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Coxsackievirus pathogenesis in the neonatal central nervous system: virus dissemination, the host response to infection, and the autophagic process during viral replication

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Coxsackievirus Pathogenesis in the Neonatal Central Nervous System: 
Virus Dissemination, the Host Response to Infection, and the Autophagic 
Process during Viral Replication 

A dissertation submitted in partial satisfaction of the 
requirements for the degree Doctor of Philosophy 
in 
Biology 
by 
Jenna M. Tabor-Godwin 

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2011
The Dissertation of Jenna M. Tabor-Godwin is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego
San Diego State University
2011
DEDICATION

This dissertation is dedicated to my mom and my fiancé. To my mom, thank you for always listening and steering me in the right direction. To my fiancé, thank you for always reminding me of my goals and giving me that extra push when I really needed it.
EPIGRAPH

“The important thing is not to stop questioning.”

Albert Einstein
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LIST OF ABBREVIATIONS

3-MA  3-methyladenine
Bcl-2  B-cell lymphoma 2
CAII  Carbonic anhydrase II
CAR   Coxsackievirus adenovirus receptor
CCPA  2-chloro-N\textsuperscript{6}-cyclopentyladenosine
CNS  Central nervous system
CVB3  Coxsackievirus B3
DMSO Dimethyl sulfoxide
eGFP  Enhanced green fluorescent protein
GFAP  glial fibrillary acidic protein
hrs  Hours
IHC  Immunohistochemistry
LC3  Light chain 3
LCMV  Lymphocytic choriomeningitis virus
LysM Lysozyme M
Mbp  Myelin basic protein
mL  Milliliter
NP  Nucleoprotein
NPSCs  Neural progenitor and stem cells
OB  Olfactory bulb
pfu  Plaque forming units
PI  Post-infection
PI3  Phosphatidylinositol 3
qRT-PCR  Quantitative real time polymerase chain reaction
Rm  Rapamycin
RMS  Rostral migratory stream
SVZ  Subventricular zone
TTR  Transthyretin
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ABSTRACT OF THE DISSERTATION

Coxsackievirus Pathogenesis in the Neonatal Central Nervous System: Virus Dissemination, the Host Response to Infection, and the Autophagic Process during Viral Replication

by

Jenna M. Tabor-Godwin

Doctor of Philosophy in Biology

University of California, San Diego 2011
San Diego State University 2011

Professor Ralph Feuer, Chair

Coxsackievirus is a common viral pathogen in newborn infants that is capable of causing pancreatitis, myocarditis, and meningitis in the acute stages of infection. Due to the ability of the virus to target the brain, a neonatal mouse model was developed in order to study the effects of coxsackievirus B3 (CVB3) in the central nervous system (CNS). A novel population of myeloid cells was observed that rapidly entered the neonatal...
CNS through the choroid plexus, which mediates the blood-cerebral spinal fluid barrier, specifically after CVB3 infection. These myeloid cells were highly susceptible to infection, based on the expression of eGFP from recombinant eGFP expressing coxsackievirus (eGFP-CVB3). They were also found to express nestin, a neural stem cell marker, and move from the choroid plexus into the parenchyma of the brain, as observed by serial immunofluorescence images. Therefore, these cells may provide an innovative method of viral dissemination in the neonatal CNS. In addition, a unique chemokine induction profile was detected in the choroid plexus following CVB3 infection which may contribute to myeloid cell infiltration and subsequent choroid plexus damage. These results with CVB3 were then compared to lymphocytic choriomeningitis virus (LCMV), a contrasting neurotropic RNA virus in the neonatal CNS, and several differences in stem cell tropism, the immune response and pathology were found. The neonatal mouse model also revealed that neural progenitor and stem cells (NPSCs) are highly susceptible to CVB3 infection. Since several picornaviruses have recently been shown to induce autophagy in order to aid viral replication, we investigated the role of autophagy during acute CVB3 infection in NPSCs. We revealed that CVB3 infection in NPSCs does not induce autophagy, in contrast to cardiomyocytes. Thus, we hypothesize that the role of autophagy during CVB3 infection is cell-type specific. Taken together, these results show that acute CVB3 infection in the neonatal CNS elicits a unique and multifaceted response from the host.
INTRODUCTION OF THE DISSERTATION
The Molecular Biology of Coxsackievirus

Coxsackievirus B3 (CVB3) is a member of the picornavirus family and the enterovirus genus, which is characterized by a relatively small (~7000 base pairs) positive-strand RNA genome. These viruses attach to receptors on the host cell surface, including the Coxsackievirus Adenovirus Receptor (CAR) and Decay Accelerating Factor, which mediate their entry (5). They are then endocytosed and released into the cytoplasm upon acidification, where translation of the viral genome and viral replication occur. The viral genome encodes a single polyprotein that is translated from an internal ribosome entry site located within the 5' untranslated region. The viral polyprotein is then post-translationally cleaved into eleven different proteins: four structural proteins (VP1-4) that comprise the viral capsid, and seven non-structural proteins (2A-C and 3A-D), including the RNA-dependent RNA polymerase (3D), that assists the virus in hijacking the molecular machinery of the host cell (24). These virus-mediated effects include increasing membrane permeability (viral protein 2B), inhibition of intracellular transport (viral protein 3A), host shut-down of cap-dependent translation (viral protein 2A) and inhibition of host transcription (viral protein 3C) (14). The positive strand RNA genome is then transcribed by the RNA-dependent RNA polymerase (viral protein 3D) to produce negative strand RNA genome, which is used as a template for viral replication (12). As the infection progresses, enteroviruses are capable of causing potent cytolytic effects. Eventually the cell is lysed and the newly synthesized virions are released.
Coxsackievirus Tropism and Pathogenesis

CVB3 is a very common viral infection, as approximately 75% of individuals in developed countries harbor enterovirus antibodies (21). In the acute or early stage of infection, the virus is capable of causing pancreatitis, myocarditis, and meningitis (18) (13) (15). More specifically, CVB3 targets acinar cells in the pancreas and cardiomyocytes in the heart (20). In the brain, CVB3 exhibits tropism for infiltrating nestin+ myeloid cells, neural progenitor and stem cells (NPSCs) and immature neurons (22) (9).

CVB3-mediated disease is particularly prevalent in newborn infants and the young (17). Although it has been proposed that the increased susceptibility in the young might be due to an immature immune response, a further explanation is that the virus preferentially targets cells that are actively progressing through the cell cycle (8). Whereas, the large number of proliferating cells found in neonates might serve to boost the number of target cells for ongoing CVB3 infection (11).

Recently, CVB3 has been linked to lasting sequelae in all of its target organs through the presence of viral genome. These include several long-term diseases, such as dilated cardiomyopathy in the heart and diabetes in the pancreas (4) (25). In the central nervous system (CNS), CVB3 infection has been linked to demyelinating diseases, learning disabilities, and schizophrenia (6) (3) (19). Thus, a greater knowledge of the acute phase of CVB3 infection in the CNS might be beneficial in illuminating mechanisms of virus dissemination and persistence.
Coxsackievirus Infection in Neural Progenitor and Stem Cells

Our laboratory has recently been determined that neural progenitor and stem cells (NPSCs) are highly susceptible to CVB3 infection. This was found in vivo through the use of our neonatal mouse model of CVB3 infection, and in tissue culture of primary NPSC cultures (9) (23). We have previously shown that nestin+ neural stem cells with type B stem cell morphology found within the subventricular zone (SVZ), a neurogenic region of the brain, were very susceptible to CVB3 infection and expressed high levels of viral protein (10). With these in vivo results in mind, we cultured primary NPSCs, or undifferentiated neurospheres, in order to more easily follow the course of infection.

We have recently determined that NPSCs were highly susceptible to both eGFP-CVB3 and dsRED-CVB3, a recently created recombinant virus expressing dsRED marker protein (23). Hence, we are able to follow live CVB3 infection with either eGFP or dsRED in these cultures. In addition, neurospheres have the ability to differentiate into precursors of all three cell lineages of the CNS: neurons, astrocytes, and oligodendrocytes (7). We have observed that these differentiated precursor cells are still susceptible to infection, although they support lower levels of CVB3 viral replication as compared to undifferentiated NPSCs. Furthermore, undifferentiated NPSCs have been found to support persistent CVB3 infection in tissue culture. We are currently analyzing what affects persistent infection may have on NPSC differentiation.
The Trojan Horse Model of Viral Dissemination

We hypothesize that CVB3, as well as other viruses, may spread throughout the body by means of trafficking immune cells (Figure I). This paradigm centers on the localization of the virus to its receptor in the tight junction (Figure IA). For example, the main receptor for CVB3, CAR, localizes to the tight junction, as well as the receptors for adenovirus, reovirus and hepatitis C virus (2). In addition, the presence of virus may induce the release of immune cell specific chemokines that aid in the recruitment of immune cells. Consequently, these cells move toward (Figure IB) and through (Figure IC) the tight junction, where virions are ready and waiting due to the localization of their receptor. The immune cells may become infected upon extravasation through the tight junction (Figure ID), thus spreading viral infection throughout the surrounding tissue in a “Trojan horse” fashion.

Our data showing myeloid cell infiltration after CVB3 infection in the neonatal CNS fits this model (22). We have demonstrated CAR localization in the tight junctions of the epithelial cells of the choroid plexus, where myeloid cells enter the CNS. Myeloid cell infection is not observed until the cells are in proximity of the tight junction. In addition, we detect infected myeloid cells moving further into the parenchyma of the brain. Further studies to analyze this model will involve labeling virions directly to see if they are associating with CAR in the tight junction. The phenomenon of infection, myeloid cell recruitment and virus dissemination has also been described for cytomegalovirus, in which a virally encoded chemokine homolog induced
myeloid cell recruitment into areas of viral infection. Thereafter, the infiltrating myeloid cells were readily infected and assisted in the dissemination of the virus throughout the tissue (16).

There are several scenarios in which viral localization to the tight junction, and thus trafficking immune cells, may be beneficial to viral dissemination. Targeting the tight junction puts the virus in the right location, while infecting immune cells gives the virus access to proliferative targets that may be more susceptible to infection. Finally, by infecting immune cells themselves the virus may be temporally shielded from the immune response, including neutralizing antibodies. The route of CVB3 dissemination from the gut to the brain, following fecal/oral infection, is currently unknown. We suggest that virion binding to tight junctions followed by the Trojan Horse model may be a unique strategy employed by CVB3 and other viruses. For reovirus, which can also spread from the intestine to the CNS, studies in receptor knock-out mice have shown that the virus loses its ability to disseminate to the CNS when the receptor is absent (1). Thus, localization of the viral receptor in the tight junction may be necessary for infection of immune cells and the circulation of virus throughout the host.
Figure I: Model for virus binding to the tight junction and handoff to trafficking immune cells.

(A) Virus (green) localizes to its given receptor (red) in the tight junction and the presence of virus may induce chemokine secretion (dark blue) from the epithelial or endothelial cells (grey). (B) Immune cells responding to chemokine secretion (light blue) move towards and (C) through the tight junction. (D) When moving through the tight junction the immune cells are exposed to virus and become infected, thus disseminating viral infection in the host.
REFERENCES


myeloid cells responding to coxsackievirus infection assists in the dissemination of virus within the neonatal CNS. J.Neurosci. 30(25):8676-8691.


CHAPTER I:

A Novel Population of Myeloid Cells Responding to Coxsackievirus Infection

Assists in the Dissemination of Virus within the Neonatal CNS
Development/Plasticity/Repair

A Novel Population of Myeloid Cells Responding to Coxsackievirus Infection Assists in the Dissemination of Virus within the Neonatal CNS

Jenna M. Tabor-Godwin, Chelsea M. Ruller, Nolan Bagalso, Naili Am, Robb R. Pagariqan, Stephanie Harkins, Paul E. Gilbert, William B. Klosses, Natalie A. Gude, Christopher T. Cornell, Kelly S. Doran, Mark A. Sussman, J. Lindsay Whitton, and Ralph Feuer

Enterovirus Infection in newborn infants is a significant cause of aseptic meningitis and encephalitis. Using a neonatal mouse model, we previously determined that coxsackievirus B3 (CVB3) preferentially targets proliferating neural stem cells located in the subventricular zone within 24 h after infection. At later time points, immature neuroblasts, and eventually mature neurons, were infected as determined by expression of high levels of viral protein. Here, we show that blood-derived Mac-1 mononuclear cells were rapidly recruited to the CNS within 12 h after intracranial infection with CVB3. These cells displayed a myeloid-like morphology, were of a peripheral origin based on green fluorescent protein (GFP)-tagged adoptive cell transplant examination, and were highly susceptible to CVB3 infection during their migration into the CNS. Serial immunofluorescence images suggested that the myeloid cells enter the CNS via the choroid plexus, and that they may be infected during their extravasation and passage through the choroid plexus epithelium; these infected myeloid cells ultimately penetrate into the parenchyma of the brain. Before their migration through the ependymal cell layer, a subset of these infected myeloid cells expressed detectable levels of nestin, a marker for neural stem and progenitor cells. As these nestin-positive myeloid cells infected with CVB3 migrated through the ependymal cell layer, they revealed distinct morphological characteristics typical of type B neural stem cells. The recruitment of these novel myeloid cells may be specifically set in motion by the induction of a unique chemokine profile in the CNS induced very early after CVB3 infection, which includes upregulation of CCL2. We propose that intracranial CVB3 infection may lead to the recruitment of nestin-positive myeloid cells into the CNS which might represent an intrinsic host CNS repair response. In turn, the proliferative and metabolic status of recruited myeloid cells may render them attractive targets for CVB3 infection. Moreover, the migratory ability of these myeloid cells may point to a productive method of virus dissemination within the CNS.

Introduction

Enterovirus infections have been previously associated with a wide range of neurological disorders in a clinical setting, including persistent encephalitis (Berger et al., 2006), white matter damage (Verbeek-MacLeod et al., 2006), and acute disseminated encephalomyelitis (Saitoh et al., 2004). Coxsackievirus B3 (CVB3) infections are relatively common and cause a number of human enterovirus-associated diseases, including pancreatitis, myocarditis, and aseptic meningitis (Whitton et al., 2005). Newborns in particular are highly susceptible to infection and disease (Romero, 2008). We have previously described the ability of CVB3 to infect neural stem cells in the neonatal CNS, induce apoptosis within infected neurons, and establish a persistent infection (Feuer et al., 2003, 2005, 2009). The choroid plexus was previously shown to be an early site of CVB3 replication (Feuer et al., 2003). However, a close examination of the infected choroid plexus at the cellular level has not previously been undertaken.

The choroid plexus remains a poorly understood organ in the CNS which has recently been shown to harbor previously unidentified host functions (Emerich et al., 2005). One of the critical functions of the choroid plexus is to regulate the production of CSF in the CNS. The choroid plexus also forms the blood–CSF barrier in the CNS (Ransohoff et al., 2003). The blood–CSF barrier is distinct from the blood–brain barrier (BBB) in that entry is controlled by the tight junctions of the choroid plexus cuboidal epithelium, as opposed to the endothelial cell layer comprising...
the B8R. Furthermore, the blood-CSF barrier may be an important entry point for activated immune cells (Ransohoff et al., 2003). Certain substances, such as trastuzumab, may be actively transported by the choroid plexus into the CSF (Dickson et al., 1996). However, many other functions have recently been described for the choroid plexus, including the production of growth factors (Shingo et al., 2003) and an active involvement in neurogenesis (Falk and Friesen, 2002).

Here, we more closely determined the involvement of the choroid plexus during the early stages of CVB3 infection in the neonatal CNS. The choroid plexus epithelial cells appeared to be spared from CVB3 infection. Instead, infected cells morphologically similar to myeloid cells were found clustered throughout the choroid plexus tissue and within the lateral ventricle. By analyzing serial immunofluorescence sections by microscopy, we carefully characterized the phenotype of myeloid cells undergoing extravasation through the columnar epithelium of the choroid plexus and eventually entering the parenchyma of the neonatal CNS. These cells were seen in abundance only after infection, suggesting that initial CVB3 infection in the CNS may induce chemoattractant molecules into the surrounding area. Furthermore, many recruited myeloid cells expressed neutin, a marker for neural stem and progenitor cells (Lendahl et al., 1990). We also examined the early induction of chemokines which might be responsible for the recruitment of these unique myeloid cells, and their possible fate as they enter the parenchyma of the neonatal CNS. We hypothesize that CVB3 may induce the migration of these unique myeloid cells by the upregulation of chemokines, including CCL12, to aid virus dissemination within the neonatal CNS.

Materials and Methods
Isolation and production of a recombinant coxsackievirus. The generation of a recombinant coxsackievirus expressing eGFP has been described previously (Feneu, 2002). Briefly, the CVB3 infections cells (pH3) (obtained from Dr. Kirk Knowlton at University of California at San Diego) was engineered to contain a unique SFI site which facilitates the insertion of any foreign sequence into the CVB3 genome (mPKS). For the recombinant CVB3 expressing dsRED (mPKS-2), dsRED gene was amplified from a dsRED expression plasmid (Clontech Laboratories) using dsRED sequence-specific primers with flanking SFI sites. The PCR product was cloned into mPKS1. Following transfection of HeLa RW cells with dsRED-CVB3 plasmid, infections virus was generated. All virus stock were grown on HeLa RW cells maintained in DMEM (Gibrogen) supplemented with 10% fetal bovine serum. Virus titrations were performed as described previously.

Mice and viral inoculations. Mice experimentation conformed to the requirements of the Animal Care and Use Committee and the National Institutes of Health. BALB/c and C57Bl6 mice were obtained from the Scripps Research Institute. Animal facilities or Harlan Sprague Dawley. Breeding pairs were checked every day, and 1-d-old pups were infected intracranially with 2 x 10^3 pfu or 1 x 10^5 pfu eGFP-CVB3, 5 x 10^7 pfu wild-type CVB3 (WT-CVB3), 5 x 10^7 pfu lymphocytic choriomeningitis virus (LCMV), or mock-infected with MEM. The procedure for intracranial (i.c.) inoculation of 1-d-old pups has been described previously (Feneu et al., 2003, 2006). Pups were killed at various time points between 12, 24, and 48 h postinfection (PT) by euthanization/CO2, followed by immediate decapitation. The brains were fixed by immersion in 10% neutral-buffered formalin for 2-4h, paraffin-embedded, and immunostained or stained with hematoxylin and eosin (H&E).

Adaptive transfer of GFP-expressing bone marrow-derived cells or newborn liver derived cells into recipient animals infected with CVB3. Adult bone marrow-derived cells (BMDMs) were isolated from the femurs of adult male GFP transgenic mice and washed three times with PBS solution. Alternatively newborn liver from actin promoter-GFP transgenic mice were homogenized into a single cell suspension and washed three times with PBS. A total of 1 x 10^7 GFP-expressing donor cells were injected intracranially (i.c.) into 1- to 5-d-old C57Bl6 recipient mice (Mason et al., 2005). Either simultaneously or 1 d later, recipient mice were infected intracranially with dSRED-CVB3 (2.5 x 10^5 pfu) or mock-infected with MEM. On days 1, 2, and 6 PI, the brain and livers from recipient mice were harvested, paraffin-embedded, and inspected for the presence of peripheral GFP-expressing donor cells and virus protein expression (dSRED) by immunofluorescence microscopy.

In situ hybridization. Our in situ hybridization procedure has been described previously (Feneu et al., 2003). Briefly, a digoxigenin-labeled antisense RNA (421 bases) probe for the 5' untranslated region of CVB3 was generated using the MAXscript In situ Transcription Kit (Ambion Inc.), as described by the manufacturer. In situ hybridization procedures were performed using the mNiAqacer On Situ Hybridization Kit (Ambion Inc.), as described by the manufacturer. The radio-labeled probe (10^7 cpm) was applied to deparaffinized sections, and the sample was sealed in a humidified chamber and incubated at 46°C for 18 h. After washing and RNase A treatment, slides were immersed in photographic emulsion, held at 4°C for 6 d, then developed. Finally, slides were stained with hematoxylin (1 min) and eosin (1.5 min), and mounted with Cytoseal (VWR International).

Myeloid cell quantification of the choroid plexus. Oil images (100 x) of H&E-stained brain sections were taken using a Zeiss Axiosvert 200 inverted microscope with an attached Zeiss ICM Color Camera. Brain sections of BALB/c mice infected with eGFP-CVB3, and C57Bl6 mice infected with GFP-CVB3, WT-CVB3, and LCMV at 12, 24, and 48 h postinfection were used. The brain sections with the most intact choroid plexus were used for imaging. Three images of the most representative regions were taken of each choroid plexus, using two animals per time point. Myeloid cells in and around the choroid plexus were counted based on morphology and location. Data were entered into GraphPad Prism 3.0 software, analyzed, and displayed graphically.

