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Coxsackievirus and Idiopathic Heart Failure: Differentiation, Dissemination, and Death

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2013

The Dissertation of Jon Sin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2013

DEDICATION

I dedicate this dissertation firstly to God for the abundant blessings he has placed in my life.

Secondly, I dedicate this dissertation to my family. To my mom, my dad and my brother, you have encouraged me and supported me for as long as I can remember.

EPIGRAPH

Science becomes dangerous only when it imagines that it has reached its goal.

–George Bernard Shaw

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LIST OF ABBREVIATIONS

BAF	Bafilomycin-A1
BW	Body weight
CAR	Coxsackie adenovirus receptor
CSC	Cardiac stem cell
CVB	Coxsackievirus B
DAF	Decay accelerating factor
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
eGFP	Enhanced green fluorescent protein
EMV	Extracellular microvesicle
FACS	Fluorescence-activated cell sorting
GFP-CVB	eGFP-expressing CVB
H&E	Hematoxylin and eosin staining
HW	Heart weight
IP	Intraperitoneally
ISO	Isoproterenol
KO	Knockout
LC3	Microtubule-associated protein-1 light chain 3
MEF	Mouse embryonic fibroblast
MI	Myocardial infarction

PFU	Plaque forming units
PI	Post-infection
qPCR	Quantitative real time polymerase chain reaction
Sca-1	Stem cell antigen-1
TL	Tibia length
VEGF	Vascular endothelial growth factor
WT	Wildtype

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ABSTRACT OF THE DISSERTATION

Coxsackievirus and Idiopathic Heart Failure:

Differentiation, Dissemination, and Destruction

by

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University of California, San Diego, 2013

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Coxsackievirus B (CVB) most commonly causes a self-limited febrile illness in infants, but in severe cases can manifest in acute systemic inflammatory diseases including meningo-encephalitis, pancreatitis, and myocarditis. Chronic consequences of mild CVB infection are unknown, though there is an epidemiologic association between early subclinical infections and late heart failure, raising the possibility of subtle damage leading to late-onset dysfunction, or chronic ongoing injury. Huang and colleagues described a

mouse model where neonatal exposure to doxorubicin depleted cardiac stem cells (CSCs) and resulted in cardiac dysfunction in adult animals that was only elicited by a prolonged exercise challenge or surgical myocardial infarction. Additionally, Feuer et al have shown that CVB infects and destroys actively proliferating neural stem cells. Here we report that cultured CSCs from neonatal mouse hearts were also susceptible to CVB infection. To test the hypothesis that CVB might deplete CSCs during self-limited infection, we inoculated neonatal mice with CVB and assessed the effects of viral infection on CSCs. We observed infected CSCs in the neonatal heart as well as a 50% reduction in CSCs in adult animals previously exposed to CVB. Our observations indicate that CVB can target and infect CSCs in the neonatal heart, subsequently exhausting them. Shortly after infection, we observed the induction of premature differentiation in the surviving CSCs which contributed to the sustained depletion of CSCs in adults. When these animals were physiologically stressed as adults via exercise of pharmacological stimulation, they exhibited cardiac hypertrophy and fibrosis. This supports the hypothesis that predisposition to heart failure can be a sequel to asymptomatic CVB infection. We hypothesize that asymptomatic CVB infection may result in sensitivity to cardiac injury and that this is due to depleted CSCs. Additionally, because CVB is known to upregulate host autophagy after infection, we explored how autophagy might be involved in CSC depletion and viral dissemination. We found that CVB induced the release of extracellular microvesicles that contained large amounts of virus. Because viral particles would be sequestered within host-derived membranes, we hypothesize that

these vesicles may be a novel mode of host cell escape that bypasses humoral immune defenses.

INTRODUCTION OF THE DISSERTATION

The Molecular Biology of Coxsackievirus

Coxsackievirus type B (CVB) is a non-enveloped positive sense single-strand RNA virus of the *Picornaviridae* family and *Enterovirus* genus and possesses a very small genome of 7.5-kilobase pairs. Like other enteroviruses, CVB replicates in the cytoplasm and commonly is released from the cell as it dies (cytopathic effect); however it has been recently suggested that the virus can subvert the host autophagy pathway to facilitate viral replication possibly by using autophagosome-like vesicles as manufacturing sites while simultaneously being masked from the innate immune system.[1, 2] CVB's genome contains an internal ribosomal entry site within the 5' untranslated region which encodes a single polyprotein via cap-independent translation. This polyprotein is post-translationally cleaved into four capsid proteins (VP1-4) as well as seven non-structural proteins (2A-C and 3A-D) which include proteins involved in shutting down cap-dependent translation (2A) as well as suppressing innate immunity (3C).[3, 4] Also, more recently, 2BC and 3A have been shown to be involved in manipulating host autophagic machinery which would indicate an involvement of autophagy in the infection process.[5] Because CVB is non-enveloped, the virus is inherently cytolytic, causing the host cell to burst in order to release free virions. However, autophagy is now being viewed as a possible mechanism by which picornaviruses can escape from the cell without destroying it.[1]

Coxsackievirus and Heart Disease

CVB is a common human pathogen that typically causes mild symptoms such as fever, rash, and upper-respiratory symptoms that are self-limiting. Though the virus can also cause severe inflammatory diseases including myocarditis, the manifestation of a cardiac disease phenotype has been documented to be extremely rare (5% of infected patients).[6] Additionally, it has been documented that a large percentage of individuals (~70%) with end-stage idiopathic dilated cardiomyopathy have detectable levels of CVB RNA in the myocardium without any history of antecedent viral myocarditis.[7, 8] These findings raise the possibility that mild infection with CVB can cause a subtle but lasting injury, although it is unclear whether this is immune-mediated or due to direct injury. To our knowledge, a mechanistic basis for a link between neonatal CVB infection and adult-onset dilated cardiomyopathy has not been previously characterized.

Cardiac Stem Cells

Until recently, the heart was viewed as a terminally differentiated organ predominantly comprised of a fixed number of non-renewable cardiomyocytes. It was not until a distinct population of resident stem cells was identified in the heart that this notion was challenged. These cardiac stem cells (CSCs) were previously described to have large nuclei, scant cytoplasm, and expressed hematopoietic markers such as CD117 (c-Kit) and Sca-1.[2, 9-11] When these cells were isolated and cultured, they were capable of differentiating into all four cell

types of the cardiac lineage, which are cardiomyocytes, smooth muscle cells, endothelial cells, and fibroblasts.[2, 12, 13] CSCs have also been shown to play a beneficial role in adult cardiac repair as local injection of isolated GFP-expressing CSCs has been shown to preserve myocardial muscle mass and reduce scar formation after experimental myocardial infarction in mice. Immunohistochemistry revealed GFP-positive myocytes in the myocardium bordering the infarct, suggesting migration and differentiation of CSCs.[14] However, the low number of surviving CSCs following re-introduction into the infarcted heart is not consistent with the substantial cardiac repair observed; therefore it has been suggested that the rescuing effects of the paracrine factors released by CSCs may outweigh their ability to differentiate and integrate into the damaged myocardium.

The Impact of Coxsackievirus Infection on the Cardiac Stem Cell Pool

Due to their role in cardiac development as well as in cardiac maintenance, a perturbation of the CSC pool could conceivably have deleterious effects on the developing heart. It has previously been demonstrated that in mouse pups, exposure to the chemotherapeutic drug doxorubicin (DOX) before postnatal day 21 resulted in a decreased number of CSCs due to early senescence.[15] Although DOX-treated animals exhibited normal cardiac function at 8 weeks of age, endurance exercise led to the development of pathological hypertrophy and fibrosis. This model seemed to mirror the late consequences of childhood doxorubicin-induced cardiotoxicity observed in humans which

manifests in weakening of the myocardium, an attenuation of cardiac pump performance, and progression to congestive heart failure.[15] Because of the detrimental effects of a reduced CSC pool (demonstrated in the DOX study) and studies by Feuer et al describing CVB's tropism towards neural stem cells, we investigated the effect CVB had on CSCs.[16, 17] We developed an in vivo mouse model of CVB infection of CSCs and observed that not only did CVB infect CSCs, but infection caused a sustained reduction in the CSC pool. This exhaustion of CSCs predisposed the adult animal to stress-induced cardiac hypertrophy, left ventricular dilation, and the accumulation of fibrotic tissue; the early stages of dilated cardiomyopathy.

The Effects of Coxsackievirus-induced Autophagy on the Host Cell

Autophagy is a process by which a cell degrades old or damaged proteins and organelles. The bulk degradation/recycling pathway begins with the formation of a phagophore which elongates around the materials targeted for degradation. Once fully enclosed, the double-membrane autophagosome is directed to the lysosome and subsequently fuses with it forming the autolysosome. Acidic hydrolases degrade the cargo within the autolysosome, resulting in the release of free amino acids which can be reused by the cell. Normally homeostatic autophagy can serve to clear out unnecessary cellular components as well as to liberate nutrients during fasting. Autophagy is also known to play a crucial role in the removal of invading pathogens (xenophagy). As previously mentioned, CVB has been reported to upregulate host cell

autophagy upon infection.[17-23] Though the natural assumption would be that autophagy is upregulated by the cell to remove the virus, others have suggested that CVB subverts autophagy by using autophagosomes as a factory for viral replication.[18, 20] The role of autophagy during the course of infection and its subsequent effects have been the subject of much recent investigation but is not completely understood. We found that when mouse embryonic fibroblasts with impaired autophagy were infected with CVB, viral dissemination was heavily impacted resulting in fewer infected cells and much lower viral titers in the media. Upon closer inspection we noticed that infected wildtype cells released tiny microvesicles containing high amounts of viral protein. These particles may represent a novel route by which naked viruses such as CVB can exit the cell without the need for a lytic phase. Because the host cell is not readily destroyed, the virus has the benefit of prolonged replication. In addition, since CVB coats itself with host components, this could presumably facilitate immune evasion as well.

CHAPTER I:

Coxsackievirus B Targets Cardiac Stem Cells During Acute Infection of the Neonatal Myocardium

INTRODUCTION

Cardiac stem cells (CSCs) are unique pluripotent cells that reside in the myocardium. These cells have classically been shown to express hematopoietic markers such as c-Kit and stem cell antigen-1 (Sca-1) but lack bone marrow marker CD45 and hematopoietic marker CD34.[2, 9-11] CSCs are capable of differentiating into all mature cell types of the cardiac lineage which include cardiomyocytes, vascular smooth muscle cells, vascular endothelial cells, and cardiac fibroblasts.[2, 12, 13] Interestingly, niches of CSCs can be found in the adult heart raising speculation regarding their precise role in an organ predominantly consisting of myocytes long thought to be post-mitotic and terminally-differentiated. Several investigators have reported the recruitment of cardiac progenitors to sites of injury following experimental ischemia/reperfusion.[5, 16] Additionally, when adult CSCs were isolated from mice, expanded and subsequently injected into the border zones of infarct regions in recipient mice subjected to experimental myocardial infarction, those recipients showed significantly improved cardiac function, and increased survival compared to control animals.[24] In fact, CSCs have shown so much regenerative promise in animal heart disease models that clinical trials have begun and already show functional improvements in heart failure patients receiving CSC injections at the site of injury.

Though the potential benefits of CSCs have been described in multiple studies, their natural biological role is somewhat elusive, in that the regenerative

capacity of the heart and its plasticity as a whole is extremely limited. Huang et al had previously shown that neonatal exposure to the chemotherapeutic drug doxorubicin (DOX) induced premature senescence of CSCs, resulting in a significant reduction of the stem cell pool during adulthood. Aside from the significant decline in CSCs, under basal conditions these animals exhibited no abnormal phenotype through 11 weeks of postnatal development and adulthood. However, when the mice were subjected to exercise or surgically-induced cardiac stress, DOX-treated animals developed pathological hypertrophy characterized by left ventricular enlargement and fibrosis. These data suggest that CSCs may be involved in cardiac adaptation to increased load.

In light of previous studies demonstrating a preferential targeting of neural stem cells by CVB, and a more recent report that CVB infects bone marrow stem cells, we sought to determine if CVB had a broader stem cell tropism and examined the ability of CVB to infect CSCs.[16, 17, 25, 26] We developed an in vivo model of asymptomatic juvenile infection in BALB/c mice. The purpose of this model was two-fold in that it was developed to not only confirm susceptibility of CSCs to CVB infection, but also to determine if a mild CVB infection (e.g. no acute pathology or lasting inflammation) could diminish the CSC pool leading to cardiac deficits during adulthood, similar to CSC depletion via DOX exposure. Therefore, juvenile mice were infected with 10^7 pfu CVB intraperitoneally (IP) at postnatal day 3 and hearts were harvested 2 days postinfection (PI) for histological analysis. We found that CSCs were preferentially infected by CVB,

accompanied by cellular growth arrest and premature differentiation rather than lytic cell death. We hypothesize that this unusual consequence of infection impairs the capacity for the CSC population to self-renew.

METHODS

Generation of Recombinant eGFP-Expressing Coxsackievirus B

Generation of eGFP-expressing CVB (GFP-CVB) has been previously described.[27] Briefly, a plasmid was derived from an infectious CVB3 clone isolated from the heart of an acute myocarditis patient (pH3). This plasmid was engineered to contain an Sfi1 restriction site (pMKS1). The eGFP gene was amplified from an eGFP expression plasmid (Clontech, Palo Alto, CA) using eGFP primers with flanking Sfi1 sites. These PCR products were subsequently cloned into pMKS1. This construct was transfected into HeLa RW cells and lysates containing infectious virus were collected as viral stocks. Viral concentrations were measured via plaque assay.

Cardiac Stem Cell Isolation and Infection

CSCs were isolated using the Millipore Cardiac Stem Cell Isolation Kit (Millipore #SCR061) according to the manufacturer's protocol. To determine CVB replication in cultured CSCs, cells were seeded in 6-well plates at a concentration of $1x10^4$ cells/well. After 3 days, cells were infected with GFP-CVB at MOI 1 or MOI 1000. Viral titers were measured in culture supernatants obtained each day following infection. For western blots, CSCs were seeded in 6-well plates at a concentration of $1x10^6$ cells/well. After 3 days, cells were infected with GFP-CVB at MOI 100. 3 days postinfection (3d PI), cells were lysed in RIPA buffer and proteins were used for western blot analysis.

Mice and Viral Inoculations

Animal experiments were performed in compliance with the San Diego State University Animal Research Committee and the National Institutes of Health. BALB/c mice were obtained from the Scripps Research Institute animal facilities. Breeding pairs were monitored daily and three day-old pups were infected intraperitoneally with 10^7 pfu GFP-CVB or with equivalent volumes of DMEM for mock-infected controls. Mice were anesthetized with isoflurane and were sacrificed via cervical dislocation. Hearts were removed and rinsed in cold PBS. For histology, hearts were fixed by immersion in 10% neutral-buffered formalin for 24 hours and then transferred to 70% ethanol for an additional 24 hours. The tissue was then paraffin-embedded.

Virus Titration via Plaque Assay

Viral titration has been described previously.[27] Briefly, six-well dishes were plated with HeLa RW cells and grown to 100% confluency. Samples were serially diluted (10-fold dilutions) in DMEM and incubated on cell monolayers for 1H with gentle rocking every 15min. Virus was immobilized by overlaying equal parts molten agar and 2X DMEM. 48H later, solidified agar plugs were overlaid with fixing solution (75% methanol +25% acetic acid) for 10min at room temperature. Plugs were then removed with a metal spatula and fixed cells were stained with 0.25% crystal violet for 20min. Cells were then rinsed and plaques were counted.

Histology

Paraffin-embedded hearts were cut into 4µm thick sections. Sections were then deparaffinized with xylene and rehydrated in stepwise decreasing ethanol concentrations followed by PBS and distilled water. Antigen recovery was performed by boiling sections in sodium citrate buffer (10mM sodium citrate + .05% Tween20, pH 6.0). For immunostaining, the following primary antibodies were used: anti-CD3 (1:100, Biocare Med #CP215A), anti-Iba1 (1:500, Wako #JNH4100), rabbit anti-GFP (1:100, Invitrogen #A6455), goat anti-c-Kit (1:40, R&D #AF1356), rat anti-Sca-1 (1:25, Cedarlane cat #CL8934AP), mouse anti-Ki67 (1:200, Vector #VP-K452), rabbit anti-SM22 (1:2000, Abcam #ab14106), rabbit anti-Von Willebrand Factor (1:100, Sigma #F3520), rabbit anti-CD31 (1:100, Abcam #ab28364), mouse anti-sarcomeric actin (1:100, Sigma #A2172). Perkin Elmer proprietary blocking solution (Perkin Elmer #NEL702001) was used for antibody dilution and tissue blocking.

Whole Heart Dissociation for FACS Analysis

Extracted hearts were placed in isolation buffer and excess blood was expressed by gentle compression with forceps. Hearts were then minced into ~2mm^3 pieces in Millipore Isolation Buffer. Heart pieces were resuspended in Millipore Cardiac Tissue Dissociation Buffer and incubated at 37^{o}C shaking at 200 rpm. After 45 minutes of incubation, tissue was mechanically dissociated for 5 minutes by repeated trituration with a wide-bore pipette tip. Tissue was then incubated for 15 more minutes and then mechanically dissociated for 5 minutes.

Large tissue pieces were removed by filtering suspension through Millipore 100um Steriflip unit. Cells were fixed in 10% formalin in PBS. For FACS analysis the following antibodies were used: PE-anti-Sca-1 (1:100, BD #552108), FITC-anti-Sca-1 (1:100, BD #557405), APC-anti-CD45 (1:100, Caltag #MCD4505), PE-anti-CD34 (1:100, Caltag #RM3604).

