UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Mechanisms of Coxsackievirus B3 Dissemination and Persistence in the Host: Consequences for Neurodegenerative Disease

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

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2012

The Dissertation of Scott Michael Robinson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2012

DEDICATION

I wish to dedicate this dissertation to all those who provided me all the support that I definitely couldn't have completed this degree without. I am extremely grateful to my family, Mom, Dad, Shannon, Shane, Grandma and Grandpa. You truly are the amazing grace that saved me. After all you did for me; I understand the saying that "blood is thicker than water." I am also grateful to my physical therapist, Phil Lancaster who pushed hard with my parents so that I may walk again and so much more. I am very grateful for Ralph Feuer providing me a lab and plenty of funding to pursue my research. Also, thank you Ralph, for also showing me the other parts that are necessary for a successful career in scientific research. Finally, this is dedicated to my other therapists who worked on my recovery, professors at SDSU who let me work in their labs, friends and acquaintances who prayed for me to recover, and Anca Segall who allowed me to return to her lab when I was still recovering from my injuries and get back into the PhD program at San Diego State University with University of California, San Diego. Without all of you giving me the opportunity, I would not have been able to complete my PhD.

EPIGRAPH

"'Tis a lesson you should heed:

Try, try, try again.

If it first you don't succeed,

Try, try, try again."

William E Hickson: ***Oxford Dictionary of Quotations (3rd edition)*****. Oxford University Press. 1979. pp. 251**

TABLE OF CONTENTS

LIST OF ABBREVIATONS

CNS	Central nervous system
CV	Coxsackievirus
CVB3	Coxsackievirus B3
eGFP	Enhanced green fluorescent protein
EAE	Experimental Autoimmune Encephalomyelitis hrs
	Hours
ICCS	intraCellular Cytokine Staining
IHC	Immunohistochemistry
LC3	Light chain 3
MOG	Myelin oligodendrocyte glycoprotein
MOI	Multiplicity of Infection
NPSCs	Neural progenitor and stem cells pfu
	Plaque forming units
PI	Post-infection
RT-PCR	Reverse transcriptase polymerase chain reaction

LIST OF FIGURES

ACKNOWLEGEMENTS

Chapter 1, in part is currently being prepared for submission for publication of the material. Robinson SM, Rhoades RE, Okonkwo U, Lacharite R, Pagarigan RR, Whitton JL, and Feuer R. The dissertation author was the primary investigator and author of this material.

Chapter 2, in part is currently being prepared for submission for publication of the material. Robinson SM, Tsueng G, Sin J, Rahawi S, Hilton B, Wolkowicz R, Cornell CT, Whitton JL, Gottlieb RA, Feuer, R. The dissertation author was the primary investigator and author of this material.

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Bachelor of Science in Biochemistry – May 1999
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2008-2010 National Institute of Health: Research Supplements to Promote Diversity in Health-Related Research - PA-05-015 (1R01NS054108-01A2S1)

1999 Bachelor of Science from SDSU with distinction in the major

PUBLICATIONS

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MANUSCRIPTS SUBMITTED/IN PREPARATION

Tabor-Godwin JM, Ruller CM, **Robinson SM**, Knopp KA, Buchmeier MJ, Doran KS, Feuer R. Distinct neural stem cell tropism, early immune activation, and choroid plexus pathology following coxsackievirus infection in the neonatal central nervous system. Submitted/In Revision.

Robinson SM, Rhoades RE, Okonkwo U, Lacharite R, Pagarigan RR, Whitton JL, Feuer R. Molecular analysis of recombinant coxsackievirus B3 instability in vivo reveals the nature of viral replication during persistent infection. In Preparation.

Robinson SM, Tsueng G, Sin J, Rahawi S, Hilton BJ, Wolkowicz R, Cornell CT, Whitton JL, Gottlieb RA, Feuer R. Monitoring virus-induced intracellular membrane remodeling and virus dissemination in real time utilizing a novel recombinant coxsackievirus B3 expressing "fluorescent timer" protein. In Preparation.

SCIENTIFIC PRESENTATIONS

2012	Student Research Symposium, SDSU, San Diego, CA. Oral Presentation Title: A Novel Recombinant Coxsackievirus B3 Expressing Timer Protein Assists in Tracking Virus Infection in Real Time.
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2010	Society for Neuroscience Meeting, Convention Center, San Diego, CA. Poster Presentation Title: Enhanced Susceptibility or Resistance to Neurodegeneration / Autoimmune Disease in Mice Infected with Coxsackievirus Shortly after Birth.
2010	Student Research Symposium, SDSU, San Diego, CA. Oral Presentation Title: Timer-CVB3: A Recombinant

	Coxsackievirus Which Utilizes "Fluorescent Timer" Protein to Track the Progression of Infection in Real Time.
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Approach For Calculating Coxsackievirus Replication Rates During Persistence in the Heart.

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PROFESSIONAL RESEARCH EXPERIENCE

September 2007- September 2012, Doctoral Dissertation Research

San Diego State University; Department of Cellular and Molecular Biology

Laboratory of Dr. Ralph Feuer

Techniques Performed: In-vitro transcription, lipofection of HeLa cells, viral preparation using in-vitro transcribed RNA and a DNA plasmid with the viral genome following the CMV promoter, PCR, infection of mice by intraperitoneal injection and intracranial injection, sacrifice of mice and harvesting of brain, spinal cord, pancreas, and spleen, embedding harvested tissue in paraffin and cutting slides, embedding harvested tissue in freezing media and cryo-cutting slides, immunohistochemical slide staining, haemotoxlin-eosin slide staining, tissue culture with HeLa cells, plaque assay titration of virus solutions and tissue homogenates using HeLa cells, fluorescence microscopy, in-vitro immunostimulation of harvested tissues, surface staining, intracellular cytokine staining and FACS analysis of harvested tissues

August 2000-September 2002, Master's Thesis Research

University of Chicago;

Laboratory of Dr. Tao Pan and Dr. Joe Piccirilli

Project: Studied RNaseP activation in the nucleus and cytoplasm following transcription; Studied the Azoarcus ribozyme; experimented on its catalytic specificity to determine if it bound more stably to substrate or product; Experimentation also included assaying of the Azoarcus ribozyme's temperature preference for catalyzing reactions

Techniques Performed: In-vitro ribozyme reaction, RNA agarose gel electrophoresis

1998-1999, Undergraduate Research

San Diego State University

Laboratory of Dr. Anca Segall

Project: Synthesized DNA which could block cleavage by Integrase; analyzed the formation of pre-cleavage DNA-protein complexes

Techniques Performed: Synthesis of oligonucleotides with phosphoramidate internucleotide linkage, polyacrylamide gel purification of oligonucleotides, EMSA, Radioactive labeling of DNA with T4 polynucleotide kinase, PCR, in-vitro site-specific recombination

1997-1989, Oligonucleotide Synthesis Technician

San Diego State University

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Techniques Performed: Synthesized and purified many oligonucleotides and poured sequencing gels

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TEACHING EXPERIENCE

Fall 2007	Teaching Assistant Cell and Molecular Biology, San Diego State University
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ABSTRACT OF THE DISSERTATION

Mechanisms of Coxsackievirus B3 Dissemination and Persistence in the Host: Consequences for Neurodegenerative Disease

by

Scott Michael Robinson

Doctor of Philosophy in Biology

University of California, San Diego 2012

San Diego State University 2012

Professor Ralph Feuer, Chair

Kirk Knowlton, MD, Co-Chair

Enterovirus infections are quite common in the United States, causing approximately 10-15 million symptomatic infections every year. Coxsackievirus B3 (CVB3), a member of the picornavirus family and enterovirus genus, hasbeen shown to be responsible for viral myocarditis, aseptic meningitis, and pancreatitis in humans. The mechanism responsible for CVB3 persistence in the host (chronic / sporadic viral replication or viral latency) remains unclear. Therefore, we utilized the inherent long-term instability of our recombinant coxsackievirus expressing the enhanced green fluorescent protein (eGFP- CVB3) in two mouse models (BALB/c and B cell knockout mice) to determine the mechanism of virus persistence in the heart. These studies may help to determine if antiviral treatment during persistent infection may be productive in patients suffering from chronic viral myocarditis. Also, we genetically engineered a unique molecular marker – "Fluorescent timer" protein - within our infectious viral clone and isolated a high-titer viral stock (Timer-CVB3) following transfection in HeLa cells. "Fluorescent timer" protein changes its fluorescence from green to red over time. Timer-CVB3-infected HeLa cells, neural progenitor and stem cells (NPSCs), or C2C12 myoblast cells slowly changed fluorescence from green to red over 72 hours, as determined by fluorescence microscopy or flow cytometric analysis. The slow change in fluorescence for Timer-CVB3-infected HeLa cells could be interrupted by fixation, suggesting that the fluorophore associated with "fluorescent timer" protein was stabilized by formalin cross-linking reactions. Induction of a type I interferon response reduced the progression of cell-to-cell infection in both HeLa cells and NPSCs. Time lapse photography of partially differentiated NPSCs infected with Timer-CVB3 revealed substantial intracellular membrane remodeling and the assembly of discreet virus

replication organelles which changed fluorescence color in an asynchronous fashion within the cell. Unexpectedly, Timer-CVB3 infection of partially differentiated NPSCs or C2C12 myoblast cells induced the release of numerous extracellular microvesicles containing viral material which may represent a novel route of dissemination. Through the use of this novel recombinant virus, we hope to gain a better understanding of CVB3 tropism, alterations in host cell organization, and virus dissemination within the host. Finally, we wished to determine if a previous CVB3 infection during the neonatal period may provide a "fertile field" for the acceleration or exacerbation of subsequent neurodegenerative and autoimmune disease in the aging host. We hypothesized that a previous neurotropic infection which persists in the host may aggravate a subsequent neurological disease in a mouse model of Alzheimer's disease, and separately in a mouse model of multiple sclerosis. The induction of key inflammatory and cytokine genes during persistent infection, and the nature of the neurological disease may be important determinants for enhancement or reduction of disease following neonatal CVB3 infection.

INTRODUCTION OF THE DISSERTATION

Enteroviruses (EV) are among the most common and medically-important human pathogens, are a frequent cause of central nervous system (CNS) disease [1]. Worldwide distribution of EV infection is revealed by the detection of EV-specific antibodies in the sera of approximately 75% of individuals within developed countries. For example, in 1996, approximately 10-15 million diagnosed cases of EV infection occurred in the US alone [2]. Few studies have been done to determine if non-polio enteroviruses, or close relatives, have the ability to persist and cause long term damage in the CNS [3] [4], or whether previous infection of neurons may indirectly lead to the degeneration of aging motor neurons.

Coxsackieviruses (CV), members of the enterovirus genus, are significant human pathogens, and the neonatal CNS and heart are major targets for infection. CV infection may cause severe morbidity and mortality, particularly in the very young. CV infection during pregnancy has been linked to an increase in spontaneous abortions, fetal myocarditis [5], and neurodevelopmental delays in the newborn [6]. Infants infected with CV have been shown to be extremely susceptible to myocarditis, meningitis and encephalitis with a subsequent mortality rate as high as 10%. Adult infection and subsequent viral myocarditis has also been described, and a substantial proportion of patients suffering from chronic viral myocarditis may eventually develop dilated cardiomyopathy, a condition underlying almost half of all heart transplants. Severe demyelinating diseases may occur following

infection, including acute disseminated encephalomyelitis [7] and acute transverse myelitis [8]. For example, a recent report describes the case of a 19 year old nursing student who experienced cognitive problems and lapsed into a coma. By immunohistochemistry utilizing biopsied tissue, she was found to suffer from viral encephalitis and persistent coxsackie B virus infection of the CNS. Also, a number of delayed neuropathologies have been associated with previous CV infection, including schizophrenia [9] [10], encephalitis lethargica [11], and amyotrophic lateral sclerosis [12] [13].

In general, early damaging events on the CNS by viral infection may result not only in severe physical and intellectual disability, but also in less obvious neurological deficits. For example, children who were thought to have fully recovered from neonatal CNS virus infections exhibited some deficiency in scholastic performance [14]. Thus, the enduring harmful effects of childhood infections on the CNS may be greatly under-appreciated. Picornaviruses including polioviruses, CVs, and other unclassified enteroviruses, frequently infect the CNS [15]. Although these infections often are considered acute and self-limiting, evidence is emerging that these viruses – or at least viral RNAs – may persist for months or years after the initial infection. For example, ~50 years after the primary infection, a large percentage (~30%) of polio victims are now experiencing new symptoms (post-polio syndrome), which some have correlated with the presence of viral RNA in the CNS [16]. **If these viruses persist, neurotropic infection might provide a chronic inflammatory stimulus leading to regional cytokine induction and activation of**

autoreactive T cells through molecular mimicry and bystander activation [17] [18]. This may be especially true for viruses such as CV, which have the ability to infect stem cells [19] and neurons [20].

Type B coxsackieviruses (CVB) include six serotypes, each being associated with acute disease in humans, including acute viral myocarditis and pancreatitis. Intriguingly, persistent CVB infections have been associated with autoimmune-type diseases such as chronic myocarditis, diabetes, and chronic inflammatory myopathy. In all cases, virus persistence in target tissues is associated with chronic disease. Persistent viruses, especially those such as CVB, may provide chronic inflammatory events leading to regional cytokine induction and activation of autoreactive T cells through a variety of potential mechanisms, including molecular mimicry and bystander activation [18] [17]. **It is not clear what effect persistent infection and the associated inflammatory events might have on resident stem cells, either during development, or in the adult.** The lack of infectious virus has been used as proof by others to suggest that CVB-associated diseases, which include myocarditis, may occur through autoimmune mechanisms. However, an alternative model is that CV may remain "latent" with periodic reactivation, similar to other latent viruses, such as herpes virus [21] [22] [23]. The sporadic expression of viral proteins may lead to a chronic immune response and subsequently, immuno-pathology [24]. *How does CVB, an inherently lytic virus, persist in vivo?* Recent publications suggest the presence of CVB in the heart relies upon the generation of non- cytolytic variants with 5' terminal mutations and deletions [25] [26] [27] [28].

However, it is not clear how attenuated, non-cytolytic variants might outcompete wild type virus in heart tissue. Studies in other models suggest that CVB persistence is not facilitated by genetic alterations in the virus that give rise to replication-defective forms, but rather is due to the genetic stability of double-stranded viral RNA complexes [29]. *Can CVB3 be reactivated from cells harboring viral RNA?* Little is known about the stability of CVB RNA in the absence of viral replication, nor about the possibility that CVB can be reactivated from tissues harboring latent viral RNA. Recently, we have published studies characterizing the ability of CVB3 to remain in an apparently latent state in quiescent tissue culture cells [30] [31]. Following stimulation of quiescent cells by injury, or by the addition of growth factors, viral protein expression was detected and infectious virus was produced, suggesting that latent CVB3 may be reactivated in response to cellular activation. Published evidence suggests that this phenomenon may be important *in vivo*, as well. For example, CVB3 undergoes a burst of replication in B cells proliferating within germinal centers in the spleen [32]. Also, mice deficient for $p56^{lck}$ activation enzyme support only low levels of CVB3 production and are protected from CVB3-mediated myocarditis [33].

Recently, our laboratory has shown that coxsackievirus B3 (CVB3) targets proliferating cells in regions of the neonatal CNS supporting neurogenesis [19]. Nonetheless, infected migratory neuronal progenitor cells were able to differentiate into mature neurons. Many neurons eventually underwent caspase-3 mediated apoptosis at later stages of disease [34].

Intriguingly, viral RNA was detected in the CNS of surviving animals in the absence of infectious virus. The detection of CVB3 RNA in target tissues may have great significance for CVB3-mediated disease, especially since the long term presence of replication-restricted CVB3 RNA in the heart utilizing transgenic mice has been directly associated with dilated cardiomyopathy in a previous study by Wessely et. al. [35]. Also, previous studies have shown that CVB3 may produce viral proteases which may cleave essential proteins such as dystrophin, thereby exacerbating the disease process in the heart [36].

Establishment of viral persistence in the neonatal mouse CNS following inoculation with a non-lethal dose of CVB3. Our recent results suggest that neonatal mice inoculated with a lethal dose with a recombinant CVB3 expressing the enhanced green fluorescent protein (eGFP-CVB3) showed reduced levels of neurogenesis and profound CNS developmental delays at early time points up to day 5 PI [37].

We speculated whether CVB3 infection may lead to lasting consequences in the CNS within surviving mice. Therefore, we inspected the later stages of infection, ongoing neuropathology, and long-term consequences on CNS development in neonatal mice inoculated with a non- lethal dose of virus. As shown previously (20), three day-old mice inoculated with eGFP-CVB3 (2×10^7 pfu IC) survived acute infection and established a persistent infection in the CNS. High viral titers were observed up to day 5 PI (***Figure 1A***; top graph). By day 10 PI and beyond, infectious virus could no longer be detected by conventional plaque assay. However, viral RNA was

observed by real time RT-PCR up to day 90 PI, although RNA abundance decreased substantially over time (***Figure 1B***; middle graph).

Existing evidence favors a role of persistent CVB3, defined by long-term retention of viral RNA in the absence of infectious virus, in contributing to the chronic phase of disease [38] [39] in the heart. Supporting this view, a near equal ratio of plus to minus viral genome progressed over time until by day 30 PI, as determined by real time RT-PCR (***Figure 1C***, bottom graph).

These results suggest that CVB3 may persist as a double-stranded viral RNA complex during the establishment of persistence. However, it remains unclear what regulates the switch from acute to persistent infection, and whether CVB3 persistence is distinguished by sporadic viral replication or true viral latency.

We will utilize the inherent instability of our recombinant eGFP-CVB3 to serve as a measuring device for determining viral replication status during the persistent phase of infection. The stability of the eGFP insert within our recombinant virus will be evaluated over time by RT-PCR, or by plaque assay expression of eGFP, in two mouse models: BALB/c mice which harbor viral RNA in the heart and CNS in the absence of infectious virus; and B cell knockout (BcKO) mice which support a chronic infection due to the lack of a neutralizing antibody response.

We will follow the progression of eGFP-CVB3 infection in each organ over time in the host by analyzing the generation of deleted viral variants.

Also, we have recently created an additional recombinant CVB3 expressing Timer fluorescent protein (Timer-CVB3). Timer protein is a mutant (V105A, S197T) of the dsRed, red fluorescent protein. The dsRed protein is known to fluoresce green immediately after translation. However one to five hours after synthesis, the green flourophore is modified to fluoresce red. The engineered mutations in Timer protein result in this autocatalysis being substantially delayed. Therefore, Timer protein fluoresces green for the first day after it is made. Two to three days later, it is modified to fluoresce red. This three day time course for the conversion from green to red fluorescence allows determination of the order of viral infection by Timer-CVB3 if examination is made within three days initial infection.

The pancreas is a primary target organ for CVB3 infection, and early viral replication here may seed other organs such as the heart and CNS. Importantly, understanding factors contributing to pancreatic infection may help to reduce early viral replication and virus dissemination within the host. For example, transgenic mice expressing the antiviral molecule interferon- within the pancreas showed reduced viral titers and substantially less myocarditis [40]. Our preliminary observations suggest that different regions of the pancreas may be preferentially susceptible to infection, with the head of the pancreas (attached to the duodenum) most susceptible to infection. We hypothesize that hormones (cholycystokinin-8 – CCK8 and secretin) released in the vicinity of the head of the pancreas during feeding may regulate viral replication. Therefore, we will utilize Timer-CVB3 to study the progression of

infection in the pancreas of C57 BL/6 mice following intraperitoneal inoculation. These mice will then be sacrificed and their pancreases harvested. Using Timer-CVB3, I also will examine the pancreas of persistently- infected mice to observe potential virus reactivation following consumption of a meal. We hypothesize that after a period of food restriction, consumption of a meal may be a signal for the pancreas to release hormones involved in pancreatic digestive function, such as CCK8 and secretin.

Finally, **we wish to determine if a previous early neurotropic viral infection which persists and causes inflammation for extended periods, may provide a "fertile field" for accelerated or exacerbated neurodegenerative and autoimmune disease in the aging host** [41] [42]. We hypothesize that CVB3 may permanently modify the immunological make-up of the CNS, perhaps altering the expression inflammatory genes and partially exposing the blood- brain-barrier to self-reactive T cells. Similarly, CVB3 infection of the heart has been shown to lead to a higher expression of immunoproteasomes and inflammatory chemokines (34). We have recently determined that pups infected with CVB3 may suffer from hydrocephalus, and that CVB3 infection may induce choroid plexus pathology (Tabor-Godwin JM, Ruller CM, **Robinson SM**, et al; Manuscript Submitted/In Revision). Nonetheless, the choroid appeared to recover from infection as judged by carbonic anhydrase staining, a marker for choroid plexus function (***Figure 2***; white arrows). Our ultimate goal is to utilize our neonatal mouse model of CVB3 infection to study the link between microbes and chronic disease. We expect that prior

infection and chronic inflammation may be involved in setting a fertile ground for numerous autoimmune-like conditions, including diabetes, myocarditis, and demyelinating diseases. We hope that our studies may help predict the lasting neurological sequelae of a previous viral infection on the developing host.

Alzheimer's disease may be influenced by the possible induction of inflammation in the central nervous system (CNS). In a study of patients who had been diagnosed with mild Alzheimer's disease symptoms, it was found that those patients who showed higher levels of the inflammatory cytokine, TNF-α, suffered a greater decrease in cognition (32). Animal models of Alzheimer's disease have utilized mice expressing the human APP cleaved into the 41 amino acid β-amyloid peptide (hAPP751 transgenic mice) which has been shown to induce plaque formation in the murine CNS (33). Since CVB3 may increase the expression of inflammatory chemokines, a prior viral infection which persists in the CNS may accelerate plaque formation in hAPP751 transgenic mice.

Multiple Sclerosis (MS) is an autoimmune disease thought to be caused by the entry of pathogenic, self-reactive immune cells into the CNS, and targeting of myelin sheaths surrounding neurons. Experimental Autoimmune Encephalitis (EAE) is a relatively common mouse model of multiple sclerosis utilized to study immune activation and demyelination. When serum from mice producing myelin-specific autoantibodies was injected intraperitoneally (IP) into C57 BL/6 mice suffering from CVB3 infection in the CNS, an increase in

demyelination was observed [43]. We hypothesized that CVB3 infection of the CNS could make the signs of disease associated with EAE much more severe, and perhaps inhibit the ability of the mouse to recover from the neurological damage. However, CVB3 infection has also been shown to suppress the immune response and inhibit $CD8^+$ T cell activity by blocking MHC class I presentation of antigen. Hence, immune suppression following CVB3 infection may ameliorate, rather than exacerbate a subsequent autoimmune disease such as EAE.

In conclusion, defining the mechanism of CVB3 persistence utilizing novel recombinant viruses may be of great benefit in understanding disease progression and potential treatment regimes. Also, unrecognized persistent viral infections may play a role in modulating subsequent unrelated diseases which may come about later in life. We feel that our pediatric model of CVB3 infection will allow us to carefully evaluate the disease outcome in a controlled laboratory setting.

