Review

Virus-Host Interactions of Enteroviruses and Parvovirus B19 in Myocarditis

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Abstract
Viral diseases are a major threat to modern society and the global health system. It is therefore of utter relevance to understand the way viruses affect the host as a basis to find new treatment solutions. The understanding of viral myocarditis (VMC) is incomplete and effective treatment options are lacking. This review will discuss the mechanism, effects, and treatment options of the most frequent myocarditis-causing viruses namely enteroviruses such as Coxsackievirus B3 (CVB3) and Parvovirus B19 (PVB19) on the human heart. Thereby, we focus on: 1. Viral entry: CVB3 use Coxsackievirus-Adenovirus-Receptor (CAR) and Decay Accelerating Factor (DAF) to enter cardiac myocytes while PVB19 use the receptor globoside (Gb4) to enter cardiac endothelial cells. 2. Immune system responses: The innate immune system mediated by activated cardiac toll-like receptors (TLRs) worsen inflammation in CVB3-infected mouse hearts. Different types of cells of the adaptive immune system are recruited to the site of inflammation that have either protective or adverse effects during VMC. 3. Autophagy: CVB3 evades autophagosomal degradation and misuses the autophosomal pathway for viral replication and release. 4. Viral replication sites: CVB3 promotes the formation of double membrane vesicles (DMVs), which it uses as replication sites. PVB19 uses the host cell nucleus as the replication site and uses the host cell DNA replication system. 5. Cell cycle manipulation: CVB3 attenuates the cell cycle at the G1/S phase, which promotes viral transcription and replication. PVB19 exerts cell cycle arrest in the S phase using its viral endonuclease activity. 6. Regulation of apoptosis: Enteroviruses prevent apoptosis during early stages of infection and promote cell death during later stages by using the viral proteases 2A and 3C, and viroporin 2B. PVB19 promotes apoptosis using the non-structural proteins NS1 and the 11 kDa protein. 7. Energy metabolism: Dysregulation of respiratory chain complex expression, activity and ROS production may be altered in CVB3- and PVB19-mediated myocarditis. 8. Ion channel modulation: CVB3-expression was indicated to alter calcium and potassium currents in Xenopus laevis oocytes and rodent cardiomyocytes. The phospholipase 2-like activity of...
PVB19 may alter several calcium, potassium and sodium channels. By understanding the general pathophysiological mechanisms of well-studied myocarditis-linked viruses, we might be provided with a guideline to handle other less-studied human viruses.

Relevance of CVB3 and PVB19 in Viral Myocarditis

Myocarditis is the process of inflammation in the myocardium. One cause for myocarditis are viruses, that infect the host's heart. The severity of viral myocarditis (VMC) varies between a light illness with flu-like symptoms and gastrointestinal illness to severe dilated cardiomyopathy (DCM) and sudden cardiac death [1]. In western countries the most prominent cause for myocarditis is chronic viral infection and evidence is emerging that also the newly emerged SARS-CoV-2 can cause myocarditis [2, 3]. Dadashi et al. recently conducted a thorough review and meta-analysis of the prevalence of most common viruses in viral myocarditis including 75 studies from 1973 to 2018 performed in highly industrialized nations. In conclusion, the viruses with the highest prevalence rates were parvovirus B19 (PVB19) with 25.0% and non-polio enteroviruses with 18% [4]. However, the relevance of PVB19 is questionable, as the prevalence of PVB19 in healthy hearts is similar to hearts with diagnosed myocarditis (MC) or DCM [5]. Anyhow, it is proposed that a high viral load of over 500 ge (genome equivalents) and active replication of PVB19 could result in myocarditis in about 64.7% of myocarditis patients [6]. Additional co-infection with another cardiotropic virus in parallel to a PVB19 infection might even elevate the chance of myocarditis development [7, 8]. Kuhl et al. were able to support this hypothesis: They observed a clear difference between latent and transcriptionally active PVB19 in human endomyocardial biopsies. Thus, the shift from latent PVB19 to transcriptionally active, potentially caused by transactivating viruses (e.g. human herpes virus 6) and/or immune suppression, can result in the emergence of myocardial dysfunction [9].

Since viruses are the main cause for myocarditis, it is important to investigate and find treatments against the viruses that most commonly cause myocarditis, namely PVB19 and enteroviruses. By understanding these viruses, parallels to other, even less understood viruses, might be drawn. Hence, this review focuses on the effects of enteroviruses such as Coxsackievirus B3 (CVB3) and PVB19 on the human heart in order to understand underlying mechanisms of the pathogenesis of viral myocarditis and to discuss novel treatment options. Since there is a current lack of efficient treatment and cure against myocarditis viruses [10, 11], it is even more important to understand the common modi operandi of the respective viruses.

Viruses use the host cell to their advantage to reproduce and distribute viral particles by reprogramming intracellular mechanisms of the host cell. These mechanisms include immune responses, autophagy, formation of viral replication sites, cell cycle alterations, apoptosis, energy metabolism, and electrophysiological alterations that will be addressed in the next sections.

Viral genomes of Enteroviruses (Coxsackievirus B3) and Parvovirus B19

Viral genomes are customarily small and therefore code only for a few structural proteins forming the viral capsid and non-structural proteins which control the host cell-virus interactions. These interactions serve to promote viral replication and virus assembly and they vary depending on the virus type [12, 13]. PVB19 is an ssDNA virus while CVB3 is an ssRNA virus. Both are non-enveloped viruses and are considered comparatively small with genome sizes of 7.4 kb (CVB3) and 5.6 kb (PVB19) respectively [14, 15].
**CVB3**

The picornaviral genome is coding for the structural proteins VP1-VP4 and for the non-structural proteins 2A-3D (Fig. 1) [16, 17]. As previously mentioned, the structural proteins form the capsid. Differential proteolysis of the polyprotein gives rise to non-structural proteins, such as viral proteases 2A, 3C, and 3CD, a viral polymerase (3D), a viroporin (2B), and proteins for viral encapsulation (2C), membrane permeabilization (3C), and viral replication (2BC) [17]. The genomic ssRNA is not capped at the 5’ end but the viral 3B protein, also called virion protein genome linked (Vpg), is recruited to the 5’ end, which serves as a primer for viral replication [18]. Moreover, the CVB3 genome inherits an internal ribosome entry site (IRES) at the 5’ end that mediates translation of the viral RNA [19].

