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Intricacies of cardiac damage in coxsackievirus B3 infection: Implications for therapy

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Abstract

Heart disease is the leading cause of death in humans, and myocarditis is one predominant cause of heart failure in young adults. Patients affected with myocarditis can develop dilated cardiomyopathy (DCM), a common reason for heart transplantation, which to date is the only viable option for combatting DCM. Myocarditis/DCM patients show antibodies to coxsackievirus B (CVB)3 and cardiac antigens, suggesting a role for CVB-mediated autoimmunity in the disease pathogenesis; however, a direct causal link remains to be determined clinically. Experimentally, myocarditis can be induced in susceptible strains of mice using the human isolates of CVB3, and the disease pathogenesis of postinfectious myocarditis resembles that of human disease, making the observations made in animals relevant to humans. In this review, we discuss the complex nature of CVB3-induced myocarditis as it relates to the damage caused by both the virus and the host's response to infection. Based on recent data we obtained in the mouse model of CVB3 infection, we provide evidence to suggest that CVB3 infection accompanies the generation of cardiac myosin-specific CD4 T cells that can transfer the disease to naïve recipients. The therapeutic implications of these observations are also discussed.

Keywords

Heart; viral myocarditis; coxsackievirus; autoimmunity

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1. Introduction

Dilated cardiomyopathy (DCM), a myocardial disease that commonly results in congestive heart failure, is a challenging clinical entity with no known cure or specific causes, and it is a major indication for heart transplantation. The five-year survival rate of patients with DCM is less than 50% [1]. DCM can develop in individuals affected with myocarditis, for which various non-infectious and infectious agents have been implicated. Prominent among the infectious causes for myocarditis are enteroviruses like coxsackievirus B3 (CVB), a *bona fide* pathogen of the cardiovascular system. In the U.S., approximately five million enteroviral infections are attributed to CVB1-5. A proportion of these (12%) may have myocardial involvement in which CVB1, CVB3 and CVB5 serotypes are commonly implicated [2, 3]. Serologically, CVB3-reactive antibodies are found in about 50% of DCM patients, while enteroviral genomic material can be detected in up to 70% [4-8], suggesting that CVB3 infection is an important environmental predisposing factor for the development of DCM. In this review, we discuss the mechanisms related to the initial damage caused by the virus and how such damage can later be precipitated by the host's response to infection, leading to the establishment of self-destructive (autoimmune) phenomena and their implications for therapy in those affected.

2. Virus life cycle

Coxsackievirus, a member of the genus enterovirus, is a positive-sense single-stranded RNA virus belonging to the *Picornaviridae* family [9, 10]. Six serotypes have been identified (CVB1 to 6) and our focus is CVB3. The CVB3 viral genome consists of 7400 bases, and a single open reading frame flanked by 5’ and 3’ non-translated regions (NTRs) at the termini. Additionally, multiple secondary stem-loop structures can be formed in the 5’ NTR, which is known to harbor molecular determinants of viral pathogenicity [11, 12]. However, for replication of the viral genome, both the 5’ and 3’ NTRs can act as binding sites for a viral genome-linked protein (VPg), also called 3B [13, 14]. The viral genome encodes for a large polyprotein, which is proteolytically cleaved to produce structural and nonstructural (NS) proteins (Fig. 1; [15]. While structural proteins are required for virus assembly, NS proteins mediate the processing of viral polyprotein and replication of the viral genome [15-17]. The CVB3 genome lacks a 5’ 7-methyl guanosine cap structure, which is typically seen in most eukaryotic and many positive-sense viral RNAs and is needed to facilitate translation [18, 19]. Instead, the 5’ NTR, which accounts for 10% of the viral genome (742 out of 7400 nucleotides [nts]), contains an internal ribosome entry site (IRES) and mediates translation of positive-sense viral RNAs [20, 21].

For any productive infection, viruses have to enter host cells, multiply, and release progeny of infectious virions from the infected cells. The usual target tissues for CVB3 are heart and pancreas, although other organs such as brain, prostate, testis, liver, lung, and intestine can be infected [15, 22, 23]. Virus entry into the target tissues is mediated by two receptors: decay accelerating factor (DAF/CD55) and coxsackievirus and adenovirus receptor (CAR; Fig. 1) [24, 25]. Most tissues express DAF, a glycosyl-phosphatidylinositol-anchored membrane protein. The initial attachment of the virus occurs first through DAF, resulting in the rearrangement of cytoskeletal actin that involves activation of Abl and Fyn kinases [25].

This process facilitates movement of CVB3 along the apical surface of the cell membrane, which provides access to CAR in the tight junctions of epithelial cells [26, 27]. In contrast to DAF, CAR acts as an internalization receptor in the target cells, where virus interacts with CAR's two extracellular Ig domains, D1 and D2 [24]. This interaction triggers Fyn-mediated phosphorylation of caveolin-1, leading to endocytosis of the virus [26, 27] and subsequent uncoating of the RNA genome (positive-strand) into the cytoplasm.