RESULTS

Establishing a mouse model of subclinical CVB infection to examine CSC alterations

To study subclinical neonatal infection, mice were infected with 1 x 10^7 pfu GFP-CVB at postnatal day 3. This inoculum of virus was well-tolerated and infected mouse pups showed no overt health abnormalities; suckling and weight gain were similar to the mock-infected controls. There was no significant difference in heart weights between CVB and mock-infected animals at any timepoint (Fig. 1.1A). Despite the absence of overt illness, a large amount of infectious virions and viral genomic RNA was detectable in the heart up to 9d PI, confirming cardiac tropism of the virus (Fig. 1.1B and C). Immunostaining for CD3 and Iba-1 (T-cells and macrophages respectively) showed that no detectable immune cell infiltration was elicited by the viral infection (Fig. 1.1D). At 2d PI, hematoxylin-eosin (H&E) staining showed that both groups had normal arrangement of myofibrils, and Masson's trichrome staining revealed no myocardial scarring during the acute phase of infection (Fig. 1.1E).

CVB infects CSCs both in vitro and in vivo

We first verified the susceptibility of CSCs to CVB infection in vitro by infecting CSCs isolated from 14d old neonates. These cells were infected with GFP-CVB at either MOI 1 or MOI 1000 GFP-CVB and examined for viral protein expression (eGFP expression). At 1d PI we observed eGFP-positive cells at

both MOIs (Fig 1.2A). As the course of infection progressed, cells exhibited cytopathic effect, detached from the surface of the culture dish and fragmented into apoptotic bodies. Additionally, we observed a population of low eGFP-expressing morphologically normal cells. At later time points, eGFP was not detected and infected cultures developed a flattened morphology consistent with differentiation (not shown). Curiously, CSCs isolated from adult animals expressed no eGFP, nor exhibited any visible cytopathic effects suggesting that adult CSCs were resistant to infection. Plaque assays of the supernatants from infected CSC cultures revealed that CSCs from neonatal animals supported much higher viral titers than those from the adult animals (Fig. 1.2C). These differences are also consistent with clinical studies suggesting that infants are much more susceptible to CVB infections than adults.[28-30] This differential susceptibility to infection was not attributable to differences in the expression of coxsackie adenovirus receptor (CAR), implicating developmentally acquired host factors other than CAR expression that affect viral tropism and confer resistance (Fig. 1.2B). Next, to confirm that CVB infects CSCs in vivo, immunostaining was performed on the hearts of mouse pups infected with our subclinical dose of GFP-CVB. Hearts were harvested 2d PI and paraffin-embedded for histological analysis. Viral protein (eGFP) colocalized with c-Kit and Sca-1, indicating infection of CSCs (Fig. 1.2D and E). Interestingly, viral protein staining was not observed in the surrounding myocardium, suggesting that CSCs are preferentially infected.

Sca-1 expression increases in the infected neonatal myocardium

Having established that CSCs could be infected by CVB in cell culture and in the hearts of mice, we next sought to determine what effects CVB had on CSCs immediately following infection. As our early in vitro CSC infections revealed, highly infected cells exhibited cytopathic effects and cytolysis. However in the same culture, there were cells that survived infection and appeared to take on a differentiated morphology. These data suggested that there was heterogeneity in the effects of CVB infection in vitro. To further investigate the outcome of infected CSCs, immunohistochemistry was performed on the hearts of infected mouse pups 2d PI. At this timepoint, total number of c-Kit$^+$ cells was not significantly different between infected and control animals (Fig. 2.1A). Surprisingly, we observed a large increase in the number of Sca-1$^+$ cells (Fig. 1.3A). To determine if these Sca-1 cells were recruited from the peripheral blood as a response to virally-mediated cardiac damage, cell suspensions derived from enzymatically dissociated infected hearts were analyzed with flow cytometry for co-expression of Sca-1 and bone marrow marker CD45 or hematopoietic marker CD34. These results confirmed the increase in Sca-1$^+$ cells in infected hearts; however, the Sca-1$^+$ cells were largely negative for CD45 or CD34, indicating they were cardiac-derived rather than from the peripheral blood (Fig. 1.3B and C). Coupled with our previous histology results which revealed an absence of immune infiltration, these results also further ruled out an

inflammatory response elicited by the virus, and support our assertion that this concentration of virus causes a very mild subclinical infection.

CVB-infection induces premature differentiation of cardiac stem cells

Though the precise role of Sca-1$^+$ cells in the heart following CVB infection remains elusive, Sca-1 has been shown to function in suppressing cell cycle progression. Recent literature has suggested that in certain precursor cell types, Sca-1 is expressed in very low levels to prevent uncontrolled cell proliferation and premature senescence. Not until the cell enters a differentiation program is Sca-1 upregulated leading to the cell's complete withdrawal from the cell cycle which is a necessary prequel to differentiation.[31, 32] If Sca-1 similarly plays a role as a late stem cell marker in CSCs, then its upregulation could indicate a large increase in the number of stem cells undergoing differentiation in response to CVB infection. A reduction in the number of Ki67$^+$ cycling cells in hearts from infected animals 2d PI was observed relative to mock-infected controls (Fig. 1.4A and B). Costaining for Ki67 and c-Kit showed a significant reduction in the number proliferative c-Kit cells in the hearts of infected pups (Fig. 1.4C). When staining for both Ki67 and Sca-1, we observed a sharp increase in Sca-1 expression although the Sca-1$^+$ cells were predominantly Ki67 negative, consistent with Sca-1's proposed role as a cell cycle suppressor (Fig. 1.4D). To determine if the Sca-1$^+$ cells expressed markers of differentiation, infected hearts were double-stained for Sca-1 and mature cardiac lineage markers SM22, Von Willebrand factor, and sarcomeric actin (vascular smooth muscle, vascular

endothelial, and cardiomyocyte respectively). Figures 1.5A and B show colocalization of Sca-1 with SM22 and Von Willebrand factor. However, as seen in Figure 1.5C, Sca-1 did not colocalize with sarcomeric actin suggesting exclusive commitment of Sca-1^{+} cells to a vascular cell lineage in vivo. Unexpectedly, when isolated CSCs were infected in vitro, they exhibited a sharp increase in alpha-actin expression (cardiomyocyte marker) but showed no increase in SM22 expression (Fig. 1.5D). We expect that the surrounding microenvironment in vivo may influence cell lineage commitment patterns.

DISCUSSION

CVB infections are a common human pathogen. Because most CVB infections are subclinical, it is difficult to determine what percentage of the adult population has been exposed to CVB, with reports claiming between 0-39.1% exposure in various groups tested.[7, 8] However, CVB is linked to several chronic diseases including Type I diabetes, and idiopathic heart failure. Notably, CVB genomic RNA can be recovered from heart tissue of 70% of patients with idiopathic dilated cardiomyopathy.[7, 8] A connection between subclinical juvenile infection and late-onset heart failure has never been established.

A wide body of literature has shown that CVB exhibits tropism for primitive cell types including neural stem cells and bone marrow cells.[17, 25, 26, 33] It has also been reported that CVB may preferentially infect proliferating cells.[16, 33] Taken together, these findings support the notion that cardiac stem cells would be susceptible to CVB infection in early postnatal life. CVB infection of the adult heart may not deplete CSCs because stem cells that remain later in life tend to be quiescent, becoming activated only in response to cardiac stress.

Using a BALB/c mouse model of juvenile CVB infection, we were able to confirm that CVB infects CSCs and that they seem to be preferentially targeted relative to other cell types in the heart including cardiomyocytes. However, CVB exposure did not appear to produce any substantial cytolytic effects on infected CSCs in vivo but rather seemed to induce premature differentiation. It is unclear what the ultimate fate of these CSCs is following infection. Under normal

conditions, the CSC pool would expand and differentiate as needed during the course of cardiac development; however we do not know if CVB-infected CSCs that undergo this premature differentiation program can still integrate into the myocardium and function normally. It was recently reported that CVB can cause a non-cytolytic carrier-state infection in HL-1 myocytes.[34] Considering that HL-1 cells are an atrial cardiomyocyte tumor cell line, these cells may share similarities with CSCs. We hypothesize that CVB may have evolved to subvert stem cell differentiation pathways for its own replicative benefit. We observed that after CVB exposure in vivo, CSCs showed a strong tendency to differentiate into vascular cells. If these cells which line blood vessels still harbored low levels of virus, then they could presumably continue releasing virions directly into the blood.

Still, differentiation may simply be an off-target effect of CVB infection. CVB and many other viruses block host cap-dependent translation, leading to cell cycle arrest. Additionally, CVB has been documented to upregulate autophagy, a cellular process which has been shown to be essential during differentiation.[35, 36] Taken together, differentiation is a logical sequel to CVB infection of stem cells, assuming the host cell does not get destroyed due to viral load. Whether or not CSC differentiation confers any type of advantage for the virus, premature differentiation of stem cell pools would pose its own set of issues. CSCs must migrate, signal, and differentiate in a coordinated manner during the course of cardiac growth, so a disruption of this program could be

potentially hazardous. Additionally, CSCs serve a role in homeostasis and so a perturbed CSC pool could impair cardiac maintenance in response to mild damage or stressors. It is important to resolve exactly how early CVB infection impacts the CSC population and determine the ultimate consequences on the heart throughout life.

Chapter 1, in part is currently being prepared for submission for publication of the material. Sin, J., Puccini, JM., Huang, C., Konstandin, M., Gottlieb, RA., Feuer, R. The dissertation author was the primary investigator and author of this material.

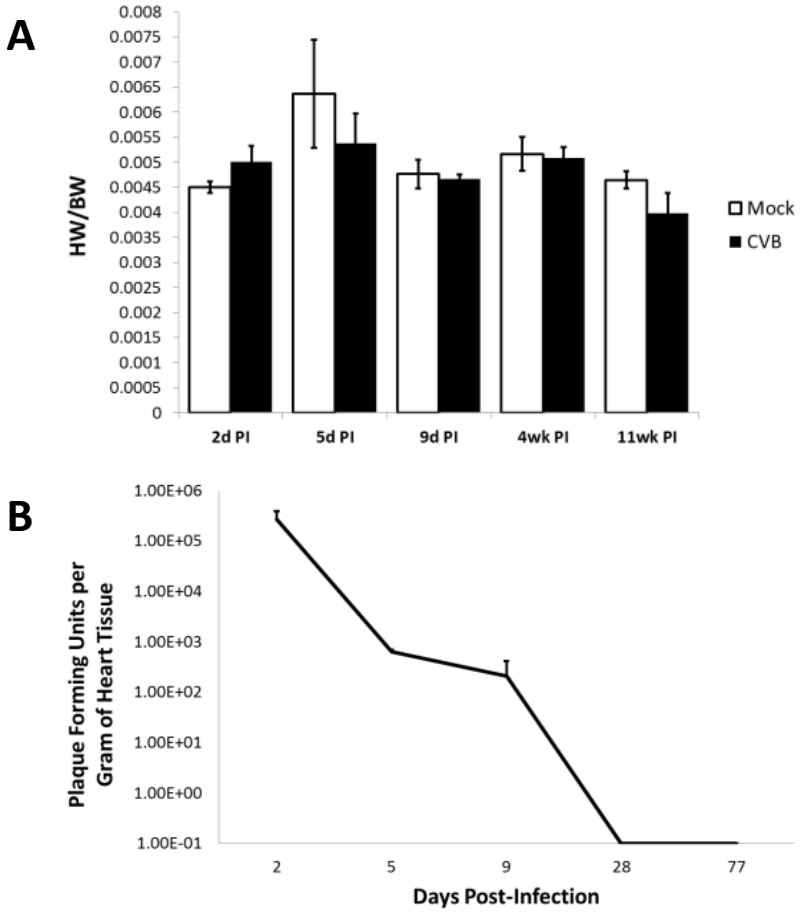
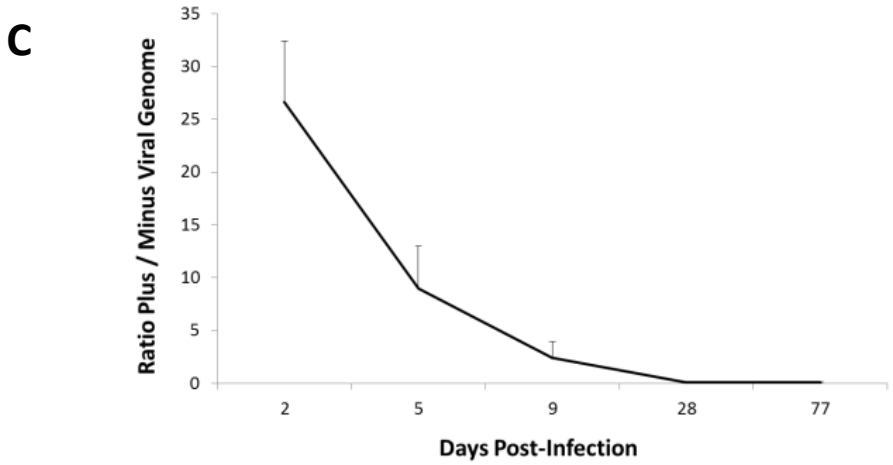


Figure 1.1: Validation of subclinical CVB infection mouse model. 3d old BALB/c pups were infected IP with 10^7 pfu GFP-CVB or mock infected with DMEM. A) Analysis of heart weight to body weight ratios (HW/BW) showed no significant differences between infected and mock controls at either early or late timepoints. B) High amounts of infectious virus were detectable in the hearts of infected mice up to 9d PI, but were undetectable at 28d and 77d PI as measured by plaque assay. C) q-PCR on infected heart homogenates confirmed the presence of viral RNA which was detectable until 9d PI, but was not observed at 28d and 77d PI. D) Histology on mouse hearts 2d PI and 77d PI showed an absence of recruited $CD3^+$ T-cells (indicated by white arrows) and $IBA1^+$ macrophages in the infected myocardium. Hematoxylin & eosin (H&E) staining confirmed normal myofibrillar arrangements and Masson's Trichrome (MT) staining showed an absence of fibrosis.



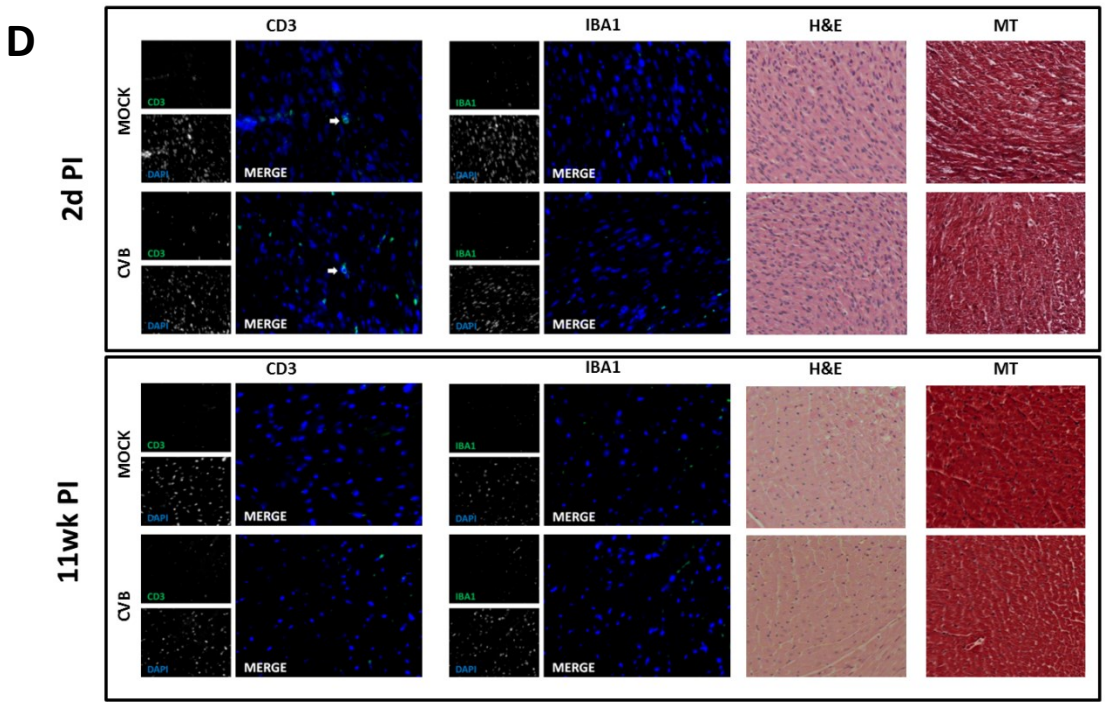


Figure 1.1: Validation of subclinical CVB infection mouse model (cont.).