**PVB19**

The PVB19 genome is flanked by two identical inverted terminal repeats (ITRs), that each form a hairpin. PVB19 expresses the three non-structural proteins NS1, a 11 kDa, a 7.5 kDa protein, and the structural proteins VP1 and VP2, which form the capsid (Fig. 2) [20-22]. VP1 makes up 4% and VP2 96% of the viral capsid [21]. The viral capsid proteins are transported inside the host cell nucleus, because VP2 harbors a nuclear localization signal. The same applies to the NS1 protein that inherits two nuclear localization signals [23]. The function of the 7.5 kDa protein is still unknown. The 11 kDa protein localizes in the cytoplasm. It is 10 times more abundant than the full-size NS1 and induces apoptosis in erythroid progenitor cells. Inhibition of its mRNA via antisense oligos shows significant decrease in apoptosis in PVB19-infected erythroid progenitor cells [24]. The NS1 protein is found to have multiple functions: DNA binding and cleavage, helicase activity, DNA damage response, cell cycle arrest, transactivation and apoptosis [22, 25].

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**Fig.1.** Genome of Picornaviruses and the known functions of the translated proteins.
Disease Progression of Viral Myocarditis: The Fight between Virus and the Immune System

Viral Entry

The mechanisms in which a viral infection results in myocarditis are diverse and depend on the virus. Enteroviruses and adenoviruses directly infect cardiac myocytes, whose lysis and resulting immune system activation leads to myocarditis. In contrast, patients with fulminant PVB19 myocarditis suffer cardiac endothelial cell infection, predominantly in venules, and to a lesser degree in small arteries, and arterioles [26].

The progression of myocarditis can be divided into three main phases [5, 10, 27]: Phase 1: Viral entry and activation of the innate immune system, phase 2: Activation of the adaptive immune response, and phase 3: Recovery or disease progression.

CVB3. Enteroviruses enter the body via the respiratory or gastrointestinal tract. The heart is infected secondarily. Phase 1 starts with the entry of the virus into the target cells, which takes minutes up to hours. Enteroviruses and adenoviruses enter cardiomyocytes by binding to the cell-specific surface proteins called Coxsackievirus-Adenovirus-Receptor (CAR) and Decay Accelerating Factor (DAF) (see Fig. 3A) [28, 29]. Inhibition of enterovirus binding to CAR and DAF by soluble recombinant CAR or DAF is shown to alleviate cardiac dysfunction and reduce viral load in mice with enteroviral myocarditis [30, 31].

PVB19. PVB19 primarily uses the receptor globoside (Gb4), also called P antigen receptor protein, to facilitate its entry into the target cell (see Fig. 3A). Humans and knockout cells deficient for Gb4 were shown to be resistant to PVB19 infections [22, 32, 33]. However, recently it has been demonstrated that Gb4 is not necessary for the initial entry of PVB19 but rather it is relevant in a post-entry step of infection [33]. Gb4 is activated and expressed more increasingly in vascular endothelial cells by tumor necrosis factor-α (TNF-α) stimulation, thus possibly being enhanced during inflammation [34]. Potentially, PVB19 infection progresses in inflamed endothelial tissue, which should be further studied. There are also other co-

Fig. 2. Genome of Parvovirus B19 and the known functions of the translated proteins.
receptors like Ku80, and integrin α5β that could facilitate and mediate PVB19 entry into the cell [22, 35, 36]. Additionally, entry of PVB19 may be enhanced via antibody-dependent mechanisms, but this hypothesis is not sufficiently investigated, yet [37, 38].

Inside the host cell, the viral genome is transported into the nucleus through nuclear pores and its genetic information is replicated. Subsequent translation into functional proteins is required for the generation of new viral particles and is performed by host cell ribosomes. These proteins include structural proteins like capsid proteins, and non-structural proteins, that manipulate the host cell function to the advantage of the virus. During the time of viral replication, the viral proteases initiate cell apoptosis and necrosis [26, 39, 40].

**Activation of Innate Immune System**

**CVB3.** Within 1 to 7 days post infection cardiac toll-like receptors (TLRs) recognize general infection patterns and initiate the release of pro-inflammatory cytokines like interleukin-1β (IL-1β), IL-6, IL-18, TNF-α, and type I and type II interferons (IFNs) that are secreted by myocytes, endothelial cells, fibroblasts, and dendritic cells (see Fig. 3B) [41, 42]. TLR 3, TLR 7, TLR 8, and TLR 9 play a particularly large role in viral myocarditis, as TLR 3 recognizes viral dsRNA, TLR 7/8 ssRNA, and TLR 9 bacterial and viral CpG DNA motifs [41]. TLR 3, TLR 4 and TLR 9 activation after recognizing pathogen-associated-molecular-patterns (PAMPs) during VMC was shown to worsen the severity of inflammation in CVB3-infected mouse hearts. In line, specific inhibition of TLR 3 and TLR 4 function by MiR-146a, an anti-TLR9 oligodeoxynucleotide, or Lupeol alleviated the CVB3-triggered cardiac inflammation, making TLR-specific therapy a possible option for VMC patients [43-45]. Secreted cytokines appear to be either beneficial or detrimental during viral myocarditis, depending on the cytokine, timing of secretion or administration, and secreted amount. Blocking IL-1β signaling in CVB3-mediated myocarditis reduces inflammation, fibrosis, and cardiac remodeling, therewith preventing progression towards chronic myocarditis in