The positive-strand RNA translates into a large polyprotein by a 5' cap-independent mechanism, whereby the IRES region of 5' NTR acts as a 'ribosome landing pad' [20, 21]. The polyprotein is then proteolytically cleaved by two viral proteases – 2A protease $^{(pro)}$ and $3C^{pro}$ – to generate three protein clusters, P1, P2, and P3, through *cis*-cleavage [28]. These protein clusters undergo a series of *trans*-cleavage steps to yield individual proteins: P1 – structural or capsid proteins (viral protein [VP]1, VP2, VP3, and VP4); P2 – $2A^{pro}$, 2B, 2C; and P3 – 3A, 3B, $3C^{pro}$, and 3D polymerase $^{(pol)}$ [Fig. 1;[28, 29]. In the meantime, $2A^{pro}$ and $3C^{pro}$ can shut off host protein synthesis by their proteolytic activity, allowing the virus to take control of infected cells. For example, $2A^{pro}$ cleaves eukaryotic initiation factor (EIF)-4γ, poly (A) binding protein, and cytoskeletal dystrophin, in addition to triggering the caspase-3-dependent apoptotic pathway [30-32]. Similarly, $3C^{pro}$ also can cleave host transcription factors (TATA-binding protein, cAMP response element binding protein, octamer-binding transcription factor), and Bax and Bid proteins, resulting in sequestration of mitochondrial cytochrome c into cytoplasm and activation of the apoptotic pathway [33-36]. While both 2B and 3A proteins inhibit vesicular transportation from the Golgi complex and exocytosis of cellular proteins, protein 2C causes disassembly of the Golgi complex and the endoplasmic reticulum [ER; [37-39]. The $3D^{pol}$ (RNA-dependent-RNA polymerase) generated from the initial translation adds two uridine residues to VPg by a process called uridylation, thereby acting as a primer for the synthesis of negative-strand RNA from the poly (A) tail [40, 41]. The negative strand then acts as a template for multiple copies of positive-strand viral genome to be synthesized by $3D^{pol}$; the positive strands are then packaged with the structural proteins and form infectious virions. The viral protein 2B, also called viroporin, oligomerizes and enters the cell membrane to form channels through which virions are released by cell lysis [42].

During the replicative cycle of the virus, however, dsRNAs can be generated utilizing the negative- and positive-strand viral genomes [43]. But the viral 2C protein, exhibiting nucleoside triphosphatase (NTPase) activity, dissociates dsRNA by unwinding [44, 45]. Variants of CVB3 with lower cytolytic activity can persist in the host as dsRNAs [43], but their identity as infectious viral particles has not been reported. Whether enteroviral RNAs like CVB3 present in DCM patients as reported in the literature [46-48] represent dsRNAs also remains to be investigated.

3. Pathogenesis of CVB3 infection

Studies from various experimental mouse models of CVB3 infections suggest that the disease course of myocarditis assumes two distinct stages that occur in continuum [49, 50]: the acute phase (14-18 days postinfection), in which infectious virus is present, causing damage to cardiomyocytes; and the chronic phase (beyond 18 days postinfection), in which

inflammation persists, although the extent of virus replication is much reduced due to selection of a defective virus [50, 51]. This is consistent with the observation that infectious CVB3 cannot be isolated in cardiac tissues from patients with DCM [52, 53]. To understand the immune pathogenesis of viral myocarditis, numerous mouse models have been developed involving the use of various strains of CVB3 [54-58]; Table 1].

3.1.Virus-induced cardiac damage

CVB3 replication can induce myocardial injury through apoptosis and necrosis of cardiomyocytes. The NS proteins generated from translation of the viral genome can significantly affect the structure and functions of cellular proteins (Fig. 2). Some of these include:

Shutdown of host proteins and cleavage of transcription factors—Notably, $2A^{pro}$ protein cleaves cytoskeletal dystrophin, and dystrophin-associated glycoproteins such as α-sarcoglycan, β-dystroglycan, and extracelluar laminin-2 [30, 59, 60]. Dystrophin is critical for connecting with the contractile protein F-actin within the cardiomyocytes [61, 62]. Dystrophin deficiency may be relevant to human disease because patients affected with familial DCM show lack of dystrophin production [61, 62]. Likewise, viral protease $3C^{pro}$ can alter translation of cellular proteins by cleaving transcription factors such as TATA-binding protein, cAMP responsive element, and octamer-binding protein [34-36].

Cell cycle arrest—Arrest in cell cycle progression has been reported in CVB3 infection, due to reduced synthesis or proteasome-mediated degradation of cyclin-D1 following the degradation of EIF-4γ by viral protease $2A^{pro}$ [63].

Inhibition of vesicular transport—By blocking the transportation of proteins from the ER to the Golgi complex, viral protein 3A can inhibit exocytosis of secretory cellular proteins [37, 38].

Apoptosis—Cardiomyocytes infected with CVB3 can show DNA fragmentation and apoptotic bodies as early as nine hours postinfection, and viral proteases ($2A^{pro}$ and $3C^{pro}$) are believed to mediate this process [64, 65].

Cell lysis—Being a cytolytic virus, CVB3 can injure the cell membrane, leading to lysis of cells by increasing the membrane permeability and pore formation [42, 66].

While the above mechanisms point to the possibility that the virus can directly damage cardiomyocytes as long as it is present in the tissues during acute infections, the pathomechanisms underlying the persistence of inflammation during the later stages of the disease process in the absence of detectable infectious viral particles remain elusive. Nonetheless, CVB3 RNA can be detected in the chronic stages in infected animals through 21 days postinfection, and the viral genomes may contain deletions of 7 to 49 nucleotides in their 5’ termini [51]. Likewise, viral genomic material containing 5’ terminal deletions was detected from heart tissue from a fatal case of enterovirus-associated myocarditis [67]. Whether such defective viruses can reactivate and contribute to tissue damage like that caused by wild type virus has not been proved as viruses with 5’ terminal deletions were

non-cytolytic. However, evidence suggests that the defective virus can replicate in cell culture; mice inoculated with this virus showed the presence of viral RNA for up to 25 weeks, but their hearts or pancreases were normal [51]. Thus, the notion of immune-mediated damage is gaining more attention in explaining the disease pathology in the chronic stages.

