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REVIEW

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An overview of the immune mechanisms of viral myocarditis

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Summary

Viral myocarditis has been identified as a major cause of dilated cardiomyopathy (DCM) that can lead to heart failure. Historically, Coxsackieviruses and adenoviruses have been commonly suspected in myocarditis/DCM patients in North America and Europe. However, this notion is changing as other viruses such as Parvovirus B19 and human herpesvirus-6 are increasingly reported as causes of myocarditis in the United States, with the most recent example being the severe acute respiratory syndrome coronavirus 2, causing the Coronavirus Disease-19. The mouse model of Coxsackievirus B3 (CVB3)-induced myocarditis, which may involve mediation of autoimmunity, is routinely used in the study of immune pathogenesis of viral infections as triggers of DCM. In this review, we discuss the immune mechanisms underlying the development of viral myocarditis with an emphasis on autoimmunity in the development of post-infectious myocarditis induced with CVB3.

KEYWORDS
autoimmunity, immune mechanisms, viral myocarditis

1 | INTRODUCTION

Myocarditis is inflammation of the myocardium, the muscular layer of the heart wall, and is generally diagnosed by endomyocardial biopsy and cardiac magnetic resonance imaging (CMRI) analysis.[1] It has been identified as the third leading cause (6%) of cardiovascular deaths in young athletes, next only to coronary artery abnormalities (17%) and hypertrophic cardiomyopathy (36%).[2,3] Lymphocytic myocarditis is one form that can be seen in a broad range of patients; symptoms include chest pain and heart failure, and the disease can result in sudden cardiac death in young adolescents.[3] Children admitted with acute viral illness may have cardiac abnormalities, and myocarditis can be detected in up to one-third of patients,[3] raising the question whether some of these individuals will eventually develop dilated cardiomyopathy (DCM) as adults. Furthermore, in contrast to other autoimmune diseases that are more common in females than males, the incidence and severity of most cardiovascular diseases, such as atherosclerosis, myocardial infarction, myocarditis, DCM, and heart failure, are more common in males than females, with the exception of hypertension.[4] Most individuals affected with myocarditis do recover, but a proportion of these patients (up to 20%) can develop chronic myocarditis, leading to DCM and congestive heart failure.[5]

Excluding any known cause of myocardial damage, the DCM disease process is defined by the presence of decreased fractional shortening or ejection fraction and increased left ventricular end-diastolic diameter, and is usually associated with cardiomyocyte loss.[6] However, if myocarditis is identified as the cause of cardiac dysfunction in

Abbreviations: ANT, Adenine nucleotide translocator; APN, adiponectin; BCKD, branched chain ketoacid dehydrogenase; CAR, coxsackievirus-adenovirus receptor; CCL, chemokine ligand; CCR, chemokine receptor; CHOP, C/EBP homologous protein; CMRI, cardiac magnetic resonance imaging; COVID-19, Coronavirus disease-19; CR, complement receptor; CTLA, cytotoxic T-lymphocyte associated protein; CVB, Coxsackievirus B; CXCL, C-X-C motif chemokine; CXCR, C-X-C motif chemokine receptor; DAF, decay-accelerating factor; DAMPS, damage-associated molecular patterns; DC, dendritic cells; DCM, dilated cardiomyopathy; dsRNA, double stranded RNA; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; Foxo3a, Forkhead box o3; Gpat-1, glycerol-3-phosphate acyltransferase 1; GPX-1, glutathione peroxidase 1; H1R, histamine 1 receptor; IRAK, interleukin-1 receptor-associated kinase; IRF, interferon regulatory factor; ISG15, interferon-stimulated gene of 15 kDa; MAVS, mitochondrial antiviral signaling protein; MDA-5, melanoma differentiation-associated protein 5; MIP-1α, macrophage inflammatory protein -1 alpha; MMP, matrix metalloproteinase; MyD88, myeloid differentiation factor 88; NF-kB, nuclear factor kappa B; NLRP3, NLR Family Pyrin Domain Containing 3; NO, nitric oxide; NOD, nucleotide-binding oligomerization domain-containing protein; PAR, protease-activated receptor; PD-1, programmed death-ligand 1; PKC-θ, protein C kinase theta; PTX-3, pentraxin 3; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SCID, severe combined immunodeficiency disorder; SERCA2a, Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a; STAT, signal transducer and activator of transcription; TF, transcription factor; TGF, transforming growth factor; Th, T helper; TNFR, tumor necrosis factor receptor; TnI, cardiac troponin I; TSP-2, thrombospondin-2; β_1AR, Beta 1 adrenergic receptor.

a DCM patient, it is termed inflammatory cardiomyopathy, a syndrome that can result from various causes.[3] Clinically, DCM can be regarded as an end-stage disease, since a proportion of DCM patients (~50%) undergo heart transplantations due to the lack of effective therapeutic options.[5] In this review, we discuss the mechanisms by which virus infections can lead to DCM, with an emphasis on autoimmunity driven by virus-independent events,[7] based on observations made in various animal models of CVB3 infection.

2 | VIRUSES AS TRIGGERS OF MYOCARDITIS

Various etiological agents – both infectious and non-infectious – have been implicated in the causation of myocarditis (Figure 1A).[8,9] As to viruses, enteroviruses like Coxsackievirus B3 (CVB3) and adenovirus belonging to the *Picornaviridae* and *Adenoviridae* families, respectively, are commonly suspected in the U.S in all age groups in patients with chronic myocarditis/DCM.[3,10] Recent data, however, have revealed infections also caused by other viruses.[11,12] For example, parvovirus B19 of the *Parvoviridae* family has increasingly been detected in pediatric myocarditis patients.[13-16] Parvovirus B19 was also found to be a predominant virus in German patients with idiopathic DCM, while HCV was found in Japanese patients with DCM and hypertrophic cardiomyopathy, as evidenced by the detection of HCV antibodies.[3,17] Likewise, HHV-6 belonging to the *Herpesviridae* family was detected in up to 43% of endomyocardial biopsies, leading to the suggestion that HHV-6 be considered in pediatric patients with inflammatory cardiomyopathy or idiopathic DCM.[18] Other viruses implicated in the causation of viral myocarditis include HCV (*Flaviviridae* family), HIV (*Retroviridae* family), EBV and CMV (*Herpesviridae* family), and influenza virus (*Orthomyxoviridae* family).[8,9,19] More recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belonging to the *Coronaviridae* family, causing the Coronavirus disease-19 (COVID-19) has been implicated in the causation of viral myocarditis.[20-23]