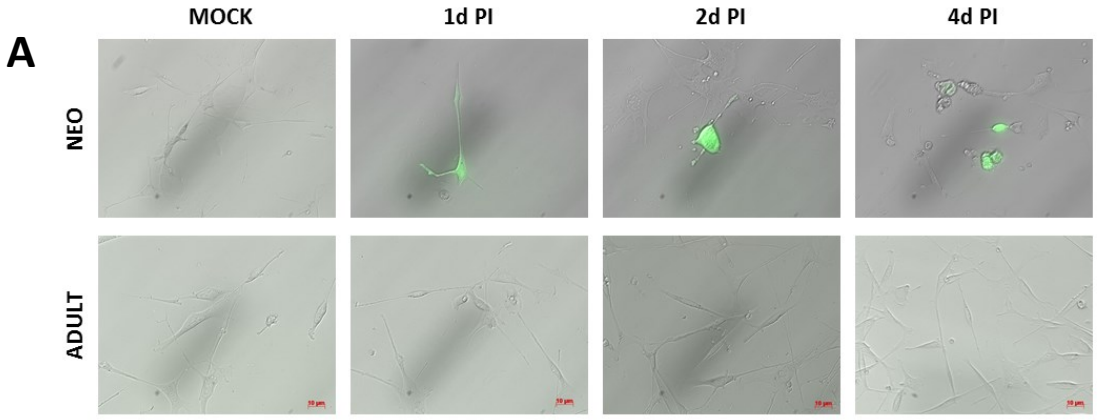


Figure 1.2: CVB infects cardiac stem cells. CSCs were isolated from neonatal mice or adult mice and were subsequently infected with GFP-CVB at MOI 1 or MOI 1000. A) CSCs from neonates infected at MOI 1000 exhibited green fluorescence starting 1d PI confirming infection. Interestingly, CSCs isolated from adult hearts showed no visible sign of infection. B) Immunostaining for coxsackie adenovirus receptor (CAR) revealed CAR expression in both neonatal and adult CSCs. C) Plaque assays performed on media from infected CSC cultures showed much larger amounts of infectious virus in the media from infected neonatal CSCs compared to adult CSCs. D and E) Costaining for viral eGFP and CSC markers in infected hearts 2d PI revealed colocalization of virus with (D) c-Kit (indicated by white arrows) and (E) Sca-1, confirming in vivo infection of CSCs.

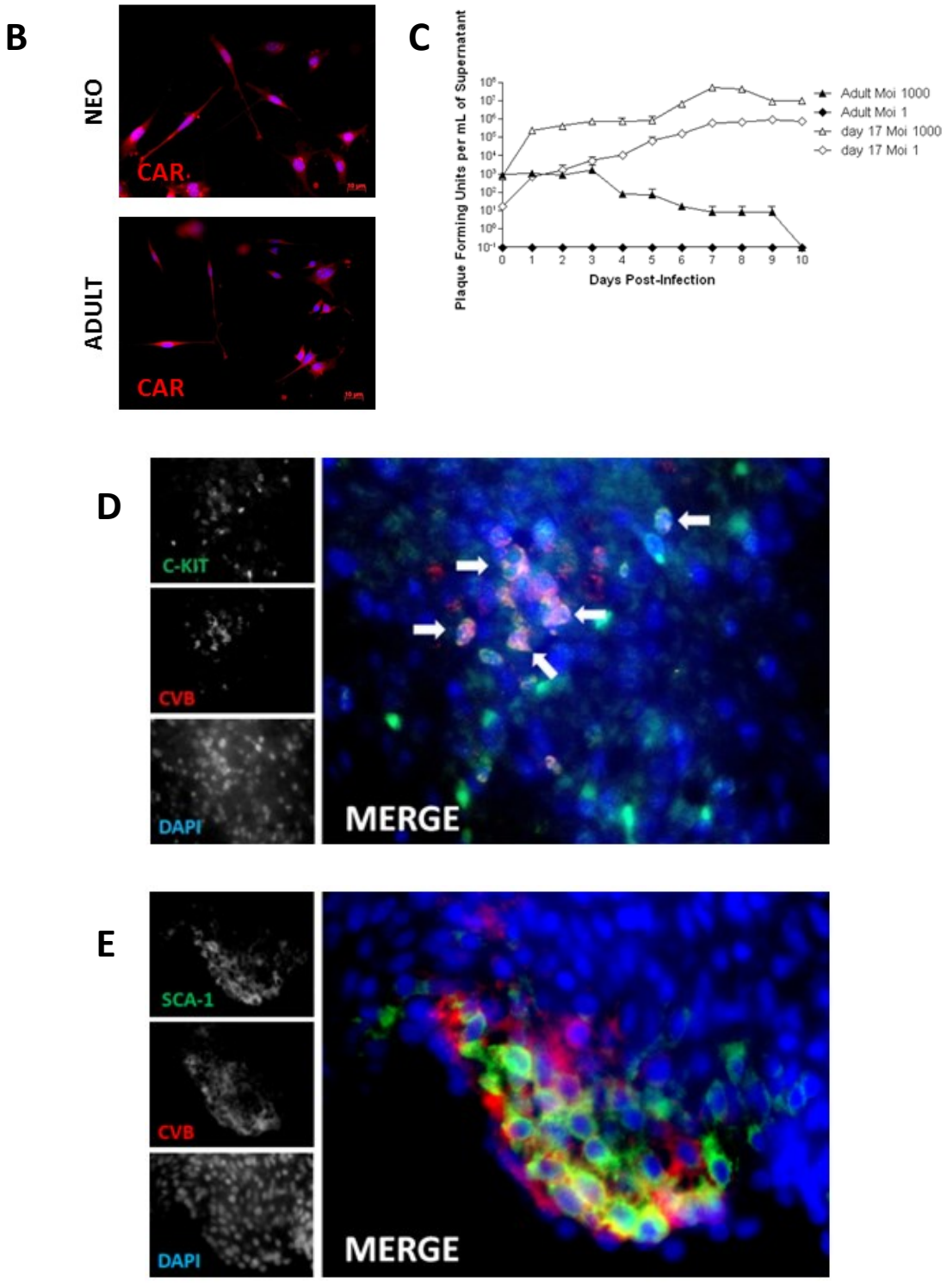


Figure 1.2: CVB infects cardiac stem cells (cont.).

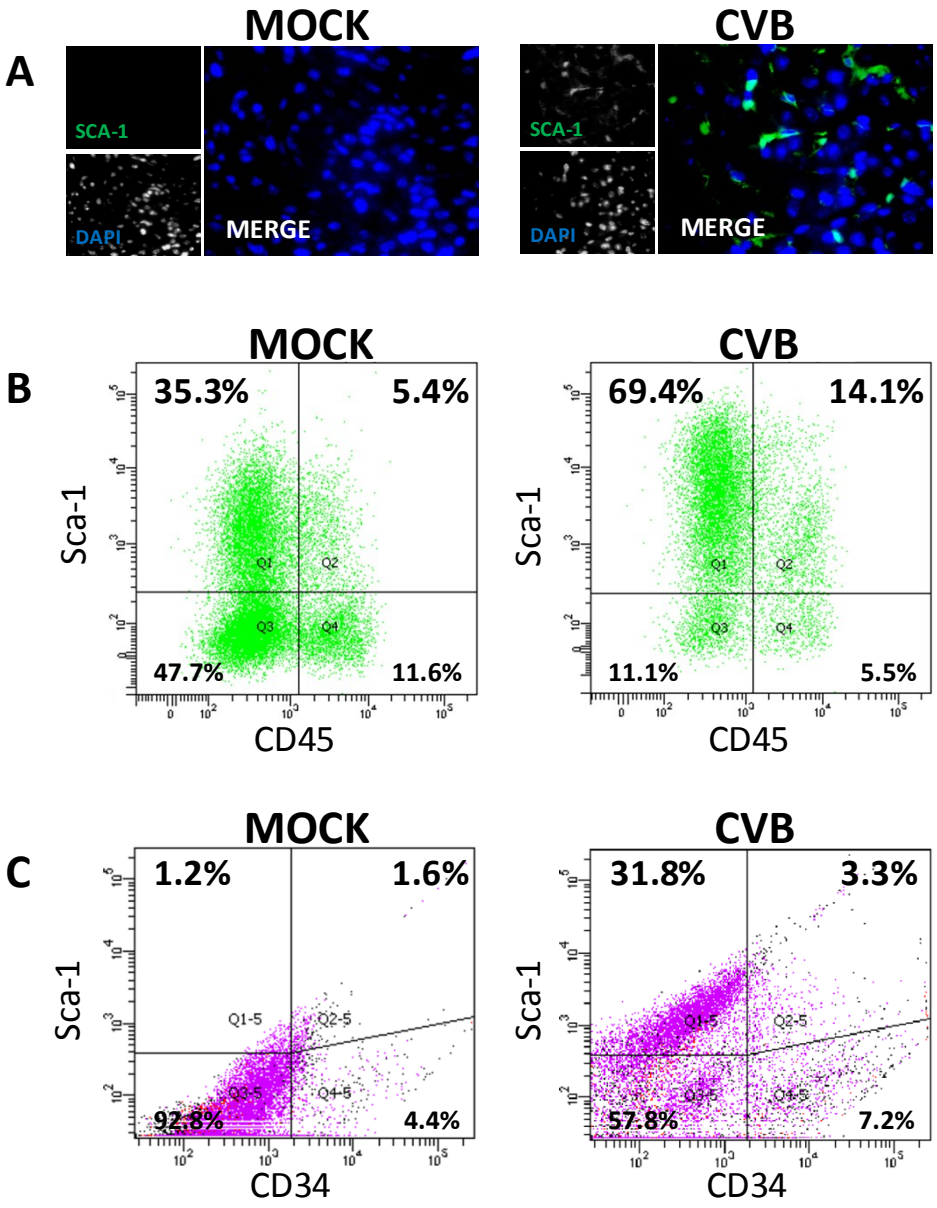


Figure 1.3: Acute CVB infection triggers increase in myocardial Sca-1 expression. A) 2d PI, immunohistochemistry revealed an increase in Sca-1 expression throughout the myocardium. B and C) FACS analysis of whole dissociated mouse hearts 2d PI confirmed an elevation of Sca-1^{+} cells in number and intensity, however these cells were (B) CD45^{-} and (C) CD34^{-} (bone marrow and hematopoietic markers respectively) ruling out peripheral blood origin.

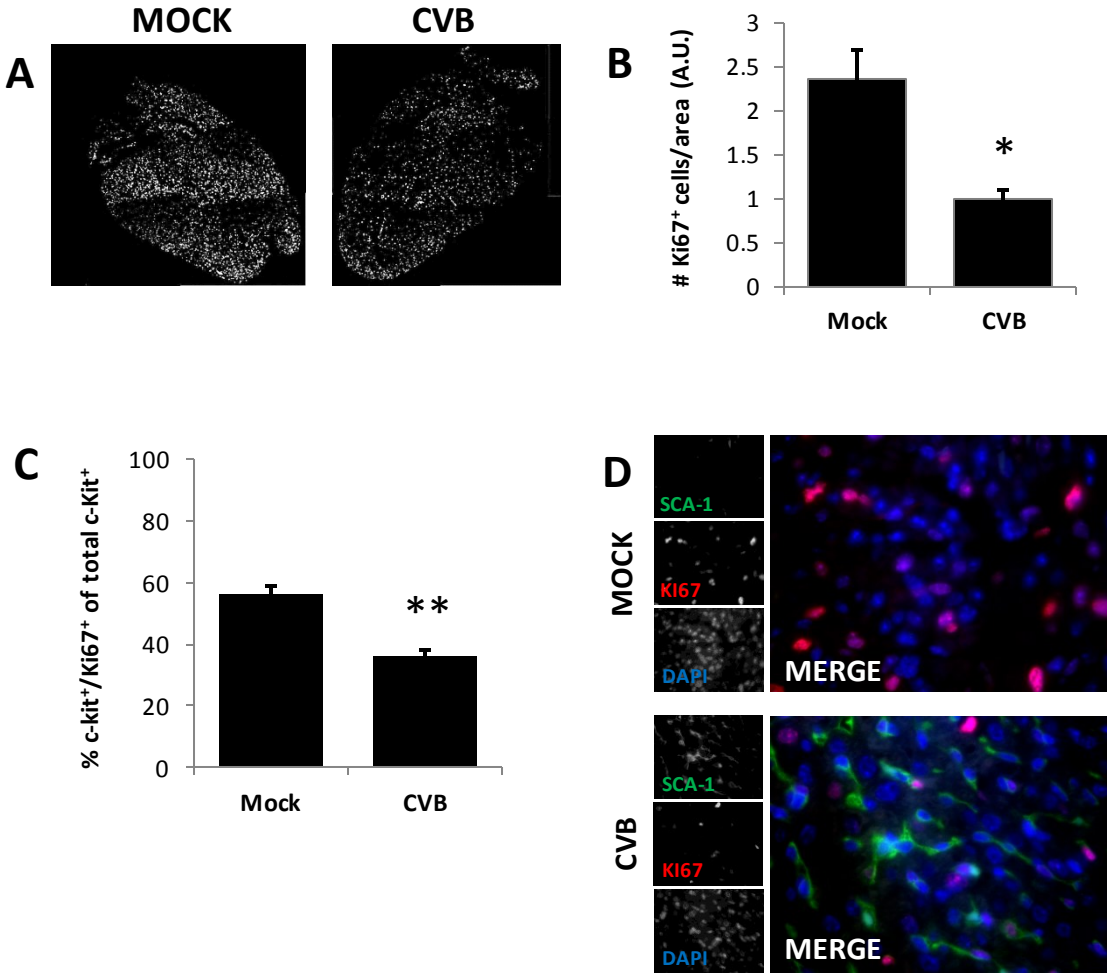


Figure 1.4: CVB infection triggers growth arrest in CSCs. A) Immunohistochemistry revealed a sharp decline in Ki67$^+$ cells in the infected neonatal myocardium 2d PI relative to mock controls. B) Quantification of Ki67$^+$ cells. C) Costaining for c-Kit and Ki67 showed a significant decrease in the percentage of cycling c-Kit cells in the infected heart 2d PI. D) Costaining for Sca-1 and Ki67 again showed a marked increase in the number of Sca-1$^+$ cells in the infected heart 2d PI, however these cells were predominantly negative for Ki67, indicating that they were non-proliferative. [*p<.05, **p<.01]

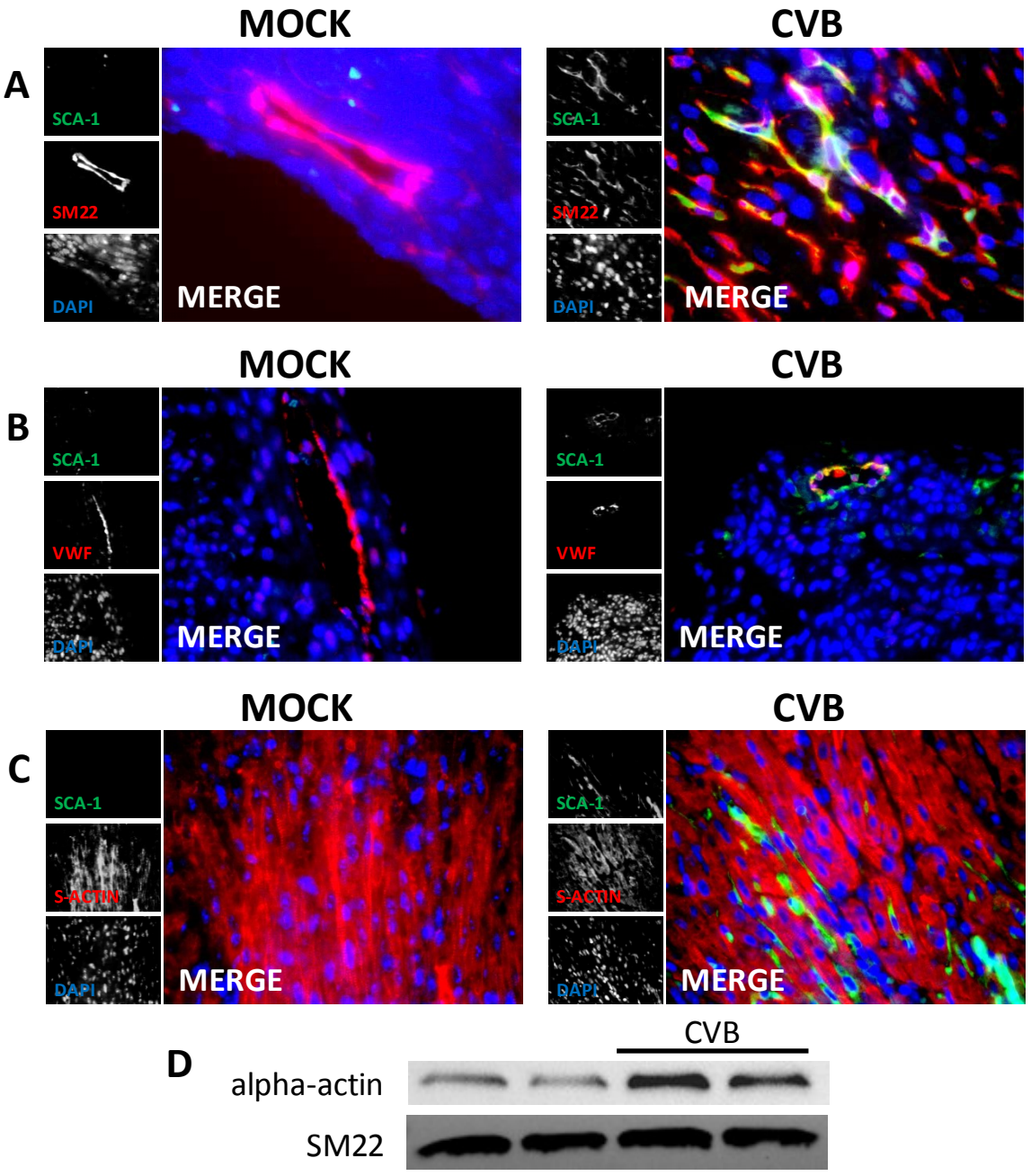


Figure 1.5: CVB infection induces premature differentiation of CSCs. A, B, and C) 2d PI, immunohistochemistry revealed colocalization of Sca-1 with (A) SM22 and (B) Von Willebrand Factor but not with (C) sarcomeric actin (smooth muscle, endothelial, and myocyte markers respectively). D) Western blots performed on isolated CSCs 3d PI revealed a significant upregulation of myocyte marker alpha-actin, but no change in SM22.