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**Fig. 3.** Most relevant effects of PVB19 and CVB3 on viral myocarditis outside of the host cell. Viral effects (red boxes) and respective possible treatment options (green boxes) are described. PVB19, Parvovirus B19; CVB3, Coxsackievirus B3, CAR, Coxsackievirus-Adenovirus-Receptor; DAF, Decay Accelerating Factor; MMP, Matrix-Metallo-Proteinase; ECM, extracellular matrix; TGF-β, Transforming Growth Factor-β.
mice [46]. In Balb/c mice and human myocardial fibroblasts infected with CVB3, exogenous administration of IFN-β had alleviating effects [47, 48]. In clinical studies, administration of IFN-β to patients with myocardial enteroviral or adenoviral persistence and left ventricular (LV) dysfunction is shown to be compliant. Additionally, complete viral clearance in all patients, accompanied with improved LV function and survival rate was observed [49, 50].

Absence of IFN-γ does not have any significant effects on CVB3-infected mice, while overexpression of IFN-γ had alleviating effects on murine CVB3-induced myocarditis [51, 52]. IFN signaling leads to the Janus Kinase (JAK)-signal transducer and activator of transcription (STAT) pathway activation. Inhibiting this pathway via Suppressor of Cytokine Signaling, SOCS-1 and SOCS-3, increases cardiac damage and elevates viral replication [53, 54].

Another important pro-inflammatory cytokine is TNF-α. Mice that are administered or express TNF-α show enhanced cardiac damage, but also enhanced viral clearance after CVB3 or encephalomyocarditis virus (EMCV) infection [55, 56]. Additionally, complete systemic depletion of TNF-α is shown to be detrimental in murine EMCV myocarditis [57]. Hence, the dose and the timing of TNF-α administration or inhibition is critical for myocarditis attenuation.

Through these cytokines, cells of the innate immune system including natural killer cells (NK cells), natural killer T cells (NKT cells), macrophages, monocytes, and dendritic cells are recruited to the infected area [42]. The presence of NK and NKT cells is assumed to be beneficial during the early phase of viral myocarditis [58, 59]. Other cells of the innate immune system appear to rather have a negative effect on infected tissue. This notion is supported as depletion of the innate immune system cells via Mac-1 antibodies reduces viral load and inflammation in EMCV-caused myocarditis in mice. Mac-1 antibodies inhibit macrophages, monocytes, and NK cells, which underlines the observed effects [60]. Membrane-bound dipeptidase 2 (DPEP2) suppresses macrophage inflammation by inhibiting NF-κB signaling, thereby having protective effects in CVB3-induced myocarditis [61].

Extracellular matrix (ECM) molecules are also upregulated by cytokines. ECM components are key regulators in cardiac remodeling and of inflammatory processes as they prime the site of infection accessible for leucocytes [62]. Matrix metalloproteinases (MMPs) are enzymes that regulate components of the ECM in order to remodel the tissue in physiological and pathophysiological processes including inflammation and tissue injury (see Fig. 3C). They can cleave cytokines, chemokines, and growth factors, therefore modulating them appropriately in response to the predominant tissue condition [63, 64]. MMP-2, -3, -8, -9, and -13 as well as some of their inhibitors called tissue inhibitors of metalloproteinases (TIMPs) namely TIMP-1 and -2 are found to be upregulated in CVB3-infected mice [65]. The enhanced production of e.g. MMP-9 in the myocardium is discussed to be activated by TLR activation in myofibroblasts. This TLR activation leads to the secretion of chemokines and to the activation of heart-infiltrating leukocytes directly worsening cardiac inflammation. Furthermore, activated leukocytes produce MMP-9, which is a strong trigger for the promotion of fibrosis [27]. Lowering the MMP level by overexpression of inhibitors like TIMP-1, plasminogen activator inhibitor-1 (PAI-1), or carvedilol decreases cardiac inflammation, cardiac necrosis and fibrosis, and reduces cardiac dilation and dysfunction in CVB3-infected mice underscoring the role of MMPs in the pathogenesis of viral myocarditis [65, 66]. However, MMPs have to be targeted selectively. Lack of MMP-8 has no effect on viral load, but lack of MMP-9 or MMP-3 worsens the condition of CVB3-infected mice including higher myocardial damage, dysfunction, viral load, and immune cell infiltration [67, 68].

**PVB19.** So far, there are no studies that investigate the role of TLRs in PVB19-mediated myocarditis. In PVB19-infected cultured endothelial cells and patients with cardiac persistence of PVB19 administration of IFN-β led to normalization of endothelial function and decrease in viral load [69, 70]. The in-vitro experiments indicate that these ameliorating effects are due to replication suppression of transcriptionally active PVB19 intermediates [69].
The Adaptive Immune System

CVB3. In phase 2 the adaptive immune system responds to the viral infection (see Fig. 3B). This phase is characterized by high viral replication and inflammatory processes. Therefore, the most severe tissue damages occur in this phase [1, 71, 72]. Phase 2 includes elevated release of cytokines especially TNF, IL-1a, IL-1b, IL-2, IFN-γ and the recruitment of virus-specific T- and B-lymphocytes, which are accompanied by cellular and humoral antibodies to detect and destroy the infection [10]. Heart-reactive autotboomides are produced in most myocarditis patients that detect and immobilize own cardiac proteins. Autoantibodies targeting myosin, β1-adrenergic receptors, cardiac troponin I, cardiac mitochondria, and others are produced [73]. Removal of antibodies via immunoadsorption in DCM patients in pilot studies improved cardiac function and reduced cardiac inflammation [74, 75]. In contrast, in chronic DCM patients, troponin I autoantibodies had a cardio-protective effect [76]. The role of the immune system on CVB3 VMC is summarized in a facilitated review article by Corsten et al. [42]. They are able to conclude that overall NK cells, M2 macrophages, T-helper 2 cells, regulatory T cells, and B cells are cardioprotective while M1 macrophages, T-helper 1 cells, cytotoxic T cells and γδ+ T cells have adverse effects during VMC. These autoimmune responses as well as the more protuberant effects of the virus itself, like viral protease activity, lead to damage in the ECM proteins, contractile apparatus, and interstitial cells [10]. In addition, T-helper 17 cells are described to contribute to CVB3 replication by producing autoantibodies e.g. against cardiac ANT promoting heart failure in myocarditis [77-79]. Integrin CD11b appears to contribute to T-helper 17 cell induction [80]. Reducing T-helper 17 cells in VMC could be a promising target for therapy. In contrast, regulatory T cells and mesenchymal stromal cells, that have immunomodulatory effects, are beneficial in CVB3-mediated myocarditis. They reduce the recruitment of monocytes to the heart. Transferring regulatory T cells or mesenchymal stromal cells, or using agonists that promote production of these cells could be a promising therapy concept as well [81-83].