FIGURES

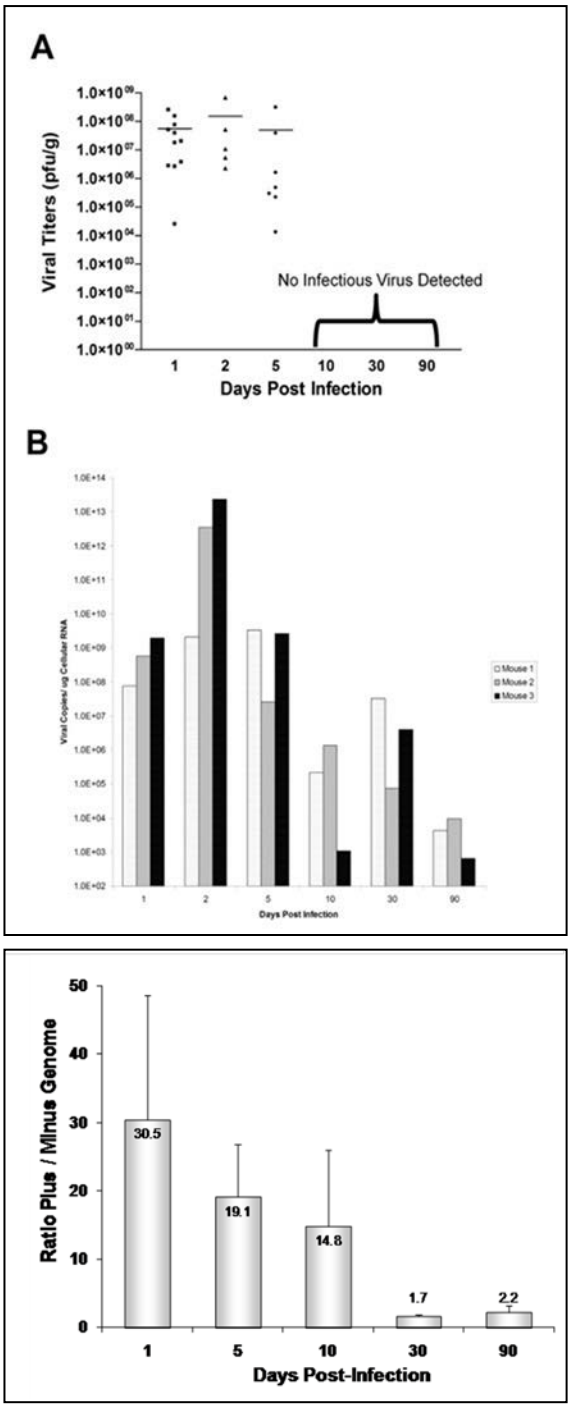

Figure 1. CVB3 RNA, but not infectious virus, persists in the neonatal CNS.

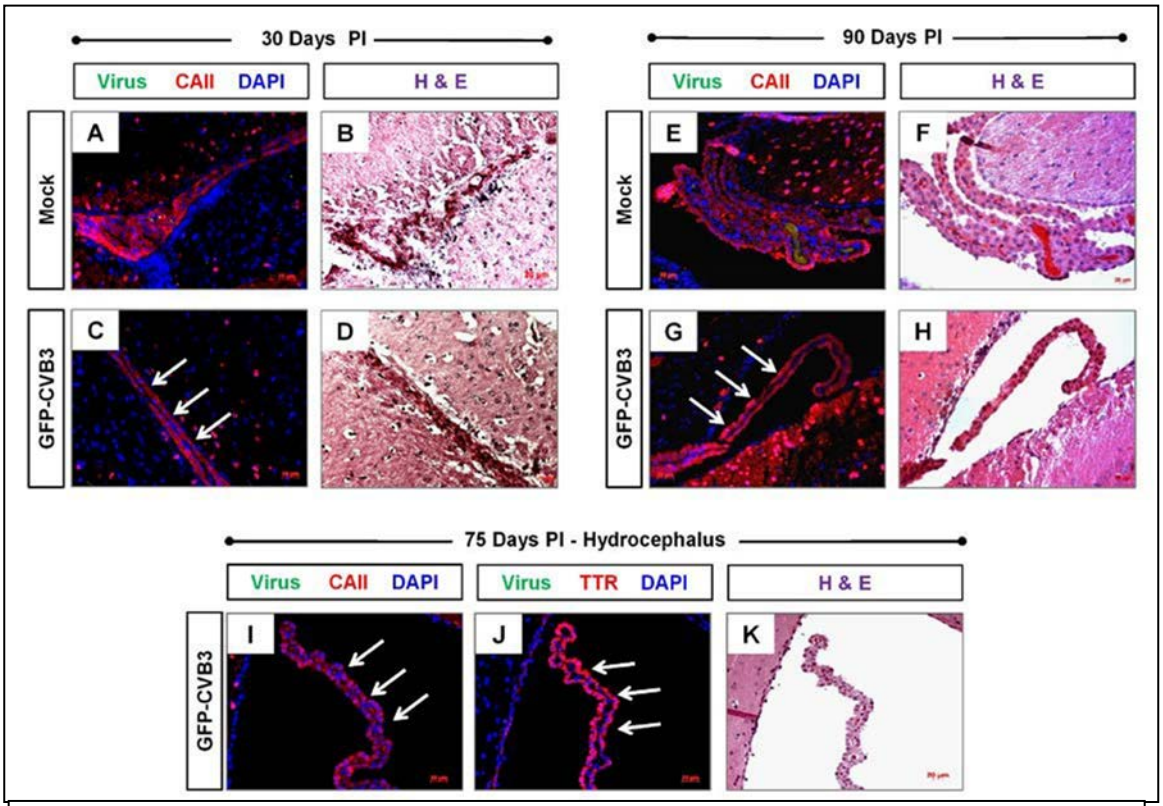

Figure 2. Recovery of carbonic anhydrase II expression in the choroid plexus at late time points following GFP-CVB3 infection.

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CHAPTER I:

Molecular Analysis of Recombinant Coxsackievirus B3 Instability In Vivo Reveals the Nature of Viral Replication during Persistent Infection

INTRODUCTION

Previously, we generated a recombinant coxsackievirus expressing eGFP (eGFP-CVB3) to study viral tropism and pathogenesis. The eGFP gene was inserted immediately before the open reading frame of the single viral polyprotein. The eGFP gene also included an artificial viral protease cleavage site to release the protein from other viral proteins following translation [1]. We generated eGFP-CVB3 stocks after transfection of viral RNA generated by *in vitro* transcription.

The percentage of viral plaques expressing eGFP decreased in a continuous and reproducible manner by passage six suggesting instability of the recombinant virus. Our viral stock (passage two) demonstrated a relatively pure population of recombinant virus (greater than 97% expressed eGFP). The resulting recombinant coxsackievirus efficiently infected HeLa cells and expressed high levels of eGFP. We examined the number of passages before our recombinant virus no longer expressed eGFP (***Figure 1***). eGFP-CVB3 was passaged continuously in three separate cultures by serial infection of HeLa RW cells at a constant multiplicity of infection (MOI) of 0.1 (***Figure 1***; Low MOI), or alternatively at a much greater MOI (10.0) (***Figure 1***; High MOI). Supernatants were harvested after twenty-four hours, and viral titers were determined by plaque assay. Before plaque assay plates were fixed and stained, the percentage of eGFP expressing plaques were determined by fluorescence microscopy.

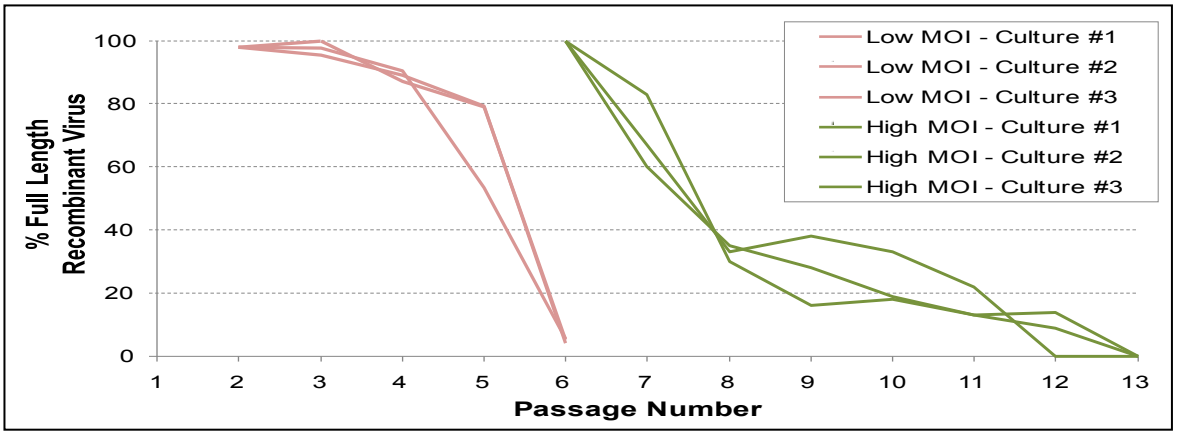


Figure 1. Loss of the eGFP insert over time with serial passage of recombinant virus in vitro.

Although stable to a certain number of passages, eGFP-CVB3 eventually failed to express the foreign protein. At passage five, the number of plaques expressing eGFP in all three independent cultures dropped below 85%. By passage six, less than five percent of plaques were eGFP positive.

Similar results obtained from three independent cultures suggest that recombinant CVB3 may delete foreign inserts in a highly reproducible manner. Intriguingly, the loss of eGFP expressing plaques could be delayed by simply starting the infections of HeLa cells with a greater multiplicity of infection (moi = 10.0) (***Figure 1***; green lines).

We summarized the observed *in-vitro* results for the retention of the eGFP insert within the recombinant virus in ***Figure 2***. For cultures inoculated with a low MOI (0.1), by definition one virus infects every 10 cells. After initial infection, the virus undergoes multiple rounds of replication followed by virion release. For cultures inoculated with a high MOI (10.0), by definition ten viruses infect one cell, and the lack of new target cells limits subsequent viral replication.

Therefore, low MOI passage results in the recombinant CVB3 undergoing multiple rounds of viral replication, as compared to high MOI passage resulting in recombinant CVB3 undergoing a single round of viral replication. As a result, the eGFP insert is preferentially retained within recombinant CVB3 at high MOI passage. These results suggest that the presence of the eGFP insert in the eGFP-CVB3 genome is inversely proportional to the amount of viral replication.

METHODS

Infection of Mice: BALB/c, C57, and BcKO mice were infected with eGFP-CVB3 when they are 7 weeks old, the mice were IP injected with either $1x10^5$ or $1x10^8$ pfu of eGFP-CVB3. The mice were sacrificed at several indicated times and heart and pancreas were harvested, and examined for eGFP expressing, infectious virus by plaque assay in HeLa cells and have RNA isolated from them. The isolated RNA were examined by RT-PCR.

Plaque Assay: A solution of HeLa cells were counted to determine its concentration of cells/mL in 1X DMEM with 10% FBS. The solution was diluted to a concentration of $1x10^5$ cells/mL. This cell solution was used to pour six well plates with 3 mL of the cell solution in each of the wells. The plate were incubated at 37°C, 5% CO_2 overnight. The next day, the solution being titered for virus concentration was diluted as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Then, the plate media was removed, each well was washed in 4 mL of 1X PBS and let stand in 1 mL of 1X PBS. This PBS was removed and 400 μL of one of the dilutions was added to one of the wells. After all six wells have had 400 μL one of the dilutions added, the plate was rocked to cover the well and incubated at 37°C, 5% CO_2 for 1 hour being rocked every 15 minutes to cover the wells. After this 1 hour for infection, the wells were filled with 4 mL of 0.6% Agar, 1X DMEM with 2.5% FBS and 1X Penicillin/Streptomycin (P/S). Then the plate was incubated at 37°C, 5% CO_2 for 48 hrs. Then, the plate was stained by first adding 2 mL of (3:1) Methanol: Acetic Acid (fixing solution) to each well and the plate was set at room temperature for 30 minutes. After fixing, the fixing solution was poured off and the agar plugs were removed from each well and 1 mL of 0.25% crystal violet was added to each well and the plate

remained at room temperature for 1 hour. After staining, the crystal violet was removed from each well to a waste container and the plate was washed with Reverse Osmosis (RO) H_2O 7 times. Then the plate was dried-down on a paper towel on the bench top at room temperature overnight. After the plate was fully dried it was looked at and anywhere on the plate's well that isn't colored purple will be counted as one plaque. The concentration of the virus equals (# of plaques in well/ solution dilution for that well)/.4 mL = virus titer (pfu/mL).

Tissue Homogenization: Tissues harvested from sacrificed mice were divided in half and one half and added into a 2mL sterile, screw-cap tube with half of the tube filled with sterile silica zircondium beads. Until homogenization takes place, the samples were stored in the -80^{o}C freezer. For homogenization, 1mL of sterile 1xDMEM was added to the tube and homogenization was carried out on a bead-beater homogenizer. The tube filled with beads, tissue, and 1xDMEM was homogenized for 2 min total time at homogenization intensity in sets of 30 seconds homogenization, followed by 1 minute incubation on ice.

RNA Isolation: 250µL of the homogenized tissue(s) were transferred to a 1.5 mL or 2 mL snap cap tube.

750µL of Trizol LS from Invitrogen were added and the solution(s) was mixed well.

The mixed solution(s) remained at room temperature for 5 minutes.

200mL of Chloroform were added and the solution(s) was shaked for 15 seconds.

The mixed solution(s) remained at room temperature for 15 minutes.

The tube(s) were centrifuged at 12,000 x g for 15 minutes.

New snap cap tubes were prepared for each sample and 500µL of isopropanol was added to them.

After the tube(s) has spun-down, 500µL of the aqueous layer in the tube was transferred to the new tube(s) with the isopropanol and mixed by inversion at least 10 times.

Tube(s) were incubated at -20^{o}C for 1 hour.

Tube(s) were centrifuged at 13.2kRPM for 12 minutes.

They were vacuum aspirated to remove the isopropanol.

1mL of 75% (v/v) ethanol in DEPC H_2O was added to wash the pellet in the tube(s).

Tube(s) were centrifuged at 13.2k RPM for 5 minutes.

Tube(s) were vacuum aspirated.

Tube(s) were air-dried on the bench top for 30 minutes.

Dried RNA pellet(s) were resuspended by adding 20µL of DEPC H_2O to the tube(s).

Tube(s) will be incubated at 56^{o}C for 10 minutes.

The RNA solution(s) will be stored at -80^{o}C.

RT-PCR: RT-PCR is the coupling of cDNA preparation and PCR protocols using the RNA isolated from the mouse tissue homogenates.

cDNA preparation: Solution of RNA isolated from the harvested tissue were examined spectroscopically for absorbance of 260 nm light and quantitated using the extinction coefficient 40 µg/mL/OD. 1µg-100ng of the RNA was combined with 0.5µL of 50µM solution of one of two primers used for PCR, 1µL of 10mM dNTP's, and DEPC H_2O for a total volume of 13µL. Another 13µL mix was set up with the other primer to get cDNA of both the sense and the antisense RNA. These mixes were incubated at 70^{o}C for 5 minutes, followed by incubation on ice for 2 minutes.

Then, 5µL of 5X First Strand Buffer from the Invitrogen Super Script RT reverse transcriptase kit, 1µL of 0.1M DTT, 1µL of RNase Out (40 U/µL) from Invitrogen, and 1µL of Super Script RT (200 U/µL) was added to each 13µL premix that had been made and then heated and cooled. The full 20µL reaction mix was incubated at 52°C for 1 hour. Then the reaction was heat-inactivated by incubation at 70°C for 15 minutes.

After the reaction mixture is allowed to cool to 4°C, 1µL of RNase H (4 U/µL) from Invitrogen was added and incubated at 37°C for 20 minutes. Then, the reaction was heat-inactivated by incubation at 65°C for 20 minutes. The finished cDNA reaction mixture with the cDNA was stored at -20°C.

PCR: The basic formulas for PCR reaction were 100ng of template DNA mixed with 1X PCR Buffer –Mg (Invitrogen), $MgCl_2$, dNTP's, Forward Primer, Reverse Primer, and Platinum Taq polymerase (Invitrogen). For PCR from cDNA, 5 µL of the cDNA reaction was mixed with the other PCR components in place of the 100ng of template DNA.

The melting temperatures (MT) for the primers were determined using the formula: MT= 100.5 + (0.41*(#GC nucleotides/total # nucleotides)) – (820/total # nucleotides) + 16.6*(log(monovalent cation)). Then the thermocycling will be set as follows:

95° for 5 minutes

- 95°C for 30 seconds
- MT-5°C for 30 seconds
- 72°C for 1 minute (for each 1,000 base pairs of product)

} 30-40 times

72°C for 5 minutes

4°C hold.

Ideally, Forward and Reverse primers have MT's within 5°C of each other. If not the annealing temperature (second step of the PCR cycle) that were used will be 2°C below the lowest MT of the two primers.

RESULTS

The eGFP insert was lost from the viral genome during continuous passage. Although a loss of eGFP expression indicates a deletion event within the insert most probably had taken place, we tested whether this was due to the complete or partial loss of the foreign insert. By RT-PCR analysis, a deleted variant having a similar size PCR product as the parental virus (pMKS1) had overtaken the recombinant viral population with each new passage. Paralleling the results determined by the eGFP plaque assay count, RT-PCR results demonstrated preferential selection of the deleted genotype by passage six.

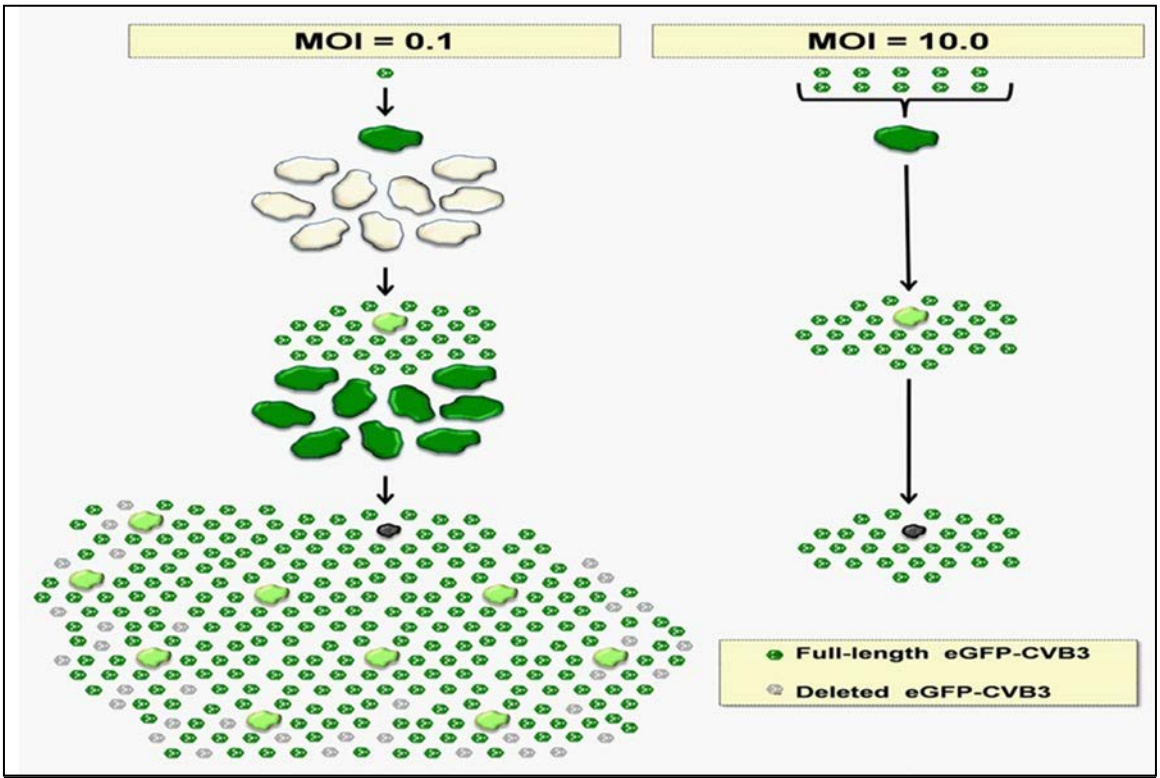


Figure 2. Interpretation of Observed In Vitro Infection – Different Multiplicities of Infection.

Sequence analysis of the deleted genotype revealed that the entire eGFP open reading frame and an additional three amino acids found in the parental pMKS1 vector

were absent (***Figure 3***). Ten additional engineered codons present in pMKS1 (although not found in wild type CVB3) remained intact. Also, the entire open reading frame remained in-frame, suggesting that the deleted CVB3 genotype remained viable. These results were consistent between individual cultures, suggesting that the loss of the eGFP insert occurs in a highly reproducible and predictable manner, and that the new deleted CVB3 genotype may quickly outcompete recombinant eGFP-CVB3.

Viral RNA from 3 month post infected heart tissue was obtained and sequenced to discover that the eGFP gene was also deleted *in vivo* in an identical fashion (data not shown). Also, the eGFP insert has been observed to be lost from the recombinant virus within 45 days post-infection in the CNS (data not shown). We hypothesize that differential viral replication in both target organs contributes to the length of time that the recombinant virus remains intact.

Figure 3. Alignment of wild type, recombinant and deleted recombinant viruses

Wild type CVB3 exhibited a higher replicative fitness as compared to recombinant virus. We hypothesized that these deleted viral genotypes may act similar to wild type (wt) virus in terms of the viral fitness rates. Potentially, wt virus, or deleted genotypes generated *in vivo*, may outcompete recombinant virus due to their faster growth kinetics in the absence of the artificial insert. Therefore, we tested the ability of wtCVB3 to outcompete recombinant eGFP-CVB3 in a standard fitness assay. Both populations of virus were mixed together equally (1:1) or at a ratio of 99:1 (eGFP-CVB3:wtCVB3) and

the mixture was used to infect HeLa RW cells (MOI = 0.1). Infection of HeLa RW cells was done in triplicate for each mixture. Supernatants were quantified for virus by plaque assay and analyzed for eGFP expression. Samples were passaged continuously until either wild type or recombinant virus had gained dominance in tissue culture (***Figure 4***).

An equal mixture of wild type virus with recombinant virus led to the prevalence of wtCVB3 within the first passage, thereby demonstrating the higher fitness rate of wtCVB3, as compared to recombinant virus. Skewing the ratio of recombinant virus to wtCVB3 a hundred-fold (99:1 mixture) temporarily kept recombinant virus levels high; however by passage 3, greater than 99% of isolated virus was wt CVB3 (no eGFP expression).

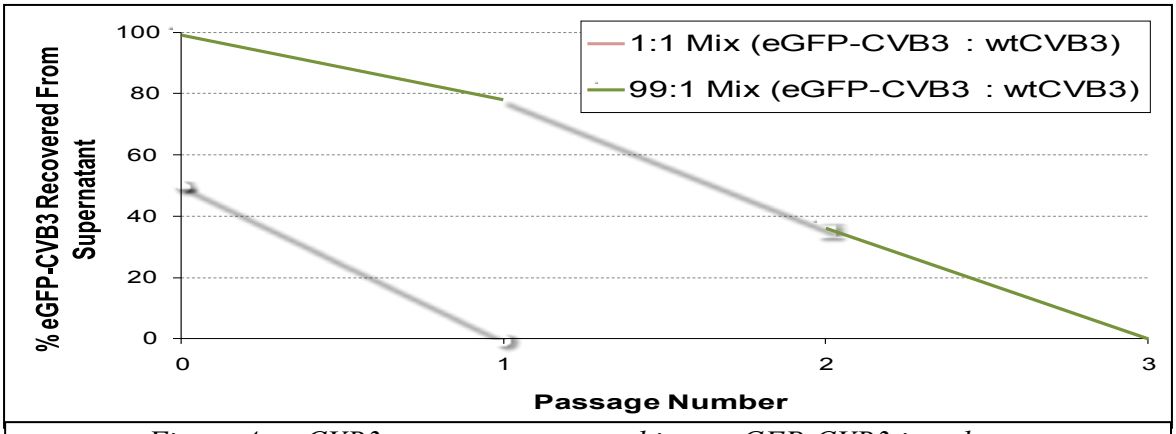


Figure 4. wtCVB3 outcompetes recombinant eGFP-CVB3 in culture.