To study the pathogenesis of viral myocarditis, mouse models of CVB3 infection are commonly employed. The most common form of viral myocarditis in humans is lymphocytic myocarditis associated with cardiac necrosis, which may lead to self-limiting acute disease or a life-threatening fulminant form of myocarditis. It results in left ventricular dysfunction and heart failure, and a proportion of these patients also may develop DCM.[19,24] Essentially, the susceptible mouse strains A/J (H-2^a) and Balb/c (H-2^d) develop acute myocarditis in about 10 to 14 days post-infection. The disease is characterized by massive inflammatory infiltrates and necrosis associated with left ventricular dysfunction.[25] At about 30 days or later, it progresses to DCM, which is associated with chronic myocarditis, myocardial fibrosis, and cardiac dysfunction.[25,26] While the acute phase is marked by high virus titers

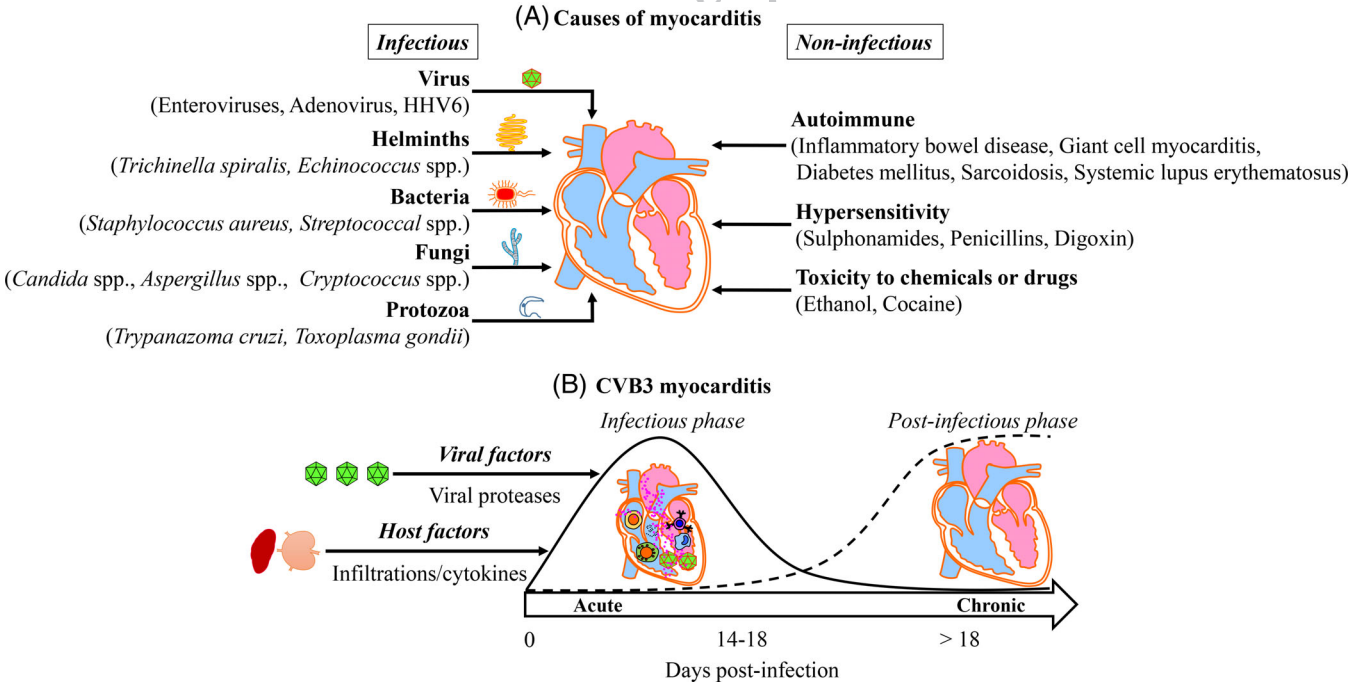

FIGURE 1 Major causes of myocarditis with an emphasis on Coxsackievirus B3 (CVB3) pathogenesis. A, Causes of myocarditis. Myocarditis can be induced by various infectious and non-infectious agents; major causes are shown with examples. B, CVB3 myocarditis. CVB3 infection in susceptible mouse strains is manifested by infectious (viral, up to ~14 to 18 days) and post-infectious (non-viral, beyond ~18 days) phases that occur in continuum in infected animals. In addition to necrosis and apoptosis of cardiomyocytes, the infectious phase is marked by myocardial inflammation (solid curve) contributed by cardiac innate and adaptive immune cells, but infiltrates progressively decrease by ~3 weeks post-infection. As the animals pass through acute myocarditis, inflammation subsides and the healing process begins, occurring in association with cardiac remodeling events, formation of fibrosis, collagen deposition, left ventricular wall hypertrophy, and cardiac dysfunctions. All these features are suggestive of DCM that occurs over a period of weeks to months (dotted curve)

and virus-induced injury, infectious virus is completely cleared from blood and peripheral tissues in the chronic phase; however, viral nucleic acid and capsid protein VP1 can persist in the heart, spleen, and lymph nodes.[27] Additionally, despite the absence of infectious virions, chronic inflammatory changes may continue to persist in this phase.[27] In contrast to susceptible mouse strains, C57Bl/6 (H-2^b) mice are relatively resistant to infection and do not develop chronic myocarditis.[19,26] However, disease resistance can be broken by treatment with IL-1β and tumor necrosis factor alpha (TNF-α),[28] suggesting that innate inflammatory cytokines may be critical to controlling infection in resistant strains. Isolated reports indicate that murine adenovirus-1 can also induce myocarditis in mice.[29,30] For example, myocarditis can be induced in C57Bl/6 mice age-dependently, causing neonatal mice to develop lethal infection; on the other hand, while hearts in adult mice may contain IFN-γ-secreting T cells,[29] infection is not lethal. We routinely use the CVB3 infection model in A/J mice. The mouse model of CVB3 myocarditis has a few attractive features. First, human isolates of CVB3 (eg, Nancy strain) induce myocarditis in mice, the histological features of which resemble human disease.[31,32] Because enterovirus-reactive antibodies and enteroviral nucleic acids have been detected in up to 70% of DCM patients,[10] and because enteroviruses like CVB3 exhibit tropism in cardiac tissue,[10,26] the CVB3 infection model is more suitable for studying the immune pathogenesis of DCM.[31,32] Second, the disease course assumes acute (viral) and chronic (non-viral) phases that occur in continuum (Figure 1B).[10,33] This pattern may reflect the events of human disease, in that patients with chronic myocarditis/DCM show serological and molecular evidence of enteroviral infections, but infectious virions, if any, are rarely or not detected.[26,31] Finally, various tools are readily available to study the pathomechanisms in CVB3 infections.[34,35]

