Tampere University

Iiris Mustonen

INFECTION MECHANISM OF COX-SACKIEVIRUS B

Life cycle and strategies for immune evasion

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ABSTRACT

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Coxsackieviruses B (CVBs) are small yet highly contagious viruses that cause several acute and chronic diseases. Structurally CVBs are small, nonenveloped, positive-sense single-stranded RNA viruses. The virus spreads through the fecal-oral route, and it can cause different diseases from mild cold-like symptoms to severe diseases. CVB infection is one of the major causes of infection-induced myocarditis. It is also strongly linked with type 1 diabetes.

This study aims to overview the virus's infection mechanism and its counteractions in the cell and immune system. The infection mechanisms include the invasion of the virus, replication, assembly, and release. The virus enters the cell through coxsackievirus- and adenovirus receptors and utilizes the host's machinery for replication and translation. The virus effectively interferes with the host cell by disturbing its metabolisms and preventing transcription.

The virus is recognized by innate immunity through pattern recognition receptors. Detection drives the proper response of adaptive immunity, where B cells have the greatest impact. Nevertheless, CVB has clever ways to evade immunity. Successful evasion can lead to persistent infection as well as to effective spreading of the virus to secondary tissues. Chronic diseases are a result of persistent infections and damaging immune responses. Myocarditis and type 1 diabetes are examples of chronic diseases associated with CVB infection and pancreatic β-cells are demonstrated to be especially susceptible to coxsackievirus B infection. Understanding the infection mechanism of the virus is crucial for designing effective ways to prevent or treat the outcomes of the infection.

Key words: Coxsackievirus B, infection mechanism, immune evasion, type 1 diabetes

TIIVISTELMÄ

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Coxsackie B -virukset ovat pieniä mutta helposti tarttuvia viruksia, jotka aiheuttavat sekä akuutteja että kroonisia sairauksia. Rakenteellisesti Coxsackie B -virukset ovat vaipattomia RNA-viruksia, joilla on positiivinen yksijuosteinen RNA-genomi. Virus leviää ulosteen ja suun kautta, ja sen aiheuttamien sairauksien oireet vaihtelevat lievistä flunssan kaltaisista oireista vakaviin sairauksiin. Coxsackievirus B on yksi infektion seurauksena kehittyvän sydänlihastulehduksen merkittävimmistä aiheuttajista. Virustartunta on yhdistetty myös tyypin 1 diabeteksen puhkeamiseen.

Tämän tutkielman tavoitteena on antaa yleiskuva viruksen infektiomekanismista ja menetelmistä vaikuttaa isäntäsolun ja immuunipuolustuksen toimintaan. Infektiomekanismi sisältää viruksen tunkeutumisen isäntäsoluun, sen genomin replikaation ja translaation, uusien virusten kokoamisen sekä vapauttamisen solusta joko solun hajottavaa, tai ei-hajottavaa reittiä. Virus pääsee soluun coxsackie- ja adenovirusreseptorin kautta. Virus hyödyntää isäntäsolun komponentteja replikaatioon ja translaatioon. Virus häiritsee isäntäsolua esimerkiksi estämällä sen omaa transkriptiota ja sotkemalla sen metaboliareittejä.

Immuunijärjestelmä tunnistaa viruksen synnynnäiseen immuniteettiin kuuluvien toistokuvioita tunnistavien reseptorien kautta. Viruksen tunnistaminen aiheuttaa adaptiivisen immuunivasteen aktivoimisen, jossa B-soluilla on suurin merkitys virusta vastaan puolustautuessa. Viruksella on kuitenkin monia nokkelia tapoja välttää tunnistetuksi tuleminen. Mikäli virusta vastaan ei synny tarvittavaa immuunivastetta, voi virusinfektio pitkittyä ja levitä sekundäärisiin elimiin. Krooniset sairaudet ovat seurausta pitkittyneistä infektioista ja vahingollisesta immuunivasteesta. Sydänlihastulehdus ja tyypin 1 diabetes ovat esimerkkejä kroonisista sairauksista, joiden kehittymiseen on liitetty Coxsackievirus B -infektio. Varsinkin haiman β-solujen on todistettu olevan erityisen alttiita virusinfektiolle. Infektiomekanismin kokonaisvaltainen ymmärtäminen on välttämätöntä, jotta virusinfektion ehkäisemiseksi ja lieventämiseksi voidaan kehittää hoitomenetelmiä.

Avainsanat: Coxsackievirus B, infektiomekanismi, immuunipuolustuksen välttäminen, tyypin 1 diabetes

PREFACE

This document is a Bachelor's thesis done in the Faculty of Medicine and Health Technology of Tampere University

I want to thank my instructor, Saana Soppela, for her wise comments and guidance. I also want to thank the Protein Dynamics group at Tampere University for introducing me to the exciting world of viruses.

Tampere, 27.4.2022

Iiris Mustonen

TABLE OF CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

ADE antibody-dependent enhancement

APC antigen -presenting cell

CAR coxsackievirus-adenovirus receptor

CVA coxsackievirus A

CVB coxsackievirus B

DAF complement decay-accelerating factor

eIF4G eucaryotic translation initiation factor 4 gamma 1

IFN interferon

Ig immunoglobulin

IRES internal ribosomal entry site

IRF interferon regulatory factor

MAVS mitochondrial antiviral signaling protein

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

PRR pattern recognition protein

RIG-I retinoic acid-inducible gene I

RLR RIG-I like receptors

T1D type 1 diabetes

TLR toll like receptor

1. INTRODUCTION

Coxsackieviruses belong to the enterovirus genus of the *Picornaviridae* family. Coxsackieviruses consist of coxsackieviruses A (CVA) and B (CVB). CVB has six serotypes: coxsackievirus B1-B6. Different serotypes are associated with different diseases. For example, all of them are associated with myocarditis whereas CVB4 is linked with pancreatitis and T1D (Lasrado et al. 2020).

Structurally CVBs are small, nonenveloped, positive-sense single-stranded RNA (ssRNA) viruses. The CVB genome is approximately 7.5kb and consists of structural and non-structural proteins. Together VP1-VP4 will form a protomer, which will form an icosahedral capsid with a pseudo T=3 symmetry. VP1-VP3 constructs the outer capsid, whereas VP4 is an internal component. The rest of the genome encodes non-structural proteins essential for viral replication and protein synthesis as well as interference with the host cell. (Garmaroudi et al. 2015)

CVB spreading occurs through the fecal-oral route, and it can cause different diseases from mild cold-like symptoms to severe illnesses. CVB infection is one of the major causes of infection-induced myocarditis and aseptic meningitis. It is also strongly linked with type 1 diabetes (T1D) (Marjomäki and Flodström-Tullberg 2022; Oberste and Pallansch 2008; Sesti-Costa et al. 2017).