Immunofluorescence staining. eGFP (from eGFP-CVB3 or GFP-expressing donor cells) or dsRED expression was observed in unstained paraffin-embedded sections overlaid with PBS. For immunofluorescence staining, paraffin-embedded sections (5 and thickness) were deparaffinized with three washes in xylene and serial washes in 100%, 95%, 70% ethanol, followed by a wash in PBS and distilled water. Thereafter, high temperature antigen unmasking in 0.01 M citrate buffer, pH 6.0, was performed. The Mouse on Mouse Kit (Vector Laboratories Inc.) was used for primary antibodies derived from mouse. Two main immunofluorescence staining protocols were used. For GFP (1:100, ABS080; Milipore Corporation; or 1:100, ab3970, Abcam), nestin (1:50, MAAB53; Milli- pore Corporation); pellodo K07 (neat, RTU-K67-M1M1, Novocastraz Laboratories) RC2 (1:50, Developmental Studies Hybridoma Bank, Iowa City, IA), doubletortin (1:100, AER513; Millipore Bioscience Research Reagents), ZO-1 (1:100, MAB1520; Millipore Bioscience Research Reagents), CD11b (1:200, ab53187; Abcam Inc.), phospho-p44/42 (p89K/2; Th220/Try240) MAP Kinase 1:100, #9101, Cell Signaling Technology Inc.), Le-CD45 LS (1:500, #550455; Clone 2F10, BD Biosciences Inc.), Musabili (1:100, AER5977; Millipore Corporation), Iba1 (1:100 or 1:200, #416-2001, Wako Pure Chemical Industries, Ltd.), Mac-3 (1:200, #550292; BD Biosciences Inc.), pellodo C11 (neat, #22644A; Biocare Medical Inc.), and laminin (1:200, L. 9395; Sigma-Aldrich Inc.) antibodies; sections were blocked with 10% normal goat serum for 30 min, primary antibody was put on overnight at 4°C, goat anti-rabbit, anti-rat, or anti-mouse conjugated to biotin at 1:100, or bovine anti-chicken FITC (sc7300, Santa Cruz Biotechnologies) was applied for 30 min. Finally sections were incubated with streptavidin-Alexa Fluor 488 or streptavidin-Alexa Fluor 594 at 1:500 for 30 min, respectively. For CD11b (1:100, ab52628; Abcam Inc.), CD13 (1:200, ab19988; Abcam Inc.), CD54 (1:50, ab158; Abcam Inc.), CD11b (1:200, #550455; Abcam Inc.), CD31 (1:200, sc14462; Santa Cruz Biotechnologies Inc.), CCL12/MCP-5 anti-MCP-5 (K19 sc-9718, Santa Cruz Biotechnologies Inc.), e-Kit (1:20, AF1536; R&D Systems), Sc-I (1:20, R&D Systems Inc.), SC-230 (1:20, BD Biosciences Inc.), mGAR (1:100, Santa Cruz Biotechnologies Inc.), Transferrin Signal Amplification (TSA) Kit #45 (Invitrogen Inc.) was used according to manu-
manufacturer’s protocol. Briefly, sections were quenched with 3% H2O2 for 1 h, blocked in 1% Blocking Solution for 1 h, incubated in primary antibody in 1% Blocking Solution overnight at 4°C, goat anti-rabbit antibody conjugated to biotin at 1:100 in 1% Blocking Solution was applied for 2 h, streptavidin-HRP was applied at 1:100 in 1% Blocking Solution for 1 h, and last sections were incubated with tyramide-SNARF at 1:100 in Amplification Buffer for 20 min. Specificity controls for immunostaining included sections stained in the absence of primary antibody, or in the presence of rabbit IgG control antibody at 0.1 μg/ml (Vector Laboratories, Inc.), rat IgG K type control (1:100, Clone 13-34, #59972, BD Biosciences, Inc.), or mouse IgG2a, negative isotype control (1:20, Clone D07, CRL-6001, Millipore Bioscience Research Reagents). For detection of DNA/nuclei, sections were overlaid with VectorShield Mounting Medium with DAPI (4′,6-diamino-2-phenylindole dihydrochloride) (Vector Laboratories). Sections were observed by fluorescence microscopy (Zeiss Axiovert 200 inverted microscope) for GFP (green), the indicated cellular marker (red) and DAPI nuclear staining (blue). Green, red, and blue channel images were merged using Axiovision software.

Confocal microscopy images. Confocal images were captured using a Zeiss Laser Confocal Scanning Microscope (LCSM) using the latest Zen 2009 Zeiss software suite (Carl Zeiss Inc.). All serial optical 8 bit image sections [obtained with a 63× Plan-Apochromat (1.4 numerical aperture) objective at 0.3 mm interval step slice] were imported and spatially reassembled using Imaris software (Bitplane Inc.) to generate a three-dimensional representation of the tissue of which single snapshots and movies were created. Maximum projected two-dimensional images were created in Image Pro Plus (Media Cybernetics Inc.) or ImageJ (National Institutes of Health, Bethesda, MD) software for the montage image panel presentation.

Quantitative real-time reverse transcriptase PCR for CBV3. 5′-untranslated (UTR) CBV3-specific primers (forward primer 5′-CACTCGTGATACGTTCTCTCTCTCTACCT3′; reverse primer 5′-GAGGGCATCCCTGATGGCC3′), and a FAM/TAMRA-labeled probe (5′-CTCGGCACACCCACGGTCT3′) were used for the Taqman method of quantitative real-time reverse transcriptase (qRT)-PCR. A total of 1 μg of total RNA from the samples was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen Inc.) according to the procedure described by the manufacturer. Separate RT reactions were performed for the reverse primer or forward primer to quantitate the number of positive and negative-sense viral genomes, respectively. RT reactions were treated with 1 μl of RiboLock R (Invitrogen Inc.) to remove RNA complementary to the cDNA. PCR amplification was done using Platinum Quantitative PCR SuperMix-UDG ready to use cocktail (Invitrogen Inc.) containing all components except the amplification primers and FAM/TAMRA-labeled probe, as described by the manufacturer. Quantitative analysis of viral RNA was performed using a Bio-Rad IQ5 Real-Time PCR System using 96 well optical reaction plates heated to 50°C for 2 min to digest dUTP containing contaminants, followed by 95°C for 2 min to desensitize UNG and activate Platinum TaqDNA polymerase. Forty cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 30 s were carried out. To normalize the amount of RNA in each sample, separate RT reactions using random primers instead of CBV3-specific primers were performed and amplified for 18S ribosomal RNA. The 18S ribosomal products were amplified with 18S forward (5′-CGCTACTACCCCATCAAGGA3′)- and reverse (5′-GCTGAGGTTCTGGGAGGCT3′) primers, and included a FAM/TAMRA (5′-TACTGCAGCAGAGGGCTCAGC3′) probe. The standard curve was generated using in vitro transcribed pBS-CBV3 RNA. The standard curve was based on Cq values (cycle number at which fluorescence crossed the threshold) and Cq values from unknown samples (normalized for 18S ribosomal RNA signal) were compared with the standard curve to determine viral RNA copy numbers.

Quantitative real-time reverse transcriptase PCR for LCMV. LCMV glycoprotein (GP)-specific primers (forward primer 5′-CATTCATCCGCGCATTATTCAGG3′; reverse primer 5′-GAACCTGCTGCTTCTCGCAAAC3′) and a FAM/TAMRA-labeled probe (5′-TTTTGACCGGCGCATGTTCTG3′) were used for Taqman method of quantitative real-time RT-PCR. RT-PCR was performed as described for CBV3. Forty cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 30 s were carried out. To normalize the amount of RNA in each sample, separate RT reactions using random primers instead of LCMV-specific primers were performed and amplified for 18S ribosomal RNA. The 18S ribosomal products were amplified with 18S forward (5′-CGCTACTACCCCATCAAGGA3′)- and reverse (5′-GCTGAGGTTCTGGGAGGCT3′) primers, and included a FAM/TAMRA (5′-TACTGCAGCAGAGGGCTCAGC3′) probe. The standard curve was generated using the LCMV p60-5 GP plasmid, a generous gift from Dr. Juan Carlos de la Torre (Department of Immunology and Microbial Sciences, The Scripps Research Institute). The LCMV GP was isolated from the plasmid using restriction enzyme digest (BfiI and EcoRI) and gel purified. Isolated GP DNA was then quantified using a spectrophotometer, converted to copy number, and serially diluted (10-fold dilutions). The standard curve was based on Cq values. Cq values from unknown samples (normalized for 18S ribosomal RNA signal) were compared with the standard curve to determine viral RNA copy numbers.

Microarray analysis. Total RNA was isolated from homogenized brain samples using the Qiagen RNeasy Kit. RNA was sent to the BIOGEM Microarray Core at University of California at San Diego and analyzed for quality. Samples were reverse transcribed to cDNA and then in vitro transcribed to cRNA with the incorporation of biotin. cRNA was hybridized to Illumina Whole Mouse Genome 6 Samples chips, which analyze ~45,200 genes. Hybridized chips were incubated with Cy3- and Cy5-labeled cRNA, and then read using the Illumina BeadArray Station. Normalized gene expression was used to examine differences and similarities in the following samples: GFP-CBV3, WT-CBV3, LCMV, and mock-infected C57BL/6 mice at 12 h postinfection. Relative chemokine/cytokine expression levels were identified via microarray analysis. More specifically, gene expression levels in the brain of GFP-CBV3 or LCMV-infected neonatal mice were compared with mock-infected control mice. The fold change in gene expression levels for known chemokines compared with mock-infected control mice was analyzed to determine possible chemotractant molecules associated with myeloid cell recruitment through the choroidal plexus specifically following coxsackievirus infection.

Apoptosis staining. Detection of cells undergoing apoptosis was evaluated using the ApopTag Red Apoptosis Detection Kit (Millipore Inc.), as specified by the manufacturer. Briefly, paraffin-embedded sections were deparaffinized and pretreated with protease K (20 μg/ml) for 15 min. Equilibration buffer was added directly onto the specimen, after which terminal deoxynucleotidyl transferase (TdT) enzyme in reaction buffer was added for 1 h at 37°C. Sections were washed in working strength Stop/Wash buffer for 10 min. Presoaked working strength anti-digoxigenin conjugate (rhodamine) was added to the sections and incubated at room temperature for 30 min. The samples were washed with PBS and observed by fluorescence microscopy, as described above.

Statistical analysis. Statistical significance was determined using one-way ANOVA with Newman–Keuls post hoc comparison for myeloid cell quantification (see Fig. 3H). A 6 × 2 ANOVA with group (BALB/c-eGFP-CBV3, C57BL/6-eGFP-CBV3, C57BL/6-WT-CBV3, C57BL/6-LCMV, C57BL/6-MOCK, and C57BL/6–CCL12) as a between-group variable and time (12 h, 24 h, 48 h) as a within-group variable, and average number of myeloid cells per animal as the dependent variable; revealed a significant main effect of group F(5,50) = 8.97, p < 0.001, a significant main effect of time F(2,100) = 17.25, p < 0.001, and a significant group × time interaction F(10,170) = 3.95, p < 0.05. A Newman–Keuls post hoc comparison test of the main effect of group revealed that the average number of myeloid cells per animal in BALB/c-eGFP-CBV3, C57BL/6-eGFP-CBV3, C57BL/6-WT-CBV3, and C57BL/6–CCL12 was significantly higher (p < 0.05) from C57BL/6–MOCK, while C57BL/6-LCMV was the only group not significantly higher. In addition, there was no difference observed between the C57BL/6–WT-CBV3 and C57BL/6–CCL12 groups. A Newman–Keuls post hoc comparison test of the main effect of time revealed that the average myeloid cells per animal was significantly higher (p < 0.05) at the 24 h time point compared with both the 12 and 24 h time points. A Newman–Keuls post hoc comparison test of the group × time interaction revealed that the average number of myeloid cells per animal in C57BL/6–eGFP-CBV3 was significantly higher (p < 0.05) from C57BL/6–MOCK and C57BL/6–
LCMV at the 12 h, 24 h, and 48 h time points. For apoptosis quantification in the choroid plexus (see Fig. 11.4), a one-way ANOVA with group (C57BL/6−eGFP−CVB3, C57BL/6−eGFP−CVB3, and BALB/c−eGFP−CVB3) as a between-group variable, and Apoptag signal per choroid plexus as the dependent variable, revealed significant between-group differences \( F_{2,14} = 4.84, p < 0.05 \). A Newman–Keuls post hoc comparison test revealed that Apoptag signal per choroid plexus was significantly higher \((p < 0.05)\) in C57BL/6−eGFP−CVB3 and BALB/c−eGFP−CVB3 compared with C57BL/6−eGFP−CVB3 and C57BL/6−eGFP−CVB3.

**Results**

Myeloid cells migrating into the CNS are highly susceptible to infection

Previously, we determined the ability of CVB3 to target neurogenic regions of the CNS at early time points following infection. However, the choroid plexus was also highly susceptible to infection. Upon closer inspection, the choroid plexus epithelial cells appeared to be spared from CVB3 infection. Instead, H&E staining revealed the presence of cells with myeloid-like morphology which appeared to infiltrate through the vascular stroma (Fig. 1A, black arrows). These cells were seen in abundance only after infection, suggesting that initial CVB3 infection may induce chemotactic molecules into the surrounding area. In addition, the presence of red blood cells external to the vascular regions of the choroid plexus suggested some hemorrhaging due to infection. In contrast, no infiltration of myeloid-like cells was observed for mock-infected control sections (Fig. 1B). Higher magnification of the infected choroid plexus revealed the extravasation of myeloid-like cells through the tight junctions of the choroidal epithelial cells (Fig. 1C, black arrows) and penetration through the ependymal cell layer (ECL) of the CNS (Fig. 1G, notched cyan arrow). These cells accounted entirely for the presence of viral signal in the choroid plexus and lateral ventricle, as shown by in situ hybridization using a CVB3-specific 5'-UTR probe (Fig. 1D, black signal), although viral signal was also identified (as published previously) near the ECL. In contrast, choroid plexus epithelial cells were generally spared from infection. Similar results were obtained when sections were inspected for viral protein expression (Fig. 1E, eGFP signal).

**Infected myeloid cells extravasate through the choroid plexus and into the ependymal cell layer**

Higher magnification of fluorescent microscopic images revealed the apparent stepwise migration of infected myeloid cells from the vascular stroma of the choroid plexus, through the epithelial cell layer, across the CSF of the lateral ventricle, and into the parenchyma of the brain (Fig. 1F, eGFP signal). These results suggest a route of entry for CVB3 through the blood–CSF barrier of the neonatal CNS using cells of the myeloid lineage, similar to what has been observed for HIV and the blood–brain barrier (Egenina et al., 2006). Extravasation of infected myeloid cells through the choroid plexus (CP) columnar epithelium was observed in sections immunostained with laminin and zona occludens 1 (ZO-1) antibodies (Fig. 2A, B, respectively). In higher-magnification images, infected myeloid cells were observed in the process of extravasation through laminin 1 (red) basement membrane outlining the capillaries of the choroid plexus (Fig. 2C, small gray arrows). Despite the early age of infected pups, ZO-1 staining (red) identified the tight junctions of the columnar epithelium which organizes the blood–CSF barrier in the CP (Fig. 2D). The blood–CSF barrier appeared to be predominantly intact, and infected myeloid cells could be seen trafficking through the columnar epithelium (Fig. 2D, small gray arrows). The acti-
Ki67 was routinely cytoplasmic in infected myeloid cells (Fig. 2G, white arrows), as opposed to the normal nuclear distribution within uninfected cells found nearby (Fig. 2G, notched cyan arrows). Similar, the majority of infected myeloid cells expressed high levels of pERK1/2 in the cytoplasm, although other infected myeloid cells showed little to no expression (Fig. 2H, small white arrows). Both cytoplasmic and nuclear distribution of pERK1/2 has been described previously (Rochaio et al., 2009). These results indicate that the majority of myeloid cells were undergoing proliferation or were highly activated, which might assist CBV3 replication. High expression levels of the CBV3 receptor [murine c-csk and adenovirus receptor (mCAR)] was observed in regions within the tight junctions of the columnar epithelium (overlapping with ZO-1 and in the SVZ (Fig. 2I)). In addition, detectable levels of mCAR were seen on infiltrating myeloid cells (Fig. 2J, white arrows).

We more carefully inspected the process of myeloid cell extravasation by confocal microscopy and IMARIS 3D analysis (Fig. 3). The progressive infiltration of infected myeloid cells through the basement membrane was observed in lamina (red)-stained sections of the choroid plexus at 12 and 24 h PI. Confocal microscopy revealed the progressive entry of infected myeloid cells through the tight junctions of the choroid plexus columnar epithelium. In addition, a 3D IMARIS movie representation was created at 12 and 24 h to inspect infected myeloid cell entry (Supplemental Movies 1 and 2, respectively, available at www.imaris.com as supplemental material). Higher magnification (Fig. 3, zoom 1) revealed that myeloid cells expressed high levels of viral protein (green) only upon entry through the basement membrane. No eGFP" myeloid cells were seen within the capillaries of the choroid plexus, indicating that infection of these cells occurred during myeloid cell extravasation (Fig. 3, gray-scale images of each color channel). IMARIS 3D analysis with a diminishing lamina label more clearly outlined the entry of infected myeloid cells through the basement membrane of the choroid plexus (Fig. 3, zoom IMARIS 3D with diminishing lamina label). Higher-magnification images showed the process of diapedesis of infected myeloid cells into the surrounding lateral ventricle (Fig. 3, zoom 2—white arrows).

Myeloid cells may be recruited through the blood-CSF barrier by a unique set of chemokines which include CCL12 expression within the choroid plexus

RNA viruses are known to upregulate key chemokines, cytokines and type I interferon response genes (Takemasa and Akira, 2010).

We expected that novel chemotactic factors may be upregulated to induce myeloid cell recruitment in the neonatal CNS specifically in response to CBV3 infection. Such chemotactic molecules may also have a direct role on the recruitment of bone marrow stem cells into damaged tissues (Dar et al., 2006). We used Illumina BeadArray Technology (MouseWG-6 v2 Expression Beadchip) and the BeadChip BioMedical Genomics Microarray Facility (Department of Medicine, UCSD) to identify novel genes upregulated in the neonatal CNS during nestin + myeloid recruitment. RNA was isolated from mock-infected neonatal mice, or neonatal mice infected with eGFP-CBV3, wtCBV3 and LCMV at 12 h PI. By comparing CBV3-infected, LCMV-infected and mock-infected animals using Illumina’s genome-wide gene expression profiling, we were able to analyze gene expression alterations for nearly 46,000 transcripts. A number of chemokines were shown to be upregulated following CBV3 infection compared with mock-infected control mice (Fig. 4A). Chemokines upregulated in response to eGFP-CBV3 infection...
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Figure 3. Progressive extravasation of infected myeloid cells through the basement membrane as determined by confocal microscopy and IMARIS 3D analysis. Neonatal mice were infected intracranially with eGFP-CVB3, and the brains were harvested 12 and 24 h PI. The kinetics of myeloid cell migration through the basement membrane (outlined by laminin staining in red) was observed by confocal microscopy and IMARIS 3D analysis at 12 h (top) and 24 h PI (bottom). Low magnification of immunofluorescence images showed infected myeloid cell migration (green) through the tight junction of the choroid plexus epithelium. Gray-scale images of all three colors (virus, green, laminin, red, DAPI, blue) at 12 and 24 h PI revealed the intensity and organization of the myeloid cell infiltration in greater detail. To better visualize myeloid cell entry, IMARIS 3D with diminishing laminin label (diminishing red) was performed on a higher magnification (zoom 1) for both 12 and 24 h PI images. Higher magnification of zoom 1 revealed the extravasation of infected myeloid cells through the basement membrane (zoom 2, white arrows). Original images were obtained with a 63 x Plan-Apochromat objective at 0.5 mm interval step sizes (as described in Materials and Methods).

included CCL7, CCL9, and CCL4. In particular, extremely high levels of CCL12 were induced following eGFP-CVB3 infection. However, cytokines and interferon response gene expression were not significantly different between the treatments, except for a moderate increase in IRF-1 and OAS1 g for eGFP-CVB3-infected samples (data not shown). Next, we wanted to determine the location and level of CCL12 protein in the neonatal CNS by immunofluorescence staining. CCL12 (red) was highly expressed in eGFP-CVB3-infected samples as early as 12 h PI (Fig. 4B). Furthermore, higher-magnification images indicated that protein expression was localized to the to the choroid plexus and ependymal cell layer (Fig. 4D). In contrast, little or no CCL12 signal was observed in mock-infected samples (Fig. 4C,E).

Myeloid cell infiltration is specific to coxsackievirus infection. To determine whether myeloid cell infiltration was specific to CVB3 infection, an additional RNA virus, LCMV, was analyzed concurrently with CVB3-infected samples at 12, 24, and 48 h PI. Myeloid cell infiltration was observed in the choroid plexus of both neonatal BALB/c and C57BL/6 mice infected with GFP-CVB3 (Fig. 5A,B, respectively). Higher magnification, showed the characteristic myeloid cell infiltration (Fig. 5C, black arrows). A relatively low inoculum (5 × 10^3 PFU, i.c.) of wild-type CVB3 (wt CVB3) also led to a limited induction of myeloid cell infiltration (Fig. 5D). In contrast, little or no myeloid cell infiltration was observed for either LCMV or mock-infected neonatal C57BL/6 mice (Fig. 5E,F, respectively). Also, intracranial administration of CCL12 was found to induce the recruitment of myeloid cells independent of CVB3 infection, albeit at a lower level (Fig. 5G).