PVB19. PVB19-mediated myocarditis exerts endothelial damage similar to CVB3-mediated myocarditis. PVB19 infection also leads to activation of the cytokines TNF-α and IL-6. Further, PVB19 infection is associated with the upregulation of adhesion molecules, which causes accumulation of T-lymphocytes in the myocardium, and induction of apoptosis in endothelial cells, which could ultimately lead to ischemia and cardiomyopathy [26].

Recovery or Disease Progression

CVB3 and PVB19. In phase 3, the disease can progress either towards viral clearance and recovery or towards irreversible chronic DMC. Around 60% to 70% of myocarditis patients recover from the infection with healed injury due to an intact immune system [84]. In severe injuries, caused by apoptosis or necrosis of the myocardium, the contractile myocardium tissue cannot heal due to lack of cardiac precursor cells that could generate more cardiomyocytes. In these cases, pathologic remodeling and fibrosis occurs causing DCM or heart failure (see Fig. 3C). Transforming Growth Factor-β (TGF-β) signaling is indicated in ischemic heart diseases and myocarditis to be the key in the transition from acute inflammation to fibrosis [85]. Concordantly, inhibition of TGF-β in mice with induced experimental autoimmune myocarditis prevents myocardial fibrosis [86]. TGF-β signaling is demonstrated to affect heart-infiltrating CD133+ hematopoietic cells in mice with induced autoimmune myocarditis which then generate myofibroblasts leading to fibrosis [86]. The myofibroblasts excessively produce collagen and tip the MMP and TIMP balance, thus disrupting the ECM. This leads to cardiac fibrosis and ultimately DCM [87]. In very severe cases, calcification of the myocardium can occur leading to a poor prognosis for the patient [88].

Moreover, even after viral clearance, it is possible that myocardial inflammation still persists. This may be due to cross-reactive antibodies produced against the virus but also recognize antigens of the hosts. This mechanism is called molecular mimicry [89].
**Immunomodulatory Treatment against Viral Myocarditis**

It becomes evident that the immune reaction plays a crucial role in fighting against the viral infection and at the same time causing pathologic cardiac phenotypes. Immunomodulation in combination with antiviral drugs as treatment option in VMC could be promising, but studies are still lacking [90]. So far, infectious myocarditis has to be ruled out to start immunosuppression, while in some non-infectious myocarditis scenarios immunosuppressive treatment is mandatory [2, 91]. IFN-β treatment against CVB3 myocarditis is considered promising as mentioned previously. Recently, it has been shown that the use of the mineralocorticoid receptor antagonist eplerenone during the first acute phase of infection is beneficial in murine CVB3-mediated myocarditis by reducing infiltration of monocytes and macrophages, oxidative stress, apoptosis, and fibrosis [92]. Cannabidiol also has immunomodulatory effects and is shown to reduce T cell-mediated inflammation and had alleviated effects in mice with experimental autoimmune myocarditis [93]. A recent phase 3 study using intravenous immunoglobulin therapy (IVIG), shown effective in several autoimmune and autoinflammatory diseases, showed no noteworthy effect in PVB19-mediated myocarditis [94, 95]. Hence, immunomodulation as therapy in viral myocarditis has to be further evaluated.

**Autophagy in CVB3-Mediated Myocarditis**

Autophagosome formation is an innate cell response for pathogen removal or for protein recycling and degradation. However, some viruses can evade autophagosomal degradation and misuse autophagy to their advantage: CVB3 seems to have the ability to stimulate autophagosome formation as shown in HeLa cells and in murine cardiomyocyte cell line HL-1. The CVB3 3C protease inhibits lysosome-autophagosome fusion by cleaving synaptosomal-associated protein 29, which is part of the SNARE complex connecting lysosomes and autophagosomes [96, 97]. Additionally, CVB3 uses autophagic components for the formation of extracellular microvesicles (EMVs) which contain CVB3 virions. This indicates that CVB3 uses EMVs for viral release (see Fig. 4B) [97, 98]. In addition, formation of these autophagosomes appears to be relevant for CVB3 replication, by using them as replication organelles (ROs). Consistently, in electron microscopy, the ROs of CVB3 resemble autophagosomal structures [99-101]. These RO structures might promote the formation of double membrane vesicles (DMVs). Thus, inhibition of autophagosome formation e.g. by 3-methyl-adenine or small interfering RNAs that target autophagosome formation-promoting genes is shown to reduce viral replication of CVB3, poliovirus, and rhinoviruses 2 and 14 in-vitro [99, 102]. Inhibition of autophagosome formation seems to be an interesting therapy approach that needs further investigation in more relevant disease models for myocarditis.