The infection tends to be more severe for younger individuals, and even fatal for neonates. Primary infection occurs in the respiratory or gastrointestinal tract, but the spreading virus can cause secondary infections in other tissues (Baggen et al. 2018). Even though most of the infections are asymptomatic or comparable with common cold symptoms, persistent chronic infections in secondary organs are more severe. Spread infection in the heart, brain, pancreas, and liver, for instance, can lead to myocarditis, meningitis, pancreatitis, or hepatitis (Marjomäki and Flodström-Tullberg 2022). CVB outbreaks typically during warmer months like in the summer or early fall. CVB2, CVB4, and CVB5 are reported in cyclic outbreak peaks, whereas CVB3 and CVB1 have a more constant prevalence. CVB6, on the other hand, is rarely reported (Oberste and Pallansch 2008).

CVBs are small yet highly contagious viruses that cause several acute and chronic diseases. Despite their size, they have clever ways to interfere with the host and evade the immune system. Understanding the infection mechanism is crucial for designing effective ways to prevent or treat the outcomes of the infection. This thesis will review the infection mechanism of coxsackievirus B: its life cycle and its comprehensive influence on the host cell and immune system.

2. HOW CVBS CAUSE AN INFECTION

Coxsackieviruses are primarily human pathogens. They are highly contagious and are generally spread through respiratory secretions and fecal-oral routes (Oberste and Pallansch 2008). Many CVB infections occur during the first years of life (Marjomäki and Flodström-Tullberg 2022). They are lytic viruses, which means that cell death enables the viral material to spread to nearby cells and to the bloodstream. Yet, nonlytic spreading has also been observed. (Netanyah et al. 2020) The infection mechanisms include the invasion of the virus, its replication, assembly, and finally release. The life cycle of enteroviruses is extensively illustrated in Figure 1. CVBs also have multiple ways to interfere with the host cell improving its survival and replication.

Because coxsackieviruses are spread through the fecal-oral route, the primary infection site localizes in the gastrointestinal tract. The secondary target tissue is serotype dependent, but overall tissue tropism for coxsackievirus B includes the heart, pancreas, liver, brain, pharynx, and gastrointestinal tract itself. The heart and pancreas are the most remarkable for chronic diseases. (Marjomäki and Flodström-Tullberg 2022)

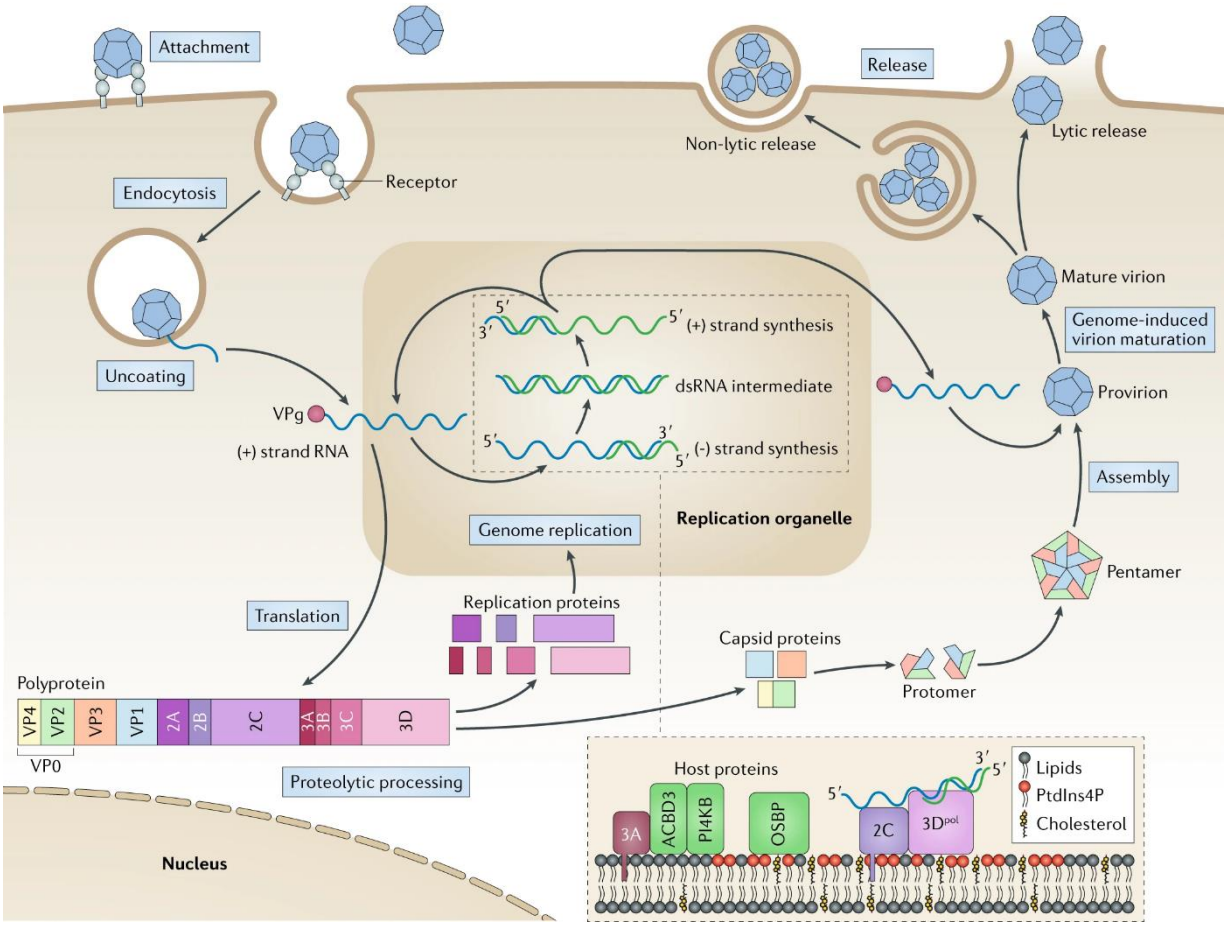

Figure 1. Overview of enteroviruses life cycle. Adapted from (Baggen et al. 2018)

2.1 Entry and uncoating

The infection begins with the invasion of the virus into the cell. The virus ends up in the primary site through receptor-mediated endocytosis. The entry to the cell occurs through a specific coxsackievirus and adenovirus receptor (CAR). CAR mRNA is expressed in tissues linked with CVB symptoms such as the human heart, pancreas, and brain. The receptor helps in the uncoating of the virus capsid and the release of the viral genome. All the CVB serotypes seem to bind CAR. (Carson 2001)

Localization of CAR receptor has been studied since it has been found in tissues linked with CVB infection. In epithelial cells, CAR functions a cell adhesion molecule located in tight junctions. However, it has several other functions as well including cell signaling (Carson 2001). The CAR expression has been thought to be induced by an infection since the tissues normally lacking CAR are infected with CVBs (Baggen et al. 2018).

