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NOVEL VACCINES AND ANTIVIRAL TREATMENTS FOR ENTEROVIRUS INDUCED INFECTIONS AND DISEASE

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Novel vaccines and antiviral treatments for enterovirus induced infections and disease
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To my grandparents and parents

And

To all patients who suffer from T1D

Popular science summary of the thesis

Chances are you were infected with a virus!

According to the Enterovirus Foundation, enteroviruses are estimated to impact almost 50 million individuals globally every year. In many cases people do not even know they were infected. However, there are some groups that are more sensitive to infection, causing more severe outcomes. This thesis focuses in two of these groups. The first is people with cystic fibrosis, a genetic disease that impacts the lungs, making these people prone to virus infections and unable to clear pathogens. The second group is those prone to develop type 1 diabetes, an autoimmune disease that disrupts insulin production. There is evidence suggesting that enterovirus infections could be involved in the development or acceleration of type 1 diabetes.

The aim of this thesis was to address how we could help these groups and explore possible preventions as well as treatments which would target enterovirus infections. One way could be vaccination, given in childhood to support the body and protect against these viruses. Another way could be an antiviral treatment, so even if infection happens a supporting treatment is in place.

In the first study of this thesis, we explored how common infections with an enterovirus called coxsackievirus B (CVB) are among people with cystic fibrosis, by checking for CVB virus neutralising antibodies . We then checked whether a CVB vaccine given to CF mouse model could protect them against a CVB infection. We found that CVB infections are common among individuals with cystic fibrosis and that the CVB vaccine protected the CF mice against infection, concluding that the CVB vaccine could be beneficial for protection against infection among people with cystic fibrosis.

In the second study, the same vaccine was given to mice that are prone to develop diabetes before they were infected with CVB. The virus made the unvaccinated mice developed diabetes faster, but not those that were given the CVB vaccine. This suggests that CVB vaccination could be beneficial for people prone to developing type 1 diabetes by protecting or delaying their development of the disease through blocking virus infections. Moreover, we showed that the vaccine is safe for individuals with autoimmune disease.

In the last study, we investigated an antiviral drug called Vemurafenib and found that it can protect against CVB infection in both cells and mice. This indicates that Vemurafenib has the potential to become a treatment for enterovirus infections.

This thesis work shows that vaccines used are safe and able to protect against CVB infection. Moreover, we explored new antiviral treatments which protected against virus infection at the site of infection (gut) and in cells that produce insulin.

Abstract

Enterovirus infections are common around the world and can impact people's lives in various ways, causing pancreatitis, myocarditis, common cold and more. This thesis is focused on testing the effectiveness of new vaccines and an antiviral agent in the context of cystic fibrosis (CF) and type 1 diabetes (T1D).

Many enteroviruses cause respiratory infections. Due to their problematic lungs, patients with CF are extra susceptible to infections including those by viruses. In **Paper I,** we demonstrated that enteroviruses known as coxsackieviruses (CVB) are common in people with CF. We also showed that mice carrying the CF mutation most common in humans respond to a newly developed CVB vaccine by producing neutralising antibodies leading to protection against CVB infection. Poliovirus is another enterovirus and the inactivated poliovirus vaccine used in the Swedish national vaccination programme is similar to the new CVB vaccine. To understand how the CVB vaccine might work in people with CF, we measured neutralising antibodies against poliovirus in serum samples from patients. The results suggested that most individuals were able to establish robust immunity to poliovirus, indicating that the new CVB vaccine would provide similar immunity in the CF population.

Paper II and **Paper III** focused on vaccine responses and virus infections associated with T1D. It has been suggested that enteroviruses are involved in T1D development. Establishing vaccination coverage and antiviral treatments might therefore be beneficial for susceptible individuals. In **Paper II**, we showed that a CVB vaccine is safe and does not accelerate autoimmune disease in a diabetes prone host. Moreover, we demonstrated that the CVB vaccine protected mice from infection-induced acceleration of autoimmune diabetes. This and previous studies by the group, paved the way for a clinical trial with an equivalent vaccine, PRV-101. In **Paper III** we investigated the antiviral properties of Vemurafenib, a cancer drug, as potential antiviral treatment against enteroviruses. We showed that this drug prevents infection of cells at the primary site of infection (epithelial cells lining the gut), as wells as insulin-producing cells, demonstrating the potential for next generation anti-enterovirus treatments.

This thesis provides insight into the development of new vaccines and antiviral agents, mainly against CVBs with the goal of improving the lives of those affected by CF and preventing the development of T1D.

List of scientific papers

I. Stone VM, Utorova R, **Butrym M**, Sioofy-Khojine AB, Hankaniemi MM, Ringqvist EE, Blanter M, Parajuli A, Pincikova T, Fischler B, Karpati F, Hytönen VP, Hyöty H, Hjelte L, Flodström-Tullberg M. Coxsackievirus B infections are common in Cystic Fibrosis and experimental evidence supports protection by vaccination. iScience. 2022 Sep 5;25(10):105070.

II. Stone VM, **Butrym M**, Hankaniemi MM, Sioofy-Khojine AB, Hytönen VP, Hyöty H, Flodström-Tullberg M. Coxsackievirus B Vaccines Prevent Infection-Accelerated Diabetes in NOD Mice and Have No Disease-Inducing Effect. Diabetes. 2021 Dec;70(12):2871-2878.

III. **Butrym M**, Blanter M, Byvald F, Vasylovska S, Ringqvist EE, Parajuli A, Lau J, Marjomäki V, Stone VM, Flodström-Tullberg M. Vemurafenib prevents coxsackievirus B replication in intestinal epithelial cells and pancreatic beta cells. Manuscript.

Scientific papers not included in the thesis

I. Lim CH, Puthia M, **Butrym M**, Tay HM, Lee MZY, Hou HW, Schmidtchen A. Thrombin-derived host defence peptide modulates neutrophil rolling and migration in vitro and functional response in vivo. Sci Rep. 2017 Sep 11;7(1):11201.

II. Zarychta-Wiśniewska W, Burdzinska A, Zagozdzon R, Dybowski B, **Butrym M**, Gajewski Z, Paczek L. In vivo imaging system for explants analysis-A new approach for assessment of cell transplantation effects in large animal models. PLoS One. 2017 Sep 20;12(9):e0184588.

III. Burdzinska A, Dybowski B, Zarychta-Wiśniewska W, Kulesza A, **Butrym M**, Zagozdzon R, Graczyk-Jarzynka A, Radziszewski P, Gajewski Z, Paczek L. Intraurethral co-transplantation of bone marrow mesenchymal stem cells and muscle-derived cells improves the urethral closure. Stem Cell Res Ther. 2018 Sep 21;9(1):239.

IV. Puthia M, **Butrym M**, Petrlova J, Strömdahl AC, Andersson MÅ, Kjellström S, Schmidtchen A. A dual-action peptide-containing hydrogel targets wound infection and inflammation. Sci Transl Med. 2020 Jan 1;12(524):eaax6601.

V. Strömdahl AC, Ignatowicz L, Petruk G, **Butrym M**, Wasserstrom S, Schmidtchen A, Puthia M. Peptide-coated polyurethane material reduces wound infection and inflammation. Acta Biomater. 2021 Jul 1;128:314-331.

VI. Puthia M, Petrlova J*, Petruk G*, **Butrym M**, Samsudin F, Andersson MÅ, Strömdahl AC, Wasserstrom S, Hartman E, Kjellström S, Caselli L, Klementieva O, Bond PJ, Malmsten M, Raina DB, Schmidtchen A. Bioactive Suture with Added Innate Defense Functionality for the Reduction of Bacterial Infection and Inflammation. Adv Healthc Mater. 2023 Sep 9:e2300987. * Authors contributed equally.

Contents

List of abbreviations

2A-C, 3A-D	viral non-structural proteins
$2A^{pro}$, $3C^{pr}$, $3CD^{pro}$	viral proteinases
3Rs	principals: replacement, reduction, refinement
ACBD3	acyl-CoA-binding domain-containing protein
$BRAF^{V600E}$	oncogenic mutation in cutaneous melanoma
BSL2	biosafety level 2
Caco-2	human colorectal adenocarcinoma cell line
CAR	coxsackievirus-adenovirus receptor
CD20	cluster of differentiation 20
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CF	Cystic Fibrosis
CFTR	transmembrane conductance regulator protein
CFTR	transmembrane conductance regulator gene
$Cftr^{tm1EUR}$	mouse model, F508del
cGAS	cyclic GMP-AMP synthas
CPE	cytopathic effect
CVB	coxsackievirus B
DAF	decay-accelerating factor
DC	dendritic cells
dsRNA	double stranded RNA
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EndoC	pancreatic human beta cells
ER	endoplasmic reticulum
EV	enteroviruses

EV-A71	enterovirus A71
EV-D68	enterovirus D68
F508del	deletion of phenylalanine at position 508
GAD	glutamic acid decarboxylase
GADA	glutamic acid decarboxylase antibodies
GI	gastro-intestinal tract
GMK	green monkey kidney cell line
H1	human embryonic stem cell line H1 (WA01, WiCell)
hESC	human embryonic stem cells
HLA	human leukocyte antigen
HT-29	human colorectal adenocarcinoma cell line
i.p.	intraperitoneal
ICAM-1	intercellular adhesion molecule 1
ICAM-5	intercellular adhesion molecule 5/telencephalin
IFN-α	interferon alpha
IFN-β	interferon beta
IFN-γ	interferon gamma
IFN-λ	interferon lambda
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-1β	interleukin 1β
IL-18	interleukin 18
IL-4	interleukin 4
IL-6	interleukin 6
INS-1	rodent beta cell lines
IPV	inactivated polio vaccine

LN	lymph nodes
MDA5	melanoma differentiation-associated protein 5
MEM	minimal essential medium
MHC class I	major histocompatibility complex class I
MOI	multiple of infection
NOD	non-obese diabetic
ORF	main open reading frame
ORF2	short open reading frame
OSBP	oxysterol-binding protein
PFA	paraformaldehyde
PFU	plaque-forming unit
PI4KB	phosphatidylinositol 4-kinase-β
PLX4032	Vemurafenib, inhibitor of B-Raf^{V600E}
PLX7904	paradox-breaker RAF inhibitor
PP	Peyer's patches
PRR	pattern recognition receptors
PRV-101	CVB vaccine admitted to clinical trials
PtdIns4P	phosphatidylinositol-4-phosphatate
RIG-I	retinoic acid-inducible gene I
RO	replication organelle
SC-islets	stem cell-derived islets
SOCS-1	suppressor of cytokine signaling-1
ssRNA	single stranded RNAsingle stranded RNA
T1D	Type 1 Diabetes
TCRα^{tm1Mom}	TCRα-knock out mouse
Tfh	T follicular helper
Th1	Type 1 T helper
TLR	toll-like receptors

TNF-α	tumour necrosis factor alpha
UTR	untranslated region
v/v	volume/volume
VP1, VP, VP0 (VP2 and VP4)	viral structural proteins
WHO	World Health Organization
wt	wild type

Introduction

1 Literature review

1.1 Enteroviruses

Enteroviruses belong to the *Picornaviridae* family and the *Enterovirus* genus (1). Out of fifteen species in this genus, seven are known to infect humans: *Enterovirus A-D* and *Rhinovirus A-C* (1). The best known and studied enterovirus is poliovirus (2). Apart from poliovirus there are other well described non-polio enteroviruses such as coxsackie-, echo-, entero-, and rhinoviruses (1). Enteroviruses are approximately 30nm, non-enveloped and positive single stranded RNA (ssRNA) viruses (2).

Enteroviruses are common human pathogens which in many cases cause mild symptoms like a common cold or no symptoms at all (2, 3). Nevertheless, there are certain groups which are more susceptible to infections, such as infants, children, and patients with immune deficiencies (2). These groups can develop serious conditions such as aseptic meningitis, neonatal sepsis like disease and encephalitis (2-4). Moreover, enterovirus infections can result in acute flaccid paralysis, hand-foot-and-mouth disease, pancreatitis, myocarditis, and other diseases (5-14).

