

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

ENTEROVIRUS INFECTIONS IN TYPE 1 DIABETES AND CYSTIC FIBROSIS – ANTIVIRAL DEFENCE AND VIRAL IMMUNE EVASION STRATEGIES

Emma Svedin



**Karolinska
Institutet**

Stockholm 2017

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

Printed by E-Print AB 2017

© Emma Svedin, 2017

ISBN 978-91-7676-614-9

Enterovirus Infections in Type 1 Diabetes and Cystic Fibrosis - Antiviral Defence and Viral Immune Evasion Strategies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

This thesis will be defended in public in lecture hall 4Z, Alfred Nobels Allé 8, Karolinska University Hospital, Huddinge

Friday the 12th May 2017, at 9:30

By

Emma Svedin

Principal Supervisor:

Malin Flodström Tullberg, Professor
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Co-supervisor(s):

Lena Eliasson, Professor
Lund University
Department of Clinical Sciences
Islet Cell Exocytosis

Opponent:

Thomas Michiels, Professor
Université Catholique de Louvain
De Duve Institute

Examination Board:

Bryndis Birnir, Professor
Uppsala University
Department of Neuroscience

Anna Smed Sörensen, Docent
Karolinska Institutet
Department of Medicine, Solna

Anna Överby Wernstedt, Docent
Umeå University
Department of Clinical Microbiology

To my family

ABSTRACT

Enteroviruses are common viruses which cause infections in humans that usually result in mild flu-like symptoms before viral clearance. However, in some cases these infections can progress to more severe diseases such as myocarditis, pancreatitis and hepatitis.

Coxsackievirus induced hepatitis in infants may become so severe that the outcome is fatal. In addition, infections with enteroviruses belonging to the group B Coxsackieviruses have been implicated in the etiology of type 1 diabetes. Enterovirus infections are also commonly observed in patients with cystic fibrosis, resulting in lung exacerbations and morbidity.

Proper antiviral response mechanisms are crucial for the prevention of viral replication and spread, as well as the inhibition of virus induced cellular damage. Recently a novel group of interferons, called type III interferons, were discovered and shown to have antiviral properties predominantly in cells of epithelial origin. In Paper I we show that type III interferons protect primary human hepatocytes from Coxsackievirus infection. Given the importance of interferons in preventing early viral replication, many viruses have developed mechanisms to inhibit their induction. In Paper II, we showed that Coxsackieviruses inhibit the induction of type III interferons in infected cells. In addition, we demonstrated that this inhibition was caused by the proteolytic activity of the viral protease 2A^{pro}. The exact role of enterovirus infections in type 1 diabetes development is still under speculation. Coxsackieviruses encodes several viral proteins that have been shown to interfere with cellular function and signaling pathways. In Paper III, we used primary human pancreatic islets and an insulin-secreting cell line to identify mechanisms by which Coxsackieviruses can cause beta cell dysfunction. We found that the viral proteins 2A^{pro}, 3A and 3C^{pro} could, independently of one other, affect exocytosis with 2A^{pro} and 3C^{pro} targeting calcium influx while 3A inhibited exocytosis via a calcium independent mechanism. An impaired antimicrobial defense has been observed in patients with cystic fibrosis. This could explain why common respiratory infections are often prolonged and more severe in these patients. By using a mouse model for cystic fibrosis, we showed in Paper IV that the most common mutation resulting in cystic fibrosis, F508del, caused an impaired adaptive immune response with a delayed production of neutralizing antibodies to Coxsackievirus.

In conclusion, the studies performed in this thesis add to our understanding of innate and adaptive immune response mechanisms during Coxsackievirus infections. In addition they demonstrate a role for viral proteins in circumventing host antiviral immune responses, thus causing cellular damage, which could contribute to disease pathology. Increasing the knowledge of host-pathogen interactions may help to develop new treatments that could prevent severe Coxsackievirus infections.

LIST OF PUBLICATIONS

- I. Katharina Lind, **Emma Svedin**, Renata Utorova, Virginia M Stone, and Malin Flodström-Tullberg. Type III Interferons are Expressed by Coxsackievirus Infected Human Primary Hepatocytes and Regulate Hepatocyte Permissiveness to Infection. *Clinical and Experimental Immunology*. 2014, 177:687-695
- II. Katharina Lind, **Emma Svedin**, Erna Domsgen, Sebastian Kapell, Olli Laitinen, Markus Moll, and Malin Flodström-Tullberg. Coxsackievirus counters the host innate immune response by blocking type III interferon expression. *The Journal of General Virology*. 2016 vol. 97 (6) pp. 1-12
- III. **Emma Svedin**, Erna Domsgen, Sebastian Kapell, Anna Edlund, Lena Eliasson, Malin Flodström-Tullberg. Coxsackievirus affects multiple steps in the insulin secretion pathway leading to impaired insulin release by infected beta cells. *Manuscript*
- IV. **Emma Svedin**, Renata Utorova, Michael H. Hühn, Pär Larsson, Manasa Gamriella, Katharina Lind, Terezia Pincikova, Gerald M. McInerney, Bob Scholte, Lena Hjelte, Mikael C. I. Karlsson, Malin Flodström-Tullberg. Enterovirus challenge reveals an association between a common polymorphism in CFTR and impaired adaptive antiviral defense. *Manuscript*

PUBLICATIONS NOT INCLUDED IN THE THESIS

- SI. Olli H. Laitinen, **Emma Svedin***, Sebastian Kapell*, Anssi Nurminen*, Vesa P. Hytönen and Malin Flodström-Tullberg. Enteroviral proteases: structure, host interactions and pathogenicity. *Reviews in Medical Virology*. 2016; 26: 251-267
* Contributed equally
- SII. Terezia Pincikova, **Emma Svedin**, Erna Domsgen, Malin Flodström-Tullberg, and Lena Hjelte. Cystic fibrosis bronchial epithelial cells have impaired ability to activate Vitamin D. *Acta Paediatrica*. 2016, 105, pp, 851-853
- SIII. Morten Gram Pedersen, Vishal Ashok Salunkhe, **Emma Svedin**, Anna Edlund, and Lena Eliasson. Calcium Current Inactivation Rather than Pool Depletion Explains Reduced Exocytotic Rate with Prolonged Stimulation in Insulin-Secreting INS-1 832/13 Cells. *PloS one*. 2014, vol. 9 (8) p. e103874
- SIV. The nPOD-V Consortium, Sarah J Richardson, Pia Leete, Shalinee Dhayal, Mark A Russell, Maarit Oikarinen, Jutta E Laiho, **Emma Svedin**, Katharina Lind, Therese Rosenling, Nora Chapman, Adrian J Bone, Alan K Foulis, Gun Frisk, Malin Flodström-Tullberg, Didier Hober, Heikki Hyöty, and Noel G Morgan. Evaluation of the fidelity of immunolabelling obtained with clone 5D8/1, a monoclonal antibody directed against the enteroviral capsid protein, VP1, in human pancreas. *Diabetologia*. 2013 vol. 57 (2) pp. 392-401
- SV. Pär G Larsson, Tadepally Lakshmikanth, **Emma Svedin**, Cecile King, and Malin Flodström-Tullberg. Previous maternal infection protects offspring from enterovirus infection and prevents experimental diabetes development in mice. *Diabetologia*. 2013 vol. 56 (4) pp. 867-874
- SVI. Michael H Hühn, Stephen A McCartney, Katharina Lind, **Emma Svedin**, Marco Colonna, and Malin Flodström-Tullberg. Melanoma differentiation-associated protein-5 (MDA-5) limits early viral replication but is not essential for the induction of type 1 interferons after Coxsackievirus infection. *Virology*. 2010 vol. 401 (1) pp. 42-48

CONTENTS

1	Introduction	1
1.1	Enteroviruses.....	1
1.1.1	Coxsackievirus group B.....	1
1.2	Basic Immunology	3
1.2.1	The innate immune system	3
1.2.2	The adaptive immune system	5
1.2.3	Viral immune evasion strategies.....	7
1.3	Type 1 diabetes	7
1.3.1	Autoimmunity	7
1.3.2	Environmental factors in T1D	8
1.3.3	The islets of Langerhans	9
1.4	Cystic fibrosis	10
1.4.1	CFTR.....	11
1.4.2	Impaired immune functions in CF.....	12
1.4.3	The CFTR ^{tm1EUR} mouse model.....	12
2	Aims of the thesis	15
3	Material and methods.....	17
3.1	Virus strains	17
3.2	Cell lines.....	17
3.3	Primary human cells	17
3.4	Animals	18
3.5	Hormone secretion.....	18
3.6	Patch Clamp	19
4	Results and Discussion.....	20
4.1	Paper I	20
4.2	Paper II.....	21
4.3	Paper III.....	24
4.4	Paper IV	26
5	Concluding remarks	29
6	Acknowledgements.....	30
7	References	33

LIST OF ABBREVIATIONS

ANO	Anoctamin
APC	Antigen-presenting cell
BCR	B cell receptor
CAR	Coxsackie and adenovirus receptor
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CVB	Coxsackievirus group B
DAF	Decay-accelerating factor
DC	Dendritic cell
ds	Double stranded
eIF4G	Eukaryotic initiation factor 4G
ECMV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
EV	Enterovirus
GAD	Glutamate decarboxylase
GLUT	Glucose transporter
HCV	Hepatitis C virus
hGH	Human growth hormone
IA-2	Islet antigen-2
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNLR	Interferon lambda receptor
IPS1	Interferon- β promoter stimulator 1
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
LGP2	Laboratory of genetics and physiology 2
MDA5	Melanoma differentiation-associated protein 5
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88

NF- κ B	Nuclear factor kappa light chain enhancer of activated B cells
NK	Natural killer
NOD	Non-obese diabetic
NOS2	Nitric oxide synthase 2
OAS	2'-5'-oligoadenylate synthase
ORCC	Outward rectifying chloride channel
PAMP	Pathogen associated molecular patterns
PCBP	Poly C binding protein
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
R848	Resiquimod
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I like receptor
Rnase L	Ribonuclease L
RP	Reserve pool
RRP	Readily releasable pool
S1P	Sphingosine-1-phosphate
SNP	Single nucleotide polymorphism
ss	Single stranded
T1D	Type 1 diabetes
TCR	T cell receptor
TD	T dependent
TI	T independent
TLR	Toll like receptor
TRIF	TIR-domain containing adapter-inducing IFN β
VP	Viral protein
ZnT8	Zink transporter 8

1 INTRODUCTION

Our body is constantly fighting a never-ending battle against pathogens that are trying to invade our body. To defend ourselves, we have evolved several protective mechanisms that together constitute the immune system. The discovery that viruses are infectious agents which are capable of transferring and spreading disease was made in late 1800 when Dmitry Ivanovsky showed that a bacteria-free filtrate from an infected tobacco plant could still transfer disease to a healthy plant (1). A few years later, Martinus Beijerinck repeated these experiments and concluded that the disease must be caused by a new type of infectious agent which was subsequently named “virus” after the Latin word for poison (1). Today, over 5000 virus species have been discovered. Viruses can infect all living things including animals, plants and bacteria. However, as they are small parasites they cannot reproduce by themselves and require the replication machinery of living cells in order to spread (2).

Increasing our knowledge regarding host antiviral immune response mechanisms and how viruses circumvent these mechanisms, thereby causing cellular damage will hopefully provide clues on how viruses are triggering disease and insight on how to develop new antiviral therapies. This thesis is based on four papers in which I have studied the importance of type III interferons in regulating permissiveness to a Coxsackievirus infection in primary human hepatocytes (**Paper I**) and how Coxsackieviruses utilize virus encoded proteins to evade the type III interferon response (**Paper II**). In addition, I have looked at how Coxsackievirus encoded proteins can cause beta cell dysfunction after infection (**Paper III**) and how the adaptive immune response to Coxsackieviruses is affected by mutations in the cystic fibrosis transmembrane conductance regulator protein (**Paper IV**). A brief introduction to the field will follow to broaden the understanding of the concepts presented in **Papers I-IV**.

1.1 ENTEROVIRUSES

All viruses can be subdivided into different classes based on their similarities. One of the most common types of viral infection in humans is that caused by viruses belonging to the enterovirus (EV) genus. These viruses carry their genome as a small positive single stranded RNA molecule and usually infect via the enteric, i.e. intestinal, route (2). The RNA genome is surrounded by a capsid which is highly resistant to environmental exposure such as drying and low pH from stomach acid (1). EVs can use a broad range of molecules on the cell surface as receptors for viral entry (3). Due to the specificity for certain receptors, different viruses within the EV genus usually have distinct target organs which they can infect and induce pathology.

1.1.1 Coxsackievirus group B

Coxsackievirus group B (CVB) belongs to the EV genus and comprises of six different serotypes, CVB 1-6 (4). CVBs infect cells via the Coxsackievirus and adenovirus receptor (CAR) (5), which is part of the tight junction complex that mediates cellular adhesion in a

number of tissues such as heart and epithelial cells of the gut (6-8). In addition, studies have shown that certain serotypes of CVB can also infect via decay acceleration factor (DAF), which is a protein that regulates activation of the complement system (9, 10). Infections with CVBs are very common and are usually associated with mild flu-like symptoms. However, they can in some cases give rise to more severe diseases such as myocarditis, hepatitis, pancreatitis and meningitis, with CVB infections being the most common cause of aseptic meningitis in children (2). In addition, several studies have suggested that CVBs may be involved in the etiology of type 1 diabetes (T1D) (11, 12).

1.1.1.1 CVB replication and host cell interactions

After CVB enters the host and has attached to the viral receptors CAR or DAF on target cells, it is internalized via endocytosis (13). Once inside the cell, the CVB genome is uncoated and exposed to the cytoplasmic contents. The positive RNA strand can immediately be translated by the cellular ribosome into a single viral polyprotein. The polyprotein encodes four structural proteins (VP1-4) and seven non-structural proteins (2A-C and 3A-D) which are all important for viral replication (14). Among the non-structural proteins are two viral proteases $2A^{pro}$ and $3C^{pro}$ which are responsible for viral maturation and, upon translation, will cleave the viral polyprotein into separate functional viral proteins (15-17). Once the viral proteins are formed, the virus can start to replicate in the cytoplasm. This replication takes place on rearranged cellular membranes that are derived from the endoplasmic reticulum (ER) and Golgi (14).

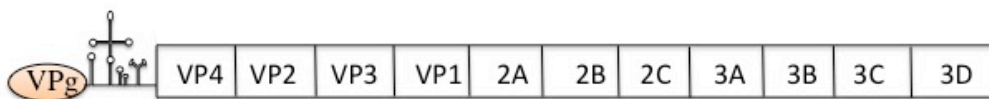


Figure 1: The CVB polyprotein

Although EVs are small viruses that only carry a very limited number of proteins, several of these viral proteins have been shown to have multiple functions within the infected cell. For example, in addition to cleaving the viral polyprotein, the proteases can also target several cellular proteins. One target for $2A^{pro}$, is the eukaryotic initiation factor 4G (eIF4G) which is needed for translation of 5' capped cellular RNA (18). Since viruses do not have a 5' cap but instead bind to the ribosome via an internal ribosome entry site (IRES), CVB is able to inhibit cellular protein translation and thus promote the production of viral proteins. $3C^{pro}$ has been shown to cleave poly C binding protein (PCBP) and through this, it can regulate the switch from viral translation to viral replication (19). Furthermore, it has been reported that $2A^{pro}$ directly contributes to disease pathology by cleaving dystrophin, resulting in CVB induced cardiomyopathy (20, 21). Other viral non-structural proteins with membrane binding capacity, like 2B and 3A, can also affect host cell functions by recruiting cellular membranes to generate viral replication organelles in the cytoplasm. 2B and 3A target both COPII and COPI coated vesicles and inhibit ER to Golgi transport (22, 23). In addition, it has been suggested that 3A is important in promoting the binding of the viral polymerase 3D to the

membrane of these replication organelles (23). However, the actual need for 3A in this process has been debated (24).

1.2 BASIC IMMUNOLOGY

To prevent surrounding pathogens from infecting and causing disease, we have developed specialized mechanisms to protect ourselves. Collectively, these specialized mechanisms are called the immune system (25). The first part of the immune system includes physical barriers like the skin and mucosa which prevent pathogens from entering our bodies. If a pathogen still manages to break through these barriers and enter the body there are two immune response arms that can respond, the fast acting innate immune response and the more slowly activated but highly specific adaptive immune response.

1.2.1 The innate immune system

The innate immune system provides the first line of defense if a pathogen manages to breach the physical barriers and cells belonging to the innate immune system can immediately recognize and respond to a pathogen. Cells that can mount an innate immune response include macrophages, dendritic cells (DCs), neutrophils and natural killer (NK) cells, which are constantly present in the tissue, as well as infected nucleated cells. These cells express germ line encoded pattern recognition receptors (PRRs) that are activated in a non-specific manner via the recognition of general structures found on pathogens, so called pathogen associated molecular patterns (PAMPs) (26). Recognition of such danger signals induces the production of cytokines that then attract further immune cells to site of infection and also induce the phagocytosis of pathogens. Thus the early activation of the innate immune response is important for containing infections and preventing spread. In addition, cells of the innate immune system also have an important role in activating the adaptive immune system as they are able to process and present antigens to T cells (26). Due to the large variety of pathogens, cells have developed a broad range of PRRs which are expressed both intracellularly, for recognition of e.g. viruses, and on the cell surface for detection of extracellular pathogens such as bacteria.

1.2.1.1 Recognition of viruses and signaling pathways

Two major groups of PRRs that are important for the recognition of RNA viruses are the Toll like receptor (TLRs) and RIG-I like receptors (RLRs) (27, 28). As many as 10 different TLRs have been discovered in humans but only TLR3 and TLR7-9 are expressed in an intracellular location on endosomes and endolysosomes (29). Even though TLR2 and TLR4 have also been shown to recognize viruses, they seem to be mainly important for the recognition of enveloped viruses (30, 31). TLR3 recognizes dsRNA and is important for the detection of influenza A virus and CVB3 (32) (33). TLR3 also serves as a sensor of the synthetic RNA molecule, Polyinosinic:polycytidylic acid (poly I:C) (34). TLR7-8 recognize ssRNA (35) and are also thought to be important in the recognition of influenza A virus and CVB (36, 37). In addition, they are stimulated by small synthetic antiviral molecules such as resiquimod

(R848) (38). TLR9 is activated by CpG molecules, which originate from by DNA viruses and bacteria (39).