Total RNA from these cultures was isolated and the appearance or disappearance of either wt or recombinant virus was analyzed by RT-PCR. wtCVB3 predominated quickly in both initial mixtures, as determined by RT-PCR and agarose gel electrophoresis (data not shown). These results indicate the inherent competitive advantage of smaller viruses by genome size (wtCVB3 and deleted recombinant CVB3) over the full-length

recombinant virus having a relatively large insert which would be expected to slow down viral replication kinetics.

Determining the intrinsic in vivo decay rate of full-length eGFP-CVB3 in two distinct models of viral persistence utilizing both BALB/c and B cell knockout (BcKO) mice. We utilized two contrasting models in order to more carefully characterize the persistent phase of coxsackievirus infection in the heart and CNS. Infection of BALB/c mice with wtCVB3 leads to acute infection, followed by viral RNA persistence in the absence of detectable levels of infectious virus [2] (***Figure 5***). This model of virus persistence is thought to parallel the condition in

humans whereby viral RNA can be detected in the heart tissue of patients suffering from viral myocarditis, yet may restricted at the level of RNA synthesis [3].

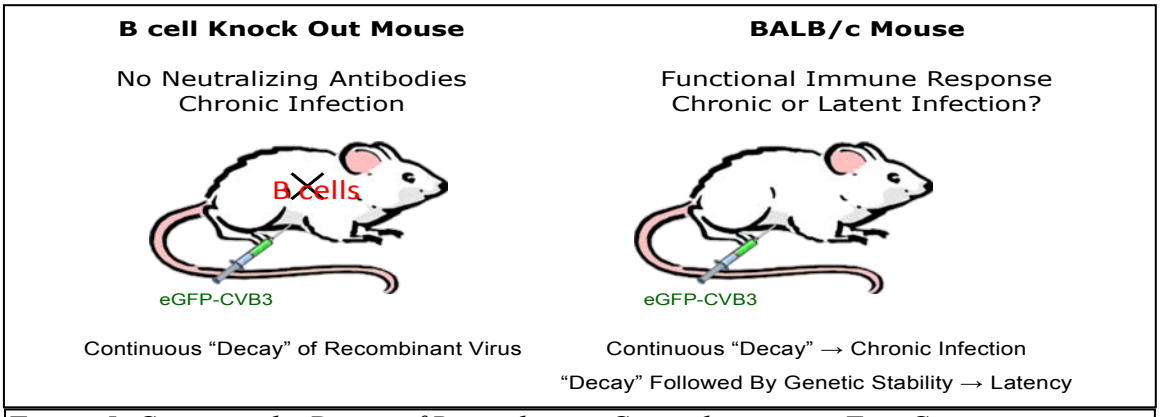


Figure 5. Compare the Decay of Recombinant Coxsackievirus in Two Contrasting Models of Persistent Infection

In contrast, infection of BcKO mice leads to a chronic infection presumably due to the lack of a neutralizing antibody response, and infectious virus is detected months after initial infection [4]. Therefore, BcKO mouse model would be expected to provide an informative control for continuous viral replication inherent during chronic infection. We

will test both mouse models for their ability to retain the eGFP insert over the course of acute infection, and into the persistence phase of infection.

These two models differ in that CVB3 undergoes continuous replication within BcKO mice, yet may persist in BALB/c mice through an undetermined mechanism. Potentially, viral RNA persistence in BALB/c mice may represent a latent-type infection (***Figure 6***; green line with squares), although viral genomic RNA is typically thought to be extremely labile in the absence of ongoing synthesis. Alternatively, a low level of viral replication (yet at below detection limits) may be occurring (***Figure 6***; blue line with diamonds). A third possibility is that sporadic reactivation during latency might occur (***Figure 6***; red line with triangles). Discriminating between these three possibilities may be extremely relevant in understanding disease progression associated with CVB3 persistence.

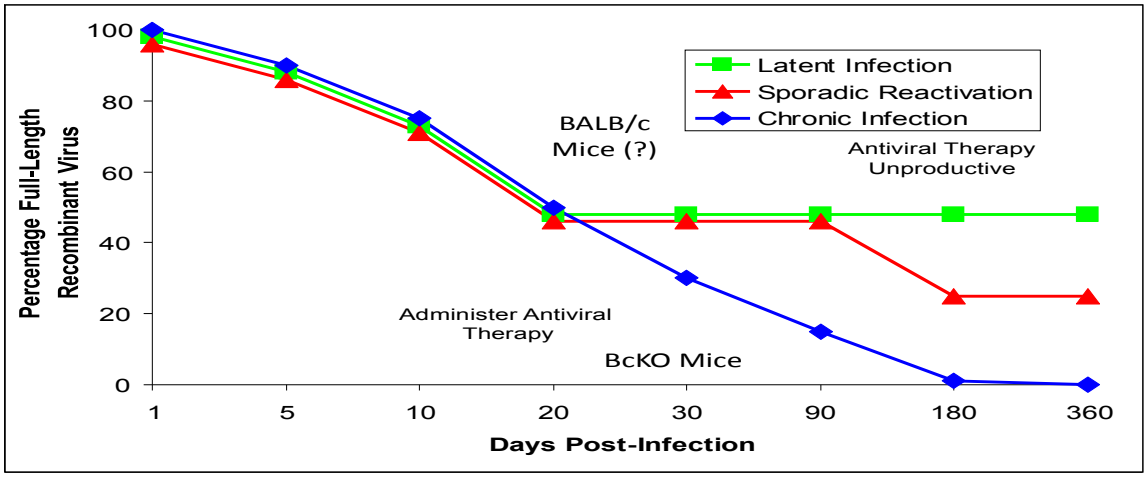


Figure 6. Predictions for the Decay of Recombinant Coxsackievirus in Two Contrasting Models of Persistent Infection

Also, further clarification of the nature of CVB3 persistence may be important for predicting the success antiviral drugs, such as ribavirin, to treat individuals long after acute infection has ended. The ability of persistent CVB3 to increase replication rates or

possibly become reactivated in response to a certain stimuli has clear importance in a clinical setting. We hypothesized that a portion of full-length recombinant CVB3 may be retained in tissues supporting persistent infection even after longer time periods (30 days), perhaps suggesting a latent-type infection.

Infectious virus was cleared from BALB/c mice but persists in BcKO mice. First, we evaluated the amount of infectious virus in the pancreas and heart of BALB/c mice following eGFP-CVB3 inoculation (intra-peritoneally – IP) (***Figure 7***).

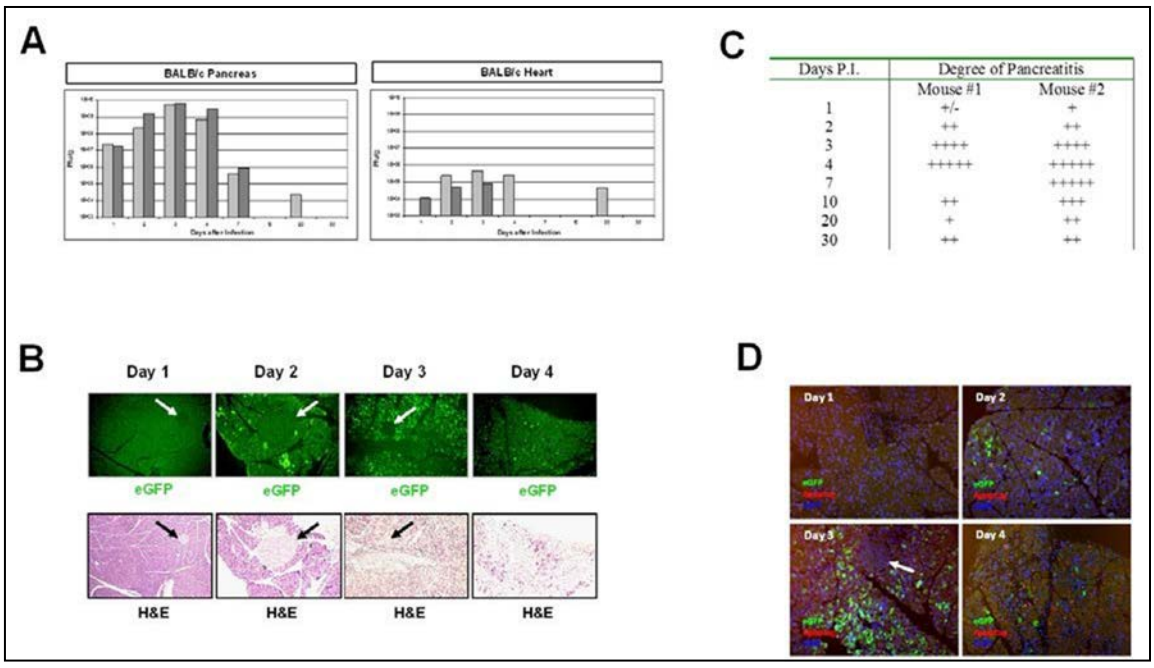


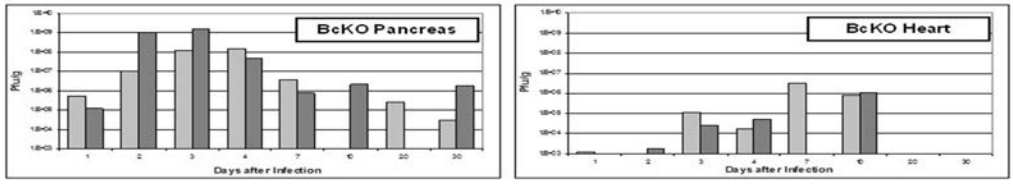
Figure 7. Viral titers and pathology in the pancreas of BALB/c mice following infection with eGFP-CVB3

Relatively high levels of infectious virus was observed in the pancreas; however, by day 10 PI, little to no infectious virus could be detected in either the pancreas or heart (***Figure 7A***). High levels of virus protein expression (eGFP) was seen in the acinar cells of the pancreas (***Figure 7B***). In contrast, no viral protein expression was detected in the islet cells (***Figure 7B***; white arrows).

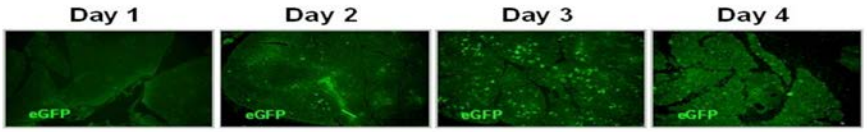
By day 3 PI, pancreatitis was observed by H&E staining. The degree of pancreatitis was quantified over time. Interestingly, low level pancreatitis could be observed up to day 30 PI (***Figure 7C***). Colocalization of virus protein expression and ApopTag staining in the pancreas suggested that viral infection induced apoptosis in the acinar cells which may contribute to the observed pancreatitis (***Figure 7D***).

The amount of infectious virus was also quantified in the pancreas and heart of BcKO mice (***Figure 8***). The relatively levels of infectious virus in BcKO mice was considerably lower at the peak time point (day PI) of pancreas infection compared to BALB/c mice (***Figure 8.A***), suggesting differential viral replication kinetics between the two mouse models of infection. However, viral replication in the pancreas continued up to day 30 PI. Viral protein expression could be seen in the pancreas of BcKO mice, however these levels were reduced compared to BALB/c mice (***Figure 8.B***). Also, a high level of pancreatitis in BcKO mice was identified for up to day 30 PI (***Figure 8.C***).

A



B

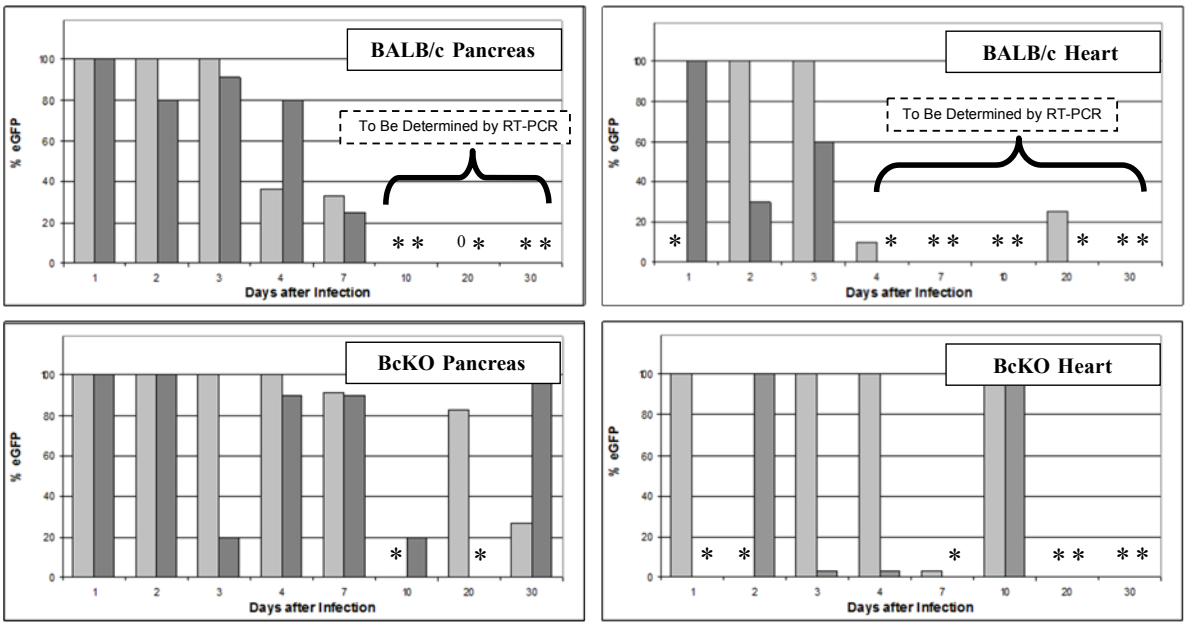


C

Days P.I.	BcKO	
	Mouse #1	Mouse #2
1	+/-	++
2	++	+++
3	++++	++++
4	+++++	+++++
7		+
10	+++++	++++
20	+++	+++
30	+++++	

Figure 8. Viral titers and pathology in the pancreas of BcKO mice following infection with eGFP-CVB3

BALB/c and BcKO mice over time. This was done by either counting the percentage of $eGFP^+$ viral plaques by fluorescence microscopy, or in the case of samples positive for viral RNA - utilizing RT-PCR analysis. A step-wise loss of eGFP expression was observed in the pancreas and heart tissues of BALB/c mice over time (**Figure 9**). By day 7, nearly 20% of viral plaques isolated from pancreatic tissue failed to express eGFP.



0 – No eGFP-expressing plaques

* – No infectious virus detected

Figure 9. Loss of the eGFP insert over time based on the percentage of eGFP expressing plaques.

In the heart of BALB/c mice, the loss of the eGFP insert appeared accelerated, perhaps a reflection of the heart being considered a secondary site of infection. RT-PCR analysis of viral RNA from harvested tissues utilizing primers flanking the insertion site revealed the complete loss of the eGFP insert by day 7 in BALB/c mice (data not shown).

In BcKO mice where viral growth kinetics was reduced, a delayed loss of eGFP expression was determined by plaque assay although a considerable degree of variability was observed. We hypothesize that the variability in the retention of the eGFP insert in BcKO mice may be partly due to the lack of B cells involved in initial viral replication and dissemination of virus within the host. Hence, the accelerated eGFP "decay rate" in BALB/c mice, as opposed to BcKO mice, may reflect the need for CVB3 to target B cells for efficient virus spread within the host during acute infection.

Given the chronic nature of CVB3 infection in BcKO mice, one might predict a complete loss of the eGFP insert if extended time points (day 90 PI) were inspected, although the day 90 time point was not analyzed for this experiment. Based on our results observed during passage of eGFP-CVB3 in HeLa RW cells, these results support the conclusion that the presence of the eGFP insert over time may be inversely proportional to the amount of replication the virus undergoes within the host.

DISCUSSION

Utilizing the inherent instability of our recombinant coxsackievirus expressing the enhanced green fluorescent protein (eGFP-CVB3) to determine whether virus persists in the heart through chronic/sporadic viral replication, or alternatively through true viral latency. By recognizing and calculating the inherent stability of our recombinant eGFP-CVB3, we use this internal "decay rate" as a molecular time clock to estimate CVB3 replication rates during persistent infection in the heart and CNS. In addition, we will sequence flanking regions of deleted viral variants to determine intrinsic viral genomic instability and improve viral vector stability.

Based on our preliminary data described in **Figure 10**, the low initial inoculum of recombinant virus used to infect mice (10^5 pfu IP) may have contributed to the accelerated loss of the eGFP insert in BALB/c mice **Figure 10**; purple line represented by 10^5 pfu.

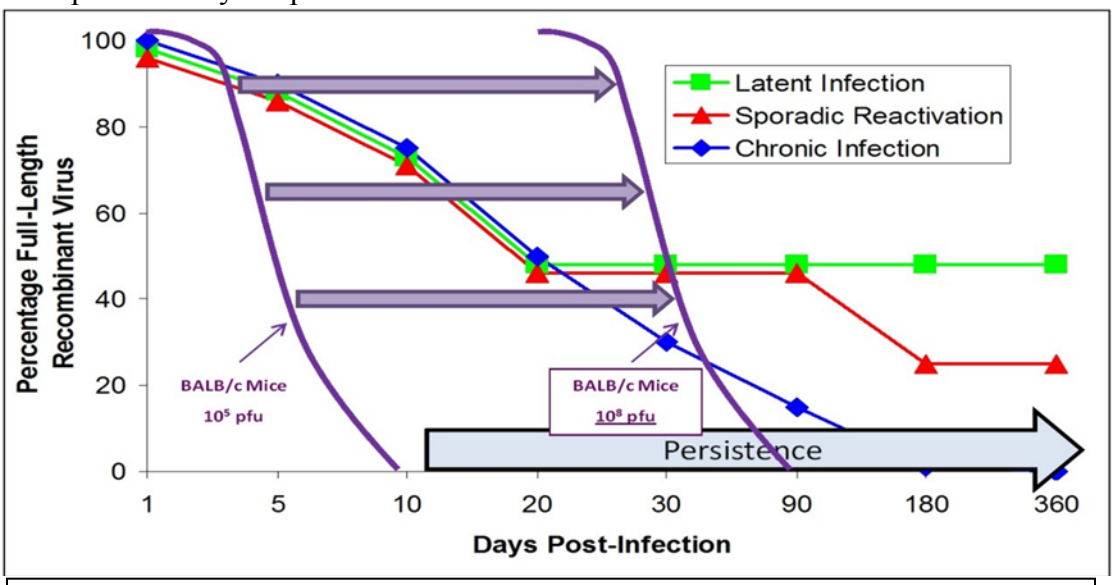


Figure 10. Resetting the molecular time clock baseline in vivo with higher initial inoculum of eGFP-CVB3.

The *in-vivo* decay rate of eGFP-CVB3 for low dose infection showed complete deletion of the recombinant virus prior to the persistent phase of infection. Therefore, we could not determine the internal decay rate of eGFP-CVB3 after day 7 in the host. We hypothesized that mice infected with an initial high inoculum ($1x10^8$ pfu IP) may harbor full-length recombinant virus at a later time point when virus persistence is established. Following the decay rate in these mice might reveal the replication kinetics of the virus based on the ongoing

decay rate **Figure 10.**; purple line represented by 10^8 pfu.

BALB/c, C57BL/6 and BcKO mice were infected with a high inoculum of recombinant virus (108 pfu IP; n=4 for each mouse strain and time point), and the pancreas and hearts were inspected for full-length or deleted forms of the virus at day 30 and 90 PI (***Figure 11***). Intriguingly, virus persistence was observed in the heart as well as the pancreas of BALB/c and C57 BL/6 mice. This observation is relevant for a separate study where we hope to determine if virus replication can be increased in response to food intake. In mice given a high inoculum, recombinant virus isolated from BALB/c and C57 BL/6 mice retained the eGFP insert at day 30 PI as determined by RT-PCR. In contrast, recombinant virus in BcKO mice had lost the eGFP insert by day 30 PI, reflecting the chronic pattern of infection in these mice. However at 90 days PI, both the BALB/c and C57 BL/6 mice showed complete deletions in isolated recombinant virus as determined by RT-PCR. Therefore, the virus is presumed to have undergone replication in these immunocompetent mice at some point during persistent infection, even though no infectious virus could be detected at either the day 30 or day 90 time point.

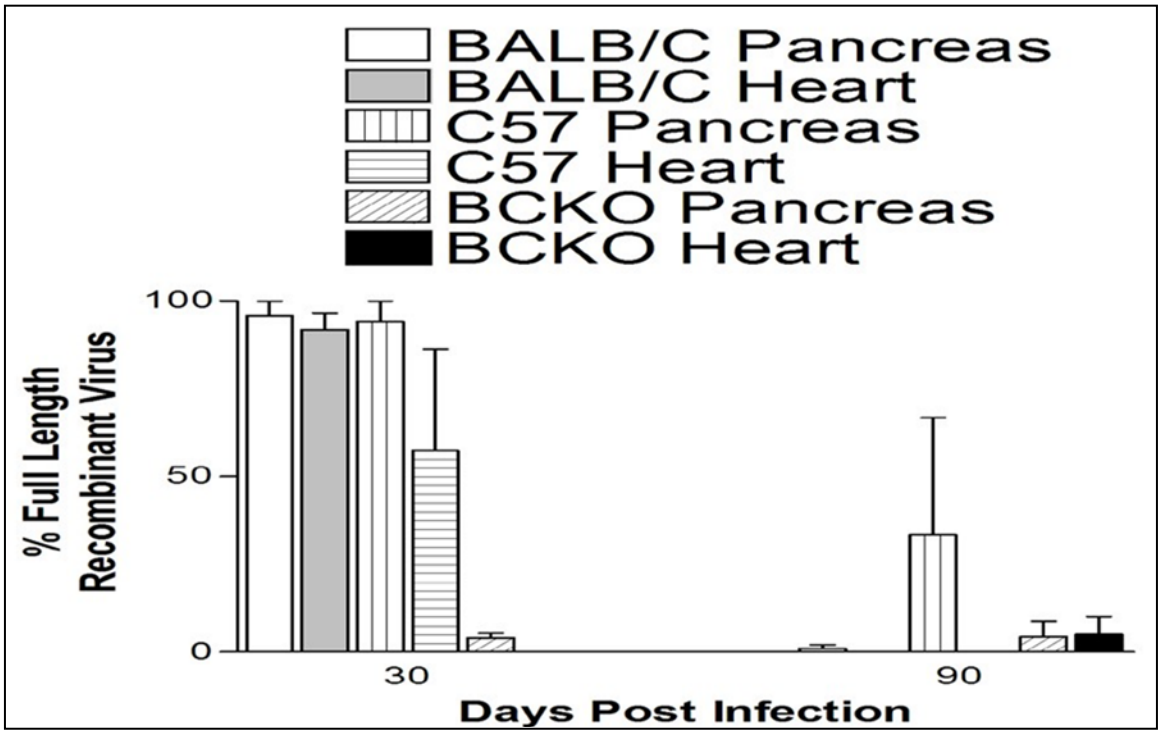


Figure 11. Mice infected with high Inoculum: Continued decay of recombinant coxsackievirus after day 30 post-infection.