CAR contributes to the uncoating of the virus capsid. Xu et al. showed that binding of the virus to CAR induces several conformational changes through interactions with the lipid “pocket factor” in the VP1. Binding will lead to the formation of an uncoating intermediate A particle and eventually release the viral RNA. Prominently, the uncoating could happen at neutral pH (Xu et al. 2021). Overall, enteroviral uncoating is suggested to occur with the help of both, the receptor and low endosomal pH (Baggen et al. 2018; Wells and Coyne 2019).

The expression of CAR reduces with aging which could explain the number of infections in newborns and young individuals, and correlate with the severity of the infection. The expression of the receptor depends on the tissue. For example, Carson et. al reported that CAR expression in the hearts of adult mice did not reduce with time, as it did in skeletal muscle. The linkage between CAR expression and vulnerability of the infection, however, must depend on multiple factors. For instance, pancreatic β-cells do not express CAR, but they are damaged in CVB infection. Also, lymphocytes can be infected, without expressing CAR. However, only the expression of mRNA cannot prove the presence of CAR protein. Therefore, only the presence of CAR cannot explain how the virus infects. (Carson 2001)

However, a coreceptor called decay-accelerating factor (DAF/CD55) is often needed for successful binding. As the primary infection site for CVBs is in the gut or respiratory epithelium, CAR receptors are hidden from the virus in the tight junctions. DAF, in contrast, is an abundant element on the cell membrane. DAF assists in exposing the CAR receptor to the virus. Therefore, interaction with DAF is essential for CAR-virus interaction. At least CVB1, CVB3, and CVB5 are shown to bind to DAF. (Baggen et al. 2018)

Coyne and Bergelson have extensively studied the coxsackievirus entry and uncoating. They showed that DAF, together with actin cytoskeleton remodeling by Abi and Fyn kinases carry the

virus to a tight junction and enable interaction with CAR. DAF begins to cluster after interaction with the virus, which will activate a nonreceptor tyrosine kinase Abi inside the cell. Abi signaling through Rac remodels the actin cytoskeleton and moves the DAF-CVB complex to CAR. Virus entry occurs through Fyn mediated caveolin phosphorylation. Therefore, Fyn kinase is essential for inducing the final passage into the cell. In conclusion, they propose that DAF-induced signaling is essential for cell entry at least in polarized epithelial cells. (Coyne and Bergelson 2006)

The specific mechanism of how the genome is released into the cytosol remains unsolved. Coyne et al. suggest that the cell entry and genome release occur separately on different sides of the cell. They also thought that the uncoating would be complete only when in the endoplasmic reticulum. Baggen et al. suggest that enteroviral genome release would happen through the formation of pore on the membrane od the endosomal vesicle. Other references mainly mention the genome release into cytoplasm or ER without discussing the specific mechanism (Baggen et al. 2018; Garmaroudi et al. 2015; Laitinen et al. 2016; Wells and Coyne 2019). Nonetheless, the CVB genome will end up in the cytosol for replication.

2.2 Translation and replication

Cytosolic CVB genome will utilize host ribosomes for its translation. Translation will provide the structural proteins for virion assembly and nonstructural proteins for replication and interference with the host. The internal ribosome entry site (IRES) mediates the translation together with host proteins. Examples of those proteins are illustrated in Figure 1. (Baggen et al. 2018). The polypeptide consists of three regions, P1-P3 where P1 consists of structural proteins, and P2-P3 nonstructural proteins. Laitinen et al. suggest that the polyprotein is not fully translated at once. Cysteine proteases would cleave the polyprotein before its completely translated. They implicate that $2A^{pro}$ would cut the P1 region from the rest after the protease located in the P2 region is translated. $3C^{pro}$, on the other hand, eventually cleaves almost all the structural and non-structural proteins from the polypeptide regions to functional proteins. The structure of the genome is illustrated in Figure 2. The proteases $2A^{pro}$ and $3C^{pro}$ are overall responsible for cleaving the polyprotein, but they have a major impact on the host cell and immunity as well, as discussed later.

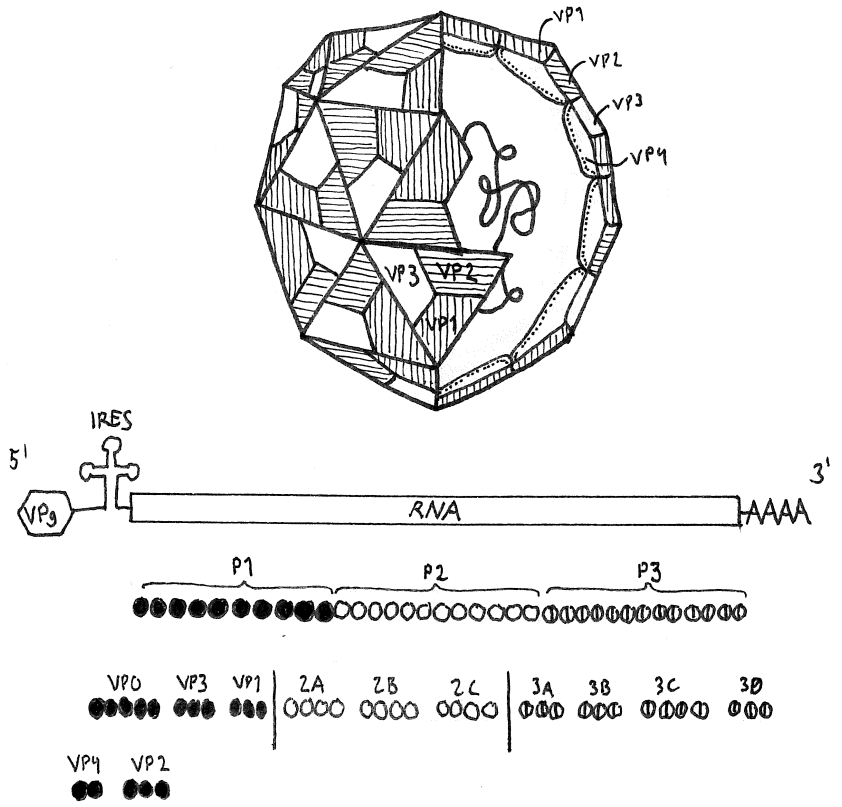

Figure 2. Structure of the viral capsid, RNA, and polyprotein. The capsid assemblies from VP1-VP4 proteins, where the VP4 is an internal component. The RNA has the small peptide VPg and IRES (internal ribosomal entry site) on its 5' end, and poly-A tail on its 3' end. The polyprotein constructs form P1-P3 regions, where P1 can be cleaved to structural proteins and P2-P3 to non-structural proteins. VP0 is further autocatalyzed to VP4 and VP2.

