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# Impaired Cardiac Function in Viral Myocarditis

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## 1. Introduction

Viral myocarditis, the inflammation of the myocardium caused by viral infection, is an important cause of dilated cardiomyopathy (DCM) – a major cause of morbidity and mortality worldwide (Mason, 2003; Esfandiarei & McManus, 2008; Cooper, 2009). In North America, viral myocarditis and DCM together account for 20% of the sudden deaths and heart failure in children and adolescents (Okuni *et al.*, 1975; Drory *et al.*, 1991). To date, there is no effective therapeutic against these diseases. Patients diagnosed with late stage DCM are limited to supportive treatments such as ventricular assist device implantation and heart transplantation.

The clinical presentation of viral myocarditis comes in various severities. Most people have contracted and subsequently recovered from multiple viral infections of the heart without overt symptoms. Yet, retrospective studies revealed that ~20% of subclinical cases later develop congestive heart failure. In addition, some may experience acute fulminant viral myocarditis or persistent chronic myocarditis symptoms. About one-third of these patients with viral myocarditis subsequently develop DCM (Esfandiarei & McManus, 2008). A combination of new diagnostic technologies for viral myocarditis such as cardiovascular magnetic resonance techniques with conventional diagnostics including clinical presentation, histopathological examination, cardiac antibody assessment, and viral polymerase chain reaction (PCR), now helps better define disease stage and its respective management protocol (Baughman, 2006).

The presence of viral genome in the myocardium is associated with significantly worse outcome over two years (Why *et al.*, 1994). Analysis of human failing hearts by PCR unveiled trails of previous viral infection. The identified viruses include enterovirus, adenovirus, parvovirus B19, herpes simplex virus 6, cytomegalovirus, hepatitis C virus, and human immunodeficiency virus, which are clinically associated with viral myocarditis (Grist & Reid, 1997; Calabrese *et al.*, 2010). Among them, coxsackievirus B3 (CVB3), an enterovirus in the picornavirus family, is highly implicated in clinical cases of viral myocarditis, particularly in neonates and young children, and is the most thoroughly studied causative agent in experimental viral myocarditis models (Froeschle *et al.*, 1966; Abelmann, 1971; Reyes & Lerner, 1985; McManus *et al.*, 1988). CVB3 replicates rapidly in short infection cycles that begin with viral receptor engagement and subsequent internalization, followed by translation of viral RNA, amplification of viral genome, viral assembly, and complete with viral progeny release.

CVB3 infection of myocarditis susceptible mice results in severe heart failure. The disease progression of viral myocarditis in the experimental infection model can be classified into three phases: acute (viremia), subacute (inflammatory), and chronic (remodeling) phases. The acute (viremia) phase is signified by active viral replication and direct virus-induced cardiomyocyte damage. The subacute (inflammatory) phase is characterized by the infiltration of immune cells that helps viral clearance but nonetheless adds to myocardial damage. The chronic (remodeling) phase is featured by the continual efforts of the impaired heart to meet the hemodynamic demand by remodeling the myocardium. Cardiac hypertrophy is triggered during remodeling to compensate for reduced contractile function due to myocyte loss and interstitial fibrosis in the earlier phases. However, such an adaption is unsustainable in the longer term in face of increasingly hostile environments, i.e. reduced blood supply and increased reactive oxidative stress, thus leading to cardiomyocyte death and triggering further inflammation and fibrosis. The pathological remodeling process eventually leads to DCM and heart failure.

This book chapter focuses on the virus-host protein interactions in cardiomyocytes during viral myocarditis. We discuss the role of virus-induced protein cleavage and dysregulation of the host protein degradation systems in the pathogenesis of viral myocarditis and its subsequent progression to DCM.

## **2. Host protein cleavages by coxsackieviral proteases contributing to cardiac dysfunction**

### **2.1 Viral proteases and dilated cardiomyopathy**

CVB3 encodes two viral proteases, 2A and 3C, both of which are cysteine proteases that have chymotrypsin-like activity and play critical roles in successful viral replication (Chau *et al.*, 2007). First, the viral proteases are required to process the large viral polyprotein, a product of mono-cistronic translation of RNA genome, into the individual functional, structural and non-structural proteins. Second, the viral proteases facilitate viral replication by cleaving a number of host proteins that are involved in various cell functions such as transcription, translation, cell signaling, and cellular structure.

Viral myocarditis is originally thought to be an immune response driven disorder. The initial observation that established the importance of direct virus-mediated myocardial damage in the pathogenesis of viral myocarditis was made in severe combined immunodeficient (SCID) mice, which lack functional T and B lymphocytes and yet developed early and severe myocyte damage upon enterovirus challenge (McManus *et al.*, 1993). The significance of viral proteins in the development of viral myocarditis and DCM was further explored by Dr. Knowlton's research group. First, they showed that the cardiac-specific expression of a replication-restricted CVB3 mutant genome in transgenic mice, which only allows the expression of viral proteins without generating viral progenies and subsequent immune response, results in DCM phenotype (Wessely *et al.*, 1998). Then, they demonstrated that mice with cardiac-restricted expression of viral protease 2A display a severe DCM phenotype (Xiong *et al.*, 2007). These findings suggest that viral proteases play an important role in the development of viral-induced dilated cardiomyopathy.