CHAPTER II:

Mild Neonatal Coxsackievirus B Infection Predisposes to Late Pathologic Cardiac Remodeling

INTRODUCTION

CVB is an enterovirus that most commonly causes a mild self-limited infection characterized by fever, upper-respiratory symptoms, and rash. In rare cases it can give rise to severe inflammatory diseases such as pancreatitis, meningo-encephalitis, and myocarditis. Studies of patients with end-stage idiopathic dilated cardiomyopathy have revealed detectable CVB RNA in the hearts of ~70% of the cases, despite the absence of a history of viral myocarditis.[7, 8] This observation has raised the concern that mild CVB infection may still have long-term cardiac sequelae. The possible mechanism is controversial, although some have suggested autoimmune mechanisms which include superantigen expression, bystander damage, or molecular mimicry.[37] Dilated cardiomyopathy is the final common pathway of many pathologic processes, in which an enlarged ventricular cavity and thinned ventricular wall, result in poor contractility (low ejection fraction) and in many cases diastolic dysfunction due to fibrosis. This may be preceded by many years of compensated cardiac hypertrophy, characterized by thickening and enlargement of the ventricles, before decompensation develops. While CVB myocarditis is clearly linked to heart failure, the notion that mild CVB infection in childhood could predispose to heart failure in adults is novel. Based on the observation that neonatal exposure to the cardiotoxic anthracycline doxorubicin (DOX) resulted in late-onset heart failure through depletion of cardiac stem cells, we hypothesized that neonatal CVB infection might have similar sequelae.

In this work, we established a model of mild neonatal CVB infection which did not cause myocarditis or immune cell infiltration in the heart, but which triggered premature differentiation of CSCs and profoundly reduced their number. Similar to DOX-induced senescence, virally-mediated premature differentiation reduced the number of CSCs available later in life. The role of CSCs in postnatal development and physiologic remodeling is poorly understood, but observations drawn from the juvenile DOX model suggest that CSCs are important for vascular development in the growing heart as well as in response to physiologic hypertrophy (during exercise) and in postinfarction remodeling.[15] If neonatal CVB infection depleted the CSC population, it could result in stress-induced cardiomyopathy similar to the juvenile DOX model. We suggest that a mild CVB infection in early childhood might impair the heart's ability to undergo physiologic remodeling, leading to dilated cardiomyopathy later in life.

To examine late consequences of our neonatal CVB infection, we infected 3d old pups with a subclinical dose of GFP-CVB and examined hearts at 11wk PI. We observed a significant decrease in the number of CSCs present in these adult hearts. Additionally, after subjecting the mice to cardiac stress either via exercise or pharmacological induction, adult mice that had sustained neonatal CVB infection developed marked left ventricular hypertrophy and chamber dilation. These results provide evidence that mild CVB infections may predispose the heart to pathologic remodeling later in adult life.

METHODS

Generation of Recombinant eGFP-Expressing Coxsackievirus B

Generation of eGFP-expressing CVB (GFP-CVB) has been previously described.[27] Briefly, a plasmid was derived from an infectious CVB3 clone isolated from the heart of an acute myocarditis patient (pH3). This plasmid was engineered to contain an Sfi1 restriction site (pMKS1). The eGFP gene was amplified from an eGFP expression plasmid (Clontech, Palo Alto, CA) using eGFP primers with flanking Sfi1 sites. These PCR products were subsequently cloned into pMKS1. This construct was transfected into HeLa RW cells and lysates containing infectious virus were collected as viral stocks. Viral concentrations were measured via plaque assay.

Mice and Viral Inoculations

Animal experiments were performed in compliance with the San Diego State University Animal Research Committee and the National Institutes of Health. BALB/c mice were obtained from the Scripps Research Institute animal facilities. Breeding pairs were monitored daily and three day-old pups were infected via intraperitoneal injection (IP) with 10^7 pfu GFP-CVB, or equivalent volume of DMEM for mock-infected controls. Animals were sacrificed 2, 5, 7, 28, and 77 days postinfection (PI). Hearts were fixed by immersion in 10% neutral-buffered formalin for 24 hours and then transferred to 70% ethanol for an additional 24 hours. The tissue was then paraffin-embedded and stained.

Exercise challenge

10-11wk PI, neonatally-infected mice were subjected to exercise challenge which consisted of daily swimming in heated water tanks with a gentle pump-driven current to encourage active swimming. After each swimming session, animals were allowed to recover under a heating lamp. A 7d training period consisted of 5min swimming on day 1, then increased daily (15, 30, 45, 60, 75 and finally 90min). Following this initial week of training, mice underwent 90min daily swimming exercise for 14d. Upon completion of 14d of full swimming (21d total), hearts were excised for determination of heart weight (normalized to tibia length) and histology. Hemodynamic analysis was not possible due to quarantine restrictions imposed on the CVB-exposed mice.

Beta-adrenergic stimulation

Isoproterenol hydrochloride was resuspended in sesame oil to prolong uptake. 13wk PI, neonatally-infected mice were IP-injected with 40mg/kg/d isoproterenol hydrochloride daily for 10 days. Following the final day of injections, hearts were excised for determination of heart weight (normalized to tibia length) and histology.

RESULTS

Neonatal CVB infection depletes CSC pools in adult mice

In our previous neonatal studies we found that CVB infected CSCs, causing growth arrest and early differentiation. Both these effects would reduce the number of self-renewing CSCs. To determine if early CVB exposure resulted in a decline in CSC numbers, we infected mice on postnatal day 3 with 10^7 pfu of CVB and examined c-Kit$^+$ CSCs in heart tissue at 2d, 5d, 9d, 4wk, and 11wk PI. At 2d and 5d PI, there was a trend for an increase in the number of c-Kit$^+$ cells although this did not reach statistical significance. However, by 11wk PI, a statistically significant 50% decrease was observed (Fig. 2.1A). Sca-1, another classical cardiac stem cell marker which we consider to signify progression towards differentiation (compared to c-Kit), increased transiently and then was undetectable after 5d PI (not shown). This decline in CSCs persisted despite resolution of infection documented by plaque assay to detect infectious virions and by qPCR for viral RNA (Fig. 1B and C). These results coupled with our previous findings show that CVB reduces the pool of self-renewing CSCs in part by triggering premature differentiation, and this stem cell depletion persists into adulthood despite clearance of the virus and in the absence of ongoing inflammation.

Subclinical neonatal CVB infection predisposes to stress-induced heart failure during adulthood

Despite a reduction in the number of CSCs, the CVB-infected neonates grew normally and were phenotypically normal at 11wk PI compared to mock-infected controls. There was no difference in heart size (HW normalized to BW), and there was no evidence of myocarditis, myofibrillar disarray, or fibrosis, which would be indicative of cardiac damage (Fig. 1.1D). To determine if physiologic stress could induce heart failure in our CVB-infected animals, neonatally-infected mice were subjected to exercise challenge which consisted of 90min of continuous swimming for 14 consecutive days. We noted that the mice that had previously been infected did not swim as vigorously as the mock-infected controls (not shown). Following swimming, we observed a significant increase in HW/TL of the infected group compared to mock controls (Fig. 2.2A). Transverse cross sections from these hearts revealed enlarged left ventricles with thinning of the muscular wall (Fig. 2.2B). H&E showed the presence of myofibrillar disarray and Masson's trichrome staining revealed fibrosis in a number of the infected mice after the exercise challenge, although it was difficult to quantify (Fig. 2.2C and D). Taken together, these data support our hypothesis that mild neonatal CVB infection can result in pathologic remodeling in adult mice after exercise challenge.

Swimming is considered a "physiologic" stress. To determine if a pathologic stress elicited more robust pathologic remodeling, we administered

the beta adrenergic agonist isoproterenol (ISO).[38] At 13wk PI, neonatally-infected mice were treated with the drug for 10 days. As expected, all animals (CVB-infected and mock-infected) exposed to ISO for 10d developed cardiac hypertrophy and fibrosis. However, the previously infected animals had a significantly greater increase in heart mass than mock-infected mice (Fig. 2.3A). These observations support our hypothesis that subclinical infection with CVB predisposes to pathologic remodeling.

CVB-mediated CSC loss impairs vascular remodeling during increased cardiac load

Cardiac vascular cells are highly dynamic and responsible for adaptive restructuring of the vascular network. This remodeling is accomplished both through angiogenesis (branching of pre-existing blood vessels) and vasculogenesis (de novo blood vessel formation). These processes are critical for the heart to respond to various stimuli including stressors. With the induction of physiological stress, cardiac contractility increases, thereby increasing oxygen demand. Vascular remodeling is necessary to sufficiently perfuse the myocardium with oxygenated blood. Stem cells have been documented to be intimately involved in vascular remodeling through differentiation and release of paracrine factors such as vascular endothelial growth factor (VEGF).[15] To examine if cardiac vasculature was affected by loss of stem cells due to CVB, we stained heart sections from ISO-treated animals for blood vessel marker CD31. At baseline (13wk PI), there were no significant differences in blood vessel

density between infected and mock-infected animals. Treatment with ISO resulted in increased CD31 staining in mock-infected mice, consistent with increased vascular remodeling in response to the increased workload imposed by ISO treatment. However, there was no increase in CD31 staining in neonatally-infected mice after ISO exposure (Fig. 2.3C). This indicates that under increased load, the heart remodels its vasculature to meet demand, but neonatal CVB infection impairs this compensatory function, presumably through depletion of CSCs.

Because CSCs are known to release several factors including VEGF that stimulate blood vessel formation, we next sought to determine if the production of VEGF was impaired in the heart. Western blots of heart homogenates prepared from mock and neonatally-infected mice at 13wk PI were probed for VEGF. We found that VEGF expression was significantly lower in hearts of infected animals compared to mock-infected controls (Fig. 2.3D). This is further evidence that mild neonatal CVB infection has lasting effects on the ability of adult mice to undergo compensatory vascular remodeling under cardiac stress. We attribute this to a lack of resident CSCs which are necessary to support blood vessel formation and remodeling.

DISCUSSION

Epidemiological studies reveal an association between CVB infections and heart failure; however, a mechanistic link has not been found. To our knowledge, ours is the first model showing that mild neonatal CVB infection can predispose to pathologic remodeling in adult mice. We found that premature differentiation of CSCs observed during acute infection caused a marked and sustained reduction in CSC numbers later in life despite an absence of any detectable infectious virions or viral RNA in the hearts of adult animals. We also did not observe any physiological abnormalities or cardiac damage under basal conditions. It was only after we subjected these mice to increased cardiac workload that we elicited pathologic hypertrophy evidenced by left ventricular enlargement, chamber dilation, and histologic features of myofibrillar disarray and fibrosis.

This propensity for pathologic remodeling in the CVB-exposed mice may be due to an impairment of vascular remodeling in response to increased cardiac demand, a process which requires the participation of CSCs. We did not observe differences in cardiac vascular density between mock and CVB-infected adult mice before ISO challenge. These findings point to an impairment of compensatory remodeling rather than pre-existing deficiencies in vascular architecture. However, we did not measure coronary blood flow or analyze 3-D vessel network architecture. It is possible subtle differences in vasculature are present.

A similar phenomenon was observed in the case of DOX-mediated CSC depletion. Low dose DOX exposure in juvenile mice resulted in depletion of CSCs through early senescence. However, adult mice had normal cardiac physiology and histologic findings. Stressors such as swimming exercise elicited pathologic remodeling, and experimental myocardial infarction resulted in more severe injury and poorer survival. The poor outcomes in DOX-exposed mice were similarly attributed to an impaired capacity for vascular remodeling due to depleted CSCs. Stem cells have been shown to play a crucial role during vascular remodeling, by stimulating angiogenesis through the release of paracrine factors, and differentiating to give rise to new blood vessels. In our studies of the late consequences of neonatal CVB infection, we observed a depletion of CSCs which would be available to participate in vasculogenesis, as well as reduced expression of the stimulatory paracrine factor VEGF in the heart. Loss of CSCs and VEGF likely impacted adaptive vascular rearrangement in response to cardiac stress.

These subtle cardiac alterations may go undetected under normal circumstances, but may emerge in the setting of increased cardiac stress, whether elicited by high-level exercise, chronic hypertension, or an ischemic event. Given the high prevalence of CVB exposure in the population, many sedentary individuals may be asymptomatic. There are many unanswered questions that this animal model may help to address. For instance, does CVB infection affect stem cells at any age, or is there a high-risk window in early

childhood? Anecdotally, we observed that CVB efficiently infected CSCs obtained from young mice (17d), but only poorly infected CSCs obtained from adult animals. Moreover, these studies utilized a strain of CVB3 that was obtained from a patient with myocarditis. It is likely that only some CVB strains pose a risk for CSC depletion and late cardiac sequelae. Given the decades-long interval between childhood exposure and development of cardiac symptomatology, it will be challenging to prove causality and to determine the age threshold for vulnerability. However, these studies raise concern that mild CVB infection in childhood may not be completely benign. Prevention of these late effects will depend upon introduction of a safe and effective vaccine and/or the development of rapid diagnosis and utilization of antiviral therapy in children exposed at a vulnerable age.

Chapter 2, in part is currently being prepared for submission for publication of the material. Sin, J., Puccini, JM., Huang, C., Konstandin, M., Gottlieb, RA., Feuer, R. The dissertation author was the primary investigator and author of this material.

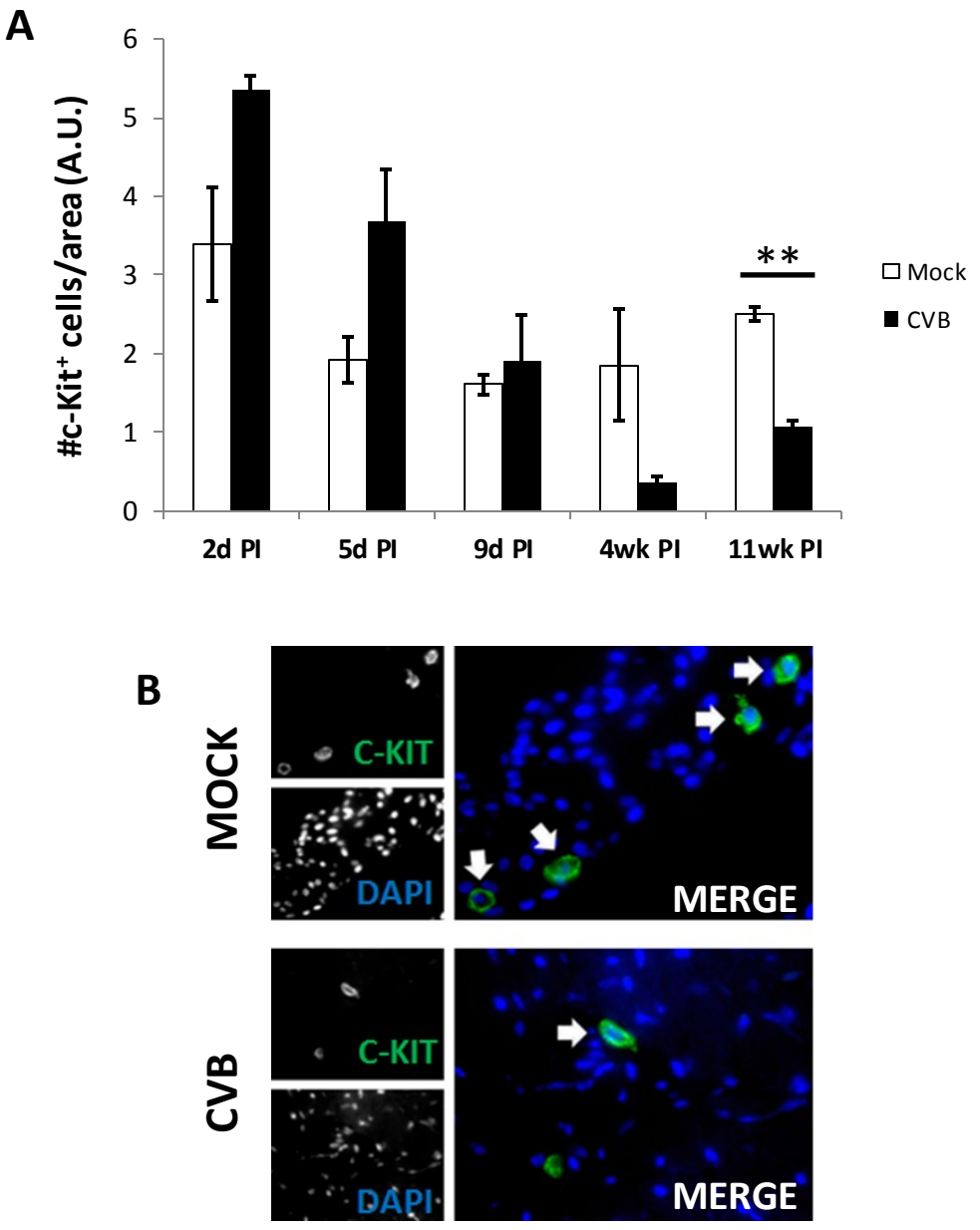


Figure 2.1: Neonatal CVB infection leads to exhausted CSC pool during adulthood. A) CSC counts of neonatally-infected mice over time. Previously infected adults had significantly fewer CSCs 11wks PI. B) Representative fields of heart sections 11wks PI immunostained for c-Kit (c-kit cells indicated by white arrows). [**p<.01]