Also, we will carry out parallel studies in our pediatric model of CNS infection in mice inoculated with eGFP-CVB3 (intra-cranially – IC) to determine the mechanisms of viral persistence in the brain. Our continued molecular analysis of deleted eGFP-CVB3 variant kinetics will assist in evaluating the virus replication rates specifically within the CNS as well as the heart. We hypothesize that the loss of full-length recombinant virus during persisting in the CNS may indicate ongoing viral replication in this model, as well. We will infect BALB/c mice and follow the establishment of persistent infection. After days 1, 5, 10, 30 and 90, we will purify the viral RNA from persistently-infected CNS tissue and determine the presence or absence of the eGFP insert. Alternatively if the eGFP insert has been deleted over time, the result may reflect active viral replication in the CNS. The results would be useful because it would provide more information on how

CVB3 behaves during persistent infection in the CNS. For example, ribavirin is a commonly used antiviral drug which inhibits the ability of the nucleic acid polymerase to replicate the viral genetic information. Since this drug only functions during viral replication, it wouldn't be useful to stop a latent virus. These studies may eventually help in devising relevant antiviral strategies for patients, particularly during the persistent phase of CVB3 infection in the heart and CNS.

As shown in **Figure 10**, the experiments done using low inoculum of eGFP-CVB3 do not allow for the determination of whether CVB3 replicates during persistent infection. The results show that the eGFP insert is mostly lost from recombinant virus by day 7 PI. Low inoculum infection cannot be used to determine if CVB3 undergoes replication during persistent infection. High inoculum infection was used instead to determine if eGFP-CVB3 retains the eGFP insert during viral persistence. We have chosen two points in time (day 30 and day 90) in mice infected with a high inoculum whereby replication or latency can be determined. However, quantifying the rate of decay has been difficult due to the stochastic nature of recombinant virus deletions in vivo. The stochafstic nature is reflected in the relative variability of eGFP loss in BcKO mice over time (**Figure 11**), as compared to similar experiments done in HeLa cells (***Figure 1***). Nonetheless, our recent results provide clear evidence that CVB3 undergoes extensive viral replication during persistent infection, as shown in ***Figure 11***.

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CHAPTER II:

Monitoring Virus-Induced Intracellular Membrane Remodeling and Virus Dissemination in Real Time Utilizing A Novel Recombinant Coxsackievirus B3 Expressing "Fluorescent Timer" Protein

TITLE PAGE

Monitoring Virus-Induced Intracellular Membrane Remodeling and Virus Dissemination in Real Time Utilizing A Novel Recombinant Coxsackievirus B3 Expressing "Fluorescent Timer" Protein

Running Title: Monitoring Virus Spread with Timer-CVB3

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ABSTRACT

Coxsackievirus B3 (CVB3), a member of the picornavirus family and enterovirus genus, causes viral myocarditis, aseptic meningitis, and pancreatitis in humans. We genetically engineered a unique molecular marker, "fluorescent timer" protein, within our infectious CVB3 clone and isolated a high-titer recombinant viral stock (Time1r-CVB3) following transfection in HeLa cells. "Fluorescent timer" protein undergoes slow conversion of fluorescence from green to red over time, and Timer-CVB3 can be utilized to track infection and cell-to-cell spread in real time. Timer-CVB3-infected HeLa cells, neural progenitor and stem cells (NPSCs), or C2C12 myoblast cells slowly changed fluorescence from green to red over 72 hours, as determined by fluorescence microscopy or flow cytometric analysis. The slow change in fluorescence for Timer-CVB3-infected HeLa cells could be interrupted by fixation, suggesting that the fluorophore associated with "fluorescent timer" protein was stabilized by formalin cross-linking reactions. Induction of a type I interferon response reduced the progression of cell-to-cell infection in both HeLa cells and NPSCs. Time lapse photography of partially differentiated NPSCs infected with Timer-CVB3 revealed substantial intracellular membrane remodeling and the assembly of discreet virus replication organelles which changed fluorescence color in an asynchronous fashion within the cell. Unexpectedly, Timer-CVB3 infection of partially differentiated NPSCs or C2C12 myoblast cells induced the release of numerous extracellular microvesicles containing viral material which may represent a novel route of dissemination.

Through the use of this novel recombinant virus, we hope to gain a better understanding of CVB3 tropism, alterations in host cell organization, and virus dissemination within the host.

AUTHOR SUMMARY

Enteroviruses are significant human pathogens, causing aseptic meningitis and encephalitis. The mechanisms of enterovirus dissemination in the host and cell-to-cell spread may be critical factors influencing viral pathogenesis. Here, we have generated an informative recombinant coxsackievirus expressing "fluorescence timer" protein (Timer-CVB3) which assists in following the progression of infection in the host. Unexpectedly, we observed the shedding of microvesicles containing viral material in partially-differentiated progenitor cells infected with Timer-CVB3. These extracellular microvesicles may be released in high levels following cellular differentiation, and may play a role in virus dissemination. Timer-CVB3 will be a valuable tool in monitoring virus spread in the infected host.

INTRODUCTION

Enteroviruses (EV) are among the most common and medically-important human pathogens, and a frequent cause of central nervous system (CNS) disease [1]. Worldwide distribution of EV infection is revealed by the detection of EV-specific antibodies in the sera of approximately 75% of individuals within developed countries. For example, in 1996, approximately 10-15 million diagnosed cases of EV infection occurred in the US alone [2].

Coxsackieviruses (CV), members of the enterovirus genus, are significant human pathogens, and the neonatal CNS and heart are major targets for infection. CV infection may cause severe morbidity and mortality, particularly in the very young. CV infection during pregnancy has been linked to an increase in spontaneous abortions, fetal

myocarditis [3], and neurodevelopmental delays in the newborn [4]. Infants infected with CV have been shown to be extremely susceptible to myocarditis, meningitis and encephalitis with a subsequent mortality rate as high as 10%. Adult infection and subsequent viral myocarditis has also been described, and a substantial proportion of patients suffering from chronic viral myocarditis may eventually develop dilated cardiomyopathy, a condition underlying almost half of all heart transplants. Severe demyelinating diseases may occur following infection, including acute disseminated encephalomyelitis [5] and acute transverse myelitis [6]. For example, a recent report describes the case of a 19 year old nursing student who experienced cognitive problems and lapsed into a coma. By immunohistochemistry utilizing biopsied tissue, she was found to suffer from viral encephalitis and persistent coxsackie B virus infection of the CNS. Also, a number of delayed neuropathologies have been associated with previous CV infection, including schizophrenia [7] [8], encephalitis lethargica [9], and amyotrophic lateral sclerosis [10] [11]. Previously, we have shown that CVB3 preferentially targets neural progenitor and stem cells (NPSCs) [12]. Lasting consequences may be observed in the CNS [13], and NPSCs may represent a site of virus persistence in surviving mice infected shortly after birth [14] [15].

We wished to more carefully observe cell-to-cell infection in the context of an ongoing Type I interferon response in order to visualize the dynamics of virus dissemination simultaneously with counteracting and protective antiviral responses generated in neighboring cells. Also, virus dissemination within the host may be an important consideration in predicting eventual pathogenesis in the host. Identifying the sequence of infection upon initial virus exposure may be challenging although critical in

preventing the natural disease course. For example, CVB3 has been shown to target the pancreas at early time points following infection. In addition to initiating acute pancreatitis, early virus replication in the pancreas may shed additional virions which may disseminate in the host eventually reaching the heart or CNS. Organ-specific expression of interferon-□ within the pancreas was previously shown to reduce initial CVB3 replication and limit acute myocarditis in the host [16]. Therefore, hindering the normal progression of virus dissemination by targeting key regions may be protective in the host.

In order to track virus dissemination both in cultured cells and *in vivo*, we generated a recombinant CVB3 expressing "fluorescent timer" protein (Timer-CVB3). "Fluorescent timer" protein encodes a mutant form of the red fluorescent protein, drFP584 (V105A, S197T) [17]. drFP584 protein has been shown to fluoresce green immediately following translation. However one to five hours after synthesis, the green flourophore is modified to fluoresce red. The engineered mutations in "fluorescent timer" protein resulted in this autocatalysis process being substantially delayed and fluoresces green for a greater period of time following translation. Over time, "fluorescent timer" protein is modified to fluoresce red. This progressive time course of conversion from green to red fluorescence may assist in determining the sequence of infection and virus spread in real time within the host. Through the use of this novel recombinant virus, we hope to gain a better understanding of CVB3 tropism, spread, and pathogenesis in our animal model of infection.

MATERIALS & METHODS

Ethics Statement. This study was carried out in strict accordance with the requirements pertaining to animal subjects protections within the Public Health Service Policy and USDA Animal Welfare Regulations. All experimental procedures with mice were approved by the San Diego State University Institutional Animal Care and Use Committee (Animal Protocol Form #10-05-013F), and all efforts were made to minimize suffering.

Generation of Timer-CVB3 Stock: The Timer gene was amplified with primers containing Sfi1 restriction site sequences. Following amplification, the PCR product was cut with Sfi1 and cloned into our parental CVB3 vector (pMKS1) linearized with Sfi1.The Timer-CVB3 plasmid contains the CMV promoter followed by the complete genome of CVB3. This plasmid was precipitated by ethanol incubation at -20^{o}C for 12 hours. After centrifugation and washing in 70% ethanol, the DNA pellet was air-dried at room temperature for 20 minutes. The pellet was then resuspended in sterile, nano-filtered water at a concentration of 1 microgram/ microliter.

HeLa cells were transfected with 2.8 microgram of the sterile Timer-CVB3 plasmid using Lipofectamine 2000 (Invitrogen). Following transfection of Timer-CVB3 plasmid in HeLa cells, infectious virus was produced, isolated and quantified by plaque assay. After transfection, the transfected cells were incubated at 37^{o}C, 5% CO_2 for 3 hours. The transfection solution was removed and the wells of the plate were washed in 3 mL of 1xDMEM with 10% FBS. Wash was removed and 2 mL of 1X DMEM with 10% FBS was added and the transfected cells were incubated at 37^{o}C, 5% CO_2 for 1day.

After 1 day incubation, another 2 mL of 1X DMEM with 10% FBS was added to each well of transfected cells on the plate. The plate was incubated at 37°C, 5% CO_2 for 2 days.

Transfected cells were observed under fluorescence microscope until the cells were observed to first fluoresce green followed by fluorescing red. Supernatants were transferred to a 50 mL conical, screw-cap tube, and 2 mL of 1X DMEM was pipetted on top of the cells in the now empty tissue-culture plate. The 50 mL screw-cap tube was centrifuged at 2000 RPM for 2 minutes. The supernatant was transferred to a new 50 mL conical screw-cap tube. The wells with the 1X DMEM in them had the cells still attached to the surface removed by cell-scraping. This cell solution was transferred to the pellet in the first 50 mL conical, screw-cap tube, and the pellet was resuspended in the solution. The cells in the cell solution were then lysed by freeze thaw 3 times: 5 minutes of freezing in 95% ethanol with dry-ice, 30 minutes in ice water, and 5 minutes at room temperature with rocking. The freeze-thawed solution was then centrifuged at 2000 RPM for 2 minutes. The supernatant from this tube was transferred to the original supernatant from the first centrifugation. This solution was then vacuum-filtered through a filter with 0.2 μm pore. This virus solution was labeled passage 1 and used to infect HeLa cells in a 150 mL flask.

The 80% HeLa cell monolayer inside the flask was washed two times in 10 mL of 1X PBS. Then, 8 mL of Timer-CVB3 (Passage 1) was added to HeLa cells in the 150 mL flask. The flask was rocked every 15 minutes to fully cover the cells and incubated at 37°C, 5% CO_2 for 1 hour. Then, 18 mL of 1X DMEM with 10% FBS was added and the flask was incubated at 37°C, 5% CO_2 for 48 hours. The supernatant and cells were

separated and cells scrapped into 10 mL of 1X DMEM were lysed by freeze thaw as previously stated to get the virus solution, labeled passage 2. This virus solution was titrated by plaque assay using HeLa cells at 37^{o}C, 5% CO_2 for 48 hours and stored at -80^{o}C.

Plaque Assay: A solution of HeLa cells was counted to determine its concentration of cells/mL in 1X DMEM with 10% FBS. The solution was diluted to a concentration of $1x10^5$ cells/mL. This cell solution was used to pour six well plates with 3 mL of the cell solution in each of the wells. The plate will be incubated at 37^{o}C, 5% CO_2 overnight. The next day, serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}were made. Then, the plate media was removed, each well was washed in 4 mL of 1X PBS. 400 µL of each dilution was added to one of the wells. After all six wells have had 400 µL one of the dilutions added, the plate was rocked to cover the well and it will be incubated at 37^{o}C, 5% CO_2 for 1 hour being rocked every 15 minutes to cover the wells. After 1 hour for infection, the wells were filled with 4 mL of 0.6% Agar, 1X DMEM with 2.5% FBS and 1X Penicillin/Streptomycin (P/S). Then the plate was incubated at 37^{o}C, 5% CO_2 for 48 hrs, followed by staining by first adding 2 mL of (3:1) Methanol: Acetic Acid (fixing solution) to each well. The plate was set at room temperature for 30 minutes. After fixing, the solution was poured off and the agar plugs were removed from each well. 1 mL of 0.25% crystal violet was added to each well and the plate was incubated at room temperature for 1 hour. After staining, the crystal violet was removed from each well to a waste container and the plate was washed with water 7 times. The plate was dried-down on a paper towel on the bench top at room temperature overnight. After each plate was

fully dried, plaques were counted. The concentration of the virus equals (# of plaques in well/ solution dilution for that well)/.4 mL = virus titer (pfu/mL).

Flow Cytometry: Hela RW cultures were plated in a 6-well plate at a concentration of 10^5 cells/ml and allowed to adhere to the plate overnight. Cells were mock-infected, infected with eGFP-CVB3 or dsRED-CVB3 at an MOI of 0.1. Alternatively, cells were infected with Timer-CVB3 at an MOI of 0.01 or 0.1. After 12, 24, 36, 48, or 72 hours PI, cells were fixed in 4% para-formaldehyde and washed three times in PBS. Cells were analyzed using a BD FACSAria located in the SDSU Flow Cytometry Core Facility.

***Isolation, culture, and infection of neurospheres*:** Neurospheres were isolated and cultured as previously described [12]. Neurospheres were vigorously dissociated and resuspended in complete NPSC culture medium to a concentration of 10^5 cells/mL in a 24 well plate and infected with Timer-CVB3 at an MOI of 0.1. Cultures were inspected daily using a Zeiss Axio Observer D.1 inverted fluorescent microscope. CVB3 carrier cultures derived from frozen stocks were infected in parallel. Parallel infections were also performed on NPSCs continuously grown and passaged in NPSCs media containing 10ug/ml Poly IC.

Alternatively, 10^5 cells/ml of NPSCs were differentiated in a 4-well chamber slide as previously described [12] and in a u-dish (iBidi, Inc., cat#81156). After differentiation for 5 days, the cultures were infected with Timer-CVB3 at an MOI of 0.1. Following infection, differentiated cells in the u-dish were monitored daily for the expression of Timer protein. Once the expression of timer protein was observed, the culture was imaged at 10 minute intervals, holding the field of image, magnification, channel exposure times, and focus consistent. The focus was adjusted occasionally as needed.

***Immunofluorescence microscopy*:** At 3 days post-infection, differentiated cultures in the chamber slides were fixed in 4% para-formaldehyde and washed three times in PBS. Fixed cells were permeabilized with 0.25% TritonX-100 in PBS, washed three times, blocked with 10% normal goat serum (NGS) and immunostained using the following antibodies: Nestin (Covance Inc.; Cat# PRB-315C) at 1:1000, neuronal class III□-tubulin (Covance Inc.; Cat# PRB-435P) at 1:1000, GFAP (Sigma Inc; Cat# G 9269) at 1:1000, MBP (Chemicon Inc.; Cat# AB980) at 1:1000. Primary antibodies were diluted in 2% Normal Goat Serum (NGS) in PBS (150-200 □l per slide) in humidified chamber and incubated overnight. Slides were washed with PBS for 5 min (3X). Secondary antibodies (at 1:1000) conjugated to Alexa-Fluor-647 (Invitrogen; Cat# A21245), were diluted with 2% NGS in PBS (150-200 □l per slide) and incubated overnight. Following incubation with the secondary antibody, slides were washed with PBS for 5 min (3X), and mounted in Vectashield with DAPI. Three to five representative images of the cultures were taken for each sampling time point at multiple magnifications.

***Infection, Imaging, and Image Analysis of HeLa RW cell cultures*:** HeLa RW cultures were plated in a 6-well plate at a concentration of 10^5 cells/ml, treated with either 10ug/ml Poly IC, 50u/ml IFN-□, or media alone and allowed to adhere to the plate overnight. Timer-CVB3 was then added at an MOI of 0.01 or 0.1. Eight images of each culture were taken at 100x total magnification per condition, per day. Samples for viral titering were taken immediately following imaging. For each image, the number of red, yellow, green, or round but colorless cells were counted using the Image J Cell Counter plug-in.

RESULTS

Generation and characterization of a novel recombinant CVB3 expressing Timer protein (Timer-CVB3) in cultured HeLa RW cells. The Timer protein slowly turns from green to red fluorescence allowing for the temporal discrimination of recently-infected and previously-infected cells [18]. As shown in ***Figure*** 1, the gene encoding Timer protein followed by an artificial viral protease cleavage site was inserted into the backbone of our infectious CVB3 plasmid. A high- titered stock of Timer-CVB3 was generated and utilized to infect HeLa RW cells. Following the infection of HeLa RW cells, Timer protein expression was observed by fluorescence microscopy. Similar to previous studies, the transition from green to red fluorescence was observed over the course of 48 hours post-infection (PI). After 20 hours, infected cells expressed high levels of green fluorescent Timer protein. By 43 hours PI, infected cells fluoresced both green and red. After 48 hours, all infected cells fluoresced red. These results demonstrate that Timer-CVB3 can be used as a molecular time clock to mark and follow infected cells over time. Also, cytopathic effects (cpe) were observed in HeLa cells at an early time point (20 hours PI) reflecting the relatively high multiplicity of infection (moi = 10.0) used in the experiment.

HeLa RW cells were infected with Timer-CVB3, dsRED-CVB3, or eGFP-CVB3 and monitored by flow cytometry, a more sensitive and quantitative readout of viral protein expression (***Figure*** 2). As expected, dsRED-CVB3 and eGFP-CVB3-infected HeLa cells expressed high levels of their respective fluorescent reporter proteins by flow cytometric analysis. In contrast, Timer-CVB3-infected HeLa cells slowly changed

fluorescence from green to red over 48 hours PI. Intriguingly, the slow change in fluorescence for Timer- CVB3-infected HeLa RW cells could be arrested in the presence of formalin, suggesting that the tertiary structure of Timer protein can be stabilized by formalin cross-linking reactions. Unexpectedly, an accelerated conversion of Timer protein to red fluorescence was observed in HeLa cells infected with a greater moi (moi = 0.1). Increased reactive oxygen species has been shown to accelerate Timer protein conversion, and the high levels of oxidative stress induced following infection [19] (especially with a greater initial viral inoculum) may influence the conversion rate of Timer protein.

Timer-CVB3 plaques using a standard virus plaque assay were expected to reveal a "bull's-eye" pattern by fluorescence microscopy, whereby initial infection is represented by red fluorescence and newly-infected cells via cell-to-cell spread is represented by green fluorescence. The progression of infection was followed over time in HeLa cells infected with Timer-CVB3 and overlayed with 0.2% agar which hindered virion diffusion in the culture medium. As expected, a change in fluorescence was observed in agar-overlayed HeLa cells at 44 hours PI, although only a portion of the virus plaque could be imaged at low magnification (***Figure 3A***). In contrast, cell-to-cell spread was not observed in HeLa cells infected with Timer-CVB3 (moi = 0.1) and grown in complete media lacking an agar overlay, although individual cells fluoresced green and yellow at 24 hours PI (***Figure 3B*** and ***Figure 3D***). By 48 hours PI, the majority of HeLa cells grown in complete media fluoresced red (***Figure 3C*** and ***Figure 3E***).

Neural progenitor and stem cells (NPSCs) were recently shown to be highly susceptible to CVB3 infection [12] [14] [20]. NPSCs grow in culture as free-floating clusters of stem cells or "neurospheres". We anticipated that the outer shell of stem cells

may first become infected with CVB3, after which infection may extend by cell-to-cell spread. Therefore, NPSCs were infected with Timer-CVB3 (moi = 0.1), and the progression of infection was monitored over time by fluorescence microscopy (***Figure 3F***, ***Figure 3G***, ***Figure 3H***, and ***Figure 3I***). By 32 hours PI, Timer protein expression outlined the progressive infection of outer shell of stem cells (yellow-fluorescing), and inner (green fluorescing) stem cells within the neurosphere (***Figure 3J***, white arrow). By 72 hours PI, red and yellow fluorescing cells were seen and signs of cpe were readily evident. Intriguingly, pretreatment of NPSCs with poly IC, a synthetic analogue of double-stranded RNA known to interact with toll-like receptor 3, reduced the progression of infection as determined by Timer protein expression (***Figure 3K***, ***Figure 3L***, and ***Figure 3M***). These results suggest that NPSCs can respond to immunostimulatory molecules and mount a protective antiviral response following CVB3 infection. Also, no Timer protein signal was observed by fluorescence microscopy for mock-infected NPSCs (***Figure 3N***). Our recent results suggest that CVB3 may establish a carrier-state infection in NPSCs (unpublished data). Carrier state infection may be the result of continuous virus replication, or alternatively, by sporadic reactivation. The utilization of Timer-CVB3 may be useful in distinguishing these two models of carrier-state infection by inspecting Timer protein fluorescence. However, timer protein expression was below detection limits despite the presence of infection virus (as determined by plaque assay) in Timer-CVB3-infected NPSC carrier state cultures (***Figure 3O***).

Host cells infected with RNA viruses induce protective molecules such as MAVS through RIG-I and MDA5 activation which act as pattern recognition receptors. The host cell undergoes an antiviral state and produces Type I interferons such as interferon-

□□□ IFN□□ and IFN□ which act to protect neighboring cells. The Type I IFN response enables neighboring cells to express protective molecules such as RNAse L which limits the spread of viral infection. We inspected the progression of Timer-CVB3 infection in HeLa cells activated by the Type I IFN response to determine the pattern of virus spread within neighboring cells in the context of an active host antiviral response.