RLRs include three members, retinoic acid inducible gene I (RIG-I), melanoma differentiation associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) which are all expressed in the cytoplasm (40). Both MDA5 and RIG-I recognize dsRNA and poly I:C. However, the two differ in the RNA patterns that they bind. RIG-I recognizes non-self 5'-triphosphorylated dsRNA and short poly I:C stretches (41-43) and its importance in the recognition on many different virus families, such as paramyxo-, flavi- and picorna-viruses has been demonstrated as well (44-46). MDA5 recognizes long dsRNA stretches and poly I:C (43, 47) and is important for the recognition of picornaviruses such as encephalomyocarditis virus (ECMV) and CVB (47-49). LGP2 also binds dsRNA but lacks the ability to signal downstream on its own. Instead, it seems to function by regulating RIG-I and MDA5 signaling (50, 51). The exact mechanism by which LGP2 regulates this pathway is, however, unknown.

Viral sensing via these PRRs leads to the activation and induction of intracellular signaling pathways that promote an immune response. TLR3 signals via TIR-domain containing adapter-inducing IFN β (TRIF) while TLR7-9 signal via myeloid differentiation factor 88 (MyD88). MDA5 and RIG-I signal via the common adaptor protein interferon- β promoter stimulator 1 (IPS1) (39, 52). Signaling via these adaptor proteins induces the activation and translocation of the transcription factors nuclear factor kappa light chain enhancer of activated B-cells (NF- κ B) and IFN regulatory transcription factor 3 and 7 (IRF3 and IRF7) to the nucleus, which results in the subsequent production of proinflammatory cytokines and interferons (IFNs) (53).

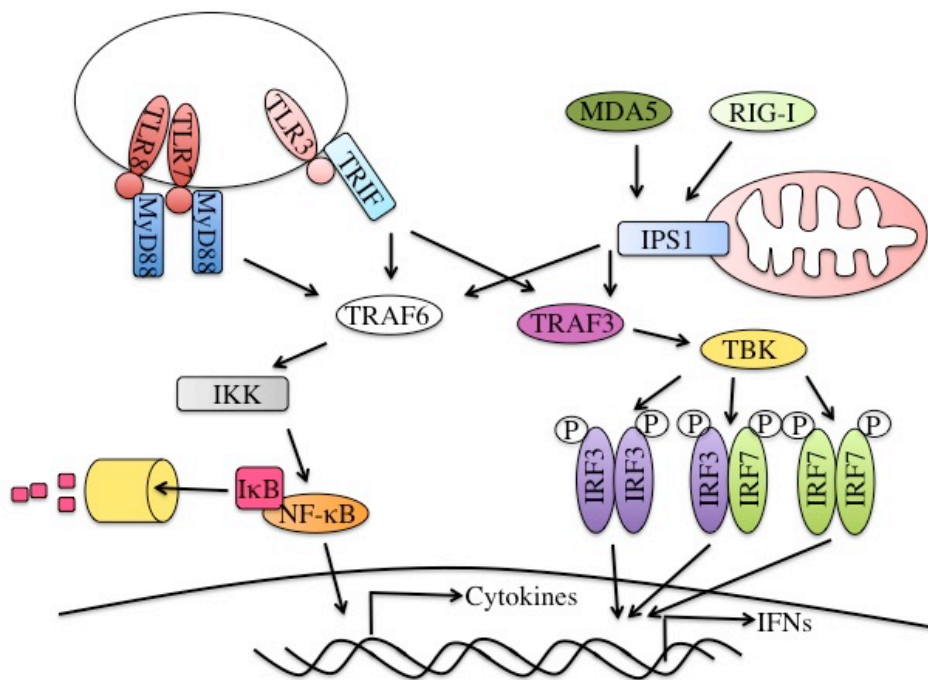


Figure 2: Model of signaling pathways involved in sensing CVBs

1.2.1.2 Interferons

IFNs were discovered 60 years ago as proteins that can interfere with viral replication (54). Since then many studies have shown the importance of IFNs in protecting the host during early virus replication by inducing an antiviral state in neighboring cells. In addition, they have been shown to affect and shape the adaptive immune system by directing B cell and T cell responses (55-57). Today IFNs are divided into three groups based on their sequence homology. Type I IFNs consist of several isoforms of IFN α and one single isoform of IFN β , as well as a number of additional subgroups whose functions in viral infection are less clear. Most cells can produce type I IFNs in response to viral infection which provide protection against a number of viruses (58, 59). Type II IFNs consists of only one type of IFN, IFN γ . IFN γ is important in regulating the response of immune cells and is mainly produced by T cells and NK cells. It activates macrophages and upregulates major histocompatibility complex (MHC) class I and class II expression (60). The type III IFNs (IFN λ) consists of four different isoforms, IFN λ 1 (IL-29), IFN λ 2 (IL28a), IFN λ 3 (IL-28b) and IFN λ 4 (61-63). Type III IFNs are mainly produced by cells of epithelial origin but can also be produced by certain types of immune cells (64, 65). IFN λ s have both similar functions and antiviral properties to those of IFN α and IFN β . However, type I IFNs signal via the IFN α -receptor (IFNAR) which is expressed on all cells (66), while type III IFNs signal via the IFN λ -receptor (IFNLR) the expression of which appears to be limited to cells of epithelial origin and specific immune cells (64, 67, 68). Thus, the antiviral effects of type III IFNs appear to have greater organ selectivity and seem to be important mainly in the promotion of antiviral effects at viral entry sites such as mucosal surfaces. Type III IFNs are important in protection against hepatitis C virus (HCV) and CVB in hepatic cell lines and human islets (69, 70). However, some differences in the responsiveness to IFN λ between human and mouse hepatocytes have been demonstrated (71).

Once IFNs are produced they are secreted and bind to receptors on cell surfaces. This binding activates intracellular signaling pathways that lead to the upregulation and expression of many different IFN stimulated genes (ISGs) which in turn will promote the cell to enter an antiviral state (72). The exact mechanisms through which several of these ISGs provide protection against infection are unknown. However, the function of the 2'-5'-oligoadenylate synthase (OAS) pathway has been demonstrated. IFNs induce OAS upregulation which can bind dsRNA, resulting in the activation of Ribonuclease L (RNase L). Activated RNase L targets both viral and cellular ssRNA for degradation (73). Another ISG which is quickly upregulated after IFN stimulation is IRF7 which is briefly described in 1.2.1.1. IRF7 is needed for the production of IFN α and is stimulated by IFN β production (74, 75).

1.2.2 The adaptive immune system

The main cells of the adaptive immune system are the B cells and T cells, which are involved in antibody production and direct cell mediated killing of infected cells. The adaptive immune response offers a much more specific recognition of antigens than the innate immune response due to the fact that each lymphocyte undergoes somatic rearrangement of the B cell

and T cell receptor (BCR and TCR respectively) (76). This creates a vast number of different receptors that are able to recognize distinct antigens rather than a group of patterns. As the adaptive immune response requires firstly that the antigen is recognized by the correct BCR or TCR, followed by the initiation of clonal expansion, it is activated more slowly than the innate immune response. However, the adaptive immune response is more efficient than the innate immune response when it comes to eliminating the pathogen. Adaptive immunity is also important for the production of long lasting memory cells, which are the basis for immunological memory and an important function of modern vaccines.

1.2.2.1 T cells

There are two main types of T cells, CD8⁺ and CD4⁺, which are usually referred to as cytotoxic T cells and T-helper cells, respectively. CD8⁺ T cells are important for cell-mediated toxicity while CD4⁺ T cells are important for proper antibody responses. CD8⁺ T cells are activated by antigens presented on MHC class I molecules, which are expressed by all nucleated cells. CD4⁺ T cells are activated by antigens presented on MHC class II which has a more restricted expression and is found on so called professional antigen presenting cells (APCs) like DCs, macrophages and B cells (77). Antigen presentation on MHC class I or MHC class II, in combination with the expression of co-stimulatory molecules, leads to T cell activation. Once activated, the cells undergo clonal expansion, thus generating a large number of cells with the same receptor specificity. Recognition of antigens on infected cells by activated CD8⁺ T cells induces the killing of the cell through the release of perforin and granzyme, which stimulates apoptosis and the fragmentation of the cellular and viral genomes. In addition, activated T cells also secrete cytokines that further stimulate the upregulation of MHC class I expression on cells, and the recruitment and activation of macrophages. Activated CD4⁺ T cells help to modify the response of other immune cells and can induce upregulation of co-stimulatory molecules to further activate CD8⁺ cells. More importantly, the interaction between the TCR on activated CD4⁺ T cells with antigens presented on B cells, activates B cell proliferation and the production of antibodies and induces antibody class switch (77).

1.2.2.2 B cells

The main function of B cells is to produce antibodies in response to pathogens. B cells can be activated in a T cell independent (TI) or T cell dependent (TD) manner (78, 79). Activation of B cells without T cell help is thought to occur via the crosslinking of BCR upon encounter with antigen. This activation mainly produces antibodies of the IgM type and does not result in the induction of long-lived memory B cells. TD activation requires the interaction of a B cell with TCR. This interaction will also promote CD40-CD40L interactions, which induce antibody class switching, and thus modify the effector function of the antibody (80). There are five different classes of antibodies, IgA, IgD, IgE, IgM, and IgG. IgM and IgD are the earliest antibodies expressed after infection, although IgG is the most predominant form. The antibodies aid in clearing the infection by promoting phagocytosis, antibody-dependent cellular cytotoxicity, neutralization of the pathogen and complement mediated killing (81).

1.2.3 Viral immune evasion strategies

Given the importance that the immune system has in preventing viral replication and spread, it is not surprising that several pathogens have developed mechanisms to inhibit activation of the immune response and to avoid recognition by the immune system. As described in *1.1.1.1*, CVBs target several cellular proteins to promote viral replication. In the same way many viruses can also target proteins to prevent their recognition by the immune system. Several studies have shown that viruses within the EV genus can target a number of proteins found in the IFN pathway (82). For example, both 2A^{pro} and 3C^{pro} can inhibit the induction of both type I and type III IFNs by directly targeting IPS1 and TRIF, and thus inhibiting MDA5/RIG-I and TRL3 signaling in infected cells (83, 84). In addition, 3A has been shown to inhibit the secretion of cellular proteins and cytokines to reduce immune responses (85, 86). This viral protein also mediates MHC class I down-regulation (87) thereby escaping recognition of T cells and T cell mediated cell death.

1.3 TYPE 1 DIABETES

Until the discovery of insulin in early 1920s, type 1 diabetes (T1D) was inevitably fatal. T1D results from an inability to properly regulate blood glucose levels due to a lack of the insulin producing beta cells. Insulin is a hormone that is needed for the upregulation of glucose transporters primarily on muscle cells and adipose tissue, which mediates the uptake of glucose from the bloodstream after food intake. T1D is the most common chronic disease in children and disease onset peaks between 5-7 years of age (88). The incidence of T1D in different countries varies drastically with 0.1 in 100 000 cases in low incidence countries such as Venezuela and up to 60 in 100 000 cases in high incidence countries like Finland (89, 90). Patients with T1D require lifelong supplementation of exogenous insulin. Even though the treatment for T1D has long been known and practiced, the cause(s) of T1D development is/are yet to be understood.

1.3.1 Autoimmunity

It has long been suggested that the selective loss of beta cells in T1D is mediated by an autoimmune attack (91). This is supported by observations showing that over 90% of patients with T1D have autoantibodies to beta cell antigens such as glutamate decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), zinc transporter 8 (ZnT8) and insulin (92). Indeed, detection of two or more autoantibodies in serum is associated with a marked increase in the risk of developing T1D and the appearance of autoantibodies usually precedes T1D onset by months up to several years (93-95). Furthermore, studies have shown that many islets from T1D patients are insulitic, which is usually defined by the presence of a lymphocytic infiltration of the islets. However, the criteria for diagnosing insulitis in humans has been debated lately, and when comparing human insulitis to that observed in the Non Obese Diabetic (NOD) mouse, a model for autoimmune T1D where islets are heavily infiltrated by autoimmune cells, the insulitis observed in humans is usually very mild (96). The matter is further complicated by studies showing that different islets from the same patient may or may not be insulitic (97). Still, if autoantibodies play a pathogenic role in T1D or whether they

result from ongoing beta cell destruction is not known. A study looking at pancreatic tissue from organ donors which were positive for one to four autoantibodies, showed that insulinitis was detected in less than 10% of the islets in two out of three subjects which had at least three autoantibodies, but not in the other autoantibody positive subjects. However, none of the subjects in the study showed a decrease in beta cell mass (98).

1.3.2 Environmental factors in T1D

During recent years there has been a rapid increase in T1D incidence with the most dramatic rise seen in children under the age of 5 (88, 89). This escalation seems to occur in individuals carrying genes that were previously deemed to confer a low risk for T1D development. In fact, 85% of new T1D cases have no history of T1D in the family (99). The rapid increase in T1D suggests that there might be an augmented role for environmental factors in T1D development. Some environmental factors that have been suggested to regulate susceptibility to T1D include dietary factors (100-102), microbiotic alterations in the intestine (103, 104) and viral infections (11, 105, 106).

Many studies have suggested infections with EVs are a potential trigger for T1D. It was already proposed in 1969 that CVBs could be involved in T1D development when Gamle *et al* published that antibodies to CVBs were found to be more frequent in newly diagnosed T1D patients compared to controls (107). This hypothesis was further supported in 1979 when Yoon *et al* isolated CVB4 from a diabetic boy, which in turn could induce diabetes in mice (108). Since then many studies have tried to prove (or disprove) the role of virus infections in T1D etiology. Indeed, many studies have found EV RNA in blood samples, as well as the presence of EV viral protein 1 (VP-1) in pancreatic and intestinal biopsies, to be more frequent in T1D patients compared to controls (109-115). However, not all studies have been able to confirm the finding that EVs are present in the intestine of patients with T1D (116). A potential disadvantage with the studies that have looked at viral protein in the pancreas is that many of them have been conducted with pancreatic biopsies taken during autopsies, some of which were collected several years after T1D diagnosis. These may not necessarily be representative of what happens at disease onset. However, a novel study recently collected biopsies from six living T1D patients at time of diagnosis (117). The study showed that all patients were positive for EV in the pancreas at T1D onset. In four out of these six patients, they were also able to retrieve a virus sequence that corresponded to EVs (115).

How EVs could contribute to T1D is still under speculation. A Finnish study recently associated CVB1 infections with the development of beta cell autoimmunity in children (118). Also, viral infections have previously been shown to cause autoimmune diseases and the proposed EV involvement in T1D shares many similarities with chronic myocarditis and dilated cardiomyopathy, where autoimmunity is associated with EV infection (119-123). EV-induced myocarditis appear to be caused by a persistent, low grade viral infection of the heart in which the virus mutates to create a 5' terminally deleted virus (124, 125). Indeed, EV have been demonstrated to have a tropism for beta cells (126). In addition, EVs seem to have the

ability to cause a persistent low grade infection in human pancreatic islets *in vitro* (127, 128). The finding that only very few cells are VP1 positive in islets from T1D patients further supports the theory that the virus is causing a low grade and possibly persistent infection (112, 115). As several recent studies have shown that many insulin positive cells remain in T1D individuals, with sometimes as much as 60% of the beta cell mass remaining (129), it is also possible that a low grade infection persists in insulin positive cells and causes beta cell dysfunction. Indeed CVB causes beta cell dysfunction when infected with EVs *in vitro* (111, 130). In addition, insulin positive islets isolated from T1D donors that stained positive for VP-1 showed impaired insulin release upon glucose stimulation, suggesting that EVs also cause beta cell dysfunction after infection *in vivo* (111, 131). However, if viruses are involved in T1D development and through which mechanisms they may contribute to disease remains unclear.

1.3.3 The islets of Langerhans

The hormone insulin is produced by the beta cell which reside in the islets of Langerhans located within the pancreas. Islets of Langerhans are clusters of endocrine cells mainly comprising alpha cells, beta cells and delta cells (132). The beta cell is the most abundant cell type and secretes insulin in response to elevated levels of glucose in the blood, for example after eating a meal, to decrease blood glucose concentrations. In contrast, alpha cells produce glucagon which increases blood glucose concentrations. To aid these functions, the islets of Langerhans are highly vascularized with thin blood vessels and even though they only constitutes for 1-2% of the total pancreatic mass, they receive 5-10% of the pancreatic blood flow in order to properly regulate blood glucose levels and maintain homeostasis (133).

1.3.3.1 Regulation of insulin secretion

Glucose is taken up from the blood by sensitive glucose transporters (GLUT1 and GLUT2), which are expressed in the beta cells in humans and rodents, respectively, (134, 135). The internalized glucose is used to generate ATP which triggers the depolarization of the cell by closing ATP sensitive potassium (K) channels. This in turn results in the opening of voltage gated calcium (Ca^{2+}) channels. Beta cells express many different types of Ca^{2+} channels; L-type, N-type, P/Q-type, R-type and T-type which are activated at different voltages to regulate Ca^{2+} influx (136). The increased levels of intracellular Ca^{2+} will result in the fusion of the insulin vesicle with the cell membrane, a process known as exocytosis, and insulin will be released into the bloodstream and transported to target organs like the muscles and liver.

Insulin is released in a biphasic manner (137). The first phase is induced very rapidly, as quick as 1 minute after a rise in blood glucose levels, and lasts for around 5-10 minutes. The second phase is activated after 10 minutes and can be sustained for hours until normoglycemia is restored. The nature of this biphasic pattern is thought to be a result of two different pools of insulin granules namely the readily releasable pool (RRP) and the reserve pool (RP) (138, 139) that are present in each individual beta cell. Around 1-5% of insulin granules belong to the RRP which are already docked to the plasma membrane and primed

making them available for the immediate release of insulin upon an increase in intracellular Ca^{2+} levels. The RP requires mobilization of insulin granules to the plasma membrane and priming before they can be released (140).