The replication, on the other hand, occurs in replication organelles, originating from host membranes during the infection. The replication mechanisms are thought to be conserved within enteroviruses. The nonstructural proteins are essential for replication. Viral proteins 2AB and 3A contribute to the formation of replication organelle. Viral proteins $3CD^{pro}$, 3AB, and 2C together with host RNA-binding proteins mediate the replication. Replication begins from the primer, 5' linked VPg. A small viral peptide which is cleaved from 3B. Transcript of – sense ssRNA will act as a template for further polyprotein synthesis as well as for transcribing more + sense RNA. (Baggen et al. 2018; Garmaroudi et al. 2015)

2.3 Assembly and release

The structural proteins VP0, VP1, and VP3 will assemble into protomers and further pentamers to construct the capsid. Virion matures when replicated + sense ssRNA is inserted, and VP0 is pro-

cessed to VP4 and VP2 by autocatalysis. (Baggen et al. 2018; Wells and Coyne 2019). Nevertheless, the specific mechanism behind virion assembly is poorly known. Baggen et al. report that the assembly could occur at the same time as replication, through interaction between 2C of the replication complex and VP3. One explanation is that no packaging signals have been detected. But it should be noted that this information is derived from articles covering broadly enteroviruses rather than specifically CVBs.

The release of the viral progeny has been thought to be highly lytic. Meaning that cell death will enable the release of mature virions to nearby cells and circulation. Lysis would occur through apoptosis or necrosis (Garmaroudi et al. 2015). Nevertheless, a nonlytic way has also been brought up regularly. There is evidence that enteroviruses can also spread in a non-lytic manner. Netanyah et al. proved that Echovirus 16 infected cells do release endosomal vesicles containing viral material from human pancreatic β-cells. Nonlytic spreading could occur before the final cell lysis. In conclusion, lytic spreading does occur, whereas the mechanisms behind the nonlytic spreading remain undetermined. There is no consensus on which enteroviruses do spread in a nonlytic way and whether it is associated with certain tissues or cell types.

After the release, mature virions spread into surrounding tissue and circulation. The virus causes acute and chronic conditions. If the primary infection is not successfully cleared, it will predispose to infection in secondary tissues. Acute inflammation causes common cold symptoms, pancreatitis, and hepatitis. Chronic disease, on the other hand, requires persistent, low-level infection to develop. With CVBs, the pancreas and heart are susceptible to persistent infections which will lead to more severe conditions like dilated cardiomyopathy. (Garmaroudi et al. 2015)

2.4 CVB interference with the host cell

CVB must interfere with the host to improve its own purpose. For example, taking over the host cell translation machinery is essential for the virus's life cycle. Viral proteases can cleave several host cell proteins as well. It should be noted that interactions with the host cell and immunity are complex and often cross-impact each other's, and these are not the only ways how CVB infection affects its host.

The role of $2A^{pro}$ in translation was already discussed, but the protease affects multiple other proteins as well, pushing the maximal success for the virus. First, it drives the host cell translation down proposing the viral translation. Eucaryotic translation initiation factor 4 gamma 1 (eIF4G) is essential for the cap-depended mRNA translation. As the host cell-produced mRNAs are always capped, the cleavage of the eIF4G will reduce the translation of cellular mRNA. Thus, the translation of viral noncapped RNA, which translation begins through IRES instead of eIF4G is increased. (Garmaroudi et al. 2015)

$2A^{pro}$ halts the host metabolism indirectly as well. It cleaves other host proteins, like nuclear pore complexes to further promote viral replication. Cleaving the complexes able the transfer of nuclear proteins in the cytoplasm, where the virus can employ them for replication. $3C^{pro}$ alters the host cell as well. Viral protein 3CD cleaves nuclear TATA boxes, which inhibits the nuclear transcription in the first place. $2A^{pro}$ protease activity is required in helping viral protein 3CD entrance to the nucleus. 3CD cleaves other transcriptional proteins as well, including transcriptional activating factor p53. (Laitinen et al. 2016)

Heart tissue is especially vulnerable to 2A protease. Substrates of $2A^{pro}$ include dystrophin, which is a cohesive protein in cardiac- and skeletal muscles holding together sarcolemma and myofiber. Considering heart tissue, $2A^{pro}$ is also responsible for cleaving serum response factor, which is a transcription factor for driving heart cell functions. These contribute to the susceptibility of heart tissue to persistent infections leading to chronic diseases. (Laitinen et al. 2016)

And, of course, the cytopathic effect leading to the final lysis of the cell and release of virions is a major change in the host cell. Some of the effects on the host cells are discussed more in detail under the immunity headline because their main target is the immune system. In conclusion, the virus has multiple ways to promote its own replication by reducing the host cell's ability. Interference is necessary since the virus needs the host cell's proteins. The mechanism presented here covered mainly the effects of viral proteases, which do not completely cover the overall effects on the host by CVB.

3. IMMUNITY AND EVASION STRATEGIES

The immune system is designed to fight against foreign pathogens and viruses are not an exception. The immune system can be divided into two branches: innate immunity which is responsible for immediate response and specific adaptive immunity which is activated by innate immunity.

CVBs cause robust immune responses. The free viruses are exposed to immune cells. Likewise, the infected cells are susceptible to immune response. If the viral material is not trapped inside replication machinery, for example, it is free to be presented on the cell surface as a mark of infection. Nearby signs of infection trigger the immune system to alarm yet healthy cells to be hostile to the virus. Therefore, the virus's opportunities to spread depending on the type and robustness of the immune response. But it should be noted that the inflammation reaction can also be the cause of tissue damage. However, even if the individual infected with CVBs has already produced specific CVB antibodies, they do not protect fully from the infection or virus replication. But antibodies can

block the spreading of the virus to secondary sites and even leave the infection unnoted. Therefore, severe outcomes like diseases are not developed. (Oberste and Pallansch 2008).

From the virus's point of view, the immune response should be avoided as efficiently as possible. Therefore, the virus has developed various mechanisms to evade and interfere with the immune system. The overall immune response and evasion strategies together with the consequences of unsuccessful clearance will be discussed herein in more detail.

3.1 Innate immunity

Innate immunity is the rapid and nonspecific response to foreign material such as viruses. The first line of defense in innate immunity is physical barriers that prevent the virus entrance. These include the epithelium, secretions, and low pH. If the virus is still able to enter the body, innate immunity will activate. Major factors of activated innate immunity are lytic and phagocytic cells (NK cells and sensor cells) together with cytokine secretion. Eventually, innate immunity activates the adaptive immune response, which is responsible for the specific response against the virus.

