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Cellular and Immunological Regulation of Viral Myocarditis

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1. Introduction

Since the discovery of coxsackievirus type B3 (CVB3) more than 50 years ago there has been considerable progress in the understanding of viral heart disease. Coxsackievirus was first discovered as a filterable agent associated with a paralytic syndrome, so named for its identification in Coxsackie, New York (coxsackievirus type A) (Dalldorf and Sickles, 1948). Coxsackievirus type B (CVB) was isolated the following year from patients with aseptic meningitis and by the mid-1950s an association with acute myocarditis in humans was apparent (Melnick et al., 1949). Many other viruses have since been shown to cause myocarditis and its long term sequelae, arrhythmias, dilated cardiomyopathy (DCM) and heart failure. By example, adenovirus, herpes viruses, and influenza can cause myocarditis in humans and models of heart failure. The viral agent most often reported as being the cause of viral myocarditis is CVB3. For example, CVB3 RNA can be detected in the heart muscle of 10 - 35 % of DCM patients (Feldman and McNamara, 2000; Hosenpud et al., 2001), depending on the study cohort.

1.1 Myocarditis etiologies

Inflammation of the heart muscle can have many causes, not least of which is viral infection. Cocaine [(Jentzen, 1989) reviewed in (Maraj et al., 2010)], virus induced, autoimmune (possibly due to resolved microbial infection), and even vaccine induced myocarditis (Cassimatis et al., 2004) have all been reported with a wide degree of occurrence. Though very rare, the vaccine induced aetiology is classified as idiopathic due to the poorly understood mechanism of inflammation. This manifestation of myocarditis is so rare it is probably very complex in cause and mechanism, requiring both environmental and genetic susceptibility factors of the host to trigger myocardial immune invasion. The subject of this chapter is one of the most common forms of myocarditis, and perhaps the most studied: myocarditis due to viral infection. In such cases, myocyte dropout and provisional matrix fibrosis, focal lesions of immune cell invasion are marked and key observations in the diagnosis of these cases.

1.2 Clinical presentation

With an experimental understanding of CVB3 induced myocarditis comes a better understanding of the breadth of symptoms presented by the admitted patient, before,

during and after a hospital visit. The patient is most often admitted to hospital with general chest pain or discomfort, combined with other symptoms of an infection, which are often suspected as other ailments (Brady et al., 2004). These symptoms of infection can be so apparent that they divert the attention of the clinician away from cardiac dysfunction. Further work-up of the case reveals elevated creatinine phosphokinase and troponin with rhythmic disturbances and reduced cardiac output, often presented by shortness of breath with minimal exertion (Brady et al., 2004). Most cases resolve with little or no supportive therapy, but a minor percentage of cases progress to dilated cardiomyopathy and congestive heart failure, for which there is no cure other than heart transplantation.

1.3 Diagnosis: the Dallas criteria

The Dallas criteria for myocarditis are a set of histology based criteria proposed by a publication in 1987 by Aretz et al. (Aretz et al., 1987). These criteria rely upon histological observations made of left ventricular biopsies, obtained by either the Cordis or Scholten bioptomes. Several biopsies of the ventricle are required for a definitive diagnosis and there is a high possibility of false negatives using this method. Though not necessarily a problem inherent in the method itself but the need for a large number of samples due to the often localised and focal nature of the disease. The chances of false negatives combined with procedural discomfort are driving forward a need to develop new and less invasive procedures like blood-based biomarker-detection.

The Dallas criteria for diagnosis of myocarditis require an inflammatory infiltrate with associated myocyte necrosis, or otherwise damage that is not characteristic of ischemia. Less intense immune infiltration with little or no evidence of myocyte destruction is classified as borderline myocarditis (Aretz et al., 1987).

Understanding the etiology of myocarditis will improve the success rate of diagnosis, which in turn, improves management of the disease and prognosis (Baughman, 2006; Felker et al., 2000). The Dallas criteria were a good stepping stone toward a standardised diagnostic method, however there remain significant drawbacks to this biopsy based histological criteria. It has been demonstrated that myocarditis could only be accurately diagnosed in 25 % of single biopsies obtained from patients who had died of myocarditis (Chow et al., 1989; Hauck et al., 1989), and more than 5 biopsies are required to diagnose myocarditis in two thirds of patients (Hauck et al., 1989), and 17 biopsies are required for an 80 % level of confidence in diagnosis (Hauck et al., 1989; Schultz et al., 2009). Therefore only positive samples obtained by endomyocardial biopsy should be considered diagnostic. These poor rates of definitive diagnosis are a product of the small size of the bioptome sample from a relatively large area afflicted with a disease that is often focal and of relatively narrow localisation.

The Dallas criteria were a good start for a disease that had no diagnostic criteria at the time of publication (1987). To this day, the Dallas criteria are used to diagnose myocarditis from endomyocardial biopsies, though the existence of sensitive methods for disease detection, particularly in the blood, biomarker discovery should make this methodology supplementary or even obsolete. Therefore, there is a need for the development of sensitive methods for the differential and definitive diagnosis of non-ischemic cardiomyopathies like myocarditis. This chapter will not cover diagnosis or new detection strategies any further as these topics are covered in greater detail, elsewhere in this book.

2. Viral replication as the central regulator of viral myocarditis

The advent of virus replication in the heart of an individual is thought to occur secondarily to a more general and systemic virus infection (Carthy et al., 1997; Mena et al., 2000; Vuorinen et al., 1994), the spleen, gut, pancreas and lymph nodes are often infected; patients often report malaise, fatigue and more general 'flu-like' symptoms about 1- 2 weeks prior to chest pains and cardiac rhythm disturbances. Immune infiltration in the heart has taken hold by the time the patient has been seen by a physician and any evidence of direct virus-induced damage has been superseded by T cell invasion at the foci of infection. It is not surprising that there have been many reports that claim the predominant source of damage is immune infiltration of the myocardium, as inflammation is what defines this disease. Autoimmune etiologies due to an antiviral response gone awry have been proposed as one of the major causes of pathology (Lv et al., 2011). Regardless, there are numerous publications that suggest a large amount of damage is inflicted by the virus itself, in the murine model, early post-infection, that is correlated with the number of myocarditic lesions spatially coincident with positivity by *in situ* hybridization for CVB3 genome (Cheung et al., 2008; Chow et al., 1992; Marchant et al., 2009a; McManus et al., 1993). These observations are supported by findings in autopsy cases, particularly in infants (Iwasaki et al., 1985a; Iwasaki et al., 1985b).