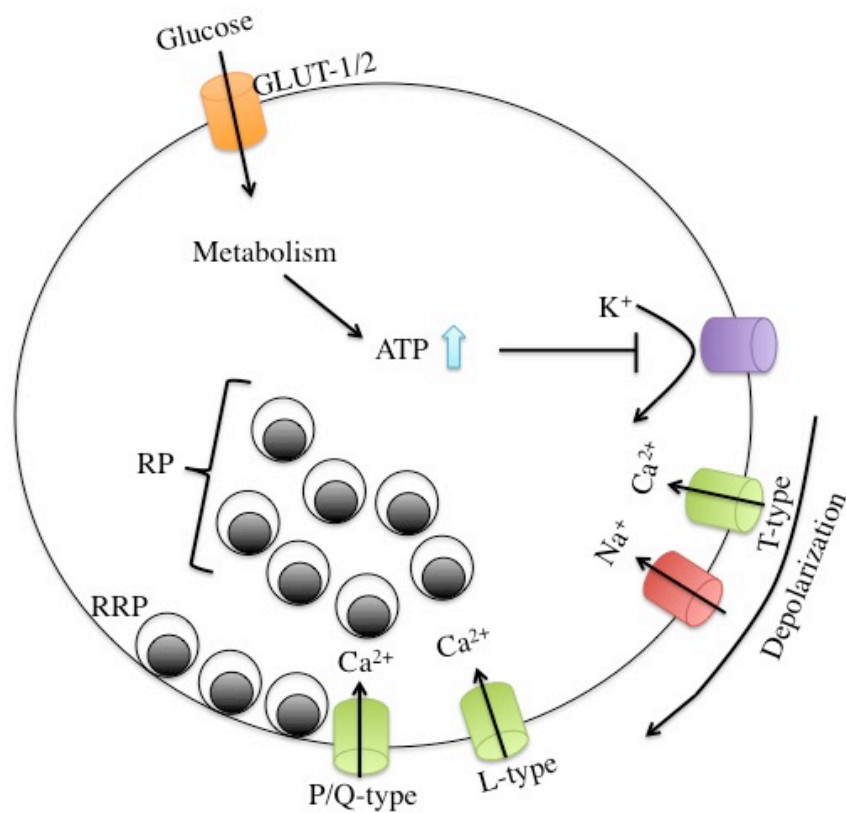


Figure 3: Simplified schematic showing signaling pathways involved in insulin secretion

1.4 CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common life shortening recessive genetic disorder in Caucasians with an incidence ranging from 1:1300 in Ireland to 1:25000 in Finland (141). Symptoms of CF in children have been described for many hundreds of years. However the cause for the disease was not known until the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene was discovered in 1989 (142, 143). CF is a multi-organ disease. One of its most prominent features is the abnormal water and ion transport in cells lining the respiratory tract which results in the accumulation of up in the lungs (144). The inability to properly clear this thick mucus from the lungs is thought to be the underlying cause of decreased bacterial clearance and chronic respiratory infections, eventually leading to respiratory failure and early mortality. Before the discovery of antibiotics *Staphylococcus aureus* was the main cause for early mortality and the majority of children died before 5 years of age (145, 146). Today, due to improved treatments, life expectancy has increased to over 40 years (147).

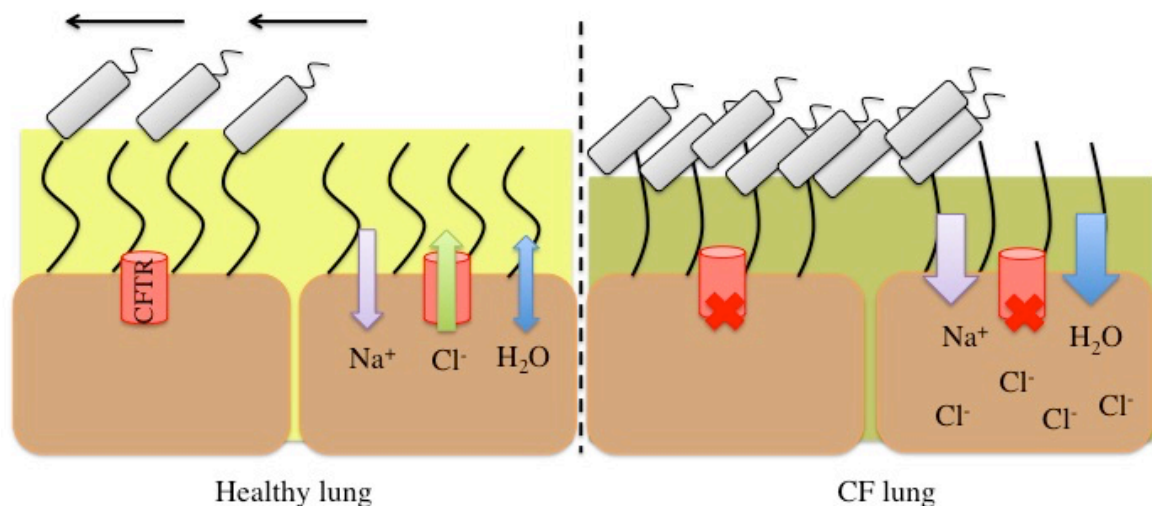


Figure 4: Impact of CFTR expression in lung epithelial cells. In the lung, CFTR is expressed on the apical side of epithelial cells and it is important for controlling electrolyte balance. In CF patients, the defective transport of chloride ions causes the accumulation of chloride inside the cells and increased influx of sodium and water. This results in dry, viscous mucus and decreased cilia movement making the environment favorable for bacterial colonization.

1.4.1 CFTR

CFTR encodes a chloride channel which is located in the cellular membrane and is needed for maintaining the properly balance of electrolytes (142, 143). Several hundred different mutations in *CFTR* contributing to CF have been identified. The mutations can be divided into six different classes depending on how the mutation affects the protein (148). In class I and class II there is a complete lack of protein synthesis (class I) or defective protein processing (class II), whereas class III-VI mutations rather affect the function or the expression of the chloride channel in the cell membrane. The most common mutation found in humans is caused by a deletion of a phenylalanine residue at position 508 (F508del) that results in the degradation of the protein before the protein reaches the cell surface. F508del is a class II mutation and affects 70-88% of patients with CF (149).

1.4.1.1 *CFTR* expression and function

CFTR is expressed in many organs of epithelial origin such as the lung, pancreas, intestine and reproductive organs and is necessary for their normal function (150). The defective ion secretion caused by dysfunctional CFTR does not just cause viscous mucus in the lungs but also leads to the obstruction of pancreatic ducts and vas deferens resulting in pancreatic insufficiency and the need for pancreatic supplements, as well as infertility (151, 152). CF also gives rise to meconium ileus bowel obstruction which affects 10% of neonates with CF (153). In addition to its importance in regulating electrolyte balance, CFTR has also have been shown to regulate the function of other proteins and ion channels such as Syntaxin 1a (154), ORCC (155, 156) and Anoctamin 1 (ANO1) (157). More recent studies have also

noted the importance of CFTR in the immune system and several studies have suggested that immune cells express CFTR (158-162), but little is known of the exact function of CFTR in different lymphocyte populations. Today novel treatments for CF are being developed in form of correctors, such as Lumacaftor, to correct CFTR folding and increase protein stability and potentiators, such as Ivacaftor, which potentiates channel opening probability and aims to restoring CFTR function (163, 164).

1.4.2 Impaired immune functions in CF

In addition to the decreased mucus clearance in the CF lung which results in bacterial infections, CF patients appear seem to be more prone to severe respiratory viral infections, which are prolonged in duration (165), suggesting that CFTR affects the immune response. Of note, these viral infections also seem to predispose the CF lung to bacterial colonization (165-169), thus contributing to pulmonary exacerbations and morbidity (165, 170). An overall dysfunctional antimicrobial immune response has been observed in CF lung epithelial cells with lower nitric oxide synthase 2 (NOS2) and OAS production leading to impaired activation of IRF1 (171). In addition, lower levels of TLR4 have been observed (172, 173). A dysregulated immune response has also been documented in the intestine and pancreas of CF patients (174-176).

Impaired function of immune cells in CF has also been observed. Lymphocytes express a number of chloride channels which are needed for the secretion of cytokines and regulation of immune responses. Since CFTR is expressed in lymphocytes, it would not be surprising if it affected their function. Indeed, CF patients have a skewed Th2 response and defective T helper cell function (177, 178) and this is postulated to be due to an intrinsic defect of the T cell population (179). In addition, DCs have been shown to have an impaired function due to low levels of sphingosine-1-phosphate (S1P) and macrophages have a decreased phagocytic capabilities (162, 180, 181).

Although many studies show that CF is associated with a diminished immune function, the CF lung displays a proinflammatory phenotype (175, 182, 183). This may, in part, be due to the constant stimuli of pathogens in the lung but may also be caused by the defective cells themselves. For example, even though neutrophils were shown to be functionally abnormal in protecting against pathogens, neutrophil airway mediated inflammation is dominating lung disease in CF (184-186). Thus the defects within the leukocyte population could promote the inflammatory milieu in the lung and contribute to lung damage.

1.4.3 The CFTR^{tm1EUR} mouse model

Several models have been developed to study the effects of CF mutations *in vivo*. Among the more recent models are ferrets and pigs, which seems to resemble the human disease in many aspects (187-189). However, the mouse has been by far the most used model to study different CF mutations as they are relatively easy to manipulate. The CFTR^{tm1EUR} mouse model carries the F508del mutation and thus mimics the most common form of CF in humans (190). The mouse has a similar phenotype to human CF with electrophysiological

abnormalities of the gastrointestinal tract and failure to reproduce (190). However, the most common feature of human CF, namely mucus accumulation and progressive inflammation in lung, are not observed in the CFTR^{tm1EUR} mouse. Still, when challenging these mice with environmental factors such as respiratory pathogens, the pathology was more severe compared to control littermates (191) suggesting that, as in humans, environmental exposure can worsen CF pathology.

2 AIMS OF THE THESIS

The overall aim of this thesis was to increase our knowledge of innate and adaptive immune response mechanisms to CVB infections. In addition, we aimed at determining the role of CVB encoded proteins in evading immune response mechanisms and causing beta cell dysfunction.

Specific aims:

- To assess whether type III IFNs are induced by CVB infection in primary human hepatocytes and regulate permissiveness to CVB infection (**Paper I**)
- To study if CVB has evolved strategies to inhibit type III IFN production by infected cells (**Paper II**)
- To investigate the mechanisms of CVB-mediated inhibition of insulin secretion from infected cells (**Paper III**)
- To study how the F508del mutation in *CFTR* affect the immune response to a common EV infection (**Paper IV**)

3 MATERIAL AND METHODS

Many different experimental techniques were used when performing the experiments included in **Papers I-IV**. In this section I have chosen to discuss some of the most important methods that were employed.

3.1 VIRUS STRAINS

In all of the papers included in this thesis (**Paper I-IV**), I have used the CVB3 Nancy strain, and on some occasions the CVB4 E2 strain (in **Paper III**). These two CVB strains have been well characterized and are used widely throughout the field. The strains were propagated in HeLa cells and viral supernatants were centrifuged to remove cellular debris. In the functional studies that I performed in **Paper III**, the virus stocks were purified. Virus purification was performed by ultracentrifugation of virus suspension through a 40% sucrose cushion. This was important so as to avoid contamination from HeLa the cells that died during virus propagation and may release cytokines and other factors into the virus suspension that might affect cell function during infection.

3.2 CELL LINES

Different cell lines were employed in the experiments performed in **Papers I-III**. In **Paper I** we utilized HepG2 and Huh7.5 cells which are both derived from human liver. We used these cells as models to investigate whether type III IFNs protect against CVB infections prior to the experiments that were performed with primary human hepatocytes. In **Paper II**, HeLa cells were used as we know from previous experiments that they are susceptible to CVB infection and they mount an intact type III IFNs response upon stimulation with poly I:C. In **Paper III**, experiments were carried out with the INS1-832/13 cell line (192). This cell line is derived from the INS1 cell line and have been stably transfected with the human proinsulin gene. INS1-832/13 cells have a stable insulin responsiveness to glucose stimulation, thus making their use favorable when studying the effect of insulin secretion over other insulinoma cell lines such as RIN cells, which are not glucose-responsive (193). A disadvantage with the INS1-832/13 cell line is that it is derived from rat and not from human. Differences in the types of Ca^{2+} channels which are important for triggering exocytosis has been documented between human and mouse beta cells, as well as in INS1-832/13 cells (194-197). This is important to remember when interpreting the results.

3.3 PRIMARY HUMAN CELLS

The use of primary human cells may sometimes be a better option when performing experiments. However, access to human material and ethical implications make these experiments more challenging. Additionally, humans are not genetically uniform and depending on the hypothesis you want to address, the results can vary greatly due to the genes present, which may significantly alter your results. However, if your goal is to study how specific genetic variations in the human population affect experimental outcomes, then the

use of primary human cells is preferable to the use of cell lines. In **Paper I**, we used primary human hepatocytes for our experiments and in **Paper III** we used isolated human islets.

3.4 ANIMALS

When studying complex immune pathways where multiple cell types depend on one another for accurate input signals and synergy, the use of cell lines is not possible. As such, animal models are utilized to study these more intricate interactions. It is, however, very important to adhere to strict ethical guidelines when performing animal experiments to minimize suffering. Mice in particular are attractive models due to the ease with which they can be genetically manipulated and their fast reproductive capacity. Today, there are many different mouse models available and they provide useful tools for studying complex diseases. In **Paper IV** I have used the CFTR^{tm1^{EUR}} mouse model which carries the most common mutation resulting in CF in humans, F508del (190). By using the CFTR^{tm1^{EUR}} mouse I hoped to gain insight into what function CFTR have in immune cell populations by studying the immune response to a common EV infection. All experiments performed in this thesis were carried out according to Swedish law.

3.5 HORMONE SECRETION

Glucose stimulated insulin secretion assays was performed with human islets and INS1-832/13 cells in **Paper III**. Important factors to consider when using isolated islets for functional experiments include that the isolation procedure and transport of the islets may affect their viability and ability to secrete insulin. In addition, upon arrival at the Karolinska Institutet, islets were kept in culture for 2-9 days to recover before the start of experiments. Islets are normally highly vascularized and without this system *ex vivo*, the lack of oxygen transport to the center of the clusters may cause hypoxia. Thus, I included only islets that maintained function and could respond to glucose stimulation in my experiments when studying insulin secretion (as statistically determined by T test).

With the INS1-832/13 cells I used the human Growth Hormone (hGH) assay to measure hormone release after glucose stimulation. This was due to the fact that transfection efficiency of INS1-832/13 cells was low (around 10-15%). As a result of this, when performing measurements of insulin release, 85% of the insulin released would originate from cells that were not expressing the viral proteins. To circumvent this problem, viral proteins, or control plasmid, were co-transfected with hGH plasmid. Since hGH is secreted by the same vesicles as insulin, hGH will be released from transfected cells upon glucose stimulation allowing us to study hormone secretion in only the cells overexpressing viral proteins with an hGH ELISA (Figure 5).

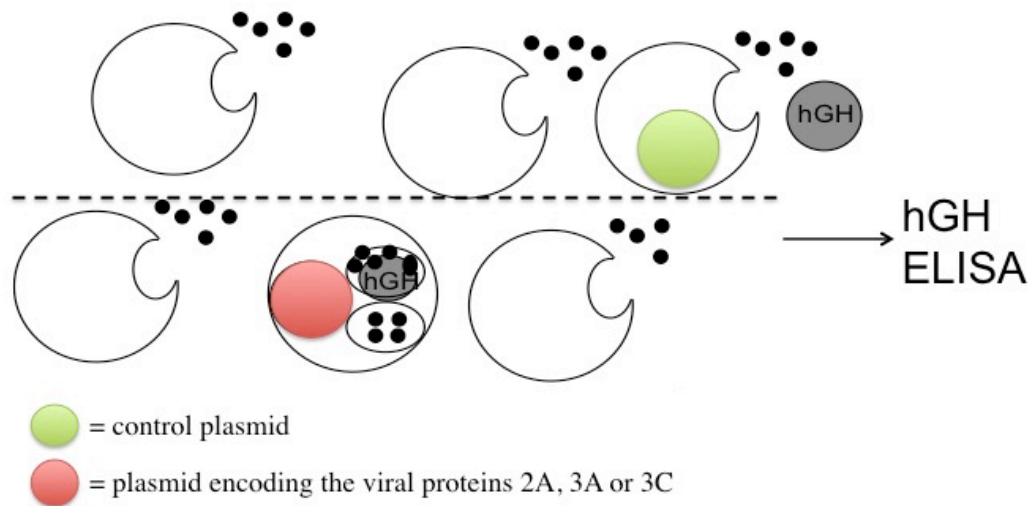


Figure 5: Diagram illustrating the hGH assay. Due to low transfection efficiency, hGH was used to measure hormone release after glucose stimulation in transfected INS1-832/13 cells. Cells were co-transfected with hGH (grey) and control plasmid (green) or plasmids encoding the different viral proteins 2A^{pro}, 3A or 3C^{pro} (red). hGH is loaded into the same vesicles as insulin and co-secreted with insulin upon glucose challenge. The release of hGH from transfected cells was measured with ELISA.