Untreated HeLa cells, or HeLa cells treated with IFN-□ or Poly IC were infected with Timer-CVB3 at a low (moi = 0.01) or high moi (moi = 0.1), and the progression of infection was monitored by fluorescence microscopy (***Figure 4***). Untreated HeLa cells infected with Timer-CVB3 at a low moi initially fluoresced green at 24 hours PI (***Figure 4A***). By 32 hours PI, yellow and green cells appeared, and the majority of HeLa cells fluoresced yellow or red by 48 hours PI. In both IFN-□ and poly IC-treated HeLa cells infected at a low moi, infection appeared delayed at 24 hours PI. Also, fewer infected cells were observed at 32 hours PI for IFN-□-treated HeLa cells, and these cells predominantly fluoresced green. By 48 hours PI, both IFN-□ and poly IC-treated HeLa cells fluoresced yellow and red similar to untreated HeLa cells. These results suggest that the Type I IFN response partially protected HeLa cells from a low inoculum of Timer-CVB3 and initially controlled the progression of infection. In contrast, little protection was observed in HeLa cells infected with a higher inoculum of Timer-CVB3 (***Figure 4B***). This modest protection against CVB3 may be partly due to the relatively weak Type I IFN response previously observed in HeLa cells, and due to the ability of CVB3 to inactivate key antiviral molecules. For example CVB3 has been shown to cleave both MAVS and TRIF, known to be critical components of the innate immune response [21].

The results showing a modest reduction in Timer-CVB3 progression in HeLa cells infected with a low or high viral inoculum were quantified using Image J analysis (***Figure 5A*** and ***Figure 5B***, respectively). Despite the delay of Timer-CVB3 infection as determined by fluorescence microscopy following IFN-□ or poly-IC treatment, no significant difference in viral titers over time was observed in HeLa cells infected with a low or high viral inoculum (***Figure 5C*** and ***Figure 5D***, respectively). These results suggest that monitoring the progression of infection by fluorescence using Timer protein may be a more sensitive method of determining the efficacy of an antiviral response within infected cells.

Previously, we described the ability of CVB3 to preferentially replicate in undifferentiated NPSCs [12]. However, a limited amount of viral replication could also be observed in differentiated NPSCs comprised of a mixed culture of progenitor cells, and early neuronal, astrocytic, and oligodendritic cell lineages. Also, the relationship between CVB3 infection and autophagy in differentiated NPSCs proved to be quite distinct and unique as compared to either undifferentiated NPSCs or cardiac cell lines [20]. We wished to follow the progression of Timer-CVB3 infection in mixed cell lineages comprising differentiated NPSC cultures. NPSCs were differentiated for 5 days and infected with Timer-CVB3. After 48 hours PI, cells were fixed and stained for progenitor (nestin$^+$), neuronal (neuronal beta-tubulin III$^+$), astrocytic (GFAP$^+$), and oligodendritic (MBP$^+$) cell lineage markers (***Figure 6***). Four-color fluorescence microscopy identified recent and matured virus protein expression in combination with each cell lineage marker, and DAPI was utilized to identify cell nuclei. The distribution of recent and matured virus protein appeared similar in nestin$^+$ progenitor cells and in the

three cell lineages, suggesting that each cell lineage was equally susceptible to Timer-CVB3 infection upon initial infection. Interestingly, cell-associated and cell free microvesicles comprising recent and matured virus protein were readily observed at higher magnification in these cultures. Previously, we described cellular blebbing in differentiated NPSCs infected with eGFP-CVB3 by Hoffman modulation contrast microscopy [12]. We hypothesized that these cell-associated microvesicles may represent the cellular blebbing phenomena observed in infected differentiated NPSCs, as described previously.

In order to better observe these cell-associated microvesicles, time-lapse videos were constructed from merged fluorescence images (recent and matured virus protein; HMC) of differentiated NPSCs infected with Timer-CVB3. Merged images were taken every 15 minutes for 7 hours to construct the time-lapse videos (***Supplemental Video 1***). The conversion of "fluorescent timer" protein was observed over time simultaneously with intracellular membrane remodeling in Timer-CVB3 infected cells. These intracellular microvesicles may represent virus replication organelles recently described by others for CVB3 [22]. Intriguingly, the presence of numerous extracellular microvesicles were identified in supernatants of the cell culture. Many extracellular microvesicles contained viral material as evident by the detection of red fluorescing timer protein. Some extracellular microvesicles comprising viral material attached to uninfected neighboring cells. The static results of the time-lapse video for Timer-CVB3=infected differentiated NPSCs or C2C12 myoblast cells are represented in ***Figure 7***. The progression of infection as determined by "fluorescent timer" protein in differentiated NPSCs was captured in images take at 47, 48, and 53 hours PI (***Figure 7A***,

Figure 7B, and ***Figure 7C***, respectively). Higher magnification showed the compartmentalization of distinct intracellular membrane remodeling of varying fluorescence following Timer-CVB3 infection (***Figure 7D***), and the accumulation of extracellular microvesicles (***Figure 7E,*** white arrows) some of which fluoresced red (***Figure 7E,*** red arrow).

Of note, differentiated C2C12 cells infected with Timer-CVB3 also gave rise to red fluorescing extracellular microvesicles, beginning at 3 days, and at 5,7, and 9 days PI (***Figure 7F***, ***Figure 7G, Figure 7H***, and ***Figure 7I***, respectively). Mouse C2C12 cells are a myoblast cell line capable of differentiation into myocyte cells. Higher magnification revealed the presence of red fluorescing microvesicles near a green fluorescing cell, suggesting a new infection event taking place (***Figure 7J,*** red arrow). By 9 day PI, high magnification showed red fluorescing microvesicles near more defined myocyte cells with signs of cpe (***Figure 7K,*** red arrow). The presence of numerous extracellular microvesicles comprising viral material in two contrasting progenitor cell models following differentiation suggests that the differentiation process may play a role in their formation and release following infection.

High magnified image frames from the time-lapse video shown in ***Supplemental Video 1*** showed the progression of an intracellular membrane complex which may represent a CVB3 replication organelle described previously by others (***Figure 8***). As shown in frame 9, the apparent loss of a red fluorescing intracellular membrane complex may represent microvesicle egress (***Figure 8B-J***; red arrow). A high magnification time-lapse video of this area (***Supplemental Video 2***) and other areas (***Supplemental Video 3***

and ***Supplemental Video 4***) reveal the possible egress of additional microvesicles from the infected cell.

DISCUSSION

Tracking viral infection may be an important consideration in understanding viral dissemination and pathogenesis. For example, vaccinia virus has been shown to undergo repulsion of superinfection by the early expression of A33 and A36 protein which allows the virus to increase the rate of spread and maximize the replication rate substantially [23]. Also, human T-lymphotropic virus-1 (HTLV-1) utilizes the virological synapse, a specialized area of cell-cell contact promoting transmission [24]. Much remains to be determined with regards to cell-to-cell spread of CVB3 and virus dissemination within the host. Therefore, we engineered the "fluorescent timer" protein into our infectious clone of CVB3 in order to monitor virus dissemination both in culture and *in vivo*.

By tracking the progression of virus infection utilizing Timer-CVB3, we have observed some intriguing aspects of virus replication in partially differentiated progenitor cells. First, we observed extensive intracellular membrane remodeling in partially differentiated NPSCs which reflects the formation of viral replication organelles described by others [22]. The conversion of "fluorescent timer" protein from green to red monitored by fluorescence microscopy revealed distinct regional replicative organelles fluorescing at an asymmetrical pattern. We suggest that ongoing translation within virus replication organelles and the lack of virus protein diffusion within the cytoplasm due to compartmentalization reflects the localized conversion of "fluorescent timer" protein from green to red as shown by time-lapse video. These consequences have enabled the

direct visualization of intracellular membrane remodeling of the host cell by CVB3 in real time. The induction of CVB3 replication organelles may also reflect the ability of enteroviruses to hijack the autophagy pathway and maximize viral replication [25] [26] [27] [28].

Unexpectedly, we observed the generation of extracellular microvesicles containing viral material which may represent a novel strategy of virus shedding and transmission to neighboring cells. Previous publications have suggested the possible contribution of extracellular microvesicles or exosomes to virus dissemination [29] [30]. Also, the generation of autophagic vesicles following enterovirus infection may lead to fusion with the plasma membrane and possible extracellular release of virions the cytosolic lumen [31]. We suggest that the extensive intracellular membrane remodeling observed in differentiated NPSCs and C2C12 cells may result in extracellular release similar to the formation of exosomes observed in tumor cells and other cell lineages. It remains to be determined if the autophagic pathway contributes to this phenomenon, although we have recently observed a reduction in the level of autophagy in differentiated NPSCs following CVB3 infection [20]. We speculate that the previous observed reduction in autophagy in differentiated NPSCs following CVB3 infection may represent extracellular shedding of autophagosomes specifically within progenitor cells undergoing differentiation.

A possible limitation of observing timer protein fluorescence in infected tissue is that cells need to be observed directly. Timer protein progression cannot be observed by immunohistochemistry because the antibody specific for Timer protein will not allow for fluorescence color change over time. However, the results of flow cytometry data using

HeLa RW cells infected with Timer-CVB3 and fixing with formalin at various time points over 72 hrs show that it is possible to stop timer protein progression at a specific point in time by fixing with paraformaldehyde. When observed at the end of the time course, timer protein fluorescence reflects the amount of time between when the tissue was infected and when fixed. In order to observe the fluorescence of timer protein directly in tissues, sections will need to be frozen, fixed and cut into sections using a Cryocut.

The pancreas is a primary target organ for CVB3 infection, and early viral replication here may seed other organs such as the heart and CNS. Importantly, understanding factors contributing to pancreatic infection may help to reduce early viral replication and virus dissemination within the host. For example, transgenic mice expressing the antiviral molecule interferon-□ within the pancreas showed reduced viral titers and substantially less myocarditis [16]. Our preliminary observations suggest that different regions of the pancreas may be preferentially susceptible to infection, with the head of the pancreas (attached to the duodenum) most susceptible to infection. We hypothesize that hormones (cholycystokinin-8 – CCK8 and secretin) released in the vicinity of the head of the pancreas during feeding may regulate viral replication. Therefore, future experiments will utilize Timer-CVB3 to study the progression of infection in the pancreas of C57 BL/6 mice following intraperitoneal inoculation. Using Timer-CVB3, we also will examine the pancreas, heart, and CNS in order to determine the dynamics of virus protein translation during persistent infection. Also, the possibility remains that other cells in the pancreas may be sequentially infected with Timer-CVB3.

For example, initial infection may occur in endothelial cells or macrophages, prior to infection of acinar cells.

Timer-CVB3 may also assist in characterizing persistent infection in the heart or CNS, whereby the nature of persisting viral material is unclear. For example, new viral protein expression during sporadic infection or virus reactivation might be expected to produce green fluorescence in cells harboring viral infection, while a carrier-state infection may produce red and green fluorescence. Future studies will focus on characterizing extracellular microvesicles for the presence of infectious virus and well-known markers for exosomes and autophagosomes. Also, we will determine the ability of other cell types to generate extracellular microvesicles following CVB3 infection.

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FIGURE LEGENDS

Figure 1. HeLa cells infected with Timer-CVB3 slowly turn from green to red. The gene for "fluorescent timer protein" was inserted into our infectious plasmid clone for CVB3 (pMKS1). Upon HeLa cell infection with recombinant CVB3 expressing "timer protein" (Timer-CVB3), we expected the slow conversion of the green fluorescing form of timer protein to red (shown diagrammatically). HeLa cells infected a high inoculum of Timer-CVB3 (moi = 10.0) initially fluoresced green (recent viral protein) at 20 hours PI as determined by fluorescence microscopy. By 43 hours PI, both green and red fluorescence (matured viral protein) was observed in infected HeLa cells. By 48 hours PI, the majority of cells fluoresced brightly in the red channel. At all time points, signs of cytopathic effect (cpe) were observed, reflecting the high inoculum of virus added to HeLa cells. Many of the cells showing cpe failed to express significant amounts of detectable "timer protein" by fluorescence microscopy.

Figure 2. HeLa Cells infected with Timer-CVB3 slowly change fluorescence from green to red as determined by flow cytometric analysis. HeLa cells were either "Mock" infected with 1xDMEM, O.1 MOI of eGFP-CVB3, 0.1 MOI of dsRed-CVB3, or the indicated MOI of Timer-CVB3. After the indicated times post-infection, the supernatant solution and the cells were scrapped from the plate surface, centrifuged at 2000 RPM for 2 minutes and resuspended in a solution of 4% paraformaldehyde in 1X PBS for fixation overnight. The cells were then centrifuged at 2000 RPM for 2 minutes and then resuspended in 0.1% BSA in 1X PBS and the cell solutions were stored at 4^{o}C until cell

solutions were analyzed on a FACS-Aria machine. Quadrants were set so that the cells from the mock-infected solutions were all in the double negative quadrant.

Figure 3. Progression of Timer-CVB3 plaque progression and viral spread in HeLa cells and NPSCs. *(A)* The conversion of "fluorescent" timer protein was observed during plaque formation in HeLa RW cells infected with Timer-CVB3 and overlayed with agar. A "bulls-eye" pattern was seen by fluorescence microscopy whereby matured viral protein (red) was detected in early infected cells, and recent viral protein (green) was detected in cells infected at a later point in time. *(B), (D)* HeLa cells grown in culture and infected with Timer-CVB3 at 24 hours PI fluoresce yellow and green (recent viral protein). *(C), (E)* HeLa cells infected with Timer-CVB3 at 48 hours PI fluoresce yellow and red (matured viral protein). *(F), (G), (H), (I), and (J)* Progression of NPSCs infected with Timer-CVB3 was observed by fluorescence microscopy. *(K), (L), (M), (N)* NPSCs pretreated with poly IC showed reduced infection with Timer-CVB3. *(O)* Carrier-state NPSCs infected with Timer-CVB3 failed to express detectable levels of timer protein.

Figure 4. Progression of Timer-CVB3 infection in HeLa cells pretreated with IFN-□ or P oly IC. At the low MOI of 0.01 (A), more cells are observed to round up/detach in the untreated HeLa cell culture as compared to the IFN or poly IC treated cultures after 24hrs of infection. At this time point, more green cells are observed in the untreated condition as compared to either of the treated conditions. At 32hrs post infection, more yellow cells are observed in the untreated and poly IC treated cultures than in the IFN treated cultures. More cells rounded/detaching cells and green cells are

observed in the IFN treated cultures at 32hrs pi than were seen at 24 hrs pi. At 48hrs pi, all cultures contain a mixture of red, green, yellow, and rounded/detaching but colorless cells. At the higher MOI of 0.1 (B), the cultures appear to be very similar across the three different conditions at each of the three time points.

Figure 5. IFN-□ treatment restricted the progression of Timer-CVB3 infection in HeLa cells at low multiplicities of infection. *(A)* HeLa cells treated with IFN-□ or Poly IC showed fewer signs of cytopathic effect (cpe) and fewer green cells by fluorescence microscopy following infection with Timer-CVB3 as compared to untreated cultures at 24 hours PI. For HeLa cells given a relatively low amount of viral inoculum (moi = 0.01), untreated cultures showed the highest percentage of green cells. By 32 hours PI, the number of cells exhibiting cpe was similar across the three conditions, and the percentage of cells expressing green timer protein was similar between the untreated and the poly IC-treated cultures. In contrast IFN-□-treated HeLa cells continued to show reduced numbers of green cells. By 48 hours PI, the majority of the infected HeLa cells expressed both early and late viral proteins (yellow signal), and no significant differences were observed between the three conditions. *(B)* The effects of interferon were minimized at the higher MOI as the cell counts and estimated percentages indicated conditions were more similar than different. A similar but extremely muted trend was observed at this MOI. *(C)* At the lower MOI, viral titers were similar early on, but at 48hrs post infection, the presence of IFN allowed the virus to reach higher titers than in the untreated or poly IC treated cultures; however these differences were not significant.

(D) At the higher MOI, viral titers were again similar early one; however, higher viral titers were not observed in the IFN treated cultures.

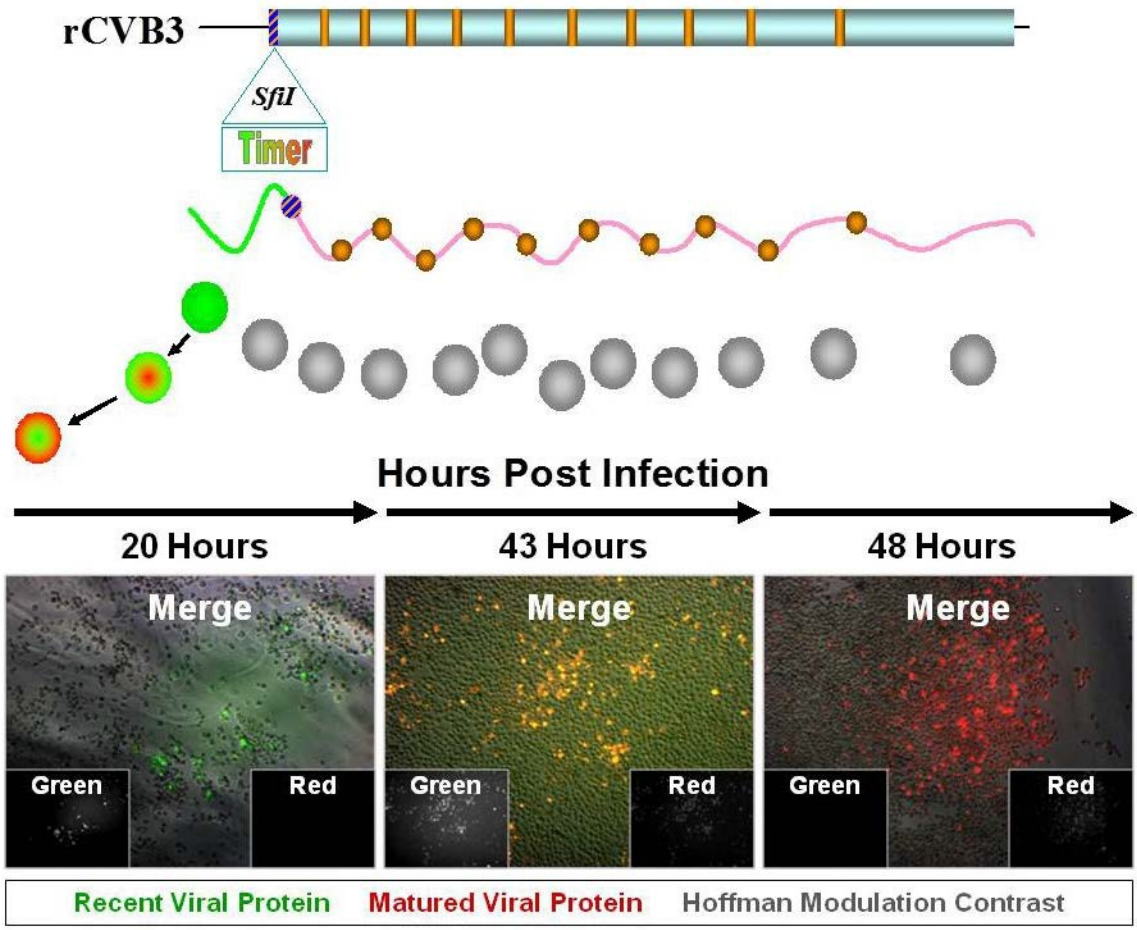
Figure 6. Inspection of recent and matured virus protein expression in differentiated NPSCs infected with Timer-CVB3. NPSCs cultures were differentiated for five days, and then infected with Timer-CVB3 for three days, followed by immunostaining for lineage markers (in white) exhibit early and late viral protein expression. Timer protein produced early on in the course of infection had sufficient time to mature into the red fluorescing form (matured viral protein) marking the cells which were infected early on. Newly produced virus which still fluoresces green demarcates the cells which were infected later in the course of infection (recent viral protein).

Figure 7. Shedding of extracellular microvesicles containing viral material following Timer-CVB3 infection of differentiated cells. *(A-C)* Timer protein can be observed changing from green to red over the span of 6 hours in infected differentiating NPSCs. *(D)* Digital magnification and enhancement of images B and C reveal the release of extracellular microvesicles (white arrows) some containing timer protein (pink arrow) between 48hrs post infection and 53 hours post infection *(E)*.

Figure 8. Time lapse photography frames showing microvesicle egress from a Timer-CVB3-infected cell. Close inspection of images *(A)* taken at 10 minute intervals during the six hour span in which the infected cultures were observed revealed an intracellular

membrane complex of green and red fluorescence when digitally enhanced *(B-I)*. The intracellular membrane complex eventually is released from the infected cell in frame (J).