**Replication Sites of Enteroviruses and Parvovirus B19**

CVB3

Some positive stranded RNA viruses, that include enteroviruses, alter the host cell membrane in order to generate DMVs as replication sites [103, 104]. In these DMVs, components of a replicase complex are anchored. They create a protected environment against the detection of dsRNA intermediates by the host cell innate immunity. As a consequence, virions can mature in DMVs finally allowing for non-lytic release of the virus [105]. The organelles that are misused to promote DMV formation are called replication organelles (ROs). Pathways of autophagy, lipid metabolism and secretion are found to contribute to RO biogenesis [106]. DMVs of enteroviruses are not connected to other cellular membranes. Those DMVs exist in open vase-like structures, and form multilamellar vesicles in later infection stages [107]. Enteroviral ROs originate from the endoplasmic reticulum (ER) and
Golgi membranes [108]. In polioviruses, the 3A and 2BC non-structural proteins induce DMV formation from ROs [109]. CVB3 can either abuse components of the autophagy pathway for the formation of DMVs or it can create replication sites in an autophagy-independent manner [110]. Enteroviruses can use their viral proteins to recruit several host proteins to promote RO biogenesis. Inhibiting RO formation via small molecule compounds is suggested to be a promising treatment target, as these agents are able to reduce viral replication [106]. For example, using an inhibitor against phosphatidylinositol 4-kinase beta (PI4KB), which is a RO inducer, leads to a decrease in viral RNA replication. This decrease can be rescued by a mutation in the 3A protein of CVB3, which leads to RO-independent virus replication in the Golgi [111]. Activation of the AMP-activated protein kinase (AMPK) via Metformin reduces lipid accumulation and therewith disturbs RO formation in CVB3-infected HeLa cells [112]. Therefore, inhibiting the formation of ROs and DMVs appears to be an attractive treatment option. Upon virion assembly, the virion is released either via cell lysis or via extracellular microvesicles (EMVs). The EMVs contain autophagosomal markers. This could indicate that EMVs either originate from autophagosomes or that they use the autophagosomal pathway for virion release [98].

**PVB19**

PVB19 uses the host cell nucleus as the site for viral replication, transcription, and encapsidation. As previously mentioned (see section 2), PVB19 proteins have nuclear localization signals that guide the proteins inside the host nucleus. They depend on the host cell DNA replication system as PVB19 does not encode a polymerase [22]. Thus, PVB19 attenuates the host cell cycle to their advantage where certain host cell replication factors are present and active as discussed in the next section.
Cell Cycle Arrest Promotes Viral Replication

**CVB3**

Viruses can induce cell cycle arrest to promote viral replication (see Fig. 4A). Coxsackievirus infection is shown to inhibit host cell proliferation. In HeLa and neuronal progenitor cells CVB3 causes cell cycle arrest at G1/S phase by increasing the ubiquitin-dependent proteolysis of cyclin D1 and p53 [113, 114]. Inhibition of ubiquitin-dependent proteolysis via MG-132, lactacystin, and pyrrolidine dithiocarbamate reduces CVB3 replication in CVB3-induced myocarditis in mice [115, 116]. Moreover, it is shown that the CVB3 VP1 protein can induce cell cycle arrest at the G1 phase in cardiac myocytes by inducing the heat shock protein 70 which in turn downregulates cycline E and upregulates p27kip1 [117]. Data indicate that in the G1 or G1/S phase coxsackieviruses are able to produce more viral polyproteins and viral progeny than in quiescent cells at G0 phase [118]. Apparently, the cell cycle status plays a crucial role in enteroviral replication, persistence, and reactivation. However, in adult cardiomyocytes cell cycle arrest may be less relevant as in other cardiac cells, due to an almost complete stop of cell division.

**PVB19**

The NS1 protein (endonuclease activity) of PVB19 participates in viral DNA replication as it binds to the viral DNA to initiate DNA replication [119]. It is shown that the inhibition of the endonuclease activity of NS1 by certain flavonoid compounds inhibits PVB19 DNA replication, thus making NS1 a promising treatment target [120]. DNA replication of paroviruses mainly occurs in the S-phase of the host cell replication cycle, in which cellular S-phase replication factors are provided, which the virus can use for its own replication. Knocking down S phase factors such as minichromosome maintenance complex protein 2 (MCM2) or MCM5 shows significant decrease in PVB19 DNA replication in erythroid precursor cells (EPCs). Concordantly, NS1 of PVB19 is able to arrest cell cycle in late S phase in EPCs [121].

In conclusion, inhibiting viral protein activity that participates in viral replication can be promising treatment targets. Manipulating cell cycle regulation in favor for the host cell could also be an approach but has to be thoroughly evaluated due to possible teratogenic and carcinogenic effects.

Viral Regulation of Apoptosis

**Enteroviruses (CVB3)**

Enteroviruses are typically known to be lytic viruses. This means that for viral release, they often destruct host cell membranes, which leads to cell death. However, evidence is emerging that enteroviruses can also release virions in a non-lytic way in vesicles also discussed in section 5 [122]. Lytic viruses are known to be able to regulate cell apoptosis to their advantage, i.e. by inhibiting apoptosis in order to efficiently replicate inside the host cells in early stage of infection. In later infection phases enteroviruses induce apoptosis to facilitate the release of the virus via cell lysis [123, 124].