Phagocytes direct the cell-mediated innate immunity response. Together with cytokines, a complement system is responsible for the humoral side of innate immunity. The complement system can be activated by antibodies or immune complexes for instance. Its function is as said, to complement the immune response. Activation leads to opsonizing and killing viruses together with induction of inflammation reaction. DAF (complement decay-accelerating factor) mentioned as a CAR coreceptor has a role in complement regulation. DAF inhibits the formation of complete complement activation and therefore protects cells from the outcome, such as lysis.

Viral activation of innate immunity requires the detection of the virus. Pathogen-associated molecular patterns on the virus surface can be recognized by pattern recognition receptors (PRRs) on the membranes of various cells.

Toll-like receptors (TLRs) are an example of those receptors. They are single-pass transmembrane proteins that can start intracellular signaling through different adaptors. Some TLRs can detect viral structures. These TLRs are mainly present in immune cells like macrophages and dendritic cells. Eventually, downstream signaling cascades will activate transcription factors including interferon regulatory factors (IRFs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), that will ultimately activate type 1 interferons and proinflammatory cytokines. (Murphy and Weaver 2017).

RIG-I-like receptors (RLRs) on the other hand are PRRs that recognize cytosolic viral RNA. Because viral replication occurs in the cytoplasm, they have an important role in detecting viruses. Contrary to TLRs which are mainly expressed in immune cells, RLRs are broadly found amongst a variety of cell types. Type I interferons can induce their expression. Cytosolic helicases RIG-I and

MDA5 are examples of RLRs. RIG-I detects ssRNA and MDA5 dsRNA. Eventually, the signaling pathway of both helicases interacts with the mitochondrial antiviral protein (MAVS), functionally located in mitochondrial membrane. MAVS will activate NF-κB and interferon regulatory factor 3 (IRF-3). These transcription factors will induce the transcription of type I interferons and proinflammatory cytokines. (Feng et al. 2014; Sesti-Costa et al. 2017)

Type I interferons include interferon-α and interferon-β. Upon activation by TLRs and RLRs as described above they trigger the antiviral state which aims to suppress the infection together with the activation of interferon-stimulated genes. To achieve that, viral transcription is blocked, viral translation is suppressed, antigen presentation to the adaptive immune system is increased, and immune cells including dendritic cells, macrophages, NK cells, and lymphocytes are recruited and activated. In other words, they recruit the adaptive immunity to finish the clearance of the infection. (Murphy and Weaver 2017).

3.2 CVBs and innate immunity

TLRs that have been shown to detect RNA also do contribute to CVB infection. TLR-3, TLR-7, and TLR-8 on the endosomal membrane release proinflammatory cytokines during infection. TLR-3 detects dsRNA and can be found in pancreas. Whereas TLR-7/-8 ssRNA and can be found in B cells in addition to macrophages and dendritic cells. (Murphy and Weaver 2017). TLR-4 on the extracellular membrane has been linked with CVB3 and CVB4 infections, even though it is not generally thought to interact with virus infections. TLR4 induced proinflammatory cytokines have been detected in endomyocardial tissues and pancreas and therefore are related to the infection in secondary tissues. (Wells and Coyne 2019)

TLR3, however, has a significant role in detecting CVBs. Double-stranded RNA for the activation of TLR-3 can be either from endocytosis or replicative intermediate. TLR3 is noteworthy for a couple of reasons. It can be detected in the intestinal epithelium; the primary infection site of CVBs. Downstream signaling of TLRs usually begins with MyD88, but TLR-3 alone can signal through TRIF (Murphy and Weaver 2017). Sesti-Costa et. al have studied the importance of the effect originating from TLR3 activation, concluding that innate immune response to CVB3 is dependent on TLR3. They showed that TLR3 can have a major impact on limiting the virus replication. CVB3 infected TLR3 knockout mice were not able to clear the infection. Wild-type mice, on the other hand, could eradicate the virus. Interestingly, the same effect did not apply to pancreases. Nonetheless, TLR3 activation has key effects on the adaptive immunity as well, triggering the proper response for fighting the infection. The effects on adaptive immunity along with the importance of TRIF will be discussed later.

RLRs counteract with CVBs as well. There is no consensus whether the RIG-I would be a major factor in CVB infection. It recognizes cellular DNA from viral RNA by 5' end-capping of mRNA's that is transcribed in the nucleus. ssRNAs lacking that cap are recognized as foreign. As discussed, CVB RNA has VPg attached on the 5' end. Therefore, CVBs could not trigger the RIG-I pathway. Nevertheless, there is evidence that RIG-I does detect RNA derived from CVB3. It is thought that CVB3 produces different structures such as a double-stranded replicative form of the RNA, or larger RNA complex IRES that could be detected by RIG-I. Francisco et al. supposed that the importance of RIG-I could be dependent on the cell type. For example, the IFN-β production was reduced with RIG-I deficient macrophages but lost completely in mouse embryonic fibroblasts. According to their result, they concluded that RIG-I is important, but MDA5 is an essential factor in the detection of coxsackievirus infection. (Francisco et al. 2019)

3.2.1 Evasion from innate immunity

CVBs have clever ways to evade innate immunity. As seen, RLR activated MAVS and TLR3 activated TRIF play a significant role in triggering the proper response against the infection. The signaling from these receptors will result in the activation of different IRFs and NF-κB which will eventually adjust the cell to an antiviral state. The proteases which interfere with the host cell are also responsible for distracting innate immunity. Mukherjee et al. have proved that $3C^{pro}$ cleaves TRIF and MAVS. Wells. et al report that $2A^{pro}$ cleaves MDA5 and MAVS as well. Mukherjee proved that the cleavage does reduce the functionality, but Wells' report from 2A had no proof for the loss of functionality. (Mukherjee et al. 2011; Wells and Coyne 2019)

TRIF starts the downstream signaling after TLR3 activation. $3C^{pro}$ can cleave both, the N- and C-terminal of TRIF. Mukherjee et al. showed that the $3C^{pro}$ goes to the TRIF signalosome, where the rest of the TRIF signaling molecules cluster. The C-terminal cleavage would occur there. N-terminal cleavage was not as common, but the cleavage was proven to be possible. The C-terminal is essential for triggering the NF-κB signaling, as the cleavage was proven to halt the NF-κB -mediated activation of apoptosis. MAVS is cleaved by $3C^{pro}$ as well. MAVS localizes in the mitochondrial membrane, but cleaving causes the N terminal to disseminate from it. The C-terminal that will remain on the mitochondrial membrane after cleavage cannot induce as strong IFNβ and NF-κB compared to the original. (Mukherjee et al. 2011)

In summary, at least $3C^{pro}$ represses the formation of an antiviral state in the host cell by fleeing from TLR and RLR signaling. Disturbing the NF-κB -mediated apoptosis could additionally give the virus more time to replicate and spread more successfully. Route of release can also be a significant factor when trying to avoid the encounter with the immune system in the first place. Therefore,

the release in a nonlytic way, can be considered as an evasion mechanism as the vesicles enable the spreading hidden from the immune system.