2.1 The cytotoxic nature of enterovirus replication

Coxsackievirus B3 is a member of the *Enteroviridae* of the *Picornaviridae* family, which are fast replicating and exquisitely cytotoxic viruses. Another member of this family is poliovirus, which is also highly cytotoxic, producing a paralytic syndrome reminiscent of the aseptic meningitis and encephalitis sometimes caused by CVB3. The very nature of enterovirus replication makes these viruses very cytotoxic because the infected cell must burst to permit the release of progeny virus virions for infection of bystander cells and further replication cycles. This aspect of CVB3 replication means that every virus infected cell will die within 8 – 24 hrs of infection, releasing numerous rounds of progeny virus into the heart before the innate immune system has had a chance to control infection. The degree of immune infiltration and damage inflicted on the heart muscle is therefore a function of the amount of virus inoculum that takes hold in the heart, dictating the degree of damage done to the myocardium.

The virus polarises the protein expression machinery entirely to the benefit of the virus by a number of mechanisms and so in combination with the inability of the cell to perform its natural housekeeping functions inevitably leads to cell destruction. The viral proteases act to cleave capped cellular mRNAs thereby skewing protein translation in the cell from cap-dependent translation initiation to internal ribosome entry site (IRES) translation. Coxsackievirus B3 translates it's RNA genome via IRES translation initiation so destruction of the cell's own capped mRNAs removes any competition of the virus' own IRES RNAs for the cell's ribosomes. This usurping of control of the cell's protein expression machinery effectively brings the normal housekeeping function of the cell to a halt.

The massive amount of protein produced by the virus, devolution of the endoplasmic reticulum (ER) and other membranous structures combined with the oxidative stress placed upon the cell by virus replication, all destabilise the homeostasis of the infected cell. The large protein-nucleic acid aggregates that make up progeny virion are not normal to the cell and induce numerous UPR responses; due to their toxic nature, protein aggregates are

normally sequestered to vimentin-cytoskeleton encased aggrosomes (Garcia-Mata et al., 1999; Johnston et al., 1998). For example, the irregular folding of viral proteins can lead to sequestering of virus protein products into cellular aggresomes (Spiropoulou et al., 2003). These combined facets of CVB3 replication contribute to the inherently toxic nature of virus replication, even in the absence of the onset of programmed cell death.

2.2 Virus entry into the host cell

The replication cycle of CVB3 in the cell begins in a very similar manner to poliovirus infection via receptor mediated endocytosis into the target host cell. Coxsackievirus B3 binds its cellular receptor(s), the coxsackie-adenovirus receptor (CAR) and ,sometimes, decay acceleration factor (DAF), to achieve entry via endocytosis into the host cell. Once the virus has achieved delivery inside the cell there is massive rearrangement of internal membrane structures and then complete usurping of the host cell protein translation machinery for the purposes of virus replication and virion assembly. The RNA polarity (positive-polarity RNA) of the CVB3 genome means that viral polypeptide can be translated directly after delivery into the host cell. All of the proteins encoded by the CVB3 genome are expressed as 1 poly-protein that is then cleaved by virus proteases, 2A and 3C, into the individual viral proteins VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, and 3D.

2.3 Death and lysis of virus infected cells

Direct virus-induced damage inflicted by CVB3 upon the infected cell is through simultaneous activation of multiple cell death pathways, by direct and indirect mechanisms. For example, all of the known caspase cell death activation pathways are activated during CVB3 infection: the FAS/FADD receptor-mediated caspase- 8 pathway and the mitochondrial cytochrome c -caspase- 9 pathway are activated to ultimately trigger the death effector, caspase-3 (Carthy et al., 1998; Carthy et al., 2003). Normally, the activation of caspases during apoptosis leads to programmed cell death: cleavage of cellular DNA by endonucleases and shrinking of the cell's contents into a cytoskeleton-vimentin cage, for engulfment by phagocytes at some later time. This process is programmed, and most importantly, 'neat,' preventing the dissemination of the dead cell's contents throughout the tissue. The debris released from cells are hydrolytic and oxidative, some are apoptotic, which would lead to bystander necrosis of surrounding cells and tissue if not disposed of 'neatly' through programmed cell death. However, CVB3 infected cells do not die in a programmed and neat manner. The act of virus replication; viral proteases and activation of numerous cell death pathways all at once cause infected cells to rapidly lyse and release cellular contents, along with virus progeny virions, for further rounds of replication in surrounding bystander cells.

Necrotic cell death during CVB3 infection is most likely caused by a myriad of pathways that act in concert to result in the uncontrolled lysis of the cell for the purpose of virus progeny release. Evolution of the virus has favoured a more chaotic lysis/necrosis type death as opposed to a programmed and controlled method of killing the host cell. We will summarise the pathways of CVB3 induced cell death below:

2.3.1 Caspase-induced cell death during CVB3 infection

The caspase pathways of apoptosis were the first mechanisms of programmed cell death that were discovered [(Lazebnik et al., 1994; Miura et al., 1993; Yuan et al., 1993; Yuan and

Horvitz, 1990) reviewed in (Yuan and Horvitz, 2004)]. The first cellular pathways implicated in cell death due to CVB3 infection were also caspase mediated (Carthy et al., 1998; Colston et al., 1998), since these were the predominant mechanisms of cell death being studied at the time (Andrade et al., 1998; Atkinson et al., 1998; Barry et al., 2000). As new mechanisms of cell death were discovered, such as GSK-3β, some were attributed to virus induced cell death.

Comprehensive studies of caspase cleavage and activation during infection have demonstrated a pan-caspase activation profile during viral infection (Carthy et al., 1998; Carthy et al., 2003), from activation of the tumour necrosis factor (TNF) receptor activated caspase-8 to the mitochondrial cytochrome *c* release that results in the activation of caspase-9. All of the above mentioned pathways converge upon the activation of the effector caspase, caspase-3. The most notable caspase-8 activated pathway is triggered by Fas ligand binding and activation of Fas receptor, a member of the TNF superfamily of receptors. Various oxidative states, toxins and aberrant protein expression can lead to a permeable mitochondrial membrane that releases cytochrome *c* into the cytoplasm which leads to caspase-9 activation. Though the caspase-8 and -9 pathways are quite different in that they are extrinsic and intrinsic pathways of cell death, respectively, they both lead to cleavage and activation of caspase-3, a death effector caspase. In cells destined to die by either of these routes of apoptosis there is cleavage of poly ADP-ribose polymerase (PARP) and DNA fragmentation factor (DFF) by caspase-3. Cleavage of DFF by this caspase reveals an endonuclease that enters the nucleus and cleaves DNA, resulting in DNA fragmentation (Figure 1A).