Enteroviruses can use their proteases 2A and 3C to suppress apoptosis by cleaving host cell proteins that are important for apoptosis induction, like retinoic acid-induced gene 1 (RIG-1) [125-127], melanoma differentiation-related gene 5 (MDA-5) [128], TIR domain-containing adaptor-inducing β-interferon (TRIF) [129], and IFN-β promotor stimulator 1 (IPS-1, also called MAVS or visa) [129, 130]. Enteroviruses can also inhibit apoptosis by regulating the phosphoinositide 3-kinase / protein kinase B (PI3K / Akt) pathway. They enhance Akt phosphorylation, which decreases caspase-3- and PARP-cleavage shown in CVB3-infected cells [131]. Furthermore, inhibition of c-Jun NH₂-terminal kinase (JNK)-mediated cell death was shown in poliovirus- and enterovirus 71 (EV71)-infected cells [132, 133] (see Fig. 4B). Other possible ways of inhibiting apoptosis by enteroviruses include
the upregulation of the endoplasmic reticulum transmembrane protein homocysteine-induced ER protein (Herp). This Herp inhibition reduces cytoplasmic Ca\(^{2+}\) by inhibiting the degradation of the Ca\(^{2+}\) channels Inositol 1,4,5-triphosphate receptor (IP\(_R\)) and Ryanodine receptor (RyR), which was shown in poliovirus infection [134]. Also, polioviruses can disturb the transport of the TNF receptor to the cell membrane which inhibits TNF-mediated apoptosis [135]. In CVB3-infected HeLa cells CVB3 increases autophagosome formation by activating calpain which also works anti-apoptotic [136, 137].

Viral proteases can not only suppress aspects of apoptosis but also induce other sets of apoptosis regulators: The 3C protease of EV71 can activate caspase-8 and caspase-9, thus indirectly activating caspase-3 [138]. Furthermore, 2A and 3C can induce cell death by activating caspases that in turn cleave the DNA repair enzyme PARP [139]. Other host cell apoptosis factors that are affected by enteroviral proteases 2A or 3C are the telomere-binding protein PinX1 [140], heterologous ribonucleic acid protein A1 (hnRNP A1) [141], eukaryotic translation initiation factor 4G (eIF4G) [142], death-associated protein (DAP5) [143], and BH3-interacting domain death agonist (Bid) [142].

The 2B proteins in enteroviruses that are expressed by poliovirus, EV71 and CVB3 are viroporins that have pro- and anti-apoptotic effects, depending on the infection phase. Viroporins are proteins which act like ion channels that can semi-selectively conduct ions [144, 145]. These enteroviruses use their viroporins to alter Ca\(^{2+}\) homeostasis and thus apoptosis. The viroporins are inserted into the plasma membrane, mitochondrial membrane, golgi apparatus, ER, and in the sarcoplasmic reticulum (SR). The viroporin insertion leads to a decrease of Ca\(^{2+}\) in the targeted organelles [146, 147]. To compensate the decrease of Ca\(^{2+}\) in the organelles, the cell utilizes capacititative calcium entry (also called store-operated calcium entry (SOCE)), as a result of which the calcium ion channels in the plasma membrane open. This leads to an increase in cytoplasmic Ca\(^{2+}\) [148, 149].

Pro-survival effects occur at the onset of infection and pro-apoptotic effects at later stages, due to the gradual increase of Ca\(^{2+}\) flux from the ER to the mitochondria and into the cytoplasm [150]: When the cytosolic Ca\(^{2+}\) level is only moderately increased, Ca\(^{2+}\)-sensitive transcription factors are activated which support virus replication and activate or accelerate Ca\(^{2+}\)-dependent enzymatic processes. Uptake of Ca\(^{2+}\) by mitochondria can occur via mitochondrial Ca\(^{2+}\) uniporter (MCU), the mitochondrial NCX transporter (NCLX), and possibly through the aforementioned viroporins [151]. Increase of mitochondrial calcium concentration leads to the increase of ATP production usually to counteract a higher energy demand [152]. In addition, the CVB3 viroporin suppresses the cell death stimuli actinomycin D and cycloheximide that would usually activate caspase-3, but a clear connection between Ca\(^{2+}\)-level manipulation induced by the viroporin and the inhibition of cell death stimuli has still to be established [147]. On the other hand, high concentrations of Ca\(^{2+}\) in mitochondria lead to initiation of the intrinsic apoptotic pathway by releasing cyt c and activating caspase-9 which in turn activates caspase-3 and -7 [150, 153, 154]. Moreover, CVB3-induced myocarditis in mice exerts ER stress that promotes apoptosis and other cell death mechanisms. Inhibition of ER stress has an ameliorating effect on disease progression [155]. This can possibly be explained by viroporin expression, which disturbs ER-Ca\(^{2+}\)-homeostasis and intracellular glycoprotein trafficking in the Golgi. This causes Golgi stress and remodeling of the ER membrane [156]. By self-localizing into mitochondria, the 2B protein of EV71 is shown to directly recruit Bcl-2-associated X protein (Bax). Bax in turn alters the permeability of the mitochondrial membrane leading to increased cyt c release and caspase-3 activation [157, 158]. Thus, viroporins exert extensive effects on ion homeostasis manipulating apoptosis and inflammation signaling. Drugs, siRNA, or shRNA that can inhibit viroporin activity and expression are shown to decrease viral titer in several picornavirus-infected cells [153]. CVB3 and EV71 can additionally regulate miRNAs which has pro-apoptotic effects: CVB3 upregulates miRNA-34a in infected rodent cardiomyocytes [159]. EV71 upregulates let-7b in human neuroblastoma cells [160], and it also upregulates miRNA-146a and downregulates miRNA-370 in human rhabdomyosarcoma cells [161], thus making these miRNAs possible treatment targets.
In conclusion, enteroviruses can promote anti- and pro-apoptotic signaling. The virus can switch these opposite processes in a time / phase-dependent way: Activation of the intrinsic apoptotic pathway in CVB3-infected HeLa cells and Green Monkey Kidney cells is initiated at later stages of infection [162-164]. Agol et al. observed a pro-apoptotic phase at the very early stage of infection, followed by an anti-apoptotic stage and another switch to pro-apoptotic signaling in the late stage of infection [165]. By sequentially inhibiting or activating cell signaling, viruses can regulate apoptosis at different stages of infection [123]. One way to control this is by gradually increasing cytosolic Ca²⁺ levels through the expression of viroporins, which is initially pro-survival at moderate Ca²⁺ levels, but promotes apoptosis at later stages with high cytosolic Ca²⁺ concentrations. Furthermore, the previously discussed Herp is shown to reduce cytosolic Ca²⁺ peaks at the early infection stage, in which the cytosolic Ca²⁺ level is gradually increasing, therefore acting as a switch for Ca²⁺ level change during a virus life cycle [134]. Moreover, the aforementioned activation of PI3K / Akt survival pathway is observed to primarily occur at the early stage of infection, and only after active replication of poliovirus, apoptosis is promoted due to phosphorylation of JNK [132].

