UNEXPECTED FACTORS THAT INFLUENCE COXSACKIEVIRUS B3 REPLICATION IN MOUSE INTESTINE

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by

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DEDICATION

To my husband Wen.

For his love, friendship, humor and constant support during the challenges of graduate school and life.

To my parents Xiaoyu Wang and Lifen Fu.

For loving me unconditionally and encouraging me to go on every adventure.

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UNEXPECTED FACTORS THAT INFLUENCE COXSACKIEVIRUS B3 REPLICATION IN MOUSE INTESTINE

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Coxsackievirus is a human pathogen that frequently infect humans. Although many infections are asymptomatic, there can be severe outcomes, including heart inflammation and pancreas inflammation. Most studies with coxsackieviruses and other viruses use laboratory-adapted viral strains because of their efficient replication in cell culture. I used a cell culture-adapted strain of coxsackievirus B3 (CVB3), CVB3-Nancy, to examine viral replication and pathogenesis in orally inoculated mice. Using HeLa cell plaque assays with agar overlays, I noticed that some fecal viruses generated plaques >100 times as large as inoculum viruses. These large-plaque variants emerged following viral replication in several different tissues. I identified a single amino acid change, N63Y, in the VP3 capsid protein that was sufficient to confer the

large-plaque phenotype. Wild-type CVB3 and N63Y mutant CVB3 had similar plaque sizes when agarose was used in the overlay instead of agar. I determined that sulfated glycans in agar inhibited plaque formation by wild-type CVB3 but not by N63Y mutant CVB3. Furthermore, N63Y mutant CVB3 bound heparin, a sulfated glycan, less efficiently than wild-type CVB3 did. While N63Y mutant CVB3 had a growth defect in cultured cells and reduced attachment, it had enhanced replication and pathogenesis in mice. Infection with N63Y mutant CVB3 induced more severe hepatic damage than infection with wild-type CVB3, likely because N63Y mutant CVB3 disseminates more efficiently to the liver. This work reinforce the idea that culture-adapted laboratory virus strains can have reduced fitness *in vivo*. N63Y mutant CVB3 may be useful as a platform to understand viral adaptation and pathogenesis in animal studies.

I also explored other factors that influence CVB3 infection in mouse intestine. First, a sex bias for severe sequelae from coxsackievirus infections has been observed in humans. We sought to examine if the phenomenon can be seen in mice and to further understand the mechanisms. Here we orally infected mice with CVB3 and confirmed that CVB3 replication in the intestine is sex-dependent. CVB3 replicated efficiently in male mice intestine, but not female mice. Overall these data suggest that sex and the immune response play an important role in CVB3 replication in the intestine and should be considered in light of the sex bias observed in human disease.

Previously, our lab has shown that intestinal microbiota promote replication and pathogenesis of several viruses, including poliovirus (PV), reovirus and CVB3. With that finding, we wanted to examine how microbiota enhance CVB3 infection. Bacteria in the colon produce millimolar quantities of butyrate and other short-chain fatty acids (SCFAs) through fermentation of dietary fiber. SCFAs are among the most abundant molecules in the distal gastrointestinal tract. To determine whether bacterial-derived SCFAs such as butyrate impact

CVB3 replication in the intestine, I antibiotic treated mice and then supplied them with tributyrin, a form of butyrate that is absorbed in the distal gastrointestinal tract. I found that CVB3 replication and pathogenesis was restored in antibiotic-treated mice that received tributyrin. These results suggest that butyrate is sufficient to promote CVB3 replication. My preliminary data demonstrate that oral delivery of a histone deacetylase (HDAC) inhibitor, Vorinostat, is sufficient to restore CVB3 replication in antibiotic-treated mice, suggesting that the HDAC activity of butyrate may promote CVB3 infection.

Taken all together, I identified several unexpected factors that may influence CVB3 replication in mouse intestine although much remains open for exploration.