Two of the experimental hallmarks of apoptotic cell death are PARP fragmentation, as observed on a Western blot, and fragmented genomic DNA as viewed by agarose gel electrophoresis. It was demonstrated that inhibition of caspase-3 PARP and DFF cleavage did not inhibit virus induced cell death completely (Carthy et al., 1998), however some alleviation of cytopathic effect was notable. The broad spectrum zVAD.fmk caspase inhibitor is not 100 % effective at inhibiting all caspases, and there is the possibility that in the absence of caspase activation that a redundant mechanism of cell death becomes dominant in mediating cytotoxicity. In this scenario we would expect zVAD.fmk to appear ineffectual at inhibiting cell death. It was apparent that the caspases were activated though this was clearly not the mechanism that was entirely responsible for CVB3 induced cytopathic effect. Below we will outline more mechanisms responsible for cell death and that may run parallel to caspase mediated cytotoxicity. Later in this chapter we will also cover new systems biology approaches that could be applied toward the understanding of the dominant/ pertinent pathways of CVB3 induced cell lysis and the networks employed by the virus to drive cell death.

2.3.2 Glycogen synthase kinase-3β

In addition to the roles of caspases during cell death, the actions of glycogen synthase kinase-3 β (GSK-3β) have also been implicated in the induction of cytopathic effect by CVB3 replication (Yuan et al., 2005; Zhang et al., 2003). Phosphorylation of transcription factors by GSK-3β can lead to either the activation or down-regulation of transcription, and activation of GSK-3β has been observed relatively early in the CVB3 replication cycle (Yuan et al. 2005). The catenins are poly-phosphorylated by activated GSK-3β, leading to degradation in the proteasome (Doble and Woodgett, 2003; Harwood, 2001). During CVB3 infection, the activation of GSK-3β has been proposed to lead to instability of cell viability due to

degradation of a protein called β-catenin (Figure 1C). The catenins interact with the actin cytoskeleton and act to maintain morphological integrity by connecting the actin cytoskeleton with the cell membrane. The loss of these connections through enhanced GSK-3β activation and β-catenin degradation is consistent with the profound cell rounding that occurs during intermediate to late phases of CVB3 replication. The accumulation of β-catenin in the cytoplasm leads to translocation into the nucleus where it transactivates survival signalling via T-cell factor (TCF)/lymphocyte enhancer factor (LEF) family members (Doble and Woodgett, 2003; Hardt and Sadoshima, 2002; Harwood, 2001). Consistent with these findings, the inhibition of GSK-3β or the over-expression of β-catenin resulted in the suppression of viral progeny release, but not viral protein production (Yuan et al., 2005). Therefore GSK-3β is pivotal in regulating the rupture of infected cells for progeny release.

2.3.3 The endoplasmic reticulum unfolded protein response

CVB3 induces the unfolded protein response (UPR), which is an acute stress response that leads to ER stress (Zhang et al., 2010). Poliovirus, CVB3 and other viruses have been shown to replicate their genomes and translate protein on the surfaces of double membrane vesicles derived from the ER and autophagosomes (Suhy et al., 2000; Wong et al., 2008; Zhang et al., 2011). This activity in combination with interruption of regular protein translation and a massive upregulation of protein production by the virus probably leads to activation of the UPR. This process involves 3 different pathways of independent activation, initiated by 3 respective stress sensors in the proximal ER: ATF6a, IRE1-XBP-1, (X box binding protein 1), and PERK (PKR-like ER protein kinase). It has been shown that infection with CVB3 invokes activation of ATF6a and XBP-1, possibly through replication of the virus on the surface of double membrane vesicles derived from the ER and autophagosome (Wong et al., 2008). Other viruses that replicate in the cytosol, such as West Nile Virus, have been shown to activate this pathway to promote virus replication and cell death ((Medigeshi et al., 2007) review). The UPR response to massive viral protein synthesis and usurped cellular pathways most likely acts to initiate or augment the onset of death in the infected host cell. However if these pathways are activated too early in the virus replication cycle the cell will die before virus protein production and virus progeny can be completed and released, respectively. This would surely result in the termination of virus replication. Zhang et al. showed that expression of the UPR response protein, XBP-1 acts to delay the onset of cell lysis and release of progeny virion (Figure 1B). Therefore, pathways like XBP-1 are used by the virus to counter early destruction of the cell; so CVB3 has evolved mechanisms to prevent premature death of the infected cell. Major cellular survival pathways, like Akt, are also invoked to counter the death reflex, which we will discuss later in the chapter.

2.3.4 Cell death and lysis through activation of many simultaneous pathways

The overwhelming and rapid activation of many different pathways simultaneously leads to cell death and lysis (Figure 1), liberating progeny virion. It is possible, that cell death pathways may even be synergistic, additively driving the cell toward more rapid and thus uncontrolled death, leading to lysis. The cytochrome c- caspase-9 pathway requires a minimum of 15 hours to kill a cell that has been chemically induced, almost twice as long as it takes CVB3 to lyse an infected cell (Qu and Qing, 2004). The plant toxin abrin requires upwards of 15 – 36 hours to kill a culture of HeLa cells (Qu and Qing, 2004), the E2 protein of human papillomavirus requires 20 – 24 hrs to induce apoptosis (Desaintes et al., 1999),

whereas CVB3 will have induced apoptosis and destroyed a similar culture of HeLa cells in just 8 – 10 hours. The rapid nature of CVB3 induced cell lysis suggests the synergy between numerous death pathways 'criss-cross' and interact to enhance their collective cell-death effect.

The loss of checked and contained cell death results in necrosis and lysis of the CVB3 infected host cell. Even though the pathways of cell death are clearly defined in this arena, the precise mechanisms of initiation are still unclear, and potential pathway interactions are unknown. Many reports have indicated that a particular pathway may be involved in CVB3 induced cell death although there hasn't been 100 % recovery of cell viability demonstrated from experiments that inhibit just one pathway. However, zVAD, a pan caspase inhibitor does not completely inhibit virus induced death and lysis just as GSK3β inhibition doesn't completely inhibit cell-rounding and death. Therefore there is still much to be learned about the initiation and interaction of cell death pathways during CVB3 infection, but we may never be able to completely inhibit CVB3 induced cell death because once virus replication is initiated the cell may already be past the threshold of viability.

2.3.5 Necroptosis?

Necroptosis (necrotic apoptosis) is a tumour necrosis factor receptor activated form of programmed cell death that is activated by the same class of receptors that activate caspase-8 mediated apoptosis (Declercq et al., 2009; Hitomi et al., 2008; Yuan and Kroemer, 2010). This is a more recently discovered pathway of cell death that has not been reported in the context of CVB3 infection. However, instead of caspase-8 activation that normally results from TNF receptor ligation there is activation of a receptor interacting protein (RIP)-1/RIP-3 pathway that results in rupture of the cell through loss of membrane integrity; a mode of cell death reminiscent of CVB3 induced cell lysis. Transgenic caspase-8 null animals undergo RIP-3 necroptosis instead of apoptosis, leading to embryonic lethality and non-viability of the line. However caspase-8 -/-, RIP-3 -/- double transgenic mice are viable, suggesting that caspase- 8 holds RIP-3 'in check', preventing initiation of programmed necrosis (Kaiser et al., 2011). The necroptosis pathway has not yet been described with respect to CVB3 infection but given the similarities between CVB3 induced cell lysis and necroptosis this pathway of cell death and the RIP-1/RIP-3 proteins may provide some insight.

