemball, C.C., S. Harkins, and J.L. Whitton, *Enumeration and functional evaluation of virus-specific CD4+ and CD8+ T cells in lymphoid and peripheral sites of coxsackievirus B3 infection.* Journal of Virology, 2008. **82**(9): p. 4331-42.
160. Slifka, M.K., et al., *Using recombinant coxsackievirus B3 to evaluate the induction and protective efficacy of CD8+ T cells during picornavirus infection.* J Virol, 2001. **75**(5): p. 2377-87.
161. Sauter, P. and D. Hober, *Mechanisms and results of the antibody-dependent enhancement of viral infections and role in the pathogenesis of coxsackievirus B-induced diseases.* Microbes Infect, 2009. **11**(4): p. 443-51.
162. De Palma, A.M., et al., *Inflammatory rather than infectious insults play a role in exocrine tissue damage in a mouse model for coxsackievirus B4-induced pancreatitis.* J. Pathol., 2009. **217**(5): p. 633-41.
163. Fuse, K., et al., *Myeloid differentiation factor-88 plays a crucial role in the pathogenesis of Coxsackievirus B3-induced myocarditis and influences type I interferon production.* Circulation, 2005. **112**(15): p. 2276-85.
164. Daneman, D., *Type 1 diabetes.* Lancet, 2006. **367**(9513): p. 847-58.
165. Di Lorenzo, T.P., M. Peakman, and B.O. Roep, *Translational mini-review series on type 1 diabetes: Systematic analysis of T cell epitopes in autoimmune diabetes.* Clin Exp Immunol, 2007. **148**(1): p. 1-16.
166. Miao, D., L. Yu, and G.S. Eisenbarth, *Role of autoantibodies in type 1 diabetes.* Front Biosci, 2007. **12**: p. 1889-98.
167. Mariño, E., et al., *Marginal-zone B-cells of nonobese diabetic mice expand with diabetes onset, invade the pancreatic lymph nodes, and present autoantigen to diabetogenic T-cells.* Diabetes, 2008. **57**(2): p. 395-404.
168. Falcone, M., et al., *B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice.* J Immunol, 1998. **161**(3): p. 1163-8.
169. Pescovitz, M.D., et al., *Rituximab, B-lymphocyte depletion, and preservation of beta-cell function.* N Engl J Med, 2009. **361**(22): p. 2143-52.
170. Ueno, A., et al., *Enhanced early expansion and maturation of semi-invariant NK T cells inhibited autoimmune pathogenesis in congenic nonobese diabetic mice.* J Immunol, 2008. **181**(10): p. 6789-96.
171. Driver, J.P., et al., *Invariant natural killer T-cell control of type 1 diabetes: a dendritic cell genetic decision of a silver bullet or Russian roulette.* Diabetes, 2010. **59**(2): p. 423-32.
172. Litherland, S.A., et al., *Aberrant prostaglandin synthase 2 expression defines an antigen-presenting cell defect for insulin-dependent diabetes mellitus.* J Clin Invest, 1999. **104**(4): p. 515-23.
173. Allen, J.S., et al., *Plasmacytoid dendritic cells are proportionally expanded at diagnosis of type 1 diabetes and enhance islet autoantigen presentation to T-cells through immune complex capture.* Diabetes, 2009. **58**(1): p. 138-45.
174. Eisenbarth, G.S., *Banting Lecture 2009: An Unfinished Journey: Molecular Pathogenesis to Prevention of Type 1A Diabetes.* Diabetes, 2010. **59**(4): p. 759-74.
175. Smyth, D.J., et al., *A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region.* Nat Genet, 2006. **38**(6): p. 617-9.
176. Nejentsev, S., et al., *Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes.* Science, 2009. **324**(5925): p. 387-9.

177. Patterson, C.C., et al., *Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study.* Lancet, 2009. **373**(9680): p. 2027-33.
178. Redondo, M.J., et al., *Concordance for islet autoimmunity among monozygotic twins.* N Engl J Med, 2008. **359**(26): p. 2849-50.
179. Knip, M., et al., *Environmental triggers and determinants of type 1 diabetes.* Diabetes, 2005. **54 Suppl 2**: p. S125-36.