3.6 PATCH CLAMP

To measure Ca²⁺ currents and study the exocytosis of insulin granules in INS1-832/13 cells, I performed patch clamp studies in **Paper III** using the standard whole cell configuration. This is achieved by creating a tight seal between the pipette and the membrane wall of the cell and then applying negative pressure to remove the membrane patch within the pipette. Removal of the membrane patch allows replacement of the cytosol with the pipette solution, giving total control over the intracellular milieu. In addition, it allowed me to add active 2A^{pro} in the intracellular solution of the pipette before applying my stimulation protocols. For studies looking at the effects of 3A and 3C^{pro}, cells were transfected the day before performing experiments. Since 3A and 3C^{pro} transfected cells could be identified by GFP expression, only GFP positive cells were chosen for patch clamp studies. To study the function of Ca²⁺ channels, a protocol in which the cells were depolarized from -70 to voltages between -50mV to +50mV for 50 ms was used and Ca²⁺ currents were recorded. To study exocytosis, an increase in cell membrane capacitance was measured. Exocytosis was triggered by a train of ten 500 ms depolarizations from -70 to 0 mV. During exocytosis, insulin granules fuse with the membrane of the cell and increase the cell surface area. Membrane capacitance is calculated using the formula $C = (\epsilon * A) / d$. Since biological membranes have a specific capacitance of 9 fF/ μm^2 (ϵ) and d is the distance between the two layers of phospholipids (both of which are constants), the measured changes in membrane capacitance is proportional to the increase in cell area (A) and reflects the fusion of insulin granules.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Infections caused by CVBs are common in young children. In a proportion of infected children, CVBs cause hepatitis, which in many cases is fatal (198, 199). Proper regulation and induction of immune response mechanisms are crucial for controlling and inhibiting early virus replication. Several studies have shown the importance of type I IFNs in preventing early CVB replication and spread (200, 201). However, the ability of the type III IFNs to prevent CVB replication was, until recently, relatively undiscovered. CVBs have previously been shown to induce type III IFN expression in primary human islets after infection (70). However, whether the type III IFNs are produced by human hepatocytes in response to CVB infection had not been investigated. In **Paper I** we studied whether the type III IFNs are induced in human hepatocytes upon CVB infection and if type III IFN protects primary human hepatocytes from CVB infection.

We started by showing that primary human hepatocytes are susceptible to CVB infection and that titers of replicating virus increased over time (**Paper I**, Figure 1A). In addition, these infected hepatocytes seemed to respond to CVB infection by up regulating mRNA expression levels of IFN λ 1 and IFN λ 2 (**Paper I**, Figure 1B). Consistent with results seen in previous studies, we confirmed that human hepatocytes express both subunits of IFNLR (**Paper I**, Figure S1) and that they respond to type III IFN treatment by up regulating ISG expression (**Paper I**, Figure 3A). More importantly, pretreatment with IFN λ 1 or IFN λ 2 protected human hepatocytes from CVB replication (**Paper I**, Figure 3B). Thus, we showed that human hepatocytes respond to CVB infection by producing IFN λ 1 and IFN λ 2 and that type III IFNs promote the protection of primary human hepatocytes from CVB infection.

This article was published before the approved use many new antiviral therapies such as Harvoni in treating chronic HCV infections. Until then, pegylated IFN λ had been considered as a new potential treatment in place of pegylated IFN α for chronically infected HCV patients as it caused less severe side effects, due to the limited expression of IFNLR (202, 203). As of today, there is no specific treatment for severe hepatic infections caused by CVBs, only in life threatening conditions immunoglobulins or pleconaril can be administrated (204, 205). Few studies have looked at the effects of pegylated IFN λ on acute HCV infections in humans, since they are usually very mild and not diagnosed until the chronic phase (206). However, the use of pegylated IFN λ in treating acute CVB infections, which display more severe symptoms such as hepatitis, could be of interest to study.

A limitation with our study is that we did not investigate the induction of IFN λ 3. In addition, very little was known about the newly discovered IFN λ 4 gene at the time of publication. This could be of interest to investigate in the future since studies exist showing that single nucleotide polymorphisms (SNPs) in the IFN λ 3 and IFN λ 4 genes appear to determine whether patients are able to spontaneously clear HCV infection or if they progress to chronic

disease (207) as well as determining the effectiveness of IFN treatment (206). If SNPs in IFN λ 3 and IFN λ 4 would render children more prone to developing hepatitis after CVB infection and whether, as a result, IFN treatment would be less effective in these individuals is not known, but they are important factors to consider.

To summarize, in **Paper I** we show that type III IFNs protect human hepatocytes from CVB infection.

4.2 PAPER II

As IFNs are important for inhibiting early viral replication, it is not surprising that many viruses have evolved different mechanisms to interfere with IFN production and downstream functions (82). Studies have shown that both 2A^{pro} and 3C^{pro} of CVBs have the ability to block type I IFN production (83, 84). Whether CVBs have evolved similar mechanisms to inhibit the induction of type III IFNs had not been studied. However, given the importance of type III IFNs in protecting against CVB infection in other cells types ((70) and shown in **Paper I**), we hypothesized that CVBs have also evolved mechanisms to inhibit their production. In **Paper II** we set out to determine if CVBs inhibit type III IFN induction and if so, by what mechanism(s).

In this study we used HeLa cells, which are readily infected by CVBs, as a model system. Since CVBs are recognized by RIG-I, MDA5 and TLR3 (33, 46, 48) we wanted to look at the inhibitory effect of CVBs on both the RLR and TLR3 pathways. Thus, poly I:C was administered either exogenously, to study the type III IFN induction by TLR3 (34), or via transfection to look at type III IFN induction by RIG-I and MDA5 (208, 209). We started by confirming that HeLa cells up regulate IFN λ 1 and IFN λ 2 expression when exposed to poly I:C, via both the RLR and TLR3 pathways (**Paper II**, Figure 1A). In stark contrast, CVB3 was a poor inducer of type III IFNs (**Paper II**, Figure 1A). In addition, we showed that HeLa cells infected with CVB do not up regulate type III IFN production after poly I:C stimulation (**Paper II**, Figure 2A), suggesting that CVB is able to inhibit the pathways through which poly I:C induces type III IFN production.

Next, we wanted to study which proteins in the PRR signaling pathways are targeted by the virus. Induction of type III IFNs is dependent on activation of the transcription factor IRF3. IRF3 activation occurs after hyperphosphorylation of the protein, resulting in dimerization and translocation to the nucleus (210, 211). When comparing the phosphorylation pattern of poly I:C stimulated cells to that of CVB infected cells, we observed that no hyperphosphorylation of the IRF3 protein was observed in CVB infected cells (**Paper II**, Figure 3A). The IRF3 phosphorylation pattern was in agreement with the results published by Feng et al (84). This led us to believe that signaling molecules upstream of IRF3 were affected in CVB infected cells

Two important proteins upstream of IRF3 are the adaptor proteins IPS1 and TRIF, which mediate signaling from the PRRs MDA5/RIG-I and TLR3, respectively. Indeed, we

demonstrated that CVB infection decreased the expression of both IPS1 and TRIF (**Paper II**, Figure 4). In addition, the decreased expression of both full-length proteins correlated with the appearance of one or several cleavage products (**Paper II**, Figure 4) suggesting that CVBs inhibit type III IFN signaling pathways by cleaving these adaptor proteins.

Many EVs have been shown to induce apoptosis in infected cells resulting in caspase activation and degradation of several cellular proteins, including IPS1 (212), by the proteasome. Thus, to exclude the possibility that the observed cleavage of IPS1 and TRIF was a result of CVB induced apoptosis, we infected cells in the presence of caspase and proteasome inhibitors. This established that the expression of both IPS1 and TRIF still decreased in infected cells even when apoptosis was inhibited (**Paper II**, Figure 5) suggesting that the virus itself is responsible for mediating the cleavage of these cellular proteins.

CVB encodes two proteases, $2A^{pro}$ and $3C^{pro}$, which are capable of mediating cleavage of cellular proteins eg in (17, 20, 213). To study their respective roles in inhibiting type III IFN production, HeLa cells were transfected with the separate viral proteases before stimulation with poly I:C. We found that overexpression of $2A^{pro}$, but not $3C^{pro}$, significantly decreased poly I:C induced IFN λ 1 and IFN λ 2 expression (**Paper II**, Figure 6). In addition, when treating cell lysate with active $2A^{pro}$ we observed the same cleavage pattern of IPS1 and TRIF as in infected cells (**Paper II**, Figure 7).

In **Paper II** we show that $2A^{pro}$ is inhibiting type III IFN production. Even though the actual cleavage sites of IPS1 and TRIF that are targeted by $2A^{pro}$ were not investigated, it seemed likely that the protein cleavage was due to the proteolytic activity of the viral protease. This was further supported by the fact that we could exclude a role of cellular proteases and apoptosis in our experiments as the source responsible for cleaving IPS1 and TRIF. In addition, the same cleavage pattern was observed in CVB infected and $2A^{pro}$ treated cells and cleavage products of both IPS1 and TRIF were dependent on the concentration of active $2A^{pro}$ (**Paper II**, Figure 7).

In contrast to a study published in 2011 where it was concluded that type I IFN production is inhibited by $3C^{pro}$ mediated cleavage of IPS1 and TRIF (83), we did not see any effects of by $3C^{pro}$ in our experiments. Our results were in agreement with those published in 2014 by Feng *et al*, showing that IPS1 and MDA5 are targeted by $2A^{pro}$ in CVB infected cells (84). In addition to their findings, we also showed that TRIF was targeted by $2A^{pro}$. One explanation for these different findings made by Mukherjee *et al* and us, could be the different time points used to look at the effect of overexpressed $2A^{pro}$ and $3C^{pro}$. Since we observed that $2A^{pro}$ rapidly induced toxic effects in our transfected HeLa cells (Figure 6) we looked at the protein expression and cleavage of IPS1 and TRIF at much earlier time points than in the study published by Mukherjee *et al*. It may be that with longer overexpression studies, we might have observed some additional effects with $3C^{pro}$, but at least at early time points after infection, $2A^{pro}$ seems to play the predominant role in cleaving IPS1 and TRIF. In fact, overexpressing $2A^{pro}$ alone did not inhibit IFN λ expression to the same extent as that seen in

CVB infected cells, suggesting that there may still be additional effects by $2A^{pro}$, or other viral proteins, at later time points than those chosen for our experiments. Indeed, when looking at decreased protein expression in infected cells, TRIF seemed to be cleaved earlier than IPS1. In the paper by Feng *et al*, they also show an effect of $3C^{pro}$ in cleaving RIG-I, although this effect was not observed until 9h after infection. We also observed a decreased protein expression of both MDA5 and RIG-I in our studies, but these occurred after 4-6h of infection at the end of the viral replication cycle. Taken together, this suggests that proteins present in the type III IFN pathway are cleaved at different time points after infection by $2A^{pro}$ and $3C^{pro}$.

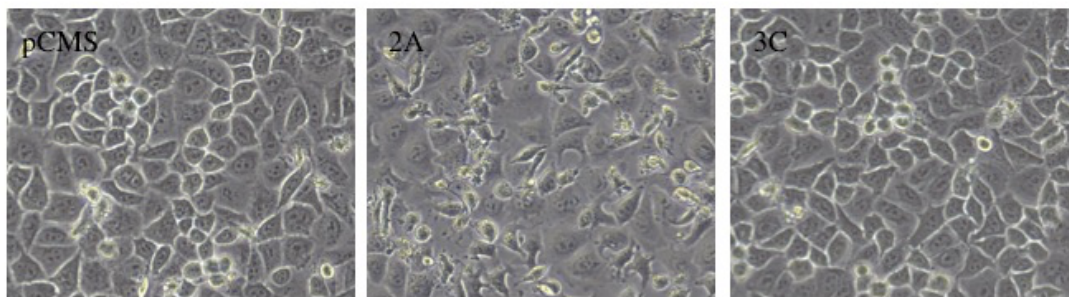


Figure 6: $2A^{pro}$ affects viability in transfected HeLa cells. Transfection with $2A^{pro}$ induces cell death in HeLa cells 24h after transfection as compared to the control plasmid, pCMS, or $3C^{pro}$.

In addition to directly cleaving proteins within the IFN pathway, both proteases have also been shown to inhibit transcription and translation in infected cells (18, 214, 215). The cleavage of eIF4G and inhibition of cellular protein production seemed to be an early event in the virus life cycle and complete cleavage of the protein occurred before 2h (unpublished data). Thus the decrease of full-length proteins could also, to some extent, be mediated by decreased protein synthesis in the cell. The effect of decreased protein expression due to translation shut down is hard to study separately as this effect is also mediated by $2A^{pro}$ and $3C^{pro}$. Of course, the almost complete and very efficient blockage of type III IFNs in infected cells could be explained by a combination of effects by both of the viral proteases on protein production as well as direct cleavage of target proteins to maximally inhibit the type III IFN response.

Only a few studies have looked into the mechanism by which different viruses inhibit type III IFNs. However, at mucosal surfaces, which are the primary infection route of CVBs, very little IFNAR is expressed and the protective effects in mucosal cells seem to be mainly mediated by type III IFNs (216). Thus, inhibition of type III IFNs may help the virus to establish infection in the intestinal mucosa and spread to cause systemic infections.

In conclusion, we show in **Paper II** that CVB inhibits the type III IFNs by targeting proteins of both the RLR and TLR3 pathways through $2A^{pro}$ mediated cleavage.

4.3 PAPER III

It has been suggested that infections caused by EVs are involved in the etiology of T1D. Indeed, several studies have reported the presence of EV protein within islets of T1D patients (110, 112, 115). The fact that only very few cells are positive for VP1 suggests that there may be a low grade and possibly persistent infection within the islets. Previous studies have shown that CVBs are able to cause beta cell dysfunction after infection *in vitro* (111, 130). However, the mechanism by which CVBs cause beta cell dysfunction is not known. CVBs encode several viral proteins that have been shown to interfere with cellular functions and signaling pathways e.g. ((217) and shown in **Paper II**). Therefore, we hypothesized that virus-encoded proteins cause the defective insulin secretion in infected islets. Thus, in **Paper III** we wanted to study the individual effects of viral proteins on insulin secretion to identify the mechanisms through which CVBs cause beta cell dysfunction.

We started by confirming the findings that infected human islets have a decreased ability to secrete insulin in response to glucose. Indeed, both CVB3 and CVB4 infected islets had a decreased insulin release compared to uninfected control islets from the same donor (**Paper III**, Figure 1B). As CVBs are able to inhibit transcription and translation in infected cells, we wanted to exclude the possibility that the decreased insulin secretion was due to differences in the expression levels of intracellular insulin in infected and control islets. Therefore, we looked at intracellular insulin protein and insulin mRNA expression levels in infected and control islets. While levels of intracellular insulin proteins did not differ, the expression of insulin mRNA was lower in infected islets (**Paper III**, Figure 1C-D). As insulin protein content was not decreased at the time of our experiment, the diminished release of insulin from infected islets was more likely due to mechanical defects in beta cell function. However, if T1D is caused by a persistent rather than an acute infection, like the one we have studied here, then insulin protein expression may decline over time due to inhibited transcription of insulin RNA, which may further contribute to a decrease in insulin secretion.

As access to human material is sparse and the islets consist of a mixture of endocrine cells we wanted to establish an infection model system in which we could perform mechanistic studies specifically in beta cells. By using the INS1-832/13 cell line, we confirmed our results seen with CVB infected islets (**Paper III**, Figure 2C-D) and showed that this is a valid model in which to study the effect of CVB induced inhibition on insulin secretion. In this cell system it was important to monitor viability during the experiments because infections with CVB have been shown to cause lysis of cells *in vitro* which would cause the unspecific release of insulin from lysed cells into the supernatant. However, at the terminal time point in our experiment, there were no obvious increases in cell death (**Paper III**, supplementary table 1). In addition, the majority of replicating virus was still inside the cells rather than in the supernatant, further supporting that the cells have not yet been lysed by the infection (**Paper III**, Figure 2A).

Next, we wanted to study the effect of separate viral proteins on insulin secretion. The viral proteins 2A^{pro}, 3A and 3C^{pro} have previously been shown to interfere with several cellular functions (17, 218). Thus, these proteins were chosen for overexpression studies in INS1-

832/13 cells. Insulin secretion is a tightly regulated process that is dependent on many intracellular signaling steps, briefly described in 1.3.3.1. Before exocytosis can occur, Ca^{2+} must enter the cell to promote the fusion of insulin granules with the cellular membrane. Therefore, we performed patch clamp experiments to look at Ca^{2+} influx in INS1-832/13 cells treated with different viral proteins. Our result showed that 2A^{pro} and 3C^{pro} inhibited Ca^{2+} influx while 3A did not (**Paper III**, Figure 4). However when looking at exocytosis in these cells all proteins were able to decrease the voltage stimulated release of insulin granules, with the most dramatic effect being on the RRP (**Paper III**, Figure 5). Beta cells express many different types of Ca^{2+} channels that are activated at different voltages to regulate exocytosis. Thus, it would be interesting in future studies to follow up on our findings and establish if the proteases target a specific channel and if so, how this inhibition is mediated. In contrast to the proteases, 3A inhibited exocytosis in a Ca^{2+} independent manner. 3A has previously been shown to inhibit protein secretion from other cell types by disrupting the of ER to Golgi transport (85). It is tempting to speculate that 3A might inhibit insulin secretion by the same mechanism, but this remains to be established.

So far we had observed that CVB seems to have the ability to inhibit insulin secretion via different mechanisms, such as the inhibition of transcription, Ca^{2+} influx and voltage stimulated exocytosis. Next, we wanted to address the individual effects of 2A^{pro} , 3A and 3C^{pro} on glucose stimulated hormone release. By using the hGH₁₋₁₇₁ assay where hGH is loaded into the same vesicles as insulin, we were able to study hormone release specifically in transfected cells (as discussed section 3.5). These experiments showed that the viral proteins have very different effects on hGH expression and release (**Paper III**, Figure 6). Both 2A^{pro} and 3C^{pro} seemed to be responsible for decreasing mRNA production in transfected cells (**Paper III**, Figure 6A) with 2A^{pro} mediating a near to complete block. The decreased levels of hGH mRNA also probably explains why 2A^{pro} and 3C^{pro} transfected cells also had lower or no expression of hGH protein (**Paper III**, Figure 6B). Interestingly, 3A increased the levels of hGH (**Paper III**, Figure 6B). When normalizing the levels of secreted protein to intracellular protein levels, it seemed like 3A has the most drastic effect on hormone secretion (**Paper III**, Figure 6C). However, 3C^{pro} appeared to increase basal levels of hormone release, indicating that beta cell function is also impaired in these cells. A disadvantage with this experiment, which demonstrated that 2A^{pro} seemed to be the main protein responsible for decreasing mRNA levels, was that we could not study the effect of 2A^{pro} on actual hGH release. One way to circumvent this problem and study the separate effect of 2A^{pro} on hormone release could be to create an IRES construct in front of the hGH in the plasmid. Since IRES is normally used by the virus to promote viral replication in cells, even when cellular protein production is inhibited, this would allow hGH production in cells where protein translation is normally inhibited. It is important to note that in our patch clamp experiments, active 2A^{pro} was administered via the pipette directly in the intracellular solution for no more than three minutes. This supports the point that direct cleavage by 2A^{pro} is responsible for inhibiting exocytosis in these experiments, rather than a decreasing in protein expression.