2.4 Virus induced cell death and lysis – 'Timing is everything'

There have been reports showing that cell death activated early in the virus life cycle significantly inhibits viral replication (Zhang et al., 2010). Release of the cell contents prior to progeny virion production and more programmed processes of cell death will halt virus replication by killing the cell prior to assembly of progeny virus virion or confinement of progeny virion into vimentin cages, respectively.

Virus replication is cytotoxic and can cause cell death before production of progeny virion, terminating the life-cycle of the virus. For example, reactive oxygen species are a byproduct of CVB3 replication very early in the life-cycle of the virus, placing stress on the cell such that the mitochondrial cytochrome *c* pathway of caspase-9 mediated cell death might be activated prior to virion assembly. To prevent premature death of the host cell coxsackievirus triggers the activation of pro-survival proteins and their associated cascades to suppress cell lysis until progeny virion can be assembled. Perhaps the most studied pro-

survival protein, Akt, is activated during the CVB3 replication cycle (Esfandiarei et al., 2007; Esfandiarei et al., 2004; Liu et al., 2008). It may appear counter-intuitive for a cytolytic virus to evolve to activate pro-survival pathways, particularly if the virus requires the cell to lyse at the end of the cycle. However, as we discussed in section 2.1 CVB3 replication is cytotoxic from the onset so pro-survival pathways may be selected evolutionarily to support the survival of the cell while viral protein synthesis and viral progeny assembly are taking place. These mechanisms of cell survival during virus infection act in favour of viral replication because they allow maximum amounts of virus progeny to be assembled prior to cell lysis and release of viral progeny. Subsequently, these same survival pathways must be inhibited to then permit cell death and lysis for release of assembled viral progeny virions.

2.5 Coxsackievirus induced cell death: many dimensions of complexity

It is now apparent that several cell death pathways and perhaps dozens of proteins are activated in a web of molecular interaction in order to short circuit normal death mechanisms, to cause rapid and aberrant rupture of the infected cell (Figure 1). New pathways of cell death, such as necroptosis, may as yet be shown to mediate death of the CVB3 infected cell. Nevertheless, there already exists a large web of seemingly disparate pathways of CVB3 induced cellular lysis. As we discussed earlier, it is difficult to determine the foundation pathway that is responsible for virally induced cell lysis of infected cardiomyocytes, and which ones are triggered, merely as a matter of coincident activation. New methods of data organisation and analysis are clearly needed as more pathways and mechanisms of CVB3 induced cell death are revealed. Knowledge of the truly pertinent and central pathways of cell death could lead to the most robust antivirals. Toward the end of this chapter we will discuss the use of systems biology to organise the myriad of pathways activated during CVB3 infection (proteolytic and kinase) and how it could be applied to elucidate the dominant pathways of virus induced cell death.

3. Inflammatory regulation of myocarditis by the host: regulatory T cells and healthy vs pathological signalling

3.1 Mouse genetic determinants of immune infiltration

Once virus infection has taken hold in the heart there is infiltration and onset of immune responses to clear infection and lay down provisional matrix in place of damaged tissue. As we discussed earlier in the chapter, the severity of the immune response varies broadly from patient to patient, from mild to massive immune infiltration that can lead to DCM and congestive heart failure. We know from the mouse models of CVB3 induced myocarditis that immune infiltration is correlated with the amount of virus inoculum, and therefore with the degree of damage that has been mediated by virus. However experiments comparing the susceptibility of various inbred strains of mice to CVB3 induced myocarditis showed that there could be a genetic predisposition to myocarditis severity (McManus et al., 1993). These data suggest that a genetic predisposition of the individual patient to mild or severe myocarditis, combined with viremia and cardiac viral load could act in concert to determine severity and number of myocarditic foci. The studies conducted with inbred mouse strains have implicated the MHC class II (H2) locus (Wolfgram et al., 1986), in addition to other genetic loci (Traystman et al., 1991) associated with the severity and susceptibility to CVB3 myocarditis, both acute, chronic and autoimmune. Not surprisingly, the H2 locus of the MHC II complex has been implicated in clearance of acute coxsackievirus infection