Myeloid cell recruitment was quantified by counting the number of cells present in high-magnification images in three independent regions of the choroid plexus for two animals per treatment (Fig. 5H). Using this quantitative methodology, both neonatal BALB/c and C57BL/6 mice infected with eGFP-CVB3 induced high numbers of myeloid cells when compared with mock-infected control mice (p < 0.05 using ANOVA with Newman–Keuls post hoc comparison). A lower number of myeloid cells was observed in neonatal mice given a substantially lower inoculum of wtCVB3 or intracranially inoculation of CCL12 chemokine, compared with the mice infected with a high dose of eGFP-CVB3. The small increase in the number of myeloid cells for either wtCVB3 or CCL12 inoculation remained signifi-
Infected myeloid cells near the choroid plexus express both neural stem/precursor cell and myeloid lineage markers. Remarkably, immunofluorescence data revealed that many CVB3-infected myeloid cells in the choroid plexus (eGFP) expressed nestin (red), a marker which identifies neural stem and progenitor cells (Lendahl et al., 1990) (Fig. 6A, C). As expected, the majority of nestin staining was observed within the SVZ. Surprisingly, numerous infected myeloid cells located within or near the choroid plexus expressed high levels of nestin. These infected myeloid cells also expressed high levels of Mac3 (red), an antigen normally found on myeloid-lineage cells (Fig. 6B, D) (Ho and Springer, 1983). Furthermore, numerous Mac3+ cells were found within the SVZ, suggesting their eventual entry into the parenchyma of the brain. Isotype control staining for both nestin and Mac3 demonstrated the specificity of our immunofluorescence methodology in these sections (Fig. 6E, F, respectively). Higher magnification of sections in Figure 6A revealed nestin+ cells morphologically similar to that of myeloid cells located adjacent, yet distinct, from the columnar epithelium of the CP and the ependymal cell layer (Fig. 7A, C, E). These nestin+ myeloid cells were not only distinct from the columnar epithelium of the choroid plexus and the ependymal cell layer, but exhibited morphology similar to that of type B neural stem cells with cellular protrusions penetrating the ECL and contacting the lateral ventricle. Identical sections used for fluorescence microscopy as described above were stained by H&E to reveal infected cells having characteristic myeloid morphology and located adjacent to the choroid plexus or within the lateral ventricle (Fig. 7B, D, F, black arrows). Interestingly, many infected cells expressed nestin in a polarized manner within discrete cellular regions in the apparent direction of cell movement. A few infected myeloid cells in other sections expressed nestin near ciliated structures or cellular processes extending into the ependymal cell layer, made apparent after H&E staining of the same section (Fig. 7G, H). These sections showed a staining pattern suggestive that functionally, nestin (an intermediate filament) may play a direct role in cell migration, which might fit well with what is known about intermediate filaments, cell motility (Helfand et al., 2003) and neuronal progenitor cell progression (Doetsch et al., 1999). We also observed a number of infected myeloid cells near the choroid plexus or within the ependymal cell layer expressing a radial glial marker (Fig. 7I, J) or a neuronal precursor cell marker (Fig. 7K, L).

Macrophages/microglia engulphed CVB3-infected myeloid cells entering the CNS

Within 12 and 24 h PI, Iba1+ macrophages/microglia were seen responding to CVB3 infection (Fig. 8). Iba1+ cells were observed near the choroid plexus and within the subventricular zone (Fig. 8A). In contrast to Mac3+ staining results, infected myeloid cells in the choroid plexus did not express Iba1, a marker for activated monocytes/microglia/macrophages (Ito et al., 2001). Instead, Iba1+ macrophages/microglia were seen engulfing multiple infected myeloid cells (Fig. 8B–D, white arrows). Macrophage/microglia engulfment of CVB3-infected cells in the CNS has been described previously; although at later time points (Fener et al., 2009). Higher magnification revealed the presence of Iba1+ microglia/macrophages (macrophage/microglia cells that are globular and swollen following phagocytosis of CNS debris) engulfing virus-infected cells in some sections (Fig. 8B). Similar results illustrat-
ing the phagocytosis of multiple infected-myoelid cells in the CNS were also seen 24 h PI following either Iba1 (Fig. 8E, G, white arrows) or CD11b immunofluorescence staining (Fig. 8F, H, white arrows).

Progressive increase in Iba1+ macrophages/microglia and engulfment of CVB3-infected myeloid cells undergoing apoptosis

We observed a progressive increase in Iba1+ macrophages/microglia within the choroid plexus over 48 h PI (Fig. 9). In contrast to the appearance of infected myeloid cells (green) as soon as 12 h PI, the kinetics of macrophage/microglia migration to the choroid plexus was delayed. Very few Iba1+ macrophages/microglia (red) were seen at 12 h PI (Fig. 9A, B). At 24 h PI, the number of Iba1+ macrophages/microglia increased at a point in time when infected myeloid cells appeared within the SVZ (Fig. 9C, D). By 48 h PI, the integrity of the choroid plexus appeared compromised, and Iba1+ macrophages/microglia were found in high numbers (Fig. 9E, F). Intriguingly, Iba1+ macrophages/microglia actively engulfed the few remaining infected myeloid cells within the choroid plexus (Fig. 9G). As shown by ApoTome analysis or “grid projection” which allows for optical sectioning on a motorized Zeiss Axio Observer fluorescence microscope, Iba1+ macrophages/microglia could be visualized engulfing multiple infected myeloid cells (Fig. 9H, z-stack image, white arrows also shown in supplemental Movie 3, available at www.fronterolab.org as supplemental material). An x,z view (top part of image) and a y,z view (right-hand part of image) in Figure 9H show the inter-
Adoptively transferred donor cells from actin promoter-GFP transgenic mice inoculated intrahepatically follow a similar path to the CNS following CVB3 infection

We wished to reveal the peripheral origin of infected myeloid cells responding to CVB3 in the CNS. Therefore, adult BMDCs, or alternatively, newborn liver-derived cells from actin promoter-GFP transgenic mice were inoculated intrahepatically into 1- or 3-d-old recipient C57BL/6 mice, as described previously (Massengale et al., 2005). These recipient mice were subsequently infected (intracranially) with a recombinant coxsackievirus expressing dsRED protein either simultaneously, or 24 h following the intrahepatic inoculation of GFP-expressing donor cells (Fig. 10A). On days 1, 2, and 6 postinoculation, the brains of recipient mice were inspected for GFP+ myeloid cells near the choroid plexus and within the parenchyma similar to those observed in Figure 1. Surprisingly, the inoculation of newborn liver-derived donor cells (the site of hematopoesis in newborn mice) routinely led to greater engraftment in the recipient liver, compared with adult BMDCs (Fig. 10B,C). Intriguingly, the appearance of numerous GFP+ myeloid-like cells within the brain was evident as soon as 24 h postinoculation (POI) in simultaneously infected mice, or alternatively, as soon at 48 h POI in mice infected 1 d after intrahepatic inoculation (Fig. 10D). GFP donor cells with myeloid morphology, similar to infected myeloid cells seen in Figure 6, were observed adjacent to the ECL within the SVZ, near the choroid plexus, and within the olfactory bulb (Fig. 10F,H,I, respectively). Furthermore, mock-infected animals failed to display an influx of GFP+ donor cells (Fig. 10E); although a few GFP+ perivascular macrophages were seen near blood vessels in the brain parenchyma (data not shown). By day 6 POI, GFP+ donor cells, including a few cells with neuronal-like homology, were observed within the cortex (Fig. 10J,K). The majority of GFP+ donor cells did not express high levels of viral protein, and the degree of infection (as judged by dsRED expression) appeared not to be as robust as with eGFP-CVB3. We attribute this discrepancy to the relatively low initial viral inoculum (2.5 × 10^6 pfu, i.e., reflective of our marginal dsRED-CVB3 working stock, and a greater technical difficulty in observing dsRED expression compared with eGFP (fluorescence requires dsRED tetramer formation, and dsRED protein has a tendency to aggregate). Our recombinant CVB3 expressing dsRED may also be of lower viral fitness compared with eGFP-CVB3 (data not shown), perhaps due to the greater toxicity of dsRED in endothelial cells. Also at later stages of infection (day 6 POI), viral protein levels may be waning in surviving cells. Nonetheless, these data suggest a peripheral origin of myeloid cells in the choroid plexus and CNS responding to CVB3 infection.

Induction of apoptosis in the choroid plexus following CVB3 infection

We determined the ability of eGFP-CVB3 to induce apoptosis within infected myeloid cells by TUNEL (ApopTag staining). The amount of ApopTag signal was quantified on a per section basis and compared with mock-infected control samples (Fig. 11A). For both C57BL/6 and BALB/c mice, high levels of ApopTag signal was observed within infected myeloid cells adjacent to the

cycle/activation, and myeloid/monocyte/macrophage markers. Infected myeloid cells consistently expressed cell cycle/activation markers. Also as described above, these cells expressed relatively high levels of nestin and Mac3. In contrast, most infected myeloid cells failed to express high levels of other informative neural stem/progenitor cell or hematopoietic cell markers.

Expression of molecular markers by CVB3-infected myeloid cells in the CNS

A summary of antibodies used for immunofluorescence staining to identify recruited myeloid cells responding to CVB3 infection at 12 and 24 h PI is shown in Table 1. In general, antibodies were used to determine neural and hematopoietic stem cell, cell
Figure 7. Infected myeloid cells in the ventricle express nestin and have the morphology of type B stem cells as they migrate through the ECL and into the parenchyma of the brain. Sagittal brain sections from infected pups were harvested 24 h Pi, deparaffinized, and immunostained with antibodies against nestin, RC2, and doublecortin (DC). A, E, Infected myeloid cells near the choroid plexus (A) (cyan notched arrow), adjacent to the ECL (white arrow), and within the lateral ventricle (E) (white arrow) expressed high levels of nestin. C, High magnification of A illustrated the cytoplasmic and extended cellular processes typical of nestin staining, extending within the ECL. B, D, F, Adjacent sections were stained with H&E to reveal the myeloid-like morphology of these nestin+ cells (black arrow) in different regions of the lateral ventricle. G, H, A small number of infected myeloid cells (G) in transit through the ECL and moving toward the SC expressed nestin (red), cyan notched arrow in high-magnification inset in cytoplasmic locations adjacent to extended cellular processes, visualized in the identical section stained by H&E in H (cyan notched arrow in high-magnification inset). Nestin expression appears to be localized in the direction of movement. J, L, A few infected myeloid cells (white arrow) near the choroid plexus and within the ECL expressed RC2, a marker for radial glial cells. K, I, Some myeloid cells (white arrow) adjacent to the ECL also expressed doublecortin, a marker for neuronal precursor cells. LV, lateral ventricle. A, B, E–H, I–L, 63× objective; C, D, 100× objective with a further 4×-fold magnification; J, 100× objective.

chloroid plexus 24 h Pi. Little to no ApopTag signal was seen in C57BL/6 mock-infected control sections. The ApopTag signal levels were statistically significant (p < 0.05) using ANOVA with Newman–Keuls post hoc comparison for infected C57BL/6 and BALB/c mice, compared with mock-infected control samples. Representative fluorescent microscopic images of infected C57BL/6 and BALB/c neonatal mice revealed high levels of ApopTag signal (red) in myeloid cells adjacent to the choroid plexus (Fig. 11 B, C, respectively). Higher magnification showed ApopTag signal predominantly within infected eGFP+ myeloid cells (Fig. 11 E, F). In contrast, little to no ApopTag signal was observed in mock-infected mice (Fig. 11 D, G).

Possible outcome of CVB3-infected myeloid cells following their migration into the parenchyma of the CNS. Our present model suggests that upon inoculation, eGFP-CVB3 virions may provisionally bind to the tight junctions of the choroid plexus epithelial cells (Fig. 11 H). The induction of key chemokines specific to CVB3 infection, including CXCL12, may play a role in the recruitment of unique myeloid cells through the choroid plexus. Upon extravasation through the tight junctions of the choroid plexus epithelial cells, eGFP-CVB3 virions may target these highly activated myeloid cells, thereby leading to their infection and rapid expression of viral proteins (including eGFP). Infected myeloid cells may eventually enter the lateral ventricle and travel through the ependymal cell layer. Presently, our ability to follow infected myeloid cells through the ependymal cell layer into the parenchyma of the CNS is limited. However, our existing data indicate several possible outcomes for these cells as they migrate into the parenchyma. (1) Infected myeloid cells may undergo apoptosis and be engulfed by Iba1+ macrophages/microglia before entering the parenchyma. (2) Infected myeloid cells may reach the parenchyma of the brain and undergo virus-induced apoptosis shortly thereafter. (3) Iba1+ macrophages/
microglia may engulf infected myeloid cells within the parenchyma. (4) Infected myeloid cells may remain for extended periods of time within the CNS and eventually contribute to CVB3 persistence, as described previously (Feuer et al., 2009). Additionally, these cells may assist in the dissemination of CVB3 deep within the CNS. (5) The production of infectious virus by these cells may contribute to the infection of type B neural stem cells, progenitor cells, or neurons; as shown previously (Feuer et al., 2003, 2005). (6) Finally, given their ability to express neural stem cell and progenitor cell markers, infected myeloid cells may exhibit long-term effects in the brain. These effects may, either indirectly or directly contribute to neurogenesis and/or CNS repair. The outcome of such a scenario might be the eventual generation of infected neurons which eventually migrate into the cortex and olfactory bulb and contribute to viral persistence. Given the plasticity of cells in a developing neonate and the unique immunological environment produced following CVB3 infection, such an outcome might be a possibility.

Discussion

Our recent studies on coxsackievirus B3 (CVB3) tropism and pathogenesis in our neonatal mouse model of CNS infection have allowed us to make some intriguing observations. First, using a recombinant CVB3 expressing GFP, we determined that this vi-
Table 1. Expression of molecular markers by myeloid cells recruited into the CNS

<table>
<thead>
<tr>
<th>Neural stem cell markers</th>
<th>Hematopoietic/monomuclear stem cell markers</th>
<th>Cell cycle/activation markers</th>
<th>Microglial/macrophage markers</th>
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<tr>
<td>Nestin</td>
<td>CD105</td>
<td>K67 (cytoplasmic)</td>
<td>Mac3</td>
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<td>+/-</td>
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<td>Microglial</td>
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<td>Res (Ki67)</td>
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The results of immunoﬂuorescence staining for neural stem and progenitor cell markers, hematopoietic/mononuclear stem cell markers, cell cycle/activation markers, and microglial/macrophage markers in uninfected myeloid cells +/− in all three time points are described. The relative level of expression for each marker is represented as a range of high expression (++++) to low expression (+). Recurrently myeloid cells expressed high levels of cell cycle/activation markers. However, few cells retained neural stem and progenitor cell markers except for nestin and Mac3 which were expressed on uninfected myeloid cells.

The choroid plexus, an invagination of modified ependymal cells forming epithelium, may be a unique microenvironment and has been implicated in regulating neurogenesis (Krizhanovsky and Ben-Arie, 2006) and the immune response in the CNS (Reboldi et al., 2009). The recruitment of myeloid-like cells appears to be CBV3-specific, given that an unrelated RNA virus [lymphocytic choriomeningitis virus (LCMV)], failed to stimulate myeloid cell recruitment through the blood–CNS barrier. Also, a unique spectrum of chemotactic factors were expressed specifically following eGFP-CBV3 infection. In particular, CCL12, a chemokine known to induce the recruitment of monocytes, was highly expressed in regions of the choroid plexus and the ependymal cell layer. Furthermore, intracranial inoculation of CCL12 into neonatal mice independently induced the recruitment of myeloid cells through the choroid plexus, albeit at a lower level compared with eGFP-CBV3 infection (Feng et al., 2011). Also, it remains to be determined whether myeloid cells recruited following CCL12 inoculation are identical to those induced following CBV3 infection. Injection of a single chemokine may not completely mimic the microenvironment induced by CBV3 necessary to attract these novel myeloid cells. Perhaps the injection of a “chemokine cocktail” might more accurately recreate the immune response induced following CBV3 infection.

The temporal distribution of these infected cells suggests their extravasation through the vascular stromal areas of the choroid plexus columnar epithelium, across the CSF of the ventricles, and the continued migration across the ependymal cell layer into the parenchyma of the brain. Although these cells may be contributing to the immune response against CBV3 infection in the CNS, few signs of inflammation other than reactive microgliosis were evident in the brain parenchyma shortly after infection (Feuer et al., 2009). Furthermore, these myeloid cells failed to express Iba1, a marker for activation microglia/macrophages. This suggested to us that infected myeloid cells seen in the choroid plexus do not differentiate into microglia or macrophages in the neonatal CNS. Also, these cells did not have long dendrites typical of dendritic cells and failed to express dendritic cell markers. Dendritic cells have been previously shown to be resistant to convivovirus infection (Kramer et al., 2008).

Intriguingly, Iba1+ macrophages/microglia which did not express nestin were observed actively engulfing numerous infected myeloid cells. Serial sections demonstrated distinct cell populations (infected myeloid cells and Iba1+ macrophages/microglia) having different migration kinetics entering the choroid plexus following CBV3 infection. Furthermore, the progression of cell migration captured by immunofluorescence microscopy highlighted the transient nature of infected myeloid cells, and illustrated their rapid movement away from the choroid plexus and into the parenchyma of the brain between 24 and 48 h PI. Also, the few remaining infected myeloid cells in the choroid plexus at 48 h PI underwent apoptosis, and these apoptotic bodies were subsequently phagocytosed by the large number of Iba1+ macrophages/microglia infiltrating into the choroid plexus at this point following infection.

Additional immunostaining revealed that the majority of myeloid cells in the choroid plexus expressed high levels of phospho-p42/44 MAP kinase (pERK1/2) or Ki67 (marker for cell proliferation), which may explain the susceptibility of these cells to infection (Feuer et al., 2002, 2004; Esfandiar et al., 2004; Feuer and Whittone, 2008). Nestin staining within infected myeloid cells was frequently localized to leading cellular processes in the direction of the ependymal cell layer. Although expression of nestin has been repeatedly used to identify migratory neural stem and progenitor cells, little is known regarding the function of nestin. Recent data suggest that nestin may participate in the phosphorylation of microtubules and direct cell migration and movement (Helfand et al., 2003; Kleberger et al., 2007).

The extravasation of infected myeloid cells through the choroid plexus columnar epithelium was observed by immunostaining of sections with laminin and ZO-1 antibodies and by confocal microscopy with IMARIS 3D analysis. Collectively, staining for laminin and ZO-1 identifies two key structural components of the choroid plexus, basement membranes and tight junctions. Laminin is a ubiquitous connective tissue glycoprotein and a major component of basement membranes. Laminin helped to identify the endothelial cell layer of the choroidal plexus and permitted us to observe the diapedesis of cells from the blood capillaries of the vascular stromal core. Infected myeloid cells were observed in the process of extravasation through laminin-/- regions. Despite the early age of infected pups (12, 24, and 48 h postbirth), ZO-1 staining outlined the tight junctions of the columnar epithelium organizing the blood–CNS barrier in the choroid plexus. Infected myeloid cells could be seen trafficking through the columnar epithelium. The great majority of infected cells expressed high levels of viral protein during or after extravasation, indicating that infection may have taken place upon entry of these cells through the blood–CNS barrier. As suggested by our own CAR immunostaining, we hypothesize that binding of CVB3 virions to CAR located within the tight junctions (Nagai et al., 2003) may assist in virus dissemination following the recruti-
Figure 10. Recruitment of intrathecally inoculated GFP-expressing donor cells into the CNS following infection with dsRED-CVB3. BMDMs or newborn liver-derived cells were isolated from either naive GFP transgenic mice. GFP-expressing cells were inoculated intrathecally into 1 or 2 or 3 day-old recipient C57BL6 mice. Recipient mice were intracranially infected either simultaneously or 24 h later, with dsRED-CVB3. The brains and livers of recipient mice were harvested 1, 2, and 3 postinoculation. Recipient mice showed varying numbers of engrafted GFP-expressing donor cells. B, C. The most successful engraftment occurred in recipient mice inoculated intrathecally with newborn liver cells (green), although the majority of recipient mice showed some level of successful engraftment. B. As soon as 24 h postinfection, GFP-expressing donor cells were observed in the CNS (representative animal shown), C. In contrast, no GFP-expressing donor cells were observed in recipient mice inoculated with newborn liver cells (green), although the majority of recipient mice showed some level of successful engraftment. C. By day 6 postinfection, GFP-expressing donor cells were observed in the entorhinal cortex (white arrow). Also, viral protein expression (red) was observed in the entorhinal cortex, although no colocalization with donor was seen. D, E. Higher magnification of J identified GFP-expressing donor cells in the entorhinal cortex with neurona-like processes (white arrow). F, G. 20× objective, F-J. 63× objective. K. 63× objective with a further ×2 magnification.

As these novel cell enter the parenchyma of the brain, they exhibit morphological similarities to type B NSCs that normally reside within the SVZ and display typical cellular processes that characteristic penetrate through the ECL contacting the lateral ventricles (Alvarez-Buylla et al., 2002). Some myeloid cells may eventually undergo apoptosis, or become phagocytosed by resident macrophages/microglia. We suggest that CVB3 infection, and the subsequent damage to the neonatal CNS, may induce myeloid cell recruitment through the blood–CSF barrier to repair the brain following injury, such as after an infection. Moreover, CVB3 may exploit this inherent repair process in the neonatal CNS by intensifying myeloid cell recruitment for the purpose of maximizing virus dissemination into the brain. Early CVB3 infection and subsequent chemokine expression may induce the recruitment of myeloid cells and the eventual systemic distribution of virus into the CNS and other tissues, as described for murine cytomegalovirus by the Mocarski group (Noda et al., 2006). Aseptic meningitis caused by coxsackievirus infection in newborns may be exacerbated by myeloid cell recruitment since these cells may provide an effective method for viral dissemination throughout the neonatal CNS. The recruitment of mobile cells, and their subsequent susceptibility to infection, may be a common theme among viruses in their drive to disseminate through the host. The benefits to viral spread using these cells within the host are manyfold in this scenario. (1) Virions might be protected from neutralizing antibodies normally found in high titer in the sera. (2) Mobility of migrating cells might provide virions with rapid transportation to normally inaccessible organs (for example, the CNS). (3) Many different genera of viruses require activated or proliferating host cells for maximal viral replication, and the metabolic activity of migratory cells may make them attractive targets for viral infection. Myeloid cells have not been previously identified as supporting neurogenesis, although some primary hematopoetic cells and CD14+ monocytes express nestin and other stem cell markers under certain culture conditions (Vitry et al., 2003; Chen et al., 2005). Recent studies have described the ability of CD14+ multipotent monocytes having the ability to differentiate in vitro into bone, skeletal muscle (Kuwana et al., 2003), cardiac muscle
Figure 11. Induction of apoptosis in the choroid plexus and the possible outcome of CVB3-infected miyeloid cells following their migration into the parenchyma of the CNS. A–D. Quantification of ApoTag signal in the choroid plexus of C57BL/6 mice infected with eGFP-CV3B, BALB/c mice infected with eGFP-CV3B, or mock-infected C57BL/6 mice 24 h PI. Infected mice showed a statistically significant increase in ApoTag labeling (asterisks) compared with mock-infected mice (p < 0.05 using ANOVA with Newman–Keuls post hoc comparison). Representative images of ApoTag signal (red) and viral protein expression (green) in the choroid plexus are shown at 20x and 63x magnifications for infected (A, C, E, F) or mock-infected (B, D) mice. DAPI-stained cells are shown in blue. E. An illustration of the possible outcome of CVB3-infected miyeloid cells is shown. Following inoculation into the subarachnoid, miyeloid eGFP-CV3B infection may lead to iC3b induction. Virions bind to CAR expressed within the tight junctions of the choroid plexus epithelium. During myeloid cell recruitment, virions may infect miyeloid cells upon extravasation across the epithelium. Eventually, CVB3-infected miyeloid cells may undergo apoptosis near the choroid plexus (F) or after entering the parenchyma of the CNS (24). Some infected miyeloid cells may be phagocytosed by astrocytes/macrophages/microglia. 4. Alternatively, some infected miyeloid cells may escape detection and assist in the establishment of persistent CVB3 infection. 5. Inflammatory virus may be released from miyeloid cells upon entry through the E3 and subsequently infect type B and eventually progenitor cells, immature and mature neurons. 4. Finally, miyeloid cells responding to infection may contribute to CNS repair/regeneration.