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LIST OF ABBREVIATIONS

CAR — coxsackievirus and adenovirus receptor

CVA — type A coxsackievirus

CVB — type B coxsackievirus

DAF — decay-accelerating factor

DMEM — Dulbecco's modified Eagle's medium

HDAC — Histone deacetylase

hpi — hours post infection

HS — heparin sulfate

IFN — interferon

IFNAR — interferon alpha/beta receptor

IM — intramuscular

IP — intraperitoneal

MOI — multiplicity of infection

NaB — sodium butyrate

PABP — poly (A) binding protein

PBS — phosphate buffered saline

PFU — plaque forming unit

PVR — poliovirus receptor

SAHA — suberoylanilide hydroxamic acid

TB — tributyrin

CHAPTER 1: Literature review

COXSACKIEVIRUS INFECTION

History and epidemiology

In 1947, there were several small outbreaks of poliomyelitis in New York. Researchers from the Wadsworth Laboratory in Albany, NY, investigated this outbreak by looking for polioviruses that could replicate in mice. They made fecal suspensions from two children that were suspected of having poliomyelitis, and inoculated these suspensions into adult and suckling mice (39, 40). They found that only the suckling mice (1-7 days old) developed paralysis while adult animals were resistant to infection. However, the damage responsible for limb paralysis was widespread lesions in skeletal muscles, which contrasted with poliovirus-induced paralysis, where damage occurs in the central nervous system. They carried out further studies and revealed that the viruses could be distinguished from poliovirus and are another large group of human viruses (39). In 1949 the new viruses were suggested to be called Coxsackie viruses, naming after the small village where the first recognized patients resided.

Coxsackieviruses are non-enveloped RNA viruses belonging to the *Enterovirus* genus within the *Picornaviridae* family. Coxsackieviruses are divided into two groups based on the diseases they cause. Group A coxsackieviruses (CVA) have 23 serotypes and infect the skin and mucous membranes while group B coxsackieviruses (CVB) have 6 serotypes infect the heart, pleura, pancreas, and liver. Some CVA strains are responsible for the outbreaks of epidemic diseases. Coxsackievirus A16 is the most common cause of hand, foot, and mouth disease (HFMD) in the United States. Coxsackievirus A6 was the most commonly reported type of enterovirus in the United States from 2009 to 2013, mostly due to a large outbreak in 2012 of severe HFMD (147). Coxsackievirus A24 has been associated with outbreaks of conjunctivitis

(117). Among the 6 serotypes of CVB, CVB3 is widely studied because it is cardiotropic and clinically important.

There are no current vaccines or treatment for coxsackievirus infections.

Diseases

CVB3 is implicated in a range of diseases including myocarditis (161, 165), type I diabetes (163, 164, 165), and aseptic meningitis (31), causing over 40,000 symptomatic infections each year in the U.S. (92). However, the majority of human CVB3 infections are mild or asymptomatic. Coxsackievirus is spread through the fecal-oral route and nearly all humans are infected with coxsackievirus by the age of 5.

Myocarditis is an autoimmune disease that leads to a significant minority of dilated cardiomyopathy (DCM) cases in the US (34, 151, 175). Myocarditis is responsible for 4-20% of sudden cardiovascular deaths among young adults, the military, and athletes (67). Although most cases of suspected myocarditis are not linked to a specific cause, viral infections like CVB3 are the most commonly identified cause of myocarditis in developed countries (34, 67). Using antiviral treatments with interferon (IFN)-β, researchers were able to reduce inflammation and DCM in animal models and patients (102, 173) suggesting that viral infections are an important cause of myocarditis.

CVB3 induces autoimmune myocarditis that progresses to DCM in susceptible strains of mice like A/J and BALB/c (54, 63). Resistant strains of mice like C57BL/6 develop acute CVB3 myocarditis, but do not progress to DCM. Acute myocarditis is characterized by a predominantly T helper (Th) 1 and Th17 response (10, 78, 131, 180). However, only mice that respond to infection and/or self-antigen (i.e. damaged self) with a Th2 response, such as BALB/c and A/J strains, develop the chronic stage of myocarditis with fibrosis and DCM (1, 2, 53, 79).

Coxsackieviruses have been suggested as one of the infectious and environmental triggers of type I diabetes (T1D) (82, 164), while researchers have also showed that CVB may have protective role in T1D development (164). Tracy *et al*. studied CVB replication in nonobese diabetic (NOD) mice to examine how infection by diverse CVB strains affected T1D incidence in a model of human T1D. 4- or 8-week-old NOD mice were inoculated with any of nine different CVB strains and the incidence of T1D significantly reduced by 2- to 10-fold over a 10-month period relative to T1D incidences in mock-infected control mice, suggesting that CVB strains have protective effect in T1D development in NOD mice.

Aseptic meningitis is a condition in which the layers lining the brain, the meninges, become inflamed. Enteroviruses are the most common cause of aseptic meningitis. Different CVB strains have been reported to cause aseptic meningitis outbreaks (4, 164, 177).