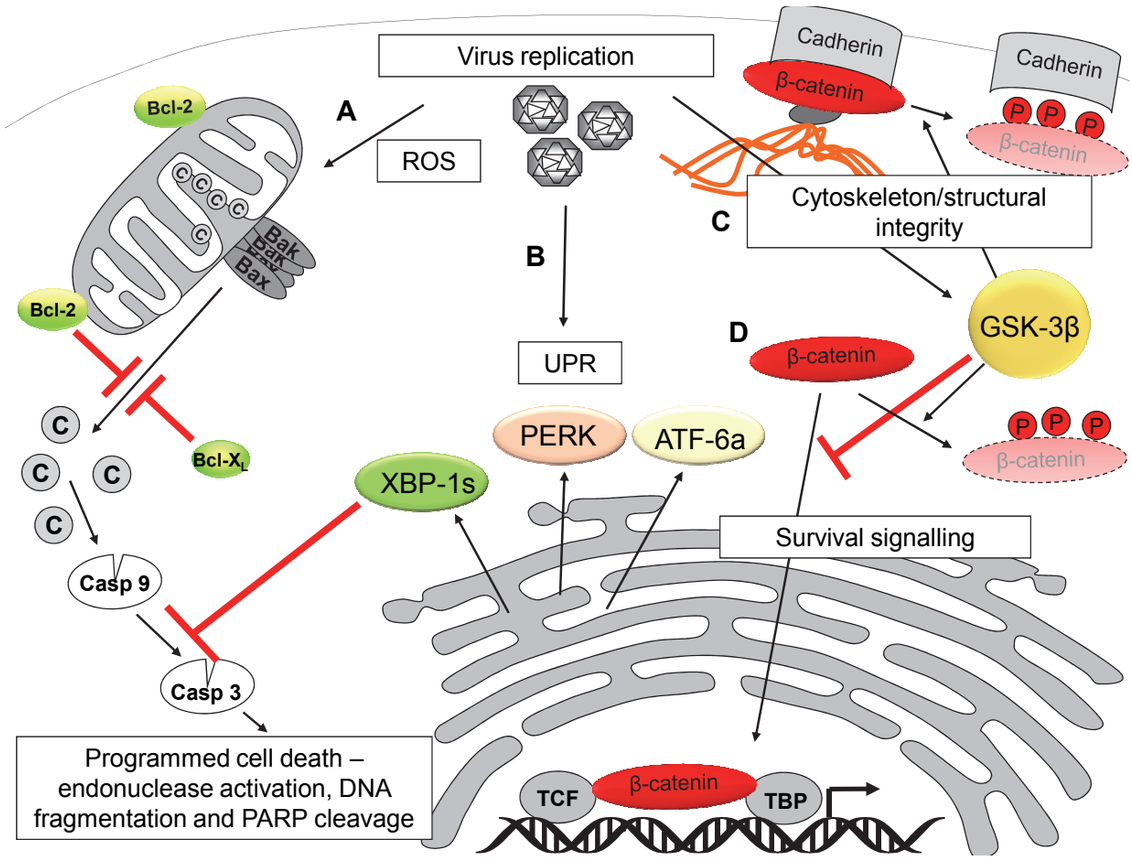

Fig. 1. Pathways of cell death in CVB3 infected cardiomyocytes. A. Coxsackievirus replication results in production of reactive oxygen species which promote the release of cytochrome c from mitochondria. The formation of Bak and Bax at the membrane surface mediate passage of cytochrome c into the cytosol. Overexpression of the anti-apoptotic proteins BCl 2 and BCl-XL during CVB3 infection significantly reduce cytochrome *c* release and activation of the caspase-9 pathway of apoptosis, which leads to caspase-3 death effector caspase activation. ROS = reactive oxygen species. B. The unfolded protein response (UPR) of the endoplasmic reticulum (ER) is activated during CVB3 infection of cardiomyocytes leading to activation of all 3 known pathways of the UPR: spliced XBP-1s, PERK and ATF6a ER stress sensors are all activated during CVB3 infection. Virus replication occurs on the surface of double membrane vesicles and membrane derived from the ER, activating the ER stress sensors. The result of XBP-1s activation is to slow the onset of cell death to prevent the cell from rupturing prior to the assembly of progeny virus virions. UPR = unfolded protein response. C and D. Glycogen synthase kinase -3β (GSK-3β) is activated during CVB3 infection resulting in phosphorylation and subsequent proteosomal degradation of its target, β –catenin, in the cytosol. C. Loss of β –catenin in the cytosol results in loss of connections between the actin cytoskeleton and cadherin receptors, via connections to α and β –catenins. D. Less β –catenin in the cytosol means that there will be less available to translocate across the nucleus to activate survival gene expression via TCF and TBP transcription factors.

(Wolfgram et al., 1986). The presence of a strong adaptive response that includes robust neutralising antibodies is required to control CVB3 infection in mice (Wolfgram et al., 1986) and patients (McKinney et al., 1987; Misbah et al., 1992), as well as other picornaviruses, like polio (MacLennan et al., 2004). Therefore, the host's own genetic makeup will determine how quickly and how completely virus infection is cleared from the body. An inability to mount a broad immune response will lead to more virus replication, more immune infiltraton of the myocardium, more matrix deposition and longer recovery times. In addition to these host dependent factors there may also be environmental factors that play roles during infection and inflammation: extrinsic source stress, pollution/allergens and population density could also contribute to susceptibility and severity of viral myocarditis.

3.2 Chronic infection and the cycle of virus-immune mediated damage

Over the last 3 decades it has become apparent that viral load is a primary determinant of viral myocarditis severity (Cheung et al., 2008; Chow et al., 1992; Lim et al., 2005; Marchant et al., 2009a; McManus et al., 1993), the virus causes extensive, early direct injury to the myocardium through its replication. The studies cited above all indicate significant injury to the myocardium prior to cardiac immune infiltration, supporting the view that considerable damage to the heart is directly mediated by the virus alone. An argument has been made previously for the importance of chronic virus replication in myocarditis and as a basis of dilated cardiomyopathy (Szalay et al., 2006) and chronic heart failure. Enhanced immunoproteasome expression in chronically CVB3 infected cardiomyocytes (Szalay et al., 2006) may increase the expression and presentation of auto-antigens, leading to more severe myocarditis through autoimmune avenues. Chronic virus replication will lead to consistent damage which will, in turn, result in provisional matrix turnover. This type of damage-repair loop will propagate the fibrosis that is evident during chronic myocarditis and DCM. As we covered in the apoptosis/death pathway sections of this chapter the release of cell constituents and debris, and progeny virus and proinflammatory cytokines, from an infected cell will lead to inflammation at the site of virus insult. Therefore, the damage inflicted by the virus itself will be exacerbated by the onset of cytolytic immune infiltration and fibrosis.

3.3 Regulatory T cells

Not all T cell infiltration and immunity are harmful as there are moderating arms of the immune response that solely act to moderate the strength of a response, preventing autoimmunity. Li et al recently reported that allografted M2 (anti-inflammatory) macrophages led to improvement of virus-induced myocarditis (Li et al., 2009) which was associated with enhanced levels of regulatory T cells (Treg). Huber et al have also reported decreased viral load and immune infiltration after adoptive transfer of a CD4+ CD25+ regulatory-like T cell population into a mouse model of CVB3 infection (Huber et al., 2006). Regulatory T cells are CD4 + cells that express the α-subunit of the IL2 receptor CD25, and are negative for CD127. The hallmark protein expressed in these cells is the transcription factor FoxP3 and they circulate as a functionally distinct T cell subpopulation, preventing autoimmune and other aberrant responses to innocuous environmental antigens (Wing and Sakaguchi). The T-reg population prevents the proliferation of self-reactive effector T cell populations and were aptly named suppressor T cells when first discovered for their ability to secrete IL10 and moderate Th1 immunity (Wing and Sakaguchi). Thus, it would be reasonable to postulate that adoptive transfer of T-regs into a mouse model of viral

myocarditis would suppress the immune response and allow virus replication to proceed unchecked.

In a study by Shi et al (Shi et al., 2010) T-reg cells were adoptively transferred into CVB3 infected mice and the effect of the transferred cells upon viral load and signalling within the heart were investigated. Included in the study were PBS injection controls, but also naïve T cells. Decreased immune infiltration and enhanced Akt activation, as compared to the naïve CD4 T cell and PBS grafted controls, were notable in the T-reg adopted mice (Figure 2). Perhaps the most surprising of the results was the decreased viral load, not only in the heart but also in the pancreas and spleen, associated with lower expression of the Coxsackie-Adenovirus Receptor (CAR). The authors adoptively transferred, not only T-reg cells, but CD25 (-) CD4 T cells (naïve) as well (Figure 2A), into a separate group of mice. In fact, the naïve CD4 T cell treated control group fared worse than either the PBS or T-reg treated groups, related to less Akt activation and higher CAR receptor expression. As a result, the naive T cell population brought on a more severe myocarditis phenotype due to higher viral loads. Taken altogether, Shi *et al.* describe the protective role that T-reg cells play in immune infiltration of the heart during viral myocarditis, suggesting that there is a balance to be struck between clearance of infection and immune-associated damage to the myocardium. In fact, in this study there was observed decreased viral loads consonant with a decrease in immune infiltration due to reduced TNF-α release and lower CAR expression (Figure 2B). The authors found that though TNF-α could enhance CAR expression in mouse cardiomyocytes the co-addition of TGF-β eliminated TNF-α-induced CAR expression. To conclude, these findings suggest a more intimate link between inflammation and virus replication than previously posited. Thus, moderating the immune response may be critical for prevention of chronic virus replication.