### **2.2 Cleavage of dystrophin during CVB3 infection may contribute to dilated cardiomyopathy**

The observation that mouse cardiac expression of viral protease 2A induces DCM has been explained based on the landmark finding that dystrophin, which links the cytoskeleton to

the extracellular matrix (ECM) by forming the dystrophin glycoprotein complex (DGC), is cleaved during CVB3 infection by protease 2A (Badorff *et al.*, 1999; Badorff & Knowlton, 2004) (Fig. 1A). Dystrophin has three domains that serve different purposes. Its N-terminal domain anchors to the actin cytoskeleton and its rod domain provides the linkage to the C-terminal domain, which binds to  $\beta$ -dystroglycan that in turn connects to the sarcolemma and the extracellular matrix (Fig. 1A). Furthermore, dystrophin-deficient mice have been shown to have an increased susceptibility to viral myocarditis and develop severe cardiomyopathy (Xiong *et al.*, 2002). Human genetic mutations of the dystrophin gene cause Duchenne Muscular Dystrophy (DMD) (Nigro *et al.*, 1990). Approximately 20% of DMD patients suffer and die from a resultant cardiomyopathy. Other mutations of the dystrophin gene also cause X-linked DCM (Ferlini *et al.*, 1999). CVB3-induced dystrophin cleavage occurs at its 3' hinge and therefore breaks its connection to the ECM. As a result, the sarcolemmal integrity is compromised and force transmission is reduced. This can further lead to cardiomyocyte necrosis due to the increased sarcolemmal permeability (Fig. 1A). Thereafter, dystrophin cleavage has been viewed as a major mechanism in enteroviral cardiomyopathy. However, dystrophin knockout mice (Mdx) display only mild cardiomyopathy phenotype, due to the compensatory upregulation of utrophin, a dystrophin homologue (Deconinck *et al.*, 1997; Grady *et al.*, 1997). Thus, other mechanisms may also contribute to the severe cardiomyopathy phenotype in viral protease 2A expressing mice.

### **2.3 Cleavage of serum response factor by viral protease 2A is associated with impaired cardiac function**

Recent efforts have demonstrated for the first time that serum response factor (SRF) is cleaved in CVB3-infected mouse hearts and cultured murine cardiomyocytes (unpublished). SRF, which belongs to the MADS-box (MCM1, Agamous, Deficiens, and SRF) protein superfamily, is a muscle-enriched transcription factor that regulates the expression of contractile and regulatory genes, as well as microRNAs (miRNAs) (Miano, 2003; Niu *et al.*, 2007; Oka *et al.*, 2007) (Fig. 1B). SRF interacts with tissue specific cofactors such as myocardin, Nkx2.5, c-Fos, and binds to the serum response element (SRE) of its target genes. It contains two major domains: the N-terminal DNA binding and dimerization domain and the C-terminal transactivation domain (Fig. 1B). Genomic studies have identified over 1200 SRE containing genes and more than 250 of these have been verified (Sun *et al.*, 2006). Cardiac contractile genes under SRF regulation include cardiac  $\alpha$ -actin,  $\beta$ -myosin heavy chain, myosin light chain, cardiac troponin I, etc. SRF is indispensable for mesoderm formation and plays a central role in cardiac development and function (Niu *et al.*, 2007). Therefore, SRF knockout results in embryonic lethality (Parlakian *et al.*, 2004). The construction of cardiac-specific inducible SRF knockout transgenic mice overcomes this problem and helps illustrate the importance of SRF in cardiac function (Parlakian *et al.*, 2005). It was shown that SRF knockout in the adult mouse heart results in damaged cardiac function, and subsequent progression to DCM. Genetic mutations of SRF in humans have not been described likely due to the associated lethality. However, the expression of alternatively spliced SRF isoforms, which was shown to have inhibitory effects on wild-type SRF, is increased in failing human and animal hearts (Davis *et al.*, 2002). Furthermore, a cleaved form of SRF lacking the transactivation domain was also found in human failing hearts as a result of caspase-3 activation during cardiomyocyte apoptosis (Chang *et al.*,

2003). This caspase-cleaved SRF fragment functions as a dominant-negative mutant that inhibits SRF-dependent activation of cardiac genes. On the other hand, SRF overexpression in transgenic mouse hearts leads to the development of cardiac hypertrophy and subsequent cardiomyopathy (Zhang *et al.*, 2001).

During CVB3 infection, SRF is cleaved into a ~47kD N-terminal fragment (SRF-N) and a ~20kD C-terminal fragment (SRF-C) by viral protease 2A (unpublished). As a result, the DNA-binding domain is detached from the transactivation domain, thus abolishing SRF function. In addition, the SRF-N fragment generated from the cleavage can compete with wild-type SRF for target gene binding, and thereby exhibits dominant-negative suppression of SRF target gene expression (Fig. 1B). This study suggests another important mechanism by which CVB3 damages cardiac function and leads to subsequent DCM. Further research using knock-in transgenic mice expressing non-cleavable SRF will help clarify the relative contribution of SRF cleavage in the pathogenesis of viral myocarditis.