(Kodama et al., 2005), and most recently the neuronal lineage (Kodama et al., 2006). Others have described the close association of perivascular macrophages with regions of neurogenesis within the SVZ (Mercer et al., 2002). However, the role of these cells during neurogenesis has not been evaluated. Bone marrow-derived cells have been shown to migrate into the CNS in response to injury and help the repair process (Brazelton et al., 2000). A recent study suggests that neuronal progenitor cells may have a cell marker, Lea (see 1), found on monocytes (Capela and Temple, 2002). The possibility remains that progenitor cells may be induced to migrate from the blood only after expression of a migratory factor or chemokine following infection with CVB3 or after CNS damage. Identifying such a migratory factor would be imperative for new treatments involving stem cell therapies. Controversy exists in the field of “transdifferentiation,” whereby some researchers have shown that BMDCs can differentiate into neurons, cardiomyocytes, and other cell lineages (Brazelton et al., 2006; Mezy et al., 2003). In contrast, others have interpreted these results differently (Castro et al., 2002). They suggest that BMDCs fuse to sick or dying neurons and repair functionality without “transdifferentiation.” Intriguing results from Natal et al. (2006) describe a population of miyeloid progenitor cells in close association with fibroblast-like cells of unknown function expressing nestin within choroid plexus stroma cultures. Furthermore, choroid plexus ependymal cells were recently shown to harbor neural progenitor cells (Itozka et al., 2006). We hypothesize that these neonatal nestin+ miyeloid cells responding to CVB3 infection may represent a unique primitive progenitor cell which might assist with CNS repair in response inflammation, damage, or infection with particular viruses. Neonatal miyeloid progenitor cells may exhibit unique characteristics and function compared with their fully differentiated counterparts within the adult host. For example, neonatal monocytes show modified expression levels of genes involved in the functional immune response when compared with adult monocytes (Strunk et al., 2004; Lawrence et al., 2007). To clarify the peripheral origin of these miyeloid cells which appear to be highly susceptible to CVB3 infection, we intracerebrally inoculated bone marrow-derived cells or newborn liver-derived cells from actin promoter-GFP transgenic mice into 1- or 3-day-old C57BL/6J recipient animals. Recipient animals were subsequently intracerebrally inoculated with a recombinant CVB3 expressing dsRED protein (dsRED-CVB3). We demonstrated the migration of miyeloid-like cells expressing GFP with similar morphological characteristics and anatomical location within the CNS, as shown for mice infected with eGFP-CV3B. In contrast, no GFP+ donor cells were observed in mock-infected neonatal mice. The migration of adoptively transferred GFP-expressing cells which traffic into the CNS following dsRED-CVB3 inoculation suggests a peripheral origin for miyeloid-like cells seen within the choroid plexus following infection.
We performed an Illumina BeadArray Whole Mouse Genome analysis of the neonatal brain following infection with two contrasting RNA viruses in hopes of identifying novel chemokines and cytokines induced specifically by CVB3 infection which might be responsible for nestin + myeloid cell recruitment into the CNS. As determined by our CDNA microarray analysis, CVB3 replication in the neonatal CNS appeared to induce a specific combination of chemokines which may lead to myeloid cell recruitment. This recruitment may be amplified in the neonatal host, where development is ongoing and cellular plasticity may be more evident.

Microarray analysis indicated that several chemokines were induced in eGFP-CVB3-infected samples at 12 h postinfection. In particular, the β-chemokines CCL12, CCL7, CCL9, and CCL4 were highly induced in response to CVB3 infection. These chemokines were not upregulated following infection with an unrelated RNA virus (LCMV), nor have they upregulated been described in the literature for other neuropathic microbes, including HIV and T. brucei, which primarily induce CCL2 (Courtois et al., 2006; Campbell and Spector, 2008). Also, CCL12 protein was expressed at high levels within the choroid plexus and the ependymal cell layer following CVB3 infection, and intracranial inoculation of CCL12 into neonatal mice increased the number of myeloid cells within the choroid plexus independent of CVB3 infection. These results suggest that the CCL12 chemokine may play an essential role in the recruitment of nestin + myeloid cells. In contrast, CCL12 expression levels previously shown to promote hematopoietic stem cell chemotaxis (Nervi et al., 2006) were relatively unchanged (data not shown). Although relatively low levels of chemokines were induced following wtCVB3 infection, we hypothesize that differential viral kinetics of the slower replicating recombinant CVB3 (eGFP-CVB3) versus wtCVB3 may be partly responsible for these differences. Nonetheless, myeloid cell recruitment was observed for both recombinant and wtCVB3. Future studies will address the role of CCL12 in myeloid cell recruitment through the choroid plexus, and the ability of these recruited myeloid cells to assist in the establishment of CVB3 persistence in the CNS.

References


Chapter 1, in full, is a reprint of the material as it appears in Journal of Neuroscience 2010. Tabor-Godwin JM.; Ruller CM.; Bagalso N.; An N.; Pagarigan RR.; Harkins S.; Gilbert PE.; Kiosses WB.; Gude NA.; Cornell CT.; Doran KS.; Sussman MA.; Whitton JL.; Feuer R., Society for Neuroscience, 2010. The dissertation author was the primary investigator and author of this paper.
CHAPTER II:

Utilizing LysM-eGFP Transgenic Mice to Analyze Peripheral eGFP+ Cells in the Neonatal CNS after Coxsackievirus Infection
INTRODUCTION

Myeloid cell infiltration after CVB3 infection in the neonatal CNS is a unique and previously unobserved phenomenon (14). Infected myeloid cells were found to express Mac3, a marker for the myeloid lineage, when they first enter the CNS through the choroid plexus. Cells were observed moving through the laminin-labeled blood vessel of the choroid plexus by confocal microscopy. When these cells moved further into the brain, they began to express nestin, a marker for neural stem cells (12). This may indicate that the myeloid cells are adapting to the unique microenvironment of the CNS. In order to determine unequivocally that these cells were entering from the periphery, LysM-eGFP mice were utilized.

LysM-eGFP mice have the eGFP gene inserted in the murine lysozyme M (LysM) locus (2). This inactivated the LysM gene, however the mice have no developmental, behavioral or reproductive abnormalities. The presence of eGFP in this locus allowed for the labeling of myelomonocytic cells with eGFP. Cells with strong eGFP expression, as determined by flow cytometry, included 52% mature neutrophils, 40% myelocytes, 4% monocytes and 3% non-myeloid cells. Furthermore, only 4.5% of eGFP-negative cells were of the myelomonocytic lineage.

Since LysM-eGFP mice label cells of the myeloid lineage, we utilized these animals to track myeloid cells from the periphery into the brain after CVB3 infection. One-day old LysM-eGFP mice were infected with dsRed recombinant CVB3 (dsRED-CVB3) and the CNS was analyzed for eGFP+ cells
by immunohistochemistry (IHC) at early times post-infection. We observed more eGFP+ cells entering the CNS in infected animals. Furthermore, these cells were found in neurogenic regions of the CNS, including the SVZ, rostral migratory stream and the olfactory bulb and were found to express dsRed viral protein. We hypothesize that CVB3 will attract eGFP+ myeloid cells into the neonatal CNS in LysM-eGFP transgenic mice and that this will be a useful model in understanding myeloid cell infiltration.
METHODS

Isolation and Production of a Recombinant Coxsackievirus

The generation of a recombinant coxsackievirus expressing eGFP has been described previously (3). Briefly, the CVB3 infectious clone (pH3) (obtained from Dr. Kirk Knowlton at University of California at San Diego) was engineered to contain a unique $Sfi$ I site which facilitates the insertion of any foreign sequence into the CVB3 genome (pMKS1). For the recombinant CVB3 expressing dsRED (dsRED-CVB3), dsRED gene was amplified from a dsRED expression plasmid (Clontech Laboratories, Mountain View, CA) using dsRED sequence specific primers with flanking $Sfi$I sequences. The PCR product was cloned into pMKS1. Following transfection of HeLa RW cells with dsRED-CVB3 plasmid, infectious virus was generated. All virus stocks were grown on HeLa RW cells maintained in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum. Virus titrations were carried out as described previously.

Mice and Viral Innoculations

Mouse experimentation conformed to the requirements of the San Diego State University Animal Research Committee and the National Institutes of Health. LysM-eGFP mice were obtained from the La Jolla Institute of Immunology and Allergy (La Jolla, CA) as a generous gift from Dr. Klaus Ley with the permission of Dr. Thomas Graff. Breeding pairs were checked every day, and one day-old pups were infected intra-cranially with $1 \times 10^7$ pfu
dsRed-CVB3 or mock-infected with DMEM. The procedure for intra-cranial inoculation of one-day old pups has been described previously (4) (5). Pups were sacrificed at various time points between 12, 24, and 48 hours post-infection (PI) by hypothermia/CO2, followed by immediate decapitation. The brains were fixed by immersion in 10% neutral-buffered formalin for greater than 4 hours, paraffin-embedded, and immunostained.

Immunohistochemistry and Microscopy

Paraffin-embedded sections (3 to 4μm thick) were deparaffinized with xylene followed by rehydration in decreasing percentages of ethanol, PBS, and distilled water. High temperature antigen unmasking was performed for 25 min with citrate-based antigen unmasking solution (Vector Laboratories, Inc.). Two main staining protocols were utilized. For GFP (1:100, AB3080; Millipore Corporation; or 1:100, ab13970; Abcam), nestin (1:50, MAB353; Millipore Corporation) and Iba1 (1:100, #016-20001; Wako Pure Chemical Industries, Ltd.) antibodies; sections were blocked with 10% normal goat serum for 30 min, primary antibody was incubated overnight at 4°C, and either goat anti-rabbit antibody conjugated to biotin or bovine anti-chicken FITC (1:500, sc2700; Santa Cruz Biotechnologies, Inc.) was applied for 30 min. Lastly, sections were incubated with streptavidin-Alexa Fluor 488 or 594 at 1:500 for 30 min. For dsRed (1:50, 632496; Clontech Laboratories) Tyramide Signal Amplification (TSA) Kit #25 (Invitrogen Inc., Carlsbad, CA) was used according to manufacturer’s protocol. Briefly, sections were quenched with 3% H2O2 for 1 hour, blocked in 1% Blocking Solution for 1 hour, incubated in primary
antibody in 1% Blocking Solution overnight at 4°C, goat anti-rabbit antibody conjugated to biotin at 1:100 in 1% Blocking Solution was applied for 2 hours, streptavidin-HRP was applied at 1:100 in 1% Blocking Solution for 1 hour, and lastly sections were incubated with tyramide-594 at 1:100 in Amplification Buffer for 20 minutes. In order to ensure antibody specificity, no primary controls were included for each antibody. For detection of DNA/nuclei, sections were overlaid with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc.). Sections were observed by fluorescence microscopy (Zeiss Axiovert 200 inverted microscope). Green, red, and blue channel images were merged utilizing AxioVision 4.8 software.
RESULTS

Peripheral eGFP+ Cell Recruitment into Neurogenic Regions of the Neonatal CNS after CVB3 Infection

In order to confirm that myeloid cells were entering from the periphery after CVB3 infection, LysM-eGFP transgenic mice were utilized. Mice were infected one-day post-birth with $10^7$ pfu dsRed-CVB3 and harvested at 12, 24 and 48 hours PI (Figure 2.1A). Viral titers were performed and showed increasing amounts of virus over time (Figure 2.1B), peaking at $1.2 \times 10^9$pfu at 48 hours PI. Immunohistochemistry (IHC) for eGFP revealed eGFP+ cells entering through the choroid plexus of infected animals at 12 and 24 hours PI (Figure 2.1C-F). Myeloid cells have previously been shown to enter the CNS through the choroid plexus, though the response was more robust (14). By 48 hours PI, many eGFP+ cells were seen in the subventricular zone (SVZ), which harbors the stem cell niche in the brain (Figure 2.1G) (13). Myeloid cells have been shown to move further into the brain after CVB3-infection, in accordance with this observation (14). Furthermore, few eGFP+ cells were observed in Mock infected animals (Figure 2.1H). In addition, eGFP+ cells were also seen in other neurogenic regions of the CNS, including the rostral migratory stream (RMS, Figure 2.1I) and olfactory bulb (Figure 2.1J). Furthermore, when IHC for viral protein (dsRed) was performed, it was found that several eGFP+ cells from the periphery were highly infected. These cells were found in both the SVZ (Figure 2.1K, M) and the RMS (Figure 2.1L, N). Thus, eGFP+ cells from LysM-eGFP transgenic mice have many similarities with myeloid cells, including their
location in the CNS and susceptibility to infection, and may represent the same cell population.

**CVB3-Infection in LysM-eGFP mice Induces a Robust Influx of Macrophages/Microglia into the CNS that Engulf eGFP+ Cells**

In order to further examine similarities between myeloid cells in non-transgenic mice and eGFP+ cells from our transgenic mouse model, Iba1 staining was performed. Iba1 is a specific marker for macrophages/microglia that is highly expressed in these cells upon activation (9). It was previously shown that infected myeloid cells become engulfed by Iba1+ cells and undergo apoptosis by 48 hours PI (14). Furthermore, CVB3-infected cells were also observed being engulfed by Iba1+ cells at 5 days PI (6), however it was not known whether these CVB3-infected cells were myeloid cells due to the limitations of the non-transgenic model. In addition, infected myeloid cells and Iba1+ cells had varying kinetics in entering the CNS, as myeloid cell infiltration peaked at 24 hours PI and Iba1+ cell infiltration peaked at 48 hours PI (14). IHC for Iba1 was performed on CVB3-infected LysM-eGFP samples 48 hours PI. Iba1+ cells were observed engulfing eGFP+ cells in the choroid plexus (Figure 2.2A-B), the SVZ (Figure 2.2C-D) and the retrosplenial cortex (RSC) (Figure 2.2E-F). In high magnification images, Iba1+ cell membrane extensions can be seen surrounding infected cells in these regions of the CNS (Figure 2.2B, D, F, white arrows). Furthermore, several Iba1+ cells that were not engulfing eGFP+ cells were observed in the meninges (Figure 2.2G). In addition, some Iba1+ cells were found engulfing eGFP+ cells in the
hippocampus (Figure 2.2H, white arrows), while others were not (Figure 2.2H, blue arrows). Taken together, these results indicate that CVB3 infection elicits a strong macrophage/microglia response in the CNS of LysM-eGFP transgenic mice, and that these cells strive to remove eGFP+ cells, which have been shown to harbor infection (Figure 2.1K-N).

**eGFP+ Peripheral Cells in the CNS of CVB3-Infected LysM-eGFP Transgenic Mice Express Nestin**

Furthermore, infected myeloid cells were found to express nestin as they moved away from the choroid plexus and further into the brain. Nestin is an intermediate filament that is used to label neural progenitor and stem cells (NPSCs) in the CNS (7). Interestingly, nestin may play a role in cell migration and movement through the phosphorylation of microtubules (8) (11). In order to determine whether eGFP+ cells from LysM-eGFP mice express nestin, IHC was performed 48 hours PI. Nestin was found in eGFP+ cells in the choroid plexus (Figure 2.3A-B, white arrows), the SVZ (Figure 2.3C-D, white arrows), and the RSC (Figure 2.3E-F, white arrows). Interestingly, eGFP+ multi-lobed cells, representative of neutrophils, were observed in the choroid plexus and SVZ (Figure 2.3B, D, blue arrows) and were not nestin positive. Thus, eGFP+ peripheral cells that enter the CNS after CVB3 infection in LysM-eGFP mice express nestin, as do CVB3-infected myeloid cells in non-transgenic mice.
DISCUSSION

LysM-eGFP transgenic mice were utilized as a model to confirm myeloid cell entry from the periphery and track infected myeloid cells as they disseminate throughout the neonatal CNS. LysM-eGFP mice underwent robust infection in the CNS with dsRed-CVB3 and eGFP+ cell migration into the CNS was observed via IHC. More eGFP+ cells were observed in infected as compared to mock infected animals, thus indicating that infiltration of eGFP+ cells was specific to infection. Since eGFP is only expressed on cells of the myeloid lineage in LysM-eGFP transgenic mice, the presence of eGFP+ cells in the CNS indicates that these cells entered from the periphery. Also, eGFP+ cells were observed in similar regions of the CNS as infected myeloid cells, including the choroid plexus and the SVZ. Furthermore, since myeloid cells are highly infected by CVB3, one would expect eGFP+ cells to also be susceptible to infection if they represent the same cell population. eGFP+ cells were found to be infected by CVB3 based on their expression of dsRed viral protein. Surprisingly, many eGFP+ cells were observed in neurogenic regions of the CNS, including the SVZ, the RMS and the olfactory bulb (1). While myeloid cells were previously observed in the SVZ, they have not been seen in the RMS and the olfactory bulb. This may represent an advantage based on using LysM-eGFP mice, as these cells may be able to be tracked further into the brain.

To analyze eGFP+ infiltrating cells further, IHC for Iba1, which labels macrophages/microglia was performed. Infected myeloid cells in non-transgenic mice have been shown to be a distinct population as compared
to Iba1+ cells, with varying kinetics and distribution (14). Iba1+ cells have also been observed engulfing infected myeloid cells, followed by eventual myeloid cell apoptosis. It was found that eGFP+ cells from LysM-eGFP transgenic mice also represented a separate population, as compared to Iba1+ cells. Iba1+ cells were found in great abundance in the CNS and were found in areas of eGFP+ cells, as well as other areas of the brain, including the meninges. Initially, we expected to see eGFP+ macrophages/microglia in the brain since LysM-eGFP mice label the myeloid lineage. However, eGFP+ cells were not found to be Iba1+. Instead, Iba1+ cells were observed engulfing eGFP+ cells in the choroid plexus, the SVZ, and the RSC. These Iba1+ cells appeared not to engulf eGFP+ cells in the meninges and the hippocampus. A possible explanation for the loss of eGFP from Iba1+ macrophages/microglia might include changes in gene expression upon entering the CNS. In addition, a recent study analyzing lymphocytic choriomeningitis (LCMV) infection in LysM-eGFP transgenic mice also did not observe eGFP+ macrophages/microglia in the CNS (10).