3.2. Immune pathogenesis

As CVB3 injures cardiomyocytes, the tissue destruction can be potentiated by inflammatory cytokines (interleukin [IL]-1, IL-6 and tumor necrosis factor [TNF]-α) produced by cells of the innate (e.g., macrophages) and adaptive (antiviral, T helper [Th]1 and Th17 cytokines) immune systems (Fig. 3). Toll-like receptors (TLRs) can influence the severity of CVB3-induced myocarditis: mice deficient for TLR-3 developed more severe myocarditis than their wild type littermates, whereas TLR-4-deficient mice developed less severe myocarditis [68, 69]. Furthermore, expression of TLRs can occur gender dependently: elevated expression of TLR-4 in the macrophages of male mice contributed to mortalities in CVB3-infected animals, but the underlying mechanisms are unknown [70, 71]. In contrast, the roles played by certain cellular components of the innate immune system, such as gamma delta (γδ) T cells and natural killer (NK)-T cells, are more complex, in that γδ T cells and NK-T cells perform opposing functions (Fig. 3). $V\gamma4^+$ γδ T cells promote CVB3-induced myocarditis by killing regulatory T (Treg) cells through CD1d-mediated lysis and support interferon (IFN)-γ- production by CD4 T cells. NK-T cells, however, favor generation of Treg cells and dampen the inflammatory response triggered by γδ T cells. γδ T cells also can induce cardiac damage by killing the virus-infected CD1d-expressing cardiomyocytes by Fas/Fas-L pathway [72-74], while NK cells are protective in CVB3-induced myocarditis: mice depleted of NK cells showed increased viral titers and exacerbated myocarditis [75, 76]. Recent studies indicate that CVB3 infection can lead to degranulation of mast cells within 6 hours postinfection and to production of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, granulocyte macrophage-colony stimulating factor and other molecules (histamine, heparin, and proteases). These mediators have the potential to precipitate the severity of viral myocarditis [77].

The relevance of the molecular mimicry hypothesis in the causation of CVB3-induced myocarditis also has been tested, but direct evidence is lacking (Fig. 3). Anti-streptococcal antibodies and anti-streptococcal T cells were shown to bind CVB3 and cardiac myosin; these responses were shown to be pathogenic [78-84]. Similarly, the phenomenon of epitope spreading also may be relevant to the pathogenesis of viral myocarditis, because pathogens like CVB3 that primarily infect hearts can lead to secondary generation of autoimmune responses resulting from release of self-antigens due to cardiac injury (Fig. 3). Either the newly released antigens are taken up by the resident antigen-presenting cells (APCs) (e.g., dendritic cells, DCs), or APCs can engulf cardiomyocytes infected with an infectious agent, leading to induction of pathogenic CD4 Th and/or cytolytic T lymphocyte (CTL) responses by cross-priming. Similarly, under the influence of Th cytokines, B cells sensitized with self-antigens can precipitate cardiac damage by producing pathogenic autoantibodies (Fig. 3). But in these scenarios, presence of efficient Treg cells can dampen inflammatory responses. For example, adoptive transfer of Treg cells prior to infection with CVB3

protects mice from developing myocarditis [85], pointing to a possibility that autoimmune responses accompany CVB3 infection.

In support of autoimmune theory, autoantibodies to various cardiac antigens – such as cardiac myosin heavy chain (Myhc)-α, adenine nucleotide translocator 1 (ANT), β-adrenergic receptor-1 (BAR), branched chain α-ketoacid dehydrogenase (BCKD), cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase (CATP), laminin, and the muscarinic receptor – have been detected in patients with myocarditis and DCM, including experimental CVB3 myocarditis models [86-93]. But the pathological significance of these autoantibodies continues to be uncertain. Evidence for the generation of autoreactive T cells in CVB3 infection was first provided based on the finding that the CTLs obtained from Balb/C mice infected with CVB3 lysed the cardiomyocytes, and the T cells transferred the disease into naïve animals, but their antigen-specificity was not known [56, 94]. To delineate the autoimmune mechanisms in CVB3 myocarditis, we recently created major histocompatibility complex (MHC) class II dextramers (the new generation MHC class II tetramers) for Myhc-α 334-352, permitting us to determine the antigen-specificity of autoreactive CD4 T cells that might be generated in infected mice [55, 95]. Myhc-α has been recognized as a major autoantigen candidate in heart autoimmunity, and CD4 T cells sensitized with Myhc-α or its immunodominant epitopes induce autoimmune myocarditis, the phenotype of which resembles postviral myocarditis in humans [96, 97]. For example, Myhc-α 334-352 and Myhc-α 614-643 induce lymphocytic myocarditis in A/J and Balb/c mice, respectively [98, 99]. Using Myhc-α 334-352 dextramers, we demonstrated that A/J mice infected with CVB3 showed generation of Myhc-α-specific CD4 T cells and that antigen-specific CD4 T cells infiltrate hearts in infected mice [55]. More importantly, using adoptive transfer protocols, we showed that autoreactive CD4 T cells generated in mice infected with CVB can transfer myocarditis to naïve mice. The pathological changes observed in these mice were restricted to hearts, as pancreases were normal [55]. In addition, T cells sensitized with irrelevant antigen and virus did not induce disease. Furthermore, by proving that the naïve repertoire does not contain a significant proportion of Myhc-α-reactive T cells, we concluded that generation of the Myhc-α-reactive T cells was secondary to the damage caused primarily by the virus [55]. We proposed that when intracellular proteins like Myhc-α are released as a consequence of the cardiomyocytolytic effect of viruses, they become visible to the immune system, leading to the induction of pathogenic autoimmune responses. This may be the reason inflammation persists in the absence of recoverable infectious virus in chronically infected mice. Similar observations are made in human DCM patients, who exhibit the signature of CVB infections, but actively replicating virions are rarely detected in cardiac tissues [100].