## 2.4 Caspase activation by viral proteases

Both viral proteases 2A and 3C lead to late onset of host cell apoptosis through the direct cleavage of caspase-8 and the activation of caspase-3, as well as the cleavage of anti-apoptotic protein Bid that leads to mitochondrial cytochrome c release and subsequent initiation of the caspase cascade (Chau *et al.*, 2007). Cardiomyocyte apoptosis is a common phenomenon in various cardiac diseases. Apoptosis, also known as programmed cell death, is the self-destruction pathway activated when host cells decide to commit suicide. Apoptosis during viral myocarditis caused by viral protease-induced caspase activation, however, is “switched on” intentionally by viral mechanisms for the release of mature viral progenies. In cell culture, caspase activation results in cytoplasmic proteolysis and DNA fragmentation. However, despite ultrastructural evidence of cytochrome c release detected in many cardiomyocytes of heart failure patients, intact nuclei are seen in all of these myocytes (Narula *et al.*, 1998). This suggests that the terminally differentiated cardiomyocytes have evolved strategies to resist nuclear fragmentation despite ongoing cytoplasmic apoptosis. In fact, the number of apoptotic myocytes in a cardiomyopathic heart ranges only from 0.07% to 0.7% as compared to 0.003% in the normal myocardium (Narula *et al.*, 1998). Nevertheless, cytoplasmic apoptosis initiated during viral myocarditis may compromise mitochondrial ATP generation, as well as cause destruction to contractile proteins which add to systolic dysfunction in the disease pathogenesis.

## 2.5 Other mechanisms

Other mechanisms that viral proteases use to impair cardiomyocyte function include the interference of host gene transcription by the cleavage of cyclic AMP response element binding protein (CREB) (Yalamanchili *et al.*, 1997), the disruption of host protein translation through the cleavage of eukaryotic translation initiation factor 4 $\gamma$  (eIF4 $\gamma$ ) (Chau *et al.*, 2007) and eIF5B (de Breyne *et al.*, 2008), the interception of cell signaling pathways via the cleavage of RasGAP (Huber *et al.*, 1999), and the weakening of the cytoskeletal network by the cleavage of cytokeratin-8 (Seipelt *et al.*, 2000). Although there are no known cardiac diseases associated with the aforementioned proteins, their cleavages exert additive effects to the final detriment of the infected cardiomyocytes.