1.1.1 Infection cycle

Enterovirus transmission occurs mainly through the gastro-intestinal (GI) tract, also known as faecal-oral route (2-4, 15-17). However, some of the viruses from the *Enterovirus* genus can be transmitted through the respiratory tract (2, 4, 14, 18-22). These are the initial/primary sites of the infections and if the virus manages to bypass the first line of immune system defences and the mucosal tissue, the virus life cycle begins. The infection starts when viruses bind to attaching and uncoating receptors presented on the cell surface (2). Each virus may require a different receptor to attach to the cell and may have different entry pathways (2). For instance, coxsackieviruses B (CVBs) can bind to complement decay-accelerating factor (DAF) (23-25) and their main receptor coxsackievirus-adenovirus receptor (CAR) (26, 27) while other viruses belonging to the *Enterovirus* genus, like rhinoviruses, can bind to intercellular adhesion molecule 1 (ICAM-1) (28). Another example is enterovirus D68 (EV-D68) which binds to alpha2,3- linked Sialic acids, and intercellular adhesion molecule 5/telencephalin (ICAM-5) was also proposed among other receptors (29, 30).

After initial binding to the expressed receptors, virus can enter the cell via endocytosis, which is followed by capsid transformation, which possibly lowers the pH inside the endosome and is assisted by host enzymes. This leads to the release of the virus genome, a positive ssRNA molecule, to the cytosol. The positive ssRNA contains a short open reading frame (ORF2) located upstream from main open reading frame (ORF) which is flanked by an untranslated region (UTR) at 5′ and 3′ ends (2). The ORFs undergo a translation step which is mediated by ribosomes. Translated ORF2 gives a single protein, ORF2p, which assists in the infection of the intestinal epithelial cells (31, 32). The translation of the main ORF results in the production of a polyprotein which is processed by viral proteinases ($2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$), leading to the production of structural capsid proteins (known as VP1, VP3 and VP0, the latter is later cleaved to VP2 and VP4) and non-structural proteins (known as 2A-C, 3A-D) (2). The viral proteinases are also involved in the cleavage of host proteins which support the virus life cycle by optimizing translation, replication, and virus spread (2, 33). Moreover, they disrupt the first line of immune defence (innate immunity, such as type I interferon and stress pathways) and are involved in causing alterations to the cellular cytoskeleton leading to changes in cellular morphology (2, 3).

Viral genome replication takes place in virus-induced structures called replication organelles (ROs) (34). The ROs are believed to originate from endoplasmic reticulum (ER) and/or Golgi apparatus membranes and are assembled by viral proteins (2BC and 3A) (34). These viral proteins with the assistance of host proteins such as acyl-CoA-binding domain-containing protein (ACBD3), phosphatidylinositol 4-kinase-β (PI4KB is responsible for the synthesis of phosphatidylinositol-4-phosphatate known as PtdIns4P), and oxysterol-binding protein (OSBP is responsible for mediating cholesterol recruitment to the ROs) create suitable lipid environments and membrane structures in which replication takes place (2, 34). As positive ssRNA enters the replication stage, the synthesis of negative ssRNA starts by creating a template for the production of new positive ssRNA. During replication an intermediate form is made, a double-stranded RNA (dsRNA) (2) .

Newly formed positive ssRNA can either act as a template for continued translation and replication or in the presence of structural proteins (pentameric shaped particles that accumulate around ssRNA) a provirion is assembled (2). This provirion undergoes maturation resulting in mature infectious virion (structural

protein VP0 is cleaved into VP2 and VP4) (35). The mature virion can be released from the cell in both a lytic and non-lytic (via extracellular vesicles) manner (2, 3).

Enteroviruses can cause acute infections (4, 36). Nevertheless, there is evidence showing that *Enteroviruses* can lead to a persistent infection (36). This could be due to host immunodeficiencies (36). It was also found that persistency can happen with or without active virus replication (37). There might be other factors causing persistent infections. For instance, persistent infections in the heart tissue could be due to restricted virus replication as well as the production of stable double-stranded RNA, which was shown in a murine model (38-40). The low ratio of positive- to negative strand RNA was implicated as a plausible cause of persistent infection in the human heart too (41). Moreover, a persistent infection was shown to be associated with terminal 5´-UTR deletions in the viral genome which was confirmed in both a murine model and human samples (42, 43). Evidence of low-grade infection in human pancreas has also been observed as virus RNA was detected but no virus was isolated from the tissue (44, 45). This could be due to a low number of produced infectious particles, which occurs in CVB5 infection for instance and possibly could contribute to persistent infection (46, 47). Infections through exosomes and other causes of persistency have also been pointed out (36, 48).

1.1.2 Tissue tropism

Based on clinical data, viruses belonging to the *Enterovirus* genus display different tissue tropisms. For instance, infections caused by CVBs are known to induce myocarditis and pancreatitis whereases EV-D68 commonly causes infections of the respiratory tract and it can impact the central nervous system (CNS) (5, 6, 8, 9, 11-13, 18, 20, 21). The close relatives to the enteroviruses, rhinoviruses, are also mainly involved in respiratory tract infections (19, 22). Secondary spread of the viruses to other organs occurs after the initial infection, due to viraemia (3). The tissue which the virus spreads to is strongly related to the expression of specific receptors for virus attachment and infection on the surface of tissue cells (2). For instance, ICAM-1 has high expression in the lungs, ICAM-5 is expressed by dendrites/soma of neurons of the telencephalon, and alpha2,3- linked Sialic acids are expressed in the lungs (30). CAR has a high expression in the heart which was confirmed in autopsy examination of human samples (49). Such examinations also demonstrated high expression of CAR in the pancreas, including islets. In both organs there were age related differences in CAR expression which could possibly explain increased susceptibility to viral infections at younger ages (mainly CVB

infections (2, 49)). Beside receptors expressed on the host cells, genomic regions in the virus were shown to be a contributing factor for virus tropism in a murine model (reference?). It was also suggested that recombination between viruses may happen which might lead to changes in tissue tropism and pathogenicity (50). There might also be other determinants for tissue tropism. In a recently published paper it was shown, in a mouse model, that even though the required receptor was not expressed in some of the organs, the tissue still had a high infection rate (48). This may occur when a virus is attached to exosomes for example, that are taken up by cells via endocytic pathways which are independent of receptor expression and lead to broader virus tropism (48).

1.1.3 Immune response to the infection

The immune response to a viral infection consists of both innate and adaptive immunity. Very briefly, the immune system is responsible for the recognition of potential pathogens (here viruses), and it eliminates the source of infection and infection, providing an immunological memory which will enable long-term protection against the pathogen. Although it sounds very simple there is a big and sophisticated machinery behind it.

1.1.3.1 Immune response in the intestine

Enteroviruses are mainly enteric viruses, which means they infect through the oral-faecal route with the primary infection site in the intestines (51). In the intestine, the first lines of defence are the epithelial cells and mucus, the latter is packed with defensins and other factors that are secreted by the epithelial cells. Infections caused by enteroviruses do not affect the gut per se. They can cause minor discomfort but seldom severe intestinal symptoms.

When viruses breach these first lines of defence and enter a cell, innate immunity is activated. The virus is recognized by pattern recognition receptors (PRR). This can happen through in an intrinsic way via cytosolic sensors or an extrinsic way through endosomal sensing (51). Cytosolic sensors can detect DNA via cGAS and RNA via RIG-I and MDA5. Such sensors are expressed across the human body including the pancreatic islets (52). A murine model lacking MDA5 shows increased risk of infection and a higher mortality rate following CVB infection (53). Endosomal sensing is mediated by toll-like receptors (TLRs). These are expressed by immune cells (such as dendritic cells and macrophages) as well as intestinal epithelial cells (51). TLRs involved in the sensing of ssRNA viruses recognise single stranded RNA (TLR7/8) and double stranded RNA (TLR3). TLR activation leads to

the initiation of a cascade of signalling pathways resulting in the production of inflammatory mediators such as interferons and cytokines in the infected cell (51).

In innate immunity, the main players are the type I and III interferons (IFN-α/β/λ), IL-6, IL-1β, IL-18 and tumour necrosis factor alpha (TNF-α) (51). These are responsible for 1) communicating with surrounding cells, 2) mediating antiviral responses including inducing the expression of proteins with antiviral activity and 3) inducing apoptosis in already infected cells, stimulating the production of chemokines to facilitate the recruitment of leukocytes, and mediating the activation of the natural killer cytotoxic response (51). Increased interferon production may also lead to increased major histocompatibility complex class I expression (MHC class I) on the surface of the infected cell. This signals that the cell is in an abnormal state via presentation of viral antigens to cytotoxic CD8+ T cells which can destroy the infected cell, hence stopping the infection (51). However, it is good to keep in mind that some of the viruses from the *Enterovirus* genome including the CVBs downregulate expression of MHC class I in infected cells (showed in various cell lines) therefore meaning CD8+ T cells are unable to act (54, 55). In such a situation, natural killer cells will respond to the infected cell by producing type II interferon (IFN-γ) rather than cytotoxic killing (56). Type III (IFN-λ) interferons were found to be expressed by infected epithelial cells (both intestinal and lung epithelial cells), hepatocytes, islets, and myeloid cells (57-60). Studies have shown that IFN-λ can attenuate virus infection (CVBs) in hepatocytes, islets and intestinal epithelial cells implicating its role in antiviral control (58-60). Unfortunately, some viruses such as CVBs can block IFN-λ expression to evade the host innate immune response (60, 61).

Lymphocytes are recruited to tissues with infected cells via chemokines produced by parenchymal cells and dendritic cells (DCs) which are located underneath the epithelium and serve as a bridge between innate and adaptive immunity. In addition to DCs, macrophages and epithelial M cells also contribute to the activation of adaptive immunity in the gut (51). Upon exposure to and acquisition of the virus particle, DCs migrate to lymph nodes where they present the processed antigen via MHC I and II to naive T cells. The lymphocytes existing in the intestinal tissue are comprised of conventional CD8+ T cells, tissue resident innate-like CD8+ T cells and double positive CD4+ CD8+ T cells (51). Displaying the antigen to the lymphocytes in the draining lymph node initiates the adaptive immune response. Presenting antigen through MHC I leads to the differentiation of naïve conventional CD8+ T cells to a memory and effector subtype. The effector

CD8+ T cells migrate to the site of infection and can eliminate the infected cells through granzyme or perforin cytotoxicity or cytokine-mediated cytotoxicity. This is orchestrated by chemokines which help with the recruitment and the trafficking of T cells to the site of the infection (62). It is not entirely clear how the intestinal T cells can control infection by some enteric viruses such as CVBs. However, it was found in murine models that protection against CVB infection might happen outside of the gut niche, showing the importance of the peripheral immune response (63). This finding is fortunate for vaccine development, showing that the peripheral immune response is also important for protection against virus infection, especially when vaccination is not administered orally (vaccination that specifically induces immunity at the local site of infection).

An antigen is also presented through MHC II to CD4+ T helper cells which are the main support for B cells (apart from differentiation into a Th1 response and interferon production). They help with antibody class switching and affinity maturation. This is an example of immune crosstalk between cellular immunity and humoral immunity (51).

B cells are responsible for producing antibodies against specific pathogens such as viruses and antibodies are key players in virus clearance. Individuals who lack immunoglobulins are highly susceptible to viruses, including enteroviruses (64). The importance of virus neutralising antibodies for protection against viruses is evident from immunoglobulin transfers (including passive maternal transfer) and vaccination. The antibodies result in lower levels of infection or complete protection from infection (65-68).