3 | CVB3-INDUCED MYOCARDITIS IN THE MOUSE

CVB3 is a single-stranded, positive RNA belonging to the genus *enterovirus* and the family *Picornaviridae*. The viral genome is 7.4 kb long, and CVB3 is one of the six serotypes of group B Coxsackieviruses.[36] The virus has tropism primarily for cardiac and pancreatic tissues, although brain and liver can be infected.[10,36] The prototype myocarditic strain of CVB3 routinely being used experimentally is the Nancy strain of CVB3, among others.[37,38] The virus lacks a 5′cap structure and instead has an internal ribosome entry site region within the 5′ non-translated region; the genetic elements in this region control viral replicative functions.[36,39] During the replicative cycle, a transitory step representing the formation of double-stranded RNA (dsRNA) may occur,[40] but the viral 2C protein possessing nucleoside triphosphatase activity can cleave dsRNA by unwinding.[41,42] The major natural route of infection for enteroviruses is the gastrointestinal tract, but viruses can infect via the respiratory tract, as well.[43] Experimentally, however, the oral route of CVB3 infection results in only mild disease, as indicated by low morbidity and mortality rates even with high doses of virus inocula, suggesting that the gut acts as a major barrier for infection.[43] Thus, for consistency and reproducibility, the intraperitoneal route is commonly employed in pathogenetic studies, and the resulting viremia induces severe pancreatitis and myocarditis in mice.

3.1 | Contribution of viral factors

Virus entry into the target cells is facilitated by two host receptors, decay-accelerating factor (DAF) and coxsackievirus-adenovirus receptor (CAR), such that the virus-bound DAF allows CAR to be exposed for final entry of the virus into the cells.[44,45] Thus, cardiomyocytes and pancreatic cells, as expected, express these receptors constitutively.[46] The importance of these receptors is also supported by the finding that animals deficient in CAR are protected from CVB3-induced pancreatitis and myocarditis.[47] Likewise, attenuated myocarditis was noted in animals receiving soluble receptors/virus receptor traps for CAR and DAF.[48-50] Because the virus is cytolytic, it spreads by lysis of the infected cells, leading to the release of RNA-packaged virions to infect other cells.[26,36]

CVB3 causes myocardial injury via apoptosis and necrosis of cardiomyocytes within 3 to 4 days post-infection (Figure 1).[27] Several viral proteases (non-structural proteins) have been shown to affect various cellular functions. Some of the mechanisms include shutdown of host proteins and cleavage of transcriptional factors; cell cycle arrest and inhibition of stress granule formation and IFN-β transcription mediated by 2A protease$^{(pro)}$; inhibition of vesicular transport by viral protein 3A; and apoptosis of infected cells by 2A^{pro} and 3C^{pro}.[51,52] More recently, CVB3 infection was found to subvert host proteolytic systems (immunoproteasomes) that favor viral replication. In addition, increased accumulation of abnormal ubiquitin-protein aggregates appear to promote cardiac remodeling events and DCM via oxidative stress response, apoptosis, and autophagy pathways.[53] Excellent reviews of these aspects of viral pathogenesis can be found elsewhere.[27,36,54]

3.2 | Contribution of host factors to the pathogenesis of viral myocarditis and progression to DCM

CVB3 infection is clearly a disease of polygenic traits, since multiple genes have produced similar phenotypes, as described above in various gene knockout models (Table 1). It must be noted, however, that most of these knockout models were produced on a C57Bl/6 genetic background; however, the wild type C57Bl/6 mice are relatively resistant to CVB3 infection and fail to develop chronic disease.[26] Thus, the information obtained from these models is more conceptually useful and relevant to the acute rather than the chronic myocarditis phase of the disease course. Upon infection, various cardiac-resident cells, such as cardiomyocytes, endothelial cells, mast cells, phagocytes, and fibroblasts, may contribute to acute inflammation by secreting cytokines such as IL-1, IL-6, TNF-α and IL-18, among others.[27,116] As viral

TABLE 1 Clinical phenotypes of Coxsackievirus B3 (CVB3) infection in mice deficient for various immune genes or cell types