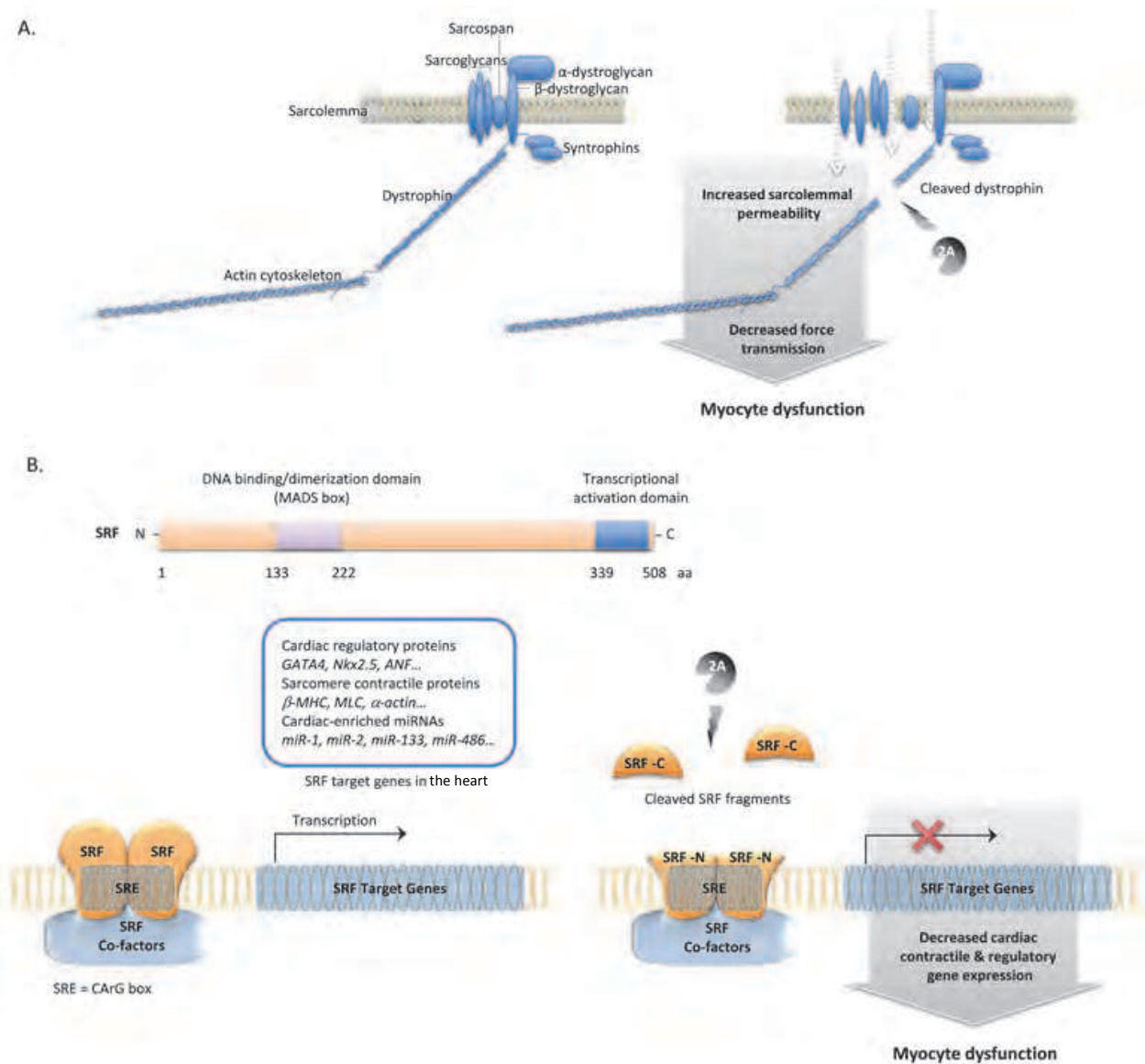


Fig. 1. Host protein cleavages by coxsackieviral proteases in viral myocarditis. A. Coxsackieviral protease 2A cleaves dystrophin at its 3' hinge. Dystrophin is a component of the dystrophin-glycoprotein complex that links the cytoskeleton to the extracellular matrix. Dystrophin cleavage contributes to myocyte dysfunction by reducing contractile force transmission and increasing sarcolemmal permeability. B. Viral protease 2A also cleaves serum response factor (SRF). SRF is a muscle-enriched transcription factor that regulates the expression of cardiac regulatory proteins, sarcomere contractile proteins, as well as cardiac-specific microRNAs (miRNAs). SRF associates with co-factors such as GATA4, Nkx2.5, MEF2, and myocardin and binds to serum response element (SRE) (also known as CARG box) to activate gene transcription. SRF cleavage results in myocyte dysfunction by the dissociation of the N-terminal DNA binding/dimerization domain from the C-terminal transcriptional activation domain, thus abolishing SRF-mediated gene expression. Furthermore, the N-terminal fragment (SRF-N) exhibits a dominant-negative effect on endogenous SRF function by competing for DNA binding.

### 3. Dysregulation of host protein degradation systems in viral cardiomyopathy

#### 3.1 Protein degradation systems

Protein degradation is an integral part in the maintenance of cellular homeostasis. It allows for the selective removal of host proteins that are misfolded, damaged, and unnecessary, and balances the ongoing protein synthesis that is driven to provide the desirable cellular function in response to environmental changes. The dynamic interaction between protein synthesis and degradation is of particular importance to cardiomyocytes, the basic contractile units of the heart, due to the plasticity of the heart. It drives the hypertrophy or atrophy of individual cardiomyocytes, which contributes to sufficient contractile force generation that meets the hemodynamic demands. Consequently, the dysregulation of protein degradation jeopardizes cardiomyocyte vitality and function and plays an important role in the development of various cardiac diseases (Zheng & Wang, 2010).

Enteroviral infection leads to the dysregulation of host protein degradation pathways, which include the ubiquitin/proteasome system (UPS) and autophagy. Such viral-induced manipulation plays key roles not only in viral propagation, but also in the pathogenesis of viral myocarditis and the subsequent development of DCM. The following sections discuss the contributions of UPS and autophagy in viral propagation and the development of cardiomyopathy.

#### 3.2 The ubiquitin/proteasome system

The ubiquitin/proteasome system is the major protein degradation pathway in eukaryotic cells that accounts for ~80% of host protein recycling (Zheng & Wang, 2010). By controlling the longevity/half-life of most proteins (predominantly short-lived but also some long-lived proteins), the UPS extends its role beyond protein recycling and regulates most aspects of cellular functions. UPS substrate selectivity is achieved by protein polyubiquitination, i.e. the attachment of ubiquitin (Ub) molecules onto the target protein. Ubiquitin is a small 76 amino acid protein modifier molecule that conjugates to target protein or to another Ub through one of its seven lysine residues. Ubiquitin linkage at different lysine residues serves different functions. For example, polyubiquitination via lysine 48 targets protein for UPS degradation, polyubiquitination via lysine 63 promotes signal transduction or targets degradation through the autophagy pathway, whereas monoubiquitination modulates protein intracellular localization and protein function.

Protein ubiquitination is regulated in a multi-step manner (Hershko & Ciechanover, 1998) (Fig. 2A). First, Ub is activated by the ubiquitin-activating enzyme (E1) using ATP. Then, it is transferred to ubiquitin-conjugating enzyme (E2). Finally, it is conjugated onto the target protein selectively brought in by the ubiquitin ligase (E3). A polyUb chain is formed by repeating the ubiquitination process. The expression of these important enzymes that regulate protein ubiquitination may change according to physiological stimuli. For instance, in cardiac atrophy mouse model, the E2 enzyme UbcH2 expression is upregulated in atrophic hearts to increase the capacity of protein degradation (Razeghi *et al.*, 2006).

The 26S proteasome is composed of the 20S proteolytic core and the 19S proteasome activator lid(s) (Luo *et al.*, 2010). The 20S proteolytic core is made up of two outer ( $\alpha$ -subunits) and two inner ( $\beta$ -subunits) rings. It contains caspase-like, trypsin-like, and chymotrypsin-like protease activities conveyed by subunits  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5, respectively. The 19S lid(s), also known as proteasome activator 700 (PA700), helps the recognition and

docking of polyubiquitinated target protein. 19S also serves to detach and hence recycle the Ub by its deubiquitinating enzyme (DUB) activity. Furthermore, 19S unfolds the target protein and feeds it to the 20S core for degradation.