B cells are exposed to antigen in the lymph nodes (such as Peyer's patches-PP or other draining lymph nodes-LN). There are two pathways of B cell activation: T cell independent and T cell dependent. In the case of T cell independent activation, the antigen is directly recognized by B cells through the B cell receptor. This leads to quick differentiation into plasmablasts which produce a large number of antibodies (with low affinity), hence combating the active infection (although this is only a temporary response) (69). B cells activated through T helper (CD4+) cells enter and initiate reactions in germinal centres of lymph nodes. PP are found to be continuously active since they are close to the gut and constantly exposed to different pathogens and gut microbiota (70). Once activated, the B cell starts to rapidly proliferate and undergo mutations (this happens in the dark zone of lymph nodes or PP). This results in the production of high affinity B cell receptors. To

assure quality of the receptor, B cells move to another region of the lymph node (light zone), where they can test the receptor against the antigen exposed on follicular dendritic cells. T helper cells (T follicular helper, Tfh) provide feedback (71) and if the receptor does not have high affinity the B cell returns to the dark zone where it continues to mutate until higher affinity is established. When the affinity is high the B cell goes through differentiation (71). This results in the formation of memory B cells, long lasting plasma cells and plasma blasts. Memory cells guard lymphoid organs whereas plasma cells migrate to the bone marrow where they continue to produce antibodies (72). The formation of memory T and B cells ensure that the adaptive immune response is faster in case of reoccurring infection (51).

Naïve B cells expressing IgM or that co-express IgM and IgD antibodies are more frequent in tonsils or other LN than in PP (73). After class switching the B cells produce IgG, IgA and IgE. In the intestinal niche IgA seems to be the most common (51).

Some enteroviruses infections are cleared without the need for antibodies, suggesting that innate immunity alone is sufficient to clear the infection. Many infections are also asymptomatic and several studies have shown that enteroviruses are detected in stool samples of healthy subjects (e.g. (74)). Often it is not known if the shedding of enterovirus in the stool is a result of an ongoing or a persistent infection.

1.1.3.2 Immune response to respiratory infections

Respiratory viral immune responses are very similar to gut immunity. Some of the viruses from the *Enterovirus* genus infect the respiratory system and cause common seasonal colds (75). Disease severity depends on age, whether a person is immunocompromised, and if they have other co-existing diseases that impact the lungs such as cystic fibrosis (CF) (76, 77). Excessive immune responses, known as a cytokine storm, led by IL-4 and IFN-γ are followed by infection in the lungs. This reaction can have detrimental effects leading to multiorgan failure. It is still unclear if the cytokine storm generated is similar across different viruses (78). Additionally, there is emerging evidence showing the crosstalk between gut microbiota and the lungs, and how the microbiota can affect airway infection or progression towards lung disease in, for example, CF (79).

1.1.4 Enterovirus rates- a global perspective

For years poliovirus (belonging to *Enterovirus* genome) was a focus for many scientists and international health agencies. The World Health Organization (WHO) set the stage for the Global Poliovirus Eradication Initiative which was one of the largest international collaborations battling virus infection. Despite a reasonably successful poliovirus eradication program, among the *Enterovirus* genome there are many non-polio enteroviruses circulating in the population (80). A recent analysis aggregates published evidence on enterovirus epidemiology and patients' cohorts. The article suggested that enterovirus infection has a high prevalence in all continents and viruses belonging to *Enterovirus B* species are the most detected worldwide, predominantly present in Asia and Europe. In terms of detection, the Enterovirus D species (EV-D68) was second in Europe. Among the most reported viruses were CVB1-5 and EV-D68. Importantly, rhinoviruses in North and South America and Oceania (in continent specific species distribution) were excluded from the analysis (74). A large-scale retrospective surveillance study which analysed data from the European Union and European Economic Area regions between 2015 and 2017 also showed a wide circulation of Enteroviruses in Europe. Among other enteroviruses, CVB5 was identified as a virus detected in children younger than 3 months, proving that the infection is common in vulnerable groups (81). Based on published papers, during the past 60 years, CVB infections were recognised in North America as a cause of many "epidemics" and outbreaks (9, 82-85). Centers for Disease Control and Prevention established surveillance monitoring to track enterovirus infection in the United States. Japan also has rigorous surveillance monitoring which enabled the analysis of data collected over 15 years, and to explore virus transmission dynamics (86). Such surveillance monitoring could help with outbreak forecasts and to deploy necessary actions for limiting outbreaks (86). Similar platforms which would collect and compare data across Europe were needed, so the European Non-Polio Enterovirus Network was established (87, 88). The data collected through the network will allow for the evaluation of the actual burden of non-polio enterovirus infections from national and global perspectives and provide prevention guidance as well as diagnostic recommendations (87, 88). This knowledge and data exchange platform is a promising tool which will deepen the understanding of non-polio enterovirus infections, predict potential outbreaks or pandemics, and provide an input into new preventions such as vaccines and treatments like antivirals.

1.2 Coxsackieviruses

Coxsackieviruses were first discovered and isolated in 1947 from two young patients suffering from paralysis who came from a city called Coxsackie, in New York, the USA (89). This nomenclature was proposed two years later by Dalldorf and the virus isolate became a prototype for group A coxsackieviruses (90, 91). Later, it was suggested that a new classification should be introduced, namely coxsackievirus group A and B. This was based on histological assessments (92). The coxsackievirus B group (known as CVB) was originally identified by Melnick et al. (93). The CVBs showed different pathology of infection which mainly impacted the brain, pancreas, and heart in a murine model. The infection of these organs caused diseases such as myocarditis, pancreatitis and pleurodynia (Bornholm disease) (8, 94-96). As of today, CVBs belong to *Enterovirus B* species and encompass six types from CVB1 through to CVB6 (1). The infections caused by CVBs have also been associated with type 1 diabetes (T1D) (44, 97-99), although their role in the disease aetiology has not been addressed. Moreover, there is evidence highlighting their impact on patients with CF and they are common among this group (100-102).

1.3 Cystic Fibrosis and Enterovirus infection

1.3.1 Aetiology

Cystic fibrosis (CF) is a monogenic disease which is estimated to impact between 145,000-187,000 individuals across the World (103). This disease is caused by mutations in the transmembrane conductance regulator (*CFTR*) gene, encoding the CFTR protein, an anion channel which enables chloride ion transport and assists bicarbonate flow across apical cell membranes (104-109). Mutations are known to impact epithelial cells in lungs, but other organs are also affected, including liver, gastrointestinal tract, pancreas, kidneys, the male reproductive tract, sweat glands and more (110-114). There have been more than 2000 *CFTR* mutation variants discovered, and 719 of these variants cause disease (110, 113, 115). The mutations are associated with varying severity of CF disease, based on either absence or reduced functionality of the CFTR protein. They have been grouped into six classes. Classes I to III contain mutations of higher severity, causing non-functional or defective CFTR channels. These types of mutations result in severe lung diseases and pancreatic dysfunction. Class IV to VI includes mutations that cause decreased or scarcened functionality of the channel and therefore milder disease. The most common mutation is a deletion of phenylalanine at position 508

(F508del), which lies in class II (104). The F508del mutation causes a CFTR trafficking defect and destruction of misfolded CFTR protein, hence a non-functional CFTR channel. The overall results of mutations in CF are excessive production of mucus in the affected organs, leading to mucinous obstruction followed by organ destruction (110, 116, 117). Due to the presence of this viscous mucus in the lungs, pulmonary infections in CF patients are very common and can be chronic leading to inflammation and destruction of the lungs (117-119).

Besides *CFTR* gene expression in epithelial cells, evidence presented in recent studies also suggests there are alterations in immune cells in individuals with CF (109, 120-122). Both neutrophils and macrophages were found to have defective pathogen clearance, even though they express a pro-inflammatory profile (121). Altered T cell functions were found in CF patients, but the B cell response remained normal (123), although there was evidence of an enhancement in B cell response in CF individuals (124). *In vivo* infection studies also reported an impaired immune response in mice harbouring mutations in the *CFTR* gene resulting in higher viraemia compared to wild type (wt) animals. Moreover, delays in antibody production were observed (65). The immune cell repertoire remained normal but a response to T-cell dependent antigen was delayed (65).

Thanks to new-born screening and recent discoveries, namely modulator therapies, patients with CF have better chances of improved quality of life and extended life spam. Nevertheless, lung infections are still a common thread among these individuals (113, 114, 125).

1.3.2 Infections in CF

People with CF suffer from re-occurring and chronic lung infections. They lead to inflammation, decline in lung function, pulmonary deterioration, pulmonary exacerbations, and bronchiectasis (110, 126-128). Therefore, individuals with CF often require prophylaxis treatment such as anti-inflammatory drugs, antibiotic treatments, vaccination (for example against influenza) or monoclonal antibody treatment. New approaches such as bacteriophage treatment are already in clinical trials (127, 129).

1.3.2.1 Viral infections

Several viruses are associated with infections in CF patients, among them influenza virus, coronaviruses, rhinoviruses, enteroviruses, and respiratory syncytial virus. These viruses target different parts of the respiratory system

(upper or lower respiratory tract) and young individuals are more prone to infections (129). For example, human rhinovirus was mostly detected in young CF patients, but its prevalence was as high as in healthy controls (130). Among other viruses belonging to the *Enterovirus* genus, coxsackieviruses (like CVBs) have appeared in a few studies, suggesting their influence and presence in patients with CF (100, 101, 131). The prevalence of CVB infections in the CF population versus healthy populations has until recently not been investigated (102).

1.3.2.2 Bacterial infections

Bacterial infections are common among people with CF and different strains are more likely to colonise different age groups. *Staphylococcus aureus* is a gram-positive coccus which is commonly isolated from the respiratory tract of children with CF. Infection with *S. aureus* has also been associated with *Pseudomonas aeruginosa* co-infection based on clinical isolation. Methicillin-resistant *S. aureus* is also recurrently present in CF lungs. *Pseudomonas aeruginosa* is a gram-negative bacterium and has been associated with more persistent infection in adults with CF and the prevalence of this virus is more frequent in this group. This aligns with the Patients Registry Report performed by the Cystic Fibrosis Foundation. Beside these pathogens there are also other bacteria involved in infecting the CF lungs (110, 132, 133). It has been suggested that *S. aureus* is the first bacteria to come which predisposes lungs to *P. aeruginosa* infection. *In vivo* studies showed the potential *S. aureus* impact on *P. aeruginosa* pathogenesis (134).

1.3.2.3 Connection between viral and bacterial infections

Early evidence showed a weak correlation between viral seroconversion and colonisation with *Pseudomonas* species (100, 128). However, more recent studies show that the link between virus infections and bacterial colonization is possible. Recent research presented a connection between early viral infection in infants followed by higher frequency of bacterial pathogens in the lower respiratory tract (130). Moreover, enhancement of bacterial biofilm was present in a co-culture with virus infection in an *in vitro* scenario (135, 136). It has been suggested that virus infections may be involved in the facilitation of bacterial infections (131, 137). Therefore possible targeting of viruses could be beneficial for controlling symptoms and disease progression (138). Although some studies did show the reduction of virus-related hospitalization due to prophylactic monoclonal treatment, the encounter of *Pseudomonas aeruginosa* or *Staphylococcus aureus*

was not delayed in infants with CF, and the infection occurred even earlier than it was initially expected (139-141). Nonetheless, viral, bacterial, and fungal infections are factors that lead to a decline in lung function as well as pulmonary exacerbations (142). Moreover, viral infection was shown to cause an acute increase in bacterial load, mainly in adults with chronic bacterial infection, compared to their baseline (141, 143). There is also evidence that virus infections are more prevalent in younger children, below 5 years of age, whereas bacterial infections are seen more regularly in children at 12 years of age or more (144). Therefore, working on various treatments that aim to prevent, minimize, or eliminate infections is crucial.

1.3.3 Vaccination in CF

Previously, there were no specific vaccine recommendation for CF patients, only the general suggestion to follow the national vaccination program (145). Since then, it has been highlighted that additional vaccines are beneficial to the CF population, including the seasonal flu vaccine and a vaccine against pneumococcus (146). Moreover, further monitoring of vaccine efficacy in the CF population would be beneficial in understanding the actual prevention against diseases (145, 146). Over the years, some studies have reported weakened responses to vaccination in people with CF (147-150). Contradictory, another study showed no significant differences in antibody responses in CF children and controls against tetanus and polio vaccine (151). Altogether, these findings suggest a need for further exploration of such impaired responses to vaccinations and the design of new vaccines, which can target other bacteria and viruses prevalent in CF.