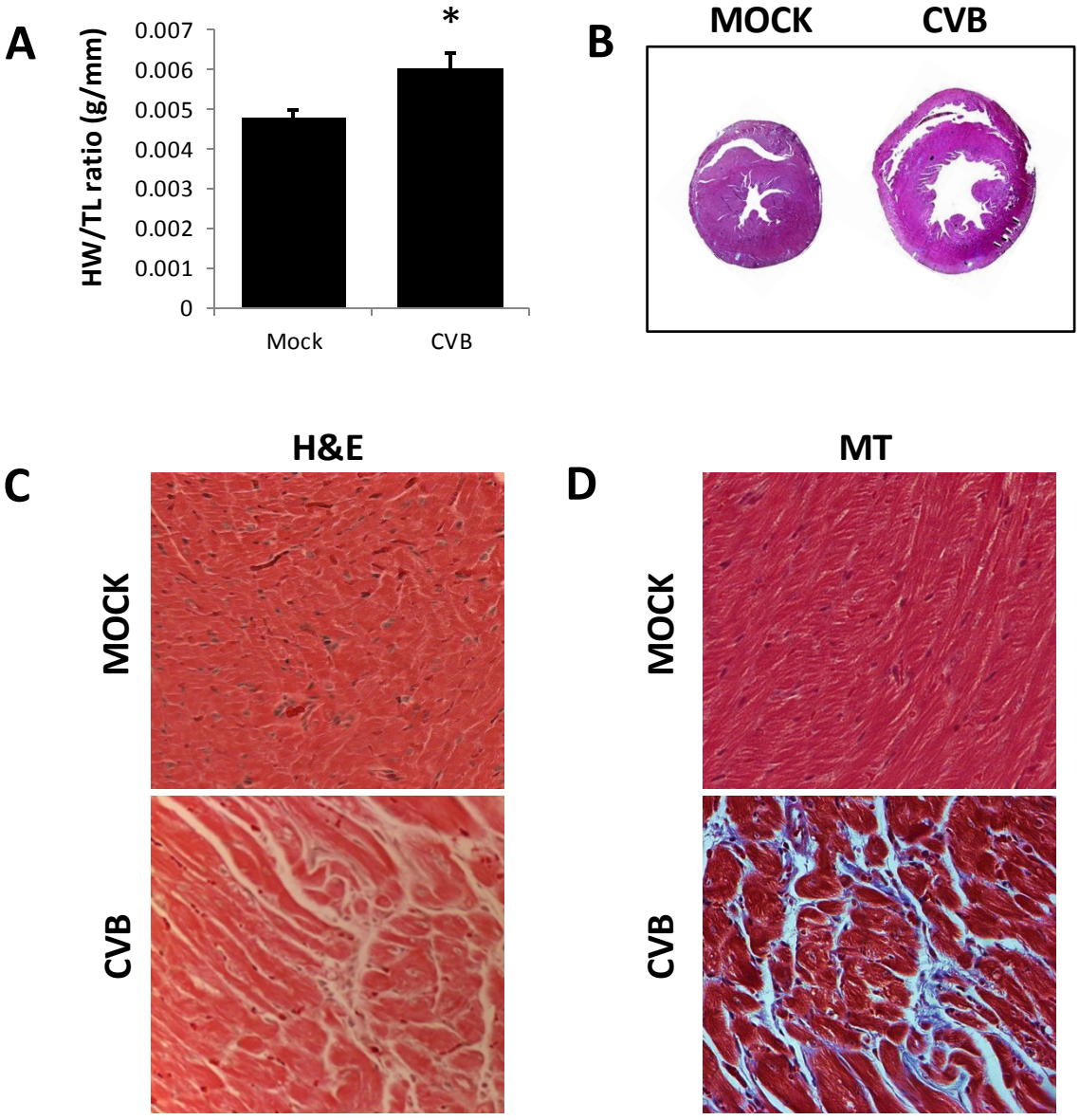


Figure 2.2: Exercise challenge induces heart failure in neonatally-infected adult mice. A) Heart weight/ tibia length ratios (HW/TL) showed increased heart mass in previously infected adult mice following swimming. B) Representative transverse heart sections from swum animals. C and D) (C) H&E staining showed myofibrillar disarray and (D) Masson's trichrome staining revealed fibrosis in the hearts of previously infected mice. [*p<.05]

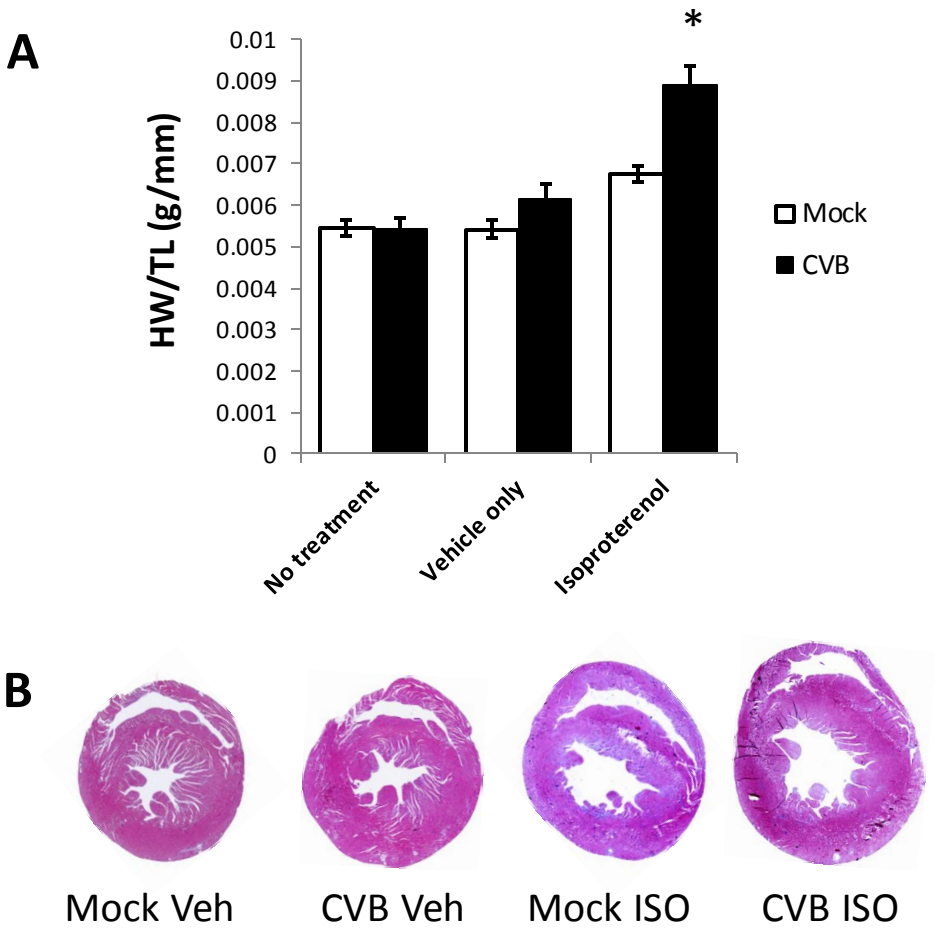


Figure 2.3: Isoproterenol induces more severe cardiac hypertrophy in previously infected mice due to impaired vascular remodeling. A) HW/TL showed heightened sensitivity to hypertrophy in neonatally-infected adult mice. B) Representative transverse heart sections from ISO and vehicle-treated mice. C) Immunostaining heart sections for CD31 revealed an inability for previously infected mice to increase vascular complexity after ISO treatment. D) Western blots performed on heart homogenates from mice 13wks PI showed significantly less VEGF in previously infected mice. [*p<.05]

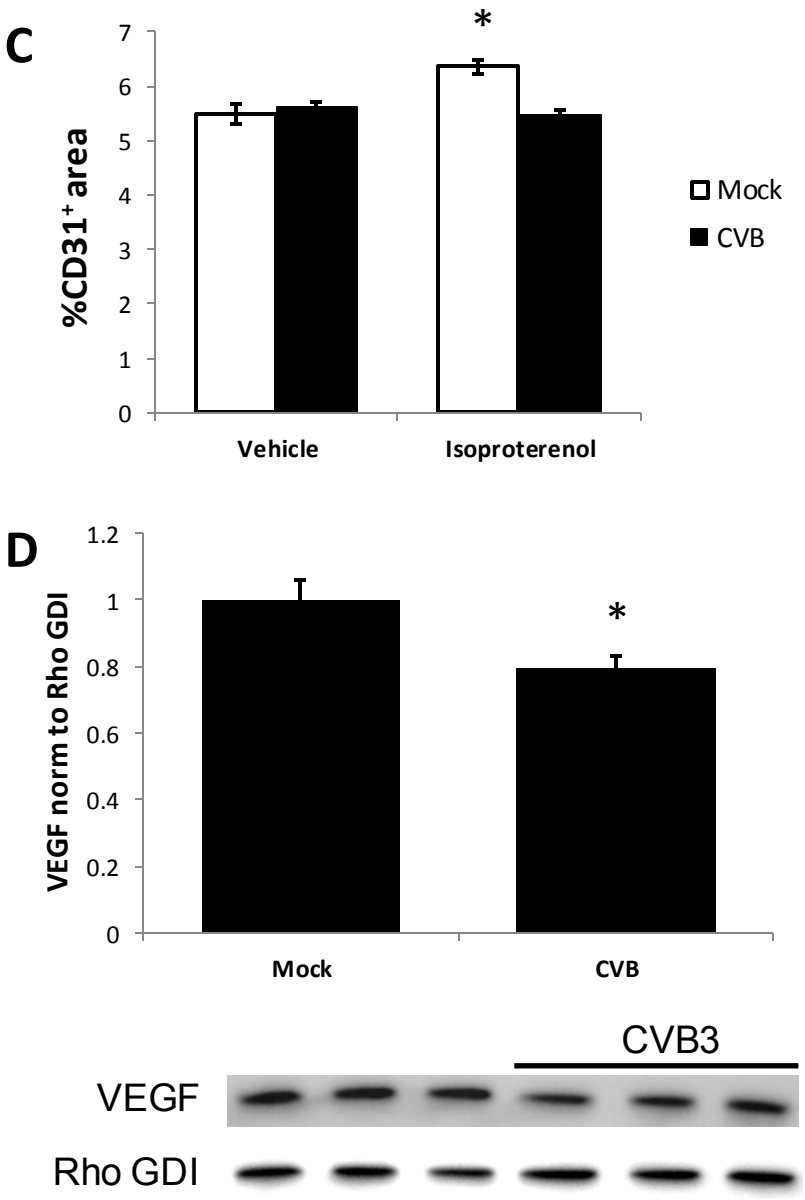


Figure 2.3: Isoproterenol induces more severe cardiac hypertrophy in previously infected mice due to impaired vascular remodeling (cont.).

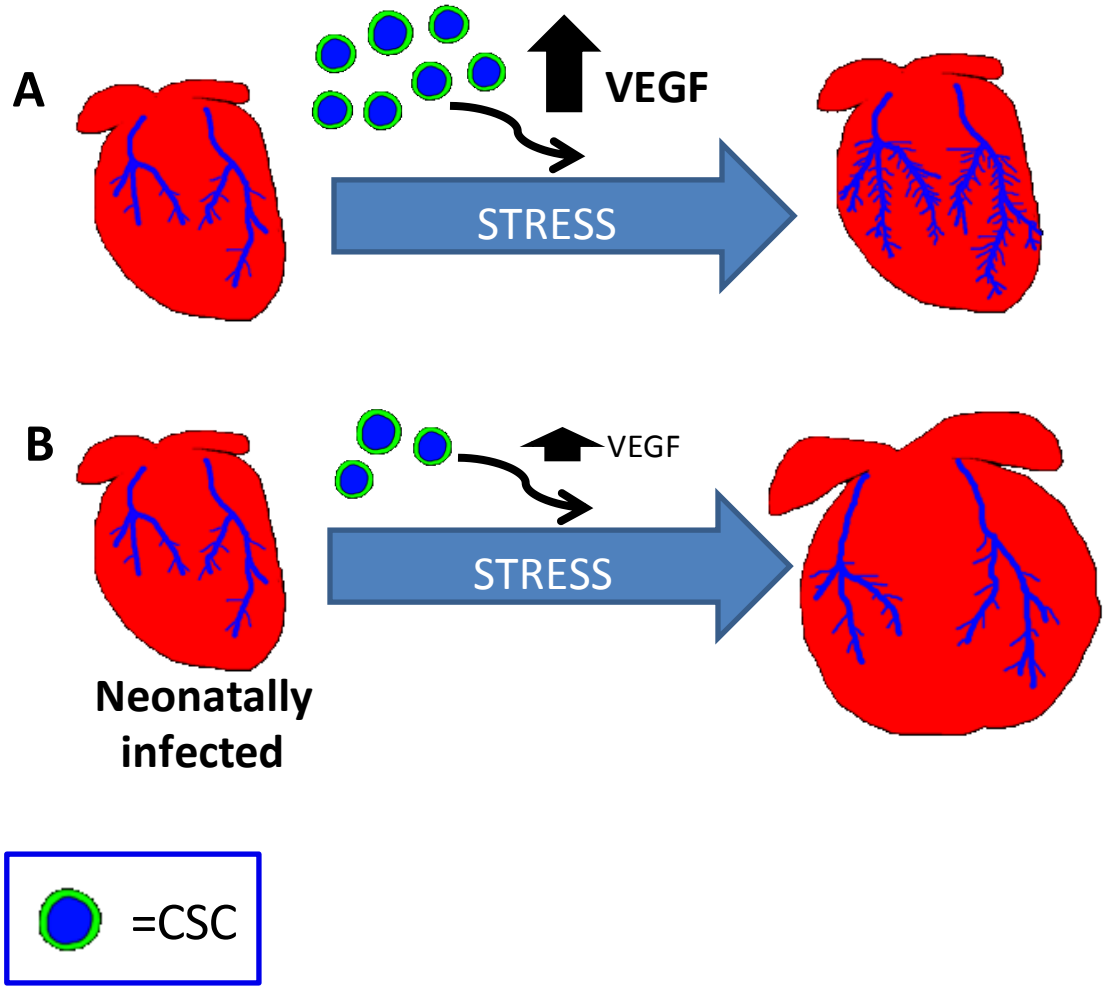


Figure 2.4: Neonatally infected adult mice cannot compensate for cardiac stress due to impaired CSC participation during vascular remodeling. A) With increased cardiac load, myocardial oxygen demand increases. CSCs are activated/recruited to stimulate angiogenesis via paracrine signaling or promote vasculogenesis via direct differentiation into vascular cells. B) Following juvenile CVB infection, CSCs are exhausted, but the heart vasculature develops normally. However, during increased cardiac load, fewer CSCs are available to participate in compensatory vascular remodeling resulting in an ischemic myocardium. This results in maladaptive cardiac remodeling.

CHAPTER III:

Autophagy and its role in Coxsackievirus B infection

INTRODUCTION

Autophagy is a cellular mechanism necessary for the removal and recycling of old and damaged organelles and proteins. In addition to disposing of native cellular components, autophagy has also been shown to be involved in the destruction of invading pathogens. It is not surprising then that host autophagy is upregulated upon infection with CVB. However, several reports have suggested that autophagy may be subverted by CVB in order to maximize viral replication. Viruses have incredibly rapid rates of evolution therefore it is feasible that after many years of CVB getting engulfed and degraded by host autophagy, it eventually developed the ability to somehow hijack the autophagosome to use as a site of replication.

The viral activation of autophagy has several implications for the host cell. Most notably in the context of progenitor cells, we have found that autophagy may be an important component of differentiation. Autophagy is upregulated during the process of differentiation, and this coincides with the cell's withdrawal from cell cycle. Autophagy could be coincidental with growth arrest as mTOR and Akt signaling suppresses autophagy. To learn whether autophagy is a cause or a consequence of the initiation of differentiation, we studied the simplified model system, the C2C12 skeletal myoblast cell line, which undergoes differentiation to myotubes under low-serum conditions. We found that blocking autophagy in C2C12 cells prevented myotube differentiation under low serum conditions. This suggests that autophagy is required during the differentiation

process. It also raises the question whether virally-induced autophagy is sufficient to induce differentiation. Intriguingly, we observed upregulation of autophagy in CSCs upon CVB infection which coincided with the premature differentiation previously seen.

It is unclear how or if at all CVB actually exploits autophagy for its own benefit. We examined the importance of autophagy during CVB infection by utilizing ATG5 knockout mouse embryonic fibroblasts (MEFs). Wildtype MEFs were highly susceptible to infection and exhibited considerable cytopathic effects while producing high amounts of virus. On the other hand, in ATG5 KO MEFs, only a small percentage of the cells became infected by CVB while the rest of the population appeared completely normal. Eventually the healthy cells overtook the population without any sign of viral spread from the initial round of infected cells, suggesting there may be some kind of impairment in viral dissemination with the ablation of autophagy.

Upon closer inspection of the wildtype MEFs, we observed small viral protein-containing extracellular vesicles budding off the surface of infected cells. However, MEFs deficient in autophagy did not generate these vesicles. It has been previously described by Kemball et al that upon infection, CVB becomes entrapped within autophagosomes.[5] However, the clearance of these autophagosomes via lysosomal degradation is blocked (presumably by viral factors). We hypothesize that after CVB infects a cell, the virus hijacks autophagy, allows itself to get engulfed, and escapes the host cell as cargo

within ejected autophagosomes. This strategy may represent a novel mode of CVB dissemination that could mask the virus from host immunity, and represents a process that to our knowledge has not previously been described for a non-enveloped virus.

METHODS

Generation of Recombinant eGFP-Expressing Coxsackievirus B

Generation of eGFP-expressing CVB (GFP-CVB) has been previously described.[27] Briefly, a plasmid was derived from an infectious CVB3 clone isolated from the heart of an acute myocarditis patient (pH3). This plasmid was engineered to contain an Sfi1 restriction site (pMKS1). The eGFP gene was amplified from an eGFP expression plasmid (Clontech, Palo Alto, CA) using eGFP primers with flanking Sfi1 sites. These PCR products were subsequently cloned into pMKS1. This construct was transfected into HeLa RW cells and lysates containing infectious virus were collected as viral stocks. Viral concentrations were measured via plaque assay.