4. Antiviral immune responses and viral clearance

Experimentally, the two phases of CVB3 infection (acute and chronic) occur in continuum, but the infectious virus particles cannot be detected in mice beyond 14 to 18 days postinfection; furthermore, successful viral clearance may require the participation of both B cells and T cells [50, 57, 94, 101-103]. The importance of antibodies in the attenuation of viral myocarditis is demonstrated by the observation that animals infected with CVB3 could generate strong virus-specific neutralizing antibodies, and that B cell-deficient mice were

unable to resolve CVB3 infection and showed elevated viral titers [102, 104, 105]. Sex hormones appear to influence the disease outcome. Male Balb/C mice infected with CVB3 showed robust disease-inducing IgG2a and IFN-γ-producing CD4 T cells (Th1-type), as opposed to females, in which dominant IgG1 and IL-4-producing CD4 T cells (Th2-type) favored disease resistance [106]. Previously, severity of CVB3-induced myocarditis was shown to be relatively high in mice deficient for CD8, but the disease was attenuated in mice deficient for CD4 [103, 107]. Conversely, mice deficient for both CD8 and CD4 were protected, suggesting that both cell types modulate the disease outcome [103].

The data available on the roles played by Th cytokines, however, are conflicting. For example, IFN-γ-knockout mice infected with CVB3 were protected from myocarditis, while reconstitution with recombinant IFN-γ restored disease susceptibility [108]. Similarly, IL-12Rβ1-deficient mice infected with CVB3 showed decreased viral replication and reduced inflammation in the hearts, whereas IFN-γ-deficiency exacerbated viral replication [69]. In other studies, however, IL-12 was found to be protective in CVB3-induced myocarditis by increasing the production of IFN-γ [109]. Furthermore, non-obese diabetic mice transgenically expressing IFN-γ specifically in the pancreas were found to be protected from CVB3 infection [110]. In contrast, IL-4-deficient C57Bl/6 and IL-13-knockout Balb/C mice developed severe myocarditis with prolonged viral persistence, suggesting that Th2 cytokines promote disease protection in CVB3 infection [111, 112]. Recent studies indicate that IL-17 produced by Th17 cells is a critical mediator of CVB3 pathogenesis, as IL-17 levels were elevated in both acute and chronic phases of viral infection [113-115]. Furthermore, blockade of IL-17 using neutralizing antibodies led to reduction in myocardial damage as well as viral replication [115, 116]. The question of whether induction of robust IL-17 responses coincides with viral clearance requires additional studies. However, neutralization of IL-22 in IL-17A-deficient mice decreased the severity of myocarditis and enhanced viral replication, raising the question whether Th17 and Th22 cells display differential effects on CVB3 pathogenesis because IL-22 can be secreted by both subsets [117].

Finally, formation of dsRNAs, although transitory, can interact with TLR-3, and TLR-7 and induce synthesis of type I interferons (IFN-α and IFN-β) by activating interferon regulatory transcription factor (IRF)-3 and IRF-7 pathways [Fig. 3; [118, 119]. IFNs can then activate two host proteins – RNA-dependent protein kinase and 2’ 5’-oligoadenylate synthetase – resulting in the synthesis of EIF-2α and ribonuclease (RNase) L [120]. While EIF-2α inhibits viral replication, RNase L promotes degradation of the viral genome, but both can trigger apoptosis [121-124]. In addition, EIF-2α can induce the transcription of apoptotic genes by enhancing the synthesis of activating transcription factor-4. Similarly, RNase L can activate c-Jun-NH2-terminal kinase and promote the transcription of apoptotic genes and/or activation of the mitochondrial death pathway [121, 122]. Thus, apoptosis of infected cells is considered to be one of the important mechanisms of viral clearance. Consistent with this notion, it has been recently demonstrated that selective ablation of type I IFN receptor in cardiomyocytes leads to exacerbation of myocarditis likely due to a delay in the clearance of virus [125].

5. Therapeutic implications

Various therapeutic strategies have been developed in the medical management of patients with myocarditis/DCM. These include the use of antiviral compounds, interferons, intravenous immunoglobulin therapy, and immunosuppressive agents such as corticosteroids, and azathioprine [Table 1; [126-128]. None of these therapeutic agents, however, have proven to be effective in preventing the disease progression nor are these generally recommended for all patients regardless of the stage of disease. Similarly, no clear consensus has emerged for the use of immunosuppressive drugs because the supporting data are lacking. These judgmental limitations are in part due to the practical difficulties to differentiate patients with or without infectious origin in the clinical settings. Thus, for these groups of patients, the use of antiviral compounds and immunosuppressive drugs has been suggested as therapeutic modalities [126-128].