1.3.4 Experimental Models of CF

There are several animal models that exist which are helpful in the exploration of new treatments and in understanding the mechanisms behind CF (152). One of the models is the $Cftr^{tm1EUR}$ mouse model, known as F508del (the most common mutation among human population). These mice do not develop lung disease but have a lower body mass compared to wild-types, and they have issue with their intestines. These features are similar to human CF disease (153-155).

1.4 Type 1 diabetes and enterovirus infection

1.4.1 Aetiology

Type 1 Diabetes (T1D) is an autoimmune disease which impacted 8.4 million individuals across the World in 2021 (156). It is estimated that by the year 2040, the number of patients suffering from T1D will increase to 13.5- 17.4 million cases (156). The main characteristic of this disease is the destruction of the insulin producing beta cells in the pancreas leading to a life-long insulin regime (157). The destruction of the beta cells is a result of immune attack towards those cells. The predominant immune cell types contributing to the inflammation found in the islets of Langerhans (known as insulitis), are CD8+ T cells and macrophages, CD4+ T cells and CD20+ B cells (158). Autoantigen specific CD8+ T cells are suggested to attack the beta cells presenting antigens such as insulin, proinsulin, GAD and post-translational modified peptides or viral antigens. (157-159). Activated T cells are also responsible for B cell activation and production of antibodies targeting beta cell antigens, so called autoantibodies (AAB) (157). Autoantibodies against insulin or GADA are considered biomarkers for T1D prediction (159, 160). A recently published manuscript suggests that virus infection, here coxsackievirus infection, induces the direct killing of beta cells rather than CD8+ T cell-mediated beta cell destruction. The study found that CD8+ T cell inefficiently mediated the killing of virus infected beta cells (55).

Only about half of the risk of developing T1D can be explained by genetics, indicating that the environmental factors play a role (161). Two haplotypes of human leukocyte antigen (HLA) genes are associated with T1D risk, *DR3* and *DR4-DQ8* (157, 161). HLA alone are not sufficient to explain the increasing rate of the disease (47, 161). Therefore, other factors may contribute to T1D.

1.4.2 Environmental factors

Environmental factors have been associated with the development of T1D. These encompass for example diet (during infancy and as well adulthood) and levels of vitamin D (157). In addition to dietary factors, gut microbiota and contact with environmental microbiota might have an impact on the pathology of T1D (162, 163). One of the most studied environmental associations are virus infections and in particular those caused by viruses belonging to *Enterovirus B* species, coxsackieviruses (CVBs) (97, 98, 157). Published evidence confirms the presence of *Enteroviruses* in individuals prior to or with T1D (44, 99, 164, 165).

Currently there are two hypotheses which supporting the relationship between virus infection and T1D development. The first hypothesis predicts that CVB infection contributes to the initiation of islet immunity, focusing mainly on the temporal association between virus infections and the development of autoantibodies (98, 166-168). The second hypothesis implies that enterovirus infections can accelerate the onset of diabetes in individuals positive for autoantibodies (169). These hypotheses suggest that T1D is not a simple genetic autoimmune disease but arises due to an interplay between genes and environmental triggers.

1.4.3 Virus infections

There are many studies showing the implications of enterovirus infection in the context of T1D (44, 45, 97-99). Both severe and persistent infections have been studied, but there is currently a tendency to shift the focus more towards a persistent infection (47). Moreover, infection followed by destruction of beta cells can be direct or indirect (170). A recent study showed that the virus can destroy beta cells in a direct way in both *in vitro* and *ex vivo* models (55).

In terms of persistency, it was shown that a persistent infection in human pancreatic ductal cells cause changes in transcriptome as well as modifications in protein expression and secretion in these cells. It has been hypothesized that this might be a result of a persistent infection and that the infection can spread to beta cells (171, 172).

An interesting aspect is also the difference between alpha cells and beta cells in the context of immune responses and the predisposition of beta cells to destruction (173). It was shown that endoplasmic reticulum (ER) stress genes, which have a higher expression in beta cells, could contribute to beta cell destruction. Moreover, differences in the expression of viral recognition and innate immune response genes were detected (173-175). In addition, beta cells do not express a subset of genes which alpha cells do, that are involved in the inflammasome machinery and antiviral responses (173, 176, 177). Taken together, this suggests that the beta cells are more likely to be susceptible to induced stress and viral infection. Other genes responsible for expressing pattern recognition receptors have a higher expression in the alpha cell compared to the beta cell, therefore suggesting they have a higher chance of clearing viruses more efficiently than beta cells (173, 178, 179). This may make beta cells more prone to persistent infections (47, 173, 180). Moreover, IFN-mediated stress response

markers are expressed in the islets of T1D donors, showing higher expression in islets with insulitis compared to the islets in which insulin is still detected. Finally, these markers were correlated with the presence of enterovirus protein VP1 (181).

Interestingly, mixed drainage between organs, hence cross talk between T lymphocytes in the lymph nodes (LNs), was recently explored (182). The occurrence of cross talk is especially prominent in LNs which drain several organs such as the pancreaticoduodenal LNs, which are present in humans (183). There are also associations between pancreatic diseases and intestinal pathologies (182). It was shown in a mouse model that gut draining lymph nodes are also responsible for draining the pancreas so possible virus infections in the gut could unleash pancreatic immunity (182). This is even more interesting considering that enteroviruses were found in the intestines of T1D individuals, which aligns with the overall virus route of infection, and gives a possible entry to the pathogenesis of T1D (184, 185). Although some studies showed that there is no association between infection and the disease (186), analysis of the virome in faecal samples from controls and individuals with AAB+ or T1D showed that enteroviruses might be associated with islet autoimmunity (167).

There is plenty of evidence suggesting that enterovirus infection is involved in T1D pathology, but there is a lack of firm evidence. Therefore, the development of potential preventions such as vaccines or treatments like antivirals may well be beneficial for patients with T1D (47, 170).

1.4.4 Experimental models

Throughout the years, many models have been created and utilized to learn and explore the pathology of and potential cures for T1D (187, 188). Among those are *in vitro* models such as pancreatic human beta cells (e.g. EndoC), rodent beta cell lines (e.g. INS-1), primary human islets of Langerhans which are available from brain dead organ donors, and stem cell-derived islets (SC-islets) (189-193). To understand more systemic mechanisms of T1D and to gain understanding of environmental triggers, *in vivo* models were established. The most common is the non-obese diabetic (NOD) mouse which spontaneously develops diabetes from 10-14 weeks of age (194). Common features of this model and human T1D include the presence of autoantibodies and immune infiltration in the pancreas before the onset of diabetes (195). This model shows that upon infection with CVBs, diabetes onset is accelerated (66, 67, 196, 197). Another well described model of virus-induced diabetes is a specially prepared transgenic NOD mouse that produces

high levels of the suppressor of cytokine signaling-1 (SOCS-1) in pancreatic beta cells (also known as the SOCS-1-tg mouse model) (198). Pancreatic beta cells of these mice are susceptible to coxsackievirus infection which mean that virus can directly destroy the beta cells leading to the onset of diabetes (66, 199).

1.5 Potential preventions and treatment for enterovirus infection

1.5.1 Vaccines

Vaccines have proven to be a very important protective shield against many infections. Throughout history, they contributed to the eradication of smallpox, massively reduced rates of poliovirus infection and recently helped with the SARS-CoV-2 pandemic. At the time of writing, the only vaccines against non-polio enteroviruses in clinical use are against EV-A71 and they are only used in Asia (200-202).

Immune responses to a vaccine mimic the response to the virus or specific virus components. Upon the introduction of the vaccine to the system (host), the dendritic cells are alerted by danger signals which then take up the antigen injected. The antigen is transported to the lymph nodes where it is presented to CD4+ T cells. The CD4+ T cells and soluble vaccine antigen activate B cells which leads to proliferation and differentiation into memory B cells, antibody producing plasmablasts and long-lasting plasma cells which migrate to the bone marrow. Vaccination also activates CD8+ T cells which divide into effector and memory cells. Thanks to this, the host will be able to react faster and stronger upon encountering the same antigen. Vaccination protects individuals from developing severe disease and in some cases, even infection (sterilizing immunity), and can stop the spread of the pathogen in the population, hence building herd immunity (203).

1.5.1.1 Vaccines against coxsackievirus infection

Based on high rates on enterovirus infections around the world, associations between enterovirus infections and severe disease, and lack of non-polio vaccines in clinical use, several initiatives have been taken to produce new enterovirus vaccines. In a large, collaborative initiative, Coxsackieviruses were selected, building on their high prevalence in different groups and because they may have an impact on T1D pathogenesis (204, 205). One of the two existing polio vaccines is an inactivated, whole-virus particle vaccine with high protection rates and therefore a coxsackievirus vaccine was made in a similar way (206, 207).

These vaccines underwent extensive pre-clinical trials where their safety and ability to protect against virus infection were measured (66, 67, 199). Selected models include mice specifically related to autoimmune disease (NOD mice) (66, 67, 199) and those susceptible to virus infection in the beta cells (SOCS-1-tg) (66, 199). To closer mimic humans, the non-human primates, rhesus macaques, were also studied (66). All studies found the new CVB vaccines to be highly immunogenic and protective against CVB infections (66, 67, 199). Moreover, vaccines prevented virus-induced diabetes in SOCS-1-tg mice (66, 199). The vaccine was also tested in a CF mouse model (102). Before entering clinical trial it was important to evaluate whether the vaccine itself can induce adverse effects (197). All together, these efforts resulted in the production of a vaccine (PRV-101) which was recently tested in healthy adults (NCT04690426)(208).

1.5.2 Antivirals

Antiviral therapies are intended to eradicate virus infections that are either ongoing or persistent. Their aim is to prevent infection or block the virus life cycle in an on-going infection. There is no universal antiviral treatment which impacts all existing viruses. This is because each virus family, and even different virus species, are different in terms of structure, viral genome, and ways of infecting, replicating, and spreading in the host.

There are two potential approaches in antiviral therapies. One aims to target viral proteins, the other targets the host factors key to virus replication and propagation (209). Examples of targeting viral proteins include the viral capsid, the uncoating stages of initial virus infection, non-structural proteins involved in the translation stage (viral proteases) and replication stages (viral polymerases) or other expressed viral proteins. Structural proteins may also be a possible aim for antiviral treatment (209). Among some host factors such as Golgi specific factors, host proteins involved in the replication stage, mainly by formatting replication organelles, are highlighted as potential antiviral targets. These include oxysterol-binding protein (OSBP) and phosphatidylinositol 4-kinase-β (PI4KB) proteins which are involved in the transport of cholesterol and the formation of PtdIns4P (phosphatidylinositol-4-phosphatate), respectively (2, 47).

Antiviral agents that target viral proteins are more likely to cause fewer side effects compared to drugs that target host factors. On the other hand, the targeting of host factors is less likely to induce drug resistance than drugs that target viral proteins (2).

In terms of drug development strategies, drug repurposing has become popular in recent years due to faster recognition of possible antivirals from already approved and used drugs (209). With increased used of artificial intelligence more advanced technologies, this process can be further improved and sped up. Besides faster recognition, cost savings and quicker re-introduction to the market are also beneficial .

1.5.2.1 Anti-enterovirus treatment

Over the years many antivirals have been suggested as anti-enterovirus therapies. Even though some of them were admitted to clinical trials, they were withdrawn due to insufficient therapeutic effect, safety concerns and high toxicity (47, 209-211). So far there is no existing anti-enterovirus therapy. Nevertheless, a recently published paper showed that treatment with pleconaril and ribavirin preserved residual insulin production in children and young adults with resent onset of T1D (212), providing rationale for further exploration and development of new antiviral drugs against enteroviruses. Such treatment could perhaps prevent or even support the treatment of T1D.