FIGURES



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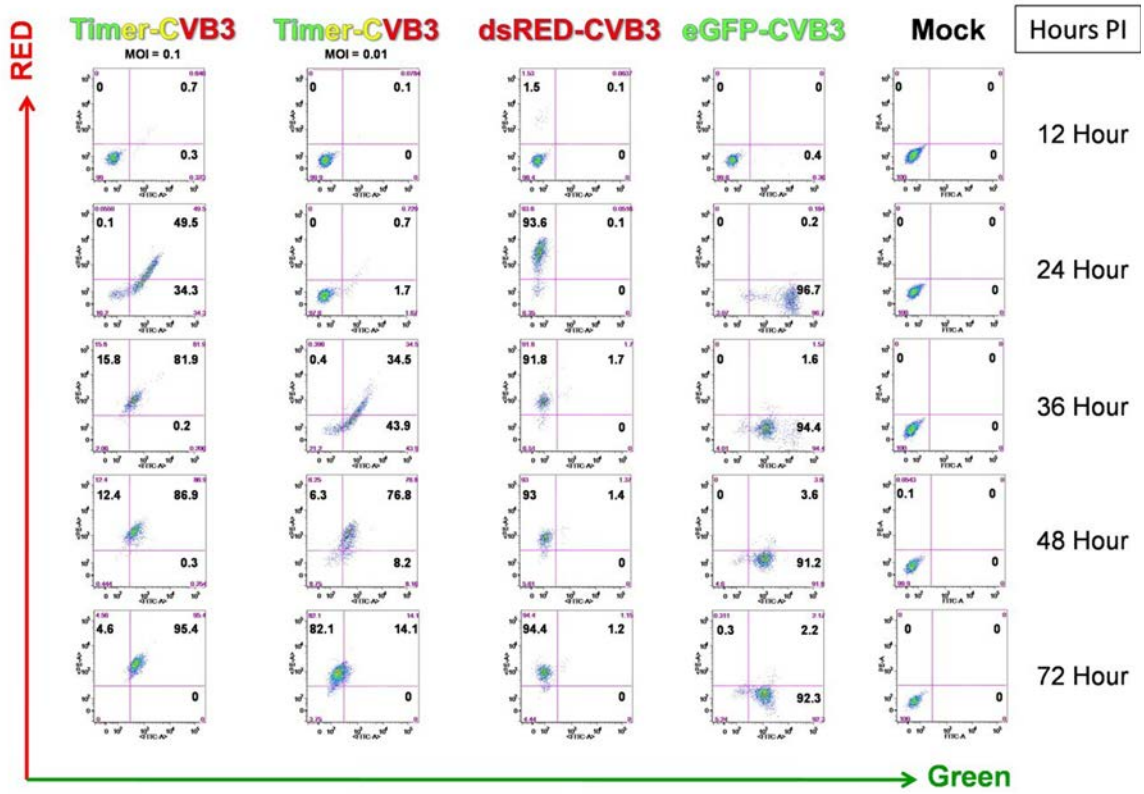

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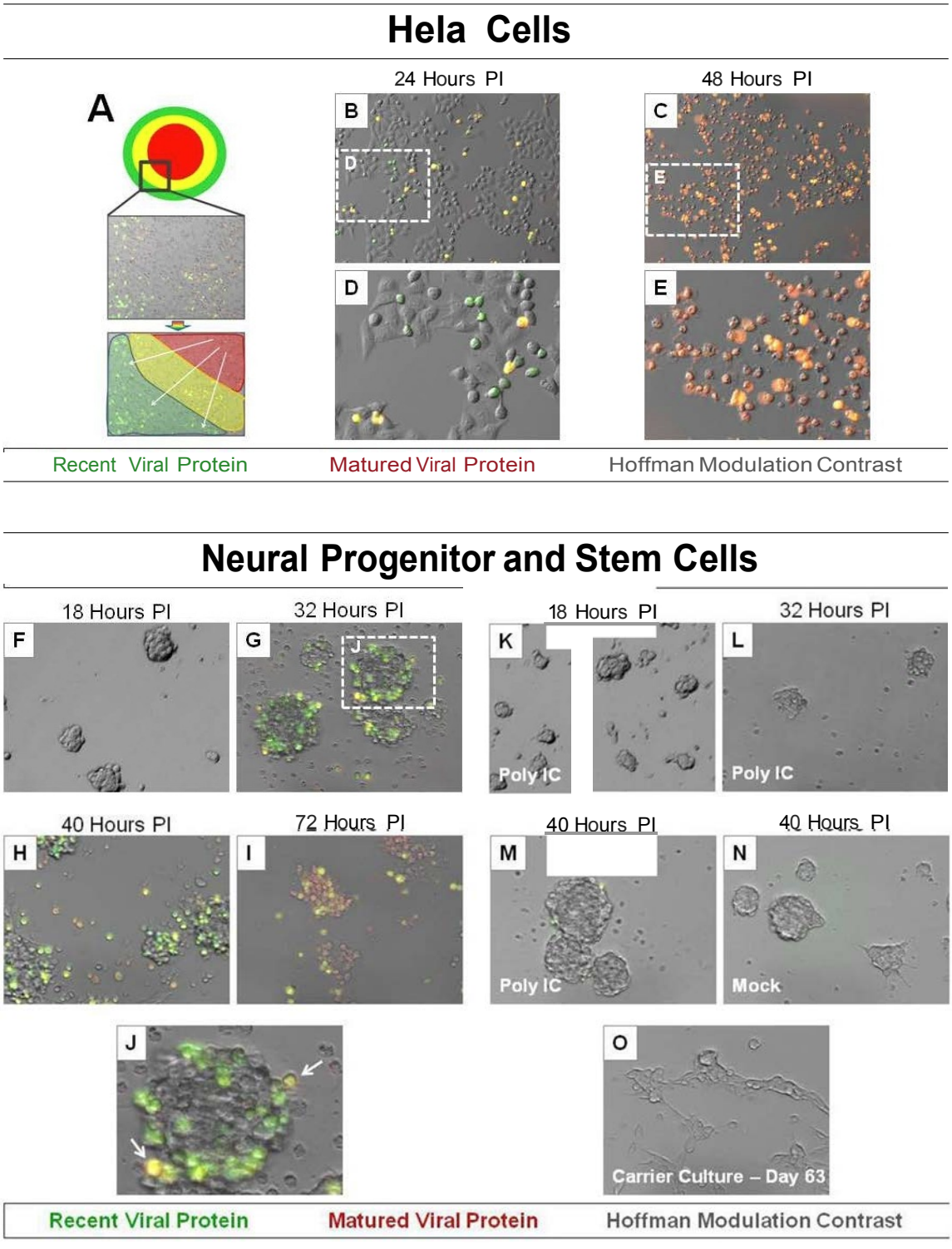

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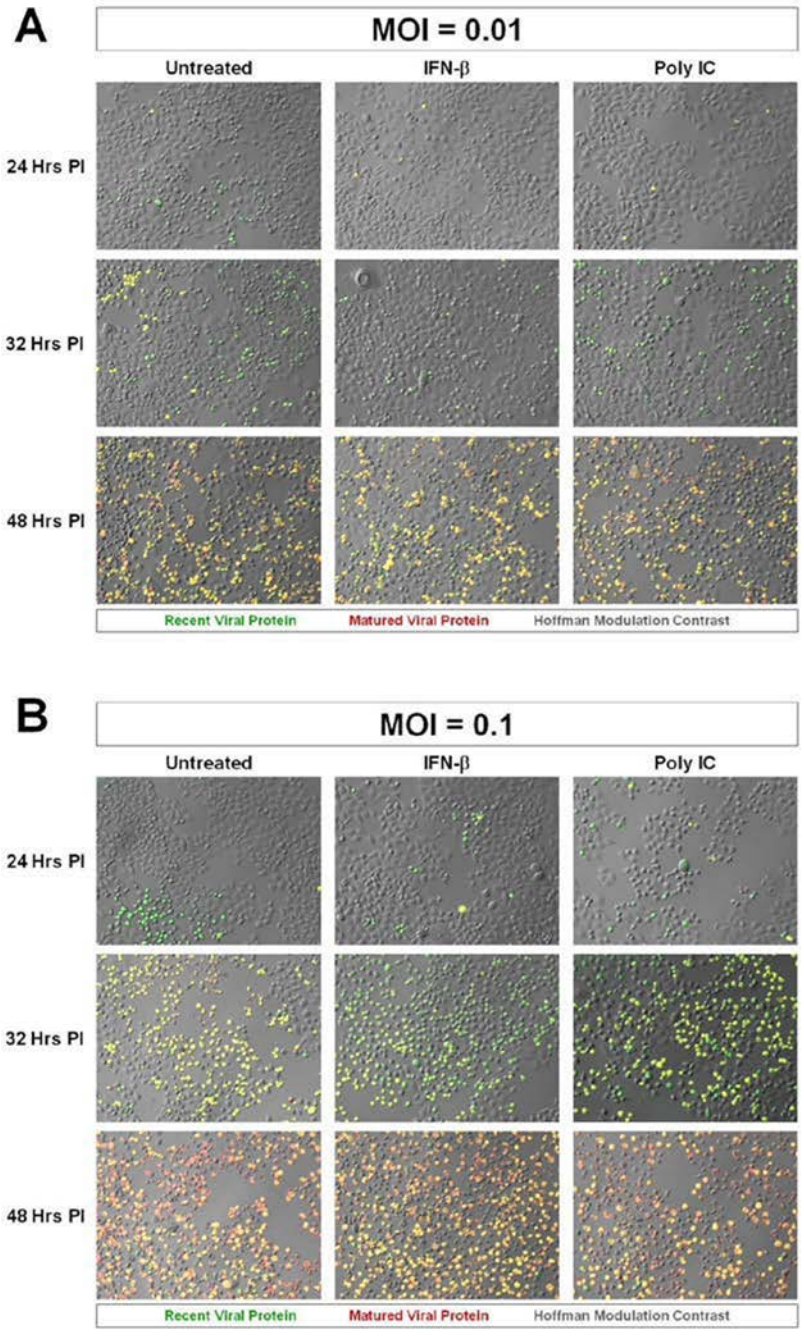
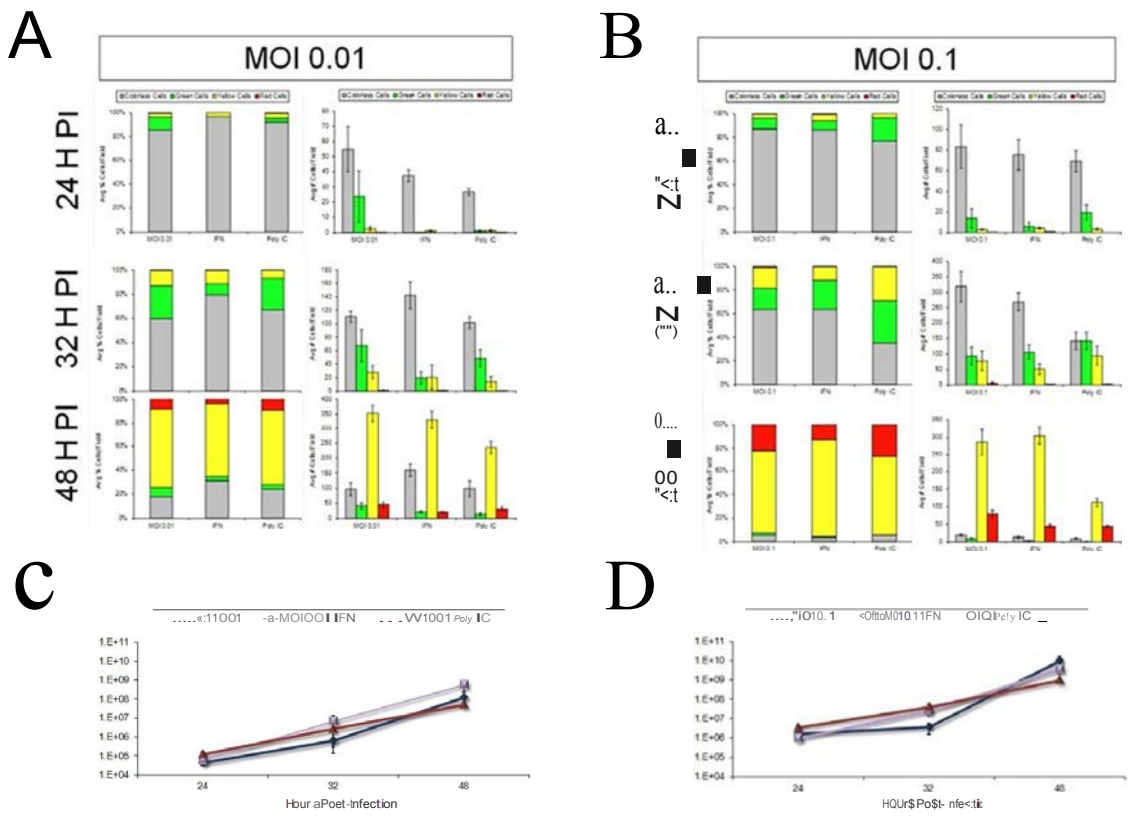


Figure 4. Progression of Timer-CVB3 infection in HeLa cells pretreated with IFN-β or Poly IC.



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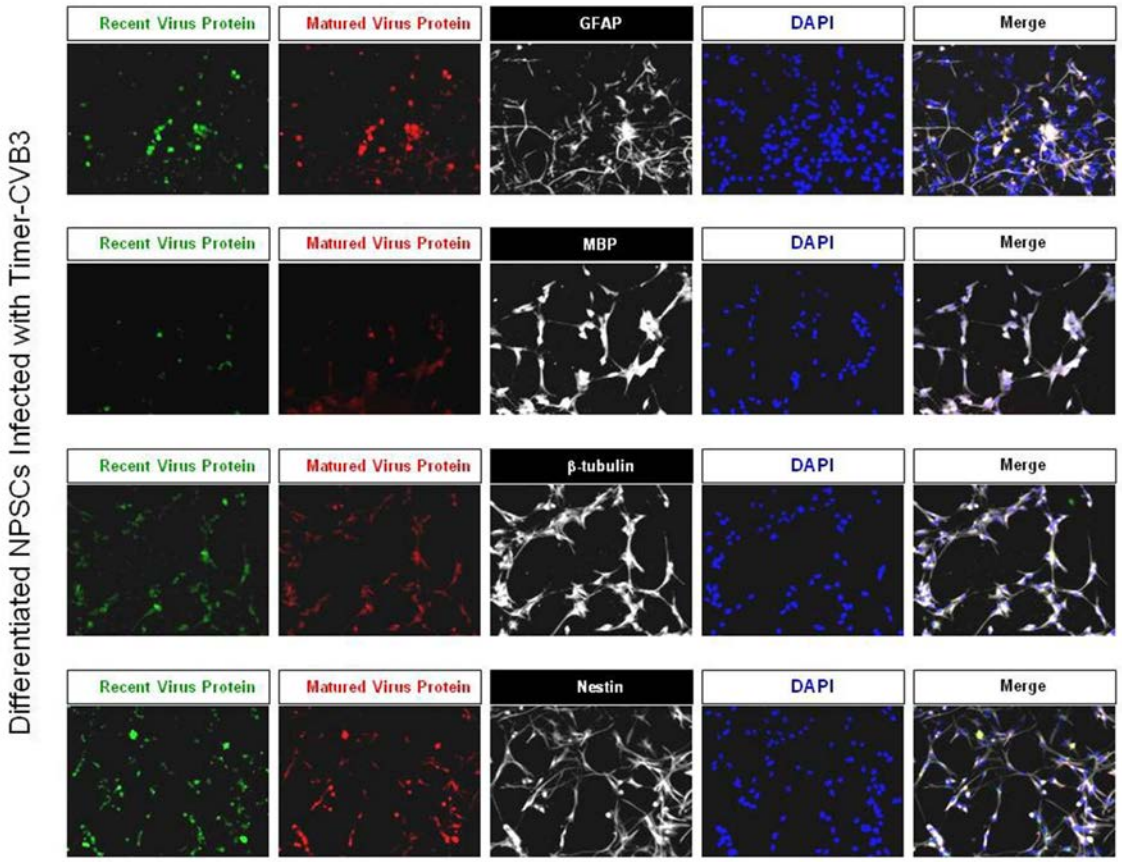

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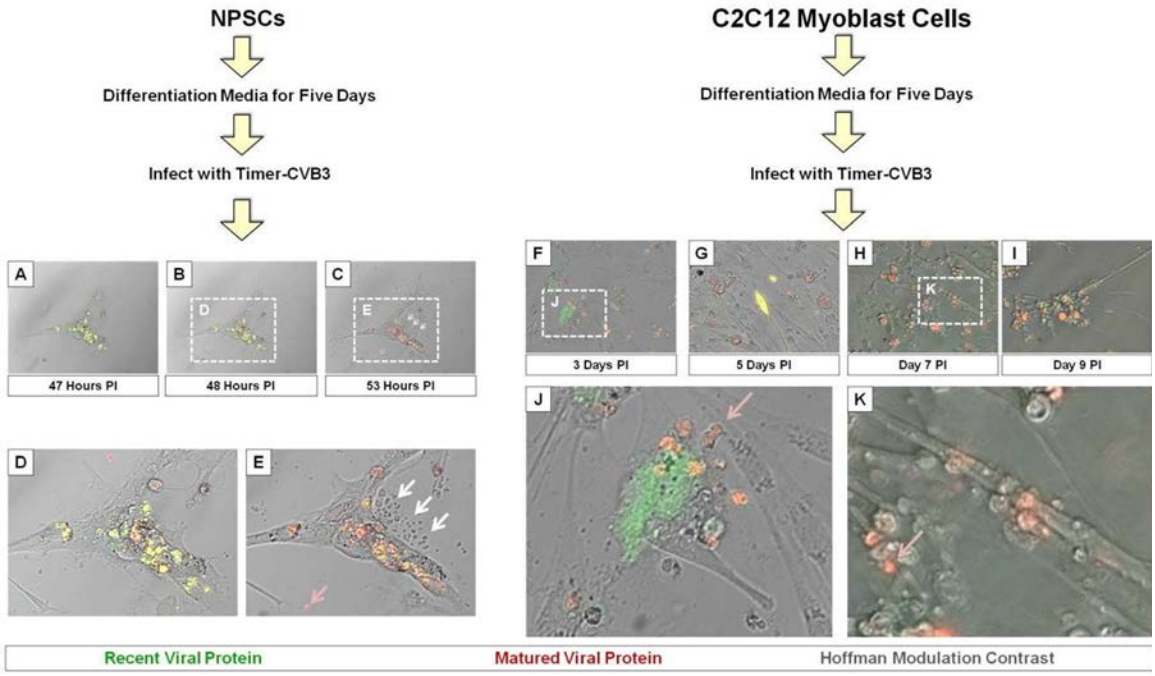

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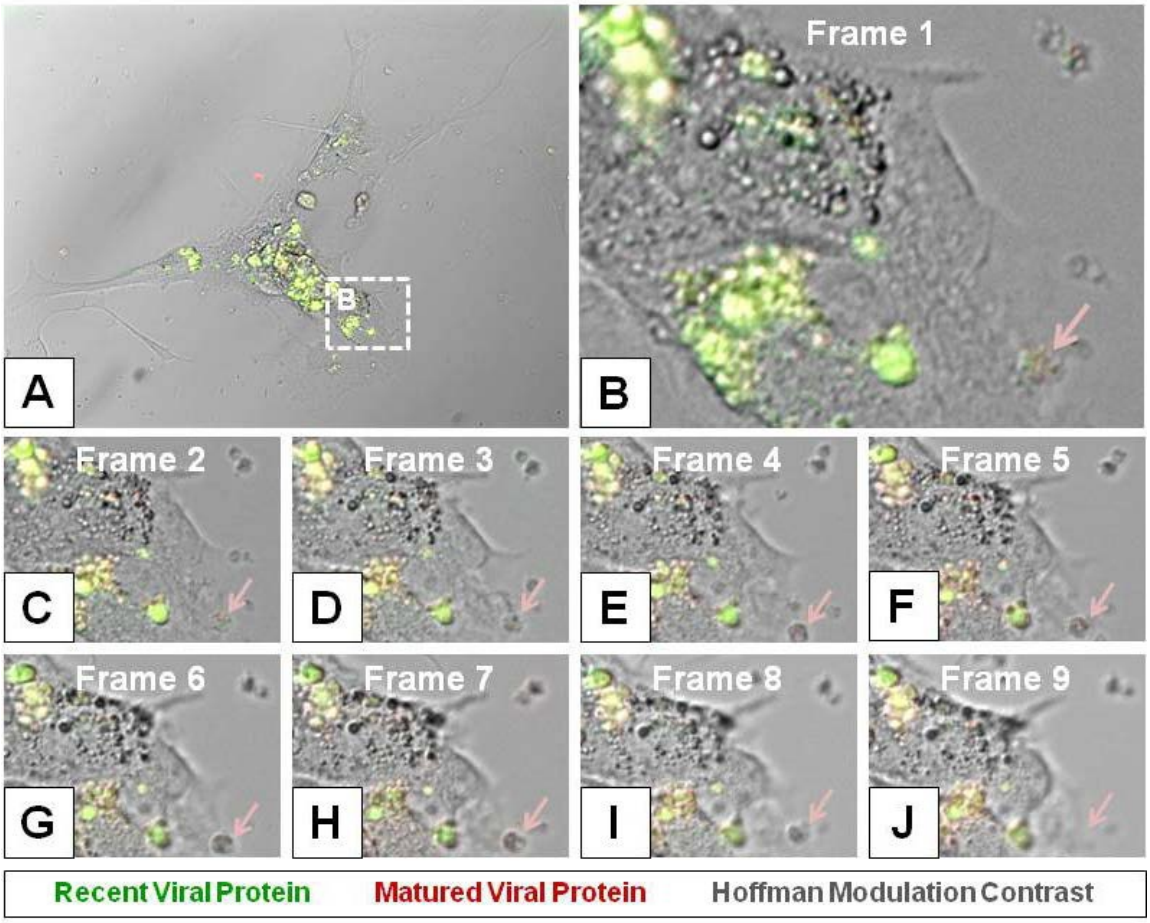