One of the most interesting findings regarding infected myeloid cell infiltration after CVB3 infection is that these cells expressed nestin, a marker for NPSCs (12). Infiltration of nestin+ myeloid cells may be a previously unobserved phenomenon in which neonates may have increased plasticity due to their young age. We performed IHC for nestin in infected LysM-eGFP transgenic mice and found cells that were positive for both nestin and eGFP in the choroid plexus, the SVZ and the RSC. Furthermore, mature eGFP+ neutrophils
were identified by their multi-lobed nuclei and were not observed to express
nestin. Therefore, based on infection, location, and nestin expression,
peripheral eGFP+ infiltrating cells from LysM-eGFP transgenic mice appear to
represent a similar population as infected myeloid cells in non-transgenic mice
and may be useful for further analysis of myeloid cell infiltration and fate in the
CNS.
FIGURES

Figure 2.1: Recruitment of eGFP+ cells to neurogenic regions of the neonatal CNS in LysM-eGFP transgenic mice after CVB3 infection

(A) LysM-eGFP transgenic mice were intra-cranially inoculated 1 day post-birth with 107 pfu of dsRED-Coxsackievirus B3 and harvested at 12, 24 and 48hrs post-infection (PI). (B) Viral titers increased over time. eGFP+ cells from the periphery were observed entering the neonatal CNS through the choroid plexus by 12hrs PI (C, E) and continued at 24hrs PI (D, F). By 48hrs PI, eGFP+ cells were observed in neurogenic regions of the brain including the subventricular zone (SVZ) (G), the rostral migratory stream (RMS) (I) and the olfactory bulb (J). Very few eGFP+ cells were observed in mock infected samples (H). Several eGFP+ cells were found to be highly infected, based on their expression of dsRED in the SVZ (K,M) and the RMS (L,M). Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications of the 20X magnification images are shown in white boxes.
Figure 2.2: eGFP+ cells in infected LysM-eGFP transgenic mice are engulfed by Iba1+ cells

eGFP+ (green) were found to be engulfed by Iba1+ (red) in the choroid plexus (A, B), the subventricular zone (SVZ) (C, D), and the retrosplenial cortex (RSC) (E, F). In high magnification images (B, D, F) Iba1+ cell membrane extensions can be seen wrapped around eGFP+ cells and are depicted with white arrows. In addition, several Iba1+ cells that were not engulfing eGFP+ cells were found in the meninges (G). Both eGFP engulfing Iba1+ cells (white arrows) and non-eGFP engulfing Iba1+ cells (blue arrows) are seen in the hippocampus (H). Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications of the 20X magnification images are shown in white boxes.
48hrs Post-dsRED-CVB3 Infection of LysM-eGFP Transgenic Mice

**Peripheral Cells**

**Iba1**

**DAPI**
Figure 2.3: eGFP+ cells in infected LysM-eGFP transgenic mice express nestin
Peripheral eGFP+ cells (green) express nestin in the choroid plexus (A, B), the subventricular zone (SVZ) (C, D), and the retrosplenial cortex (RSC) (E, F). Double-positive cells for eGFP and nestin are shown with white arrows. Cells that have multi-lobed nuclei, representative of neutrophils, are shown with blue arrows and were not found to be positive for nestin. Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications of the 20X magnification images are shown in white boxes.
REFERENCES


CHAPTER III:

Differential Neural Stem Cell Tropism and Host Gene Expression Identified for Two Contrasting Neurotropic RNA Viruses Following Infection of the Neonatal CNS
INTRODUCTION

Aseptic meningitis and encephalitis in infants are serious conditions that can lead to severe morbidity and mortality. Given the large number of viruses which can be responsible for neonatal meningitis and encephalitis, including several enteroviruses and herpesviruses, the pathogenesis and lasting sequelae of these infections can vary substantially and are not fully understood (28) (24). Symptoms often include fever, irritability and lack of appetite; and these infections have been linked to long-term consequences, such as learning disabilities, demyelinating diseases, and behavioral disorders (7) (22). In order to better understand the pathogenesis of neurotropic viruses in the newborn host, we chose to directly compare lymphocytic choriomeningitis virus (LCMV) and coxsackievirus B3 (CVB3), which both can cause meningitis and encephalitis in the neonatal host (10) (22).

CVB3 and LCMV, both neurotropic RNA viruses, were utilized for the study due to their potent ability to cause disease in our neonatal mouse model of viral infection and dissimilarities in stimulating protective T cell responses. CVB3 is a member of the picornavirus family and the enterovirus genus. Enteroviruses may be responsible for approximately 10 to 15 million symptomatic infections each year in the United States alone (32). LCMV is a rodent-borne arenavirus that is an emerging cause of neonatal meningitis, and often transmitted congenitally (3). LCMV, a non-cytolytic, enveloped virus, is comprised of a negative-sense segmented RNA genome and induces a strong cytotoxic T lymphocyte response. In contrast, CVB3, a cytolytic, non-
enveloped virus comprised of a single positive-sense RNA genome, induces a very weak cytotoxic T lymphocyte response in its host (36).

Despite considerable inherent dissimilarities between CVB3 and LCMV, including differences in T cell activation, a divergence in molecular structure, and substantial differences in virus replication; both viruses appear to target neural progenitor and stem cells (NPSCs) in the central nervous system (CNS). Our laboratory has previously shown that CVB3 preferentially targets NPSCs in culture (35) and in the neonatal host presumably due to their undifferentiated status and high proliferative index (15) (18). We have previously described the recruitment of unique myeloid cells into the CNS following CVB3 infection. (36) These cells were highly susceptible to infection and assisted in the dissemination of infectious virus into the neurogenic regions of the neonatal CNS. Also, LCMV has been shown to infect glial progenitor cells in neurogenic regions of the central nervous system (CNS), including the subventricular zone, rostral migratory stream and olfactory bulb (6) (4). We wished to inspect progenitor cell tropism of both viruses in greater detail by immunofluorescence microscopy utilizing well-characterized cell markers for neural (nestin+) and glial (Olig2+) progenitor cells.

Regardless of its prevalence in a clinical setting, CVB3 infection of the neonatal CNS has not been well studied (32). Also, while the cellular targets of infection and the subsequent immune response have been extensively studied for LCMV in the adult CNS, much remains to be determined following neonatal infection (6) (8). We hypothesize that a direct comparison of early
infection in these viruses will determine unique and previously unknown targets of infection that may have important consequences on viral pathogenesis and viral progression in the neonatal CNS. One day-old mice, known to be highly susceptible to both LCMV and CVB3 infection, were utilized for these studies (16), although LCMV can successfully infect the adult CNS as well (22). In addition, the symptoms of mice infected with LCMV vary based on the time of infection (5). The brains of one day old CVB3 and LCMV infected mice were analyzed for gene expression changes by microarray analysis at 12, 24 and 48 hours post-infection (PI) in order to reveal the neonatal host response to both neurotropic viruses. Also, we sought to examine potential pathology in the choroid plexus following infection by H&E staining, TUNEL assay, and fluorescence microscopy utilizing markers of choroid plexus function. We also analyzed the activation of immune response genes following LCMV and CVB3 infection of the neonatal CNS, including the induction of chemokines and MHC class I gene expression. Our results revealed a unique pattern of host immune activation, stem and progenitor cell tropism, and virus-mediated pathology for each neurotropic virus.
METHODS

Isolation and Production of Recombinant Coxsackieviruses

The generation of recombinant coxsackievirus expressing eGFP (GFP-CVB3) has been described previously (13). Briefly, the CVB3 infectious clone (pH3) (obtained from Dr. Kirk Knowlton at University of California at San Diego) was engineered to contain a unique Sfi I site that allows the insertion of foreign DNA into the CVB3 genome. Virus stocks were grown on HeLa RW cells maintained in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro) supplemented with 10% fetal bovine serum. Standard plaque assay on HeLa RW cells was used to titer the recombinant virus. Briefly, HeLa RW cells were grown to 70% confluency in a six-well plate, inoculated with 10^{-2} to 10^{-7} dilutions of viral stock, allowed to infect the cells for one hour, and then overlaid with 2X DMEM and 1% agar for two days. Plates were fixed with acetic acid and methanol and stained with crystal violet overnight. Plaque forming units were counted the following day.

Mice and Viral Innocations

Mouse experimentation conformed to the requirements of the San Diego State University Animal Research Committee and the National Institutes of Health. C57BL/6 mice were obtained from Harlan Sprague Dawley. Breeding pairs were checked every day, and one-day old pups were infected intra-cranially with 10^7 pfu eGFP-CVB3, 5 x 10^3 pfu LCMV, or mock infected with 1X DMEM. The procedure for intra-cranial (ic) inoculation of one-day old
pups has been described previously (15) (13). Young pups were killed by halothane treatment followed by immediate decapitation at 12, 24 and 48 hours PI. Older pups (10 days and beyond) were killed by halothane treatment followed by cervical dislocation. The brains were fixed by immersion in 10% neutral-buffered formalin for greater than four hours, paraffin-embedded, and immunostained and/or stained with hematoxylin & eosin (H&E).

**Immunohistochemistry and Microscopy**

Sagittal paraffin-embedded sections (3 to 4μm thick) were deparaffinized with xylene followed by rehydration in decreasing percentages of ethanol, PBS, and distilled water. High temperature antigen unmasking was performed for 25 minutes with citrate-based antigen unmasking solution (Vector Laboratories, Inc.). For primary antibodies derived from mouse, including mouse anti-LCMV-NP (nucleoprotein, 1:20), Mouse on Mouse Kit was utilized (Vector Laboratories, Inc.). For GFP (1:100, AB3080; Millipore Corporation; or 1:100, ab13970, Abcam), nestin (1:50, MAB353; Millipore Corporation), Olig2 (1:100, ab33427; Abcam) antibodies; sections were blocked with 10% normal goat serum for 30 minutes, primary antibody was incubated overnight at 4°C, goat anti-rabbit, anti-rat, or anti-mouse antibody conjugated to biotin, bovine anti-chicken FITC (sc2700, Santa Cruz Biotechnologies), or goat anti-rabbit Alexa Flour 594 or 488 was applied for 30 minutes. Lastly, sections were incubated with streptavidin-Alexa Fluor 488 or 594 at 1:500 for 30 min. For CCL12/MCP-5 (1:100, anti-MCP-5 (K19)-sc9718; Santa Cruz Biotechnology Inc.), Transthyretin (1:50, 11891-1-AP; Proteintech
Group, Inc.), Carbonic Anhydrase II (1:50, CA II (G-2): sc-48351; Santa Cruz Biotechnology, Inc.) and MHC Class I (1:50, 5K46: sc-71576; Santa Cruz Biotechnology, Inc.) antibodies, the Tyramide Signal Amplification (TSA) Kit #25 (Invitrogen Inc.) was used according to manufacturer’s protocol. Briefly, sections were quenched with 3% H2O2 for 1 hour (unless antibody was generated in mouse), blocked in 1% Blocking Solution for 1 hour (which was used for all subsequent incubations), incubated in primary antibody overnight at 4°C, goat anti-rabbit or appropriate secondary antibody conjugated to biotin at 1:100 was incubated for 2 hours, streptavidin-HRP was incubated at 1:100 for 1 hour, and finally sections were incubated with tyramide-594 at 1:100 in Amplification Buffer for 20 minutes. In order to ensure antibody specificity, no primary controls were included for each antibody. For detection of DNA/nuclei, sections were overlaid with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc.). Sections were observed by fluorescence microscopy (Zeiss Axiovert 200 inverted microscope). Green, red, and blue channel images were merged utilizing AxioVision 4.8 software. Whole brain images were generated from several 5X images that were automated and photo-merged using Adobe Photoshop CS2 software.

**Quantification of Infected Nestin and Olig2-Positive Progenitor Cells**

Fluorescent images (20X) of nestin/Olig2 co-localized with GFP-CVB3/LCMV-NP immunostained brain sections were taken using a Zeiss Axiovert 200 inverted microscope. Brain sections of C57BL/6 mice infected with GFP-CVB3 at 24 hours PI and LCMV at 48 hours PI were used. All three
walls of the subventricular zone (SVZ) were imaged, using three animals per time point. First, the total number of infected cells per SVZ were counted. Then, the percent of double positive cells for infection and the given progenitor cell marker was calculated for both GFP-CVB3 and LCMV. Data was analyzed in GraphPad Prism 3.0 software by Student’s T-test and displayed graphically.

**Microarray Analysis**

Total RNA was isolated using the Qiagen RNeasy Kit from homogenized brain samples. Isolated RNA was sent to the BIOGEM Microarray Core at University of California at San Diego and analyzed for quality before microarray analysis was started. Briefly, samples were reverse transcribed to make cDNA and then *in vitro* transcribed to generate cRNA, which incorporated biotin. cRNA was hybridized to Illumina Whole Mouse Genome 6 Samples chips, which hybridize approximately 45,200 genes. Hybridized chips were incubated with Cy3, washed and read using the Illumina BeadArray Station. Normalized gene expression was used to examine differences and similarities in the following samples; GFP-CVB3, LCMV, and Mock-infected C57BL/6 mice at 12, 24 and 48 hours PI. Gene expression levels based on RNA expression in the brain of eGFP-CVB3 or LCMV-infected neonatal mice were compared with mock-infected control mice. The fold change in gene expression levels between GFP-CVB3 and LCMV infected mice over mock were used to determine how these viral infections altered immune response, developmental, and CNS specific gene expression in the neonatal host.
### ApopTag Staining and Quantification

Cells undergoing apoptosis were identified using the ApopTag Red In Situ Apoptosis Detection Kit (Millipore Inc.), as specified by the manufacturer. Briefly, paraffin-embedded sections were deparaffinized and antigen unmasked with proteinase K. Equilibration buffer was added directly onto the specimen for 5 minutes, then the samples were incubated in terminal deoxynucleotidyl transferase (TdT) enzyme in reaction buffer for 1 hour at 37°C. Samples were shaken for 15 seconds, then washed in working strength Stop/Wash buffer for 10 min. Working strength anti-digoxigenin conjugated rhodamine incubated on the samples at room temperature for 30 minutes. Sections were washed with 1X PBS and observed by fluorescence microscopy. ApopTag positive cells in the choroid plexus were counted, analyzed in GraphPad Prism 3.0 software by one-way ANOVA with Newman-Keuls post-hoc analysis and displayed graphically.
RESULTS

Early Viral Protein Expression for Both LCMV and CVB3 Localized to the Choroid Plexus and SVZ of the Neonatal CNS

Both LCMV and GFP-CVB3 have been shown to be highly neurotropic and target neurogenic regions of the neonatal CNS early after infection (4) (16) (17). However, the progenitor cell type supporting LCMV infection has not been clearly evaluated by immunofluorescence microscopy utilizing informative cell markers. Therefore, we inspected and carefully compared the tropism, distribution, and kinetics of host gene expression and viral protein expression following LCMV and GFP-CVB3 infection in our neonatal mouse model. In a previous study, we observed increasing amounts of LCMV and GFP-CVB3 viral genome via real-time RT-PCR at 12, 24 and 48 hours after infection of one day old mice (36). In order to determine sites of infection in the neonatal CNS, immunofluorescence microscopy for viral protein was performed (Figure 3.1). For LMCV, an anti-nucleoprotein (NP) antibody was utilized to track infection (Figure 3.1A, E, I; red signal). NP forms a complex with the LCMV genome and is essential for viral replication (26). At higher magnification, increasing amounts of NP were found in the subventricular zone (SVZ) and choroid plexus epithelial cells of LCMV-infected animals over time (Figure 3.1B, F, J). Viral protein was also found in the olfactory bulb, retrosplenial cortex and meninges by 48 hours PI; however the majority of signal was seen within the choroid plexus and surrounding the lateral ventricle.
An anti-GFP antibody was used to detect the distribution of GFP-CVB3 (Figure 3.1C, G, K; green signal). As described previously, infected myeloid cells were observed in the choroid plexus at 12 and 24 hours PI at higher magnification (Figure 3.1D, H). The choroid plexus is responsible for generating cerebral spinal fluid as well as regulating the entry of immune cells into the CNS across the tight junctions comprising the choroid plexus epithelial cells (14). GFP-CVB3 protein expression was also found in the SVZ at these time points. Previously, we identified infected nestin+ neural progenitor and stem cells (NPSCs) in the SVZ following GFP-CVB3 inoculation (16) (17). By 48 hours PI, GFP-CVB3 protein expression was less evident in the choroid plexus, as infection spread into the parenchyma of the brain to include the retrosplenial cortex, the rostral migratory stream, olfactory bulb, and hippocampus (Figure 3.1L).

**LCMV Infection Targeted a Greater Number of Cells in the SVZ and Revealed Broader Progenitor and Stem Cell Tropism in Comparison to CVB3 Infection**

We investigated the tropism of each neurotropic virus in greater detail utilizing antibodies against informative stem/progenitor cell markers. The SVZ has been shown to comprise NPSCs which give rise to glial cells and neuroblasts migrating through the rostral migratory stream (12) (13). Nestin, an intermediate filament implicated in cell motility, was used to label NPSCs (19) (25). Olig2, a transcription factor known to play a role in glial cell differentiation was utilized to identify glial progenitor cells (27). Co-localization of viral proteins and stem/progenitor cell markers were evaluated by
immunofluorescence microscopy (Figure 3.2). The peak of infection of the SVZ as determined by viral protein expression was utilized as the optimum time point to inspect LCMV (at 48 hours PI) and GFP-CVB3 (at 24 hours PI) tropism for stem/progenitor cell populations. While Olig2 may also label mature oligodendrocytes, we limited the examination of infected Olig2+ cells to those residing within the SVZ, which does not contain mature oligodendrocytes. We found that LCMV infects significantly more cells in the SVZ than GFP-CVB3, infecting 364.5 cells compared to 102.5 cells, respectively (Figure 3.2A, p=0.0295). LCMV infected significantly more Olig2+ cells than GFP-CVB3, with 37.64% compared to 12.9%, respectively (Figure 3.2B, p=0.001). However, LCMV infected less nestin+ cells than GFP-CVB3, with 39.55% compared to 76.78%, respectively (Figure 3.2C, p=0.0302). Furthermore, there was no difference in the percent of LCMV infected Olig2+ and nestin+ cells (p=0.8558), while there was a significant difference between GFP-CVB infected Olig2+ cells and nestin+ cells (p=0.0005) by Student’s T-test. This data indicates that LCMV infects glial and neural progenitors equally, whereas CVB3 preferentially targets neural progenitors. Representative immunofluorescence images illustrated the colocalization of LCMV protein and Olig2 (Figure 3.2D, F; white arrows) or nestin (Figure 3.2H, J) within the SVZ at 48 hours PI. Similarly, representative immunofluorescence images illustrated the colocalization of GFP-CVB3 protein and Olig2 (Figure 3.2E, G) or nestin (Figure 3.2I, K; white arrows) within the SVZ at 24 hours PI.
LCMV Altered the Expression of a Greater Number of Genes at Later Time Following Infection as Compared to CVB3

In order to analyze overall changes to the host gene expression profile as a result of the neonatal CNS response to viral infection, microarray analysis was performed following both LCMV and GFP-CVB3 and the host gene expression levels were compared to mock-infected control mice (Figure 3.3). In total, LCMV infection altered the expression of 231 genes greater than or equal to a 2-fold induction, as compared to mock-infected control mice. (Figure 3.3A). Alterations in host gene expression were delayed for LCMV infection; 5 genes were upregulated at 12 hours PI. By 24 hours PI, 151 genes were upregulated; and by 48 hours PI, 135 genes remained upregulated. In total, GFP-CVB3 infection altered the expression of 117 genes greater than or equal to a 2-fold induction, as compared to mock-infected control mice. Alterations in host gene expression were more rapid following GFP-CVB3 infection, perhaps reflecting the greater inoculum of virus. However, recombinant coxsackieviruses show delayed growth kinetics and reduced morbidity, as compared to wild type coxsackievirus in our neonatal mouse model (16). The greater inoculum utilized was chosen based on previous comparisons of viral growth kinetics and mortality curves between GFP-CVB3 and wild type CVB3. At 12 hours PI, 55 genes were upregulated. By 24 hours PI, only 10 genes were upregulated; and by 48 hours PI, 66 genes remained upregulated.
The number of genes up or downregulated following infection for both viruses were grouped and shown as Venn diagrams for each time point (Figure 3.3B). Perhaps revealing distinct neonatal host responses for each neurotropic RNA virus, no more than 7 genes were up or downregulation in parallel for both viruses at any time points. Those genes either up or downregulated by both viruses included the Hox developmental genes. More specifically, Hoxa5 and Hoxb5 were upregulated at 12 hours PI for both viruses; also, insulin degrading enzyme and O-linked N-acetylglucosamine transferase were downregulated by both viruses at 12 hours PI. Purkinje cell protein 2 was the only gene upregulated by both viruses at 24 hours PI; also, Hoxa5, Hoxb5, Hoxb7, microtubule-associated protein-1B, and pre B-cell leukemia transcription factor-1 were downregulated by both viruses at 24 hours PI. By 48 hours PI, cDNA sequence BC1179090, S100 calcium binding protein-A8, S100 calcium binding protein-A9 and stefin-A1 were upregulated by both viruses; also, claudin-10, ectonucleotide pyrophosphatase / phosphodiesterase-6, G protein-coupled receptor 37-like-1, myelin basic protein, myelin-associated glycoprotein, myelin-associated oligodendrocytic basic protein, and tryptophan hydroxylase-2 were downregulated by both viruses at 48 hours PI.
Immune Response Gene Expression Varied Temporally and in Magnitude between CVB3 and LCMV

Many of the immune response genes induced by GFP-CVB3 and LCMV infection in the neonatal CNS are summarized via heat map (Figure 3.4A). GFP-CVB3 induced a narrow combination of chemokines at 12 hours PI, including CCL12 (outlined with a red box), CCL7, CCL4, CXCL4, CCL2, CXCL10, and CCL9 and CCL4. In contrast, LCMV induced a greater number of chemokines (including the chemokines described above) and high levels of many interferon response genes. In addition, relatively high levels of CXCL10, CCL5, CXCL9, OAS1g and PKR were induced following LCMV infection although at a later time point (24 hours PI). The magnitude of change for many of the immune response genes was far greater following LCMV infection, as compared to GFP-CVB3. For example, CXCL10 showed a 12.5 fold induction in the neonatal CNS following LCMV infection at 24 hours PI. Many of these chemokines have been shown previously to be induced in the adult CNS following LCMV infection (1). Also, LCMV induced high levels of the MHC genes H2-K1, H2-Q8 and beta-2-microglobulin (B2m) at 24 hours PI, while the expression of these genes following GFP-CVB3 infection were reduced in magnitude and delayed temporally (48 hours PI).

As described previously, CCL12 expression localized to infected myeloid cells, choroid plexus epithelial cells, and ependymal cells in GFP-CVB3-infected animals at early time points (12 hours PI) following infection (33). We wished to inspect the expression of CCL12 in greater detail and over
more time points following both LCMV and GFP-CVB3 infection. In order to verify the microarray results and inspect the location of CCL12 gene expression, immunofluorescence microscopy was performed on neonatal brain sections for MCP-5/CCL12 at 12 hours, 24 hours, and 48 hours PI (Figure 3.4B, C, D, respectively; red signal). CCL12 expression colocalized with sites of infected myeloid cell infiltration shortly after GFP-CVB3 infection (12 hours PI). The choroid plexus is known to be a critical entry site for immune cell infiltration into the CNS (30), and CCL12 may be a key chemokine responsible for the unique myeloid cell infiltration observed following GFP-CVB3 infection. Expression levels of CCL12 in GFP-CVB3-infected animals rapidly decreased by 24 hours and 48 hours PI. Diffuse and lower expression levels of CCL12 were observed in the neonatal CNS early (12 hours PI) following LCMV-infection, although CCL12 levels increased by 24 hours PI and remained elevated, though to a lesser extent, at 48 hours PI. No CCL12 protein was seen in mock-infected control mice at 12 hours PI, although low expression levels were observed in the choroid plexus at 24 and 48 hours PI. Thus, immunofluorescence microscopy served to confirm the results at the protein level for the microarray analysis described in Figure 3.4A.