3.3 Adaptive immunity

Slower activating adaptive immunity is responsible for the specific immune response. As in innate immunity, adaptive immunity functions in a humoral and cell-mediated fashion. Adaptive immunity functions through extracellular fluids and is antigen mediated. Multiple factors of innate immunity, such as excreted cytokines activate adaptive immunity. T and B cells can specifically bind to and destroy pathogens and infected cells. Lymphocytes can kill infected cells upon detection of the infection or opsonize or engulf viral particles from the bloodstream.

Even though lymphocytes have a major responsibility in adaptive immunity, antigen-presenting cells (APCs) are essential for functional adaptive immunity. Therefore, they link innate immunity to adaptive. APCs include, for example, dendritic cells, macrophages, and B cells. Major histocompatibility complexes (MHC) present antigens on cell membranes and therefore activate naïve T cells towards differentiation on the surfaces of APCs. Depending on the MCH class (I or II) and their coreceptors (CD4+ or CD8+) they cause distinct cell-mediated responses.

MHC class I presents intracellular components in the complexes on all eukaryotic cells. Presenting infectious agents on MCH I can trigger CD8+ cells, which will destroy the infected cell upon detection. Therefore, MHC I activate the cytotoxic, cell-mediated response. MHC class II can be found on dendritic cells, B cells, and phagocytes, for instance. MHC II presents extracellular antigens or proteins derived from endocytosis. MHC II activates CD4+ T helper cells (Th cells) which in turn orchestrate B cell differentiation and the development of the immune response.

There are several Th-cell classes: Th1, Th2, and Th17 amongst others. Th1 response is important for clearing the infection as they produce (IFN)-γ and activate CD8+ T cells. It is also often responsible for tissue damage. Th2 recognizes extracellular pathogens and activates B cells and antibody-mediated immunity. Th17 induces epithelial and stromal cells to secrete chemokines which recruit neutrophils. Usually, the Th17 response is linked with microbial infection as they induce the production of antimicrobial peptides in epithelial barriers.

The humoral, antibody-dependent response functions through B cells. B cells can produce different antigen-specific antibodies (immunoglobulins, Igs) and enable memorizing previous pathogen invasions. Different cytokines activate different immunoglobulins. Overall IgM is the initial antibody produced after encountering a pathogen. During the infection, the IgM structure can be modified to other isotypes during “class switching”. IgG is most common in the blood whereas IgA is a mucosal antibody. There are other isotypes as well, but they are not as relevant in virus infections.

3.4 CVBs and adaptive immunity

Since CVBs are prominently spread in a lytic way, adaptive immunity is essential for preventing the infection from scattering to other tissues including the pancreas and heart. In general, enteroviruses provoke a robust humoral response, where antibodies are produced even in asymptomatic infections. (Dotzauer and Kraemer 2012).

Antibody response is essential in the prevention of persistent infections. For example, B cell-deficient mice were highly susceptible to persistent and chronic conditions in the heart, pancreas, and other tissues. The significance is proved by the fact that the viral titers can be reduced by passive immunoglobulin transfer. (Kemball, Alirezaei, and Whitton 2010)

The antibody responses for CVBs begin with the IgM antibody. IgM response would arise a couple of days after infection, and the specificity would be mainly against the VP1. IgGs would appear only a bit later targeting VP1 as well. (Dotzauer and Kraemer 2012; Kemball, Alirezaei, and Whitton 2010). Different serotypes of coxsackieviruses, including CVAs, do not elicit cross-neutralizing antibodies. Meaning that the infection of one serotype would not protect from another. This is beneficial to the virus, given that the immune response tends to be robust (Kemball, Alirezaei, and Whitton 2010; Kim et al. 2001). It is expected that CVBs would induce IgA response, as the infection occurs on mucosal surfaces. However, Dotzauer et al. report that IgA response is not detected in every CVB infection. If IgA is produced, the response would arise two weeks after infection.

The T cell response is thought to be notably less significant compared to the B cell antibody response. Literature has proven that the CD8+ response to CVB3 is astonishingly weak (Kemball et al. 2009; Kemball, Alirezaei, and Whitton 2010). One explanation could be that CVBs can inhibit the MCH I presentation of antigens, which will be discussed later in more detail. Nonetheless, the dendritic cells have a major role in activating CD8+ T cells. Cytokines direct the development of Th responses, and dendritic cells can induce the production of cytokines required for Th1 response.

Sesti-Costa et al. have demonstrated that TLR3 is essential for Th1 response (which activates CD8+ cells). Th1 is often responsible for tissue damage, but together with immune response inhibition by Treg cells Th1 is important for clearing the infection. Th2 in CVB infection, on the other hand, has been shown to worsen the infection in some cases. Further, they showed that the Th1 response prevents the formation of the Th17 response. As Th17 cells cannot reduce viral replication, and therefore can lead to tissue damage, it is linked with persistent infection. It was also demonstrated that the Th17 is not remarkable for counteracting the CVB infection in a mouse model. It should be noted that TLR3 did not affect CD4+ T cell production. (Sesti-Costa et al. 2017)

However, the possible significance of TLR3 in proper response is interesting, since the CVB has been demonstrated to interfere with the TLR3 signaling as well as block the antigen presentation

in MHC I complexes (discussed below). Could this underlie the effectiveness of Th1 in the virus clearance, as its induction is prevented in multiple phases of the infection?

In contrast, CD4 response to CVBs seems to function as expected in virus infection. CD4+ T cells show an effector phenotype and the development of memory cells. Memory cells in turn can dramatically duplicate when gathering again with the pathogen. To conclude, the B cell-dependent adaptive immunity plays a major role in adaptive immune responses against CVBs. Nevertheless, T cells do contribute to the adaptive response and can have a major role in developing CVB-induced diseases. (Kemball, Alirezaei, and Whitton 2010)

3.4.1 Evasion from adaptive immunity

CVBs suppress the activation of innate immunity in several ways, which directly affect the activation of adaptive immunity. But the virus has ways to disturb the adaptive immunity factors as well.