The immunoproteasome is an alternative version of the proteasome expressed to accommodate inflammatory responses upon stimulation with interferon- $\gamma$  (Rivett & Hearn, 2004). The immunoproteasome has a 20S core that substitutes the constitutive catalytic  $\beta$ -subunits with inducible  $\beta$ -counterparts ( $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i), which offer different proteolytic function and activity to generate small peptides suitable for antigen presentation by major histocompatibility complex (MHC) class I (Griffin *et al.*, 1998) (Fig. 2A). In addition to the 19S proteasome, the immunoproteasome can also have a different lid(s) – the 11S proteasome, also known as PA28 (proteasome activator 28). Different compositions of 11S exist: heteroheptamer of PA28 $\alpha$  and PA28 $\beta$  that are induced by interferon- $\gamma$  under intensified immune response (Murray *et al.*, 2000) and homoheptamer of PA28 $\gamma$  that resides in the nucleus and assists ATP- and ubiquitin-independent proteasomal activity (Mao *et al.*, 2008). Sometimes, hybrid proteasomes with both 11S and 19S lids are also observed. However, their functions remain to be explored.

### 3.2.1 The UPS and heart diseases

UPS dysregulation is a common phenomenon in heart diseases. It is accentuated with the accumulation of Ub-protein conjugates and is associated with markedly reduced proteasome proteolytic activity in failing human hearts as compared to non-failing hearts (Predmore *et al.*, 2010). This suggests that ubiquitinated proteins in hearts are not degraded due to impaired proteasomal function. While no changes were noted in protein expression of proteasome subunits (i.e. 20S, 19S, 11S), elevated levels of protein carbonyls and 4-hydroxynonenylated proteins were observed in failing hearts. Also, oxidative modification to the 19S ATPase subunit Rpt5 was found in these failing hearts. Together, these oxidative modifications to proteasome subunits and substrate proteins may lead to impaired proteasomal function. On the other hand, microarray studies demonstrate reduced transcript levels of some 20s  $\alpha$ - and  $\beta$ -subunits in the failing hearts as compared to controls (Hwang *et al.*, 2002; Kaab *et al.*, 2004). The incongruence between protein and mRNA expression of proteasome subunits may be attributed to myocyte loss and fibrosis in the failing hearts.

Animal models of cardiac diseases also have an increased ubiquitinated protein expression, but are accompanied with changes in their proteasome expression profile. Upregulation in protein expression of proteasome subunits was observed in a left ventricular hypertrophy mouse model (Depre *et al.*, 2006). Post-translational modifications of the proteasome subunits were also reported in these hypertrophic hearts (Depre *et al.*, 2006). Treatment with proteasome inhibitor effectively prevents cardiac hypertrophy development, suggesting that the upregulation of proteasome expression is central to this physiological adaptation. Similar beneficial effects of proteasome inhibition in the regression of cardiac hypertrophy were observed in other studies (Meiners *et al.*, 2008; Stansfield *et al.*, 2008). Besides hypertrophic cardiomyopathy, the accumulation of Ub-conjugated proteins was observed in hyperglycemia-induced cardiomyopathy mouse model (Powell *et al.*, 2008). A parallel drop in the basal ATP-dependent proteasomal activity was observed in these mice. However, an increased ATP-independent chymotryptic proteasomal activity was observed, which is accompanied by an increased expression of 11S lid subunits PA28 $\alpha$  and PA28 $\beta$ , as well as

the 20S subunits  $\alpha 3$  and  $\beta 5$ . Together, these data suggest a shift to immunoproteasomal activity is induced under hyperglycemic stress conditions to help the degradation of accumulated proteins.

The difference in cardiac proteasome expression profiles between human heart failure and various cardiomyopathy mouse models can be attributed to the limited time frame of animal studies. It is likely that stressed human hearts also induce the compensatory upregulation of proteasomal expression and activity at the earlier disease stages, but fail to maintain these changes over time.

### 3.2.2 The UPS and viral myocarditis

The UPS is also dysregulated in viral myocarditis (Fig. 2A). Accumulation of Ub-protein conjugates, as in other cardiomyopathies, was observed in CVB3-infected mouse hearts and cultured cells (Luo *et al.*, 2003; Gao *et al.*, 2008; Si *et al.*, 2008). The expression of several enzymes in the UPS pathway such as E1 enzyme E1A/E1B, E2 enzyme UbCH7, and DUB UCHL1 (ubiquitin carboxyl-terminal hydrolase L1) is upregulated in CVB3-infected mouse hearts, while ATP-dependent proteasomal activity is unaltered (Gao *et al.*, 2008). *In vitro* application of proteasome inhibitors such as MG132 (Luo *et al.*, 2003), lactacystin (Luo *et al.*, 2003), pyrrolidine dithiocarbamate (PDTC) (Si *et al.*, 2005), and curcumin (Si *et al.*, 2007) effectively attenuates viral RNA replication and protein synthesis. In addition, depletion of Ub by RNA interference also inhibits viral replication (Si *et al.*, 2008). Furthermore, it was shown that viral RNA-dependent polymerase 3D is ubiquitinated during viral replication, which may help its anchorage to intracellular membrane platforms required for the assembly of viral RNA replication machinery (Si *et al.*, 2008). *In vivo* administration of proteasome inhibitor to CVB3-infected mice also improves the outcome of viral myocarditis with reduced myocardial damage and inflammatory infiltration (Gao *et al.*, 2008). The viral titer, however, is not significantly reduced in the treated mice. This suggests that proteasome inhibitor treatment ameliorates viral myocarditis via multiple mechanisms: direct viral inhibition and immunomodulation. It was further demonstrated that the expression of 11S subunit PA28 $\gamma$  plays a role in CVB3 replication (Gao *et al.*, 2010). CVB3 infection leads to the redistribution of PA28 $\gamma$  from the nucleus to the cytosol, where it interacts with host proteins, such as tumor suppressor protein p53, and promotes their degradation via UPS. Overexpression of PA28 $\gamma$  enhances viral replication while its knockdown does the opposite.