**MHC Class I was Strongly Expressed after LCMV Infection and Localized to the Lateral Ventricle**

As described above, high expression levels of the MHC class I genes (H2-K1, and H2-Q8) and B2m was observed via microarray analysis following LCMV infection. In contrast, the expression profile for these genes was
delayed temporally and greatly reduced in magnitude following GFP-CVB3 infection. Immunofluorescence microscopy confirmed the microarray results and aided in determining the location of MHC class I gene expression in the neonatal CNS following both LCMV and GFP-CVB3 infection. An antibody corresponding to the H2-K1 locus and cross-reacting with the H2-Kb haplotype of C57BL/6 mice (Clone 5K46; Santa Cruz Biotechnology, Inc.) was utilized to evaluate the upregulation of MHC class I gene expression (Figure 3.5; red signal). MHC class I gene expression was largely absent in the neonatal CNS of GFP-CVB3-infected mice at 12 hours (Figure 3.5A, white arrows) and 24 hours PI (Figure 3.5B, white arrows). At 48 hours PI, low levels of MHC class I gene expression was observed in the SVZ (Figure 3.5C). Also, a lack of viral protein colocalization with MHC class I gene expression was evident. These results parallel previous studies with CVB3 suggesting an inhibition of antigen presentation via the MHC class I pathway (9) which may contribute to evasion of CD8+ T cell immunity (23). In contrast to the results seen for GFP-CVB3, detectable levels of MHC class I gene expression was observed as early as 12 hours PI following LCMV infection (Figure 3.5A). These levels increased by 24 hours PI (Figure 3.5B), and remained high at 48 hours PI (Figure 3.5C). In addition, high levels of LCMV NP expression was observed in regions of the SVZ which expressed high levels of MHC class I gene expression. Moderate levels of MHC class I gene expression for LCMV-infected mice were also present in other regions of the brain, including the meninges, olfactory bulb, and cerebellum at all time points analyzed. Detectable levels of MHC Class I gene
expression was observed in the subventricular zone and choroid plexus of mock-infected animals at 12 and 48 hours PI, although little signal was observed at 24 hours PI.

**Developmental and CNS-Related Genes, such as Transthyretin, were Downregulated after CVB3 Infection**

To analyze developmental and CNS specific gene expression changes, microarray results were summarized via heat map (Figure 3.6A). The gene expression profile for both GFP-CVB3 and LCMV was similar at 12 and 24 hours PI. Hox gene expression was upregulated following infection of GFP-CVB3 and LCMV at 12 hours PI. However by 24 and 48 hours PI, GFP-CVB3 infection downregulated several Hox genes, including Hoxa5 (-5.8-fold and -2.9-fold, respectively), Hoxb5 (-5.0-fold and -2.7-fold, respectively), Hoxb7 (-2.6-fold and -1.4-fold, respectively). In contrast, LCMV induced these Hox genes (+5.5, +4.9, and +2.1-fold, respectively) at 48 hours PI, subsequent to a precipitous decline of Hox gene expression at 24 hours PI. Both GFP-CVB3 and LCMV downregulated expression levels of myelin basic protein (Mbp; -5.2 and -5.7-fold, respectively) at 48 hours PI. In addition, LCMV specifically downregulated glial fibrillary acidic protein (GFAP; -3.1-fold) at 48 hours PI. Intriguingly, GFP-CVB3 specifically reduced the expression of transthyretin (TTR; -3.3-fold; Figure 3.6A, red box) at 48 hours PI. The reduction of TTR expression following GFP-CVB3 infection was of particular interest given the previously observed pathology in the choroid plexus at 48 hours PI (33), and the recognition of hydrocephalus in a significant number of mice surviving GFP-CVB3 infection.
(16). TTR protein produced by the choroid plexus accounts for approximately 25% of the protein secreted in the CSF. The reduction of TTR expression may reflect virus-mediated pathology in the choroid plexus following GFP-CVB3 infection. As determined by immunofluorescence microscopy, TTR protein was expressed at high levels in the choroid plexus of LCMV or mock-infected mice at 12, 24 and 48 hours PI (Figure 3.6B, C, D; respectively). In contrast, GFP-CVB3 infected animals showed reduced levels of TTR protein in the choroid plexus at 12, 24 and 48 hours PI. The apparent lack of TTR gene expression downregulation as determined by microarray analysis at 12 and 24 hours PI following GFP-CVB3 infection might reflect reductions of TTR protein specifically within the choroid plexus in the lateral ventricle, as compared third and fourth ventricles.

**Decreased Expression of Carbonic Anhydrase II, a Functional Marker of the Choroid Plexus, Following CVB3 Infection**

Downregulation of carbonic anhydrase II, a marker of the choroid plexus functional activity, following GFP-CVB3 infection. While reduced TTR expression suggested choroid plexus dysfunction following GFP-CVB3 infection, we examined a functional maker that would clearly show a reduced capacity of the choroid plexus to produce CSF. We performed immunofluorescence microscopy using an antibody against carbonic anhydrase II (CAII) which catalyzes the reversible hydration of carbon dioxide and comprises one of the key enzymes responsible for the secretion of cerebrospinal fluid (Figure 3.7; red signal). As expected, CAII expression was
observed in choroid plexus epithelial cells of mock-infected mice at 12, 24 and 48 hours PI (Figure 3.7A, B, C). LCMV-infected mice also expressed high levels of CAII at every time point analyzed, including in cells expressing high levels of LCMV NP. Although high levels of CAII were observed in the choroid plexus and in nearby GFP-CVB3-infected myeloid cells at early time points (12 and 24 hours PI), CAII expression levels fell dramatically at 48 hours PI (Figure 3.7C; white arrows).

**Severe Pathology was Observed Specifically in the Choroid Plexus of CVB3 Infected Animals at 48 Hours Post-Infection**

The choroid plexus of GFP-CVB3-infected animals displayed severe pathology. We inspected choroid plexus pathology following infection in H&E stained sections (Figure 3.8). Also, the level of apoptosis in the choroid plexus was determined by ApopTag staining. The morphology of the choroid plexus following LCMV infection and the functional integrity of the choroid plexus epithelial cells (Figure 3.8A) appeared similar to that of mock-infected control mice (Figure 3.8E). In contrast, the presence of red blood cells within the lateral ventricle and possible hemorrhaging of the choroid plexus was observed following GFP-CVB3 infection (Figure 3.8C; blue arrows). Also, representative ApopTag staining images showed an increase in apoptosis within the choroid plexus of GFP-CVB3-infected mice (Figure 3.8D), as compared to LCMV-infected (Figure 3.8B) or mock-infected animals (Figure 3.8F). These results were quantified using ImageJ software to reveal a significantly higher degree of apoptosis in GFP-CVB3-infected mice, as
compared to LCMV-infected or mock-infected animals (Figure 3.8G; *p<0.05)). No difference in the levels of apoptosis was observed between LCMV and mock-infected control mice (p>0.05).

CVB3 Restricted Pathology in the Choroid Plexus Recovers at Later Time Points in both Hydrocephaly and Non-Hydrocephaly Animals

Lastly, we wished to determine whether the pathology and dysfunction observed in the choroid plexus of GFP-CVB3 infected animals at 48 hours PI is restored at later time points. Therefore, we performed IHC for transthyretin (TTR) or carbonic anhydrase II (CAII) and H&E staining on GFP-CVB3 and Mock infected animals at 30 and 90 days PI to analyze choroid plexus morphology and function (Figure 3.9). At 30 days PI (Figure 3.9A-D) and 90 days PI (Figure 3.9E-H), there is no difference observed in CAII expression (white arrows) in the choroid plexus between Mock (Figure 3.9A, B, E, F) and GFP-CVB3 (Figure 3.9C, D, G, H) infected animals via CAII IHC and H&E staining. Furthermore, CAII (Figure 3.9I) and TTR (Figure 3.9J) expression and choroid plexus morphology (Figure 3.9K) recovered in the brains of GFP-CVB3 infected animals undergoing hydrocephalus at 75 days PI. These results indicate that the damage observed in the choroid plexus of GFP-CVB3 infected animals is short-lived, however it cannot be ruled out that it could lead to alterations in the normal functioning of the choroid plexus/CNS that these assays were not sensitive enough to detect. Furthermore, GFP-CVB3-mediated hydrocephalus may be the result of CSF blockage of outflow in the ventricles. Alternatively,
choroid plexus recovery following GFP-CVB3 infection may result in
dysregulated or overproduction of CSF in the lateral ventricle.


DISCUSSION

Viral infection of the central nervous (CNS) may cause serious clinical disease particularly in newborn infants potentially due to their immature immune response and ongoing development. We established a neonatal mouse model to study both CVB3 and LCMV infection of the CNS. For these studies, we utilized a recombinant CVB3 expressing GFP (GFP-CVB3) to track virus spread in the neonatal CNS, and we compared these results with that of LCMV (Armstrong strain). Viral protein expression for both viruses localized to the choroid plexus and neurogenic regions near the lateral ventricle of the neonatal CNS. We observed a gradual increase in viral protein expression following LCMV infection accompanied by an increase in gene expression for many immune response and other host genes via microarray analysis. Early GFP-CVB3 infection induced the entrance of a novel population of infected myeloid cells and the induction of a family of chemokines as early as 12 hours PI.

Despite the temporal dissimilarities between the two viruses, both viruses exhibited tropism for the subventricular zone (SVZ) and stem and progenitor cell populations within the neonatal CNS. We utilized nestin and Olig2 markers to label both neural and glial progenitor cell populations, respectively. GFP-CVB3 clearly displayed a preference for neural progenitors, while LCMV infected both neural and glial progenitor cells relatively equally. Supporting our results, LCMV has been shown to infect neurons and astrocytes. Furthermore, LCMV was found to decrease the expression of GFAP,
an astrocytic cell marker. In contrast, GFP-CVB3 has more restricted tropism for neurons.

Immunofluorescence staining of nestin and Olig2 revealed an overlap signal in the neurogenic regions of the neonatal CNS (data not shown). Others have observed potential overlap between nestin and Olig2 staining (21). We estimate that approximately 25-50% of Olig2+ cells may also stain for nestin. Therefore, the total percent of neural or glial progenitor cells infected for GFP-CVB3 and LCMV (approximately 90% and 77%, respectively) may actually be lower, suggesting that additional cell types become infected in the SVZ. These additional cell types might include downstream progenitor cells or immature neurons and glial cells. Thus, a more in depth analysis of stem cell tropism using additional markers along the pathway of stem cell differentiation might be informative.

The broader tropism of LCMV may be responsible for its greater effect on gene expression in the neonatal CNS. GFP-CVB3, a highly cytolytic virus, may kill target cells in the neonatal CNS; whereas LCVM, a non-cytolytic virus, may exhibit a prolonged infection thereby inducing greater changes in gene expression. Although LCMV altered the expression of a greater number of genes as compared to GFP-CVB3, both viruses profoundly affected host chemokine and immune response genes, despite the early time points analyzed. The substantial induction of MHC Class I gene expression by LCMV might have been expected, due to the high numbers of cytotoxic T lymphocytes stimulated following viral infection as shown by many previous
studies (8) (34). In GFP-CVB3-infected mice, MHC Class I gene expression was either absent or at very low levels in the SVZ at 12 and 24 hours PI. These results parallel previous findings suggesting the potent ability of CVB3 to decrease MHC Class I gene expression by shutting down host cell translation and inhibiting surface expression (26) (9). Nonetheless, limited expression of MHC Class I was observed at later time points (48 hours PI) at a time when GFP-CVB3 virus protein expression waned in the choroid plexus, and signs of pathology were readily evident.

Choroid plexus pathology and apoptosis was apparent following GFP-CVB3 infection. Microarray analysis provided a molecular basis for this pathology by identifying a reduction in transthyretin (TTR) following GFP-CVB3 infection. TTR is synthesized in the choroid plexus and acts as a carrier for thyroid hormone thyroxine and retinol. Intriguingly, altered TTR levels have been shown to play a role in depression and schizophrenia (29) (18). Thus, a marked decrease in TTR following GFP-CVB3 infection may provide a functional link between previous viral infection and these neurological disorders (31). We further analyzed choroid plexus function by inspecting the expression levels of carbonic anhydrase II (CAII) following CVB3 infection. CAII was shown to be significantly decreased 48 hours following GFP-CVB infection, indicating potential dysfunction in the production of CSF in the choroid plexus of these mice. In contrast, a lack of choroid plexus pathology was observed following LCMV infection at every time point. Infected myeloid cells also expressed high levels of CAII following GFP-CVB3 infection. CAII exhibits the
most ubiquitous protein levels of any CA isozyme, which may include infiltrating myeloid cells (2) (12).

Many developmentally regulated and CNS specific genes were found to be altered following GFP-CVB3 and LCMV infection, including myelin basic protein. Of note, CVB3 and LCMV have both been associated with acute myelitis following infection (10) (20). We expect that the early time points analyzed may shed light and highlight the rapid induction of host response genes following infection with these neurotropic viruses. Early treatment may prevent possible neuropathology and alterations in gene expression which may be detrimental to the host. Further analysis of the effects of early viral infection on the neonatal host CNS may be beneficial in optimizing treatment regimes against these RNA viruses.

Chapter 3, in part is currently being prepared for submission for publication of the material. Tabor-Godwin, JM.; Ruller, CM.; Buchmeier, MJ.; Doran, KS.; Feuer, R. The dissertation author was the primary investigator and author of this material.
Figure 3.1: Viral proteins for both LCMV and GFP-CVB3 localize to the lateral ventricle of the neonatal CNS with varying kinetics and distribution

LCMV nucleoprotein expression (red signal) at 12 (A, B), 24 (E, F) and 48hrs (I, J) after LCMV infection in one day old mice. Infection of SVZ cells and choroid plexus epithelial cells increases over time. GFP-CVB3 infection (green signal) induces the extravasation of infected GFP-positive myeloid cells at 12 (C, D) and 24hrs (G, H) PI, as well as infecting resident cells in the SVZ. At 48hrs PI (K, L) infection moves away from the choroid plexus and further into the brain.

Nuclei/DNA in all images are stained with DAPI (blue). B, D, F, H, J, L are higher magnifications of the white box in A, C, E, G, I, K whole brains, respectively.
Figure 3.2: LCMV infects more cells in the SVZ and has broader stem and progenitor cell tropism in comparison to GFP-CVB3

(A) LCMV infected significantly more cells in the SVZ than GFP-CVB3 (LCMV mean = 364.5, GFP-CVB3 mean = 102.5, p=0.0295,*). (B) significantly more Olig2+ cells than GFP-CVB3 (LCMV mean = 37.64, GFP-CVB3 mean = 12.9, p=0.001,* ) and (C) significantly less nestin+ cells than GFP-CVB3 (LCMV mean = 39.55, GFP-CVB3 mean = 76.78, p=0.0302,* ) by Student's T-test. Representative images of infected Olig2+ (D, E, F, G, red signal) and nestin+ (H, I , J, K, red signal) cells by LCMV at 48hrs PI (D, F, H, J, green signal) and GFP-CVB3 at 24hrs PI (E, G, I, K, green signal) are shown. Double-positive cells are depicted with white arrows. Nuclei/DNA are stained with DAPI (blue signal) for all images. F, G, J, K are 63X magnifications of the white box shown in D, E, H, I at 10X magnification, respectively.
Figure 3.3: LCMV alters the expression of more genes than GFP-CVB3 and at different times after infection

(A) Overall changes in gene expression by GFP-CVB3 and LCMV greater than or equal to 2-fold compared to Mock are shown together and by time point. LCMV altered the expression of 231 genes in total, compared to the 117 genes altered by GFP-CVB3. (B) Genes with altered expression are grouped based on whether they are upregulated or downregulated. Venn diagrams depict if they are shared between or unique to both GFP-CVB3 and LCMV for each time point.
Figure 3.4: Immune response gene expression varies temporally and in magnitude between GFP-CVB3 and LCMV

(A) Microarray data was summarized by heat map, indicating an increase (red) or decrease (blue) in immune response gene expression between GFP-CVB3 and LCMV. (B-D) Immunohistochemistry for MCP-5/CCL12 (red signal) was performed for GFP-CVB3 and LCMV (green signal) and Mock infected samples at 12, 24 and 48hrs PI and confirmed the CCL12 induction observed in the microarray results. Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications (below) of the white box shown in the 20X magnification (above) are shown for all images.
Figure 3.5: MHC Class I is most strongly expressed by LCMV and localizes to the lateral ventricle

MHC class I (red signal) was largely absent from GFP-CVB3 (green signal) infected brain sections until 48hrs PI (C), at which point it was observed in the SVZ. For LCMV infected animals, low amounts of MHC Class I were seen at 12hrs PI (A), whereas signal peaked at 24hrs PI (B) and dropped by 48hrs PI (C). Signal was observed most strongly in the SVZ, but was present in other regions of the brain. MHC class I expression in Mock infected brains was strongest at 12hrs PI (A) and 48hrs PI (C), with little signal observed at 24hrs PI (B). Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications (below) of the white box shown in the 20X magnification (above) are shown for all images.
Figure 3.6: Developmental and CNS specific gene expression is similar between the two viruses until 48 hours post-infection

(A) Microarray data was summarized by heat map indicating an increase (red) or decrease (blue) in developmental and CNS specific gene expression between GFP-CVB3 and LCMV. (B-D) Immunohistochemistry for TTR (red signal) was performed for GFP-CVB3 and LCMV (green signal) and Mock infected samples at 12, 24 and 48hr PI and confirmed the GFP-CVB3 specific decrease in TTR observed in the microarray at 48hrs PI. Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications (below) of the white box shown in the 20X magnification (above) are shown for all images.
A Development/CNS Specific

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Figure 3.7: Carbonic Anhydrase II, a functional marker of the choroid plexus, is downregulated exclusively after GFP-CVB3 infection

Carbonic anhydrase II (CAII; red signal) is observed in GFP-CVB3 infected myeloid cells (green signal) as well as choroid plexus epithelial cells at 12hrs PI (A) and 24hrs PI (B). By 48hrs PI (C) CAII signal is greatly decreased (white arrows). LCMV and Mock infected sections express strong signal in the choroid plexus at all time points, despite direct infection of the choroid plexus epithelium by LCMV. Nuclei/DNA are stained with DAPI (blue signal) and 63X magnifications (below) of the white box shown in the 20X magnification (above) are shown for all images.
Figure 3.8: The choroid plexus of GFP-CVB3 infected animals displays severe pathology

(A, C, E) H&E staining at 48hrs PI revealed a smaller choroid plexus after GFP-CVB3 infection and disruption of the morphology of the choroid plexus. Specifically, the blood-cerebral spinal fluid barrier was compromised, as shown by red blood cells leaking in the lateral ventricle (C, blue arrows). (B, D, F) Representative images of ApopTag staining (red signal) for LCMV, GFP-CVB3 and Mock infected brain sections are shown beneath H&E stained sections, respectively. (G) Significantly higher levels of apoptosis were observed in the choroid plexus of GFP-CVB3 infected animals as compared to LCMV and Mock (p<0.01,*) infected brain sections. There was no difference between LCMV and Mock infected animals (p>0.05). Nuclei/DNA are stained with DAPI (blue signal) and autofluorescence (yellow signal) is observed in red blood cells. All images shown at 20X objective.
Figure 3.9: GFP-CVB3 restricted pathology in the choroid plexus recovers at later time points
At 30 days PI (A, B, C, D) and continuing to 90 days PI (E, F, G, H) the choroid plexus appears normal as determined by CAII immunostaining and H&E staining in GFP-CVB3 (C, D, G, H) infected samples compared to Mock (A, B, E, F). Infected animals undergoing hydrocephalus 75 days PI also retained expression of CAII (I) and TTR (J) with normal choroid plexus morphology (K). Areas of either CAII or TTR expression are shown with white arrows. Nuclei/DNA are stained with DAPI (blue signal) for immunostained images and all images are shown at 20X magnification.
REFERENCES


CHAPTER IV:

The Role of Autophagy after Coxsackievirus Infection in Neural Progenitor and Stem Cells
INTRODUCTION

Macroautophagy, hereby referred to as autophagy, is an essential process that is responsible for the breakdown of long-lived proteins and organelles within the cell. Autophagy is activated downstream of class III phosphatidylinositol 3 (PI3)-kinase signaling, and the growth of the autophagosome double-membrane is promoted by the association of covalently conjugated autophagy proteins (18). One such protein is light chain 3 (LC3), whereas the lipidated form of LC3, known as LC3-II, studs the inner and outer autophagosome membrane. Autophagosomes can be visualized directly by labeling LC3 with a fluorescent marker, such as green fluorescent protein (GFP), and by observing GFP-positive vacuoles within cells. In addition, the ratio of LC3-II to un-modified LC3-I, termed LC3 conversion, can be analyzed via western blotting (24). Once formed, the autophagosome may fuse with the lysosome and its contents can be degraded.