180. Hyppönen, E., et al., *Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study.* Lancet, 2001. **358**(9292): p. 1500-3.
181. Brissova, M., et al., *Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy.* J Histochem Cytochem, 2005. **53**(9): p. 1087-97.
182. Ishiki, M. and A. Klip, *Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners.* Endocrinology, 2005. **146**(12): p. 5071-8.
183. *Greenspan's basic & clinical endocrinology 8th edition.* 8th ed ed, ed. D.S. David G. Gardner. 2007, New York; London: McGraw-Hill Medical.
184. Korsgren, O., et al., *Current status of clinical islet transplantation.* Transplantation, 2005. **79**(10): p. 1289-93.
185. Elshebani, A., et al., *Effects on isolated human pancreatic islet cells after infection with strains of enterovirus isolated at clinical presentation of type 1 diabetes.* Virus Res, 2007. **124**(1-2): p. 193-203.
186. Gianani, R., et al., *Dimorphic histopathology of long-standing childhood-onset diabetes.* Diabetologia, 2010. **53**(4): p. 690-8.
187. Hyöty, H. and K.W. Taylor, *The role of viruses in human diabetes.* Diabetologia, 2002. **45**(10): p. 1353-61.
188. Gamble, D.R., et al., *Viral antibodies in diabetes mellitus.* Br Med J, 1969. **3**(5671): p. 627-30.
189. Yoon, J.W., et al., *Isolation of a virus from the pancreas of a child with diabetic ketoacidosis.* N Engl J Med, 1979. **300**(21): p. 1173-9.
190. Salminen, K., et al., *Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study.* J Med Virol, 2003. **69**(1): p. 91-8.
191. Clements, G.B., D.N. Galbraith, and K.W. Taylor, *Coxsackie B virus infection and onset of childhood diabetes.* Lancet, 1995. **346**(8969): p. 221-3.
192. Andréoletti, L., et al., *Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus.* J Med Virol, 1997. **52**(2): p. 121-7.
193. Hultcrantz, M., et al., *Interferons induce an antiviral state in human pancreatic islet cells.* Virology, 2007. **367**(1): p. 92-101.
194. Ylipaasto, P., et al., *Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets.* Diabetologia, 2005. **48**(8): p. 1510-22.
195. Horwitz, M.S., et al., *Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry.* Nat Med, 1998. **4**(7): p. 781-5.
196. Schloot, N.C., et al., *T-cell reactivity to GAD65 peptide sequences shared with coxsackie virus protein in recent-onset IDDM, post-onset IDDM patients and control subjects.* Diabetologia, 1997. **40**(3): p. 332-8.
197. Härkönen, T., et al., *Enterovirus infection can induce immune responses that cross-react with beta-cell autoantigen tyrosine phosphatase IA-2/IAR.* J Med Virol, 2002. **66**(3): p. 340-50.

198. Serreze, D.V., et al., *Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets.* Diabetes, 2000. **49**(5): p. 708-11.
199. van der Slik, A.R., et al., *KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects.* Diabetes, 2003. **52**(10): p. 2639-42.
200. Rodacki, M., et al., *Altered natural killer cells in type 1 diabetic patients.* Diabetes, 2007. **56**(1): p. 177-85.
201. Brauner, H., et al., *Distinct phenotype and function of NK cells in the pancreas of nonobese diabetic mice.* J Immunol, 2010. **184**(5): p. 2272-80.
202. Alba, A., et al., *Natural killer cells are required for accelerated type 1 diabetes driven by interferon-beta.* Clin Exp Immunol, 2008. **151**(3): p. 467-75.
203. Maruyama, T., et al., *The suppressive effect of anti-asialo GM1 antibody on low-dose streptozotocin-induced diabetes in CD-1 mice.* Diabetes Res, 1991. **16**(4): p. 171-5.
204. Ogasawara, K., et al., *NKG2D blockade prevents autoimmune diabetes in NOD mice.* Immunity, 2004. **20**(6): p. 757-67.
205. Riordan, J.R., et al., *Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA.* Science, 1989. **245**(4922): p. 1066-73.