Disease severity		
Attenuated	**Augmented**	**No change**
Genes of importance in the innate immune compartment		
TLR4/BALB/c[55]	TLR3/C57BL/6[56]	PA28α/β/C57BL/6[57]
MyD88/C57BL/6[58]	TRIF/C57BL/6[59]	MMP8/C57BL/6[60]
NOD2/C57BL/6[61]	MDA-5/C57BL/6[62]	Jα281 (NK T cell)/BALB/c[63]
IRAK4/C57BL/6[64]	MAVS/C57BL/6[65]	
TLR9/C57BL/6[66]	CR1/2/A/J[67]	
MIP-1α/C57BL/6[68]	TRIM21/C57BL/6[69]	
PAR-2/C57BL/6[70]	ISG15/C57BL/6[71]	
S100A8 and S100A9/C57BL/6[72]	NLRP3/C57BL/6[73]	
APN/C57BL/6[74]	NOS/MF1/129[75]	
Foxo3a/FVB[76]	Selenium/C3H/Hej[77]	
CHOP/C57BL/6[78]	GPX1/C57BL/6[79]	
CD1d/BALB/c[63]	PAR-1/C57BL/6[80]	
γδ T cell/B6·129P2-*Tcrd*^{tm1Mom}/J[81]	TSP-2/129SvJ EMS/Ter[82]	
	PTX3/C57BL/6[83]	
	Abcc6/C57BL/6[84]	
	MMP2/C57BL/6[85] MMP9/129/Sv[60]	
Genes of importance in the adaptive immune compartment		
MHC class II/C57BL/6[86]	SCID mice/C3H/Hej[87,88]	
Perforin/C57BL/6[68]	B cell/C57BL/6[89]	
CD4 T cell/A/J[90]	β2m/C57BL/6[91]	
CD4 and CD8 T cells/A/J[90]	CD8 T cell/A/J[90]	
TCRβ/A/J[90]		
Genes of cytokines/chemokines, and their receptors		
IL-12Rβ1/C57BL/6[92]	p75 TNFR/C57BL/6[93]	IL-12p35/C57BL/6[94]
IL-17A/BALB/c[95]	IFN-γ/C57BL/6[94]	Type II IFNR/C57BL/6[96]
IL-21R/C57BL/6[97]	IFN-γ and IL-17A/BALB/c[98]	
TNF-α/C57BL/6[93]	IFN-β/129/Sv×C57Bl/6[99]	
p55 TNFR/C57BL/6[93]	Type I IFNR/C57BL/6[96]	
	IL-10/C57BL/6[100]	
	IL-4/C57BL/6[101]	
	IL-13/BALB/c[102]	
	IL-9/BALB/c[103]	
	IL-6/C57BL/6[104]	
	gp130/gp130flox/flox:MLC2vKI/1[105] (Conditional knockout)	
	CXCL10/BALB/c[106]	
	CCL5/C57BL/6[64]	
	CX3CR1/C57BL/6[94]	
	CCR5/C57BL/6[64]	
Genes of importance in signaling		
CD45/C57BL/6[107]	STAT3/129/Sv[108]	PKC-θ/C57BL/6[109]
p56^{lck}/C57BL/6[110]		STAT4/BALB/c[92]

TABLE 1 (Continued)

Disease severity		
Attenuated	**Augmented**	**No change**
Other genes		
miR155/C57BL/6[111]	Gpat1/C57BL/6[112]	
ERβ/C57BL/6[113]	H1R/C57BL/6[114]	
	Dystrophin/C57BL/6[115]	
	ERα/C57BL/6[113]	

Note: See the list of abbreviations for their description.

replication continues, various innate (NK cells, macrophages, dendritic cells, neutrophils, NK-T cells, γδ T cells) and adaptive (T cells and B cells) immune cells infiltrate and contribute to tissue damage by secreting inflammatory cytokines and/or by cytolytic functions.

Essentially, innate leukocytes and other virus-infected cells may produce type I IFNs with the goal of preventing viral replication soon after infection. As adaptive immune cells begin to respond to viral antigens, antigen-specific lymphocytic responses are induced. Antibodies produced by B cells help to neutralize the infectious virus, clearing the virus by ~two weeks post-infection (Figure 1B). Inflammatory infiltrates become less apparent or disappear thereafter, but chronically affected animals may develop DCM progressively over a period of weeks and months (Figure 1B). It is generally held that the Th1 response is protective in acute myocarditis because it prevents viral replication.[32] IFN-γ can also protect the development of severe chronic myocarditis by reducing mast cell degranulation and fibrosis and suppressing the production of profibrotic cytokines such as TGF-β, IL-1β, and IL-4 in the heart.[117] Although Th2 responses can reduce acute myocarditis by promoting T regulatory (Treg) cells, Th2 cytokines can contribute to cardiac remodeling, leading to chronic myocarditis/DCM by promoting M2 macrophages.[32] In contrast, Th17 cytokines contribute to both acute and chronic myocarditis, including cardiac remodeling and DCM.[32,52] CVB3 may also directly promote Th17 response by inhibiting nucleoporin 98, which is required for lymphocyte differentiation.[118] Other cytokines that may contribute to cardiac remodeling events, collagen deposition, and fibrosis include TGF-β, IL-4, IL-1β, IL-33, and TNF-α.[117,119] Mast cells also may play a role in the remodeling process through the secretion of mast cell chymase, tryptase, MMP-9, and type I procollagen.[120] Although several studies have shown that myocarditis severity can be alleviated by Treg cells and IL-10-producing B cells,[121,122] it is not clear whether the regulatory cells are in fact beneficial in controlling infection because of their suppressive effects on effector anti-viral T cell responses.

3.2.1 | Role of genes of importance in the innate immune compartment in the disease amelioration or pathogenesis

Essentially, pathogen-recognition receptors, such as TLR-3, TLR-4, and melanoma differentiation-associated protein (MDA)-5, recognize positive-sense single-stranded RNA viruses like CVB3.[36] In support of this notion, mice deficient in TLR4, interleukin-1 receptor-associated kinase (IRAK)4, nucleotide-binding oligomerization domain-containing protein (NOD)2, and myeloid differentiation factor (MyD)88 have shown attenuated myocarditis phenotypes accompanied by reduced production of inflammatory cytokines (IL-1β, IL-18, TNF-α, IFN-γ), but varied amounts of type I IFNs (Table 1). Although a similar phenotype was evident with TLR9-deficiency,[66] chronic myocarditis was still noted, suggesting that the TLR9 pathway may be less critical in CVB3 infection. While absence of MIP-α, protease-activated receptor (PAR)-2, and APN led to attenuation as expected because of their innate response roles,[123,124] animals lacking the damage-associated molecular patterns (DAMPS) S100A8 and S100A9 had reduced cardiac inflammation and oxidative stress response,[72] suggesting their therapeutic importance. Likewise, animals deficient in transcription factors (TFs) Foxo3a and C/EBP homologous protein (CHOP), which have roles, respectively, in cellular proliferation and stress resistance and apoptosis and endoplasmic reticulum stress response,[125,126] had reduced cardiac inflammation, indicating that genes regulated by the corresponding TFs may be essential for immune pathogenesis.