MOLECULAR BIOLOGY AND REPLICATION CYCLE OF COXSACKIEVIRUS

Virion structure

The structure and replication cycle of the *Picornaviridae* have been well studied. The virion is a nonenveloped icosahedral particle. Viral proteins VP1, VP2, VP3, and VP4 form the protein capsid. One molecule each of VP1, VP2, VP3 and VP4 composes a protomer. The capsid is composed of 60 protomers. VP1, VP2 and VP3 appear on the surface of the capsid while VP4 is an internal protein. Inside the capsid there is a viral genome of approximately 7.5kb. The genome is a linear molecule of single stranded (ss) RNA. The ssRNA comprises an open reading frame (ORF), flanked on both 3′ (~100 bases) and 5′ (~800 bases) termini by untranslated regions (UTRs). The ORF contains 11 genes encoding proteins (58). Besides the four capsid proteins noted above, there are two viral proteases (2A, and 3C), an RNA-dependent-RNA-

polymerase (3D), two proteins involved in RNA synthesis (2B, and 2C), a primer of initiation of RNA synthesis (3AB) and a small polypeptide (VPg) of 20–24 amino acids derived from gene 3B.

Receptor binding and entry

The first step of infection of CVB3 is binding of virus to the viral receptor, coxsackievirus and adenovirus receptor (CAR) (132). CAR is a 46-kDa integral membrane protein with a typical transmembrane region, a long cytoplasmic domain, and an extracellular region composed of two Ig-like domains (13, 150). Both adenovirus (17) and coxsackievirus (72) interact with the N-terminal domain. Homologs of human CAR have been characterized in mice (15, 150), rats, pigs, dogs (40), and zebrafish. The murine and human CARs are very similar. They share 91% amino acid identity within the extracellular domain, 77% within the transmembrane domain, and 95% identity within the cytoplasmic domain. Variant isoforms of CAR, which differ only at the C terminus and which most likely result from alternative splicing, have been identified in mice (15), humans, and rats (56). In polarized cells like epithelial cells, CVB3 infection requires an additional attachment receptor, decay-accelerating factor (DAF) to reach CAR, because CAR is sequestered in tight junctions. If CAR expression is low, as in some non-polarized cell lines such as RD and CHO, CVB3 strains that do not bind DAF can evolve to use additional factors such as DAF and heparan sulfate (HS) to aid attachment efficiency (14, 26, 27, 126, 137, 143, 157). DAF belongs to a family of proteins that regulate complement activation. They bind to and accelerate the decay of convertases, the central amplification enzymes of the complement cascade (22, 107, 108, 183). Although DAF binding is likely to facilitate viral adsorption and mediate tropism, the availability of DAF receptor molecules on the host is normally not sufficient for CVB3 or EV7 to be able to enter cells (122, 130). HS, which is

a glycosaminoglycan (GAG), functions as another attachment factor for CVB3 infection. GAGs are present ubiquitously on cell surfaces (113). It has been shown that viruses from many different families interact with GAGs. Affinity of virus with GAGs affects viral pathogenicity and tissue tropism (16, 96, 153).

GAGs are long, unbranched polysaccharides. GAGs are composed of repeating disaccharides, usually an amino sugar and an uronic acid. N-acetylglucosamine and glucuronic acid form HS. N-acetylgalactosamine and glucuronic acid form chondroitin sulfate (CS) (50). GAG chain synthesis begins by the addition of an amino sugar to a tetrasaccharide that is attached via O-glycosylation to a serine residue, followed by a glycine. Mature GAG chains undergo distinct modifications, like N- and O-sulfation and epimerization, resulting in the great variety of GAGs. HS chains mainly occur as membrane proteoglycans, like glypicans and syndecans. Glypicans are heparan sulfate proteoglycans (HSPGs) and attached via a glycosylphosphatidylinositol (GPI) anchor to the cell membrane, while syndecans are type I transmembrane proteins (68). Heparin is a highly sulfated soluble subtype of HS. The difference between HS and heparin *in vivo* is that HS is an extracellular GAG that is attached to a core protein, while heparin is an intracellular GAG, synthesized in granulated cells and is cleaved from its core protein (77, 100). HS can be found on all tissues. Highly sulfated liver-specific HS provides binding sites for diverse ligands and receptors involved in many physiological events such as growth control, lipid metabolism, signal transduction and cell adhesion (49, 101). Liver HS also provides binding sites for some liver targeting pathogens, like malaria circumsporozoite (146), dengue virus (32) and HCV (12, 99).

Replication initiation and translation

After gaining entry into the cytoplasm, viral RNA is first translated and then replicated. Initially the viral genome is translated into a large polypeptide that is subsequently cleaved into individual structural, and nonstructural proteins by the virus-encoded proteases 2A and 3C (9, 47). CVB3 protein 3D is an RNA-dependent RNA polymerase (RdRp). 3D is required for synthesis of negative-strand viral RNA intermediate. The RNA intermediate then serves as a template for synthesis of multiple positive-strand progeny genomes. The positive-strand RNA genome and structural proteins are then packed together to make the complete virion. Ultimately, viral progeny is released for further infection of neighboring cells, marking the completion of the viral life cycle.