206. Farrell, P.M., *The prevalence of cystic fibrosis in the European Union.* J Cyst Fibros, 2008. **7**(5): p. 450-3.
207. O'Sullivan, B.P. and S.D. Freedman, *Cystic fibrosis.* Lancet, 2009. **373**(9678): p. 1891-904.
208. Reisin, I.L., et al., *The cystic fibrosis transmembrane conductance regulator is a dual ATP and chloride channel.* J Biol Chem, 1994. **269**(32): p. 20584-91.
209. Crawford, I., et al., *Immunocytochemical localization of the cystic fibrosis gene product CFTR.* Proc Natl Acad Sci USA, 1991. **88**(20): p. 9262-6.
210. Moran, A., et al., *Cystic fibrosis-related diabetes: current trends in prevalence, incidence, and mortality.* Diabetes Care, 2009. **32**(9): p. 1626-31.
211. de Vrankrijker, A.M.M., et al., *Respiratory syncytial virus infection facilitates acute colonization of Pseudomonas aeruginosa in mice.* J Med Virol, 2009. **81**(12): p. 2096-103.
212. van Ewijk, B.E., et al., *Prevalence and impact of respiratory viral infections in young children with cystic fibrosis: prospective cohort study.* PEDIATRICS, 2008. **122**(6): p. 1171-6.
213. Bonvillain, R.W., et al., *Post-operative infections in cystic fibrosis and non-cystic fibrosis patients after lung transplantation.* J Heart Lung Transplant, 2007. **26**(9): p. 890-7.
214. Moeller, A., et al., *Inducible NO synthase expression is low in airway epithelium from young children with cystic fibrosis.* Thorax, 2006. **61**(6): p. 514-20.
215. Zheng, S., et al., *Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis.* Immunity, 2003. **18**(5): p. 619-30.
216. Kelley, T.J. and H.L. Elmer, *In vivo alterations of IFN regulatory factor-1 and PIAS1 protein levels in cystic fibrosis epithelium.* J Clin Invest, 2000. **106**(3): p. 403-10.
217. Xu, W., et al., *Cystic fibrosis and normal human airway epithelial cell response to influenza a viral infection.* J Interferon Cytokine Res, 2006. **26**(9): p. 609-27.
218. Bonfield, T.L., M.W. Konstan, and M. Berger, *Altered respiratory epithelial cell cytokine production in cystic fibrosis.* J Allergy Clin Immunol, 1999. **104**(1): p. 72-8.

219. Elizur, A., C.L. Cannon, and T.W. Ferkol, *Airway inflammation in cystic fibrosis.* Chest, 2008. **133**(2): p. 489-95.
220. Smyth, R.L., et al., *Intestinal inflammation in cystic fibrosis.* Arch Dis Child, 2000. **82**(5): p. 394-9.
221. Teichgräber, V., et al., *Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis.* Nature medicine, 2008. **14**(4): p. 382-91.
222. Bruzzese, E., et al., *Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration.* Aliment Pharmacol Ther, 2004. **20**(7): p. 813-9.
223. Dimagno, M.J., et al., *A proinflammatory, antiapoptotic phenotype underlies the susceptibility to acute pancreatitis in cystic fibrosis transmembrane regulator (-/-) mice.* Gastroenterology, 2005. **129**(2): p. 665-81.
224. Guilbault, C., et al., *Cystic fibrosis mouse models.* Am J Respir Cell Mol Biol, 2007. **36**(1): p. 1-7.
225. Snouwaert, J.N., et al., *An animal model for cystic fibrosis made by gene targeting.* Science, 1992. **257**(5073): p. 1083-8.
226. Kent, G., et al., *Phenotypic abnormalities in long-term surviving cystic fibrosis mice.* Pediatr Res, 1996. **40**(2): p. 233-41.
227. Dickinson, P., et al., *The severe G480C cystic fibrosis mutation, when replicated in the mouse, demonstrates mistrafficking, normal survival and organ-specific bioelectrics.* Human Molecular Genetics, 2002. **11**(3): p. 243-51.
228. Zeiher, B.G., et al., *A mouse model for the delta F508 allele of cystic fibrosis.* J Clin Invest, 1995. **96**(4): p. 2051-64.
229. Colledge, W.H., et al., *Generation and characterization of a delta F508 cystic fibrosis mouse model.* Nat Genet, 1995. **10**(4): p. 445-52.