Exosome Isolation

Exosomes were isolated from cell culture media using ExoQuick-TC exosome precipitation reagent (System Biosciences #EXOTC10A-1) using the manufacturer's protocol. Briefly, media was collected from infected cell cultures and centrifuged at 3000 x g for 15min to remove cells and cell debris. Supernatant was transferred to a fresh tube and ExoQuick-TC reagent was added at 1:5 dilution. Samples were then mixed by inversion and incubated at 4^{o}C for 12H. To pellet the exosome fraction, samples were centrifuged at 1500 x g for 30min at 4^{o}C. Supernatant was then aspirated and exosomes were fixed in 4% paraformaldehyde or lysed in RIPA buffer for further analyses.

Virus Titration via Plaque Assay

Viral titration has been described previously.[27] Briefly, six-well dishes were plated with HeLa RW cells and grown to 100% confluency. Samples were serially diluted (10-fold dilutions) in DMEM and incubated on cell monolayers for 1H with gentle rocking every 15min. Virus was immobilized by overlaying equal parts molten agar and 2X DMEM. 48H later, solidified agar plugs were overlaid with fixing solution (75% methanol +25% acetic acid) for 10min at room temperature. Plugs were then removed with a metal spatula and fixed cells were stained with 0.25% crystal violet for 20min. Cells were then rinsed and plaques were counted.

RESULTS

CVB triggers differentiation of CSCs by upregulating autophagy

To assess the importance of autophagy in the process of differentiation, and to determine if autophagy is coincidental with differentiation or in fact a necessary component, we placed C2C12 skeletal myoblasts in low-serum differentiation media and attempted to block differentiation with the addition of autophagy inhibitor bafilomycin A1 (BAF). As expected, when undifferentiated C2C12 cells were incubated in differentiation media for 3d, they showed a substantial increase in the expression of myogenic differentiation marker myogenin. In contrast, when the cells were cultured in differentiation media in the presence of BAF, myogenin expression was abolished (Fig. 3.1A). Additionally, we observed that cell proliferation was halted under these conditions. These data demonstrate that autophagy is required for differentiation of C2C12 cells regardless of cell cycle status.

We next sought to determine if CVB infection could induce the differentiation of C2C12 cells. Immunostaining of infected C2C12s revealed high amounts of myogenin in infected cells (Fig. 3.1B). To investigate if blocking autophagy could also block CVB-induced differentiation, we performed western blots on CVB-infected C2C12 cells that had been exposed to BAF. As expected, western blot revealed elevated levels of myogenin in CVB-infected C2C12s, however this induction of myogenin was blocked when the cells were pre-treated

with BAF (Fig. 3.1C). These data show that CVB-mediated differentiation is an autophagy-dependent phenomenon.

Having observed that 1) autophagy is required for differentiation, 2) that CVB preferentially targets CSCs in vivo, and 3) that CVB triggers premature differentiation in CSCs, we expected high amounts of active autophagy in the CSCs of infected neonates. To confirm this, we co-stained heart sections from neonatal mice 2d PI for LC3 and c-Kit. Cells actively undergoing autophagy have an abundance of autophagosomes which appear as $LC3^{+}$ puncta in the cytosol. We considered cells to be undergoing active autophagy if they showed high numbers of punctate LC3 expression solely in the cytosol (Fig. 3.2A) as opposed to diffuse distribution of LC3 or absence of LC3 as previously described.[17] Consistent with our hypothesis, we found that hearts from infected neonates had dramatically more c-Kit^{+} cells actively undergoing autophagy compared to mock controls (Fig. 3.2B). This suggests that autophagy is upregulated in CSCs after CVB infection. As shown in Chapter 1, CVB infection in the heart was accompanied by premature differentiation of the CSCs (Fig. 1.4). While there may be many reasons that might explain the increase in CSC differentiation in CVB-infected neonates, one plausible explanation is that CVB infection triggers autophagy which in turn drives differentiation. However, more work is required to support this notion.

Autophagy is crucial for viral dissemination

Though it is clear that CVB induces autophagy in host cells, it is unknown if this cellular process is exploited by the virus or if its activation is a cellular self-defense response to infection. To determine if autophagy plays a role during CVB infection, we utilized MEFs isolated from mice lacking the *ATG5* gene. One of the early events during autophagosome formation is the conjugation of ATG5 with ATG12 which allows for the recruitment of LC3 and formation of autophagosomes. Though ATG5 knockout (ATG5 KO) animals are unable to initiate autophagy, embryos remain viable. However, immediately following birth but prior to suckling, neonates are highly susceptible to death by starvation due to their inability to generate free nutrients via autophagy. We infected wildtype (WT) and ATG5 KO MEFs side-by-side with GFP-CVB to observe the progression of infection. As can be seen in Figure 3.3A, WT MEFs were highly susceptible to CVB infection as is indicated by the wide-spread cytopathic effect and eGFP-expression that could be seen throughout the infection timecourse. The progression of CVB infection was drastically reduced in ATG5 KO MEFs wherein infection was limited to only a portion of the cells at 1d PI. This resulted in two disparate populations of cells: the infected group which exhibited marked cytopathic effect and high eGFP expression, and the uninfected group which appeared indiscernible from mock-infected cells. As time progressed, the number of infected cells did not increase and uninfected cells began to overtake the culture. Plaque assays performed on the culture supernatants 3d PI revealed

that the amount of free-floating virus was roughly 85% lower in ATG5 KO MEF cultures (Fig. 3.3B). These findings suggest that viral dissemination is impaired in the absence of autophagy.

CVB escapes the cell through autophagy-derived extracellular microvesicles

With the disruption of autophagy we found that the ability for CVB to spread is greatly diminished, being unable to escape from the first round of infected cells. Our data suggest that autophagy is integral to CVB infection; however it is unclear how autophagy is being used by the virus. Our previous MEF infections were performed at MOI 100 and we saw widespread cytopathic effect in the WT cells after 24 hours. To further dissect the process of infection, we infected MEFs with GFP-CVB at MOI 1 and examined infected cells at higher magnification. We found that infected WT cells generated tiny vesicles that budded from the plasma membrane (Fig. 3.4A). These vesicles were also $eGFP^+$ which indicated that they contained viral protein; however, we could not yet deduce if the presence of viral eGFP corresponded with CVB virions also being packaged into the vesicles. At this same timepoint, ATG5 KO cells did not form these vesicles, suggesting this process may require autophagy to occur.

Recent literature describes similar extracellular vesicles which have been dubbed “exosomes”.[39] Exosomes have been implicated in numerous contexts including in cancer biology, neuropathology, and immunology.[39-41] However, unlike most exosomes described in literature which range between 20-100nm in

size, the virally-induced vesicles we observed varied greatly in size from 200nm to 5μm.[41, 42] This larger size was consistent in all infected cell-types including MEFs, C2C12s, and neural stem cells. For this reason, we hypothesized that CVB may trigger vesicle release via a pathway distinct from the "classical" exosome pathways. However, due to their similarity to previously-described exosomes, we attempted to isolate our infection-mediated vesicles, herein referred to simply as extracellular microvesicles (EMVs), from culture supernatants with Exoquick, a commonly used proprietary exosome isolation reagent. We found Exoquick isolation to be an efficient strategy as it yielded large amounts of vesicles with minimal amounts of cellular debris (Fig. 3.4B). Additionally we observed many large $eGFP^+$ vesicles generated from infected cells.

To determine if EMVs shared any markers that have been associated with exosomes, we performed a western blot to detect flotillin-1 in exosomes isolated from C2C12 cells. C2C12 cells are highly susceptible to CVB and survive after infection, which together may explain the exuberant EMV production in this cell line. Flotillin-1 is an integral membrane protein associated with lipid rafts and has been widely cited as an exosome marker.[42] We found that EMVs indeed contained flotillin-1 suggesting that these vesicles could be related to previously described exosomes (Fig. 3.4C). However, it is possible that ExoQuick purification pulls down EMVs along with exosomes and the flotillin-1 might be contributed only by the smaller "classical" exosomes. Next, to detect for the

presence of virus in EMVs, we probed for viral capsid protein VP1. Though we expected infected cells to produce virus-containing EMVs, we saw 13-fold more VP1 in EMVs compared to the infected cells from which they were generated (Fig. 3.4D). Not only does this suggest that EMVs contain CVB, but also that CVB appears to be actively trafficked into EMVs, supporting the notion that EMVs represent a unique mode of viral dissemination.

CVB-induced EMVs are infectious

Because ATG5 KO MEFs did not appear to generate EMVs and did not support an expanding viral infection, we sought to elucidate the involvement of autophagy during CVB-mediated EMV production. We co-infected neural stem cells with DsRed-expressing CVB (DsRed-CVB) and an adenovirus expressing a GFP-LC3 fusion protein. We found that infected cells shed EMVs that were positive for both GFP and DsRed, indicating not only that they contained viral protein, but also that they could have been spawned from the autophagy pathway (Fig. 3.5A). This was further supported when we performed western blots to detect LC3 in C2C12 EMVs. LC3 is an autophagy marker that comes in two forms: inactive LC3-I which resides in the cytosol, and active LC3-II which is lipidated and directed to the autophagosomes. Due to conformational changes that arise from lipidation, LC3-II runs faster in SDS-polyacrylamide electrophoresis gels, separating from LC3-I. As can be seen in Figure 3.5B, whole infected cells contained both forms of LC3; however, EMVs almost exclusively contained LC3-II. FACS analysis revealed that CVB caused C2C12s

to release EMVs that expressed higher amounts of LC3-II compared to EMVs from mock-infected C2C12s (Fig. 3.5C). These data suggest that EMVs are formed through an autophagy pathway, and could represent ejected autophagosomes.

Our findings revealed that EMVs contain large amounts of viral protein. We next verified the presence of infectious virions in EMVs following ExoQuick isolation. GFP-CVB and DsRed-CVB constructs produce fluorescent proteins that are cleaved from the viral polyprotein and therefore are not associated with the virions themselves. For example, fluorescent proteins might be ejected from the cell simply because these proteins are unwanted. Western blots on EMVs revealed an abundance of VP1 which is a constituent of the viral capsid, and as such is a better indicator of the presence of virions. Still, infectivity was not yet established. We determined the presence of infectious virions associated with EMVs using plaque assays and found that the C2C12 exosome pellet indeed contained high amounts of infectious virions relative to the exosome-depleted cell culture supernatant (Fig. 3.5D). These data suggest that EMVs are indeed infectious. We next sought to determine if disruption of the EMV membrane altered infectiousness. Our prediction was that by liberating the virions from within the EMVs, the number of infectious entities would increase, resulting in higher viral titers when measured by plaque assay. Because cell membranes fracture upon freeze-thawing, we performed EMV disruption via three freeze-thaw cycles. Much to our surprise we found that disruption of EMVs reduced the

number of plaques by over 90% (Fig. 3.5E). This suggests that EMV formation is an integral component of CVB infection. It is possible that freeze-thawing destroyed some of the virions; however it is unlikely that this number of cycles would reduce the concentration of infectious virions substantially, *much less* by 90%.

DISCUSSION

Many viruses have been shown to upregulate host autophagy during the course of infection. Originally this process was thought to be a response mechanism from the host cell in order to engulf viral particles and destroy them to prevent viral propagation. However, certain viruses have been shown to strategically hijack autophagy to evade host immunity and increase replication.[1] Additionally, in some cases the autophagosome serves as a scaffold for viral assembly.[1, 5] CVB has also been reported to upregulate autophagy in cells; however, the importance of this process and its ultimate ramifications in the context of infection is uncharacterized.[1] We have previously demonstrated that CVB infection depletes CSCs by initiating premature differentiation (Chapter 1) and that this leaves the heart vulnerable to stress-induced pathologic remodeling (Chapter 2). It is now clear that CVB exhausts CSCs by activating autophagy, which drives differentiation. The tactic of infecting progenitor cells prior to differentiation expands the possible cell types CVB can target. Additionally, the virus can exploit the mobility of stem cells to “hitchhike” into cell niches that might normally be inaccessible due to receptor incompatibility. However, once CVB infects a CSC and subsequently migrates into a specialized cell niche, it still would lack the ability to infect the neighboring cells. The solution to this could be through coating itself with host membranes and fusing to the other cells in a similar fashion as an enveloped virus.

Though CVB elicited varying degrees of cytopathic effect in the different cell types we infected, they all shed small membrane-bound vesicles containing infectious virus. Also, we found these vesicles to be rich in LC3-II which suggests that they originate from autophagosomes. Whitton et al had previously shown that CVB upregulates autophagy in infected pancreatic acinar cells.[18, 19] However, autophagosome clearance was impaired. The accumulating autophagosomes eventually coalesced to form very large vesicles which the group called “megaphagosomes”. It is conceivable that these vesicles eventually get ejected from the infected cell and become the EMVs which we observed in these studies. This would also explain the larger size of these vesicles when compared to previously described exosomes, which may not involve the autophagy machinery.

A number of naked viruses have been described to be capable of non-lytic host-escape via autophagy. Jackson et al observed that poliovirus was similarly packaged into large autophagosome-like vesicles within the cell, but the mechanism by which the autophagosomal contents exited the cell was unclear.[1] They proposed two possible routes by which virions might escape. In the first scenario, the double-membrane structure of the autophagosome fuses with the plasma membrane, releasing free virions directly outside the cell. In the second scenario, only the outer-membrane of the autophagosome fuses to the plasma membrane, and the inner-membrane and the viral contents are released intact. However, the authors expected these structures to be fragile, rapidly breaking

down and liberating free virus. The membranous structures observed in poliovirus infection did not fully detach from the host cell, but were noted as blebs along the plasma membrane. In contrast, we observe that CVB-induced EMVs are free-floating, stable entities.

If CVB is indeed bundled in host-derived membrane packets that can separate from the cell, our observations with EMVs have interesting immunological implications. Typically non-enveloped viruses must escape the host via cytolysis, rendering the naked virus susceptible to neutralizing antibodies. Our findings suggest that CVB may evade host immunity by surrounding itself with cellular components. The findings in these studies also raise intriguing possibilities regarding viral dissemination. Our CSC infection data indicate that CSCs preferentially differentiated into vascular cells in vivo. In concert with the notion that CVB enclosed within EMVs are invisible to neutralizing antibodies, infected CSCs could mobilize to blood vessels and differentiate into vascular cells where they might shed CVB directly into the bloodstream. The host membrane exterior would prevent immune detection and effective antibody neutralization of the enclosed virions. Moreover the host membrane components of the EMVs might also facilitate viral entry into other cells via membrane fusion, or uptake by professional phagocytes such as monocytes/macrophages or dendritic cells, followed by infection and further dissemination. We have observed that the EMVs can readily infect other cells.

There currently exist several limitations to this study. We have shown autophagy to be crucial for C2C12 differentiation and that CVB upregulates autophagy in CSCs in vivo. We speculate that CVB-induced autophagy drives the premature differentiation of CSCs; however, this assumption is based on correlative data. Further experiments such as blocking autophagy specifically in infected CSCs are required to elucidate the involvement of autophagy in CVB-induced differentiation. Additionally, regarding EMVs, we cannot fully rule-out the possibility that virions are adhering to the surface of the vesicles which could contribute to their high infectiousness. We also do not know if ExoQuick may be pulling-down free virus. Our EMV disruption experiments revealed that freeze-thaw fracturing of the vesicles dramatically reduced the number of plaques formed during plaque assays. A difference in the number of plaques would be unlikely if the infectiousness of EMVs was simply due to free or adherent virions. Still, we do not have true spatial information of viral particles in relation to EMVs. This could be resolved by examining EMVs via transmission electron microscopy.

Chapter 3, in part is currently being prepared for submission for publication of the material. Sin, J., Tseung, G., Mangale, V., McIntyre, L., Gottlieb, RA., Feuer, R. The dissertation author was the primary investigator and author of this material.