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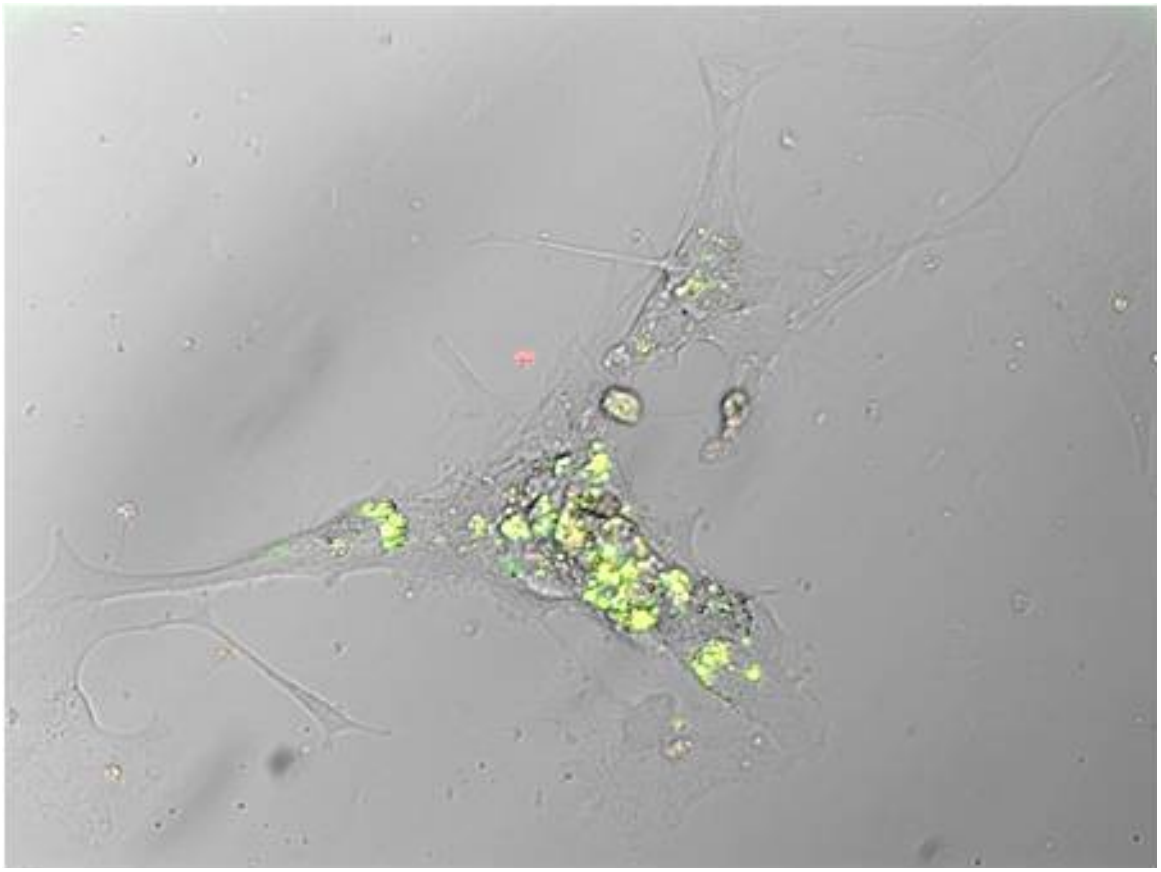
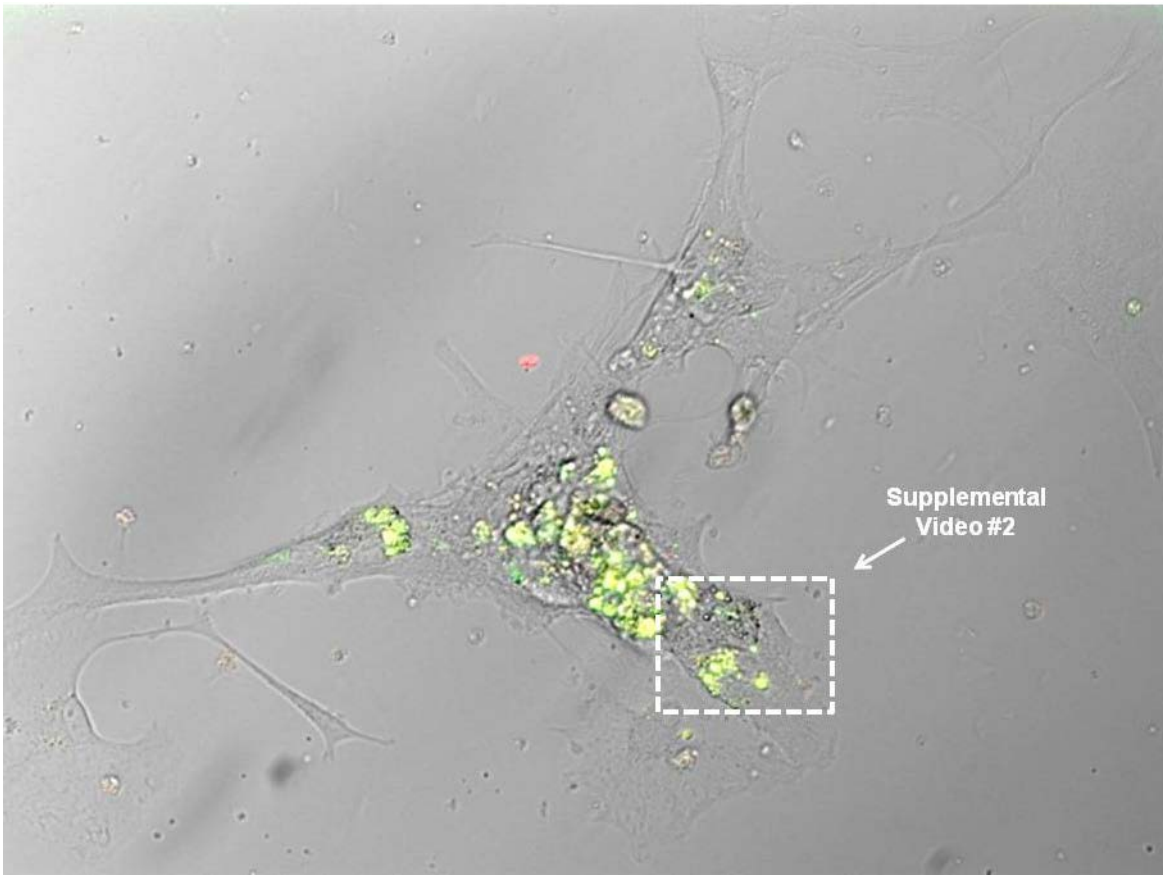
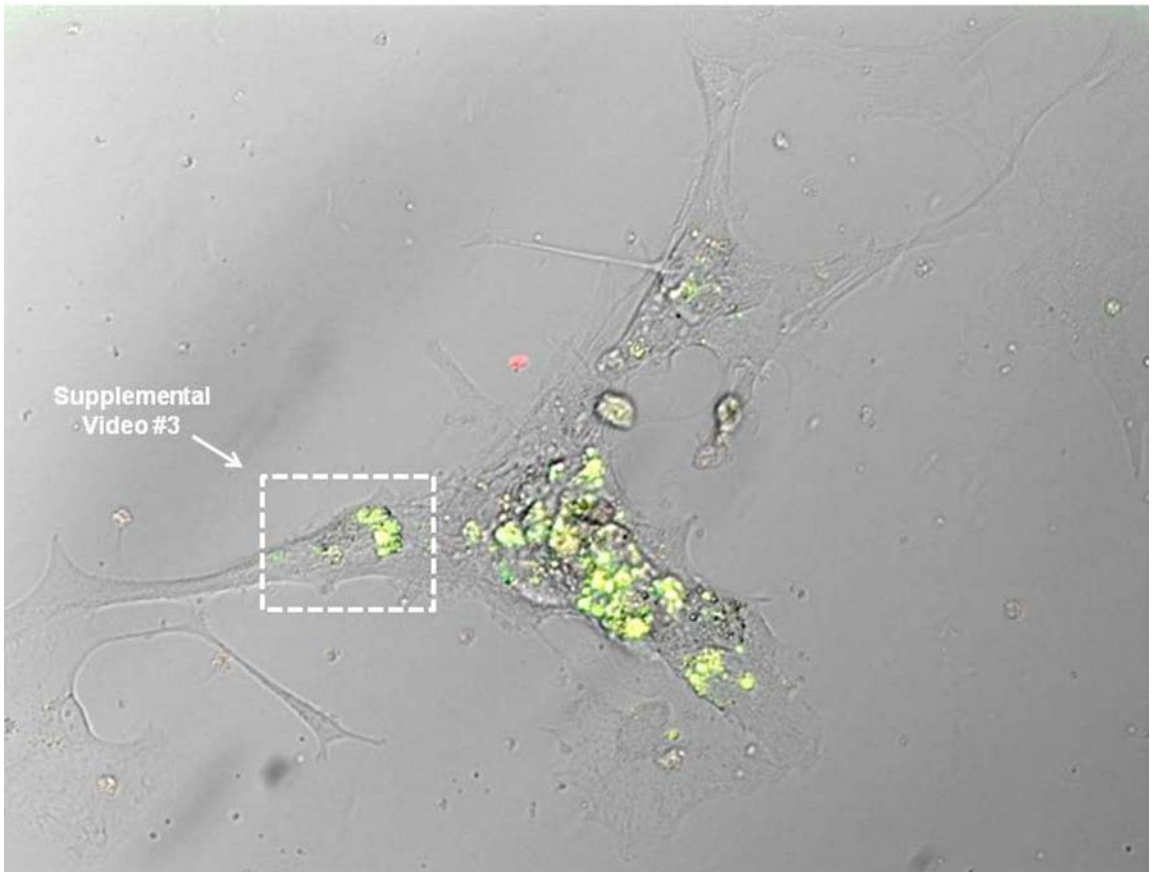


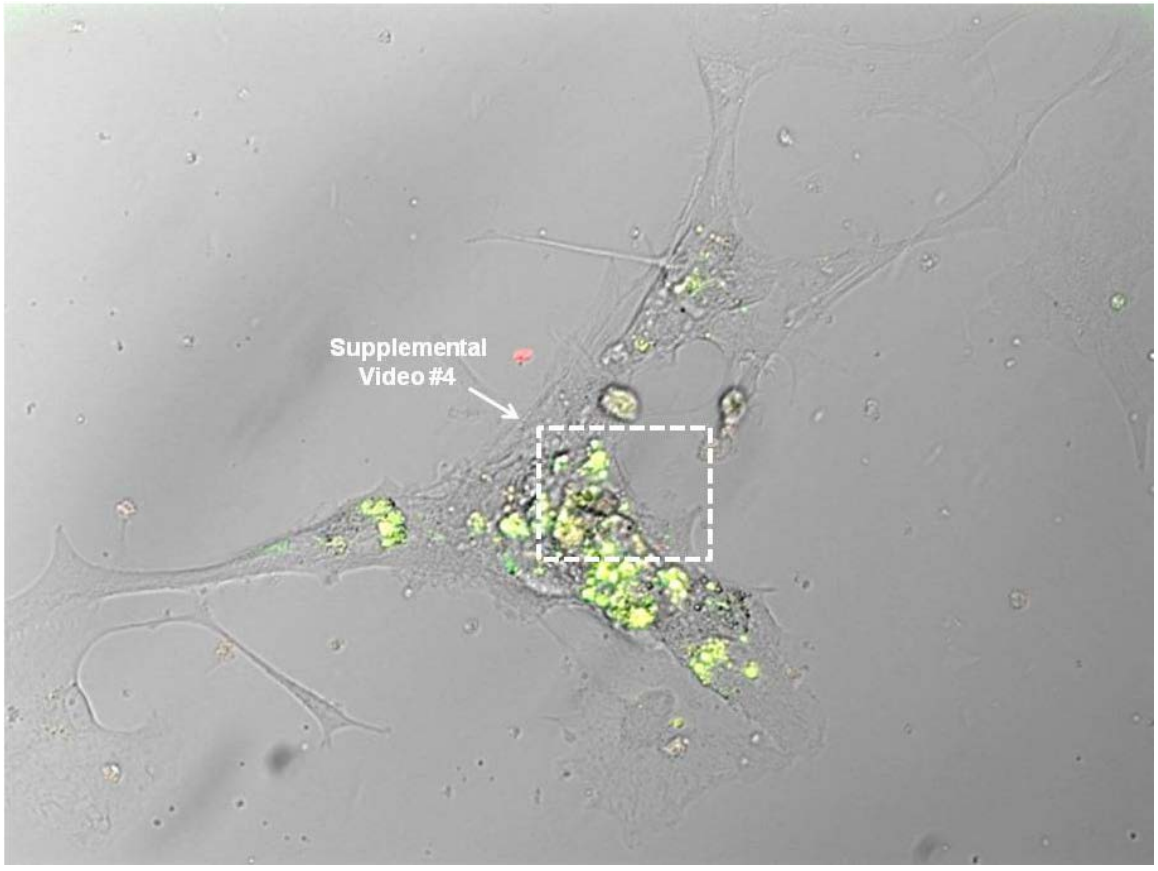
Supplemental Video 1. Time-lapse video of differentiated NPSCs infected with Timer-CVB3.



Supplemental Video 2. Time-lapse video of differentiated NPSCs infected with Timer-CVB3 at higher magnification – Region 1.



Supplemental Video 3. Time-lapse video of differentiated NPSCs infected with Timer-CVB3 at higher magnification - Region 2.



Supplemental Video 4. Time-lapse video of differentiated NPSCs infected with Timer-CVB3 at higher magnification - Region 3.

CHAPTER III:

Enhanced Susceptibility or Resistance to Neurodegeneration / Autoimmune Disease in Mice Infected with Coxsackievirus Shortly after Birth

INTRODUCTION

We have previously showed that proliferating stem cells in culture and in the CNS were preferential targets for CV infection [1,2] [3], and that CV may establish a persistent infection in the CNS [4]. During the later stages of infection, an ensuing inflammatory response and subsequent lesions remained in the adult CNS of surviving animals. High levels of type I interferons and chemokines (in particular MCP-5, IP10, and RANTES) were upregulated following infection and remained at high levels up to day 10 PI. Chronic inflammation and lesions were observed in the hippocampus and cortex of surviving mice for up to 9 months PI. Viral RNA was detected in the CNS up to 3 months PI, at high abundance (~10^6 genomes/mouse brain), and the quantity of RNA was amplified after two rounds of *in vitro* passage.

Therefore, **we wished to determine if a previous early neurotropic viral infection which persists and causes inflammation for extended periods, may provide a "fertile field" for accelerated or exacerbated neurodegenerative and autoimmune disease in the aging host** [5] [6]. We hypothesized that CVB3 may permanently modify the immunological make-up of the CNS, perhaps altering the expression inflammatory genes and partially exposing the blood-brain-barrier to self-reactive T cells. Our ultimate goal is to utilize our neonatal mouse model of CVB3 infection to study the link between microbes and chronic disease. We expect that prior infection and chronic inflammation may be involved in setting a fertile ground for numerous autoimmune-like conditions, including diabetes, myocarditis, and

demyelinating diseases. We hope that our studies may help predict the lasting neurological sequelae of a previous viral infection on the developing host.

CVB3, a neurotropic virus which preferentially infects neural stem cells and persists in the CNS, may permanently alter the expression of inflammatory genes in the CNS. We will utilize two different mouse models of disease. The first mouse model employed hAPP751 transgenic mice. These mice express human Amyloid Precursor Protein with two Swedish mutations (K670M/ N671L) and one London mutation (V717I) under the murine Thy1 promoter and reproduce aspects of Alzheimer's disease-like pathology. The second mouse model utilized the experimental autoimmune encephalomyelitis (EAE) model of autoimmune disease in the CNS, whereby immunization with a myelin peptide (MOG_{35-55}) leads to infiltration of pathogenic T cells and subsequent demyelination. In each model, 3 day-old pups were infected with a high dose of a recombinant CVB3 expressing eGFP (eGFP-CVB3).

METHODS

Staining and immune-histology: Slides were stained with hematoxylin and eosin to observe the overall morphology of the CNS. To look for amyloid plaques, the slides were stained with an anti-amyloid beta antibody (3D6).

It has been previously shown that the CNS is more susceptible to autoimmune antibodies in mice with persistent infection of their CNS with eGFP-CVB3 [7]. Since this study used autoimmune antibodies produced by a non-infected mouse, I hypothesized that neonatal mice previously infected with eGFP-CVB3 may suffer a more dramatic experimental autoimmune encephalomyelitis (EAE) disease course. To induce demyelination in these mice, I used the established method of EAE. EAE was induced in mice 3 months post CNS infection with eGFP-CVB3, and the disease course was compared with mock-infected control mice. Three day-old C57 BL/6 pups were infected with GFP-CVB3. After 90 days, EAE was induced in these mice by immunization with MOG_{35-55} peptide emulsified in complete Freund's adjuvant. EAE scores (clinical signs of disease) were evaluated over time in these mice over 45 days, after which they were killed to determine relative levels of histopathology (Luxol fast blue/PAS staining), and demyelination (immunohistochemistry for CNPase, myelin basic protein).

We hypothesized that previously infected mice will suffer from greater disease scores and more profound histopathology. However while evaluating the preliminary data; I observed that previously-infected mice were protected from the induction of EAE. Previously-infected mice showed only very small signs of EAE and completely

recovered shortly before harvesting at 45 days post-EAE induction. These preliminary data disprove my hypothesis. In fact, eGFP-CVB3 infected mice were significantly less at risk of developing EAE symptoms than mock-infected mice after EAE induction. Also, we determined if T cell responses against the MOG_{35-55} peptide were reduced in those mice previously-infected with eGFP-CVB3 utilizing intracellular cytokine staining, and determined CD4 T cell levels and interferon gamma induction. Recent studies have shown a lack of virus-specific T cell activation in mice infected with CVB3 [8], which may be involved in the observed phenomenon of apparent EAE reduction in previously-infected mice.

Induction of Experimental Autoimmune Encephalomyelitis (EAE): A (1:1) solution of 3mg/mL MOG_{35-55} plus Complete Freund's Adjuvant (4mg/mL heat killed mycobacterium tuberculosis) was mixed and emulsified by repeated pipette up, eject until the two solutions are well mixed as an emulsion. Then, 200µL of this emulsion was injected into the left thigh subcutaneously. 25µL of 20µg/mL of pertussis toxin was injected intra-peritoneally on the day of induction and 2 days later. Another 200µL of (1:1) 3mg/mL MOG_{35-55} plus Complete Freund's Adjuvant (4mg/ml heat killed mycobacterium tuberculosis) was injected into the right thigh subcutaneously one week after the initial injection into the left thigh.

The mice were monitored every day after EAE induction. They were weighed and diagnosed for EAE score. EAE scores 0 = no symptoms, 0.25 = drags tail while walks, 0.5 = partial tail curl, 1 = no tail curl, 2 = no tail curl and weak walk (hind legs often fall through wire cage top), 3= no tail curl and partial hind leg paralysis (one leg works, the other is just dragged along), 4= complete hind paralysis (both hind legs are

dragged by the mouse using front legs to move), 5= moribund (immobile, just barely alive), 6= dead. When a mouse received an EAE score of 4 it was moved to a separate cage with Napa nectar (like water) and its food was placed on the ground. Also, these mice were subcutaneously injected with 100μL of Mishell-Dutton Balanced Salt Solution every day to keep them hydrated[7]. The mice were monitored for 45 days or other indicated time after EAE induction and then harvested.

Mouse Harvest: ½ of the brain was stored in a tube with beads in it at -80^{o}C for homogenization and RNA isolation. The other ½ of the brain and portions of the spinal cord from neck to mid-thorax and lumbar spine were stored in formalin and then paraffin-embedded for sectioning and staining. To determine demyelination, slides were stained with Luxol fast blue/PAS or immunohistochemically stained with anti-myelin basic protein antibody and anti-CNPase antibody. The brains harvested from these mice were examined immunohistologically for Alzheimer's plaques and expression of inflammatory chemokines and cytokines and autophagy proteins.

RESULTS

Using a mouse model for Alzheimer's disease (hAPP751 transgenic mice – human amyloid precursor protein (K670M/ N671L/ V717I) expressed by the murine Thy1 promoter), we determined the ability of a persistent neurotropic virus to influence the outcome of amyloid plaque formation and eventual memory dysfunction associated with Alzheimer's disease. Alzheimer's disease is influenced by the amount of inflammation in the CNS. In a study of patients who had been diagnosed with mild Alzheimer's disease symptoms, it was found that those who had higher levels of TNF-α suffered worse decrease in cognition [9]. Using hAPP751 transgenic mice, determined the ability of a persistent neurotropic virus to influence the outcome of amyloid plaque formation and eventual memory dysfunction. Previous studies have demonstrated the usefulness of hAPP751 transgenic mice as a model for plaque formation [10]. CVB3 infection of the heart has been shown to lead to a higher expression of immunoproteasomes and inflammatory chemokines [11]. Therefore, CVB3 infection in the CNS might be predicted to worsen the outcome of disease in hAPP751 mice, and in patients suffering from Alzheimer's disease [10,12,13].

To study the effect of CVB3 infection in mice genetically susceptible amyloid plaque formation, I utilized hAPP751 transgenic mice [14]. I infected transgenic and non-transgenic littermate control mice with eGFP-CVB3 (10^7 pfu IC), and evaluated for survival over 7months PI. Alternatively, mock-infected transgenic mice and non-transgenic littermate control mice were observed for 7 months PI. Afterwards, the brains were harvested and examined for the amount of amyloid plaque formation by

immunohistochemistry. I predicted that mice infected with eGFP-CVB3 and having the hAPP transgene will have greater plaque formation and a lower rate of survival during the 7 months PI. Both of these examinations shed light on the relationship of viral infection to subsequent neurodegenerative disease. Since enterovirus infections are fairly common in the US, it is not unreasonable that previously-infected new-born children may harbor the lasting effects of infection. It will be very important to determine if individuals will be at greater risk for more pronounced development of neurodegenerative disease following infection. Also, it will be very valuable to examine how infection alters neurodegeneration so we can gain an understanding of how to control disease course.

These studies were initiated in collaboration with Dr. Inder Verma (The Salk Institute), Dr. Fei Liu (The Salk Institute), and Dr. Eliezer Masliah (Department of Neurosciences, UCSD, La Jolla, CA). Dr. Liu assisted in determining histopathology scores and the degree of amyloid plaques (immunohistochemistry with mAb 4G8 (1:600; Senetek)) in CVB3 infected versus mock-infected transgenic mice. Behavioral studies will be carried out in collaboration with Dr. Paul Gilbert, an Associate Professor in the department of Psychology at San Diego State University (SDSU, San Diego, CA), who has many years of expertise evaluating memory and olfactory impairment using well-established techniques. Dr. Gilbert has published in numerous journals on memory and olfactory impairment in humans [15], [16] and in animal models [17], [18], [19]. His laboratory will assist in the assembly of necessary equipment within the SDSU animal Biosafety Level-2 (BSL-2) facility to investigate

memory and olfactory deficits in hAPP751 transgenic mice infected with eGFP-CVB3.

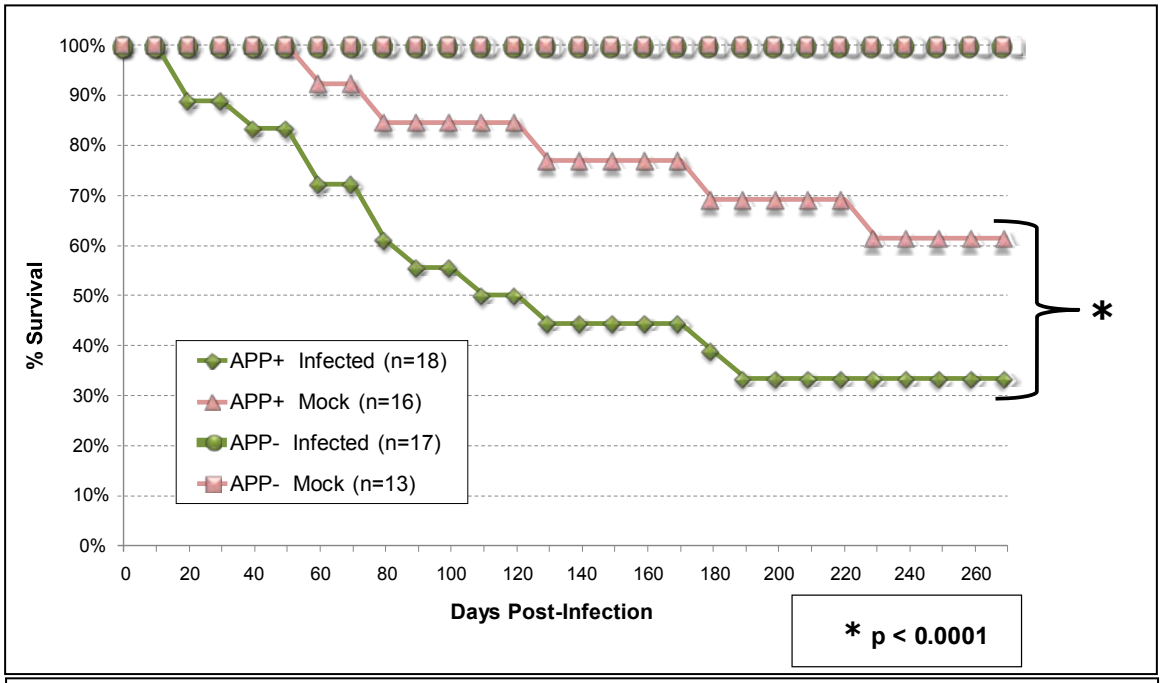


Figure 1. hAPP751 transgenic mice infected with eGFP-CVB3 show greater mortality as compared to infected non-transgenic littermate, control mice.

Our results indicate that hAPP751 transgenic mice infected with eGFP-CVB3 at 3 days-post birth have a decreased survival rate over 7 months post infection as compared to either eGFP-CVB3 infected non-transgenic littermates, or mock-infected hAPP751 transgenic mice **Figure 1**. Ongoing analysis of CNS histopathology will determine if an increase in amyloid plaque formation occurred within infected hAPP751 transgenic mice.

Other researchers have studied the importance of the cellular process of autophagy for limiting the pathology associated with Alzheimer's disease. One study showed that reduced levels of Beclin-1 expression in heterozygous knockout mice (Beclin-1 +/-) crossed with APP J20 transgenic mice (additional mouse model for

Alzheimer's disease) were observed to have less autophagy, and developed more amyloid plaques than wild type, APP transgene positive mice [20]. The J20 strain of mice is transgenic for the human APP that has the Swedish mutation (K670M/ N671L), (the same mutation as in Tg2576 transgenic mice) and the London mutation (V717I) under control of the neuron specific promoter Thy 1. Intracellular amyloid plaques were found to be reduced when autophagy deficient (Beclin-1 +/-), APP^{+} J20 mice were rescued by injection of lentivirus expressing Beclin-1 into the cortex or hippocampus of the brains of the mice.