Szalay *et al.* explored the involvement of the immunoproteasome in viral myocarditis. They found that the catalytic subunits of the immunoproteasome, LMP2 ( $\beta 1i$ ), LMP7 ( $\beta 5i$ ), and MECL-1 ( $\beta 2i$ ), are upregulated in CVB3-infected myocarditis-susceptible mouse hearts as compared to infected hearts from resistant mouse strains (Szalay *et al.*, 2006). Increased activity of the immunoproteasome in the susceptible myocardium helps generate the MHC class I-restricted peptide, boost antigen presentation and mount the subsequent adaptive immune response. A recent study demonstrates a differential immunoproteasome expression pattern between myocarditis-susceptible and -resistant mouse strains (Jakel *et al.*, 2009). In this study, immunoproteasome formation peaks early after CVB3 challenge in resistant mice, while it is postponed and expressed in greater extent in susceptible mice. The timing and magnitude of immunoproteasome activation determine in part the effectiveness of early viral clearance and the extent of direct viral-mediated damages, as well as the injury incurred during adaptive immune responses.

### 3.3 Autophagy

The other host protein degradation system is autophagy, i.e. “self-eating”. It proceeds by the engulfing of a portion of the cytoplasm including long-lived and misfolded proteins and organelles by the autophagic membranes to form double-membraned vesicles called autophagosomes, followed by their delivery to lysosomes for degradation (Levine & Kroemer, 2008) (Fig. 2B). Autophagy is activated in two parallel cascades of enzymatic actions that are similar to the process of protein ubiquitination (Ravikumar *et al.*, 2010). First, Atg12 (autophagy-related gene 12) and Atg8 (also known as LC3, microtubule-associated protein light chain 3, in mammalian cells) are activated by the E1-like activating enzyme Atg7 using ATP. Then, Atg12 is conjugated to its E2 Atg10, while Atg8 is attached to another E2 Atg3. Atg12 is then transferred to its designated partner Atg5 forming the Atg12-Atg5 complex and further matures by the conjugation to Atg16. Finally, the Atg12-Atg5-Atg16 complex acts as an E3 to help Atg8 lipidation, forming the Atg8-PE (phosphatidylethanolamine) complex. Lipidation of Atg8 helps its incorporation onto the autophagic membrane. Atg8-PE then takes part in the elongation of the autophagic membrane and its enclosure to form the autophagosome.

#### 3.3.1 Autophagy and cardiac diseases

Under baseline conditions, autophagy represents an important homeostatic mechanism. However, excessive activation of the autophagy machinery has been suggested to be involved in the pathogenesis of various disease conditions, including cardiac diseases. LC3 activation was observed early and was well-sustained in pressure-overload cardiomyopathy mouse model (Zhu *et al.*, 2007). Autophagy activation in this model promotes cardiac remodeling. Overexpression of Beclin-1, also known as Atg6, accentuates pathological remodeling and interstitial fibrosis, whereas heterozygous knockout of Beclin-1 improves systolic function and delays cardiac remodeling. In desmin-mediated cardiomyopathy mouse model, early activation of autophagy was observed well before any measurable decline in cardiac function (Tannous *et al.*, 2008). Autophagy pathway impairment by heterozygous inactivation of Beclin-1 leads to accumulation of polyubiquitinated protein aggregates, as well as acceleration to heart failure and early mortality (Tannous *et al.*, 2008). Similarly, autophagy is activated in both ischemia and subsequent reperfusion, but via two different initiation pathways (Takagi *et al.*, 2007). The AMPK pathway drives autophagy during ischemia, while Beclin-1 initiates autophagy upon reperfusion. Autophagy during ischemia is considered a cell survival response as it helps to sustain the starved cardiomyocytes during ischemia; however, autophagy during reperfusion is viewed as a pathological response as it promotes autophagic cell death.

#### 3.3.2 Autophagy and viral myocarditis

Autophagy also plays an important part in the host innate defense system by direct sequestration of invading pathogens (bacteria, fungi, and virus) for clearance through lysosomal degradation (Jackson *et al.*, 2005). In addition, autophagy helps antigen presentation to class II MHC in order to mount an adaptive immune response (Dengjel *et al.*, 2005). However, this innate defense machinery is subverted by certain viruses to facilitate their replication (Jackson *et al.*, 2005; Wong *et al.*, 2008). It was shown that LC3-PE expression, a hallmark of autophagosome formation, is increased after CVB3 infection with dramatic reorganization of intracellular membranes (Wong *et al.*, 2008) (Fig. 2B). Inhibition

of autophagy by 3-methyladenine which targets the upstream signaling class III PI3-kinase, and by siRNA knockdown of Atg7 expression effectively block viral replication (Wong *et al.*, 2008). Recent work in mouse models also suggests that autophagy is activated *in vivo* after CVB3 infection (unpublished data). LC3-PE expression is elevated in CVB3-infected organs such as the heart, liver, and pancreas. Kemball *et al.* also reported the induction of autophagosome formation in pancreatic acinar cells in CVB3-infected mice (Kemball *et al.*, 2010). This theme of virus-induced autophagy activation is further extended to coxsackievirus B4-infected rat primary neurons (Yoon *et al.*, 2008). Nonetheless, virus-induced autophagy only serves to help viral replication without increasing protein degradation as suggested by the unchanged expression level of p62, a selective autophagy substrate, after virus infection (Wong *et al.*, 2008).