Like poliovirus, coxsackieviruses rapidly shutoff host RNA and protein synthesis to facilitate their own replication. In the process of regulating viral RNA synthesis, the 5' and 3' UTRs play a major role. The viral VPg protein linked to the 5' end of the genome serves as a primer for 3D polymerase for viral RNA synthesis (178). The unusually long 5' UTR contains a number of stem-loop structures. Stem-loop I which is also called cloverleaf structure is highly conserved and plays a critical role in enterovirus genomic RNA synthesis (5). Viral translation can occur with deletion mutations of the cloverleaf region of CVB3 RNA, but RNA synthesis cannot (81). The 3' UTR of CVB3 also contains three stem-loop structures, X, Y and Z (141). They regulate viral RNA synthesis by forming unique tertiary structures. Mutations within these interaction regions affect the efficiency of positive- or negative-strand RNA synthesis (123). 2Apro, 3Cpro and 3CDpro are three proteases. They are required to cleave virus polyproteins (70, 74). 2Apro also cleaves eIF4G, which plays a central role in cap-dependent translation. EIF4G is one of the major components of the pretranslation initiation complex eIF4F, which is a

large scaffolding protein that binds to both cap-binding protein eIF4E and eIF4A and other proteins (64). Cleavage of eIF4G will result in the disruption of the preinitiation complex for cap-dependent translation of cellular mRNAs. However, this cleavage does not affect translation initiation of CVB3 RNA because viral RNAs are uncapped and contain an internal ribosome entry sites (IRES) in the 5' UTR (84). In addition, CVB3 2A protease also cleaves Poly (A) binding protein (PABP) during infection (29). PABP stabilizes the interaction of eIF4E with the cap by directly binding with the eIF4G to effectively circularize the mRNA, which, thus enhancing the rate of translation initiation (86). This cleavage results in the disruption of interaction between eIF4E at the 5' end of mRNA and the PABP on the 3' poly(A) tail to form the translation initiation loop (46, 74, 112).

CVB3 also induces cytopathic effects (CPE), which refers to degenerative change in cells associated with the host shutoff (28, 29). CPE does not essentially support viral reproduction. On the contrary, it could be a host cell response to eliminate virus replication (3). The CPE appears as morphological changes coupled with cell rounding, release of cells from the culture surface and altered membrane permeability. The generation of CPE leads to host cell lysis, mainly through apoptosis, necrosis or both. One observation from a recent study suggests that the virus-activated cell death induced by both necrotic and apoptotic branches (125). Overall, direct viral protease cleavage of structural proteins such as dystrophin along with inhibition of host cell transcription and translation likely contributes to the cellular structure disruption, and cell death of infected cells (9, 47).

COXSAKIEVIRUS ANIMAL MODEL SYSTEMS

Host range and specificity

The natural hosts of coxsackievirus infections are humans. However, monkeys and mice can be experimentally infected. The limited host range is attributed to expression of the viral receptor, CAR, which is expressed in humans, mice and monkeys.

Mouse models

Coxsackieviruses were identified when Gilbert Dalldorf and Grace M. Sickles tried to develop a mouse model for poliovirus. They inoculated suckling mice, which developed paralysis while animals more than one week old were resistant to infection.

To examine CVB3 replication and pathogenesis in mice, many studies use peripheral injection as a delivery method, which bypasses the normal oral route of infection. Some effects, such as those imparted by intestinal microbiota, can only be uncovered using the natural oral infection route (11, 85, 88, 91, 105). CVB3 does not replicate efficiently in orally-inoculated C57BL/6 mice and orally-inoculated immune-competent C57BL/6 mice do not develop disease. Our lab uses interferon α/β receptor knockout C57BL/6 mice, in which CVB3 can replicate well.

VIRAL QUASISPECIES

RNA virus replication is error prone, giving rise to genetically diverse viral populations and facilitating emergence of variants with altered properties. The high mutation frequencies of RNA viruses are due to nature of their genetic composition. RNA viruses rely on their own RdRp for replication and subsequent packaging. However, viral RdRp and reverse transcriptase lack proofreading capabilities and frequently generate mutations. The mutation rate is about one mutation per 10,000 nucleotides. Though the majority of mutations are detrimental and result in less fit or non-viable viral particles, an appropriate amount of viral-population diversity is

thought to aid viral adaptation in changing host environments and is required for full virulence *in vivo* (43, 44, 45).