Autophagy can be induced by several processes, including starvation, cell damage, and invading pathogens, known as xenophagy (26). Autophagy has also been found to play an important part in immunity through its ability to facilitate MHC-peptide and toll-like receptor-ligand interactions (10). While some microbes, such as Mycobacterium tuberculosis, are destroyed by autophagy, others, such as Dengue virus, use autophagy to their advantage (19) (28). Interestingly, some viruses, such as herpes simplex virus-1, can encode proteins that inhibit autophagy during the course of infection, thus providing increased neurovirulence (2) (31). Furthermore, viral
interactions with the autophagy pathway may occur throughout the viral life cycle and at different stages of the autophagy pathway. For example, hepatitis C virus only utilizes autophagosomes to initiate viral replication. In addition, coxsackievirus B3 (CVB3) not only induces autophagy after infection, but the virus blocks the maturation of the autophagosome, thus generating megaphagosomes (12) (22). These examples reveal a complex interplay between pathogens and autophagy that has evolved over time and appears to be specific to each microbe (29).

Enteroviruses are serious human pathogens that are responsible for a wide range of disease, ranging from simple flu-like symptoms to poliomyelitis. CVB3 has been found to cause severe morbidity and mortality by contributing to myocarditis, pancreatitis, and meningitis (3) (44). These infections are more common in neonates and may lead to long-term sequelae later in life, including dilated cardiomyopathy, learning disabilities and demyelinating disorders (34) (45) (6) (27). When tracking neonatal CVB3 infection in vivo using a recombinant eGFP expressing CVB3 (eGFP-CVB3), neural progenitor and stem cells (NPSCs) were discovered to be highly susceptible to CVB3 infection (15). This was determined through co-localization of viral GFP expression with neural stem cell markers located in the subventricular zone, a neurogenic region of the central nervous system (CNS) (16). To further analyze infection in this cell population, primary neonatal NPSCs were isolated and infected in culture. These cells generate free-floating spheres known as neurospheres that become heavily infected with CVB3 in vitro (42). When
grown in the appropriate conditions, neurospheres retain the ability to differentiate into precursors to all three cell lineages of the CNS, including neurons, astrocytes, and oligodendrocytes (13). Differentiated NPSC precursors were found to be less susceptible to CVB3 infection as compared to undifferentiated NPSCs, presumably due to their decreased proliferative status (14).

Recent publications investigating autophagy after enterovirus infection have shown that poliovirus, coxsackievirus and enterovirus 71 induce autophagy and utilize the autophagosome membrane for viral replication (39) (46) (20). In particular, previous studies have found that the poliovirus 3A and 2BC proteins, when expressed together in the absence of virus, may induce autophagy. Additionally, viral protein 2BC can induce the conversion of LC3 (21) (40). Furthermore, autophagy may play a part in the non-cytolytic release of enteroviruses from the cell (41). Similar findings were established in vivo, as CVB3 infection of the pancreas induced autophagosomes, which provided a scaffold for viral replication in pancreatic acinar cells (22). However, these previous studies have been performed in differentiated cell types, such as HeLa cells and rat primary neurons, thus leaving the role of autophagy in stem cells unexplored (49). Since NPSCs were found to be highly susceptible to CVB3 both in vitro and in vivo, the goal of our study was to determine whether autophagy was altered after in CVB3 infection in NPSCs. In addition, we wished to explore the relationship of autophagy and viral replication in NPSCs.
**METHODS**

**Isolation and Culture of NPSCs**

Mouse experimentation conformed to the requirements of the San Diego State University Animal Research Committee and the National Institutes of Health. C57BL/6 mice were obtained from the Scripps Research Institute animal facilities or Harlan Sprague Dawley (Harlan Laboratories, San Diego CA). Breeding pairs were checked every day. NPSCs were derived from isolated cortices of newborn mice, mechanically and enzymatically dissociated, and then plated as a single cell suspension in DMEM/F12 media supplemented with 2% B12 Supplement (Invitrogen), 20ng/mL EGF (Invitrogen), 20ng/mL bFGF (Preprotech), 5ug/mL Heparin (Sigma), and 0.5% pen/strep. Free-floating neurospheres were separated and transferred into new flasks every two days. Neurospheres were vigorously triturated and resuspended in culture medium to a concentration of 10^5 cells/mL in a T-25 flask (BD falcon). Neurospheres were differentiated using gelatin/fibronectin coated chamber slides in DMEM media supplemented with 1% N1 supplement, 1% FBS and 0.5% pen/strep for 5 days.

**Transfection, Infection and Treatment of NPSCs and HL-1 cells**

Undifferentiated NPSCs, differentiated NPSCs and HL-1 cells (cultured as previously described, (7)) were transfected with GFP-LC3 adenovirus for 40 hours then infected with dsRED-CVB3 at an MOI of 10. Autophagy inducers (CCPA and rapamycin, Rm) or inhibitor (3-Methyladenine, 3-MA) were
administered in fresh media at the time of dsRED-CVB3 infection at the following final concentrations: CCPA – 0.1μM, Rm – 5μM and 3-MA – 10mM. Supernatants were harvested at the indicated time points and analyzed by plaque assay to determine viral titers.

**GFP-LC3 Vacuole Quantification**

For time course experiments, NPSCs and HL-1 cells were imaged live for GFP-LC3 vacuoles at the indicated time points and 50-200 cells per well were counted in triplicate for mock and infected cultures. For non-time course experiments, cells were fixed in 4% paraformaldehyde, washed three times in 1X PBS and 200 cells per well were counted in triplicate for all treatments. For differentiated NSPC precursors and HL-1 cells, a bimodal distribution of GFP-LC3 vacuoles was observed. Therefore, transduced cells were scored as having less than or greater than 30 GFP-positive vacuoles, as previously described (4). For undifferentiated NPSCs, a bimodal distribution was not observed. Therefore, the average number of GFP-positive vacuoles per transduced cell was counted. Counts are represented as the mean plus the standard error of the mean.

**Immunofluorescence Microscopy**

NPSCs were fixed in 4% paraformaldehyde, washed three times in 1X PBS and permeabilized with 0.5% Triton X 100. Viral protein expression was determined by native dsRED (red) expression. Fixed cells were blocked with 10% normal goat serum and immunostained using the following antibodies:
neuronal class III beta-tubulin (Covance, PRB-435P) at 1:1000, GFAP (Sigma, G9269) at 1:500, and Olig2 (Abcam, ab33427) at 1:1000. Secondary antibodies were labeled with Alexa Fluor 594 or Alex Fluor 350 at (1:1000). Live and fixed cultures were imaged using a Zeiss Axio Observer D.1 inverted fluorescent microscope. Three to five representative images of the cultures were taken for each time point.

**Western Blotting**

Undifferentiated NPSCs, differentiated NPSCs and HL-1 cells were washed 1X PBS 24 hours after infection/treatment. Cells were scraped and/or centrifuged at 7,000rpm for 1 minute, based on adherence, in 1X PBS and resuspended in cell extraction buffer (Invitrogen, FNN0011) with protease inhibitors for 30 minutes on ice with periodic vortexing. Cells were then centrifuged at 13,000rpm for 10 minutes at 4°C and supernatants were transferred to a new tube. Samples were prepared for SDS-PAGE by adding 19.5μl protein extract, 7.5ul LDS sample buffer and 3ul reducing agent and heating for 3 minutes at 70°C. Gel electrophoresis was performed for 1 hour at 150V with 15μl of each sample. Proteins from the gel were transferred to a PVDF membrane for 1 hour at 30V. The membrane was blocked with 5% skimmed milk in TBST for 1 hour at room temperature. The membrane was cut in half just under the 40kDa marker. The top half was probed for rabbit-anti beta-Tubulin (50kDa, Abcam, ab6046) at (1:500) in 1% BSA in TBST overnight at 4°C. The bottom half of the membrane was probed for either endogenous LC3 (16 and 18kDa) or Bcl-2 (26kDa). Rabbit anti-LC3A/B (Cell Signaling,
# was used at (1:1000) in 5% BSA in TBST overnight at 4°C. Mouse anti-Bcl-2 (Invitrogen, 33-6100) was used at (1:500) in 1% BSA in TBST overnight at 4°C. For all primary antibodies, HRP-conjugated secondary was applied at (1:2000-5000) for 1 hour at room temperature. Washes were performed in triplicate for 5 minutes in 1X TBST between all incubations. ECL substrate (Invitrogen, WP20005) was applied for 1 minute and membranes were exposed to film. Quantification of band density was performed using Image J software and normalized to beta-Tubulin levels.

Statistics

Statistics were calculated using Graphpad Prism 3.0 software. In particular, one-way ANOVA with Newman-Keuls post-hoc analysis was used to analyze three or more groups, while Student’s T-test was utilized to compare two groups. Significance was determined by a p-value of 0.05 or lower.
RESULTS

Levels of Autophagy Increase after CVB3 Infection in HL-1 Cells

The levels of autophagy following CVB3 infection was studied first in a differentiated cell type in order to replicate previous findings by other investigators. HL-1 cells were utilized, which are a transformed cardiomyocyte cell line that have previously been shown to be highly susceptible to CVB3 infection (7) (51). Furthermore, HL-1 cells were chosen since CVB3 exhibits tropism for the heart. No previous studies have examined cardiac cells in relation to autophagy and CVB3 infection. When HL-1 cells were infected with dsRed-CVB3, there was a statistically significant increase (p=0.002) in the level of autophagy between mock and infected by 24 hours post-infection (Figure 4.1B-C). This was accompanied by a statistically significant increase (p<0.01) in viral titers over time (Figure 4.1A).

CVB3 infection and CCPA Increased the Level of Autophagy in HL-1 Cells

In order to determine whether the increase in autophagy after CVB3 infection was linked to viral replication, the level of autophagy was altered in HL-1 cells using the autophagy inducer, CCPA, and the autophagy inhibitor, 3-MA. CCPA is an adenosine A1 agonist that activates autophagy by increasing intracellular calcium levels, while 3-MA is a class III PI3-kinase inhibitor that halts autophagy but does not affect protein synthesis (47) (37). While CCPA significantly increased (p<0.01) the level of autophagy in HL-1 cells, 3-MA had no effect, most likely due to the relatively low basal level of autophagy seen in
untreated HL-1 cells (Figure 4.2A). HL-1 cells were serum starved to induce autophagy and autophagy was significantly decreased (p<0.05) in the presence of serum starvation plus 3-MA as compared to serum starvation alone and serum starvation plus CCPA (Figure 4.2B). The effectiveness of the inducer and inhibitor in the presence of dsRED-CVB3 was determined, and infection in the presence of 3-MA was shown to have a significantly lower level of autophagy (p<0.01) than infected alone and infected plus CCPA (Figure 4.2C). Thus, 3-MA and CCPA were effective at modulating autophagy in HL-1 cells.

The Induction or Inhibition of Autophagy Altered CVB3 Replication and Viral Protein Expression Levels in HL-1 Cells

CVB3 replication in the presence of CCPA and 3-MA in HL-1 cells was then analyzed. There was a significant decrease (P<0.001) in viral titers (Figure 4.3A) after 3-MA-treatment 48 hours post-infection (PI). Also, a significant increase (p<0.01) in viral titers was observed after CCPA-treatment 48 hours PI. A similar trend was observed for 24 hours PI, yet was not significant when all time points were analyzed by ANOVA with Newman-Keuls post-hoc analysis. However, when the 24 hour PI time point was analyzed alone, 3-MA-treated was significantly lower (p<0.05) than infected alone and CCPA-treated. Similar trends were observed for viral protein expression (Figure 4.3B). For example, CCPA-treated cells expressed higher levels of dsRED protein as compared to infected cells alone at 24 hours and 48 hours PI. Also, 3-MA-treated cells expressed lower levels of dsRED protein, as compared to infected cells alone.
at 24 hours and 48 hours PI. These results establish a direct relationship between autophagy and CVB3 replication in HL-1 cells.

**Decreased Levels of Autophagy in Differentiated NPSCs after CVB3 Infection**

After using HL-1 cells to confirm previous results in the literature for differentiated cell types, the level of autophagy in differentiated NPSC precursor cells was examined. NPSCs were differentiated for 5 days in differentiation media on gelatin/fibronectin coated chamber slides. These cells begin to express markers for all three CNS lineages, although continue to proliferate. Differentiated NPSC precursors showed statistically higher basal levels of autophagy, which decreased after dsRED-CVB3 infection at 8, 24 and 48 hours PI (p=0.008, p=0.0154, and p=0.0019, respectively) (Figure 4.4B). A statistically significant increase in viral titers was observed over time (Figure 4.4A; *p<0.05). By 72 hours PI, both mock and infected cultures exhibited high levels of autophagy. Interestingly, megaphagosomes were observed at 72 hours PI in both mock and infected cultures (Figure 4.4C; white arrows).

**Differentiated NPSCs Treated with Both 3-MA and CCPA Showed an Increase in Viral Titers Following Infection**

Differentiated NPSCs were treated with the identical autophagy inducer, CCPA and inhibitor, 3-MA, used in HL-1 cells. CCPA failed to increase the naturally high level of autophagy in differentiated NPSCs. In contrast, 3-MA decreased the level of autophagy as compared to mock-treated cultures (Figure 4.5A; *p<0.01). We then tested the ability of CCPA and 3-MA to alter
the level of autophagy following CVB3 infection (Figure 4.5B). While infected alone, infected plus 3-MA and infected plus CCPA all showed statistically lower levels of autophagy, as compared to mock-treated cultures (p<0.05), the drug treatments failed to alter the level of autophagy during viral infection. Surprisingly, viral titers significantly increased (**p<0.01) between infected plus 3-MA versus infected alone and infected plus CCPA. There was also a highly significantly increase (***p<0.001) between infected alone and infected plus CCPA. However, since autophagy was not altered between these groups, the relationship between autophagy and CVB3 replication was not clear. To identify whether autophagy preferentially occurred in one type of CNS precursor, immunostaining was performed using three well-known markers of neuronal and glial cells. Neuronal class III beta-Tubulin labels neuronal precursors, GFAP labels astrocyte precursors and Olig2 labels oligodendrocyte precursors. We then analyzed the level of autophagy in each cell type by evaluating colocalization of GFP-LC3 with each marker (Figure 4.5C and 4.5D). A decrease in the level of autophagy was observed for all cell lineages when comparing mock and infected cells, although only GFAP expressing cells showed statistical significance (*p<0.05). Also, each cell lineage exhibited differing levels of autophagy, although only the lower levels observed in the oligodendrocyte precursors showed statistical significance (*p<0.05).
No Change in the Level of Autophagy in Undifferentiated NPSCs after CVB3 Infection

The level of autophagy after CVB3 infection in undifferentiated NPSCs was then analyzed. Surprisingly, no change in the level of autophagy was observed over time between mock and infected NPSCs (Figure 4.6B, C). While there were increasing viral titers over time, these levels were not significantly different due to large variation between samples (Figure 4.6A).

Undifferentiated NPSCs Exhibit a Higher Basal Level of Autophagy as Compared to HL-1 Cells

Due to the difficulty of counting GFP-LC3 vacuoles in spherical, non-adherent undifferentiated NPSCs (Figure 4.6C), western blotting for endogenous LC3 was performed (Figure 4.7A). While the ratio of LC3-II to LC3-I significantly increased (p<0.01) at 24 hours PI in HL-1 cells, no change was observed in undifferentiated NPSCs 24 hours PI (Figure 4.7B), confirming our results counting GFP-LC3 vacuoles (Figure 4.6C). Furthermore, there was no significant difference between infected HL-1 cells and either mock or infected NPSCs. These results suggested a higher basal level of autophagy in undifferentiated NPSCs. To ensure that differences in the level of autophagy were not due to differences in viral replication between HL-1 cells and NPSCs, viral titers were performed (Figure 4.7C). No significant difference was seen in dsRED-CVB3 titers between HL-1 cells 24 hours PI (mean = 4.08 x 10^6 pfu/mL) and NPSCs 24 hours PI (mean = 1.91 x 10^6 pfu/mL).
Rapamycin Induced Autophagy in Undifferentiated NPSCs yet Did Not Alter CVB3 Replication

In order to determine whether undifferentiated NPSCs were able to undergo autophagy, an inducer of autophagy, rapamycin (Rm), was utilized and levels of autophagy were analyzed by western blotting for endogenous LC3 (Figure 4.8A). Rm induces autophagy by interacting with the mammalian target or rapamycin, mTOR, which is a negative regulator of autophagy upstream of class III PI3-kinase (33). Rm was found to strongly induce autophagy (p<0.001) in both the presence and absence of dsRED-CVB3 infection (Figure 4.8B). Interestingly, dimethyl sulfoxide (DMSO) alone, the vehicle control for Rm, appeared to induce autophagy in comparison to NPSCs treated with sterile water for both mock and infected. There was not a statistically significant increase between water and DMSO treated samples when ANOVA with Newman-Keuls post-hoc analysis was performed for all groups. When Student’s T-test was performed between mock plus water and mock plus DMSO there was a statistically significant increase (p=0.0063), but not between infected plus water and infected plus DMSO (p=0.0716). Viral titers were performed for infected plus water (mean = 50,000 pfu/mL), infected plus DMSO (mean = 74,167 pfu/mL) and infected plus Rm (mean = 58,333 pfu/mL) (Figure 8C). No differences (p=0.2983) in viral titers were observed between any of the treatments. Thus, CVB3 replication did not appear to be altered by the induction of autophagy in undifferentiated NPSCs.
The Addition of Fibroblast Growth Factor Decreased the Level of Autophagy in Differentiated NPSCs

The effect of basic fibroblast growth factor (FGF) on differentiated NPSCs was then examined. FGF is a component of the undifferentiated NPSC media and is typically removed to assist in differentiation. However, a recent study has shown that the addition of FGF to mesencephalic neural progenitor cell cultures may inhibit autophagy-mediated cell death (5). At 8 hours PI, a significant decrease (p<0.01) in the level of autophagy was observed after dsRED-CVB3 infection, as determined by counting GFP-LC3 positive vacuoles (Figure 4.9B, C). However, infected plus FGF was not significantly different from infected. LC3 western blotting was then performed on mock and infected differentiated NPSCs in the presence and absence FGF at 24 hours PI (Figure 4.9A). A similar trend of decreased (p<0.001) autophagy after dsRED-CVB3 infection was observed by western blotting, thus confirming previous results counting GFP-LC3 vacuoles (Figure 4.9D). In addition, infected plus FGF exhibited a significantly lower (P<0.05) level of autophagy than infected alone and mock plus FGF by 24 hours PI. Viral titers were than examined and revealed no difference (p=0.6047) between infected (mean = 25,667 pfu/mL) and infected plus FGF (mean = 31,500 pfu/mL) (Figure 4.9E), therefore indicating that decreasing the level of autophagy did not alter viral replication in differentiated NPSCs.
Bcl-2 Levels Remain Unchanged in HL-1 Cells, Undifferentiated NPSCs and Differentiated NPSCs after CVB3 Infection

B-cell lymphoma 2 (Bcl-2) levels were analyzed before and after infection in all three cultures analyzed (HL-1 cells, differentiated NPSCs, and undifferentiated NPSCs). Similar to the effects of FGF, Bcl-2, an anti-apoptotic protein, has been found to prevent autophagy mediated cell death in neural progenitor cell cultures when overexpressed (5). Bcl-2 expression may be induced downstream of FGF signaling, though a previous study did not see a difference in endogenous levels when FGF was added (11). No change was observed in Bcl-2 levels 24 hours PI by western blot analysis for HL-1 cells (p=0.9648, Figure 10A), undifferentiated NPSCs (p=0.3877, Figure 4.10B) or differentiated NPSCs (p=0.1138, Figure 4.10C) after the results were normalized to beta-Tubulin expression. These results indicate that the differences in the role of autophagy after CVB3 infection in undifferentiated or differentiated NPSCs does not appear to be mediated by Bcl-2. The differential role of autophagy after CVB3 infection is summarized for each cell type in Table 4.1, which also reviews the relationship between autophagy and CVB3 replication.
DISCUSSION

CVB3 is a common childhood enterovirus that can infect and kill NPSCs, thus leading to CNS developmental defects (35). Antibodies against enteroviruses have been detected in approximately 75% of the population and previous infection has been linked to a number of long-term diseases, including schizophrenia and other neurological diseases causing damage to the white matter of the CNS (36) (32) (43). Several publications have found that enterovirus infection induced autophagy and that this induction benefited viral replication, thus highlighting the possibility of using autophagy inhibitors as antiviral treatments (21). These previous studies have been performed in differentiated cell types, thus bringing into question whether autophagy has a function in viral replication in stem cells. Therefore, the goal of this study was to determine the role of autophagy after CVB3 infection in NPSCs.

In order to confirm previous results in differentiated cell types, HL-1 cells were analyzed. Autophagy was induced in HL-1 cells after dsRED-CVB3 infection and this induction was directly linked to CVB3 infection. However, autophagy was not induced until 24 hours PI. In HeLa cells and HEK293A cells, induction was seen as early as 7 hours post-CVB3 infection, which corresponds to roughly one round of viral replication (46). Since dsRED-CVB3 replicates more slowly than wild-type CVB3, this may be responsible for the delayed induction of autophagy after dsRED-CVB3 infection in HL-1 cells. Conversely, in primary rat neurons the level of autophagy began to increase by 16 hours
post-CVB3 infection and continued to increase at 24 hours (49). While 16 hours PI was not analyzed in HL-1 cells, the induction of autophagy in rat primary neurons is more similar to that observed in HL-1 cells as compared to HeLa and HEK293A cells. Therefore, cell type, which can also influence the rate of viral replication based on proliferation, may influence when autophagy is induced as well (14). Additionally, this is the first time that autophagy has been analyzed after CVB3 infection in a cardiomyocyte cell line. Since CVB3 is the most common infectious cause of myocarditis and may lead to pancreatitis and meningitis, understanding how the virus replicates in heart cells may be extremely important in designing potential treatment regimes (9) (22) (25). Thus, novel autophagy-regulating treatments to control CVB3 replication in the heart could be of great clinical importance. Further investigation of the role of autophagy during CVB3 replication in additional cell types, as well as in the host, may be of particular interest.