Although the dominant factor in virus suppression during T-reg treatment was most likely down-regulation of CAR expression, we propose that the alteration in signaling evoked by T-regs is also playing a significant role in modulating virus replication. The basis of our argument is that CAR expression was suppressed 3-fold *in vivo* and 5-fold in a dividing cardiomyocyte cell line *in vitro*, but the suppression of viral load in the Treg group was almost 2-logarithms reduced as compared to the control group. Although the correlation between cell receptor expression and virus infection may not be linear, the decreased levels of CAR receptor may not explain the entire antiviral effect of T-regs in the myocardium during virus infection. It is entirely possible that the signalling environment altered by the adoptive transfer of T-regs may have also contributed to a less favorable environment for virus replication. The authors reported activation of Akt in the myocardium of the T-reg treated groups, which suggests that the activation of other signalling proteins may have been altered by T-reg transfer. We have reported that Akt activation is required for successful CVB3 replication (Esfandiarei et al., 2007; Esfandiarei et al., 2004; Esfandiarei et al., 2006), a pro-survival protein required by the virus to optimize the longevity of the infected cell to promote optimal progeny virus production. On the other hand, the Akt activation reported by Shi *et al.* may merely be a reflection of a healthier environment created by allografted T-reg cells in the myocardium. It is quite clear that an unbraked immunologically active environment supports enhanced cellular signalling driven by viruses for successful replication (Esfandiarei et al., 2006; Marchant et al., 2009a; Marchant et al., 2008). For example, we have previously reported that the powerful immune-stimulating protein p38 is required for effective virus replication in a CVB3 myocarditis mouse model (Marchant et al., 2009a). The activation of p38 MAP kinase was not investigated by Shi *et al.*,

but we would predict less net activation of p38 in the presence of T-regs, and thus an environment that is less conducive to virus replication. Although p38 MAP kinase activation is required for suppressor (T-reg) T cell activity and function (Adler et al., 2007), we propose that adoptive transfer of T-regs may have decreased the immune infiltrate in the myocardium with less activation of p38 MAP kinase.

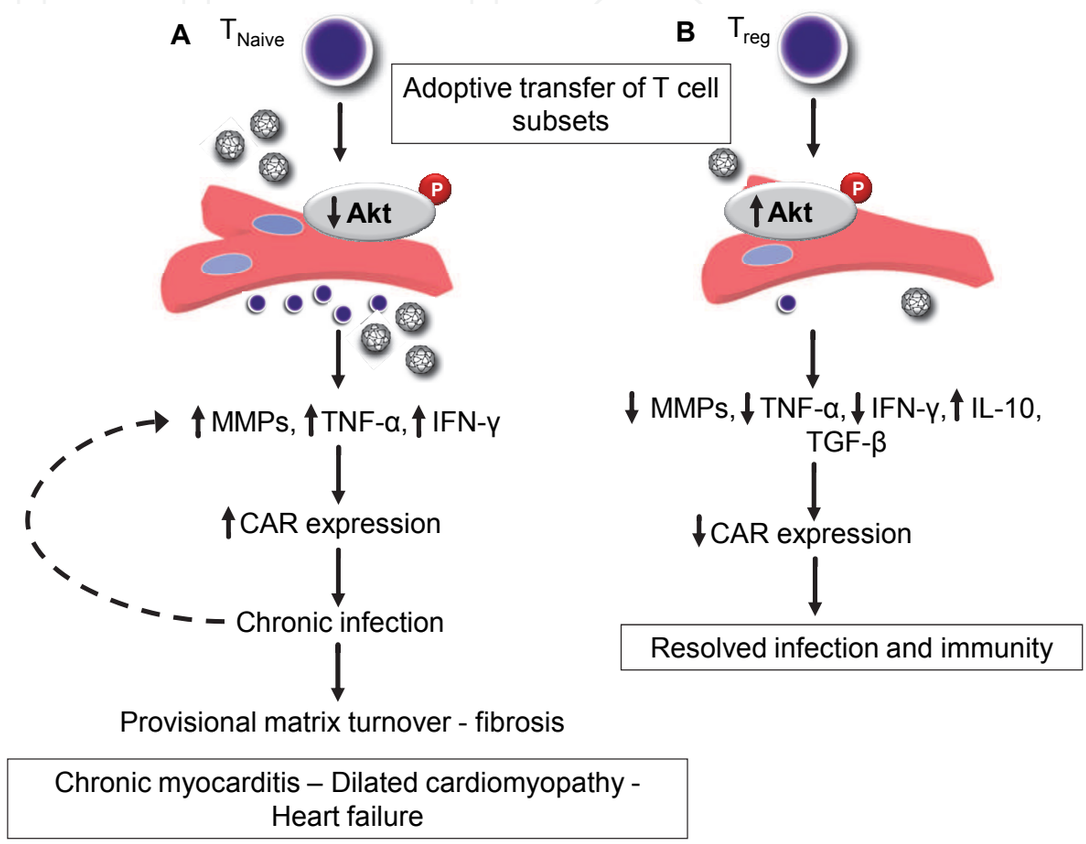

Fig. 2. Adoptive transfer of T cells into CVB3 infected mice has assisted in the elucidation of the beneficial arms of the immune response that aid in clearance of virus from the heart. A. Transfer of naïve T cells or control treatment with PBS, followed by infection with CVB3 results in high viral load in the heart followed by a large influx of T cells. An indication of myocarditis in the mouse model is the detection of decreased Akt activation, and MMP, TNF-α, and IFN-γ expression. Shi et al demonstrated that TNF- α induces coxsackie-adenovirus receptor (CAR) expression in myocardium during CVB3 replication which may promote chronic virus replication which could lead to dilated cardiomyopathy and heart failure through successive rounds of provisional matrix deposition and degradation. B. Adoptive transfer of syngeneic regulatory T cells reduces the immunologically reactive environment which results in less MMP, TNF-α, and IFN-γ expression. The result is lower CAR expression, lower viral loads and enhanced Akt activation, leading to a healthier, less immunologically reactive environment.