On the contrary, increased susceptibility to CVB3 myocarditis was noted in animals deficient in TLR3 and TRIF (adapter protein for TLR3), as well as MDA-5 and mitochondrial antiviral signaling (MAVS) protein occurring in association with skewed Th2 and Th17 responses, and reduced type I IFN secretion, suggesting their importance in disease protection.[56,59,62,65] Although animals lacking complement receptor (CR)1/CR2, TRIM21 (cytosolic ubiquitin ligase), a protein that has a synergistic function with the complement system,[127] and the IFN-stimulated ubiquitin-like protein ISG15 showed enhanced severity of myocarditis,[67,69,71] increased myocardial damage in NLRP3-deficient mice suggests that the NLRP3 inflammasome activation may serve a protective function in CVB3 infection.[73] Because of the anti-microbial properties of nitric oxide (NO), animals lacking NOS2 might have displayed increased CVB3 severity as predicted,[75] but a similar outcome in animals deficient in selenium and GPX-1 (selenium-dependent enzyme) may mean that anti-oxidants also play disease-protective roles.[77,79] A deficiency of the enzyme PAR-1 (coagulation protease), which is implicated in the innate response,[123] led to increased disease susceptibility.[80] Although increased disease severity in thrombospondin (TSP)-2-deficient animals confirms its role in tissue repair and chronic inflammation,[82,128] more severe cardiac

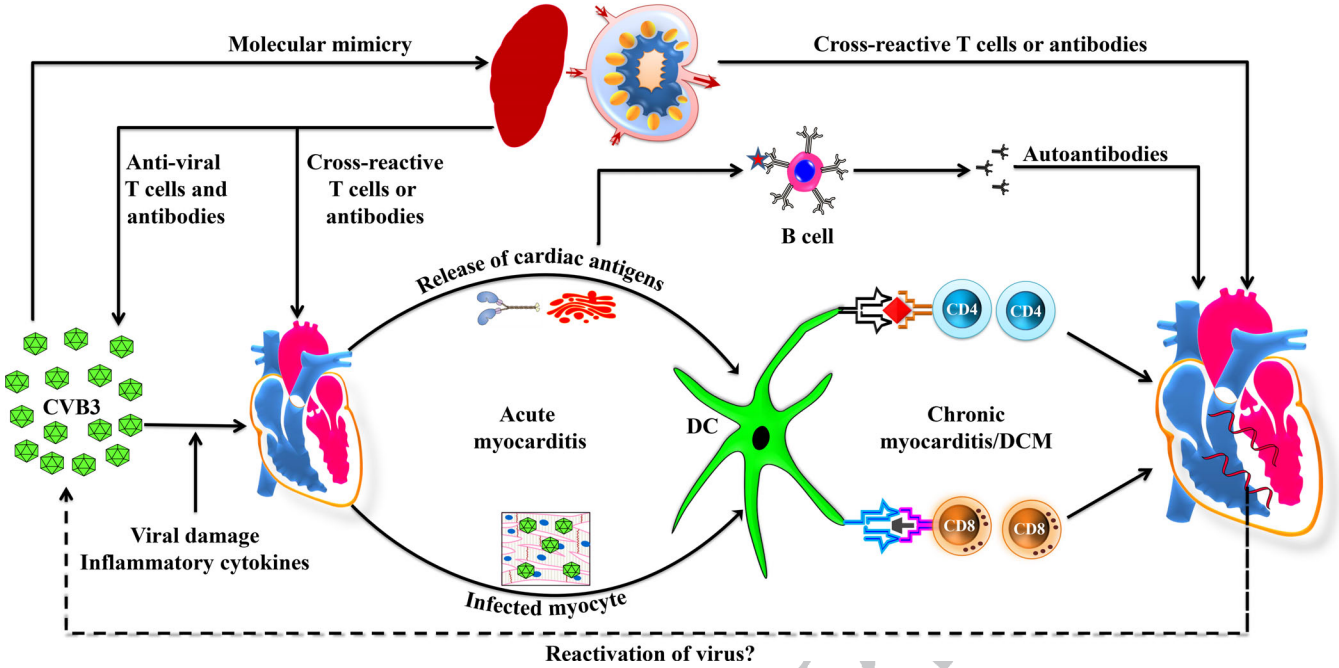

FIGURE 2 Potential autoimmune mechanisms in the development of post-infectious myocarditis induced with CVB3. Various virus and host factors contribute to acute myocarditis. As anti-viral T cell and antibody responses are induced, infectious virions may be cleared. While most animals succumb to acute infection, surviving animals may develop chronic myocarditis/DCM. Because of the cytolytic properties of virus, intracellular and surface cardiac antigens could be released, which induce the formation of autoreactive T cells and antibodies that can infiltrate hearts. Alternatively, antigens from dying or dead infected cardiomyocytes could also be engulfed by resident APCs and induce self-reactive T cell response by the cross-priming pathway. While autoantibodies promote tissue destruction via complement activation, autoreactive CD4 and CD8 T cells mediate tissue destruction by secreting cytokines and cytolytic mechanisms, respectively. Alternatively, the CVB3 proteome may have sequences similar to cardiac antigens, and such mimicry epitopes can contribute to chronic myocarditis through the generation of cross-reactive T cell and/or autoantibody responses. In all these circumstances, as inflammation sets in, it is possible that new antigens can be periodically released as a result of epitope spreading. Continuation of such a vicious cycle may be a key mechanism underlying the development of chronic myocarditis in CVB3 infection. It is unknown whether residual viral RNA, if any, can reactivate to the extent of producing wide-spread viral damage in chronically infected animals (dotted arrow). In this scenario, however, memory B cell and T cells are expected to react swiftly to viral antigens and clear the virus. Thus, the benefits of the virus-reactivation phenomenon may outweigh the ill effects of the virus

inflammation in mice deficient in pentraxin (PTX)3,[83] an acute phase inflammatory glycoprotein,[129] but not the proteasome regulator PA28αβ,[57] supports the role of immunoproteasomes in the induction of anti-viral responses. Likewise, increased susceptibility of animals deficient in the multidrug-resistant associated protein, ATP-binding cassette sub-family C member (Abcc) 6, suggests its protective functions, but the underlying mechanisms remain to be investigated.[84] However, matrix metalloproteinases (MMPs) appear to play a complex role, in that MMP2 and MMP9 mediate protective functions in CVB3 infection, whereas MMP8 is dispensable.[60,85]