As discussed above, the T cell activation through MHC I complex is surprisingly faint. One reason contributing to it that is the virus's ability to block the antigen presentation through MHC I complexes. This blocking significantly reduces the induction of primary (cytotoxic) CD8+ T cell response. Therefore, it is more challenging for the immune system to detect and destroy virus-infected cells, as intracellular pathogenic material is not detected. Yet, the CD4+ T cells together with B cells can trigger a proper response for clearing the infection as discussed above. (Kemball et al. 2009)

Another significant aspect affecting adaptive immunity is the formation of enhancing antibodies. There are several different viruses capable of inducing antibody-dependent enhancement (ADE) and enteroviruses belong to these viruses. But in short, non-neutralizing antibodies are produced, which can improve the virus entrance to immune cells. Those antibodies can be against an area of the capsid which do not contribute to the virus-host interaction. In addition, the number of neutralizing antibodies may be suboptimal leading to unwanted outcomes. ADE may lead to enhanced viral infection and can disturb the overall immune response towards the virus (Hober et al. 2012). ADE is linked with the shift of the Th1 response to a Th2 response (Kemball, Alirezaei, and Whitton 2010). ADE has been thought to contribute to developing type 1 diabetes, which is discussed briefly below. Yet, discussing this mechanism behind ADE is beyond the scope of this thesis

4. SECONDARY INFECTION

If CVB infection is not successfully eradicated on the primary site, it can spread into secondary tissues. Infection in secondary tissues can be acute as well, but in severe cases, the spreading virus will persist. Persistence means that the virus remains in cells with a lower level of replication, and action in a manner where the cell remains viable yet infected. (Chapman and Kim 2008)

The major secondary tissues for CVBs are the heart and pancreas. The diseases linked with these tissues include myocarditis, dilated cardiomyopathy, and type 1 diabetes (T1D) amongst others. Persistent infection plays a role in these chronic diseases. At least one-fifth of myocarditis and cardiomyopathy are linked with CVBs. The association of CVBs is clear with enterovirus-induced cardiomyopathy in children and adults. The reasons behind the disease include viral infection itself as well as virus-induced autoimmune responses. Persistent infection plays a role here as well. The connection between CVB and heart infections has been extensively reviewed elsewhere. (Chapman and Kim 2008; Kim et al. 2001)

Even though the connection between CVBs and cardiomyopathy is confirmed, the development of chronic diseases involves several factors all affecting each other (Kim et al. 2001). Therefore, CVB infections do not directly mean that a chronic condition will arise. This is especially significant when researching the linkage between T1D and CVBs.

4.1 Brief overview of type 1 diabetes and CVBs

CVBs association with diabetes has been investigated for tens of years. The mechanism behind T1D is complex and yet unspecified. There is evidence of several associations for CVBs, especially CVB4 being one of the major environmental factors exposing to T1D (Hober et al. 2012).

Type 1 diabetes (T1D) is an autoimmune disease that is characterized by the loss of production of insulin. The insulin producing-cells, β-cells in the Langerhans islets of the pancreas become disabled as the outcome of the disease. As a result, the patients must uptake insulin throughout their lives. The causes behind the disease remain unclear. Genetic susceptibility to the disease has been pointed out, but different environmental factors have a distinguished effect. CVBs are one major environmental factor linked with T1D, as various data indicate their responsibility for developing autoimmunity in β-cells. (Netanyah et al. 2020)

Hober et al. have summarized that the development of the disease is caused by overall interactions between the virus, insulin-producing β-cells, immunity responses, and the host cell's ability to act upon an infection. They emphasized the significance of persistent infection and ADE. They brought

up the formation of autoimmunity reaction through a prolonged immune response. Repeated infections in β-cells and constant production of IFN-α can cause the formation of autoreactive cytotoxic T lymphocytes instead of antiviral ones. One of the earliest consequences of the CVB infection in pancreas has been thought to be the infection of monocytes, enabled by the ADE effect. Infected monocytes further increase the virus spreading to the β-cells and cause more serious damage. (Hober et al. 2012)

But why pancreatic β-cells are particularly susceptible to severe CVB infection? Richardson et al. have studied the factors affecting susceptibility. As the pancreatic β-cells are highly differentiated, their neogenesis considerably decreases after the age of 10. Therefore, the terminally differentiated cells are made to be highly sensitive to interferons to enable a robust response to infections. The virus cannot be spread to secondary tissue without triggering an immune response (IFNs), so the pancreatic cells are prepared and thus could counter the virus infection. Nevertheless, IFNs cannot block the entrance. If the IFN triggered alert for the virus is not efficient enough, it can lead to a persistent condition where the cell is infected yet viable. (Richardson and Morgan 2018)

Richardson et al. hypothesized that β-cells have the necessary host cell features for efficient viral replication and possible persistence. They also demonstrated that a specific isoform of CAR receptor, CAR-SIV is highly expressed on β-cells. As other studies support, they implicate that the CAR-SIV is located on the insulin secretory granules instead of the plasma membrane. Therefore, the CAR-SIV receptors will end up in the extracellular side of the plasma membrane as the insulin is released. The viruses can bind to the receptor and as the membrane is recycled back to the cell by endocytosis, the virus comes along. Thus, making the β-cells prone to CVB infection. (Richardson and Morgan 2018)

Interestingly, β-cells are one example of cells that exhibit nonlytic spreading. T1D patients do not show the traditional tissue damage caused by lytic spreading and acute infection. The spreading would occur through the exocytotic granules. At least CVB3 has been shown to exit from HeLa cells in extracellular vesicles, and enteroviruses overall have shown to exit β-cells in EVs. The significance relies on the way to evade the recognition by the immune system when being spread inside vesicles without pathogenic surfaces. (Netanyah et al. 2020)

In conclusion, several factors contribute to the development of T1D. CVB infection is associated with the disease through various mechanisms. The factors represented in this brief overview are just a small part of them all. Despite all the effort put into the research, the direct connection between CVB and T1D cannot be pointed out. Perhaps the constantly growing knowledge will eventually enable us to understand the mechanisms behind T1D. Nonetheless, preventing the persistent CVB infection could have a major impact on the prevalence of the disease.

CONCLUSION AND FUTURE PROSPECTS

To conclude, this thesis summarized coxsackieviruses B infection mechanisms, its effect on the immune system, and evasion strategies. CVBs can infect several various tissues and effectively alter the host cell mechanisms as well as trick the immune response.

It is crucial to be able to understand the infection mechanism and its effects if we wish to prevent or cure the outcomes of the infections. Apart from Poliovirus and Enterovirus 71 in China, there are no vaccines for enteroviruses (Baggen et al. 2018). This is surprising since the acute and chronic diseases linked with enteroviruses are serious and rather common. But vaccine candidates are currently developed. There are some antiviral drugs targeting various parts of the virus's lifecycle as well (Richardson and Morgan 2018). Even if the prevention of the virus infection could not fully protect from developing diseases such as T1D, they could decrease their occurrence significantly.