3.4 ERK and p38 MAP kinases

Activation of p38 MAP kinase is required for virus replication to take place, such that inhibition of p38 MAP kinase is an effective antiviral strategy *in vitro* (Si et al., 2005) and *in vivo* (Marchant et al., 2009a). The activation of ERK MAP kinase was first reported in the context of CVB3 infection both *in vitro* (Luo et al., 2002) and *in vivo* (Opavsky et al., 2002). The activation of this molecule by virus binding the CAR receptor may be involved in triggering endocytosis uptake of CVB3 during entry (Marchant et al., 2009b). The activation of p38 MAP kinase was later shown to be required for cell lysis and release of progeny virion. Si et al reported that inhibition of p38 MAP kinase could block progeny virus production but not virus protein expression (Si et al., 2005). Underlining the importance of p38 during virus replication was a relatively recent study that investigated the role of p38 during the life cycle of several different viruses. The activation of p38 MAP kinase was demonstrated as a common and dominant signalling molecule during the replication of many different types of viruses (Marchant et al., 2010). This molecule's intimate link to the immune response is perhaps not surprising; one might expect that Akt activation, p38 inhibition and T-reg immune control lead to improved outcome and result in lower viral load and immune infiltration.

With regard to new treatment strategies, this does not necessarily mean that we should be injecting T-reg cells into afflicted patients, but methods should be pursued that mediate the immune response to swing the pendulum in favor of viral clearance and reparation, and away from immune infiltration of the myocardium that favors higher viral loads. The work of several recent studies [(Li et al., 2009; Shi et al., 2010) reviewed in (Yajima and Knowlton, 2009)] have demonstrated the virus' evolved ability to bias immune activation and associated signaling queues for the benefit of virus replication. Given the results presented by Shi *et al.*, one might initially conclude that immune suppression would be a sound therapeutic strategy. However the Myocarditis Treatment Trial, wherein patients were treated with immunosuppressive agents (Mason et al., 1995), showed that such administration had no significant benefit for outcome of human myocarditis. As such, pan-immune suppression is not the way forward and better targeted methods of immune modulation are needed. Shi *et al.* demonstrated that TGF-β secreted by T-reg cells may have been responsible for the decreased CAR expression and enhanced Akt activation (Figure 1). Such suggests that we need not inhibit the immune response entirely, but rather target, modulate and encourage the arm of the immune system that promotes virus clearance. The way forward may not be adoptive transfer of T-regs, but administration of a drug that can promote T-reg differentiation and function.

3.5 The matrix metalloproteinases

The Matrix MetalloProteinases (MMPs) have been implicated in the immune response to CVB3 infection in addition to delaying matrix remodelling that takes place after acute infection.

The MMPs are collectively termed 'matrixins' and were first discovered for their ability to degrade the tails of tadpoles during frog morphogenesis (Visse and Nagase, 2003). These enzymes are zinc endoproteinases and there have since been 24 vertebrate MMP genes discovered that play a large number of roles during immune and developmental processes. There are several gene knock-out strains of MMPs that have been used to elucidate the roles of these enzymes during development and immunity. For example, MMP-8$^{-/-}$ mice are more prone to skin cancers due to delayed neutrophil infiltration and an aberrant immune response (Balbin et al., 2003). Matrix MetalloProteinase-12$^{-/-}$ mice are more susceptible to

bacterial infection due to an innate antimicrobial property of MMP-12 in intracellular compartments (Houghton et al., 2009). This MMP was shown to bind to bacterial membranes within the endosomes of macrophages and destabilise bacteria, thus acting as an antimicrobial.

3.5.1 MMPs as modulators of the antiviral immune response

MMP-9 was one of the first MMPs to be implicated in the mechanism of myocarditis and cardiac dilatation. Heymans et al first showed that inhibition of MMP action through suppression of urokinase-type plasminogen activator, a potent activator of MMPs, and exogenous expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) decreased cardiac inflammation and reduced myocardial necrosis at 7 days, decreasing cardiac fibrosis at 35 days after CVB3 infection (Heymans et al., 2006). When the activity of MMP-2 and -9 were decreased there was a concomitant recovery of cardiac function with decreased immune infiltration. However, shortly after the publication of this paper Cheung et al demonstrated that MMP-9 was in fact a necessary constituent of the antiviral immune response (Cheung et al., 2008). They demonstrated no difference in viral load between MMP-8$^{-/-}$ mice and their WT counterparts. However, MMP-9$^{-/-}$ mice had higher viral loads and virus mediated myocardial damage, during CVB3 infection. Though the antiviral mechanism of MMP-9 action was not explicitly reported, there were significantly elevated levels of interferon-β (IFN-β) in the MMP-9$^{-/-}$ mice. This MMP has been shown previously to cleave and inactivate IFN-β (Nelissen et al., 2003), and IFN-β is known to have a negative feedback effect upon IFN-γ expression (Hanada and Yoshimura, 2002; Yoshimura et al., 2003). The viral loads were similar at early time points (3 – 5 days) post-infection between the genetic MMP-9 knock-outs and WT mice, suggesting that the innate-early immune response was intact, but there were higher viral loads seen in the MMP-9$^{-/-}$ mice 9 days post-infection. These results suggest that there was a deficiency in adaptive immunity in the MMP-9$^{-/-}$ mice, consistent with IFN-β negative feedback inhibition on IFN-γ regulated adaptive immunity in the MMP-9$^{-/-}$ mice.

4. Virus induced cell signalling and global aspects of cellular signalling responses

Thus far we have discussed a large number of parallel signalling molecules and pathways that are simultaneously activated during CVB3 infection, from proteolytic death pathways to phospho-signalling kinase cascades that activate a myriad of functions. Systems biology approaches now exist whereby one may survey a large number of pathways and molecules at the same time, query these results using complex statistical methods and elucidate the pathways that are truly required for a particular pathogenic function, and determine which of those that are merely coincident to viral infection.

4.1 Defining cell signalling network models in virus-host interactions

Host cells have evolved complex systems to detect and eradicate viruses; on the other hand, viruses have evolved mechanisms to compromise essential cellular processes and suppress the host cell defence. In these complex systems, protein phosphorylation events control most aspects of cellular function and homeostasis, and the failure of control mechanisms causes disease (Cohen and Tcherpakov, 2010). Most notably, protein phosphorylation events

mediated by kinases and phosphatases are used by viruses as they are crucial for pathogen replication, propagation, and evasion from host immune responses (Ribet and Cossart, 2010). Several studies have showed that multiple phosphorylated-proteins individually regulate the events that constitute virus replication [reviewed in (Esfandiarei and McManus, 2008)], yet the phospho-proteins are part of an intracellular signal-transduction network, composed of several pathways. Thus, systems perspective studies have enabled us to address network mechanisms active during host-virus interactions, with specific emphasis on elucidating key determinants of disease severity and the effective translation of these concepts into new systems-oriented therapeutic targets.