Of note, the replication level of CVB3 has been shown to increase in autophagy-induced cells [21]. A logical conclusion of this is that CVB3 may hijack the normal function of autophagosome where the virus genome replicates. As a consequence, autophagy may not be effective at disposing of protein aggregates, such as amyloid-β aggregates, and thus, Alzheimer's plaques might be expected to be larger and more frequent.

I infected hAPP751 transgenic mice with eGFP-CVB3, and harvest their brains 7 months post infection. The harvested brains were examined for amyloid plaques by immunohistology. I predict that CVB3-infected, hAPP751 transgenic mice will have larger and more numerous Alzheimer's plaques when compared to the mock-infected, APP transgene positive J20mice. However, unanticipated results may be observed. For example, other researchers have studied Borna Disease Virus (BDV) infection of the CNS of the mouse model of human Alzheimer's disease, Tg2576 mice. BDV was found to cause inflammation in the brains of infected mice and when injected

intracranially (IC) into 14 month old mice. In infected mice amyloid plaques were reduced in regions staining positive for BDV antigen [22].

Can antiviral therapy given late (during persistent infection) lead to amelioration of disease? We will determine if Ribavirin (200 mg/kg mouse IP) administered to mice 30 days before behavioral studies are completed to determine if viral replication can be reduced during persistent infection, and if reduction in viral persistence protects these mice from the accelerated disease.

Using a mouse model for multiple sclerosis (EAE - experimental autoimmune encephalomyelitis), we ascertained if mice infected with a persistent neurotropic virus suffer more severe demyelination following EAE induction. Multiple Sclerosis (MS) is a disease of autoimmunity caused by pathogenic T cells entering the CNS and identifying the myelin sheaths surrounding neurons. Experimental Autoimmune Encephalitis (EAE) is a common laboratory method used to study demyelination in mice [23a]. Three MS treatment drugs have been discovered and tested using this mouse model for multiple sclerosis [23b].

We have previously shown that CVB3 infects neural progenitor cells [24]. A previous study reported a case of a 19 year old nursing student who started suffering cognitive problems and ended up slipping into and out of comas. She was found to be suffering from encephalitis and persistent coxsackie B virus infection in the CNS by immunohistochemistry of a small brain biopsy [25]. CVB3 infection has also been shown to inhibit CD8+ T cell activity by blocking MHC class I presentation of antigen, although $CD4^+$ T cell activity did not appear to be abrogated [26]. When serum from mice which produce myelin-specific autoantibody was injected IP into

C57 BL/6 mice infected with eGFP-CVB3, these mice suffered an increased demyelination in comparison to mice that produce myelin-specific autoantibody only [27]. Alterations in neural stem cell function following CVB3 infection could make the symptoms associated with EAE much more severe and/or inhibit the ability of the mouse to recover from autoimmune damage.

In contrast to the results observed for hAPP751 transgenic mice, mice previously infected with eGFP-CVB3 and immunized with myelin peptide appeared to be protected from signs of EAE disease, as compared to mock-infected control mice **Figure 2**. Three months after neonatal infection, EAE was induced in these mice by immunization with MOG_{35-55} peptide emulsified in Complete Freund's Adjuvant (CFA). EAE scores (signs of disease) were evaluated over 45 days, after which the mice were sacrificed to determine relative levels of histopathology (Luxol fast blue/PAS staining), and demyelination (immuno-histochemistry for CNPase, myelin basic protein).

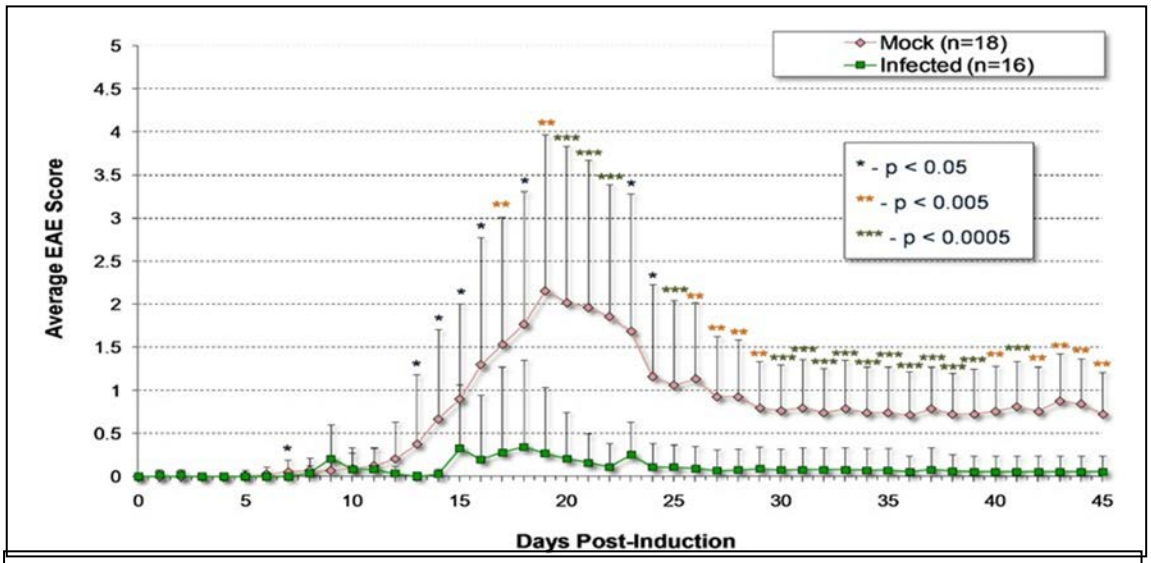


Figure 2. Mice previously infected intra-cranially with eGFP-CVB3 were less susceptible to EAE induction.

This result is very surprising. In order to examine the cells present in the tissues of the CVB3 infected and mock-infected mice, I infected the CNS of some mice with eGFP-CVB3 and mock-infected the CNS of some other mice with 1X DMEM. I then induced EAE 3 months PI. I weighed and diagnosed these mice every day after EAE induction until the EAE signs were at their peak (**Figure 3**).

Mice were sacrificed on this day post EAE induction for both the mock-infected and the CVB3 infected litters. I then harvested the spleen, brain and spinal cord from neck to mid thorax of these mice and then following the protocol for ICCS, prepared the single cell solutions to stimulate with an antigen. These cell solutions were stimulated with antigen and the examined with my fellow researcher, Laura McIntyre. Upon analysis by FACS, it was observed that CVB3 infected mice had just as many, if not more MOG stimulated $CD4^+$ T-cells as the mock-infected mice in the spleen samples. However, the CVB3 infected mice had substantially fewer MOG stimulated $CD4^+$ T-cells in the CNS samples (**Figure 4**) and also had fewer $CD4^+$ T-cells in the CNS (**Figure 5**).

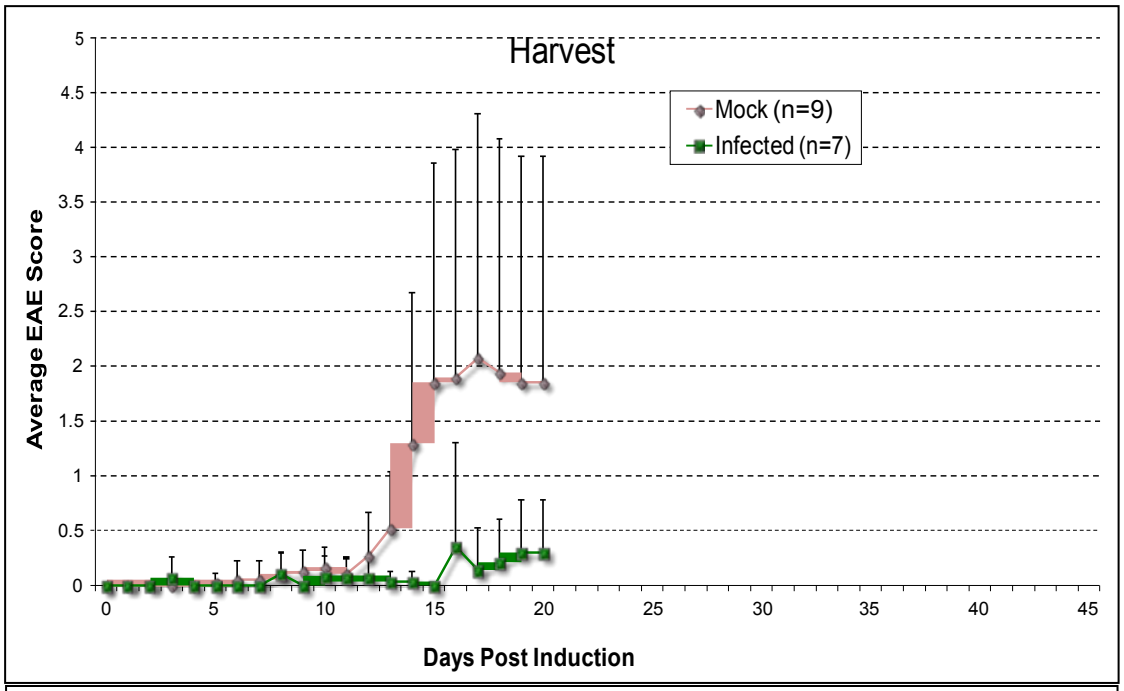


Figure 3. Tissue harvested from the CNS at the peak of EAE signs of disease.

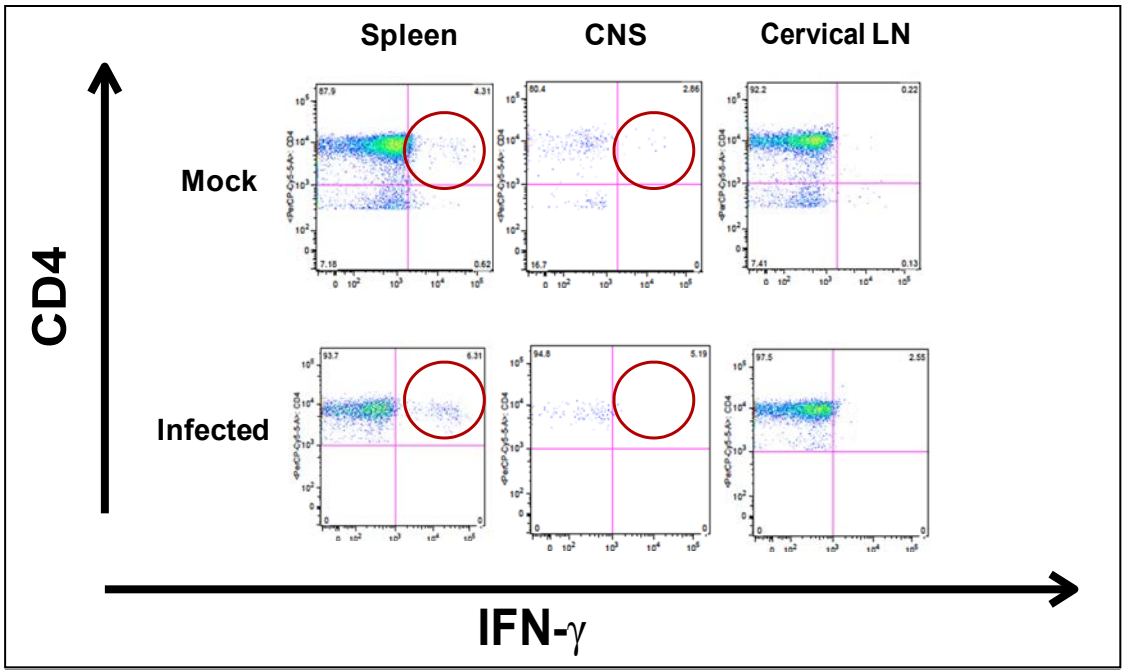


Figure 4. Fewer MOG-Specific CD4+ T cells Entered the CNS in infected Mice

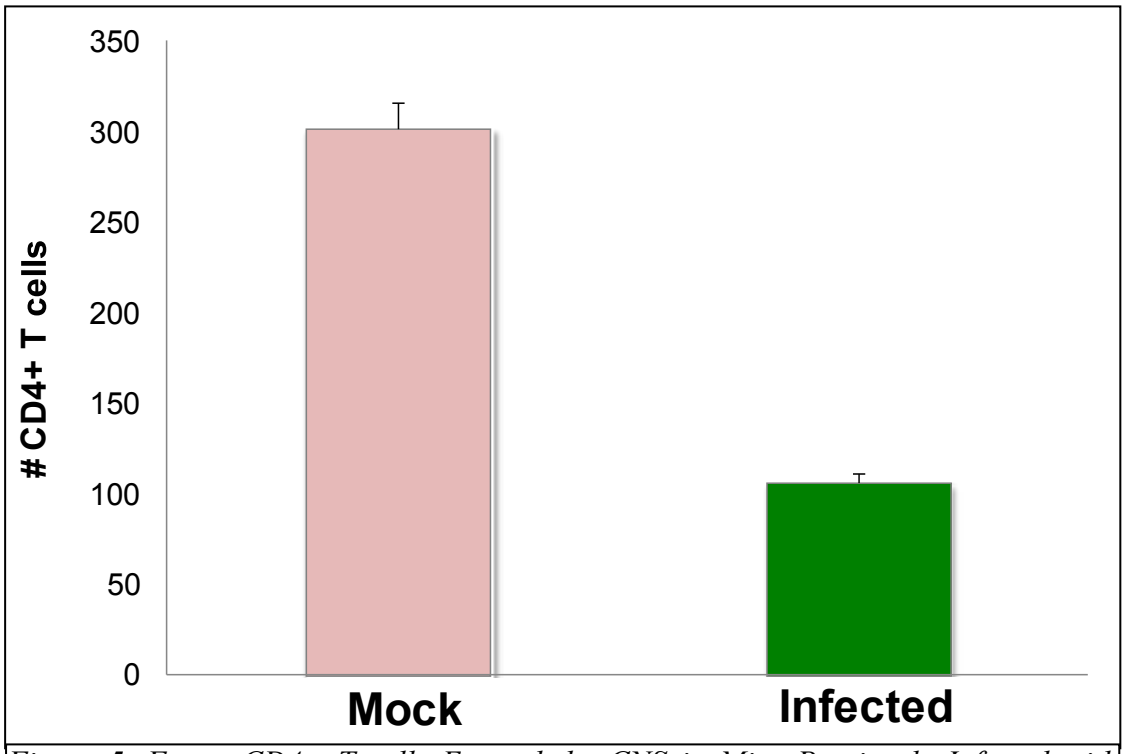


Figure 5. Fewer CD4+ T cells Entered the CNS in Mice Previously Infected with CVB3

DISCUSSION

Our results indicate that mice infected at an early age with eGFP-CVB3 showed reduced signs of EAE disease and fully recovered within 45 days, as compared to mock-infected control mice. Also, we have recently identified the presence of lymphoid structures with the meninges of persistently-infected mice which may represent tertiary lymphoid organs (TLOs) (**Figure 6**).

Previous studies on TLO function indicate that patients who have had secondary progressive multiple sclerosis (MS) have many more TLOs observed in their tissue post-mortem. TLOs have also been found in high amount in transplanted

tissue which is rejected [28] [29]. These two previously published observations make it challenging to understand how we observe TLOs present in CVB3 infected CNS, but also observe a suppression of the autoimmunity which is similar to MS. I hypothesize that the TLOs found in MS patients reflect the immune system being stimulated by autoimmune response to the myelin of the neurons in the CNS, and the TLOs found in CVB3 infected tissue reflect the immune system being stimulated by viral infection.

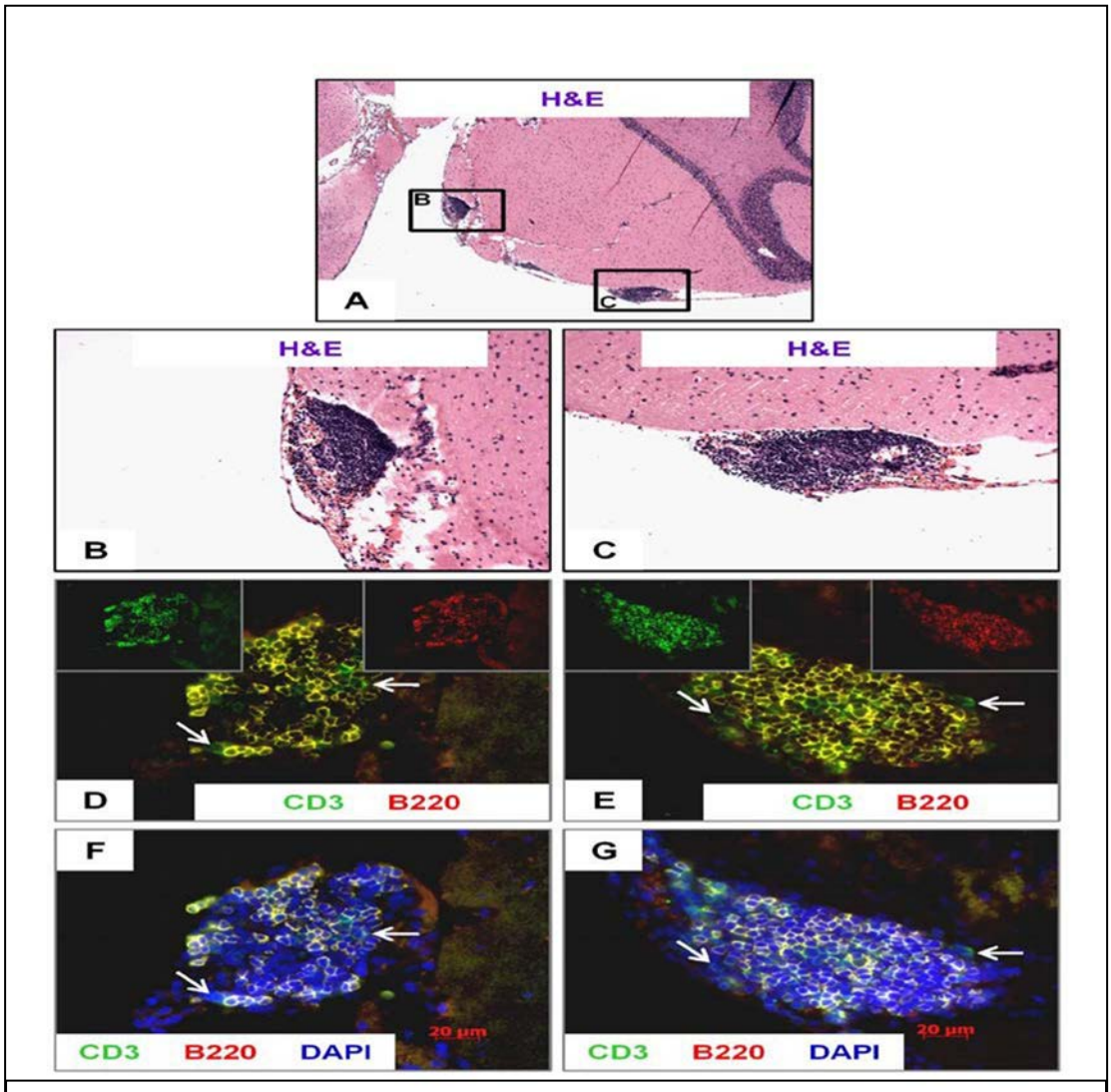


Figure 6. De novo lymphoid formation in the CNS of mice persistently-infected with eGFP-CB3.

In previous studies, infection with a non-pathogenic variant of CVB3 (H310A1) induced the activation of regulatory T cells (T_{Reg} cells) expressing IL-10 to suppress immune response [30]. H310A1 is a mutation of the H3 variant of CVB3 that has the N 173 D mutation of the VP2 viral protein.

Also, another study described the Nancy variant of CVB3 inducing T_{Reg} cells that express TGF-β to suppress the autoimmune response in a diabetic mouse model [31]. Since this study discussed CVB3 infection suppressing autoimmunity, I hypothesize that eGFP-CVB3 infection of the CNS may have results similar to those seen in the suppression of diabetes mouse model. I hypothesize that T_{Reg} cells are induced by eGFP-CVB3 infection of the CNS, and that these T_{Reg} cells will express TGF-β to accomplish suppression of EAE.

I induced EAE disease, and then examined the mice when the EAE signs were the most pronounced. The mice were harvested and the brain and cervical to mid thoracic spinal cord were processed to a single cell solution which was examined by FACS for CD4, FoxP3, IFN-γ, and TGF-β. The spleens of these mice were also harvested and examined for the same markers by FACS. The results of these FACS examinations revealed the number pathogenic T-cells ($CD4^+$, IFN-γ^+) and the number of regulatory T-cells ($CD4^+$, $FoxP3^+$) in the CNS. Our study also examined if the $CD4^+$ regulatory T-cells expressed TGF-β.

I predicted that the CNS of CVB3-infected mice will have a larger number of ($CD4^+$, $FoxP3^+$, TGF-β^+) T_{Reg} cells than the CNS of mock-infected mice. These cells expressing TGF-β may account for reduction of the amount of $CD4^+$, Ifn-γ^+ T-cells because TGF-β suppresses the proliferation of cells. I will purify these cells from

CVB3 infected mice using magnetic beads with antibodies to the surface proteins, CD4 and CD25. Some of these purified cells will be examined for FoxP3 and TGF-β to determine if they are activated T_{Reg} cells, and the percentage of the total cells activated. I will then inject these purified, active T_{Reg} cells intravenously (IV) into mock-infected mice having EAE induced 5 days beforehand. I hypothesize that this adoptive transfer will suppress EAE in the mock-infected mice similar to how adoptive transfer of active T_{Regs} cells into diabetes prone mice suppressed the development of diabetes [31].

The first limitation in our study was that the transgene positive hAPP751 mice die without viral infection at a rate of about one mouse every two months. When transgene positive mice are infected with CVB3, these mice die at a rate of one - two mice every month. As a result, at least three transgene positive, CVB3-infected mice do not survive for seven months. Therefore, we don't obtain a sufficient number of CVB3-infected, transgene positive mice to compare to mock-infected, transgene positive mice seven months after weaning. The litters that we have harvested so far have been analyzed by immunohistochemistry for amyloid plaques by our collaborator at the Salk Institute, Dr. Fei Lui. She has told me that there is no observable difference in number of plaques between the few CVB3-infected, transgene positive mice and the mock-infected, transgene positive mice that have lived for seven months after weaning – although we have a single CVB3-infected, transgene positive mouse that has been analyzed. Therefore, we will analyze sections in mice harvested at an earlier point in time (5 months PI) Two litters of hAPP751 mice have been infected with $3.5x10^6$ pfu of eGFP-CVB3 by intracranial injection. After infection, there were eight transgene

positive mice. Four months after weaning, Five months PI, there were only three transgene positive mice still alive. These mice were harvested and their brains will be examined microscopically for Alzheimer's plaques. Three APP751 mice were mock-infected by IC injection of three day old pups with 1X DMEM. One was transgene positive and all three were sacrificed four months post weaning, five months PI and harvested. Their brains will be examined microscopically for Alzheimer's plaques as the mock-infected control.

A very common cause of death after Alzheimer's diagnosis is choking or pneumonia. It is possible that the CVB3-infected, transgene positive mice exhibit increased mortality as a result of pneumonia. I will examine the lungs of CVB3-infected, transgene positive hAPP751 mice to see if there are histological signs of this ailment. I will also examine the lungs of mock-infected, transgene positive hAPP751 mice harvested at the same time, post weaning, and compare the histology of their lungs to the lungs of the CVB3 infected, transgene positive hAPP751 mice.

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