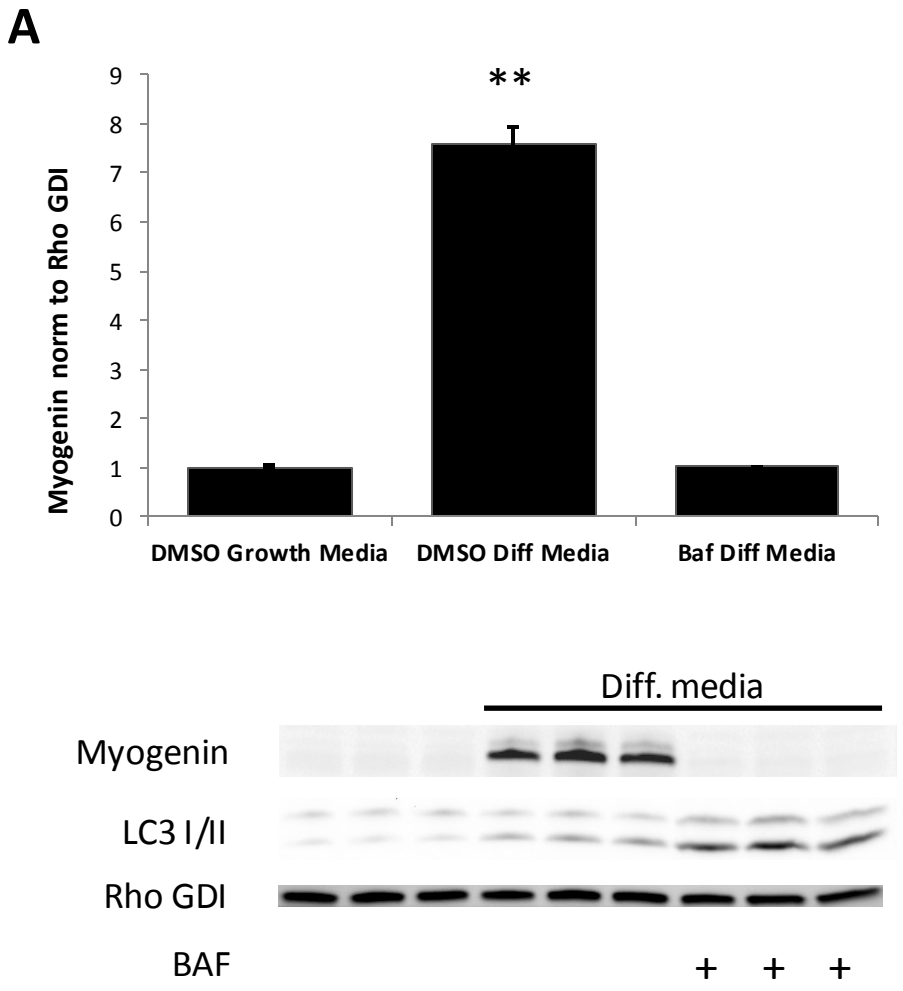


Figure 3.1: Autophagy is required for myogenic differentiation. A) C2C12 myoblasts were cultured in either full-serum differentiation media. Differentiation media induced the upregulation of myogenic differentiation marker myogenin as well as active autophagy marker LC3-II (bottom LC3 band). Myogenin expression was blocked with the addition of autophagy inhibitor bafilomycin (BAF). Due to impaired autophagic flux, LC3-II accumulated as well. B) Immunostaining revealed a marked increase in myogenin expression in infected C2C12 cells. This was recapitulated by western blot, however pre-treatment with BAF blocked this effect (C). [*p<.05, **p<.01]

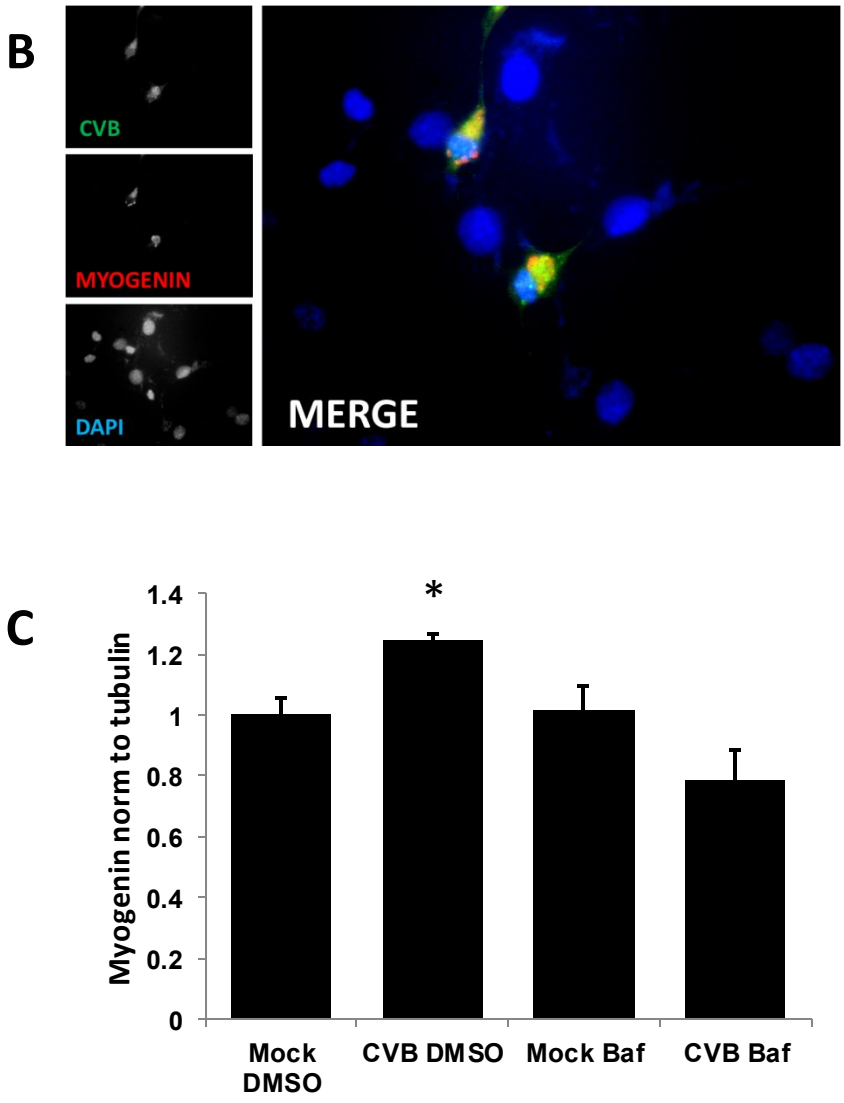


Figure 3.1: Autophagy is required for myogenic differentiation (cont.).

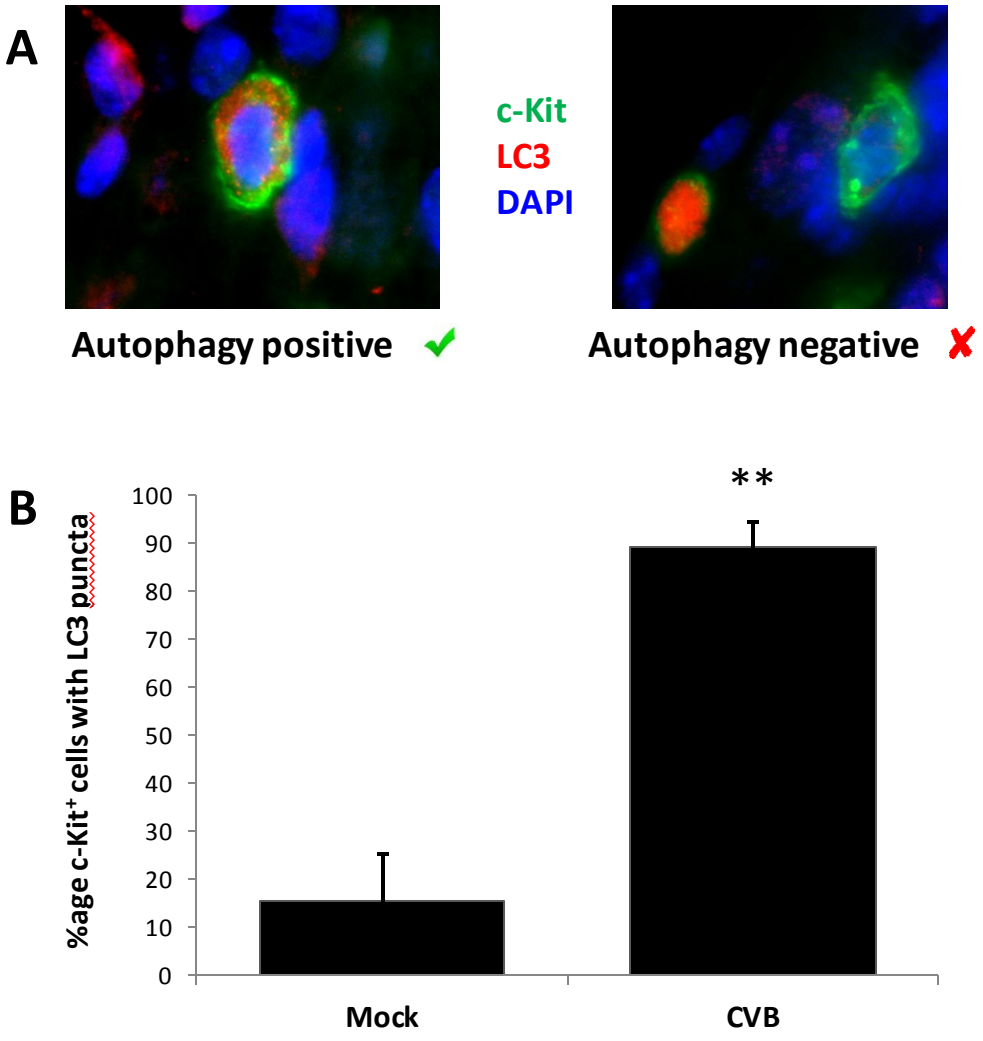


Figure 3.2: CVB infection upregulates autophagy in CSCs in vivo. Heart sections from neonates 2d PI were co-stained for c-Kit and LC3. A) Cells were considered "autophagy positive" if they showed punctate LC3 expression strictly localized to the cytosol. B) Infected animals had a higher percentage of autophagic CSCs out of total CSCs. [**p<.01]

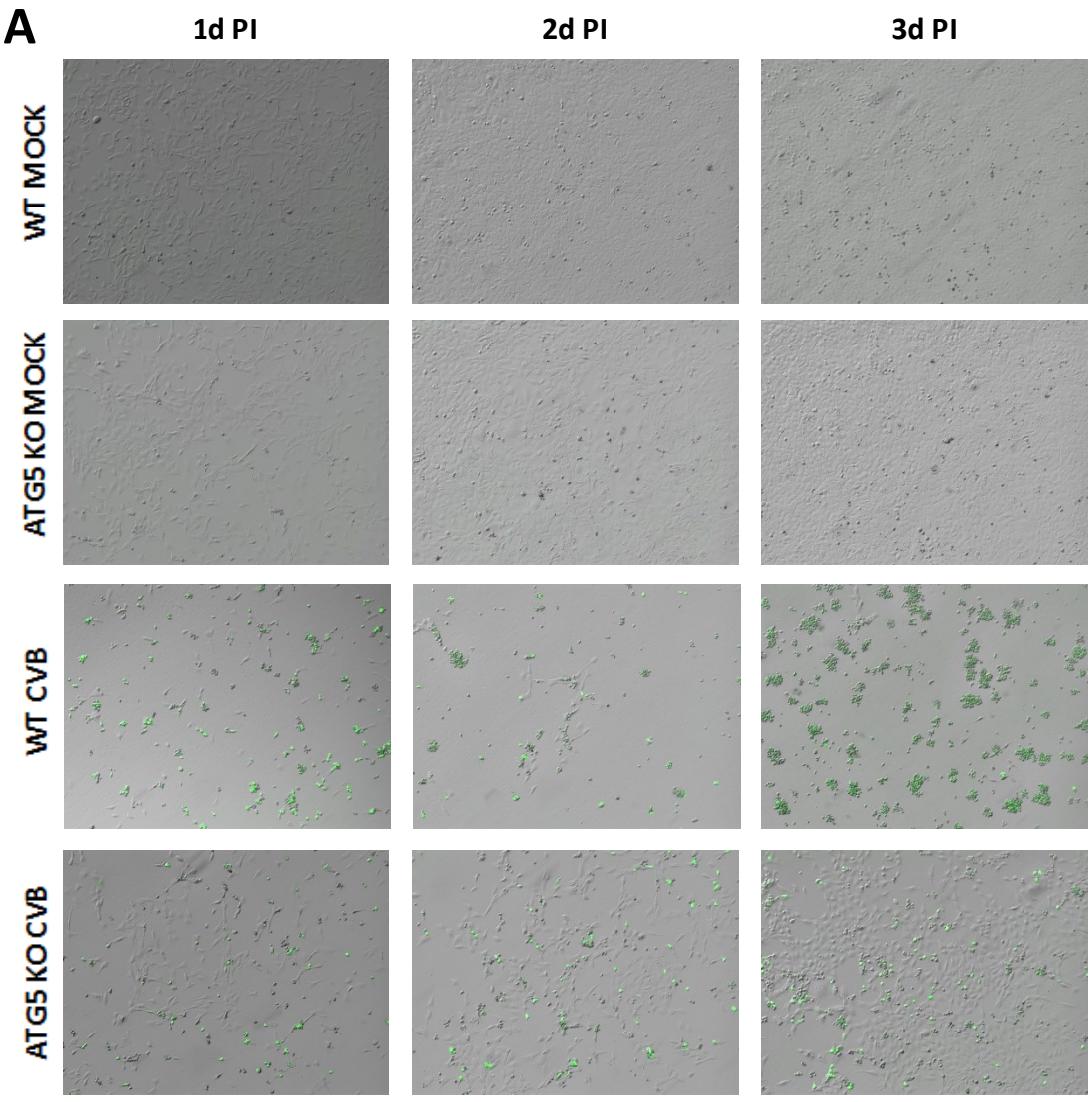


Figure 3.3: Autophagy is required for efficient viral dissemination. A) Wildtype (WT) or ATG5 knockout (ATG5 KO) mouse embryonic fibroblasts (MEFs) were either infected with CVB-GFP at MOI 100 or mock infected. WT MEFs had widespread infection after 1d PI, whereas ATG5 KO MEFs had only limited infection. B) Plaque assays on MEF culture media 3d PI showed roughly an 85% decrease in infectious virus in supernatants of infected ATG KO MEFs.

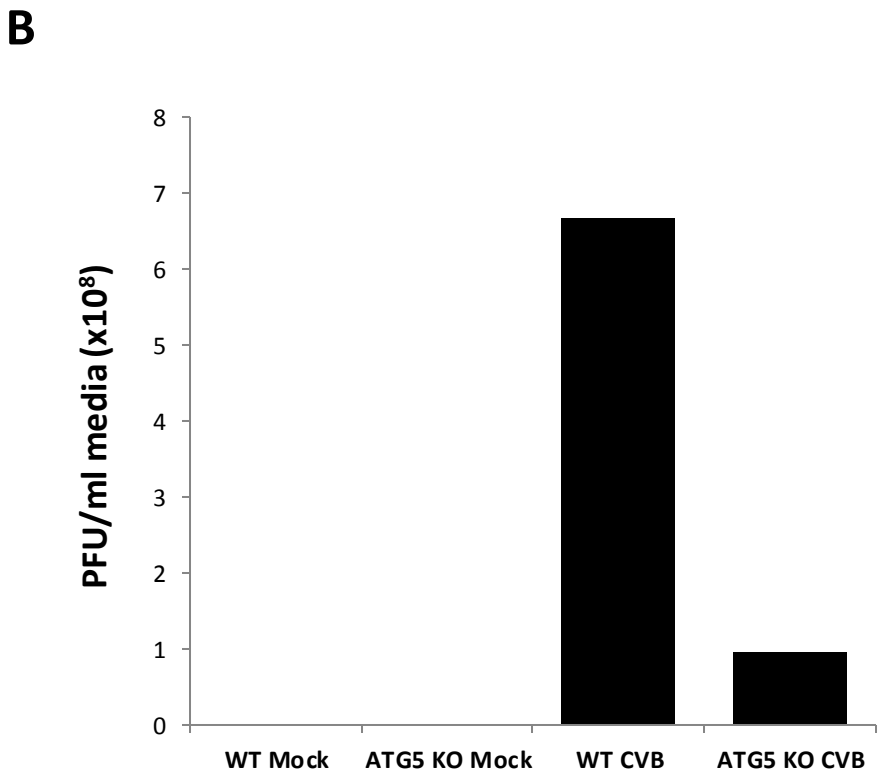


Figure 3.3: Autophagy is required for efficient viral dissemination (cont.).

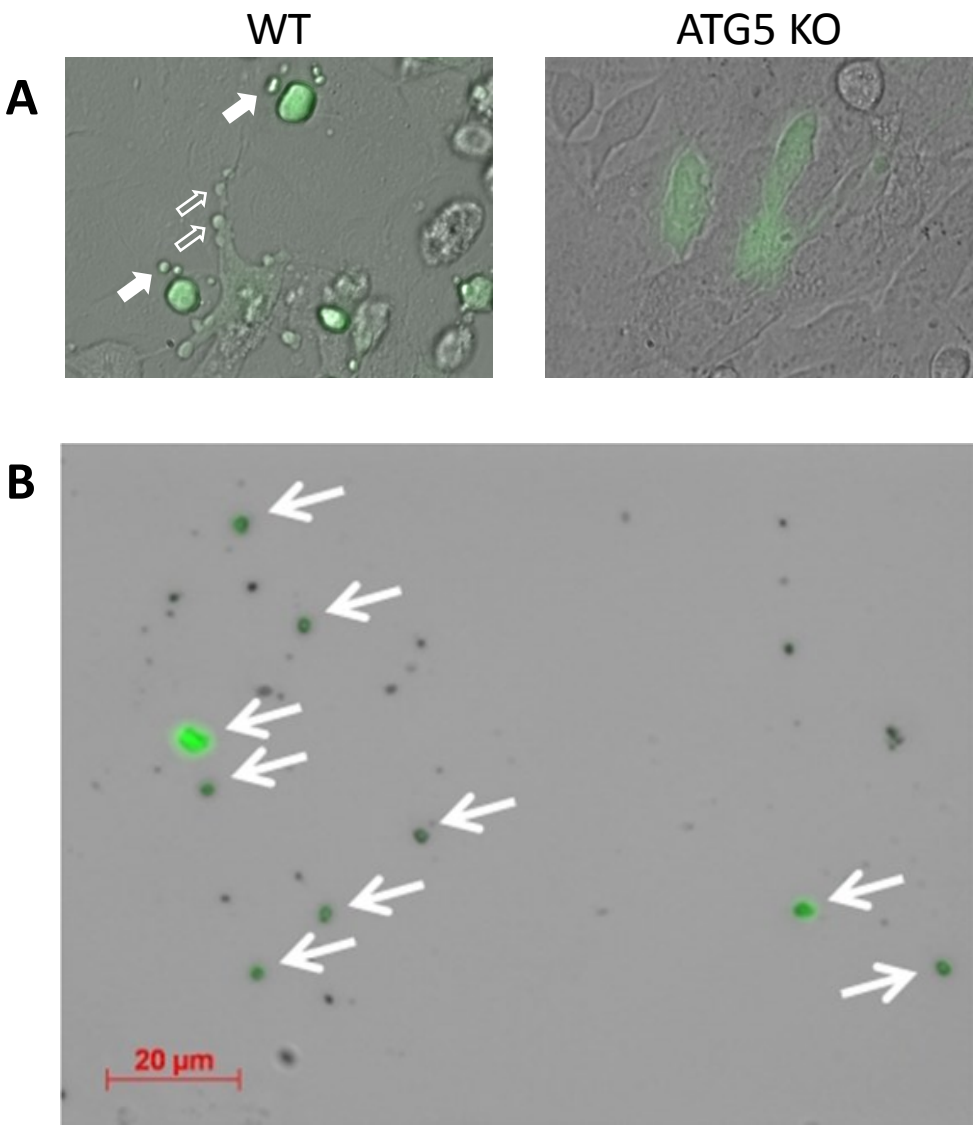


Figure 3.4: Infected cells shed extracellular microvesicles containing viral protein. A) WT and ATG5 KO MEFs were infected with GFP-CVB at MOI 1. 48H PI, WT cells formed blebs on the plasma membrane (empty white arrows) and $eGFP^{+}$ extracellular microvesicles (solid white arrows) which were not observed in infected ATG5 KO MEF cultures. B) Image microvesicles isolated from infected neural stem cells to show scale. C and D) Western blot analysis of infected C2C12 whole cell lysates vs. exosome isolates. Exosome fractions contained exosome marker flotillin-1 as well as large amounts of viral capsid protein VP1.