1.5.2.2 Emerging new treatment against enteroviruses in the context of T1D

Due to drug repurposing screening, another possible antiviral drug was identified (213). Vemurafenib (PLX4032) is a cancer drug targeting $BRAF^{V600E}$ mutation. This drug is approved and already in use in the clinics (214, 215). A few studies have proven the antiviral properties of this drug in both acute and persistent phase of infection (213, 216-218). Thanks to a recent study the possible mechanism of action was discovered, suggesting that the drug targets host protein PI4KB involved in the formatting of ROs (213). Therefore, the replication of the virus is disturbed leading to lower viral titres. It is important to address the possible antiviral effects of Vemurafenib in experimental models, by focusing on the site of infection as well as models of virus induced T1D .

2 Research aims

The overall aim of this thesis was to explore experimental *in vitro* models and pre-clinical *in vivo* disease models to assess the efficacy of novel vaccines and antivirals in preventing enterovirus induced disease.

The specific objectives of this thesis are the following:

- To examine the prevalence of coxsakievirus B (CVB) infections in people with CF and measure vaccine induced neutralising antibodies against poliovirus in this population. Additionally, to test a vaccine against CVB infection in a murine model of Cystic Fibrosis (CF) and to investigate whether the antibody response to the vaccine is T-cell dependent or independent. (**Paper I**).

- To examine the safety of a polyvalent CVB vaccine with a primary focus on islet inflammation and diabetes development, and to investigate the ability of a CVB vaccine to prevent CVB-accelerated diabetes onset in NOD mice (**Paper II**).

- To address the possibility of repurposing a cancer drug to prevent CVB infection at the site of infection and in pancreatic beta cells. (**Paper III**).

3 Materials and methods

This following section highlights the main methodology utilized in this thesis. A detailed description of each technique can be found in the papers included in this thesis (**Paper I**, **Paper II** and **Paper III**).

3.1 Virus strains

Paper I: CVB1-10796 (wild type strain from Argentina) and CVB3 (Nancy strain) were used for animal infection studies. CVB1-10796; CVB3; CVB1, CVB2, CVB3, CVB4, CVB5 and CVB6 and polioviruses 1 and 3 (the Sabin strains) were utilized for the measurement of neutralising antibody titres against these viruses.

Paper II: CVB1 was used for the animal infection studies. CVB1 and CVB1-6 were used to measure neutralising antibodies against these viruses.

Paper III: CVB3eGFP, CVB3 Nancy, CVB4-E2 and CVB3-V13 were used in the *in vitro* experiments. CVB3 Nancy was used in the *in vivo* setup.

3.2 Virus infections

Paper I:

In vivo:
CVB1: 10^6 PFU/mouse and CVB3: 10^5 PFU/mouse

Paper II:

In vivo:
CVB1: 10^7 PFU/mouse

Paper III:

In vitro:

Antiviral treatment:
CVB3eGFP: MOI 10 or MOI 0.1 for HeLa cells
CVB4-E4: MOI 150 for INS-1 cells
CVB3 Nancy: 4 x 10^3/islet, 4 x 10^4/islet, 4 x 10^5/islet

Cytopathic effect:
CVB3 V13 MOI^{-4} for HT-29 cell, MOI^{-3} for Caco-2

In vivo:
CVB3 Nancy: 10^5 PFU/mouse

In vivo infections were done by intraperitoneal injections, 200μl per injection.

3.3 Vaccines

Vaccination was performed in **Papers I** and **II**.

Formalin inactivated vaccines against CVB1 and CVB3 Nancy and CVB1-6 were propagated by our collaborators at the University of Tampere, Finland. Viruses were inactivated in 0.01% (v/v) formalin for 5 days at 37°C. The vaccines were formulated by combining virus serotype(s) with Medium M199 containing 0.1% Tween 80. CVB1 and CVB3 vaccines contained 1.8μg of virus protein per dose whereas CVB1-6 vaccine contained 1μg of each inactivated serotype per dose.

A volume of 150μl of vaccine per mouse (dose described above) was administrated by interscapular (subcutaneous) injection in the *in vivo* studies.

3.4 Antivirals

Antiviral treatments were used in **Paper III**.

Two drugs with possible antiviral activity were selected from a drug screen performed by our collaborators from the University of Jyväskylä, Finland. Vemurafenib (PLX4032) which is an approved medicine for cancer (melanoma) treatment and its analogue PLX7904.

In vitro studies: Concentrations used were 1.25/2, 5, 10 μM.

In vivo studies: A volume of 200μl with a dose of 25mg/kg was administrated via intraperitoneal injection.

3.5 Cell lines

All cells were mycoplasma free.

Papers I and **II**: Cells were used for propagating the virus as well as analysing neutralizing antibodies and virus titres.

- Vero cells, GMK and HeLa cells

Paper III: Several cell lines were used for infection and for analysing virus titres.

- Monolayer cells: HeLa cell, INS-1 832/13 cells, HT-29 and Caco-2 cells

- 3D: Stem cell-derived islets (SC-islets), propageted from human embronic stem cells (hESC) by collaborators at Uppsala University

3.6 Human samples

Serum samples from CF patients for analysis of neutralising antibodies against CVBs and polio 1 and 3 were obtained from Stockholm CF Center's clinical biobank (collected between 1992-2010). Serum samples from healthy donors were obtained from an unrelated vaccine study (collected between 2008-2016) (**Paper I**).

3.7 Animal models

In **Papers I** and **II**: Mouse models were used to study immune responses and safety after vaccination. In **Paper III** mouse studies helped to assess antiviral property and safety of Vemurafenib.

Paper I:

The F508del mouse model was chosen as this mouse is homozygous for the *Cftr*^{tm1EUR} mutation, which is the most common mutation among people with CF (153). This model enabled us to study vaccine responses and CVB infection.

The TCRα-knock out mouse is homozygous for the *TCR*α^{tm1Mom} mutation and does not express of alpha beta T cell receptors. This results in a loss of CD4-CD8+ and CD4+CD8- T cells. This model addresses the role of T-cells in the neutralising antibody response post vaccination.

Paper II:

The NOD (non-obese diabetic) mouse model was utilized in this study. This mouse model spontaneously develops autoimmune diabetes from 10-14 weeks of age. The females have a higher rate of diabetes development compared to males, with about 65-95% of female mice becoming diabetic by 30 weeks of age (195). This mouse model helped us to study the safety of the vaccine by monitoring if acceleration of the disease occurred following vaccination. Moreover, we tested weather vaccination protects from virus accelerated diabetes.

Paper III:

The NOD mouse and SOCS-1-tg models were used in this study. The SOCS-1-tg muse is a transgenic mouse model generated on the NOD background. SOCS-1-

tg mice express the suppressor of cytokine signalling (*Socs1*) under the control of the insulin promotor (198). SOCS-1 blocks the signalling of some cytokines such as interferons and beta cells that overexpress SOCS-1 do not respond to interferons during CVB infection. Hence, SOCS-1-tg mice have beta cells that are susceptible to CVB infection and upon infection they lose beta cells and develop diabetes withing 5-12 days post infection. These two mouse models helped us to address the safety and antiviral activity of Vemurafenib during systemic infection.

3.8 Animal studies

Mice used in the studies were bred in-house and housed under specific pathogen free conditions at Karolinska University Hospital, Stockholm, Sweden. Animals were kept in ventilated cages and provided with food and water ad libitum. Mice were housed up to 5 animals per cage and were not kept single housed, unless the ethical permission allowed, and it was crucial for the completion of the experiment. Permission from the veterinarian had to be obtained before animals were single housed. Experiments were performed in a BSL2 facility, and animals were allowed to acclimatize in the room for a few days prior to the experiment. Animals were randomly assigned to the groups, but researchers were not blinded to the treatment.

During animal studies, physiological and psychological well-being of mice was assessed. Animals were weighed throughout study; in some studies blood glucose levels were also monitored. In case of meeting the humane endpoint score of 0.4 or more, or if weight loss was more than 15% of their highest weight, the animals had to be removed.

At the experimental endpoint, mice were anesthetised with isoflurane, and a terminal heart puncture was performed for blood and serum collection. Mice were euthanised afterwards and organs were harvested for fixation and plaque assay.

Blood glucose monitoring:

In **Paper II** and **Paper III** blood glucose was measured. Diabetes was set at blood glucose value ≥ 18 mmol/L. In case blood glucose levels were between 13 mmol/L and 18 mmol/L the mouse had to be checked again the next day. If the value was again between 13-18 mmol/L, the mouse was identified as diabetic and had to be removed.

Blood sample and serum samples collection:

During the studies (**Papers I, II** and **III**) blood and serum samples were collected from blood drawn from the tip of the tail. Blood samples were collected in tubes containing 12mmol EDTA in the ratio 1:1. Blood for serum samples were collected in serum tubes.

Paper I: Vaccination strategy and virus challenge

TCRα^{tm1Mom} knock-out/ wild-type:
Females: 9-20 weeks of age
Vaccination: Day 0, 14 (CVB3 vaccine)
Serum collection: Day -2, 4, 5, 14, 21, 28, 35

F508del/ wild type
Females: 4-22 weeks of age
Vaccination: Day 0, 14 (CVB3 vaccine)
Serum collection: Day -2, 4, 5, 14, 28

F508del:
Males and Females: 4-22 weeks of age
Vaccination: Day 0, 21, 35 (CVB1 and CVB3 vaccine)
Virus challenge: Day 63 (CVB1: 10^6 PFU/mouse and CVB3: 10^5 PFU/mouse)
Serum collection: -4, 4, 21, 35, 63
Blood collection: Day 66 (day 3 post infection), 67 (day 4 post infection)

Paper II: Vaccination strategy and virus challenge

NOD:
Females: 4.9-7.1 weeks of age
Vaccination: two or three times, 2-3 weeks apart (CVB1-6 vaccine)
Serum collection: Day 0 and 42

NOD:
Females: 10.5-13.5 weeks of age
Virus challenge: CVB1: 10^7 PFU/mouse

NOD:
Females: 6.3-6.9 weeks of age
Vaccination: three times, 2-3 weeks apart (CVB1 vaccine)
Serum collection: Day 0 and 42
Virus challenge: weeks after final vaccination, CVB1: 10^7 PFU/mouse

Paper III: Antiviral treatment

NOD and SOCS-1-tg:
Males: 10.5 weeks of age
Antiviral treatment: Day -1, 0, 2, 3, 4
Virus challenge: Day 0, CVB3 Nancy: 10^5 PFU/mouse

3.9 Cytotoxicity studies

HeLa cell and INS-1 were exposed to increasing concentrations of Vemurafenib and its analogue for 20h and 6h or 20h, respectively. After adding the treatment to the plates, the plates were returned to the incubator and incubated at 37°C for the time indicated above. Afterwards cells were trypsinised and stained with trypan blue for viability testing (**Paper III**).

3.10 Antiviral studies

The overall idea for the antiviral treatment was to check whether it can prevent infection and/or replication of the virus. All cells used were pre-treated with Vemurafenib or its analogue for 1h at 37°C. After a series of washing, they were infected in the presence of the treatment for 1-1:30h at 37°C. After that followed another series of washing and continuation of treatment until harvesting after 6/20h or 48h for HeLa/INS-1 and SC-islet medium, respectively (**Paper III**).

Antiviral studies with HeLa cells and INS-1 cells were done in 12- or 24-well plates, respectively. Cells were harvested by using a cell scraper and both cells and supernatant were saved for plaque assay analysis.

Antiviral experiments with SC-islets were done in 24-well plates with tissue culture inserts. The handling of SC-islets required the use of stereoscope to transfer SC-islets to the inserts. SC-islet culture medium was saved for plaque assay analysis.