One limitation with our study is that we only choose to study the effect of three out of seven viral nonstructural CVB proteins, which have been shown to have an effect on cell function. Indeed, the 2B protein of poliovirus and CVB has also been shown to also inhibit protein secretion in cells (217, 219) suggesting that it also may have the ability to inhibit insulin secretion. Thus, we cannot exclude that additional viral proteins might contribute to beta cell dysfunction in infected islets.

To summarize, in **Paper III** we have shown for the first time that separate viral proteins target different steps of the insulin secretion pathway resulting in beta cell dysfunction.

4.4 PAPER IV

CF is the most common life shortening disease in Caucasians. A large proportion of the mortality rates are due to chronic bacterial infections of the lung resulting in respiratory failure. Of note, studies have suggested that viral infections seem to predispose the CF lung to bacterial infections and contribute to early mortality (166, 167). In addition, a defective antiviral response in CF patients has been observed (171). EVs, such as rhinoviruses, are common virus infections that have been shown to contribute to pulmonary exacerbations in CF (165, 220). Thus in **Paper IV** we wanted to study the effect of the most common CF mutation and how it affects immune responses to CVB infection.

We started by using an *in vivo* model for CF to study overall survival after CVB infection. To this end, we used the CFTR^{tm1EUR} mouse, which carries the most common CF mutation found in humans, F508Del (190). Our results showed that mice carrying the F508del mutation had strikingly impaired survival after challenge with CVB compared to wild-type (wt) controls (**Paper IV**, Figure 1). When analyzing early viremia from infected animals, we found no difference when comparing the wt and CFTR^{tm1EUR} mice (**Paper IV**, Figure 2A) suggesting that they had the same susceptibility to infection. However, viral replication in different organs from CFTR^{tm1EUR} mice was higher at day 5 and day 7 post infection (**Paper IV**, Figure 2B and D). These findings suggest that the CFTR^{tm1EUR} mice have an impaired ability to inhibit viral replication after infection.

The immune system is divided into the innate and adaptive arms. IFNs are an important part of the innate immune response in the inhibition of early viral replication, and they also provide activating signals for the adaptive immune response (55-57). It has been shown that type I IFNs are essential for surviving EV infections, however type II IFNs are indispensable for survival (200, 201). Thus, we looked to see if there was a defective induction of type I and type III IFNs after stimulation with poly I:C in these mice. Although we did not observe any difference in tissue specific IFN β and IFN λ production (**Paper IV**, supplementary figure 2 and Figure 3C-D), the production of IFN α in serum from CFTR^{tm1EUR} mice was significantly lower at 2h but not at 4h post injection (**Paper IV**, Figure 3B), suggesting that IFN α induction was delayed.

Since we did not observe any major differences in early viral replication between wt and CFTR^{tm1EUR} mice this may indicate that the problem lies within the adaptive immune response. An important feature of the adaptive immune response is the production of neutralizing antibodies to the virus, which aid with the clearance of the infection. Indeed, when looking at the presence of virus specific IgM and IgG antibodies in serum from infected mice, we found that no CFTR^{tm1EUR} mice had developed antibodies at day 5 post infection while more than half of the wt animals did have antibodies (**Paper IV**, Figure 4A). In addition, all wt mice had high levels of IgM and IgG antibodies at day 7 post infection while the levels in CFTR^{tm1EUR} mice were significantly lower (**Paper IV**, Figure 4A). The levels of antibodies with the ability to neutralize the virus in mice followed the same pattern as the virus specific IgM and IgG antibody responses (**Paper IV**, Figure 4B-C). The importance of these neutralizing antibodies in protecting CF mice from a lethal infection was further demonstrated in an adoptive transfer experiment (**Paper IV**, Figure 5). To exclude the possibility that the lower production of antibodies was not due to major differences in immune cell populations, we analyzed the frequency of major immune cell subsets between wt and CF mice. However, no such differences in immune cell populations were observed (**Paper IV**, Figure 6A-I). This suggests that mutations in CFTR may cause immune cell dysfunction rather than altering immune cell numbers.

To gain further mechanistic insight into which of the immune cell population that could be affected we looked at the antibody production in response to a TI or TD antigen. We observed no difference in antibody production to a TI antigen between wt and CFTR^{tm1EUR} mice (**Paper IV**, Figure 7A). However the antibody production in response to a TD antigen was significantly lower in CFTR^{tm1EUR} mice at day 7 post infection (**Paper IV**, Figure 7B). By using this approach, we were also able to follow the antibody development in CFTR^{tm1EUR} mice for a longer period of time than that which is possible in the infected mice due to the decreased survival of CFTR^{tm1EUR} mice after CVB infection. We observed that at day 12 post injection, CFTR^{tm1EUR} mice had reached the same levels of antibodies as the wt mice suggesting that there is a delayed production of antibodies rather than an overall lower antibody production in the CFTR^{tm1EUR} mice. This delayed induction of neutralizing antibodies probably allows the pathogen more time for replication and thus causes more severe infections before the immune system is able to control the infection.

Studies have shown that lymphocytes express CFTR and that defects in CFTR affect immune cell responses (221-223). In **Paper IV** we showed that antibody production to a TD antigen was impaired. The response to a TI antigen was normal in the CFTR^{tm1EUR} mice suggesting that B cell function, activation and secretion of antibodies is normal in these mice. Defects within the T cell population in CF have been reported (179). If defects in the T cell population are responsible for the delayed antibody production in response to a TD antigen is interesting for follow up studies.

Nevertheless, we cannot exclude that the delayed antibody production could be a result of factors other than a direct effect of the T cells. T cell activation is dependent on interactions

with APCs, and mainly DCs. The hypothesis that DCs are affected in CF could be supported by our data showing a delayed induction of IFN α after poly I:C stimulation. IFN α is mainly produced by pDCs early after infection (224). The data showing that IFN α induction seemed to be delayed rather than there being an overall inhibition of its production correlates well with the results demonstrating that antibody production is delayed, suggesting that they may depend on each other. Studies looking at the function of pulmonary DCs in CF mice have shown that low levels of S1P in the lung impair DC function and their T cell stimulatory capacity (162). Low levels of S1P have also been observed in the lung of the CFTR^{tm1EUR} mouse and this was shown to affect immune cell infiltrates of the lung (225). Indeed, S1P is essential for immune cell function (226) and immune cell migration (227-229). CFTR has been shown to mediate the uptake of S1P in cells (230). Normally S1P is metabolized quite fast and a decreased uptake of S1P in blood by tissue cells have been postulated to result in S1P accumulation in the circulation (231). If this occurs in the CFTR^{tm1EUR} mouse remains to be studied. However, it seems clear that CFTR has an impact in controlling levels of S1P in different parts of the body. Altered S1P levels may result in impaired priming or interaction of DC-T cells which could subsequently delay the antibody response of B cells. Given the importance of S1P for immune cell migration and activation, studying DC functions and S1P levels in the CFTR^{tm1EUR} mouse could be interesting to investigate in future studies.

To summarize our studies **in Paper IV**, we showed that CFTR^{tm1EUR} mice have a decreased survival and impaired immune response to CVB infection. In addition the antibody production to a TD antigen was delayed in CFTR^{tm1EUR} mice.

5 CONCLUDING REMARKS

In this thesis, I aimed to expand our knowledge of innate antiviral immune response mechanisms to CVB infections in primary human hepatocytes. In addition, I wanted to address strategies through which CVBs can evade the innate immune system in host cells and how CVBs cause beta cell dysfunction. Finally, I intended to study how mutations in *CFTR* affect the immune response to CVB infections.

To conclude:

- Paper I: Primary human hepatocytes respond to CVB infection by up-regulating type III IFN expression. In addition, type III IFNs induce an antiviral state in human hepatocytes by up-regulating several ISGs and attenuate CVB replication.
- Paper II: CVBs have evolved mechanisms to inhibit type III IFN production in infected cells by cleaving the adaptor proteins IPS1 and TRIF. This cleavage was mediated by the viral protease 2A^{pro}. Evading type III IFNs response mechanisms may help CVBs to establish infections at viral entry sites and subsequent viral spread thereby causing a systemic infection.
- Paper III: Insulin secretion from primary human islets and INS1-832/13 cells was inhibited by CVB infection. The viral proteins 2A^{pro}, 3A and 3C^{pro} all inhibited exocytosis and had negative effects on hGH expression (2A^{pro}, 3C^{pro}) or glucose stimulated hormone release from INS1-832/13 cells (3A, 3C^{pro}). 2A^{pro} and 3C^{pro} inhibited exocytosis by decreasing Ca²⁺ influx. Inhibiting insulin secretion from infected cells may be a potential mechanism through which CVBs could contribute to T1D after infection.
- Paper IV: Mice carrying the most common CF mutation in humans show a decreased survival rate after CVB infection. The mice had higher levels of replicating virus in several organs at days 5 and 7 post infection, which seemed to correlate with a delayed ability to produce neutralizing antibodies. A delayed antibody production to a TD antigen was also observed in CF mice, suggesting that *CFTR* may affect immune cell functions.

The work conducted in this thesis has contributed to an increase in our knowledge of immune response mechanisms during CVB infections and provides clues as to how CVBs can contribute to disease pathology. It is of importance for future studies that have the aim of developing new antiviral treatments that could prevent severe infections with CVBs and mitigate respiratory EV infections in individuals with CF.

6 ACKNOWLEDGEMENTS

The work done in this thesis would not have been possible without the help and support from numerous people to which I would like to express my gratitude

First of all I would like to thank my main supervisor **Malin Flodström Tullberg**, for taking me on as a PhD student and teaching me how to perform proper research. Thank you for always supporting me in my projects and encouraging me to develop my own ideas. It has really been fun working in your group.

My co-supervisor **Lena Eliasson**, for inviting me to your lab so that I could learn the patch clamp technique. For making me feel part of your group and for interesting discussions about beta cell function.

To the past and present members of the MFT group: **Ginny**, for making long days in PKL3 more endurable and a special thanks for all your help during the last few months. For always putting a smile on everyone's face and for trying to motivate me during tough days with the phrase "go to the gym and you will feel better" (I probably should start going to the gym). **Sebastian**, for your honest input on my projects and for sharing your ideas about new methods and research projects. For fun discussions, AW and fika (you almost managed to turn me into a full time coffee drinker). **Renata**, for all your help with the CF project, for always giving good advice about medical questions and for bringing me lots of different sweets. **Soile**, for your calming advice and keeping a positive attitude by promoting that all things will always work out in the end. **Erna**, we made a really good team working together and I could always come to you if I needed help or for discussing different ideas. I always had fun with you regardless if it was in the lab, at conferences or at parties together. **Pär**, for always making me laugh, even when working together in the dreaded isolator, and for great adventures together on different conferences around the world. **Katharina**, for not giving up on me after my first WB attempts but invited me to collaborate on your projects. Thank you for fun discussions and dinners. **Terezia**, for sharing your knowledge in CF and for interesting discussions and help when I was new to the lab. **Michael**, for helping me out and teaching me different techniques during my first year at CIM. **Olli**, for your help with the antibodies.

My collaborators in Malmö **Anna** and **Bitte**, for always making my visits to the lab fun and enjoyable. **Anna-Maria**, for helping me with all the questions about methods and cells I had during that time.

To all the co-authors on my papers for your experimental help and valuable input on the manuscripts.

Hans-Gustaf, for creating the great environment at CIM and **Anna**, **Malin** and **Johan** for keeping CIM such a wonderful place to work at.

Markus Moll, for always taking time to answer any virus or cloning related question.

Margit, Lena, Elisabeth and **Anette** for making our work at CIM run smoothly and for helping out whenever I had problems with non-working machines

The Kitchen office; **Julia, Martin C, Joana, Ben, JB, Martin I, Puran** and **Anna** for being the best office buddies ever with many laughs and discussions. A special thanks to my thesis writing buddy **Julia** for keeping track on dates and reminding me about deadlines.

Past and present members of CIM; A big thanks to everyone who shared reagents, organized AWs and helped to make CIM such an inspiring environment to work in.

People from the biomedicine program for the fun times at KI which inspired me to start a PhD, **Gustaf, Jeanette, Jacob, Sofia, My, Markus, Thomas, Daniella, Elina, Peter, Alex** and **Anna**.

Mamma och **Pappa**, for always being there for me and for your love and support.

Min syster yster **Linnea**, for at least pretending to be interested in what I am doing.

Heinrich, for increasing my work morale and for making it easier to stay and work long days in the lab. I'm looking forward to our future trips around the world together!

7 REFERENCES

1. Flint, S. J., L. W. Enquist, V. R. Racaniello, and A. A. M. Skalka. *Principles of Virology, 3rd Edition, Volume I*. ASM Press.
2. Knipe, D. M., and P. M. Howley. 2007. *Fields' Virology*. Lippincott Williams & Wilkins.
3. Rossmann, M. G., Y. He, and R. J. Kuhn. 2002. Picornavirus-receptor interactions. *Trends in microbiology* 10: 324–331.
4. DALLDORF, G. 1955. The Coxsackie viruses. *Annu. Rev. Microbiol.* 9: 277–296.
5. Bergelson, J. M., J. A. Cunningham, G. Droguett, E. A. Kurt-Jones, A. Krithivas, J. S. Hong, M. S. Horwitz, R. L. Crowell, and R. W. Finberg. 1997. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275: 1320–1323.
6. Shaw, C. A., P. C. Holland, M. Sinnreich, C. Allen, K. Sollerbrant, G. Karpati, and J. Nalbantoglu. 2004. Isoform-specific expression of the Coxsackie and adenovirus receptor (CAR) in neuromuscular junction and cardiac intercalated discs. *BMC Cell Biol.* 5: 42.
7. Cohen, C. J., J. T. Shieh, R. J. Pickles, T. Okegawa, J. T. Hsieh, and J. M. Bergelson. 2001. The coxsackievirus and adenovirus receptor is a transmembrane component of the tight junction. *Proceedings of the National Academy of Sciences of the United States of America* 98: 15191–15196.
8. Coyne, C. B., and J. M. Bergelson. 2005. CAR: a virus receptor within the tight junction. *Adv. Drug Deliv. Rev.* 57: 869–882.
9. Shafren, D. R., R. C. Bates, M. V. Agrez, R. L. Herd, G. F. Burns, and R. D. Barry. 1995. Coxsackieviruses B1, B3, and B5 use decay accelerating factor as a receptor for cell attachment. *Journal of virology* 69: 3873–3877.
10. Shieh, J. T. C., and J. M. Bergelson. 2002. Interaction with decay-accelerating factor facilitates coxsackievirus B infection of polarized epithelial cells. *Journal of virology* 76: 9474–9480.
11. Yeung, W.-C. G., W. D. Rawlinson, and M. E. Craig. 2011. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. *BMJ (Clinical research ed)* 342: d35.
12. Hober, D., and E. K. Alidjinou. 2013. Enteroviral pathogenesis of type 1 diabetes: queries and answers. *Current opinion in infectious diseases* 26: 263–269.
13. Coyne, C. B., Le Shen, J. R. Turner, and J. M. Bergelson. 2007. Coxsackievirus Entry across Epithelial Tight Junctions Requires Occludin and the Small GTPases Rab34 and Rab5. *Cell host & microbe* 2: 12–12.
14. Lin, J.-Y., T.-C. Chen, K.-F. Weng, S.-C. Chang, L.-L. Chen, and S.-R. Shih. 2009. Viral and host proteins involved in picornavirus life cycle. *Journal of biomedical science* 16: 103.
15. Kräusslich, H. G., M. J. Nicklin, C.-K. Lee, and E. Wimmer. 988AD. Polyprotein processing in picornavirus replication. *Biochimie* 1–12.
16. Nicklin, M. J., H. G. Kräusslich, H. toyoda, J. Dunn, and E. Wimmer. 1987. Poliovirus polypeptide precursors: Expression in vitro and processing by exogenous 3C and 2A proteinases. *Proceedings of the National Academy of Sciences of the United States of America* 1–5.
17. Laitinen, O. H., E. Svedin, S. Kapell, A. Nurminen, V. P. Hytönen, and M. Flodström-Tullberg. 2016. Enteroviral proteases: structure, host interactions and pathogenicity. *Reviews in medical virology* 26: 251–267.
18. Lamphear, B. J., R. Yan, F. Yang, D. Waters, H. D. Liebig, H. Klump, E. Kuechler, T. Skern, and R. E. Rhoads. 1993. Mapping the cleavage site in protein synthesis initiation