230. van Doorninck, J.H., et al., *A mouse model for the cystic fibrosis delta F508 mutation.* EMBO J, 1995. **14**(18): p. 4403-11.
231. Delaney, S.J., et al., *Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations.* EMBO J, 1996. **15**(5): p. 955-63.
232. Carvalho-Oliveira, I., B.J. Scholte, and D. Penque, *What have we learned from mouse models for cystic fibrosis?* Expert Rev Mol Diagn, 2007. **7**(4): p. 407-17.
233. Colasurdo, G.N., et al., *Respiratory syncytial virus infection in a murine model of cystic fibrosis.* J Med Virol, 2006. **78**(5): p. 651-8.
234. Wang, J.P., et al., *MDA5 and MAVS mediate type I interferon responses to coxsackie B virus.* Journal of Virology, 2010. **84**(1): p. 254-60.
235. Barral, P.M., et al., *RIG-I is cleaved during picornavirus infection.* Virology, 2009. **391**(2): p. 171-6.
236. Besch, R., et al., *Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells.* J Clin Invest, 2009. **119**(8): p. 2399-411.
237. Shigemoto, T., et al., *Identification of loss of function mutations in human genes encoding RIG-I and MDA5: implications for resistance to type I diabetes.* J Biol Chem, 2009. **284**(20): p. 13348-54.
238. Tanaka, S., et al., *Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes.* Diabetes, 2009. **58**(10): p. 2285-91.
239. Parham, P., *MHC class I molecules and KIRs in human history, health and survival.* Nat Rev Immunol, 2005. **5**(3): p. 201-14.

240. Lee, R.K., et al., *Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine-activated killer cells.* J Immunol, 1996. **157**(5): p. 1919-25.
241. Sato, K., et al., *Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta.* Eur J Immunol, 2001. **31**(11): p. 3138-46.
242. Cornell, C.T., et al., *Coxsackievirus B3 proteins directionally complement each other to downregulate surface major histocompatibility complex class I.* J Virol, 2007. **81**(13): p. 6785-97.
243. Bonaparte, M.I. and E. Barker, *Killing of human immunodeficiency virus-infected primary T-cell blasts by autologous natural killer cells is dependent on the ability of the virus to alter the expression of major histocompatibility complex class I molecules.* Blood, 2004. **104**(7): p. 2087-94.
244. Brilot, F., et al., *Coxsackievirus B4 infection of human fetal thymus cells.* J Virol, 2004. **78**(18): p. 9854-61.
245. Feuerer, M., et al., *How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets.* Immunity, 2009. **31**(4): p. 654-64.
246. Rasschaert, J., et al., *Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells.* J Biol Chem, 2005. **280**(40): p. 33984-91.
247. Hostens, K., et al., *Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release.* J Clin Invest, 1999. **104**(1): p. 67-72.
248. Rhode, A., et al., *Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development.* J Immunol, 2005. **175**(6): p. 3516-24.
249. Roep, B.O., et al., *Islet inflammation and CXCL10 in recent-onset type 1 diabetes.* Clin Exp Immunol, 2010. **159**(3): p. 338-43.
250. Hober, D. and P. Sauter, *Pathogenesis of type 1 diabetes mellitus: interplay between enterovirus and host.* Nature Reviews Endocrinology, 2010.
251. Gibson, R.L., J.L. Burns, and B.W. Ramsey, *Pathophysiology and management of pulmonary infections in cystic fibrosis.* Am J Respir Crit Care Med, 2003. **168**(8): p. 918-51.
252. Flodström, M., et al., *A critical role for inducible nitric oxide synthase in host survival following coxsackievirus B4 infection.* Virology, 2001. **281**(2): p. 205-15.
253. Kelley, T.J. and M.L. Drumm, *Inducible nitric oxide synthase expression is reduced in cystic fibrosis murine and human airway epithelial cells.* J Clin Invest, 1998. **102**(6): p. 1200-7.
254. Sharer, N., et al., *Mutations of the Cystic Fibrosis Gene in Patients with Chronic Pancreatitis.* N Engl J Med, 1998. **339**(10): p. 645.
255. Cohn, J., et al., *Relation between Mutations of the Cystic Fibrosis Gene and Idiopathic Pancreatitis.* N Engl J Med, 1998. **339**(10): p. 653.