Experimentally, the use of animal models offers the advantage of testing the efficacy of therapeutics under defined conditions. To this end, as exemplified in Table 1, a variety of compounds such as antiviral drugs, small molecules, and natural medicines have been shown to attenuate the severity of acute CVB3 myocarditis through multiple mechanisms. Likewise, immunologically, costimulatory molecules, such as B7, CD40 and cytotoxic T-lymphocyte antigen (CTLA)-4, have been successfully targeted for therapy, in addition to the use of cytokines (Table 1). The observations made in these animal models are relevant to CVB3 infections in humans because human isolates of CVB3 induce disease in mice with comparable pathologies, and the disease patterns also are similar, in that the disease course assumes two stages [50, 57]. While the acute phase is accompanied by multiplication of virus and cardiac inflammation, the chronic phase is devoid of actively multiplying viruses, yet inflammation continues to persist. In these circumstances, autoimmunity becomes a prime suspect. Our recent data showing the appearance of pathogenic Myhc-α-reactive T cells in mice infected with CVB3 is the first direct evidence to prove that autoimmunity can be an important component of CVB3 pathogenesis. Whether such an outcome is possible in humans remains to be investigated. If testing reveals this hypothesis to be true, it may provide a basis for the use of immune suppressive therapy in DCM patients who test positive for CVB3. Additionally, it also may create avenues to develop other immunologic strategies, like antigen-specific T cell therapies, to dampen autoimmune responses. Two such examples are the use of altered peptide ligands and induction of tolerance by administering soluble antigens via oral or nasal routes [129-132]. As a proof of principle for the latter approach, mice tolerized with Myhc-α and later infected with CVB3 have shown decreased severity of myocarditis, an effect mediated through T cells but not antibodies [129]. Thus, in the long term, we propose that antigen-specific T cell-based therapies may be a viable alternative to the use of chemotherapy and heart transplantations for myocarditis/DCM patients.

Conversely, it may be possible to prevent the occurrence of CVB3-mediated myocarditis through vaccination, but no commercial vaccines currently are available for use in humans. Nevertheless, experimentally, various vaccination strategies, such as the use of CVB3 virus-like particles, attenuated viral strains, and priming and boosting with CVB3 DNA and recombinant viral protein, respectively, have been shown to effectively confer varying degrees of protection [133-136].

CVB3, a *bona fide* pathogen of cardiovascular system, is ubiquitously present in the environment, making it possible that most humans may have a chance of exposure to this virus at some point in their lifetime. Although, the disease induced with CVB3, in particular, myocarditis may go unnoticed, but chronically, few of these individuals may develop DCM. Due to the lack of effective chemotherapy options, heart transplantation has become a critical necessity for patients with end-stage disease. For such patients in whom no infectious virions are present in cardiac biopsies, it becomes difficult to justify the use of antiviral drugs. The pathogenesis of CVB3 infection is complex in that tissue destruction involves the mediation of both the virus, through its lytic properties, and the host, via immune-mediated damage, importantly through the induction of pathogenic autoreactive T cell responses, as we have recently demonstrated in the mouse model. Thus, we have begun to provide answers for previously unanswered questions about why inflammation persists in the absence of detectable infectious virions, which if otherwise present, would be expected to continue to damage the cardiac tissue. Our data may support the notion that the immune-mediated damage is superimposed on the initial tissue destruction caused by the virus, but once autoimmune response sets in, the disease process becomes perpetual, at which stage, immune-suppressive therapies may become the only therapeutic option. Finally, it is to be noted that enteroviral infections have been shown to be associated with various other chronic disease conditions, such as Type I diabetes, coeliac disease, and asthma [137-141]. Whether their pathogeneses involve the mechanisms similar to myocarditis triggered by CVB3 may be a worthy area for further investigations.

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List of abbreviations

ANT	adenine nucleotide translocator 1
APCs	antigen-presenting cells
BAR	β-adrenergic receptor-1
BCKD	branched chain α-ketoacid dehydrogenase
CAR	coxsackievirus and adenovirus receptor
CATP	cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase
CTL	cytolytic T lymphocyte
CTLA	cytotoxic T-lymphocyte antigen
CVB	coxsackievirus B
DAF	decay accelerating factor
DCM	dilated cardiomyopathy
DCs	dendritic cells

EIF	eukaryotic initiation factor
IL	interleukin
IRES	internal ribosome entry site
IRF	interferon regulatory transcription factor
MHC	major histocompatibility complex
Myhc	cardiac myosin heavy chain
NK	natural killer
NS	nonstructural
NTPase	nucleoside triphosphatase
NTRs	non-translated regions
nts	nucleotides
RNase	ribonuclease
Sh	short hairpin
Th	T helper
TLR	Toll-like receptors
TNF	tumor necrosis factor
Treg	regulatory T
VPg	viral genome-linked protein
γδ	gamma delta

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Highlights

- Coxsackievirus B3 (CVB3) is a common suspect in patients with chronic myocarditis/DCM
- Autoimmunity is suspected, but a direct causal link remains elusive clinically
- Damage caused by autoreactive T cells may be a key mechanism in chronic viral infections