- factor eIF-4 gamma of the 2A proteases from human Coxsackievirus and rhinovirus. *The Journal of biological chemistry* 268: 19200–19203.
19. Perera, R., S. Daijogo, B. L. Walter, J. H. C. Nguyen, and B. L. Semler. 2007. Cellular protein modification by poliovirus: the two faces of poly(rC)-binding protein. *Journal of virology* 81: 8919–8932.
20. Badorff, C., N. Berkely, S. Mehrotra, J. W. Talhouk, R. E. Rhoads, and K. U. Knowlton. 2000. Enteroviral protease 2A directly cleaves dystrophin and is inhibited by a dystrophin-based substrate analogue. *The Journal of biological chemistry* 275: 11191–11197.
21. Luo, H., J. Wong, and B. Wong. 2010. Protein degradation systems in viral myocarditis leading to dilated cardiomyopathy. *Cardiovasc. Res.* 85: 347–356.
22. Rust, R. C., L. Landmann, R. Gosert, B. L. Tang, W. Hong, H. P. Hauri, D. Egger, and K. Bienz. 2001. Cellular COPII proteins are involved in production of the vesicles that form the poliovirus replication complex. *Journal of virology* 75: 9808–9818.
23. Hsu, N.-Y., O. Ilnytska, G. Belov, M. Santiana, Y.-H. Chen, P. M. Takvorian, C. Pau, H. van der Schaar, N. Kaushik-Basu, T. Balla, C. E. Cameron, E. Ehrenfeld, F. J. M. van Kuppeveld, and N. Altan-Bonnet. 2010. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell* 141: 799–811.
24. Dorobantu, C. M., H. M. van der Schaar, L. A. Ford, J. R. P. M. Strating, R. Ulferts, Y. Fang, G. Belov, F. J. M. van Kuppeveld, and R. M. Sandri-Goldin. 2014. Recruitment of PI4KIII to Coxsackievirus B3 Replication Organelles Is Independent of ACBD3, GBF1, and Arf1. *Journal of virology* 88: 2725–2736.
25. Parkin, J., and B. Cohen. 2001. An overview of the immune system. *Lancet* 357: 1777–1789.
26. Turvey, S. E., and D. H. Broide. 2010. Innate immunity. *J. Allergy Clin. Immunol.* 125: S24–32.
27. Kawai, T., and S. Akira. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *International Immunology* 21: 317–337.
28. Jensen, S., and A. R. Thomsen. 2012. Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion. *Journal of virology* 86: 2900–2910.
29. Lind, K., M. H. Hühn, and M. Flodström-Tullberg. 2012. Immunology in the clinic review series; focus on type 1 diabetes and viruses: the innate immune response to enteroviruses and its possible role in regulating type 1 diabetes. *Clinical and experimental immunology* 168: 30–38.
30. Bieback, K., E. Lien, I. M. Klagge, E. Avota, J. Schneider-Schaulies, W. P. Duprex, H. Wagner, C. J. Kirschning, V. Ter Meulen, and S. Schneider-Schaulies. 2002. Hemagglutinin protein of wild-type measles virus activates toll-like receptor 2 signaling. *Journal of virology* 76: 8729–8736.
31. Rassa, J. C., J. L. Meyers, Y. Zhang, R. Kudaravalli, and S. R. Ross. 2002. Murine retroviruses activate B cells via interaction with toll-like receptor 4. *Proceedings of the National Academy of Sciences of the United States of America* 99: 2281–2286.
32. Le Goffic, R., J. Pothlichet, D. Vitour, T. Fujita, E. Meurs, M. Chignard, and M. Si-Tahar. 2007. Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells. *Journal of immunology (Baltimore, Md : 1950)* 178: 3368–3372.
33. Negishi, H., T. Osawa, K. Ogami, X. Ouyang, S. Sakaguchi, R. Koshiba, H. Yanai, Y. Seko, H. Shitara, K. Bishop, H. Yonekawa, T. Tamura, T. Kaisho, C. Taya, T. Taniguchi, and K. Honda. 2008. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. *Proceedings of the National Academy of Sciences of the United States of America* 105: 20446–20451.
34. Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell. 2001. Recognition of

- double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413: 732–738.
35. Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526–1529.
36. Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
37. Triantafilou, K., G. Orthopoulos, E. Vakakis, M. A. E. Ahmed, D. T. Golenbock, P. M. Lepper, and M. Triantafilou. 2005. Human cardiac inflammatory responses triggered by Coxsackie B viruses are mainly Toll-like receptor (TLR) 8-dependent. *Cellular microbiology* 7: 1117–1126.
38. Jurk, M., F. Heil, J. Vollmer, C. Schetter, A. M. Krieg, H. Wagner, G. Lipford, and S. Bauer. 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nature immunology* 3: 499.
39. Kawai, T., and S. Akira. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34: 637–650.
40. Yoneyama, M., M. Kikuchi, K. Matsumoto, T. Imaizumi, M. Miyagishi, K. Taira, E. Foy, Y.-M. Loo, M. Gale, S. Akira, S. Yonehara, A. Kato, and T. Fujita. 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *Journal of immunology (Baltimore, Md : 1950)* 175: 2851–2858.
41. Hornung, V., J. Ellegast, S. Kim, K. Brzózka, A. Jung, H. Kato, H. Poeck, S. Akira, K.-K. Conzelmann, M. Schlee, S. Endres, and G. Hartmann. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314: 994–997.
42. Pichlmair, A., O. Schulz, C. P. Tan, T. I. Näslund, P. Liljeström, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 314: 997–1001.
43. Kato, H., O. Takeuchi, E. Mikamo-Satoh, R. Hirai, T. Kawai, K. Matsushita, A. Hiiragi, T. S. Dermody, T. Fujita, and S. Akira. 2008. 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* 205: 1601–1610.
44. Kato, H., S. Sato, M. Yoneyama, M. Yamamoto, S. Uematsu, K. Matsui, T. Tsujimura, K. Takeda, T. Fujita, O. Takeuchi, and S. Akira. 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23: 19–28.
45. Loo, Y.-M., J. Fornek, N. Crochet, G. Bajwa, O. Perwitasari, L. Martinez-Sobrido, S. Akira, M. A. Gill, A. García-Sastre, M. G. Katze, and M. Gale. 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *Journal of virology* 82: 335–345.
46. Feng, Q., M. A. Langereis, D. Olagnier, C. Chiang, R. van de Winkel, P. van Essen, J. Zoll, J. Hiscott, and F. J. M. van Kuppeveld. 2014. Coxsackievirus Cloverleaf RNA Containing a 5' Triphosphate Triggers an Antiviral Response via RIG-I Activation. *PLoS one* 9: e95927.
47. Kato, H., O. Takeuchi, S. Sato, M. Yoneyama, M. Yamamoto, K. Matsui, S. Uematsu, A. Jung, T. Kawai, K. J. Ishii, O. Yamaguchi, K. Otsu, T. Tsujimura, C.-S. Koh, C. Reis e Sousa, Y. Matsuura, T. Fujita, and S. Akira. 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441: 101–105.
48. Feng, Q., S. V. Hato, M. A. Langereis, J. Zoll, R. Virgen-Slane, A. Peisley, S. Hur, B. L. Semler, R. P. van Rij, and F. J. M. van Kuppeveld. 2012. MDA5 detects the double-stranded RNA replicative form in picornavirus-infected cells. *Cell reports* 2: 1187–1196.
49. Hühn, M. H., S. A. McCartney, K. Lind, E. Svedin, M. Colonna, and M. Flodström-Tullberg. 2010. 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* 401: 42–48.

50. Rothenfusser, S., N. Goutagny, G. DiPerna, M. Gong, B. G. Monks, A. Schoenemeyer, M. Yamamoto, S. Akira, and K. A. Fitzgerald. 2005. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *Journal of immunology (Baltimore, Md : 1950)* 175: 5260–5268.
51. Venkataraman, T., M. Valdes, R. Elsby, S. Kakuta, G. Caceres, S. Saijo, Y. Iwakura, and G. N. Barber. 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *Journal of immunology (Baltimore, Md : 1950)* 178: 6444–6455.
52. Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology* 6: 981–988.
53. Takeuchi, O., and S. Akira. 2010. Pattern recognition receptors and inflammation. *Cell* 140: 805–820.
54. ISAACS, A., and J. LINDENMANN. 1957. Virus interference. I. The interferon. *Proc. R. Soc. Lond., B, Biol. Sci.* 147: 258–267.
55. Le Bon, A., and D. F. Tough. 2002. Links between innate and adaptive immunity via type I interferon. *Current opinion in immunology* 14: 432–436.
56. Le Bon, A., V. Durand, E. Kamphuis, C. Thompson, S. Bulfone-Paus, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. *Journal of immunology (Baltimore, Md : 1950)* 176: 4682–4689.
57. Le Bon, A., C. Thompson, E. Kamphuis, V. Durand, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *Journal of immunology (Baltimore, Md : 1950)* 176: 2074–2078.
58. de Weerd, N. A., and T. Nguyen. 2012. The interferons and their receptors—distribution and regulation. *Immunology and Cell Biology* 90: 483–491.
59. Stetson, D. B., and R. Medzhitov. 2006. Type I Interferons in Host Defense. *Immunity* 25: 373–381.
60. Schoenborn, J. R., and C. B. Wilson. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv. Immunol.* 96: 41–101.
61. Kotenko, S. V., G. Gallagher, V. V. Baurin, A. Lewis-Antes, M. Shen, N. K. Shah, J. A. Langer, F. Sheikh, H. Dickensheets, and R. P. Donnelly. 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature immunology* 4: 69–77.
62. Sheppard, P., W. Kindsvogel, W. Xu, K. Henderson, S. Schlutsmeyer, T. E. Whitmore, R. Kuestner, U. Garrigues, C. Birks, J. Roraback, C. Ostrander, D. Dong, J. Shin, S. Presnell, B. Fox, B. Haldeman, E. Cooper, D. Taft, T. Gilbert, F. J. Grant, M. Tackett, W. Krivan, G. McKnight, C. Clegg, D. Foster, and K. M. Klucher. 2003. IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nature immunology* 4: 63–68.
63. Prokunina-Olsson, L., B. Muchmore, W. Tang, R. M. Pfeiffer, H. Park, H. Dickensheets, D. Hergott, P. Porter-Gill, A. Mumy, I. Kohaar, S. Chen, N. Brand, M. Tarway, L. Liu, F. Sheikh, J. Astemborski, H. L. Bonkovsky, B. R. Edlin, C. D. Howell, T. R. Morgan, D. L. Thomas, B. Rehermann, R. P. Donnelly, and T. R. O'Brien. 2013. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 45: 164–171.
64. Sommereyns, C., S. Paul, P. Staeheli, and T. Michiels. 2008. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS pathogens* 4: e1000017.
65. Yin, Z., J. Dai, J. Deng, F. Sheikh, M. Natalia, T. Shih, A. Lewis-Antes, S. B. Amrute, U. Garrigues, S. Doyle, R. P. Donnelly, S. V. Kotenko, and P. Fitzgerald-Bocarsly. 2012. Type III IFNs are produced by and stimulate human plasmacytoid dendritic cells. *the journal of Immunology* 189: 2735–2745.

66. Navarro, S., O. R. Colamonici, and A. Llombart-Bosch. 1996. Immunohistochemical detection of the type I interferon receptor in human fetal, adult, and neoplastic tissues. *Mod. Pathol.* 9: 150–156.
67. Mordstein, M., E. Neugebauer, V. Ditt, B. Jessen, T. Rieger, V. Falcone, F. Sorgeloos, S. Ehl, D. Mayer, G. Kochs, M. Schwemmler, S. Günther, C. Drosten, T. Michiels, and P. Staeheli. 2010. Lambda interferon renders epithelial cells of the respiratory and gastrointestinal tracts resistant to viral infections. *Journal of virology* 84: 5670–5677.
68. Witte, K., G. Gruetz, H.-D. Volk, A. C. Looman, K. Asadullah, W. Sterry, R. Sabat, and K. Wolk. 2009. Despite IFN-lambda receptor expression, blood immune cells, but not keratinocytes or melanocytes, have an impaired response to type III interferons: implications for therapeutic applications of these cytokines. *Genes Immun.* 10: 702–714.
69. Robek, M. D., B. S. Boyd, and F. V. Chisari. 2005. Lambda interferon inhibits hepatitis B and C virus replication. *Journal of virology* 79: 3851–3854.
70. Lind, K., S. J. Richardson, P. Leete, N. G. Morgan, O. Korsgren, and M. Flodström-Tullberg. 2013. Induction of an Antiviral State and Attenuated Coxsackievirus Replication in Type III Interferon Treated Primary Human Pancreatic Islets. *Journal of virology* –.
71. Hermant, P., C. Demarez, T. Mahlaköiv, P. Staeheli, P. Meuleman, and T. Michiels. 2014. Human but not mouse hepatocytes respond to interferon-lambda in vivo. *PloS one* 9: e87906.
72. Rauch, I., M. Müller, and T. Decker. 2013. The regulation of inflammation by interferons and their STATs. *JAKSTAT* 2: e23820.
73. Chakrabarti, A., B. K. Jha, and R. H. Silverman. 2011. New insights into the role of RNase L in innate immunity. *Journal of Interferon & Cytokine Research* 31: 49–57.
74. Au, W. C., P. A. Moore, D. W. LaFleur, B. Tombal, and P. M. Pitha. 1998. Characterization of the interferon regulatory factor-7 and its potential role in the transcription activation of interferon A genes. *The Journal of biological chemistry* 273: 29210–29217.
75. Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434: 772–777.
76. Bonilla, F. A., and H. C. Oettgen. 2010. Adaptive immunity. *J. Allergy Clin. Immunol.* 125: S33–40.
77. Murphy, K., and C. Weaver. 2016. *Janeway's Immunobiology, 9th edition*. Garland Science.
78. Dintzis, R. Z., M. H. Middleton, and H. M. Dintzis. 1983. Studies on the immunogenicity and tolerogenicity of T-independent antigens. *Journal of immunology (Baltimore, Md : 1950)* 131: 2196–2203.
79. Parker, D. C. 1993. T cell-dependent B cell activation. *Annu. Rev. Immunol.* 11: 331–360.
80. Wykes, M. 2003. Why do B cells produce CD40 ligand? *Immunology and Cell Biology* 81: 328–331.
81. Forthal, D. N. 2014. Functions of Antibodies. *Microbiol Spectr* 2: 1–17.
82. Feng, Q., M. A. Langereis, and F. J. M. van Kuppeveld. 2014. Induction and suppression of innate antiviral responses by picornaviruses. *Cytokine Growth Factor Rev.* 25: 577–585.
83. Mukherjee, A., S. A. Morosky, E. Delorme-Axford, N. Dybdahl-Sissoko, M. S. Oberste, T. Wang, and C. B. Coyne. 2011. The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. *PLoS pathogens* 7: e1001311.
84. Feng, Q., M. A. Langereis, M. Lork, M. Nguyen, S. V. Hato, K. Lanke, L. Emdad, P. Bhoopathi, P. B. Fisher, R. E. Lloyd, and F. J. M. van Kuppeveld. 2014. Enteroviruses 2Apro targets MDA5 and MAVS in infected cells. *Journal of virology*.

85. Choe, S. S., D. A. Dodd, and K. Kirkegaard. 2005. Inhibition of cellular protein secretion by picornaviral 3A proteins. *Virology* 337: 18–29.
86. Dodd, D. A., T. H. Giddings, and K. Kirkegaard. 2001. Poliovirus 3A protein limits interleukin-6 (IL-6), IL-8, and beta interferon secretion during viral infection. *Journal of virology* 75: 8158–8165.
87. Deitz, S. B., D. A. Dodd, S. Cooper, P. Parham, and K. Kirkegaard. 2000. MHC I-dependent antigen presentation is inhibited by poliovirus protein 3A. *Proceedings of the National Academy of Sciences of the United States of America* 97: 13790–13795.
88. Harjutsalo, V., L. Sjöberg, and J. Tuomilehto. 2008. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. *Lancet* 371: 1777–1782.
89. DIAMOND Project Group. 2006. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. *Diabetic medicine : a journal of the British Diabetic Association* 23: 857–866.
90. Patterson, C. C., G. G. Dahlquist, E. Gyürüs, A. Green, G. Soltész, EURODIAB Study Group. 2009. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet* 373: 2027–2033.
91. Gepts, W. 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14: 619–633.
92. Bingley, P. J. 2010. Clinical applications of diabetes antibody testing. *The Journal of clinical endocrinology and metabolism* 95: 25–33.
93. Kukko, M., T. Kimpimäki, S. Korhonen, A. Kupila, S. Simell, R. Veijola, T. Simell, J. Ilonen, O. Simell, and M. Knip. 2005. Dynamics of diabetes-associated autoantibodies in young children with human leukocyte antigen-conferred risk of type 1 diabetes recruited from the general population. *The Journal of clinical endocrinology and metabolism* 90: 2712–2717.
94. Andersson, C., K. Larsson, F. Vaziri-Sani, K. Lynch, A. Carlsson, E. Cedervall, B. Jönsson, J. Neiderud, M. Månsson, A. Nilsson, Å. Lernmark, H. Elding Larsson, and S.-A. Ivarsson. 2011. The three ZNT8 autoantibody variants together improve the diagnostic sensitivity of childhood and adolescent type 1 diabetes. *Autoimmunity* 44: 394–405.
95. Zhang, L., and G. S. Eisenbarth. 2011. Prediction and prevention of Type 1 diabetes mellitus. *J Diabetes* 3: 48–57.
96. In't Veld, P. 2014. Insulinitis in human type 1 diabetes: a comparison between patients and animal models. *Seminars in immunopathology* 36: 569–579.
97. Krogvold, L., A. Wiberg, B. Edwin, T. Buanes, F. L. Jahnsen, K. F. Hanssen, E. Larsson, O. Korsgren, O. Skog, and K. Dahl-Jørgensen. 2015. Insulinitis and characterisation of infiltrating T cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes. *Diabetologia*.
98. In't Veld, P., D. Lievens, J. De Grijse, Z. Ling, B. Van der Auwera, M. Pipeleers-Marichal, F. Gorus, and D. Pipeleers. 2007. Screening for Insulinitis in Adult Autoantibody-Positive Organ Donors. *Diabetes* 56: 2400–2404.
99. Hämäläinen, A.-M., and M. Knip. 2002. Autoimmunity and familial risk of type 1 diabetes. *Current diabetes reports* 2: 347–353.
100. Vaarala, O., J. Paronen, T. Otonkoski, and H. K. Akerblom. 1998. Cow milk feeding induces antibodies to insulin in children--a link between cow milk and insulin-dependent diabetes mellitus? *Scand. J. Immunol.* 47: 131–135.
101. Vaarala, O., M. Knip, J. Paronen, A. M. Hämäläinen, P. Muona, M. Väättäin, J. Ilonen, O. Simell, and H. K. Akerblom. 1999. Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes* 48: 1389–1394.
102. Norris, J. M., K. Barriga, G. Klingensmith, M. Hoffman, G. S. Eisenbarth, H. A. Erlich, and M. Rewers. 2003. Timing of initial cereal exposure in infancy and risk of islet

autoimmunity. *JAMA* 290: 1713–1720.