As to innate cell populations, although deficiency of NK-T cells did not alter the disease course, CD1d-deficiency resulted in decreased severity of myocarditis as evidenced by histopathology,[63] indicating that non-NK-T cells that recognize CD1d molecules – namely, γδ T cells – may have a disease-inducing role,[81] and animals are, in fact, more susceptible to CVB3 infection in the absence of these cells. Nonetheless, the functionalities of γδ T cells appear to be dependent on specific γδ T cell populations. For example, one population of γδ ($V\gamma1^+$) T cells may promote protection, while another population of γδ ($V\gamma4^+$) T cells may be critical for disease induction.[130]

3.2.2 | Role of genes of importance in the adaptive immune compartment in the disease amelioration or pathogenesis

Lymphocytes play an indispensable role in disease protection, since SCID mice, which lack T cells and B cells, develop severe myocarditis induced with CVB3, but their phenotypes vary. While B cells offer protection because their deficiency augments the disease, T cells play a more complex role. For example, mice deficient in MHC class I (β2 microglobulin) and MHC class II molecules showed opposing phenotypes with disease-protective and disease-inducing functionalities, respectively.[86,91] β2 microglobulin knock-out mice that were deficient in CD8 T cells developed fulminant disease with a chronic course, in which virus-reactive IgG responses and IFN-γ levels were low, but these outcomes were unrelated to perforin-mediated effects.[91] A similar phenotype was also noted in CD8 T cell-deficient mice.[90] In contrast, perforin deficiency offered better protection against CVB3 infection, implying that perforin secreted by non-CD8 T cells, such as NK cells, may contribute to tissue destruction.[27] On the other hand, MHC class II-deficient mice, or CD4 T cell-deficient mice, had less severe perimyocarditis, but developed strong fibrosis, indicating that CD4 T cells may promote chronic myocarditis.[86,90]

Nevertheless, mice deficient in both CD4 and CD8 T cells were better protected, and such a phenotype was also captured in TCR-β-deficient mice.[90] Collectively, these observations suggest that multiple T cell types may be involved in both disease mediation or remediation.

3.2.3 | Knockout models of genes for cytokines/chemokines and their receptors and signaling molecules

Several complex phenotypes have been noted with the deficiency of various cytokines/chemokines and their receptors (Table 1). Diminished histological severity of myocarditis was noted in animals deficient in IL-12Rβ1, IL-17A, IL-21R, TNF-α, and p55TNFR, suggesting that these molecular pathways contribute to the development of CVB3 myocarditis.[92,93,95,97] Occurrence of augmented histologically severe myocarditis in mice lacking p75TNFR may be due to differential effects of TNFRs, since the majority of TNF-α-mediated effects are attributed to p55TNFR,[131] whereas the p75TNFR pathway can suppress inflammatory effects of TNF-α.[131] Likewise, IFN-γ also displays complex functions. While lack of IFN-γ alone or in combination with the absence of IL-17A leads to increased susceptibility to CVB3 infection, such an outcome was not evident in males deficient for IFN-γ, in which the CVB3 myocarditis severity was ameliorated, as evaluated by histology and mortality rates.[94,98] Predictably, however, in the absence of IFN-β (anti-viral cytokine) and its receptor (type I IFNR); IL-10 (anti-inflammatory cytokine); Th2 cytokines (IL-4 and IL-13); IL-6 and its receptor (gp130); Th22 cytokine (IL-9); chemokines (CXCL10 and CCL5); and chemokine receptors (CXCR1 and CCR5), animals developed severe myocarditis, as analyzed by histology and survival rates,[64,94,96,99-106] suggesting the protective functions of these cytokines/chemokines and their receptors in CVB3 infection. On the contrary, lack of IL-12p35 and type II IFNR did not impact the disease outcome, indicating their dispensability in CVB3 infection.[94,96]

As to signaling molecules, mice lacking protein tyrosine phosphatase receptor type C, also called CD45, and p56lck, but not PKC-θ, had more severe disease as determined based on histology and survival rates,[107,109,110] indicating the critical role these molecules play in T cell activation in CVB3 infection. Although signal transducer and activator of transcription (STAT)4 is specifically needed for IL-12 signaling, its deficiency did not influence the disease process,[92] but, expectedly, the absence of STAT3 led to increased disease severity as analyzed by echocardiography and histology because of its involvement in IL-6 and IL-10 signaling processes.[108] This may be the reason that similar phenotypes were noted in animals lacking STAT3, IL-6, and IL-10 (Table 1).

3.2.4 | Other knockout models

It has been recently appreciated that miRNAs have a role in the development of myocarditis based on the detection of dysregulated intracellular miRNAs in heart biopsies in patients with myocarditis and DCM.[132] These are classified into myomiRs, and those related to cardiotropic virus infections, immune status, and fibrosis.[132] Since miRNAs can epigenetically regulate cardiac functions, their ablations can lead to developmental defects in the cardiovascular system and also alter immune functions.[133,134] For example, deficiency of miR155 led to attenuated CVB3 myocarditis occurring in association with reduced $CD45^+$ infiltrations in hearts with an immune response skewed toward Th2 and M2 polarizations, suggesting that miR155 has a prominent role in disease induction with CVB3.[111] Similarly, glycerol-3-phosphate acyltransferase 1 (Gpat1) deficiency resulted in increased myocarditis severity, since this molecule can influence both innate and adaptive immune responses.[112] Likewise, animals lacking histamine 1 receptor (H1R) had severe myocarditis as analyzed by histology and survival rates,[114] indicating that H1R signaling may be critical for T cell activation in CVB3 infection. Interestingly, animals deficient in the cytoskeletal protein dystrophin had histologically more severe CVB3 myocarditis,[115] suggesting a role for dystrophin in disease-mitigation. Finally, in animals lacking estrogen receptors, increased susceptibility to CVB3 infection was found in those lacking ERα, whereas ERβ-deficient animals were better protected.[113] While these differential effects may be due to differences in their binding affinities to estrogens, ERα and ERβ may mediate opposing effects, in that ERα promotes type I IFN, NK, and NK-T cell and suppressor functions, while ERβ may be more important for inducing proinflammatory responses in CVB3 infection.[113]