**PVB19**

NS1 of PVB19 induces apoptosis in erythroid cells, hepatocytes, and monkey epithelial Cos-7 cells by using its nucleoside triphosphate-binding domain and activating TNF-α and mitochondrial / intrinsic caspase pathways. This is possibly mediated via caspase 3, -6, -8, and / or -9, p53 and its downstream cell cycle kinase inhibitors p16⁴⁶ and p21⁴⁶ / ⁴⁷ [125-127, 166, 167]. Also, the small 11 kDa nonstructural protein (see also section 2) promotes apoptosis in erythroid progenitor cells by activating caspase-10 and there is evidence that this protein may be even more pro-apoptotic than NS1 [24]. Gene expression analysis of EMBs with transcriptionally active PVB19 and clinically suspected cardiomyopathy showed decrease in pro-apoptotic marker Bax [9].

In summary, enteroviruses and PVB19 alter cell death which may be to their advantage. Treatment options could include manipulating apoptosis in favor of the host. In patients, high apoptosis rates in acute myocarditis correlates with progression towards fatal heart failure [168]. Apoptosis is correlated with disease severity while inhibition of apoptosis in virus-induced diseases is overall observed to be beneficial in animal models [169]. Inhibiting CVB3-mediated apoptosis using Phyllaemblicin B reduced myocardial damage in mice, suggesting that apoptosis inhibition could be generally beneficial when it comes to therapeutic applications [170]. In line, activating apoptosis by inhibiting the anti-apoptotic STAT3 pathway showed worsening of ventricular function and correlated with reduced survival in CVB3-induced myocarditis in BALB/c mice [171].

**Viral Effects on Energy Metabolism**

**CVB3**

Several studies show that the energy metabolism is drastically altered during viral myocarditis. In mice, reduction of mitochondrial ATP/ADP ratio is observed, presumably due to the production of autoantibodies against the adenine nucleotide translocator (ANT), which transports ADP and ATP [172]. A gene array performed for CVB3-induced myopathy in mouse hearts showed that genes involved in mitochondrial oxidative phosphorylation and fatty acid metabolism were downregulated [173]. In mouse atrial cardiomyocytes mitochondrial fragmentation was observed [174]. It can be verified that CVB3 proteins localize to the mitochondria and stimulate dynamin related protein 1 (drp1)-mediated mitochondrial fission which leads to the release of EMVs containing viral progeny and fragmented mitochondrial components. This process can be reversed by inhibiting drp1 e.g. via Mdivi-1, so that EMVs are not extruded. Instead, the EMVs could be released via cytolytic cell death, which could make the virus more vulnerable to neutralizing antibodies. Indeed,
mice show improved survival rates, reduced mitochondrial dysfunction, and reduced inflammatory cell invasion after drp1 inhibition [174, 175].

The mitochondrial respiratory chain (RC) consists of five complexes that transport electrons while creating a proton gradient which is used to produce ATP. A dysfunction in this chain leads to the misdirection of electrons and the formation of reactive oxygen species (ROS), which cause oxidative stress that damages proteins, lipids, and DNA, resulting in apoptosis [176-178]. Ebermann et al. conducted an analysis on the effects of CVB3 on each complex of the RC, comparing two mouse strains: C57BL/6 mice which are able to rapidly eliminate the virus after an acute phase of infection and recover, therefore being regarded resistant against CVB3, and A.SW/SnJ mice in which CVB3 persists and results in chronic myocarditis [179]. In brief, it was uncovered, that the CVB3-resistant C57BL/6 mouse strain had decreased complex IV activity. Interestingly, the activity of complex I and complex III, which are mainly responsible for ROS production, was increased and a higher level of ROS was found compared to the CVB3-premissive mouse strain. Also, the CVB3-resistant mice showed higher expression of Bax, Bcl-2, and caspase-3, which are part of the intrinsic apoptosis mechanism. This can possibly be explained by ROS level elevation. Removal of ROS is shown to be disturbed in C57BL/6 mice as catalase, which removes H2O2, is extremely downregulated. In contrast, in the CVB3-permissive A.SW/SnJ mice all RC complexes were inhibited, which would imply that ATP generation is also reduced. The ROS level is lower possibly due to the less-active RC and activation of ROS-removing enzymes. As a possible consequence, the activation of the intrinsic mitochondrial apoptosis pathway is reduced compared to CVB3-resistant mice. Thus, imbalanced RC, higher ROS level, and apoptosis can correlate with higher virus elimination which stops the transition from acute to chronic infection. In fact, reduction of ROS by a potent antioxidant in CVB3-infected HeLa cells fails to inhibit virus replication [116]. In iPSC-derived ventricular cardiomyocytes that express CVB3 and in patients with acute CVB3 myocarditis, ROS levels and oxidative stress was elevated [180, 181]. It would be interesting to investigate whether patients with chronic myocarditis exert reduced oxidative stress. Further, elevated expression of the NADPH homologue Nox4 is observed in CVB3-infected HeLa cells fails to inhibit virus replication [116]. In iPSC-derived ventricular cardiomyocytes that express CVB3 and in patients with acute CVB3 myocarditis, ROS levels and oxidative stress was elevated [180, 181]. It would be interesting to investigate whether patients with chronic myocarditis exert reduced oxidative stress. 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**Viral Effects on Ion Channels**

Correct and rhythmic cardiac contraction depends on multiple aspects, most importantly the intracellular Ca^{2+} cycling and the generation of action potentials (AP), because they play the crucial role in the calcium-dependent excitation-contraction-coupling in the heart [183]. The action potential in the heart is mainly formed by the currents \( I_{Na}, I_{to}, I_{Ca}, I_{Kr}, I_{Ks}, \) and \( I_{K1} \) which are conducted by cardiac ion channels [184]. A study which analyzed three patient cases with myocarditis suggests that an underlying channelopathy is one possible predisposition for triggering ventricular fibrillation and sudden death after acute myocarditis [185].