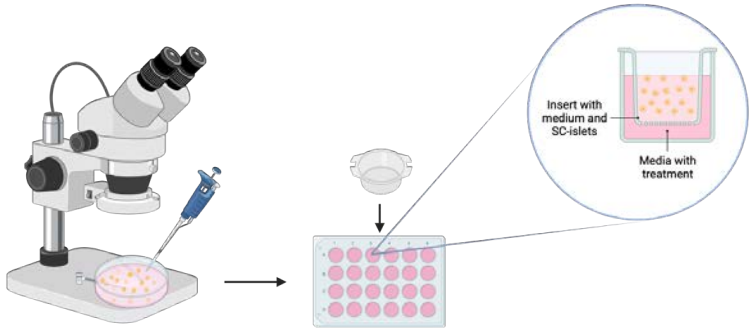

Figure 1. Schematic presentation of SC-islets antiviral treatment set-up, placing SC-islets into the insert. Created with BioRender.com.

3.11 Cytopathic effect (CPE) studies

Cytopathic effect (CPE) assay was used to determine whether Vemurafenib was able to prevent infection in the intestinal epithelial cell lines HT-29 and Caco-2 without affecting cell viability. Cells were pre-treated with the drug, infected in the presence of the drug, and incubated in the continued presence of the drug for 48h at 37°C. (**Paper III**). Once the experiment was completed, media was washed away, cells were fixed with a methanol: acetic acid glacial mix and then stained with crystal violet. Optical density was measured at 595 nm by a spectrophotometer. Cells which were protected had a preserved monolayer whereas cells affected by virus detached from the well. Therefore, a high optical density readout implicated protected cells whereas a low readout meant a lack of protection.

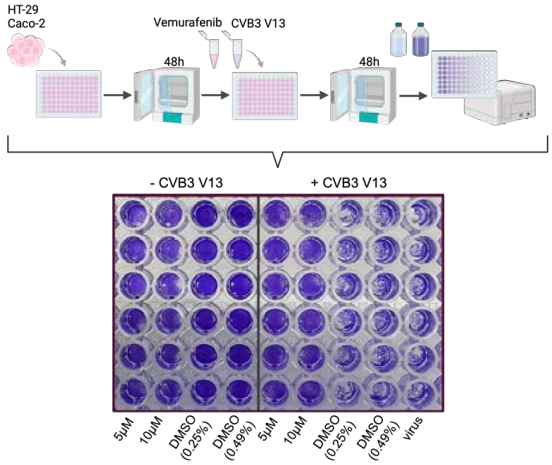

Figure 2. Schematic of CPE experimental set-up. 5 and 10µM indicates Vemurafenib treatment. Created with BioRender.com

3.12 Plaque assay

In **Papers I** and **III**, virus tires were measured by plaque assay. Samples for measurement of virus tires came from cell cultures and blood and organs obtained from mice. Organs had to undergo homogenisation followed by repeated freeze-thawing cycles to release virus particles. Cells also went through freeze-thawing cycles for the same reason.

Briefly, samples underwent a series of ten-fold dilutions and were then added to HeLa cell monolayers. After the samples were added, plates were incubated for 1h followed by washing. The cells were then covered with a 1% low-melt agarose mix containing MEM and 10% FBS and were incubated for 3 days at 37°C . Afterwards agarose was removed, cells were fixed, and the virus was inactivated by methanol: acetic acid. The plates were stained with crystal violet and virus titres were analysed by counting the number of plaques which appeared on the HeLa cell monolayer. Virus titres were presented as PFU/ml or PFU/g tissue.

Plaque assays may vary between the laboratories, by the cell lines used, plate format, medium mixture, incubation time and fixative. Nevertheless, the final results will give information on virus titres.

3.13 Neutralisation assay

In **Papers I, II** neutralising antibodies were measured. This method is used to quantify highly specific neutralising antibodies.

In **Paper I,** serum samples obtained from animals, vaccinated with CVB3 vaccine, were analysed at Centre for Infectious Medicine, Karolinska Institutet, whereas serum samples from humans collected for analysing neutralising against CVB1-6 and against polio 1 and 3, as well as serum samples obtained from CVB1 vaccinated mice, were analysed at Tampere University, Finland. Serum samples obtained in **Paper II** were also analysed at Tampere University, Finland.

Samples analysed at Tampere University, with a reduction of plaque numbers ≥ 80% comparing to pure virus suspension was considered positive for neutralising antibodies. The detection limit was set on 1:4 dilution and considered as protective titres were 1:16 (CVB1-6 human samples and mice samples) and 1:8 (polio 1 and 3).

Samples analysed at Karolinska Institutet and showing a reduction of plaque numbers ≥ 80% compared to the virus control of 30 PFU were considered positive for neutralising antibodies to CVB3.

Briefly, serum samples were diluted by either fourfold dilution or ten-fold dilution and were incubated with the virus for 1h at 37°C and then incubated overnight in RT. Thereafter, the steps followed standard plaque assay protocol.

Neutralising protocols may vary between the laboratories, by using different virus PFUs, cell lines, plate formats, medium mixtures, incubation times and fixatives. Nevertheless, the final results will give information about protective neutralising antibody tires.

3.14 Histology

In **Papers I** and **II** samples were fixed in 4% PFA and then went through dehydration steps followed by paraffin embedding. Organs were sectioned into 5μm-thick sections. For **Paper II** pancreases were sectioned and analysed by insulitis scoring. To cover a large number of islets, the pancreas blocks were sectioned at two to three levels, with at least 20 sections in between each level (100μm). Before staining, the tissue sections underwent deparaffinisation. Sections from the **Paper I** were stained with Mayer's haematoxylin and Eosin Y using a standard immunohistochemical technique.

3.14.1 Immunohistochemistry and insulitis scoring

Pancreas sections were stained with antibodies to insulin and glucagon to enable visual inspection of beta cell loss during insulitis scoring. Sections were counter stained with haematoxylin by using a standard immunohistochemical technique.

Researchers assessing insulitis were blinded to the identity of pancreas sections. The following ranking method was used: Score 0: healthy islet morphology, no mononuclear cells surrounding or infiltrating the islet. Score 1: peri-insulitis, mononuclear cells surrounding the islets in their periphery. Score 2: insulitis, infiltration of mononuclear cells into the islets. Score 3: destroyed islet, infiltrated islet with no signs of insulin staining (destroyed islets).

3.15 FACS analysis

In **Paper I** and **Paper III** FACS analysis was utilised. In **Paper I** harvested splenocytes were used to assess presence of T cells and B cells in *TCR*α^{tm1Mom} knock-out mice and their wild-type counterpart. Cells were stained with anti-CD4, anti-TCRβ or anti-B220 antibodies. Samples were acquired by BD Accuri Flow Cytometer BD. In **Paper III** FACS was used to detect GFP signal from CVB3eGFP infected HeLa cells, dsRNA staining (primary staining with anti-dsRNA

and secondary staining with anti-IgGa/A488 antibody) from INS-1 and CAR and DAF staining from SC-islets (CAR: primary anti-CAR, secondary AF488 anti-mouse; DAF: PE-labelled anti-CD55/DAF). Cells were acquired by BD Accuri Flow Cytometer.

3.16 Statistical analysis

All statistical analysis were performed using GraphPad Prism.

Paper I:

Data were represented with individual values with mean ± standard deviation, p-values < 0.05 were considered significant, and n = number of individuals or animals analysed. Used tests: chi square test for categorical variables (such as gender), Mann-Whitney U test for differences between two groups, ANOVA with Kruskal-Wallis test for comparison of more than two groups, two-way ANOVA Šídák's multiple comparison for comparison between neutralising antibody titers in the mouse model and its wild type counterpart, as well as differences in body weight, and lastly unpaired t-test for tissue scores and polio neutralising antibodies.

Paper II:

Data were represented by mean ± standard deviation, p-value < 0.05 were considered significant, and n = number of analysed animals. An unpaired t-test was used to assess insulitis score, neutralising antibody titers and age of diabetes onset (in CVB1 vaccinated and infected mice). Two-way ANOVA with Šídák's multiple comparison test was used for percentage of islets with differing insulitis scores. One- way ANOVA with Turkey multiple analysis was used for age of diabetes onset (CVB1-6 vaccination). Gehan-Breslow-Wilcoxon test was used for diabetes survival curves including assessment of survival curves after two weeks post infection when the acceleration in disease onset is expected. Lastly, Kruskal-Wallis test with the Dunn test for multiple comparisons was used for age at diabetes onset (CVB1 vaccinated).

Paper III:

Data were represented with individual values and mean ± standard deviation, p-values < 0.05 were considered significant., n = number of animals or biological replicates. One-way ANOVA with Šídák's multiple comparison was used for comparing between different treatments, non-parametric Friedman test with

Dunn's multiple comparisons was used for geometric mean fluorescence, Mann-Whitney test was used for differences between two treated groups of animals.

3.17 Ethical considerations

3.17.1 Human samples

All healthy and CF donors who donated serum samples were informed and gave consent prior participation. Experiments were conducted according to the Declaration of Helsinki. Studies with human samples were approved by reginal ethical review board in Stockholm, Sweden and were conducted in accordance with national and institutional guidelines.

3.17.2 Stem-cell-derived islets (SC-islets)

SC-islets were generated by our collaborators from Uppsala University. SC-islets were generated from the human embryonic stem cell line H1 (WA01, WiCell), which is commercially available, meaning the ethical approval is no requirement.

3.17.3 Mouse experiments

All experiments which involved animal models were conducted according to the 3Rs principals (replacement, reduction, refinement). All investigators participating in the animal studies were highly trained, they followed recommendations and studies described in the ethical application to make sure that the animals' well-being was ensured. Researchers were in close contact with a veterinarian in case abnormalities occurred. Throughout the study, animals were monitored daily and had unlimited access to food and water. Each animal experiment was well planned and justified to minimise unnecessary usage of animals. All animal experiments were approved by Stockholm Southern Animal Ethics Board.

Studies including vaccines and antiviral treatments require systemic analysis. Therefore, animals are still unfortunately used for scientific research. I believe that thanks to the contribution of animals to scientific research, we scientists have been able to answer many questions and contribute to the development of new life-saving treatments for humans.

4 Results and Discussion

Paper I

Coxsackievirus B infections are common in Cystic Fibrosis and experimental evidence supports protection by vaccination.

In **Paper I** we followed up on our previous study (65), which revealed increased mortality and viral load in the lungs of $Cftr^{tmEUR}$ mice, which carry the CFTR mutation, known as F508del, the most common mutation in CF (153, 154). A delay in initial neutralising antibody production upon CVB3 infection was also observed. This was linked to a defective antibody response to T-cell dependent antigen (65). Therefore, we wanted to explore the reason for this delay and check whether vaccination against CVBs would provide protection against virus infection in a CF model. Moreover, we wanted to find out how prevalent CVB infections are among a cohort of individuals with CF. Neutralising antibodies against poliovirus were also checked, to understand whether poliovirus vaccination gives robust (lifelong) protection in the CF population. Findings on how well CF patients respond to a enterovirus (polio) vaccine could act as a proxy to our CVB vaccine.

Enterovirus infections, like other respiratory viruses such as influenza, are common in CF (137, 219, 220). In addition, enterovirus positivity was shown to have a significant correlation with positive bacterial culture in sputum and increased the chance of admission to hospital (221). Since there was no data on how prevalent CVB infections are in the CF population we wanted to explore that first. CVB1-6 seropositivity in a CF cohort and healthy controls was measured in serum samples, by a neutralising antibody assay. We found that CVB infections are prevalent in the CF population as well as in healthy controls and that they are not more prevalent in CF (Fig.1). Most of the tested individuals were found to be positive for at least one (on average 2) CVB serotypes (Fig.S1). The mean age of the CF patients was 22.5 ± 12 years whereas the control group was 27.0 ± 8 years. The fact that CVB is not more prevalent in CF than in healthy controls could be due to individuals with CF taking greater precautions against infection thereby minimising their exposure to viral infections in general. Otherwise, this observation could be due to a delayed antibody response, therefore meaning there is less seropositivity in this group. Nevertheless, we proved that CVBs circulate among the CF population.