3.2.5 | Sex differences in immune responses to CVB3 infection

Susceptibility to CVB3 infection has been well-characterized in male and female mice, but myocarditis severity has been demonstrated to be greater in males than in females.[92] It has been suggested that elevated Th1 response accompanies CVB3 infection in male mice,[135] males produce higher levels of IL-1β, IL-18 and IFN-γ during myocarditis,[7] and infiltrations contain predominantly γδ T cells, macrophages, neutrophils, and mast cells.[32,136-138] In contrast, female mice show increased infiltrations of B cells, T cell Ig mucin 3 $(Tim\text{-}3)^+CD4^+$ T cells, and Treg-dominated Th2 response.[138,139] However, ovariectomized female mice that develop severe myocarditis as shown by histology and mortality rates may have cardiac infiltrations of IFN-γ-producing NK cells.[140] While M2 cells are shown to be protective in acute CVB3 infection,[137] IL-1β produced by $TLR4^+$ M2 cells in male mice appears to be critical for development of fibrosis and DCM in affected animals.[141] Furthermore, differential expression of TLRs may also contribute to sex differences in susceptibility to CVB3 infection in that CVB3 infection leads to the upregulation of TLR2 in females, and signaling through TLR2 may contribute to resistance to CVB3 infection in females.[142] On the other hand, TLR4, an inducer of IL-1β and IL-18, is more strongly expressed in males than females in an IFN-γ-independent manner which may contribute to susceptibility to CVB3 infection in males.[7,142,143] Although administration of estradiol into females can attenuate the severity of CVB3 myocarditis by generating Treg cells,[144] testosterone potentiates Th1 response and myocarditis severity by activating γδ T cells.[145]

Additionally, the inhibitory effect of estrogen on Th17 differentiation also may contribute to sex differences.[4] Interestingly, it has been shown recently that CVB3 can replicate better in the intestines of orally infected male mice than in female mice, and type I IFNs and sex hormones can alter both viral replication and lethality.[146] It is possible that enhanced myocarditis severity in males may be due to increased viral titer *in vivo*, but it is unknown whether viral persistence differs between the sexes during the chronic course of CVB3 infection.

All factors considered, if the infectious virus is cleared after the acute attack, it becomes hard to explain the persistence of pathological changes in chronic disease. Furthermore, virus-specific antibodies and T cells are not expected to perpetuate the inflammatory process beyond the acute phase, and the persistence of anti-viral responses, if any, should in fact help hosts to clear the virus. Thus, if inflammatory cells persist or are recruited as fresh waves into the heart, then such a reaction might be triggered by autoreactive cells.

4 | AUTOIMMUNE MECHANISMS IN THE POST-INFECTIOUS PHASE

Animals surviving acute infection can become clinically normal, but histologically, fibrosis and necrotic patchy areas become evident in the heart in the presence or absence of inflammation.[10,147] Animals progressively develop cardiac remodeling changes showing heart dysfunction as evaluated by CMRI.[148] Several lines of evidence suggest that autoimmunity can contribute to this chronic phase, which can be explained by various mechanisms as described below.

4.1 | Relevance of molecular mimicry hypothesis

Serum from CVB3-infected animals may reveal immune complexes for various antigens, such as cardiac myosin, adenine nucleotide translocator (ANT), branched chain α-ketoacid dehydrogenase (BCKD), β_1-adrenergic receptor (β_1AR), actin, laminin, tropomyosin, and heat shock proteins.[149-154] The finding that CVB3-infected animals developed autoantibodies of various IgG isotypes[27,154] suggested possible generation of autoreactive T cells, since T cell help is needed for isotype switching. In addition, ANT and anti-CVB3 antibodies can cross-react with each other, indicating a role for ANT autoantibodies in CVB3/DCM pathogenesis.[155] Similarly, neutralizing antibodies for CVB3 VP1 can cross-react with cardiac myosin, as well as other microbial antigens such as streptococcal M protein,[156] whereas myosin-reactive antibodies may react with CVB and β_1AR,[157,158] which raises a question as to their biological significance.

4.2 | Role of dual T cell receptor (TCR)-expressing T cells

Traditionally, it is held that structural similarities between self- and foreign antigens leads to the induction of cross-reactive immune responses that may include antibodies or T cells.[159] This notion has been recently revisited to suggest that the molecular mimicry hypothesis might have been misinterpreted, in that induction of cross-reactivity may involve mediation of dual TCR-expressing T cells.[159] Reports indicate that the peripheral repertoires in humans and mice may contain up to 30% and 5%-15% of dual TCR T cells, respectively.[159,160] Essentially, lack of allelic exclusion for the TCR-α allele during thymic education may cause T cells to express two TCR-α chains that can associate with a common TCR-β chain in various combinations.[159] However, regardless whether T cells expressing single or dual TCRs are expected to be deleted in the thymus, a possibility nevertheless exists that a TCR specific to foreign antigens in the dual TCR-expressing T cells may faithfully respond to infections; the other TCR, if self-reactive, may recognize self-antigens under conditions of break in self-tolerance as a result of bystander activation. But this theory requires further validation, as 95% of dual TCR-expressing cells may remain non-functional.[161] It is currently unknown whether CVB3 myocarditis involves the mediation of dual TCR-bearing T cells. Additionally, it should be noted that detection of cross-reactive antibodies or T cells may be biologically insignificant unless their functionalities are proved. These possibilities can be evaluated in experimental models in adoptive transfer settings, which remain to be tested.