**CVB3**

Steinke et al. showed in CVB3-expressing *Xenopus laevis* oocytes that, currents of the cardiac ion channels KCNQ1 (also called Kv7.1) / KCNE1 (\( I_{Ks} \)) are elevated, while the currents of the rapidly activating delayed rectifier potassium channel hERG1 (\( I_{Kr} \)) and the voltage-dependent Ca^{2+} L-type channel Cav1.2 / a2d1/ b2 (\( I_{Ca} \)) are reduced. The distribution of these channels in the cardiomyocyte membrane of CVB3-infected mice is altered, too [186]. The CVB3 proteins 2A, 2BC, 3A, and 3B have significant effects on the currents of these three ion channels. At the same time, serum and glucocorticoid-regulated kinase 1 (SGK1) is shown to be elevated, which upregulates the Rab11-dependent transport of vesicles containing ion channels like KCNQ1 / KCNE1, which would explain the enhanced KCNQ1 / KCNE1 localization in the plasma membrane (see Fig. 4C) [187, 188]. SGK1 expression is particularly elevated under stress conditions, which may explain why fibrillations in CVB3 myocarditis patients occur especially after stress or intense body labor [189]. Inhibition of SGK1 in CVB3-expressing oocytes leads to reduction of the KCNQ1 / KCNE1 current back to a healthy level, making it an interesting field of future study as a possible therapeutic treatment against pro-arrhythmic events [186]. Yang et al. investigated the effects on ion currents in rodent cardiomyocytes after inoculation with CVB3 as well [190]: The L-type Ca^{2+} channel current \( I_{Ca} \) and the outward potassium current \( I_{out} \) increased while inward rectifying potassium \( I_{K1} \) decreased. They also observed a higher expression of voltage-gated potassium channels i.e. Kv1.2, Kv2.1, and Kv4.2. Upon treatment of CVB3-infected rats with taurine and *astragalus membranaceus*, the alteration of the currents via CVB3 was prevented, thus qualifying these chemicals as promising treatment strategies.

**PVB19**

Ion channel expression and function is also relevant in vascular endothelial cells, which are infected by PVB19. They are associated with cell membrane potential generation, signal transduction, hemodynamics, and vasomotor functions. Therefore, dysfunction of ion channel expression and function can lead to vascular diseases [191]. Recent studies have shown that the VP1 region of PVB19 has a phospholipase A2 (PLA2)-like activity that can produce lysophosphatidylcholine and lysophosphatidic acid [192, 193]. Lysophosphatidylcholine can cause adverse effects on endothelial cells such as enhanced inflammation, disruption of mitochondrial integrity, induction of apoptosis and altered ion channel regulation [194]. This has a major effect on several endothelial ion channels as well. Lysophosphatidylcholine could activate the store-operated or capacitative Ca^{2+} channel \( I_{CRAC} \) in human endothelial cells, thus leading to accelerated increase of Ca^{2+} entry, which is important for endothelial vasodilation [195, 196]. Lysophosphatidic acid has a plethora of effects on ion channels including T-type Ca^{2+} channels and inward rectifier K+ and Calcium-activated K+ channels (\( \text{IK}_{Ca} \)) that are relevant in cardiovascular endothelial cells [191, 193]. Secondly, PLA2-like activity of VP1 upregulates the activity of epithelial Na+ channel ENaC which results in early swelling and later stiffening of the vascular endothelium [197]. Thirdly, downregulation of potassium channels such as Kv1.3, Kv1.5, Kir2.1 channels and inhibition of the Na+/K+-ATPase activity by VP1 leads to cell swelling and thus contributes to endothelial dysfunction [198, 199]. PLA2 also appears to be relevant for efficient transfer of the viral genome from...
lysosomes and endosomes into the nucleus for viral replication [200]. Hence, the study and development of phospholipase inhibitors as antiviral drugs against PVB19 infection could be beneficial for patient treatment.

**Conclusion**

In summary, PVB19 and enteroviruses can affect the host cells in terms of host immune system, ECM remodeling, replication, apoptosis, autophagy, energy metabolism, ion channels, and ion conductivity (see Fig. 3 and 4). To this date there are no effective treatment strategies against viral myocarditis, but in-vitro studies, animal models, and clinical trials show promising treatment targets which should be pursued further. Especially with emerging viruses such as SARS-CoV-2 which may have detrimental cardiac effects and can also cause myocarditis, it is highly relevant to find new treatment strategies against VMC [201]. To better understand the viral mechanisms and find new treatments, good disease models that resemble viral diseases in humans are also of great importance. In-vitro models for CVB3-induced myocarditis using commonly used cell lines i.e. HeLa-cells, HL-1, and H9c2 and iCell® Cardiomyocytes were shown to differ between each other after CVB3 infection regarding permissiveness, infection pattern, and mode of cell death. Therefore, in-vitro studies with commonly used cell lines have to be further evaluated whether they authentically represent the CVB3 pathogenesis. Amongst the compared cell lines, iCell® Cardiomyocytes were found to be the most suitable infection model. Infection rates were significantly higher compared to other cell lines with rates at about 100%. Cells were homogenously infected, and infected cells resembled CVB3-infected mouse hearts morphologically [202]. Another CVB3–iPS cell model recently published by Peischard et al. is also a promising disease model to analyze the effect of CVB3 in possibly any kind of cells in-vitro in a more controlled manner due to the implemented Tet-On system [181].

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