103. Wen, L., R. E. Ley, P. Y. Volchkov, P. B. Stranges, L. Avanesyan, A. C. Stonebraker, C. Hu, F. S. Wong, G. L. Szot, J. A. Bluestone, J. I. Gordon, and A. V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455: 1109–1113.

104. Giongo, A., K. A. Gano, D. B. Crabb, N. Mukherjee, L. L. Novelo, G. Casella, J. C. Drew, J. Ilonen, M. Knip, H. Hyöty, R. Veijola, T. Simell, O. Simell, J. Neu, C. H. Wasserfall, D. Schatz, M. A. Atkinson, and E. W. Triplett. 2010. Toward defining the autoimmune microbiome for type 1 diabetes. *The ISME journal* 5: 82–91.

105. Honeyman, M. C., B. S. Coulson, N. L. Stone, S. A. Gellert, P. N. Goldwater, C. E. Steele, J. J. Couper, B. D. Tait, P. G. Colman, and L. C. Harrison. 2000. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* 49: 1319–1324.

106. Hober, D., and P. Sauter. 2010. Pathogenesis of type 1 diabetes mellitus: interplay between enterovirus and host. *Nature reviews Endocrinology* 6: 279–289.

107. Gamble, D. R., M. L. Kinsley, M. G. FitzGerald, R. Bolton, and K. W. Taylor. 1969. Viral antibodies in diabetes mellitus. *British medical journal* 3: 627–630.

108. Yoon, J. W., M. Austin, T. Onodera, and A. L. Notkins. 1979. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *The New England journal of medicine* 300: 1173–1179.

109. Oikarinen, S. S., M. M. Martiskainen, S. S. Tauriainen, H. H. Huhtala, J. J. Ilonen, R. R. Veijola, O. O. Simell, M. M. Knip, and H. H. Hyöty. 2010. Enterovirus RNA in blood is linked to the development of type 1 diabetes. *Diabetes* 60: 276–279.

110. Ylipaasto, P. P., K. K. Klingel, A. M. A. Lindberg, T. T. Otonkoski, R. R. Kandolf, T. T. Hovi, and M. M. Roivainen. 2004. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia* 47: 225–239.

111. Dotta, F., S. Censini, A. G. S. van Halteren, L. Marselli, M. Masini, S. Dionisi, F. Mosca, U. Boggi, A. O. Muda, S. D. Prato, J. F. Elliott, A. Covacci, R. Rappuoli, B. O. Roep, and P. Marchetti. 2007. Coxsackie B4 virus infection of beta cells and natural killer cell insulinitis in recent-onset type 1 diabetic patients. *Proceedings of the National Academy of Sciences of the United States of America* 104: 5115–5120.

112. Richardson, S. J., A. Willcox, A. J. Bone, A. K. Foulis, and N. G. Morgan. 2009. The prevalence of enteroviral capsid protein vp1 immunostaining in pancreatic islets in human type 1 diabetes. *Diabetologia* 52: 1143–1151.

113. Oikarinen, M., S. Tauriainen, T. Honkanen, S. Oikarinen, K. Vuori, K. Kaukinen, I. Rantala, M. Mäki, and H. Hyöty. 2008. Detection of enteroviruses in the intestine of type 1 diabetic patients. *Clinical and experimental immunology* 151: 71–75.

114. Oikarinen, M. M., S. S. Tauriainen, S. S. Oikarinen, T. T. Honkanen, P. P. Collin, I. I. Rantala, M. M. Mäki, K. K. Kaukinen, and H. H. Hyöty. 2012. Type 1 diabetes is associated with enterovirus infection in gut mucosa. *Diabetes* 61: 687–691.

115. Krogvold, L. 2014. Detection of a low-grade enteroviral infection in the islets of Langerhans of living patients newly diagnosed with type 1 diabetes. *Diabetes* 1–25.

116. Mercalli, A., V. Lampasona, K. Klingel, L. Albarello, C. Lombardoni, J. Ekström, V. Sordi, A. Bolla, A. Mariani, D. Bzhalava, J. Dillner, M. Roivainen, E. Bosi, and L. Piemonti. 2012. No evidence of enteroviruses in the intestine of patients with type 1 diabetes. *Diabetologia* 55: 2479–2488.

117. Krogvold, L., B. Edwin, T. Buanes, J. Ludvigsson, O. Korsgren, H. Hyöty, G. Frisk, K. F. Hanssen, and K. Dahl-Jørgensen. 2014. Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes: experiences from the DiViD study. *Diabetologia* 57: 841–843.

118. Laitinen, O. H., H. Honkanen, O. Pakkanen, S. Oikarinen, M. M. Hankaniemi, H.

- Huhtala, T. Ruokoranta, V. Lecouturier, P. Andre, R. Harju, S. M. Virtanen, J. Lehtonen, J. W. Almond, T. Simell, O. Simell, J. Ilonen, R. Veijola, M. Knip, and H. Hyöty. 2014. Coxsackievirus B1 Is Associated With Induction of β -Cell Autoimmunity That Portends Type 1 Diabetes. *Diabetes* 63: 446–455.
119. Selin, L. K., M. F. Wlodarczyk, A. R. Kraft, S. Nie, L. L. Kenney, R. Puzone, and F. Celada. 2011. Heterologous immunity: immunopathology, autoimmunity and protection during viral infections. *Autoimmunity* 44: 328–347.
120. Fujinami, R. S., M. G. von Herrath, U. Christen, and J. L. Whitton. 2006. Molecular mimicry, bystander activation, or viral persistence: infections and autoimmune disease. *Clin. Microbiol. Rev.* 19: 80–94.
121. Baboonian, C., and T. Treasure. 1997. Meta-analysis of the association of enteroviruses with human heart disease. *Heart* 78: 539–543.
122. Cooper, L. T. 2009. Myocarditis. *The New England journal of medicine* 360: 1526–1538.
123. Caforio, A. L. P., N. J. Mahon, F. Tona, and W. J. McKenna. 2002. Circulating cardiac autoantibodies in dilated cardiomyopathy and myocarditis: pathogenetic and clinical significance. *Eur. J. Heart Fail.* 4: 411–417.
124. Kim, K.-S., S. Tracy, W. Tappich, J. Bailey, C.-K. Lee, K. Kim, W. H. Barry, and N. M. Chapman. 2005. 5'-Terminal deletions occur in coxsackievirus B3 during replication in murine hearts and cardiac myocyte cultures and correlate with encapsidation of negative-strand viral RNA. *Journal of virology* 79: 7024–7041.
125. Chapman, N. M., and K.-S. Kim. 2008. Persistent coxsackievirus infection: enterovirus persistence in chronic myocarditis and dilated cardiomyopathy. *Current topics in microbiology and immunology* 323: 275–292.
126. Roivainen, M., P. Ylipaasto, C. Savolainen, J. Galama, T. Hovi, and T. Otonkoski. 2002. Functional impairment and killing of human beta cells by enteroviruses: the capacity is shared by a wide range of serotypes, but the extent is a characteristic of individual virus strains. *Diabetologia* 45: 693–702.
127. Chehadeh, W., J. Kerr-Conte, F. Pattou, G. Alm, J. Lefebvre, P. Wattré, and D. Hober. 2000. Persistent infection of human pancreatic islets by coxsackievirus B is associated with alpha interferon synthesis in beta cells. *Journal of virology* 74: 10153–10164.
128. Yin, H., A.-K. Berg, J. Westman, C. Hellerström, and G. Frisk. 2002. Complete nucleotide sequence of a Coxsackievirus B-4 strain capable of establishing persistent infection in human pancreatic islet cells: effects on insulin release, proinsulin synthesis, and cell morphology. *Journal of medical virology* 68: 544–557.
129. Klinke, D. J. 2008. Extent of beta cell destruction is important but insufficient to predict the onset of type 1 diabetes mellitus. *PloS one* 3: e1374.
130. Roivainen, M., S. Rasilainen, P. Ylipaasto, R. Nissinen, J. Ustinov, L. Bouwens, D. L. Eizirik, T. Hovi, and T. Otonkoski. 2000. Mechanisms of coxsackievirus-induced damage to human pancreatic beta-cells. *The Journal of clinical endocrinology and metabolism* 85: 432–440.
131. Krogvold, L. 2015. Function of isolated pancreatic islets from patients at onset of type 1 diabetes: Insulin secretion can be restored after some days in a non-diabetogenic environment in vitro. Results from the DiViD study. *Diabetes* 1–24.
132. Rahier, J., R. M. Goebbels, and J. C. Henquin. 1983. Cellular composition of the human diabetic pancreas. *Diabetologia* 24: 366–371.
133. Lifson, N., C. V. Lassa, and P. K. Dixit. 1985. Relation between blood flow and morphology in islet organ of rat pancreas. *Am. J. Physiol.* 249: E43–8.
134. Newgard, C. B., and J. D. McGarry. 1995. Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annual review of biochemistry* 64: 689–719.
135. De Vos, A., H. Heimberg, E. Quartier, P. Huypens, L. Bouwens, D. Pipeleers, and F. Schuit. 1995. Human and rat beta cells differ in glucose transporter but not in glucokinase

- gene expression. *The Journal of clinical investigation* 96: 2489–2495.
136. Rorsman, P., and M. Braun. 2013. Regulation of Insulin Secretion in Human Pancreatic Islets. *Annual Review of Physiology* 75: 155–179.
137. Olofsson, C. S., S. O. Göpel, S. Barg, J. Galvanovskis, X. Ma, A. Salehi, P. Rorsman, and L. Eliasson. 2002. Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. *Pflügers Archiv : European journal of physiology* 444: 43–51.
138. Bratanova-Tochkova, T. K., H. Cheng, S. Daniel, S. Gunawardana, Y.-J. Liu, J. Mulvaney-Musa, T. Schermerhorn, S. G. Straub, H. Yajima, and G. W. G. Sharp. 2002. Triggering and augmentation mechanisms, granule pools, and biphasic insulin secretion. *Diabetes* 51 Suppl 1: S83–90.
139. Straub, S. G., and G. W. G. Sharp. 2004. Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. *Am. J. Physiol., Cell Physiol.* 287: C565–71.
140. Rorsman, P., and E. Renström. 2003. Insulin granule dynamics in pancreatic beta cells. *Diabetologia* 46: 1029–1045.
141. Farrell, P. M. 2008. The prevalence of cystic fibrosis in the European Union. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 7: 450–453.
142. Riordan, J. R., J. M. Rommens, B. S. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou, M. L. Drumm, M. C. Iannuzzi, F. Collins, and L. C. Tsui. 1989. Identification of the Cystic Fibrosis Gene: Cloning and Characterization of Complementary DNA. *Science* 1–8.
143. Rommens, J. M., and F. Collins. 1989. Identification of the Cystic Fibrosis Gene: Chromosome Walking and Jumping. 1–7.
144. Rowe, S. M., S. Miller, and E. J. Sorscher. 2005. Cystic Fibrosis. *The New England journal of medicine* 1–10.
145. Wong, J. K., S. C. Ranganathan, E. Hart, Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF). 2013. Staphylococcus aureus in early cystic fibrosis lung disease. *Pediatric pulmonology* 48: 1151–1159.
146. HOLSCLAW, D. S., H. B. ECKSTEIN, and H. H. NIXON. 1965. MECONIUM ILEUS. A 20-YEAR REVIEW OF 109 CASES. *Am. J. Dis. Child.* 109: 101–113.
147. Wat, D. 2012. Viral Respiratory Tract Infections in Cystic Fibrosis. In InTech.
148. Marson, F. A. L., C. S. Bertuzzo, and J. D. Ribeiro. 2016. Classification of CFTR mutation classes. *Lancet Respir Med* 4: e37–8.
149. Castellani, C., H. Cuppens, M. Macek, J. J. Cassiman, E. Kerem, P. Durie, E. Tullis, B. M. Assael, C. Bombieri, A. Brown, T. Casals, M. Claustres, G. R. Cutting, E. Dequeker, J. Dodge, I. Doull, P. Farrell, C. Ferec, E. Girodon, M. Johannesson, B. Kerem, M. Knowles, A. Munck, P. F. Pignatti, D. Radojkovic, P. Rizzotti, M. Schwarz, M. Stuhmann, M. Tzetis, J. Zielenski, and J. S. Elborn. 2008. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. In vol. 7. 179–196.
150. Crawford, I., P. C. Maloney, P. L. Zeitlin, W. B. Guggino, S. C. Hyde, H. Turley, K. C. Gatter, A. Harris, and C. F. Higgins. 1991. Immunocytochemical localization of the cystic fibrosis gene product CFTR. *Proceedings of the National Academy of Sciences of the United States of America* 88: 9262–9266.
151. Hill, C. M., C. J. Rolles, P. Keegan, and R. A. Chand. 1993. Pancreatic enzyme supplementation in cystic fibrosis. *Archives of Disease in Childhood* 68: 150.
152. HOLSCLAW, D. S., A. D. Perlmutter, H. Jockin, and H. Shwachman. 1971. Genital abnormalities in male patients with cystic fibrosis. *J. Urol.* 106: 568–574.
153. van der Doef, H. P. J., F. T. M. Kokke, C. K. van der Ent, and R. H. J. Houwen. 2011. Intestinal obstruction syndromes in cystic fibrosis: meconium ileus, distal intestinal obstruction syndrome, and constipation. *Curr Gastroenterol Rep* 13: 265–270.
154. Naren, A. P., D. J. Nelson, W. Xie, B. Jovov, J. Pevsner, M. K. Bennett, D. J. Benos, M. W. Quick, and K. L. Kirk. 1997. Regulation of CFTR chloride channels by syntaxin and

- Munc18 isoforms. *Nature* 390: 302–305.
155. Gabriel, S. E., L. L. Clarke, R. C. Boucher, and M. J. Stutts. 1993. CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. *Nature* 363: 263–268.
156. Schwiebert, E. M., M. E. Egan, T. H. Hwang, S. B. Fulmer, S. S. Allen, G. R. Cutting, and W. B. Guggino. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* 81: 1063–1073.
157. Edlund, A., J. L. Esguerra, A. Wendt, M. Flodström-Tullberg, and L. Eliasson. 2014. CFTR and Anoctamin 1 (ANO1) contribute to cAMP amplified exocytosis and insulin secretion in human and murine pancreatic beta-cells. *BMC medicine* 12: 87.
158. Bubien, J. K., K. L. Kirk, T. A. Rado, and R. A. Frizzell. 1990. Cell cycle dependence of chloride permeability in normal and cystic fibrosis lymphocytes. *Science* 248: 1416–1419.
159. Johansson, J., M. Vezzalini, G. Verzè, S. Caldrier, S. Bolognin, M. Buffelli, G. Bellisola, G. Tridello, B. M. Assael, P. Melotti, and C. Sorio. 2014. Detection of CFTR protein in human leukocytes by flow cytometry. *Cytometry* 85: 611–620.
160. McDonald, T. V., P. T. Nghiem, P. Gardner, and C. L. Martens. 1992. Human lymphocytes transcribe the cystic fibrosis transmembrane conductance regulator gene and exhibit CF-defective cAMP-regulated chloride current. *The Journal of biological chemistry* 267: 3242–3248.
161. Sorio, C., M. Buffelli, C. Angiari, M. Ettore, J. Johansson, M. Vezzalini, L. Viviani, M. Ricciardi, G. Verzè, B. M. Assael, and P. Melotti. 2011. Defective CFTR Expression and Function Are Detectable in Blood Monocytes: Development of a New Blood Test for Cystic Fibrosis. *PloS one* 6: e22212.
162. Xu, Y., A. Krause, M. Limberis, T. S. Worgall, and S. Worgall. 2013. Low Sphingosine-1-Phosphate Impairs Lung Dendritic Cells in Cystic Fibrosis. *American journal of respiratory cell and molecular biology* 48: 250–257.
163. Okiyoneda, T., G. Veit, J. F. Dekkers, M. Bagdany, N. Soya, H. Xu, A. Roldan, A. S. Verkman, M. Kurth, A. Simon, T. Hegedus, J. M. Beekman, and G. L. Lukacs. 2013. Mechanism-based corrector combination restores $\Delta F508$ -CFTR folding and function. *Nat. Chem. Biol.* 9: 444–454.
164. Wainwright, C. E., J. S. Elborn, and B. W. Ramsey. 2015. Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *The New England journal of medicine* 373: 1783–1784.
165. van Ewijk, B. E., M. M. van der Zalm, T. F. W. Wolfs, A. Fleer, J. L. L. Kimpen, B. Wilbrink, and C. K. van der Ent. 2008. Prevalence and impact of respiratory viral infections in young children with cystic fibrosis: prospective cohort study. *Pediatrics* 122: 1171–1176.
166. de Vrankrijker, A. M. M., T. F. W. Wolfs, O. Ciofu, N. Høiby, C. K. van der Ent, S. S. Poulsen, and H. K. Johansen. 2009. Respiratory syncytial virus infection facilitates acute colonization of *Pseudomonas aeruginosa* in mice. *Journal of medical virology* 81: 2096–2103.
167. Esther, C. R., F.-C. Lin, A. Kerr, M. B. Miller, and P. H. Gilligan. 2013. Respiratory viruses are associated with common respiratory pathogens in cystic fibrosis. *Pediatric pulmonology*.
168. Johansen, H. K., and N. Høiby. 1992. Seasonal onset of initial colonisation and chronic infection with *Pseudomonas aeruginosa* in patients with cystic fibrosis in Denmark. *Thorax* 47: 109–111.
169. Petersen, N. T., N. Høiby, C. H. Mordhorst, K. Lind, E. W. Flensburg, and B. Bruun. 1981. Respiratory infections in cystic fibrosis patients caused by virus, chlamydia and mycoplasma--possible synergism with *Pseudomonas aeruginosa*. *Acta Paediatr Scand* 70: 623–628.