Virus infection is equivalent to a network perturbation, in that viruses have evolved effective strategies to manipulate multiple signalling pathways and induce crosstalk and feedback loops among pathways to form a network (Garmaroudi et al., 2010). In fact, viruses activate signal-transduction networks through multiple independent events, which include viral docking to receptors, viral protein synthesis, viral progeny release and virus-induced inflammatory responses (Tam, 2006). Thus, to properly understand how signal-transduction networks are disrupted by viruses, a global multivariate approach is required (Ideker et al., 2001; Kleppe et al., 2006).

One of the major challenges of defining a signal-transduction network model in the context of a disease is to study a holistic picture of molecular structures, coming together to make complex and dynamic networks. New tools are required to systematically perturb and monitor signalling processes and functions within cells. Indeed, the emergence of high-throughput, extremely parallel technologies enable biologists to monitor multiple cellular components all together (Albeck et al., 2008). These tools have provided researchers the opportunity to collect comprehensive and large datasets. Nowadays, in the era of "new biology" researchers are 'drowning' in data, in that one emerging challenge is how to organize, interpret and extract pertinent information. The complex web of death pathways discussed in section 2.3 or the complex nature of the immune response discussed in section 3.0, all argue a need for higher level analysis through machine learning. Though this analysis requires skilled computational biologists, using mathematical, statistical and computational techniques to put together the biological components into functional molecular and cellular network models in a systematic fashion (Janes and Yaffe, 2006).

One work that has emerged from recent studies of networked systems in the virus-infected host cell revealed ~260 host cellular factors, affecting virus infection (Brass et al., 2008), however only a small subset of these proteins played a role in the early stage of virus infection. Interestingly, a complementary study showed how a multi-parameter approach could unravel host-virus protein interactions that likely act as a network to facilitate the early steps of HIV-1 infection (Konig et al., 2008). Recently, small-molecule inhibitor pairs were used to perturb pairs of phospho-proteins to reveal causal mechanisms within the signal-transduction network response of cardiomyocytes to coxsackievirus B3 (CVB3) infection. Hierarchical cluster analysis of the resulting dataset showed that paired-inhibitor data was required for accurate predictions of the network. In this study we also depicted a high-confidence network based on partial correlations, which identified phospho-IκBα as a central "hub" in a measured phosphorylation signature (Garmaroudi et al., 2010). Now, biologists are poised to understand the network mechanisms in virus infection, in that these networks might be used to improve patient diagnosis, monitoring, and treatment.

4.2 Proposing novel therapeutic targets for viral myocarditis through systems biology analysis of host cell signal-transduction networks

As we have already discussed, cardio-tropic viruses like CVB3 can directly and indirectly further the progression of viral myocarditis to heart failure [(McManus et al., 1993) reviewed in (Marchant et al., 2008)]. To date, there is no curative treatment beyond heart transplantation for viral myocarditis-associated heart failure. Therapies that have been used in patients with myocarditis are immune serum globulin and pleconaril (Pevear et al., 1986; Rotbart, 1999). The anti-picornaviral agent, pleconaril perturbs viral uncoating and in turn blocks viral attachment to host cell receptors. Although, directly targeting viruses has been successful in controlling viral diseases, it suffers from some serious weaknesses, including failure to eliminate chronic viral myocarditis, narrow spectrum of action, and the inherent capacity to force outgrowth of drug resistance mutations (Tan et al., 2007). Thus, the discovery of novel antiviral targets, host cell-based antiviral agents is a more promising approach and deserves more attention (Saladino et al., 2010).

At the cellular level, studies have shown that host cell phospho-proteins essential for viral replication, are potentially targetable [(Marchant 2009) reviewed in (Marchant et al., 2008)]. It is known, however, that phospho-protein networks embrace a system that contains redundant, convergent and even distinct signaling pathways (Borisy et al., 2003). Such combinative properties of signaling networks may counteract the therapeutic efficacy of highly selective drugs due to signalling pathway redundancy. Thus, combination therapy may be necessary to achieve efficacy due to less treatment resistance (Fitzgerald et al., 2006). However, motivation for this initiative, therapeutic synergy of a combination is tempered by concerns about introducing synergistic side effects. Nevertheless, an *in vivo* study that has emerged from recent studies of the healing process in a rat asthma model showed that combining drugs performed better than single ones (Lehar et al., 2009).

Together, to propose novel and promising drugs and therapies requires us to understand network mechanisms at the cellular, tissue and organism level. Using high-throughput technologies in genomics, proteomics, phosphoproteomics, metabolomics and lipidomics, we can develop interactome network models, in that these models ultimately translate to network analysis and data-driven questions in a specific disease context, that can be used to analyse a broad range of different states and disease contexts. There have been recent reports that used systems biology analysis tools in an attempt to untangle the web of kinase signalling pathways that are activated during CVB3 infection of cardiomyocytes (Garmaroudi et al., 2010). There is a current unclaimed niche of research that exists to use similar systems biology tools to analyse the pathways of cell death in order to better understand not only the dominant pathways of virus induced cell death but to also characterise the type of cell death that causes necrotic rupture of infected cells. To perturb specific molecules or biological processes in a combination as directed by network models can be a capable strategy to treat complex diseases.

5. Conclusion

In every facet of CVB3 replication there are a large number of pathways and proteins involved, forming a large network of pathways and complexity too large to analyse by conventional means. The large number of death pathways activated by CVB3 may not all be required to mediate lysis of the infected host cell. The question still remains as to whether there are pathways activated as a matter of coincidence or whether death pathways are all

consequential. There are also a large number of immune constituents involved in the clearance but also the pathogenesis of myocarditis, from naive T cells to regulatory T cell subsets and a large number of MMPs. Systems biology should prove useful in the near future to elucidate the interactions of proteins and pathways and show the truly pertinent proteins and pathways responsible for cell death, virus replication or MMP mediated tissue remodelling. New questions regarding pathway coincidence and consequence can be investigated. New methods of analysis should assist in the understanding of beneficial vs pathological arms of the immune system. With this knowledge we should be able to design highly useful and robust biomarkers, antivirals and drugs to help direct appropriate repair and remodelling.

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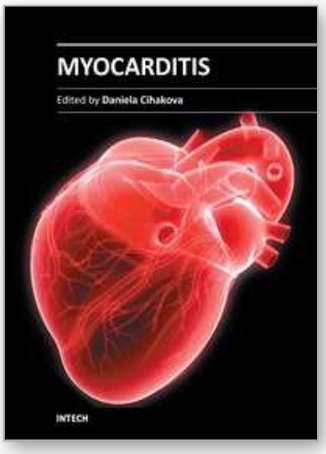

Myocarditis

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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; Chagasâ€™ 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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