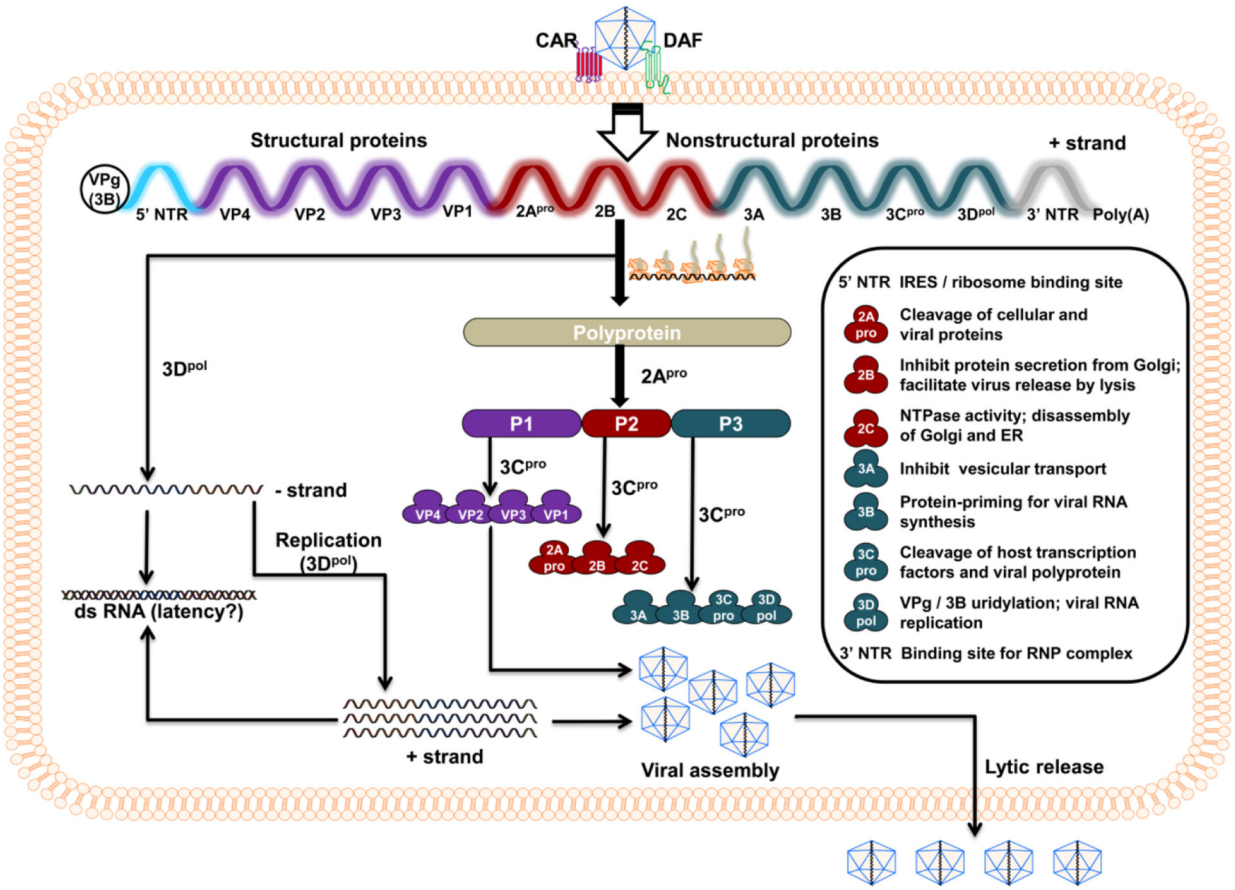

Fig. 1. The life cycle of CVB3

Virus entry into the target cells first requires interaction with DAF, which facilitates viral attachment to CAR, leading to internalization of the virus in the cytoplasm. After uncoating, the positive-sense single-stranded RNA genome is translated to yield a large single polyprotein. This process requires binding of ribosomes to IRES. The polyprotein is then proteolytically cleaved to generate structural (P1 cluster: VP1 to VP4) and NS (P2 cluster: $2A^{pro}$, 2B and 2C; P3 cluster: 3A, 3B, $3C^{pro}$ and $3D^{pol}$) proteins by $2A^{pro}$ and $3C^{pro}$, two viral proteases. While the structural proteins, also called capsid proteins, contribute to the conformation of the virus, NS proteins mediate a variety of functions as indicated in the inset. During viral replication, $3D^{pol}$ plays a critical role in the formation of negative and positive strands of viral genomes, which can be paired to form dsRNA as an intermediate stage. The progeny virus containing a single-strand positive-sense RNA genome and structural proteins is finally released through cell lysis.