The level of autophagy after CVB3 infection in undifferentiated and differentiated NPSCs was therefore investigated. While no change in the level of autophagy was observed after CVB3 infection in undifferentiated NPSCs, the basal level of autophagy was also higher in these cells. The higher basal level of autophagy may be due to the increased proliferative capacity observed in NPSCs. However, as shown by using Rm, autophagy was able to be induced in these cells. Also, no toxicity was observed following the induction of autophagy (data not shown), illustrating that undifferentiated NPSCs do have a functional autophagy pathway. However, inducing
autophagy with Rm did not alter levels of viral replication in NPSCs. CCPA was also added to undifferentiated NPSCs but was not successful at inducing autophagy. The lack of autophagic induction seen with CCPA may be due to the absence of a functional adenosine A1 receptor in NPSCs (38). In addition, 3-MA was shown to be toxic in undifferentiated NPSCs, as previously reported (50) (5).

In differentiated NPSC precursors, the level of autophagy was decreased after CVB3 infection in all three CNS cell types. However, mock-infected GFAP+ precursor cells displayed a significantly higher level of autophagy then infected GFAP and mock Olig2 positive precursor cells. Consequently, there may be a preference for autophagy to occur in mock-infected GFAP positive astrocyte precursors. The possibility remained that NPSCs may have a predisposition to differentiate into astrocytes based on plating density, and this effect could alter our results (1) (5). Furthermore, the addition of FGF was found to decrease the level of autophagy in differentiated NPSCs in both the presence and absence of infection. Even so, levels of viral replication remained unchanged in FGF-treated differentiated NPSCs.

We hypothesized that NPSCs may be downregulating autophagy after CVB3 infection due to an increase in apoptosis. For example, an inverse relationship between autophagy and apoptosis was recently been found in rat primary neurons after CVB4 infection (48). However, while apoptosis did increase over time by TUNEL analysis, the increase was not significant (data
not shown). Active caspase-3 was also analyzed, but was found in every cell. Caspase-3 activation may play a non-apoptotic role in differentiation as seen for bone marrow stromal stem cells (30). Conversely, the possibility remains that all potential death pathways, including autophagy, may be inhibited in infected differentiated NPSC precursors in order to allow the virus to persist and/or the host to retain a vital cell population within the CNS. The inability of CCPA and 3-MA to modulate autophagy in differentiated NPSC precursors in the presence of CVB3 infection would support this hypothesis. Thus, NPSCs do not follow the common paradigm concerning the induction of autophagy after CVB3 infection in differentiated cell types. Also, we show for the first time the effects of autophagy induction on CVB3 replication in HL-1 cells.

In order to further investigate why autophagy was not induced in NPSCs, Bcl-2 was examined for its ability to inhibit autophagy mediated cell death in neural progenitor cell cultures, similar to what has been shown for FGF (4). In addition, Bcl-2 overexpression may inhibit autophagy in HL-1 cells when targeted to the endoplasmic reticulum (5). Bcl-2 has also been shown to be induced after CVB3 infection (8). Therefore, if basal levels of Bcl-2 were shown to be higher in NPSCs as compared to differentiated cell types this may inhibit and/or decrease the level of autophagy. We also hypothesized that CVB3 infection might further increase Bcl-2 expression. However, Bcl-2 expression remained unchanged after CVB3 infection in HL-1 cells, undifferentiated NPSCs and differentiated NPSCs. Even so, the signaling pathways involved in modulating autophagy after CVB infection in NPSCs
merit further investigation. In order to obtain a global analysis of what signaling pathways may be activated and modulating the induction of autophagy in NPSCs, whole genome microarray will be performed in future studies.

Also, viral proteins such as 3A and 2BC which has been shown to induce autophagy, may be unable to do so in NPSCs. This could be due to a variety of reasons. For example, proteins influencing autophagy may be downregulated in NPSCs or non-functional due to differences in post-translational processing or localization within this cell type. Expressing fluorescent tagged versions of the CVB3 viral proteins 3A and 2BC within NPSCs may assist in answering these questions. These studies may also shed light on the mechanism of autophagy induction by these viral proteins, which remains unknown.

Moreover, since only extracellular viral titers were examined in this study, it will be interesting to examine intracellular viral titers and the viral genome in the presence of autophagy inhibitors or inducers following CVB3 infection. For example, a large decrease in extracellular versus intracellular poliovirus was observed previously when autophagy was inhibited. Also, exocytosis of poliovirus protein and LC3-positive vesicles was previously shown by electron microscopy (21). Because of these previous findings, autophagy has been implicated as a mechanism for the non-cytolytic release of virus from the cell, and thus provides a means for enteroviruses to persist. Since persistent CVB3 infection has been documented in both the myocardium and
the CNS, the role of autophagy during this process may be of great importance (23) (17). While it is unknown what cell types in the CNS may mediate persistence, NPSCs may be an attractive candidate due to their ability to support persistent infection \textit{in vitro} (42). Given the unusual autophagic response seen in NPSCs after CVB3 infection, it will be beneficial to see how autophagy may affect CNS persistence and pathogenesis \textit{in vivo}.

Chapter 4, in part is currently being prepared for submission for publication of the material. Tabor-Godwin, JM.; Tsueng, G.; Sayen, R.; Gottlieb, RA.; Feuer, R. The dissertation author was the primary investigator and author of this material.
Figure 4.1: Levels of autophagy increase after CVB3 infection in HL-1 cells

(A) CVB3 titers were determined by plaque assay. No titers were found in mock-infected cells. Viral titers at 8 hours and 24 hours PI were significantly higher (p<0.01, *) than 1 hour PI, with the initial inoculum subtracted. (B) Quantification of cells with high levels of GFP-LC3 autophagosome vacuoles was performed by counting 50 transduced cells per well, with 3 wells per treatment (represented as the mean ±SEM). High levels of GFP-LC3 autophagosome vacuoles were defined as greater than 30 punctate per cell. Infected cells at 24 hours PI were significantly different (p=0.002, *) than mock-infected cells. No difference was observed between mock and infected cells at 1 hour and 8 hours PI. (C) Representative 63X images are depicted for all time points and treatments.
Figure 4.2: CVB3 infection and CCPA increase the level of autophagy in HL-1 cells

(A) The level of autophagy increased (*p<0.01) following the addition of CCPA (inducer of autophagy) to HL-1 cells, as compared to mock or 3-MA (inhibitor of autophagy). (B) A significant decrease (*p<0.05) in the percentage of transduced HL-1 cells with high levels of GFP-LC3 vacuoles was observed after serum starvation (SS) in the presence of 3-MA, as compared to SS alone or SS in the presence of CCPA. (C) A significant decrease (*p<0.01) in the percent of transduced HL-1 cells with high levels of GFP-LC3 vacuoles was observed after dsRED-CVB3 infection in the presence of 3-MA, as compared to dsRED-CVB3 infection alone or in the presence of CCPA.
Figure 4.3: The induction or inhibition of autophagy alters CVB3 replication and viral protein expression levels in HL-1 cells

(A) Supernatant samples from infected cultures were taken at the indicated time points, and plaque assay was performed to determine viral titers. At 48 hours post-treatment, viral titers in 3-MA-treated HL-1 cells were significantly lower (p<0.001,**) than in untreated cultures. In contrast, viral titers in CCPA-treated HL-1 cells were significantly higher (P<0.01,*) than in untreated cultures. A similar trend, although not statistically significant, was observed at 24 hours post-treatment. (B) Representative fluorescent images of dsRED-CVB3-infected HL-1 cells showing reduced viral protein expression levels in 3-MA-treated HL-1 cells and increased levels in CCPA-treated HL-1 cells at 24 hours and 48 hours post-treatment.
Figure 4.4: Decreased levels of autophagy in differentiated NPSCs alter CVB3 infection

(A) Viral titers were determined by plaque assay. Viral titers in infected cells were significantly higher at 8 hours and 24 hours (p<0.05,*) than 1 hour. No viral titers were found in mock infected cells. (B) At 1 hour, 8 hours and 24 hours PI, a significant decrease in GFP-LC3 autophagosome vacuoles were observed following infection, as compared to mock-infected cells (p=0.008, p=0.0154, and p=0.0019, respectively). (C) Representative 63X images are depicted for all time points and treatments showing decreased GFP-LC3 autophagosome vacuoles in differentiated NPSCs infected with dsRed-CVB3. Megaphagosomes are depicted (white arrows) in 72hr PI images.
Figure 4.5: No synergistic reduction in naturally high levels of autophagy within differentiated NPSCs after treatment with an inhibitor of autophagy plus CVB3 infection

(A) The level of autophagy in differentiated NPSCs decreased (*p<0.01) in the presence of 3-MA, as compared to Mock or CCPA treatment. (B) No change in the level of autophagy (left y-axis) was observed in infected differentiated NPSCs following 3-MA or CCPA treatment. Mock-treated differentiated NPSCs were significantly different (*p<0.05) than all other treatments. Viral titers (right y-axis) were significantly different (**p<0.01) between 3-MA + Infected versus Infected alone and CCPA + Infected and a highly significantly different (**p<0.001) between Infected and CCPA + Infected, despite the similar level of autophagy in all of these treatments. (C) All three cell lineages showed a trend toward a reduction in the level of autophagy following infection, as compared to mock-infected. However, only GFAP+ cells were significantly different (*p<0.05) in the level of autophagy following infection, as compared to mock-infected. Mock-infected Olig2+ cells had a significantly lower (*p<0.05) level of autophagy as compared to mock-infected GFAP+ cells. (D) Representative images of transduced cells (green) expressing each CNS lineage marker (blue).
Figure 4.6: No change in the level of autophagy in undifferentiated NPSCs after CVB3 infection

(A) Viral titers were determined by plaque assay. Viral titers increased over time, but were not significantly different due to large variance. (B) No difference in the level of autophagy between mock and infected undifferentiated NPSCs was observed for any of the time points analyzed, as determined by Student’s T-test. (C) Representative 63X fluorescent images are shown for all time points and treatments.
Figure 4.7: Undifferentiated NPSCs exhibit higher basal level of autophagy, as compared to HL-1 cells

(A) HL-1 cells and undifferentiated NPSCs were infected with dsRED-CVB3 for 24 hours and protein extracts from lysed cells were analyzed for endogenous LC3-I and LC3-II levels by western blot analysis. Protein loading was determined by endogenous beta-Tubulin levels. (B) A statistically significant increase (*p<0.01) was observed between Mock HL-1 cells and the following three groups - Infected HL-1 cells, Mock NPSCs, and Infected NPSCs. However, no statistically significant difference was observed between Infected HL-1 cells, Mock NPSCs, and Infected NPSCs, thus indicating a higher basal level of autophagy in NPSCs regardless of viral infection. (C) There was no statistical difference (p=0.0734) in viral titers between Infected HL-1 cells and Infected NPSCs.
Figure 4.8: Rapamycin induced autophagy in undifferentiated NPSCs but did not alter CVB3 replication

(A) Undifferentiated NPSCs were treated with dimethyl sulfoxide (DMSO, vehicle control) or DMSO + Rapamycin (Rm) for 24 hours. Protein extracts from lysed cells were analyzed for LC3-I and LC3-II levels by western blot analysis. Protein loading was determined by endogenous beta-Tubulin levels. (B) Mock + Rm and Infected + Rm had significantly higher (*p<0.001) levels of autophagy by LC3-II to LC3-I ratio than all other groups, yet were not significantly different (p>0.05) from each other. While Mock + DMSO and Infected + DMSO had a higher LC3-II to LC3-I ratio than Mock + H2O and Infected + H2O, respectively, they weren’t significantly different (p>0.05). (C) No statistically significant difference (p=0.2983) was found in viral titers between Infected + H2O, Infected + DMSO or Infected + Rm.
Figure 4.9: The addition of fibroblast growth factor decreased the level of autophagy in differentiated NPSCs

(B) Differentiated NPSCs were transduced by Adeno-GFP-LC3 and fixed 8 hours after infection and/or treatment with fibroblast growth factor (FGF). Mock treatment was statistically higher than all other treatments (*p<0.01). No statistical difference was observed between any other treatments. (C) Representative 63X fluorescent images are depicted for all treatments. (A) Protein extracts from lysed cells were analyzed after 24 hours for endogenous LC3-I and LC3-II levels by western blot analysis. Protein loading levels were determined by endogenous beta-Tubulin levels. (D) A statistically significant decrease in LC3-II/LC3-I ratios (*p<0.001) was observed between Mock and the three additional groups – Infected, Mock + FGF, and Infected + FGF. Infected and Mock + FGF treatments were significantly higher (**p<0.05) than Infected + FGF. (E) No difference (p=0.6047) in viral titers was observed between Infected and Infected + FGF.
Figure 4.10: Bcl-2 levels remain unchanged after CVB3 infection

(A) HL-1 cells, (B) undifferentiated NPSCs and (C) differentiated (Diff) NPSCs were infected with dsRED-CVB3 for 24 hours. Protein extracts from lysed cells were utilized for Bcl-2 by western blot analysis. Protein loading levels were determined by endogenous beta-Tubulin levels. The quantification of Bcl-2 signal was performed using Image J software and normalized to beta-Tubulin levels for each cell type analyzed. No statistical difference in the level of Bcl-2 was observed following infection of HL-1 cells (p=0.9648), NPSCs (p=0.3877), or Diff NPSCs (p=0.1138), as compared to mock-infected cells.
Table 3.1: Overall summary of autophagy induction in NPSCs after CVB3 Infection

Results are summarized by cell type in regards to whether autophagy is induced after CVB3 infection, if autophagy can be induced in the particular cell type, and how autophagy affects viral replication. While HL-1 cells conform to previous results in the literature, as autophagy is induced after CVB3 infection and facilitates viral replication, there is a divergent relationship between autophagy and CVB3 in both undifferentiated and differentiated NPSCs.

<table>
<thead>
<tr>
<th>Does the level of autophagy change after CVB3 infection?</th>
<th>HL-1 Cells</th>
<th>Undifferentiated NPSCs</th>
<th>Differentiated NPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>An increase in the level of autophagy</td>
<td>Yes</td>
<td>No change</td>
<td>Yes – A decrease in the level of autophagy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Are inducers of autophagy able to induce autophagy in this cell type?</th>
<th>HL-1 Cells</th>
<th>Undifferentiated NPSCs</th>
<th>Differentiated NPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No – But differentiation itself induces autophagy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Is there a relationship between autophagy and CVB3 replication?</th>
<th>HL-1 Cells</th>
<th>Undifferentiated NPSCs</th>
<th>Differentiated NPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct relationship</td>
<td>Yes –</td>
<td>No – Increasing the level of autophagy with rapamycin does not alter viral titers</td>
<td>No – Decreasing the level of autophagy with FGF does not alter viral titers</td>
</tr>
</tbody>
</table>
REFERENCES


Coxsackievirus B3 (CVB3) is a common viral infection that can have lasting and deadly effects on its host. CVB3 has potent tropism for the CNS and can cause meningitis and encephalitis during acute infection (3). Interestingly, CVB3 has been linked to several long-term sequelae in the CNS, including learning disabilities, schizophrenia and demyelinating disease (2) (10) (14). Our laboratory has determined that CVB3 infects neural progenitor and stem cells (NPSCs) in the CNS both in vitro and in vivo during acute infection (13) (7). Persistent infection in vivo has been characterized by a lower brain wet weight, the presence of viral RNA, and chronic inflammation and pathology (8). While it is unknown what cell type in the CNS harbors persistence, NPSCs have been shown to undergo persistent CVB3 infection in vitro (13).

In order to better understand viral dissemination in the CNS, a non-transgenic mouse model of CVB3 infection was initially developed (6). It was found that CVB3 induces a unique population of highly infected myeloid cells that enter the CNS at early times post-infection (PI) through the choroid plexus (Chapter 1). These cells were observed moving through the laminin-labeled blood vessel of the choroid plexus and were found to express Mac3, a marker for the myeloid lineage. These results indicated that myeloid cells were entering from the periphery.

This observation was investigated further through the use of LysM-eGFP transgenic mice in our neonatal mouse model of CVB3 infection (Chapter 2). These mice have their myeloid cell lineage labeled with eGFP, thus any eGFP+
cells found in the brain will have entered from the periphery (5). eGFP+ cells were found in the CNS of infected LysM-eGFP mice and in the same neurogenic regions as myeloid cells from our non-transgenic model, including the choroid plexus and the subventricular zone (SVZ) (4). These results using LysM-eGFP transgenic mice indicate that cells of the myeloid lineage enter from the periphery after CVB3 infection. Furthermore, cells were also found in other neurogenic regions of the CNS, including the rostral migratory stream (RMS) and the olfactory bulb, which has not previously been observed. Therefore, this highlights the ability of this transgenic mouse model to track myeloid cells after entry in the CNS.

Myeloid cells from both the transgenic and non-transgenic model were found to be positive for nestin, a marker for neural stem cells (9). This may be a result of these cells exhibiting plasticity and adapting to the microenvironment of the CNS. It remains unknown whether these cells will be able to act as neural stem cells and produce mature neurons within the CNS. Tracking eGFP+ cells in CVB3-infected LysM-eGFP transgenic mice and analyzing their morphology and marker expression may help answer this fascinating question.

Myeloid cells in both models were also observed moving further into the parenchyma of the CNS. Since CVB3 is capable of persisting in the CNS in vivo as well as persistently infecting NPSCs in vitro, it is interesting to speculate as to whether myeloid cells may contribute to CVB3 persistence. Possibilities include that myeloid cells may spread virus to regions of the CNS that harbor persistence, or it may simply work to increase viral replication to a level that is
needed to allow the virus to persist. Further studies analyzing myeloid cell infiltration in relation to persistence are warranted.

Additionally, CVB3 was shown to induce a unique chemokine profile 12 hours post-infection (PI) that may play a role in myeloid cell infiltration. In particular CCL12, or monocyte chemoattractant protein-5, was highly upregulated at both the RNA and protein level. Intra-cranial injection of CCL12 in non-transgenic mice revealed a partially recovering of myeloid cell infiltration in the absence of CVB3 infection. Future experiments will focus on injecting differential chemokine cocktails in order to fully recover myeloid cell infiltration in both non-transgenic and LysM-eGFP transgenic mice.

CVB3-induced myeloid cell infiltration also greatly influenced gene expression and morphology in the host (Chapter 3). In order to compare these changes to a virus that also infects the neonatal CNS, yet does not induce myeloid cells, lymphocytic choriomeningitis (LCMV) was investigated (11). Microarray analysis revealed differences in the immune response and developmental genes. Furthermore, CVB3 downregulated several developmental and CNS specific genes at 48 hours PI, and a smaller choroid plexus with disrupted morphology was observed. Given the robust myeloid cell infiltration though the choroid plexus, it is not surprising that damage occurs. The choroid plexus was found to recover by immunohistochemistry (IHC) markers at later time points, even in animals that showed lasting pathology through the development of hydrocephalus. However, using more sensitive
tools to examine the integrity of the choroid plexus, such as Evans blue dye, would be beneficial.

By comparing CVB3 and LCMV, we found that both viruses infected the SVZ. Since the SVZ harbors adult stem cells within the CNS, we sought to examine which stem cells were undergoing infection. We demonstrated that CVB3 preferentially infected neural stem cells, while LCMV infected both neural and glial stem cells. These results coincided with known features of CVB3 and LCMV tropism in the mature CNS. For example, CVB3 has been shown to infect neurons, while and LCMV infects both neurons and astrocytes (6) (1). Whether viral infection alters the course of differentiation of these stem cells remains unknown. Interestingly, GFAP, an astrocyte marker, was found to be downregulated specifically by LCMV infection 3.1 fold. Thus, LCMV infection may be having an effect on glial stem cell differentiation. Furthermore, the effect may be direct or indirect, as the virus was found to induce potent changes in the immune response which may affect differentiation as well. Further studies to analyze viral effects on stem cell differentiation and how these may affect CNS functionality are underway.

Interestingly, several studies have recently shown that coxsackievirus, as well as other picornaviruses, use autophagy to promote viral replication (15) (16) (12). Given the ability of CVB3 to infect NPSCs, we examine whether autophagy was induced after infection in these stem cells and if it played a role in viral replication during acute infection (Chapter 4). Based on our in vitro studies, autophagy was not induced in NPSCs or differentiated NPSC
precursors, nor was a link to viral replication observed. This could be a result of differing signaling pathways or viral protein localization in NPSCs as compared to differentiated cell types.

In addition, while autophagy is not induced during acute CVB3 infection in NPSCs, it may play a role in persistent infection. Since NPSCs have been shown to harbor persistent CVB3 infection, autophagy may help mediate this process (13). Interestingly, during poliovirus infection autophagy inhibitors have been shown to decrease extracellular poliovirus production more than intracellular poliovirus production, thus providing an attractive model for non-cytolytic virus release from the cell (12). Whether autophagy modulates CVB3 persistence can be evaluated in vitro by using autophagy inducers and inhibitors in persistently infected NPSCs and determining if there is a change in CVB3 replication. To further analyze this in vivo, infecting autophagy knock-out mice with CVB3 and determining if they develop the same hallmarks of persistent infection as non-transgenic mice would be beneficial.

Acute CVB3 infection in the neonatal CNS is a complex interplay between the virus and the host. Dissecting infection using our neonatal mouse model in transgenic and non-transgenic mice has led to the discovery of a novel population of highly infected myeloid cells and a heightened awareness of the host response to infection. Furthermore, our in vitro model of viral infection in NPSCs has revealed a unique relationship between CVB3 replication and autophagy in stem cells. A more in depth knowledge of early
viral infection, in the context of viral dissemination, the host response and the autophagic process, may lead to a better understanding of persistent infection and clues as to combating the long lasting effects of CVB3 infection in the CNS.
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