170. Wang, E., C. Prober, B. Manson, M. Corey, and H. Levison. 1984. Association of Respiratory Viral Infections With Pulmonary Deterioration in Patients With Cystic Fibrosis. *The New England journal of medicine* 1–6.
171. Zheng, S., B. P. De, S. Choudhary, S. A. A. Comhair, T. Goggans, R. Slee, B. R. G. Williams, J. Pilewski, S. J. Haque, and S. C. Erzurum. 2003. Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis. *Immunity* 18: 619–630.
172. John, G., A. O. Yildirim, B. K. Rubin, D. C. Gruenert, and M. O. Henke. 2010. TLR-4-mediated innate immunity is reduced in cystic fibrosis airway cells. *American journal of respiratory cell and molecular biology* 42: 424–431.
173. John, G., S. Chillappagari, B. K. Rubin, D. C. Gruenert, and M. O. Henke. 2011. Reduced surface toll-like receptor-4 expression and absent interferon- γ -inducible protein-10 induction in cystic fibrosis airway cells. *Experimental lung research* 37: 319–326.
174. DiMagno, M. J., S.-H. Lee, Y. Hao, S.-Y. Zhou, B. J. McKenna, and C. Owyang. 2005. A proinflammatory, antiapoptotic phenotype underlies the susceptibility to acute pancreatitis in cystic fibrosis transmembrane regulator (-/-) mice. *Gastroenterology* 129: 665–681.
175. Smyth, R. L., N. M. Croft, U. O'Hea, T. G. Marshall, and A. Ferguson. 2000. Intestinal inflammation in cystic fibrosis. *Archives of Disease in Childhood* 82: 394–399.
176. Bruzzese, E., V. Raia, G. Gaudiello, G. Polito, V. Buccigrossi, V. Formicola, and A. Guarino. 2004. Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. *Aliment Pharmacol Ther* 20: 813–819.
177. Knutsen, A. P., R. G. Slavin, S. T. Roodman, K. R. Mueller, and N. L. Marino. 1988. Decreased T helper cell function in patients with cystic fibrosis. *Int. Arch. Allergy Appl. Immunol.* 85: 208–212.
178. Tiringier, K., A. Treis, P. Fucik, M. Gona, S. Gruber, S. Renner, E. Dehlink, E. Nachbaur, F. Horak, P. Jaksch, G. Döring, R. Cramer, A. Jung, M. K. RoCHAT, M. Hörmann, A. Spittler, W. Klepetko, C. A. Akdis, Z. Szépfalusi, T. Frischer, and T. Eiwegger. 2013. A Th17- and Th2-skewed Cytokine Profile in Cystic Fibrosis Lungs Represents a Potential Risk Factor for Pseudomonas aeruginosa Infection. *Am J Respir Crit Care Med* 187: 621–629.
179. Mueller, C., S. A. Braag, A. Keeler, C. Hodges, M. Drumm, and T. R. Flotte. 2011. Lack of Cystic Fibrosis Transmembrane Conductance Regulator in CD3 +Lymphocytes Leads to Aberrant Cytokine Secretion and Hyperinflammatory Adaptive Immune Responses. *American journal of respiratory cell and molecular biology* 44: 922–929.
180. Del Porto, P., N. Cifani, S. Guarnieri, E. G. Di Domenico, M. A. Marigliò, F. Spadaro, S. Guglietta, M. Anile, F. Venuta, S. Quattrucci, and F. Ascenzioni. 2011. Dysfunctional CFTR alters the bactericidal activity of human macrophages against Pseudomonas aeruginosa. *PloS one* 6: e19970.
181. Simonin-Le Jeune, K., A. Le Jeune, S. Jouneau, C. Belleguic, P.-F. Roux, M. Jaguin, M.-T. Dimanche-Boitre, V. Lecreur, C. Leclercq, B. Desrues, G. Brinchault, J.-P. Gangneux, and C. Martin-Chouly. 2013. Impaired functions of macrophage from cystic fibrosis patients: CD11b, TLR-5 decrease and sCD14, inflammatory cytokines increase. *PloS one* 8: e75667.
182. Bonfield, T. L., M. W. Konstan, and M. Berger. 1999. Altered respiratory epithelial cell cytokine production in cystic fibrosis. *J. Allergy Clin. Immunol.* 104: 72–78.
183. Elizur, A., C. L. Cannon, and T. W. Ferkol. 2008. Airway inflammation in cystic fibrosis. *Chest* 133: 489–495.
184. Alexis, N. E., M. S. Muhlebach, D. B. Peden, and T. L. Noah. 2006. Attenuation of host defense function of lung phagocytes in young cystic fibrosis patients. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society* 5: 17–25.
185. Moriceau, S., G. Lenoir, and V. Witko-Sarsat. 2010. In cystic fibrosis homozygotes

- and heterozygotes, neutrophil apoptosis is delayed and modulated by diamide or roscovitine: evidence for an innate neutrophil disturbance. *Journal of Innate Immunity* 2: 260–266.
186. Koller, B., R. Bals, D. Roos, H. C. Korting, M. Griese, and D. Hartl. 2009. Innate immune receptors on neutrophils and their role in chronic lung disease. *European journal of clinical investigation* 39: 535–547.
187. Sun, X., Z. Yan, Y. Yi, Z. Li, D. Lei, C. S. Rogers, J. Chen, Y. Zhang, M. J. Welsh, G. H. Leno, and J. F. Engelhardt. 2008. Adeno-associated virus-targeted disruption of the CFTR gene in cloned ferrets. *The Journal of clinical investigation* 118: 1578–1583.
188. Rogers, C. S., Y. Hao, T. Rokhlina, M. Samuel, D. A. Stoltz, Y. Li, E. Petroff, D. W. Vermeer, A. C. Kabel, Z. Yan, L. Spate, D. Wax, C. N. Murphy, A. Rieke, K. Whitworth, M. L. Linville, S. W. Korte, J. F. Engelhardt, M. J. Welsh, and R. S. Prather. 2008. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *The Journal of clinical investigation* 118: 1571–1577.
189. Fisher, J. T., Y. Zhang, and J. F. Engelhardt. 2011. Comparative Biology of Cystic Fibrosis Animal Models. In *Methods in Molecular Biology*. Methods in Molecular Biology vol. 742. Humana Press, Totowa, NJ. 311–334.
190. van Doorninck, J. H., P. J. French, E. Verbeek, R. H. Peters, H. Morreau, J. Bijman, and B. J. Scholte. 1995. A mouse model for the cystic fibrosis delta F508 mutation. *The EMBO journal* 14: 4403–4411.
191. Davidson, D. J., J. R. Dorin, G. McLachlan, V. Ranaldi, D. Lamb, C. Doherty, J. Govan, and D. J. Porteous. 1995. Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. *Nat Genet* 9: 351–357.
192. Hohmeier, H. E., H. Mulder, G. Chen, R. Henkel-Rieger, M. Prentki, and C. B. Newgard. 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K⁺ channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49: 424–430.
193. Poitout, V., L. K. Olson, and R. P. Robertson. 1996. Insulin-secreting cell lines: classification, characteristics and potential applications. *Diabetes & Metabolism* 22: 7–14.
194. Braun, M., R. Ramracheya, M. Bengtsson, Q. Zhang, J. Karanauskaite, C. Partridge, P. R. Johnson, and P. Rorsman. 2008. Voltage-Gated Ion Channels in Human Pancreatic - Cells: Electrophysiological Characterization and Role in Insulin Secretion. *Diabetes* 57: 1618–1628.
195. Braun, M., R. Ramracheya, P. R. Johnson, and P. Rorsman. 2009. Exocytotic properties of human pancreatic beta-cells. *Annals of the New York Academy of Sciences* 1152: 187–193.
196. Schulla, V., E. Renström, R. Feil, S. Feil, I. Franklin, A. Gjinovci, X.-J. Jing, D. Laux, I. Lundquist, M. A. Magnuson, S. Obermüller, C. S. Olofsson, A. Salehi, A. Wendt, N. Klugbauer, C. B. Wollheim, P. Rorsman, and F. Hofmann. 2003. Impaired insulin secretion and glucose tolerance in beta cell-selective Ca(v)1.2 Ca²⁺ channel null mice. *The EMBO journal* 22: 3844–3854.
197. Nitert, M. D., C. L. F. Nagorny, A. Wendt, L. Eliasson, and H. Mulder. 2008. CaV1.2 rather than CaV1.3 is coupled to glucose-stimulated insulin secretion in INS-1 832/13 cells. *Journal of molecular endocrinology* 41: 1–11.
198. Wang, S. M., C. C. Liu, Y. J. Yang, H. B. Yang, C. H. Lin, and J. R. Wang. 1998. Fatal coxsackievirus B infection in early infancy characterized by fulminant hepatitis. *J. Infect.* 37: 270–273.
199. Lin, T.-Y., H.-T. Kao, S.-H. Hsieh, Y.-C. Huang, C.-H. Chiu, Y.-H. Chou, P.-H. Yang, R.-I. Lin, K.-C. Tsao, K.-H. Hsu, and L.-Y. Chang. 2003. Neonatal enterovirus infections: emphasis on risk factors of severe and fatal infections. *The Pediatric Infectious Disease Journal* 22: 889–894.

200. Deonarain, R., D. Cerullo, K. Fuse, P. P. Liu, and E. N. Fish. 2004. Protective role for interferon-beta in coxsackievirus B3 infection. *Circulation* 110: 3540–3543.
201. Wessely, R., K. Klingel, K. U. Knowlton, and R. Kandolf. 2001. Cardioselective infection with coxsackievirus B3 requires intact type I interferon signaling: implications for mortality and early viral replication. *Circulation* 103: 756–761.
202. Muir, A. J., M. L. Shiffman, A. Zaman, B. Yoffe, A. de la Torre, S. Flamm, S. C. Gordon, P. Marotta, J. M. Vierling, J. C. Lopez-Talavera, K. Byrnes-Blake, D. Fontana, J. Freeman, T. Gray, D. Hausman, N. N. Hunder, and E. Lawitz. 2010. Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. *Hepatology* 52: 822–832.
203. Ramos, E. L. 2010. Preclinical and clinical development of pegylated interferon-lambda 1 in chronic hepatitis C. *Journal of Interferon & Cytokine Research* 30: 591–595.
204. Moreau, B., C. Bastedo, R. P. Michel, and P. Ghali. 2011. Hepatitis and Encephalitis due to Coxsackie Virus A9 in an Adult. *Case Rep Gastroenterol* 5: 617–622.
205. Bryant, P. A., D. Tingay, P. A. Dargaville, M. Starr, and N. Curtis. 2004. Neonatal coxsackie B virus infection—a treatable disease? *Eur. J. Pediatr.* 163: 223–228.
206. Sheahan, T., N. Imanaka, S. Marukian, M. Dorner, P. Liu, A. Ploss, and C. M. Rice. 2014. Interferon Lambda Alleles Predict Innate Antiviral Immune Responses and Hepatitis C Virus Permissiveness. *Cell host & microbe* 15: 190–202.
207. O'Brien, T. R., R. M. Pfeiffer, A. Paquin, K. A. Lang Kuhs, S. Chen, H. L. Bonkovsky, B. R. Edlin, C. D. Howell, G. D. Kirk, M. H. Kuniholm, T. R. Morgan, H. D. Strickler, D. L. Thomas, and L. Prokunina-Olsson. 2015. Comparison of functional variants in IFNL4 and IFNL3 for association with HCV clearance. *Journal of Hepatology* 63: 1103–1110.
208. Andrejeva, J., K. Childs, D. Young, T. Carlos, N. Stock, S. Goodbourn, and R. Randall. 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibits activation of the IFN- α promoter. *Proceedings of the National Academy of Sciences of the United States of America* 1–6.
209. Gitlin, L., W. Barchet, S. Gilfillan, M. Cella, B. Beutler, R. A. Flavell, M. S. Diamond, and M. Colonna. 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proceedings of the National Academy of Sciences of the United States of America* 1–6.
210. Lin, R., C. Heylbroeck, P. M. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Molecular and cellular biology* 18: 2986–2996.
211. Panne, D., S. M. McWhirter, T. Maniatis, and S. C. Harrison. 2007. Interferon regulatory factor 3 is regulated by a dual phosphorylation-dependent switch. *The Journal of biological chemistry* 282: 22816–22822.
212. Barral, P. M., J. M. Morrison, J. Drahos, P. Gupta, D. Sarkar, P. B. Fisher, and V. R. Racaniello. 2007. MDA-5 is cleaved in poliovirus-infected cells. *Journal of virology* 81: 3677–3684.
213. de Breyne, S., J. M. Bonderoff, K. M. Chumakov, R. E. Lloyd, and C. U. T. Hellen. 2008. Cleavage of eukaryotic initiation factor eIF5B by enterovirus 3C proteases. *Virology* 378: 118–122.
214. Yalamanchili, P., K. Harris, E. Wimmer, and A. Dasgupta. 1996. Inhibition of basal transcription by poliovirus: a virus-encoded protease (3Cpro) inhibits formation of TBP-TATA box complex in vitro. *Journal of virology* 70: 2922–2929.
215. Kuyumcu-Martinez, N. M., M. E. Van Eden, P. Younan, and R. E. Lloyd. 2004. Cleavage of poly(A)-binding protein by poliovirus 3C protease inhibits host cell translation: a novel mechanism for host translation shutoff. *Molecular and cellular biology* 24: 1779–1790.

216. Pott, J., T. Mahlaköiv, M. Mordstein, C. U. Duerr, T. Michiels, S. Stockinger, P. Staeheli, and M. W. Hornef. 2011. IFN-lambda determines the intestinal epithelial antiviral host defense. *Proceedings of the National Academy of Sciences of the United States of America* 108: 7944–7949.
217. Doedens, J. R., and K. Kirkegaard. 1995. Inhibition of cellular protein secretion by poliovirus proteins 2B and 3A. *The EMBO journal* 14: 894–907.
218. Cornell, C. T. C., W. B. W. Kiosses, S. S. Harkins, and J. L. J. Whitton. 2006. Inhibition of protein trafficking by coxsackievirus b3: multiple viral proteins target a single organelle. *Journal of virology* 80: 6637–6647.
219. de Jong, A. S. A., H.-J. H. Visch, F. F. de Mattia, M. M. M. van Dommelen, H. G. H. Swarts, T. T. Luyten, G. G. Callewaert, W. J. W. Melchers, P. H. P. Willems, and F. J. F. van Kuppeveld. 2006. The coxsackievirus 2B protein increases efflux of ions from the endoplasmic reticulum and Golgi, thereby inhibiting protein trafficking through the Golgi. *The Journal of biological chemistry* 281: 14144–14150.
220. Wang, E. E., C. G. Prober, B. Manson, M. Corey, and H. Levison. 1984. Association of respiratory viral infections with pulmonary deterioration in patients with cystic fibrosis. *The New England journal of medicine* 311: 1653–1658.
221. Bubien, J. 2001. CFTR may play a role in regulated secretion by lymphocytes: a new hypothesis for the pathophysiology of cystic fibrosis. *Pflügers Archiv : European journal of physiology* 443 Suppl 1: S36–S39.
222. Di, A., M. E. Brown, L. V. Deriy, C. Li, F. L. Szeto, Y. Chen, P. Huang, J. Tong, A. P. Naren, V. Bindokas, H. C. Palfrey, and D. J. Nelson. 2006. CFTR regulates phagosome acidification in macrophages and alters bactericidal activity. *Nat. Cell Biol.* 8: 933–944.
223. Painter, R. G., R. W. Bonvillain, V. G. Valentine, G. A. Lombard, S. G. LaPlace, W. M. Nauseef, and G. Wang. 2008. The role of chloride anion and CFTR in killing of *Pseudomonas aeruginosa* by normal and CF neutrophils. *Journal of leukocyte biology* 83: 1345–1353.
224. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Brière, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nature immunology* 2: 1144–1150.
225. Veltman, M., M. Stolarczyk, D. Radzioch, G. Wojewodka, J. B. De Sanctis, W. A. Dik, O. Dzyubachyk, T. Oravec, I. de Kleer, and B. J. Scholte. 2016. Correction of lung inflammation in a F508del CFTR murine cystic fibrosis model by the sphingosine-1-phosphate lyase inhibitor LX2931. *AJP: Lung Cellular and Molecular Physiology* 311: L1000–L1014.
226. Rivera, J., R. L. Proia, and A. Olivera. 2008. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nature reviews Immunology* 8: 753–763.
227. Czeloth, N., A. Schippers, N. Wagner, W. Müller, B. Küster, G. Bernhardt, and R. Förster. 2007. Sphingosine-1 phosphate signaling regulates positioning of dendritic cells within the spleen. *Journal of immunology (Baltimore, Md : 1950)* 179: 5855–5863.
228. Maeda, Y., H. Matsuyuki, K. Shimano, H. Kataoka, K. Sugahara, and K. Chiba. 2007. Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. *Journal of immunology (Baltimore, Md : 1950)* 178: 3437–3446.
229. Idzko, M., E. Panther, S. Corinti, A. Morelli, D. Ferrari, Y. Herouy, S. Dichmann, M. Mockenhaupt, P. Gebicke-Haerter, F. Di Virgilio, G. Girolomoni, and J. Norgauer. 2002. Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *The FASEB Journal* 16: 625–627.
230. Boujaoude, L. C., C. Bradshaw-Wilder, C. Mao, J. Cohn, B. Ogretmen, Y. A. Hannun,

and L. M. Obeid. 2001. Cystic Fibrosis Transmembrane Regulator Regulates Uptake of Sphingoid Base Phosphates and Lysophosphatidic Acid: MODULATION OF CELLULAR ACTIVITY OF SPHINGOSINE 1-PHOSPHATE. *Journal of Biological Chemistry* 276: 35258–35264.

231. Thuy, A. V., C.-M. Reimann, N. Y. A. Hemdan, and M. H. Gräler. 2014. Sphingosine 1-phosphate in blood: function, metabolism, and fate. *Cell. Physiol. Biochem.* 34: 158–171.