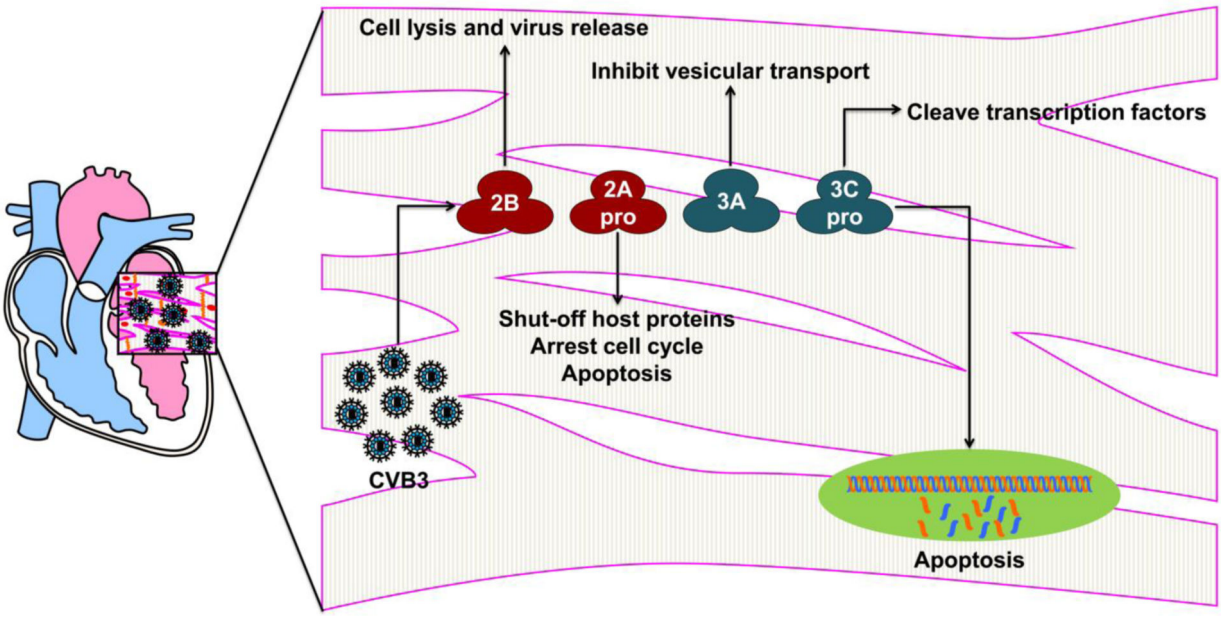

Fig 2. Pathogenic mechanism of CVB3-induced cardiac damage

In cardiomyocytes infected with CVB3, the viral genome is translated to yield several NS viral proteins, whose main functions are highlighted in the inset.

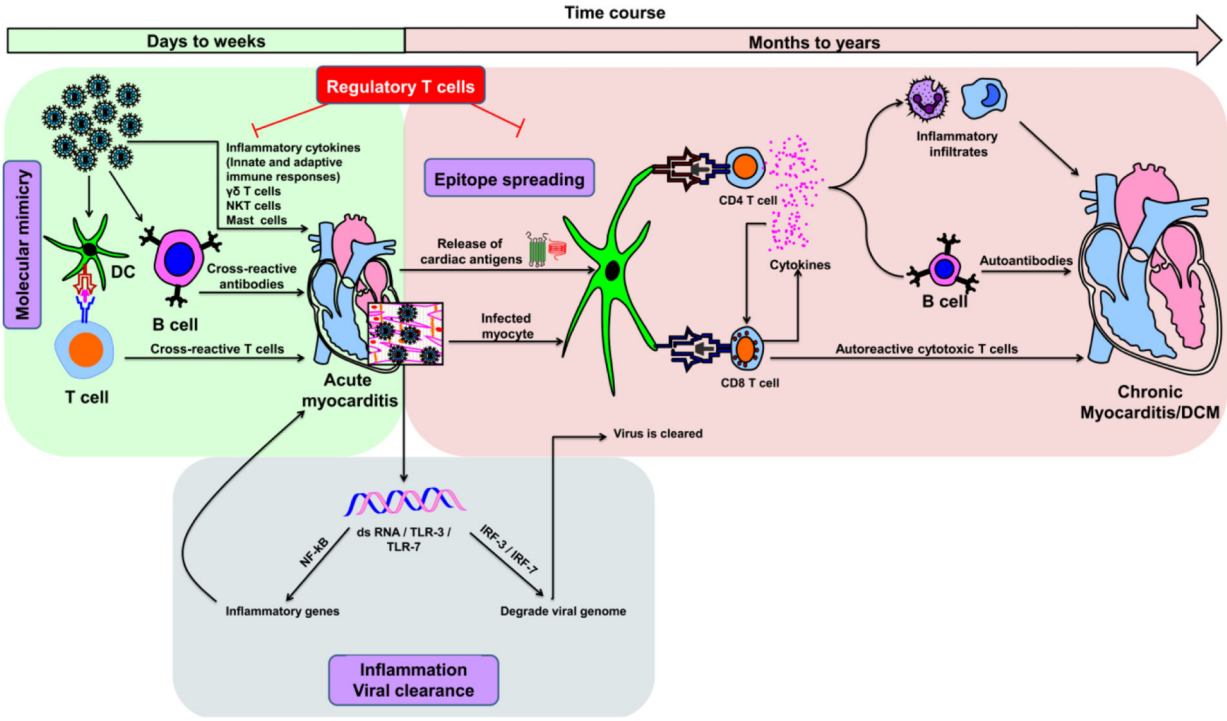

Fig. 3. Proposed mechanisms of inflammatory heart disease

Acute myocarditis can result from an overt immune response to exposure to cardiotropic pathogens, and cardiac damage can be due to the production of inflammatory cytokines of innate and adaptive immune systems. On one hand, although transitory, dsRNAs produced during the replication cycle of CVB3 can bind TLR-3 or TLR-7 and promote inflammatory response through the activation of NFkB. On the other, dsRNAs can mediate viral clearance by degrading the viral genome by activating IRF-3 / IRF-7 pathways. Various innate immune cell types, such as γδ T cells, NK-T cells and mast cells, can influence the disease-outcome. While γδ T cells and mast cells promote cardiac damage, NK-T cells can dampen such a response. Exposure to environmental microbes that carry mimicry sequences for cardiac antigens can lead to the generation of pathogenic cross-reactive T cell and antibody responses. Nonetheless, once cardiac damage sets in, intracellular proteins (e.g., Myhc-α) that were previously invisible to the immune system can be released as a result of epitope spreading, leading to their uptake by APCs (e.g., DCs) and inducing CD4 T cell responses. Alternatively, APCs can engulf infected cardiomyocytes and induce both CD4 and/or CD8 T cell responses via cross-priming. Upon activation, antigen-sensitized CD4 T cells secrete Th1 and Th17 cytokines that favor heart autoimmunity by activating macrophages and promoting neutrophil infiltration, thereby aggravating cardiac damage while providing help to CD8 T cells and B cells via cytokines and chemokines. CD8 T cells can contribute to myocarditis in two ways: **1)** secretion of cytokines similar to those secreted by CD4 T cells with identical consequences; and **2)** killing of cardiomyocytes infected with cardiotropic pathogens (e.g., CVB3) via MHC class I-dependent pathway. In the meantime, pathogen-specific T cells, neutralizing antibodies and antiviral cytokines such as IFN-α, IFN-β can be generated, which facilitate elimination of the microbe. Nonetheless, the newly generated cardiac-reactive CD4 and CD8 T cells can persist and contribute to chronic inflammation. In

these scenarios, however, presence of efficient Treg cells can dampen autoimmune responses, as they are highly critical for maintenance of self-tolerance.