Subversion of the autophagy machinery by enteroviruses may contribute to the pathogenesis of viral myocarditis beyond impacting cardiomyocyte viability. Recent research demonstrates that cellular autophagy plays a role in nucleic acid-sensing toll-like receptor 3 (TLR3) signaling, which is necessary for the antiviral interferon pathway (Gorbea *et al.*, 2010). TLR3-deficient mice show vulnerability to CVB3 infection and develop acute myocarditis (Negishi *et al.*, 2008). Dysregulation of the autophagy pathway in CVB3-infected cardiomyocytes may interfere with TLR3-mediated antiviral response, resulting in compromised viral clearance and increased myocardial damage during viremia. In addition, autophagy is known to be a pro-survival response against apoptosis. The dysregulation of autophagy may decrease the viability of virus-infected cardiomyocytes because it cannot protect the host from virus-induced apoptosis. Furthermore, angiotensin II receptors type I & II (AT1 & AT2) regulate cardiomyocyte autophagy activity (Porrello *et al.*, 2009). AT1 expression triggers autophagy in neonatal cardiomyocytes as well as subsequent autophagic cell death, while AT2 expression counteracts AT1-induced autophagic activity. Further modulation by angiotensins may have an adverse effect on virus-infected cardiomyocytes as it may further activate autophagy, thus triggering autophagic cell death.

#### 4. Potential therapeutics targeting viral proteases, UPS, and autophagy

The current knowledge of the roles of viral proteases and the host protein degradation systems in viral myocarditis may lead to new diagnostic and therapeutic approaches for the disease. Virus-induced SRF cleavage fragments may be utilized as a biomarker to detect acute phase myocarditis. Early diagnosis of viral myocarditis opens up the optimal timeframe for treatment. Successful medical interventions during acute infection can limit viral replication and its associated damage, limit viral spreading, as well as minimize the damage caused by immune activation. Since the viral proteases and the protein degradation systems all play important roles in viral propagation, a combinatorial therapy of highly specific viral protease inhibitors, proteasome inhibitors, and autophagy inhibitors during viremia would limit viral infection effectively. Furthermore, application of proteasome inhibitors and autophagy inhibitors provides additional benefits in immunomodulation to control the inflammatory response. On the other hand, viral myocarditis patients in the chronic phases may be managed differently. Since DCM patients have depressed proteasomal function, proteasome inhibitor treatment may further exacerbate myocardial damage. Moreover, long-term application of inhibitors against UPS will have adverse effects as demonstrated in the increased incidence of heart failure in cancer patients undergoing proteasome inhibitor treatment (Enrico *et al.*, 2007; Hacıhanefioglu *et al.*, 2008).