To address whether antibody production in response to a CVB vaccine is T cell-dependent we used a mouse model that is lacking TCR$\alpha\beta$ T cells and their wild-type (wt) counterpart (Fig.S2). Mice were vaccinated with a CVB3 monovalent vaccine (based on field isolated virus) on two occasions, namely on day 0 and day 14 (Fig.2A) (166, 206). The mice were monitored until day 28 following the first vaccination and serum samples were collected on days -2, 4, 5, 14 and 28. Analysis of sera showed that neutralising antibodies were present after vaccination. Nevertheless, the antibody titres in the TCR$\alpha\beta$ KO mice were significantly lower, compared to wild-type mice (Fig.2B,C). This indicates that the antibody production is in fact partly T cell dependent. As the previous study demonstrated a delay in neutralising antibody production in CVB infected mice F508del mice, which was linked to a defective antibody response to T cell-dependent antigen (65), this observation highlighted that the vaccine response in F508del mice could be similarly impaired.

As a next step we wanted to check the efficacy of the vaccine in inducing the production of virus neutralising antibodies in F508del mice. Mice were vaccinated on two occasions, day 0 and day 14 with a monovalent CVB3 vaccine (Fig.2D). The same serum collection schedule as described above was implemented. After the first vaccination, two mice had to be removed due to weight loss which exceeded the 10% limit as stated in our ethical permit. It was reported that the survival rate to maturity in this colony is 90%, therefore 10% of deaths in the colony can occur (222). Thus, we do not believe that this incident was caused by the vaccine itself especially as our previous studies showed that CVB vaccines were well tolerated in other *in vivo* models, including non-human primates, and had an excellent safety profile (66, 67, 197, 199). Collected serum was used to measure neutralising antibodies which show similar titres between F508del and wild type animals on days 4 and 5 post vaccination. Day 14 serum samples revealed that neutralising antibodies titres were lower in the CF model at this time point. After the booster dose, the titres were however at similar level between the groups (Fig.2E, F). This was confirmed using another vaccine, which also targeted CVB3 but was produced using a different CVB3 strain (Fig. 2G,H). All together these studies suggested that the used vaccines were well tolerated and were efficacious at raising neutralising antibodies in F508del mice, although the booster dose helped to increase the initially lower, neutralising antibody titres in this group.

To ensure that the vaccines protected against virus infection in F508del mice, animals were vaccinated with the CVB1 or CVB3 vaccines on three occasions, days

0, 21, 35 and serum samples were taken on days 0, 21, 35 and 63 (Fig.3A). The three-dose vaccination schedule was introduced to maximise the production of neutralising antibodies, as seen in our previous studies (67, 199). Analysis showed that vaccination worked, and animals were able to produce neutralising antibodies. The second vaccination increased neutralising antibody titres, whereas the titres remained stable after the third vaccination (Fig.3B, D). Infection was performed on day 63 post initial vaccination and animals were monitored until day 67 when the experiment was terminated (Fig.3A). Animals which were vaccinated had a stable weight after infection whereas animals which were buffer treated or untreated showed significant weight loss (Fig.4A, B). Moreover, vaccinated animals were protected from virus infection, with lower virus titres in the blood and in organs on day 3 and 4 post infection (Fig.4C, D, E, F). Vaccination also protected organs from virus induced damaged as seen by histological analysis (Figure 5). Besides protection of the liver and pancreas, protection was also visible in the lungs. Fewer hemosiderin deposits (signs of previous or ongoing wound healing) were present in vaccinated mice compared to buffer vaccinated or untreated (Fig.5, Fig.S5, Fig.6).

The vaccination of F508del mice with CVB1 and CVB3 monovalent vaccines protected animals from acute infection, viral spread to organs and virus-induced damage when compared to mice which were mock-vaccinated with buffer or left untreated. Upper respiratory tract infections caused by CVBs are rather mild and not much data exists showing their involvement in pulmonary disease (223, 224). Nevertheless, seeing the damage in the lungs of F508del mice, caused by CVB1, raises the question of which pathological mechanisms are behind it, especially in the context of CF lungs and virus infection. The prospective of possible vaccination against CVBs is interesting, especially when protection against infection and organ damage were clearly visible. Booster vaccination could be of relevance to ensure a higher level of robust (with aim of life-long) protection.

Since the current CVB vaccines used in this pre-clinical set-up were made in a similar manner to the inactivated polio vaccine (IPV) and polio vaccines are generally administered on three occasions in childhood, we wanted to check neutralising antibody titres against polio in CF patients. This could indicate the possible outcome of patients who would be vaccinated with a CVB vaccine. Poliovirus neutralising antibodies against polio 1 and polio 2 were measured in the same cohort that was used to measure CVB neutralising antibody titres. The analysis showed that both the CF cohort and healthy controls had neutralising

antibodies against poliovirus (Fig.6). The CF group had slightly lower titres, and some individuals had titres which were below those that give protective immunity (seropositivity at a ≥ 1:8 dilution). Nevertheless, there were no statistical differences between the CF and healthy groups leading to the conclusion that CF patients have a fairly good response to a formalin inactivated whole virus vaccine. This confirmed a previous finding which showed that poliovirus neutralising antibodies were present upon oral poliovirus vaccination (151). Collectively, we could hypothesise that a CVB vaccine given at early age would work similarly to the poliovirus vaccine, giving robust immunisation. Nonetheless, the measurement of neutralising antibodies should be implemented to make sure that the more susceptible population would be protected and would help to establish which vaccination schedule would be the most suitable.

All together **Paper I** showed that CVBs infections are common in a CF population as well as in healthy controls. CVB vaccination results in neutralising antibody production and provides immunity against CVBs. This was proven in our CF mouse model, which was protected from viraemia after CVB1 and CVB3 infection, viral spread to the organs, and virus-induced organ damage. Moreover, we showed that there are no significant differences in neutralising antibodies against poliovirus (induced by an enteroviral vaccine) between a CF group and healthy controls. This led us to conclude that a CVB vaccine (also an enteroviral vaccine) would give protection against CVB infections and could be beneficial for especially susceptible populations such as patients with CF.

Paper II

Coxsackievirus B Vaccines Prevent Infection-Accelerated Diabetes in NOD Mice and Have No Disease-Inducing Effect.

CVBs, that belong to the *Enterovirus* genus, are associated with T1D development, and may potentially be involved in disease onset (44, 45, 97-99, 225). Prevention of infection provided by vaccination could be beneficial for groups at risk of this disease. Our previous studies demonstrated the efficacy of CVB vaccines, both monovalent and polyvalent, in various *in vivo* models, including mice and non-human primates (66, 67, 199). Nevertheless, vaccine safety had to be further measured, especially in the context of T1D before entering clinical trials (208). We wanted to ensure that CVB vaccines do not cause any adverse effects such as

accelerating autoimmunity in diabetes prone hosts. Moreover, we also wanted to check whether CVB vaccines can prevent a different form of CVB-induced diabetes to that published in our previous work (66, 199).

There are associations between vaccination and autoimmune manifestations. They are rare, but they need to be addressed (226, 227). Therefore, it is important to test whether such adverse events can occur before a vaccine enters clinical trials. We already showed that a monovalent CVB vaccine was safe (67), but whether a polyvalent CVB vaccine itself can increase pancreatic islet inflammation, known as insulitis, in pre-diabetic NOD mice still needed to be addressed. Mice were vaccinated (CVB1-6 vaccine and buffer vaccinated) on three occasions when they were on average 5.5 weeks of age. The vaccination schedule was as follows: days 0, 14 and 28 or days 0, 21, 35. Mice were monitored until 12 weeks of age, when the experiment was terminated (Fig.1). A neutralising antibody assay using serum samples showed the presence of neutralising antibodies of each CVB type, proving that the vaccine induced antibody production. Serum samples from mock vaccinated animals did not have any neutralising antibodies (data not shown) (Fig.1A). This confirms our previous findings in our studies with a polyvalent vaccine (66). At the end of the experiment pancreases were harvested which allowed us to perform insulitis scoring (Fig.1B, C, D). Analysis showed that there were no differences in the mean insulitis score between mock vaccinated and CVB1-6 vaccinated mice (Fig.1C). The breakdown of the insulitis scores within the groups helped us to assess the level and differences in the severity of insulitis scores between the groups. Both mock vaccinated and CVB1-6 vaccinated mice had signs of insulitis but there were no significant differences between the groups (Fig.1D). These results proved that the CVB1-6 vaccine was safe in pre-diabetic NOD mice and there was no aggravation of insulitis in this model. Moreover, as previously seen (66) the vaccine did not alter weights and blood glucose levels in the mice (Fig.S1).

As a next step we tested whether the CVB1-6 vaccine accelerates diabetes onset in NOD mice. Mice were vaccinated (3 groups were created, untreated, buffer vaccinated, and CVB1-6 vaccinated) at a mean age of 5.7 weeks on two or three occasions (days 0, 21 and 35) and were monitored up to 30 weeks of age or until the development of diabetes onset (Fig.2). On day 42 post vaccination CVB neutralising antibodies were measured, and high titres were seen confirming vaccine immunogenicity (Fig.2A). The vaccine did not accelerate diabetes onset, and there were no significant differences in the mean age of diabetes onset

between the groups (Fig.2B, C). Altogether, we showed that CVB vaccination did not accelerate autoimmune disease in the NOD mouse.

As mentioned before, having proof that vaccines are both safe in the context of accelerating autoimmune diseases and that they grant high immunogenicity would help to gain more trust in the general population. Moreover, evidence that the vaccine does not accelerate disease onset suggests that a virus which is inactivated does not have diabetogenic effects (67), making it a good vaccination candidate. A similar vaccine (monovalent vaccine) showed very strong immunisation efficacy and protection again infection (67, 199).

To check whether a CVB vaccine can protect against the acceleration of diabetes onset we further utilised the NOD mouse model. First it was important to check whether infection with CVB1 can accelerate diabetes onset in our NOD mouse colony (Fig.3). Mice were either left untreated or infected with CVB1 between 10.5-15.5 weeks of age and monitored until they were 30 weeks of age or until diabetes onset. Mice infected with the virus had a significant acceleration in diabetes onset compared to controls, which was further confirmed by a significantly lower mean age at disease onset in the infected (Fig.3A, B). We then explored vaccination protection against virus-accelerated diabetes onset. Mice were vaccinated on three occasions (mice were randomly assigned to 3 groups: untreated, mock vaccinated + CVB1 infected and CVB1 vaccinated + CVB1 infected). As previously described, mice were monitored up to 30 weeks of age or until the development of diabetes (Fig.3D). Neutralising antibodies were measured to confirm vaccine immunogenicity (Fig.3C). Mice which were mock vaccinated and infected had a significant acceleration in the onset of diabetes compared to untreated and CVB1 vaccinated mice which was confirmed when looking at the age of diabetes onset (Fig.3D, E). Moreover, the exocrine tissue in the pancreas of vaccinated mice was protected from the virus induced damage that was seen in the mock vaccinated group (Fig.3F). Overall, this experiment proved that CVB1 vaccinated NOD mice were protected from CVB1-accelerated disease.

There are two possible scenarios which implicate CVBs in the development of T1D. The first suggests that enterovirus infection contributes to the initiation of pancreatic islet autoimmunity (98, 166-168). The other scenario proposes that infection can accelerate diabetes onset in individuals who are already autoantibody positive (169). These two possible scenarios imply virus (CVB) involvement in T1D pathogenesis. Even though the evidence is strong, we do not

have a definitive proof. Therefore, it is important to further explore this relationship. Creating a preventative approach, such as the use of a vaccine with high efficacy would be beneficial and perhaps provide proof of virus involvement in T1D (228).

Taken together **Paper II** shows that the polyvalent vaccine did not aggravate insulitis and did not accelerate autoimmune diabetes in NOD mice. Moreover, a CVB1 vaccine provided protection against virus acceleration of diabetes onset in NOD mice. Therefore, this suggests that such vaccination would be beneficial for susceptible populations and possible vaccination early in life could reduce virus-related T1D.

Paper III

Vemurafenib prevents coxsackievirus B replication in intestinal epithelial cells and pancreatic beta cells.