However, it should be noted that the reference material was rather small, and many important prospects had to be left undiscussed. Most of the studies are done in animal or cell models, which cannot represent the mechanisms in humans faultlessly. For example, mice are not natural hosts for CVBs. Therefore, the amount of infective virus must be considerably high for studying the infection models in mice. Even though this thesis represents all CVB serotypes, most of the results are from one serotype. For example, CVB3 was used in most of the referenced studies. Also, some aspects have not been studied CVB specifically, but rather comprehensively about enteroviruses. All these factors should be taken into consideration when reading this thesis.

5. REFERENCES

Baggen, Jim, Hendrik Jan Thibaut, Jeroen R.P.M. Strating, and Frank J.M. van Kuppeveld. 2018. "The Life Cycle of Non-Polio Enteroviruses and How to Target It." *Nature reviews. Microbiology* 16(6): 368–81. https://pubmed.ncbi.nlm.nih.gov/29626210/ (January 19, 2022).

Carson, Steven D. 2001. "Receptor for the Group B Coxsackieviruses and Adenoviruses: CAR." *Reviews in Medical Virology* 11(4): 219–26. https://onlinelibrary.wiley.com/doi/10.1002/rmv.318.

Chapman, N. M., and K. -S. Kim. 2008. "Persistent Coxsackievirus Infection: Enterovirus Persistence in Chronic Myocarditis and Dilated Cardiomyopathy." In *Group B Coxsackieviruses*, Berlin, Heidelberg: Springer Berlin Heidelberg, 275–92. http://link.springer.com/10.1007/978-3-540-75546-3_13.

Coyne, Carolyn B., and Jeffrey M. Bergelson. 2006. "Virus-Induced Abl and Fyn Kinase Signals Permit Coxsackievirus Entry through Epithelial Tight Junctions." *Cell* 124(1): 119–31.

Dotzauer, Andreas, and Leena Kraemer. 2012. "Innate and Adaptive Immune Responses against Picornaviruses and Their Counteractions: An Overview." *World J Virol* 12(3): 91–107. http://www.wjgnet.com/esps/:91-107Availablefrom:URL:http://www.wjgnet.com/2220-3249/full/v1/i3/91.htmDOI:http://dx.doi.org/10.5501/wjv.v1.i3.91.

Feng, Q et al. 2014. “Coxsackievirus Cloverleaf RNA Containing a 59 Triphosphate Triggers an Antiviral Response via RIG-I Activation.” *PLoS ONE* 9(4): 95927. www.plosone.org.

Francisco, Esther et al. 2019. “Cell-Type Specificity and Functional Redundancy of RIG-I-like Receptors in Innate Immune Sensing of Coxsackievirus B3 and Encephalomyocarditis Virus.” *Virology* 528: 7–18.

Garmaroudi, Farshid S et al. 2015. “Coxsackievirus B3 Replication and Pathogenesis.” *Future Microbiol* 10(4): 629–53. www.futuremedicine.com (April 5, 2022).

Hober, D et al. 2012. “Immunology in the Clinic Review Series; Focus on Type 1 Diabetes and Viruses: Role of Antibodies Enhancing the Infection with Coxsackievirus-B in the Pathogenesis of Type 1 Diabetes.” *Clinical and Experimental Immunology* 168(1): 47–51. https://academic.oup.com/cei/article/168/1/47/6422772.

Kemball, Christopher C. et al. 2009. “Coxsackievirus B3 Inhibits Antigen Presentation In Vivo, Exerting a Profound and Selective Effect on the MHC Class I Pathway.” *PLoS Pathogens* 5(10): e1000618.

Kemball, Christopher C., Mehrdad Alirezaei, and J. Lindsay Whitton. 2010. “Type B Coxsackieviruses and Their Interactions with the Innate and Adaptive Immune Systems.” *Future Microbiology* 5(9): 1329–47.

Kim, Kyung-Soo, Gunter Hufnagel, Nora M. Chapman, and Steven Tracy. 2001. “The Group B Coxsackieviruses and Myocarditis.” *Reviews in Medical Virology* 11(6): 355–68. https://onlinelibrary.wiley.com/doi/10.1002/rmv.326.

Laitinen, Olli H et al. 2016. “Enteroviral Proteases: Structure, Host Interactions and Pathogenicity.”

Lasrado, Ninaad et al. 2020. “Identification of Immunogenic Epitopes That Permit the Detection of Antigen-Specific T Cell Responses in Multiple Serotypes of Group B Coxsackievirus Infections.” *Viruses* 12(3): 347. https://www.mdpi.com/1999-4915/12/3/347.

Marjomäki, Varpu, and Malin Flodström-Tullberg. 2022. “Coxsackie B Virus.” *Trends in Microbiology*.

Mukherjee, Amitava et al. 2011. “The Coxsackievirus B 3C pro Protease Cleaves MAVS and TRIF to Attenuate Host Type I Interferon and Apoptotic Signaling.” www.plospathogens.org.

Murphy, Kenneth, and Casey Weaver. 2017. “The Induced Responses of Innate Immunity.” In *Immunobiology*, Garland Science, Taylor & Francis Group, LLC, 77–131.

Netanyah, Eitan et al. 2020. “Extracellular Vesicles Released by Enterovirus-Infected EndoC-BH1 Cells Mediate Non-Lytic Viral Spread.” *Microorganisms* 8(11): 1753. https://www.mdpi.com/2076-2607/8/11/1753.

Oberste, M.S., and M.A. Pallansch. 2008. “Coxsackieviruses.” In *Encyclopedia of Virology*, Elsevier, 580–87. https://linkinghub.elsevier.com/retrieve/pii/B9780123744104003721 (April 12, 2022).

Richardson, Sarah J., and Noel G. Morgan. 2018. “Enteroviral Infections in the Pathogenesis of Type 1 Diabetes: New Insights for Therapeutic Intervention.” *Current Opinion in Pharmacology* 43: 11–19.

Sesti-Costa, Renata et al. 2017. “TLR3 Is Required for Survival Following Coxsackievirus B3 Infection by Driving T Lymphocyte Activation and Polarization: The Role of Dendritic Cells.” https://doi.org/10.1371/journal.pone.0185819.

Wells, Alexandra I., and Carolyn B. Coyne. 2019. “Enteroviruses: A Gut-Wrenching Game of Entry, Detection, and Evasion.” *Viruses* 11(5).

Xu, Longfa et al. 2021. “Cryo-EM Structures Reveal the Molecular Basis of Receptor-Initiated Coxsackievirus Uncoating.” *Cell Host & Microbe* 29(3): 448-462.e5.