4.3 | Epitope spreading

Because cardiac necrosis is one of the hallmarks of acute myocarditis, it is possible that several encrypted intracellular antigens like cardiac myosin could be released and act as autoantigens. This may be the underlying reason for detection of antibodies for multiple antigens. An alternative possibility is epitope spreading, where initial release of antigens can trigger induction of autoantibodies for other antigens later in the disease course.[162] However, it is still unknown whether epitope spreading is relevant to viral myocarditis, and it may be difficult to evaluate this phenomenon, since several antigens can be released at once due to the cytolytic properties of the virus. However, it is possible that idiotypic antibodies produced in response to viral receptors can recognize other complementary self-ligands by inducing anti-idiotypic responses.[161] Interestingly, it has been demonstrated that animals immunized with the recombinant capsid protein VP1 of parvovirus B19 developed myocarditis and DCM, but the underlying mechanisms, including possible generation of anti-idiotypic antibodies, if any, were not described.[163] Nonetheless, it is possible that autoantigens released from cardiac cells can act as DAMPS leading to secretion of inflammatory cytokines. For example, cardiac myosin contains a fragment that can trigger the TLR2 pathway and enhance the production of Th17 promoting cytokines IL-6, TGF-β, IL-23, granulocyte colony-stimulating factor (GM-CSF), and Th17 cytokine-producing $CD4^+$ T cells.[164]

4.4 | Release of cryptic antigens

Cytotoxic lymphocytes generated in virus-infected animals capable of lysing cardiomyocytes were found to transfer disease to naïve mice.[37]

Two potential possibilities exist to explain this phenomenon. First, cytotoxic cells generated in CVB3-infected animals may be virus specific, but such cells can lyse cardiac myocytes possibly by cross-reactivity or through dual TCR-reactivity as reported in Theiler's encephalomyelitis virus.[165] However, supporting data are lacking in CVB3 infection. Alternatively, CVB3 infection can lead to the induction of autoreactive T cells secondary to virus damage. In our studies, we tested this hypothesis by generating MHC class II dextramers for cardiac myosin 334-352 and demonstrated that A/J mice infected with CVB3 showed the appearance of Th1 and Th17 cytokine-producing myosin-specific CD4 T cells that can transfer myocarditis to naïve animals, while pancreas remained normal.[34] We believe that such a repertoire of autoreactive T cells can be potentially generated for cryptic antigens. For example, viral proteases can cleave host proteins like dystrophin.[27,51] Whether such proteolytically cleaved proteins can become autoimmune targets is currently unknown. We are currently investigating the theme that CVB3 infection can lead to generation of myocarditogenic T cells with multiple antigen-specificities as a secondary event in CVB3 infection.

4.5 | Immune checkpoint therapies and bystander activation

It has increasingly been reported that cancer patients undergoing checkpoint inhibitor therapy develop autoimmune (lymphocytic) myocarditis possibly because gut microbes could potentially trigger such responses by cross-reactivity in genetically susceptible individuals.[166] It would be interesting to determine whether this patient population has any virus signature. If so, it might be possible to visualize a scenario in which viruses may initially trigger the generation of autoreactive T cells but remain dormant, potentially suppressed by the expression of co-inhibitory receptors on T cells (cytotoxic T-lymphocyte-associated protein [CTLA]-4 and programmed death-ligand 1 [PD-L1]). Once these checkpoints are released, autoreactive T cells may freely expand, potentially via bystander activation, and cause myocarditis.[167,168] Although speculative, this may be the underlying mechanism for the occurrence of damage in other organs, such as the gut (colitis), liver (hepatitis), pituitary gland (adrenocorticotropic hormone insufficiency), thyroid (hypothyroidism), and pancreas (type 1 diabetes), as well as acute kidney injury in a broad picture as to the reasons for occurrence of organ-specific injuries in those treated with checkpoint inhibitors.[169]

Additionally, it is to be noted that animals immunized with cardiac antigens such as myosin and cardiac troponin 1 (TNI) can develop myocarditis with histological features resembling the post-infectious phase of CVB3 infection.[170,171] Thus, we expect that cardiac remodeling events might be similar in both settings and may involve participation of residual inflammatory or resident cardiac and newly recruited immune cell populations in the reparative process that may show altered phenotypes. Evaluation of these cell populations may offer new insights into the mechanistic understanding of DCM. To this end, we are currently analyzing cardiac cell populations by single cell RNA sequence analysis in CVB3 infected mice.

5 | CONCLUSIONS

Infection with live viruses is indispensable in inducing myocardial damage unless it can be proved mechanistically that viral lysates or viral recombinant proteins can induce cardiac dysfunctions similar to virus infection. As shown in the Figure 2, CVB3, a cytolytic virus that causes damage to cardiac cells, can be potentiated by innate cytokines. Although anti-viral immune responses (T cell and antibody) may clear the virus, the reparative process may never be completely recuperated, due, in part, to the limited regenerative capacity of cardiomyocytes, especially in adults. During this process, however, it is possible that intracellular or surface antigens released as a result of viral damage may become autoimmune targets, leading to induction of pathogenic autoreactive T cells or autoantibodies. It is also possible that dying cardiac cells could be engulfed by resident APCs and trigger autoimmune responses by cross-priming. Although molecular mimicry has been proposed as a potential mechanism for induction of chronic myocarditis, supporting evidence is lacking to suggest that cross-reactive T cells or antibodies can in fact transfer disease to naïve recipients. Thus, the viable hypothesis that can be tested experimentally is whether viral infection results secondarily in the generation of autoimmune responses for multiple antigens. Determination of their appearance/disappearance or persistence may provide translational significance. For example, if autoreactive T cells persist for an extended period of time, it may be possible to investigate whether such cells can be reactivated by non-specific stimuli through bystander activation. Proving such a hypothesis may add experimental credence to the proposition that autoimmunity can be targeted for therapy in individuals affected with idiopathic DCM with virus signatures like virus-reactive antibodies.[5,172] However, in chronically infected animals, viral nucleic acid/defective virus may be present,[173] but it is not clear whether such a virus can be reactivated to induce recurrent viral damage. Finally, measurement of molecules such as cardiac troponin I and T, MB-kinase, C-reactive protein, and natriuretic peptides is practiced clinically to establish biomarkers to evaluate cardiac damage.[174,175] In these settings, while examining for viral causes, the panels may need to be expanded to include SARS-CoV-2 as a potential cardiotropic viral pathogen, where elevated cardiac injury markers may also have a prognostic value in individuals affected with the COVID-19 infection.[176-179] More recent investigations have led to the discovery of myoglobin and soluble ST2 in CVB3 myocarditis models that complemented observations made in patients with myocarditis.[180,181] These are worthy candidates to be included in the biomarker panel. These observations also support the notion that the data generated in pre-clinical settings can be related translationally to human diseases.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

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