Vaccines are a great platform for disease prevention. Nevertheless, vaccination only protects against the virus types included in the vaccine. Therefore, creating a treatment approach such as antivirals could perhaps cover a bigger spectrum of viruses. One drug with potential anti-CVB/enterovirus (EV) activity is Vemurafenib (213). This is a BRAF inhibitor used for cancer treatment (214, 215, 229, 230). A recently published study documented its antiviral properties against CVB viruses (213). Others have also reported the antiviral properties of the drug against enteroviruses and influenza virus (216-218). Since there is strong evidence of virus involvement in T1D development (44, 97, 225, 228), it was important to test whether such an antiviral treatment would provide protection in beta cells and cells that mimic the primary site of the infection. An analogue of Vemurafenib was also included.

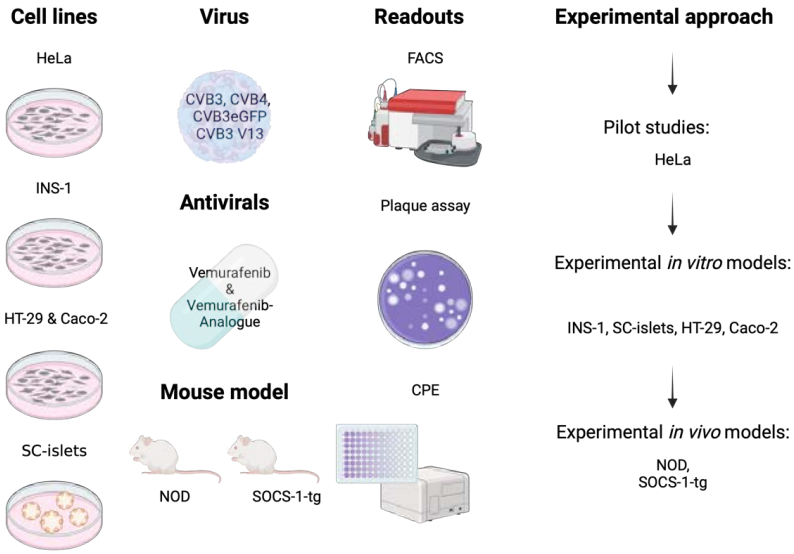

Figure 3. Antiviral experimental strategy. Created with BioRender.com

We started by checking if the antiviral drug had any cytopathic effects on treated cells. HeLa cells were treated with increasing concentrations of Vemurafenib (2μM, 5,μM and 10μM). None of the concentrations had any significant impact on viability of the treated cells (Fig.S1A). However, we noticed a small decrease in the live cell count in cells treated with the higher concentration of Vemurafenib (Fig.S1B). The Vemurafenib analogue did not show any adverse effect on treated cells (Fig.S1C, D). Treatment with both Vemurafenib and its analogue reduced both CVB3 infection (Fig.1A, D) and virial replication after 4h and 20h of treatment (Fig.1B, C, E, F), indicating promising antiviral activity in this study.

To address the potential of the antiviral properties at the primary site of infection, we deployed human intestinal epithelial cell lines (Fig.2A). In both cell lines (HT-29 and Caco-2) treated with Vemurafenib at either 5μM or 10μM concentrations, we saw a reduction in CVB3 infection at 48h post infection (Fig. 2B,C). The HT-29 cell line has a mutation which is targeted by Vemurafenib; therefore, we also saw a clear reduction in cell proliferation upon treatment with Vemurafenib. Nevertheless, we confirmed in the other cell line (Caco-2), which lacks that mutation, that treatment with Vemurafenib protects cells from CVB3 induced cytopathic effects.

Next, we tested Vemurafenib treatment in a beta cell line, INS-1. Vemurafenib did not show any cytopathic effects on the cell line after 6h and 20h of exposure (Fig.S2). We noticed in CVB3 infected cells a dose dependent reduction in dsRNA

positive cells after 6h and 20h of treatment (Fig.3A, B). We wanted to confirm this finding with stem cell derived islets (SC-islets), which mimic human islets (192). We started by measuring the expression of the receptors necessary for the initiation of CVB infection, CAR, and DAF. We found that SC-islets were positive for DAF expression alone or had co-expression of CAR and DAF (Fig.3D, E). We did not find CAR expressed alone. Unfortunately, we were unable to distinguish receptor expression between the different cell types within SC-islets. As SC-islets expressed the receptors, we were able to infect the SC-islets (Fig.3E, F). A dose response infection study helped us to identify the best virus concentration which would enable us to successfully infect the SC-islets with the least amount of virus that gives rise to infection (Fig.3F), thereby allowing us to study the antiviral properties of our drugs. We were able to test our antiviral treatment in three batches of SC-islets, using Vemurafenib or its analogue (Fig.3G). Two batches (treated either with Vemurafenib or its analogue) seemed to be protected from CVB3 infection. Another batch which was treated with Vemurafenib had a higher level of infection compared to the control. There are a few aspects which could impact an antiviral treatment. Firstly, SC-islets are 3D structures so drug penetration might not be as efficient as in monolayer cell lines in which we showed the drugs had high antiviral properties (Fig.1, 2, 3A and B). Secondly, even though we picked the lowest CVB3 concentration that we knew would productively infect (4x10^3 PFU CVB3 per islet), it could still be too much virus to detect any antiviral properties, due to viral overload. Thirdly, we have noticed on a few occasions that depending on medium used for diluting the drug, precipitation was sometimes seen (data not shown, Butrym *et al* lab observations). We hypothesise that there might be some medium components which interact with the drug, leading to precipitation which could impact the antiviral properties of the drug.

Due to a lack of cytotoxicity after antiviral treatment with Vemurafenib in the *in vitro* experiments we decided to utilise two mouse models (NOD and SOCS-1-tg) to check the safety of the drug and antiviral prevention *in vivo*. This study only focused on acute infection. Mice, 10 weeks of age, were injected daily with 25mg/kg Vemurafenib by intraperitoneal (i.p.) injection for 5 days. Treatment started the day before infection (Fig.4A). Animals were monitored for weight, blood glucose and overall health status daily. Blood was taken on days 3 and 4 post infection. The study was terminated 4 days post infection and organs were harvested. The treatment did not alter the weights and blood glucose values of the animals throughout the whole study, and no other adverse effects (standard

health monitoring) were seen (Fig.S3). Therefore, we believe that the Vemurafenib treatment was safe for the animals. Assessment of blood samples showed that there was possibly a weak antiviral effect of Vemurafenib *in vivo*. Nevertheless, they were too few animals in the study to draw strong conclusions, and the results also varied between the animals (Fig. 3B, C). Similar results were seen after organ analysis (Fig.3D and Fig.S4). The variation between the mice might come from the fact that the drug precipitated before the injection, which could impact the antiviral properties. Precipitation most likely occurred from too high a concentration of the drug which decreased its solubility. We encountered several issues with Vemurafenib solubility. Depending on the vendor, the solubility of the drug can differ quite significantly, impacting the treatment strategy. As a next step we could perhaps reduce the used concentration but increase the number of occasions in which the drug is administrated.

Currently there are no antivirals against enterovirus infection available in the clinics. Therefore, the study and exploration of new antiviral agents is important. There have been several drugs that went into clinical trials but due to insufficient efficacy or due to cytotoxicity, the trials were discontinued (3). Recent papers showed that pleconaril and ribavirin combination treatment preserved insulin production in children and young adolescents with newly develop T1D (212). Although it has not yet been demonstrated that these children and young adolescents carried a persistent enterovirus infection, this finding shows the relevance of further antiviral research and strengthens the associations between virus infection and T1D development. Altogether, **Paper III** shows that Vemurafenib has potential antiviral properties in T1D relevant models and more importantly it shows strong protection against CVB infection in a model for the primary site of the infection.

5 Conclusions

Paper I showed that CVB infections are prevalent in both CF and healthy individuals. Monovalent vaccines against CVB1 and CVB3 showed efficacy in raising neutralising antibodies and giving protection against acute infection, and protected organs from infection and organ damage. A poliovirus vaccine gave robust immunity in most individuals with CF, as based on the presence of neutralising antibodies. Altogether, these finding suggest that a CVB vaccine would be beneficial for susceptible groups such as cystic fibrosis patients.

Paper II demonstrated that CVB1-6 vaccination did not aggravate insulitis in pre-diabetic, diabetes prone NOD mice. Moreover, it did not accelerate diabetes onset in the same model. Furthermore, vaccination with CVB1 prevented from virus-induced acceleration of diabetes onset in NOD mice.

Paper III showed that the Vemurafenib treatment was not cytotoxic in the tested cells lines, and it showed antiviral properties in intestinal epithelial cell lines, which are at the primary site of CVB infection. Moreover, the drug showed antiviral properties in beta cells and SC-islets. We also recorded potential antiviral properties in the used animal model.

6 Points of perspective

This thesis is focused on evaluating preventive treatments such as vaccination and antiviral drugs in the context of different diseases. We showed that CVB are common in CF, representing a possible burden for individuals with CF. Future implementation of CVB vaccination could improve infection-control, hence reduce virus infections in CF patients. Similarly, possible benefits of such vaccination could contribute to a reduction in diabetes onset among individuals predisposed to T1D. Moreover, this thesis explored antiviral treatment, which in the case of infection could provide a complementary therapeutic through blocking further virus replication, hence supporting the immune response in the clearance of the infection. Nevertheless, there are still some questions to be answered and explored.

Paper I

This study has a few limitations that need to be taken into consideration:

It was only carried out in Sweden, so we do not know if we would have similar outcomes across the World. Therefore, performing a multicentre study could reveal if neutralising antibody tires against CVB (CVB seropositivity) are similar in CF populations in different countries. Moreover, surveillance systems of enterovirus infections could be of help, through showing the prevalence of CVB in the general population, and they may possibly include CF populations as well.

We did not acquire information about childhood poliovirus vaccination. Nevertheless, during the last few decades, polio vaccines were included in the Swedish vaccination programme which led us believe that most of the population were vaccinated. Unfortunately, we do not know whether the booster dose was administrated.

Our animal study was focused only on one CFTR mutation which restricts us from assuming that this vaccine would work in a similar way for other CFTR mutations. Therefore, vaccine efficacy testing in other mouse models carrying different CF mutations would be beneficial.

We only tested monovalent vaccines, but based on our previous study with polyvalent vaccines (66) we could assume that such vaccine would be as effective as the monovalent vaccines. Yet, future studies could explicitly evaluate this.

To confirm presence of CVB infections in CF patients, sputum samples could be collected for assessment of CVB in upper respiratory tract or bronchoalveolar lavage which would help us to measuring the prevalence in the lower respiratory tract.

Paper II:

A similar vaccine to one tested in our group (66), which contains 5 types of CVB is currently in stage I clinical trials, where the safety and immunogenicity is being tested in humans (208). Exploration of vaccination in other animal models for autoimmune diseases would be beneficial.

Paper III:

This study has a few points that should be furtherly explored:

The study will requires more follow up *in vitro* experiments where beta cells, e.g. EndoC cells, SC-islet or even human islets would be utilised. This would allow us establishing whether this new potential anti-enterovirus antiviral treatment would protect cells more relevant to T1D from CVB infection. It would be relevant to assess if the antiviral treatment could impact insulin production in these cells. The cytotoxicity in these cell lines could also be evaluated. Identification of the receptors required for infection in individual cell types in SC-islets or primary human islets would give as an overview to which cell are more susceptible to viral infection.

Since our animal studies showed mixed results after antiviral treatment, the treatment could be altered to twice daily instead of once daily. Oral admiration of the antiviral could also be explored.

Vemurafenib treatment can cause side effects. One of them which happens rarely, is pancreatitis. Therefore, this treatment should be studied in experiments where diabetes onset is measured for a longer time. This would help us assessing if such a treatment can accelerate diabetes onset.

Since the antiviral treatment is most likely taken after or during an infection, it is important to establish if indeed the antiviral treatment could reduce or clear a virus infection in the *in vivo* scenario.

Another compound (PLX7904) suggested by our collaborators displays a similar mode of action and might in addition have less side effects. This possibility could be further evaluated.

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