Table 1

List of agents tested in various mouse models of CVB3 infection

Agent	Outcome	Ref.
Anti-viral agents		
2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile	Reduced viral titers; suppressed myocardial inflammatory foci	[142]
CVB3 3C protease inhibitor	Inhibited viral replication; attenuated myocardial inflammation and fibrosis	[143]
Histone deacetylase inhibitors	Suppressed viral replication *in vitro*	[144]
Recombinant CAR4/7	Suppressed CVB3 infection; myocardial inflammation was aggravated, likely as a secondary event	[145]
Ribavirin, and recombinant IFN-α	Inhibited myocardial viral replication; reduced myocardial damage	[146]
Short hairpin (Sh) RNA specific to 2B gene	Improved survival rate, reduced viral titers; attenuated tissue damage	[147]
Sh RNA to 2C protein	Reduced viral titers, suppressed myocarditis, and proinflammatory cytokine production	[148]
Soluble CAR-Fc fusion protein	Reduced viral infection, myocardial damage, and inflammation	[149]
Soluble viral traps (CAR-DAF-Fc)	Attenuated viral infection, myocardial inflammation, and fibrosis	[150]
Pharmacological agents/herbal compounds		
20 (S)-protopanaxtriol	Antiviral effects *in vitro* and reduced disease severity	[151]
α-bromo-4-chlorocinnamaldehyde	Reduced disease severity and viral titers; inhibited nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation	[152]
β1 blocker (NO-metoprolol)	Cardioprotective	[153]
Aqueous extract of *Spatholobus suberectus Dunn*	Reduced viral titers and suppressed disease severity	[154]
Astragaloside IV	Decreased viral titers, necrosis and cellular infiltrations; attenuated myocardial fibrosis *via* inhibition of transforming growth factor beta -β1 signaling	[155]
Calycosin-7-O-P-D-glucopyranoside from *Astragalus membranaceus var. Mongholicu*	Improved survival rate; alleviated cardiac damage and reduced virus titers in the heart	[156]
Curcumin	Attenuated disease severity by inhibiting Phosphoinositide 3-kinase (PI3K)/Akt/NF-kB signaling	[157]
Phyllaemblicin B, extract from *Phyllanthus emblica*	Reduced viral titers; inhibited virus-mediated apoptosis and cardiac muscle damage	[158]
Immunological agents		
Adoptive transfer of Treg cells	Protected against disease through IL-10 production	[159]
Anti-4-1BBL	Reduced cardiac damage and inflammation	[160]
Anti-B7.1 and anti-B7.2	Anti-B7.1 prolonged the survivability of myocarditic mice; anti-B7.2 abrogated the protective effect of anti-B7.1	[161]
Anti-FAS	Reduced caspase-3 expression, viral replication and cardiomyocyte apoptosis and myocardial injury	[162]
Atorvastatin	Attenuated myocardial injury by enhancing the expression of gap junction proteins	[163]
CD40-Ig fusion protein	Relieved myocardial injury and inhibited viral replication	[164]
CpG nucleotides	Inhibited viral infection	[165]
CTLA-4-Ig fusion protein	Reduced mortality and IFN-γ production	[166]
CXCL10	Inhibited viral replication by recruiting NK cells	[167]
Galectin-9	Ameliorated the disease by decreasing Th1 cytokines and promoting Treg and Th2 phenotypes	[168]
IFN-β and IFN-α 2	Mediated anti-viral effects and protected from disease	[169]
IL-1 receptor antagonist encoded in plasmid DNA	Attenuated inflammation	[170]

Agent	Outcome	Ref.
IL-17 receptor A delivered through adenoviral vector	Decreased mortality by downregulating production of inflammatory mediators	[171]
IL-35	Protected from disease *via* reduced IL-17 production	[172]
IL-4	Improved cardiac function by promoting anti-inflammatory effects and downregulation of matrix metalloproteinases	[173]
IL-4 gene therapy	Protected from disease through Th2 polarization	[174]
miR-21	Conferred resistance by inhibiting programmed cell death 4 expression	[175]
Nasal cardiac myosin or OX40 blockade	Ameliorated disease by enhancing Treg and IL-10 production	[129]
Plasmid DNA encoding soluble TNF receptor-Fc	Attenuated inflammation and myocardial fibrosis	[176]
Polyclonal Ig therapy	Protected from disease	[177]
Proteasome inhibitors	Reduced myocardial damage	
Recombinant CVB3/IFN-γ	Conferred protection	[178]
Thrombospondin-2	Inhibited cardiac inflammation	[179]
TNF-α-induced protein 3	Alleviated the disease by inhibiting NF-kB signaling	[180]
Truncated monocyte chemoattractant protein-1	Ameliorated the disease *via* reduced infiltrations	[181]
α-Galactosylceramide	Protected from disease by modulating inflammatory cytokines and anti-vial immune responses	[182]