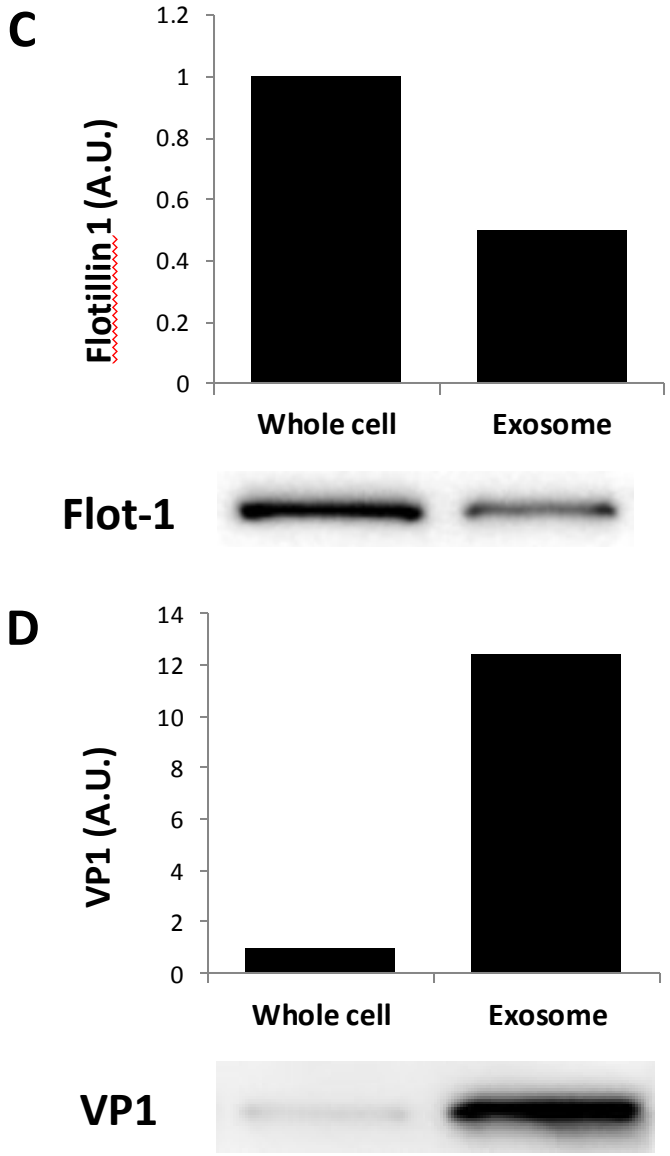


Figure 3.4: Infected cells shed extracellular microvesicles containing viral protein (cont.).

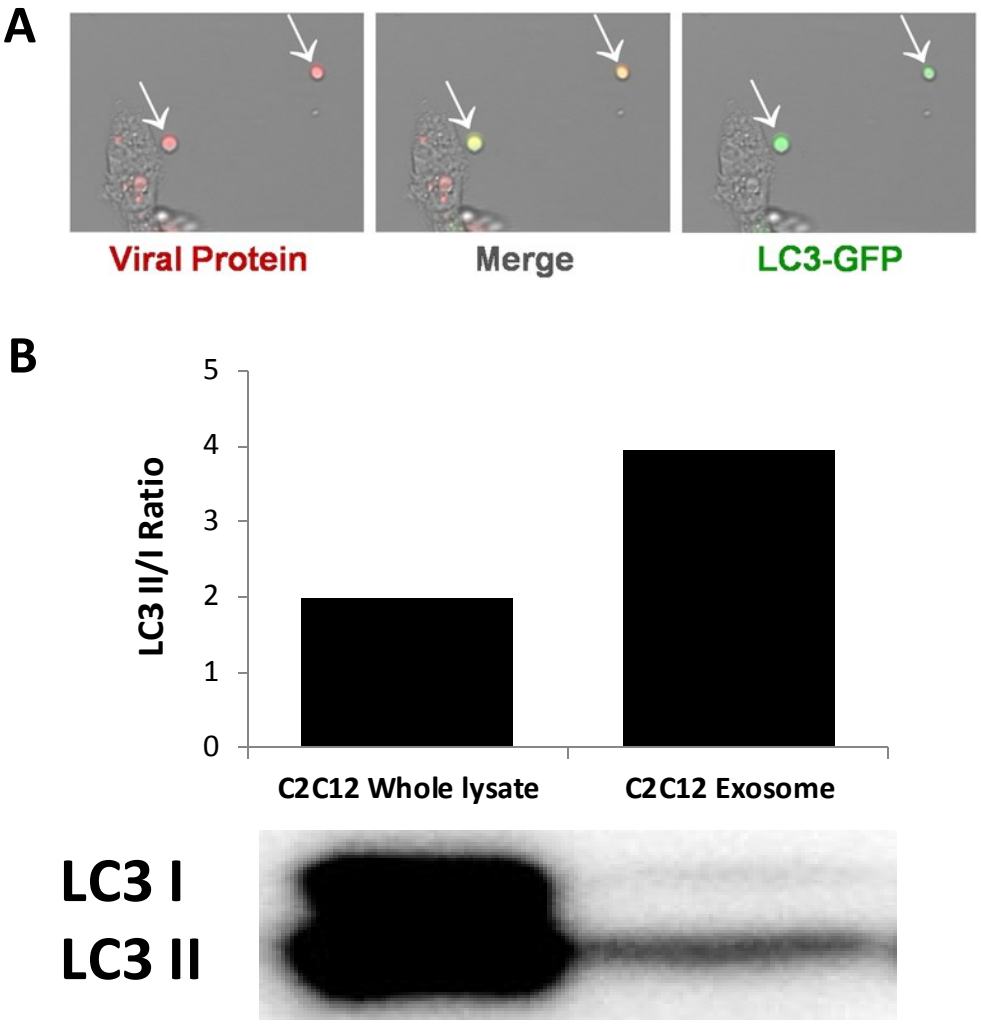


Figure 3.5: CVB-induced EMVs are infectious ejected autophagosomes. A) Neural stem cells were co-infected with DsRed-CVB and LC3-GFP adenovirus. Double-infected cells produced $DsRed^{+}/GFP^{+}$ EMVs. B) Western blots analysis of exosomes isolated from infected C2C12s revealed almost exclusive expression of LC3-II. C) FACS analysis of EMVs from infected C2C12s showed rich amounts of LC3-II in CVB-induced EMVs. D) Plaque assays on exosomes isolated from infected C2C12s revealed much higher viral titers relative to exosome-depleted media. E) Plaque assays on exosomes isolated from infected C2C12s comparing intact samples versus samples disrupted with repeated freeze-thaw cycles. Disruption of exosomes dramatically reduced the number of plaques formed.

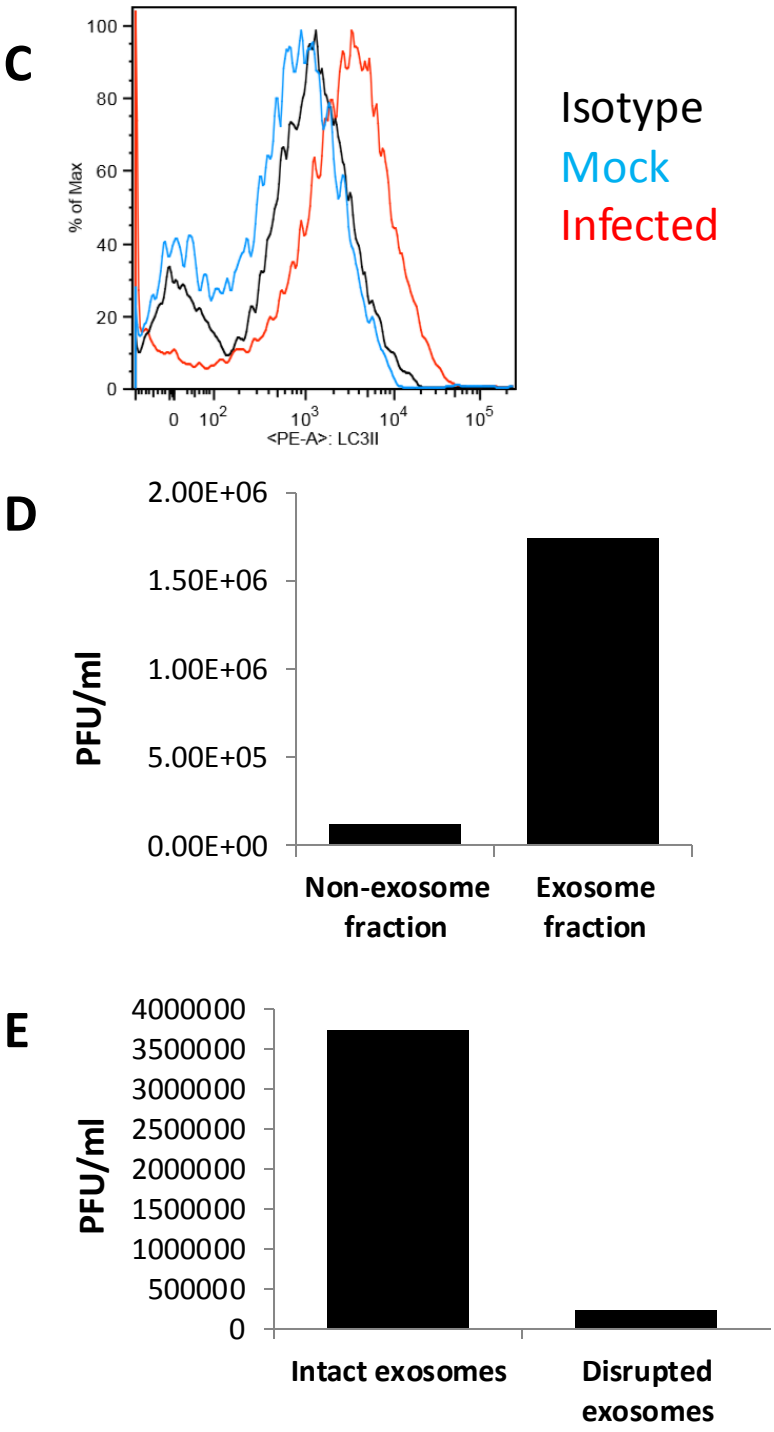


Figure 3.5: CVB-induced EMVs are infectious ejected autophagosomes (cont.).

CONCLUSION OF THE DISSERTATION

CVB is a common human pathogen that typically affects infants. It is associated with relatively innocuous infections which are limited to rash, fever, and upper-respiratory symptoms. Despite this, CVB has been linked to several severe chronic diseases, one of which is dilated cardiomyopathy. Because of the subclinical nature of acute CVB infection, patients that succumb to late dilated cardiomyopathy are often unaware of any antecedent CVB exposure; however, these patients do harbor CVB antibodies, which is evidence of past infection. It can be assumed that if there was any impetus to screen a patient for CVB, then the damage likely has already occurred. The question, then, is what is the mechanistic connection between a mild acute CVB infection and a devastating late-onset disease like dilated cardiomyopathy?

We developed a murine subclinical infection model which was characterized by normal suckling and growth of infected pups, and the absence of any cardiac damage during both early and late phases of infection. Viral tropism to the heart was confirmed by plaque assays and qPCR of heart extracts which revealed high amounts of infectious CVB virions and genomic RNA during early infection. However virus was undetectable during adult timepoints of 4wk and 11wk PI, suggesting that the virus had been cleared. We found that immediately following infection, CVB appeared to preferentially target CSCs. However, rather than triggering their destruction, as would be expected of an inherently cytolytic virus, CVB initiated premature differentiation of infected CSCs resulting in the depletion of the population. During adulthood, previously infected

animals which possessed exhausted CSC pools had an increased susceptibility to stress-induced pathologic hypertrophy. This was attributable to an inability to compensate for increased cardiac workload via adaptive vascular remodeling, a process which involves stem cell participation.

The cardiac stress we induced in our mice for these experiments can be likened to real-world challenges such as marathon running (swimming exercise) or hypertension (isoproterenol administration). However, if this sensitivity to maladaptive cardiac remodeling was coupled with an even harsher stressor such as in the case of a heart attack, then this could greatly amplify the resultant damage. Indeed, Huang's group showed that following DOX-mediated depletion of CSCs, animals developed significantly larger infarcts following surgical myocardial infarction (MI). This led to significant drop-offs in post-operative survival relative to vehicle-treated animals that also received MI. Examination of the vasculature showed less blood vessel branching into the infarct border zone in the hearts of DOX-treated animals. We would expect a similar outcome if we performed MI on mice that had been neonatally-infected with CVB, as these animals also had dysfunctional vascular remodeling when subjected to cardiac stress. Due to the relative commonness of CVB exposure, the notion that even a subclinical infection could render the heart permanently fragile is disconcerting; however it is difficult to determine how much of the CVB-exposed population is represented by our study. It is conceivable that even milder forms of CVB infection occur without manifesting in a cardiac phenotype but still cause the

infected host to seroconvert. Still, these studies may offer new insights as to how to prevent late-onset heart failure and may reveal an urgency to screen infants for CVB infection and to develop effective vaccines.

CVB infections cause a very subtle but potentially life-threatening alteration to the CSC population, and we found this to be mediated by the subversion of autophagy. CVB infection has been observed to upregulate host autophagy which is a cellular process we believe to be a crucial component for differentiation. We have also found that CVB causes the release of virus-containing extracellular microvesicles (EMVs) which appear to stem from host autophagy. This unusual phenomenon raises several intriguing questions. First, does the fact that CVB is bound by a host membrane allow for immune evasion? Being a picornavirus, CVB has classically been considered a “naked-virus”, therefore it is thought to escape the infected cell through cytolysis at which point it is exposed to neutralizing antibodies. It would be very informative to infect cultured cells with virally-induced EMVs that had been pre-treated with antisera from previously infected animals. We hypothesize that membrane-bound CVB would be masked from neutralizing antibodies and therefore infection should not be greatly affected when compared to infection with free viral stocks that had undergone freeze thaw cycles (to fracture any EMV membranes that might be present). This would offer insight regarding the role of EMVs in the context of humoral immunity.

Second, do EMVs facilitate viral entry into cells that are normally not susceptible to infection? In order to infect a cell, CVB must bind to receptors on the plasma membrane and trigger endocytic uptake of the virus. Two well-described receptors are coxsackievirus and adenovirus receptor (CAR) and decay accelerating factor (DAF). Could it be possible that viral EMVs can bypass receptor-mediated endocytosis via a membrane fusion event? We found that destroying the EMV membrane via freeze-thawing dramatically reduced the number of viral plaques formed, suggesting not only that CVB may be able to bypass receptor mediated endocytosis via EMV-to-cell fusion, but also that this mode of infection might accelerate the infection process. This would allow CVB to target many more cell types regardless of receptor compatibility. Interestingly, CVB has been known to be particularly oncolytic despite the fact that CAR is down-regulated in many cancer cell types. This suggests that CVB can utilize alternate mechanisms to infect the host. This could be investigated by infecting known CVB-resistant cells with virally-induced EMVs or free viral stocks. If EMVs could undergo membrane fusion with target cells, then this would presumably boost infectibility when compared to infection with free virus.

Third, do EMVs contain full virions or merely viral components? Because CVB is a positive-sense RNA virus, its genome behaves much in the same way as a messenger RNA, therefore if the viral RNA were able to enter the cell, that alone could propagate the virus. Imagine then that EMVs do not actually contain complete virions, but rather just the building blocks. This could be an alternative

explanation as to why intact EMVs could trigger such a great degree of infection when compared to disrupted EMVs. If intact EMVs act as a vehicle of entry for these viral pieces, then destroying the EMVs would render the viral components unable to enter the cell, and would presumably leave these components unstable.

CVB possesses many unique characteristics that reflect its highly adaptive nature. Suppressing host cell death allows the virus to prolong replication, and in the context of cardiac stem cell infection, trigger premature differentiation. By inducing differentiation, CVB can infiltrate other cell niches which were previously inaccessible. Differentiation is induced by the upregulation of host autophagy, which is also necessary for EMV production. Not only might this mask the virus from the immune system, but it also facilitates further infection. Truly CVB is an incredibly versatile machine that has evolved to exploit host processes in many unusual ways.

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