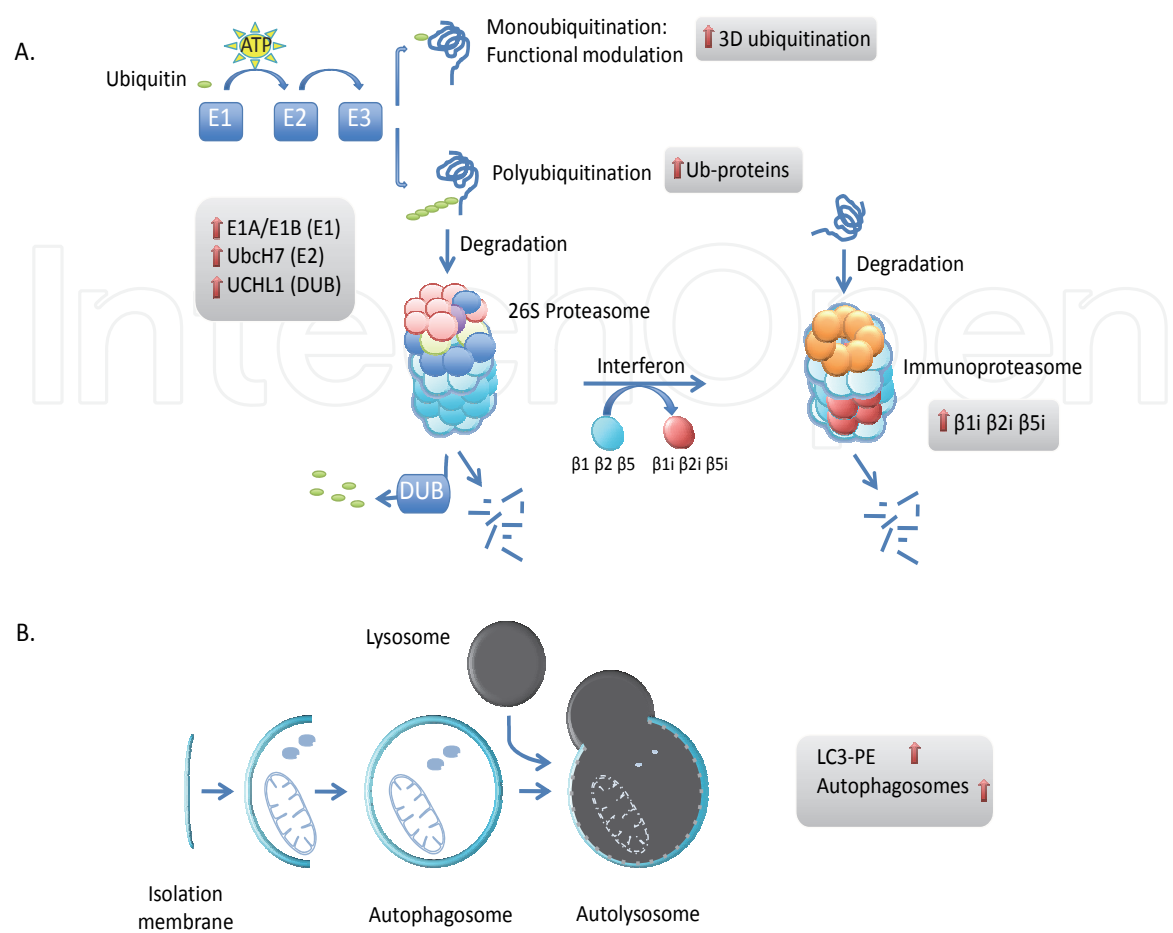


Fig. 2. Dysregulation of the host protein degradation systems in viral cardiomyopathy. The ubiquitin/proteasome system (UPS) and autophagy are the two major protein degradation mechanisms in eukaryotic cells. A. The UPS function by a cascade of enzymatic reactions (E1 - ubiquitin activating enzyme, E2 - ubiquitin conjugating enzyme, E3 - ubiquitin ligase) that conjugate ubiquitin, a small protein modifier, onto the target proteins. The type of ubiquitin conjugation linkage determines the target protein's fate: functional modulation or degradation. Polyubiquitinated target proteins are recognized by the 26S proteasome for degradation, whereas monoubiquitination serves to help endocytosis, endosomal sorting, DNA repair, histone regulation, and nuclear export. Ubiquitins are recycled by the deubiquitinating enzymes (DUBs). CVB3 infection causes the dysregulation of the UPS. An increased expression of ubiquitin-protein conjugates, E1A/E1B (E1), Ubch7 (E2), UCH-L1 (DUB) was observed in CVB3-infected mouse hearts. Proteasome inhibitor application attenuates viral replication *in vitro* and reduces myocardial lesion and fibrosis *in vivo*. B. Autophagy begins with the enwrapping of organelles and cytoplasmic proteins by the isolation membrane which elongates and encloses to form a double-membraned vesicle called the autophagosome. The autophagosome fuses with lysosomes to degrade the sequestered materials. Autophagy plays an important role in host defense by trapping and degrading invading pathogens. However, certain viruses including CVB3 evolve strategies to subvert autophagic mechanism to facilitate their own replication. Autophagosome formation is upregulated during CVB3 infection. Inhibition of the autophagy pathway has been shown to block viral replication *in vitro*.

## 5. Conclusion

Viral-induced protein cleavage and host protein degradation dysregulation play important roles in the pathogenesis of viral myocarditis and its subsequent progression to DCM. Identification of viral-induced protein cleavage fragments may allow early diagnosis of viral myocarditis, which opens up the optimal treatment window. A combination of antiviral therapies including specific viral protease inhibitors, proteasome inhibitors, and autophagy inhibitors presents new strategies for effective early viral clearance and minimization of viral-induced, inflammation-associated damage. Further studies employing system-like approaches, such as ubiquitomics, degradomics, and RNAi screens, are required to decipher the complex interactions between host and virus during different stages of viral myocarditis. Efforts in clarifying the precise functions and regulatory mechanisms of the host protein degradation systems in the disease progression of viral myocarditis will lead to novel therapeutic targets to improve treatment in different disease stages.

## 6. References

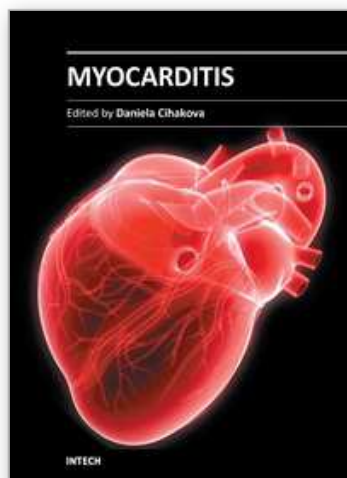
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## **Myocarditis**

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Myocarditis, the inflammation of the heart muscle, could be in some cases serious and potentially fatal disease. This book is a comprehensive compilation of studies from leading international experts on various aspects of myocarditis. The first section of the book provides a clinical perspective on the disease. It contains comprehensive reviews of the causes of myocarditis, its classification, diagnosis, and treatment. It also includes reviews of Perimyocarditis; Chagas's™ chronic myocarditis, and myocarditis in HIV-positive patients. The second section of the book focuses on the pathogenesis of myocarditis, discussing pathways and mechanisms activated during viral infection and host immune response during myocarditis. The third, and final, section discusses new findings in the pathogenesis that may lead to new directions for clinical diagnosis, including use of new biomarkers, and new treatments of myocarditis.

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