The resulting population of genetically similar genomes that can differ by one to a few nucleotides is called "viral quasispecies". The theory of viral quasispecies was devised by Eigen and Schuster in the 1970s (48). A quasispecies is a large group of self-replicating entities, of which the genotypes exist in an environment of high mutation rate, where a large fraction of offspring are expected to contain one or more mutations relative to the parent. The resulting mutations increase the diversity among the population and help the virus to adapt to the changing environment, which could be beneficial. However, in the mathematical model, Eigen and Schuster also predict an event called error catastrophe when excess mutations accumulate and the virus may lose fitness. They also predict that when there are too few mutations, it would interfere with the ability to overcome environmental pressures. The quasispecies theory changed our way to look at RNA viruses: there is no true "wild type". Instead, "wild-type" is the master sequence within a population from which other sequences are derived.

SEXUAL DIMORPHISM OF COXSACKIEVIRUS INFECTION

Sexual dimorphism in diseases

Sexual dimorphism in complex diseases, including infectious diseases, autoimmune diseases and cardiovascular diseases, presents with an irregular disease frequency in males and females. Although both sexes can be affected, one is more susceptible (115). In some cases, frequency and the severity of infectious diseases (viral, bacterial, fungal and parasitic infection) are higher in males than females (95); however, the prevalence of sexually transmitted infections (STIs), such as HIV and herpes simplex virus-2 (HSV-2), is higher among women. In addition,

autoimmune diseases (systemic lupus erythematosus [SLE], multiple sclerosis and rheumatoid arthritis) to affect more women than men (57). Furthermore, in cardiovascular diseases, recent studies have reinforced sex-related differences in the pathology of the disease, with an increased incidence and mortality in men (162).

Role of Sex hormones in sex dimorphism

Sex hormones have been the usual culprit in the explanation of sex effects in complex diseases, yet there are no specific mechanisms proposed as to how such hormones promote or protect from a disease. Genetic linkage and association studies suggest that sex hormones have effects on chromosomes and individual genes (145). The lower incidence of heart disease in women has been attributed to the cardio-protective effects of estrogen (124). However, recent clinical trials of hormone replacement therapy found an increased risk of heart disease with estrogen and progestin (a natural or synthetic substance that mimics the actions of progesterone) treatment (152), indicating that many of the effects of sex hormones on the pathogenesis of heart disease remain elusive. Furthermore, another group has shown that gonadectomy reduces CVB3-induced myocarditis in males and females, suggesting that both testosterone and estrogen can increase inflammation in the heart (79). Since there is not a direct association between sex based hormones and CVB3-induced myocarditis, Frisancho-Kiss *et al.* have reported that male BALB/c mice respond to CVB3 infection with an increased inflammatory infiltrate in the heart (53). Perhaps, differential modulation of immune responses in different sexes partly explains such a discrepancy in disease severity, wherein females exhibit more robust cell mediated, and humoral immune responses to antigenic challenges compared with males (95). Sex steroid hormones, such as 17β-estradiol (E2), progesterone and testosterone can mediate many of the sex-based differences in immune responses (60).

Immune cells such as macrophages and lymphocytes have receptors for androgens, estrogens and progesterone (60). Thus, sex hormones can interact with cells of the immune system and contribute in the regulation of their development and function, as well as participate in the outcome of diverse diseases, including infectious diseases (21).

Role of microbiota in sex dimorphism

While people commonly accept that sex hormone plays a pivotal role in sex dimorphism, evidence suggests that gut microbiota may also be involved in the process (181). Yurkovetskiy *et al*. sequenced 16S rRNA genes from male and female mice and found that the microbiota can be sex-biased. They also found that before puberty the microbiota of both genders are similar. However, the adult female microbiota is more similar to the microbiota of prepubescent mice than the male microbiota, which suggest that puberty and probably the sex hormone alters the male microbiota composition to a less diverse level. Importantly, comparison of male, female, and castrated male microbiota demonstrated that sex hormones rather than X chromosome-associated factors were important for the change in microbiota composition. From another angle, Markle *et al*. showed that the commensal microbial community can alter sex hormone levels and regulates autoimmune disease fate in individuals with high genetic risk (118). They demonstrated that early-life microbial exposures determine sex hormone levels and modify progression to autoimmunity in the nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D). Colonization by commensal microbes elevated serum testosterone levels and protected NOD males from T1D. Transfer of gut microbiota from adult males to immature females leads to elevated testosterone level and metabolism changes. The recipients showed reduced islet inflammation and autoantibody production, and robust T1D protection. In addition, these effects were dependent on androgen receptor activity.

THE ROLE OF BACTERIAL METABOLITES IN COXSACKIEVIRUS INFECTION

The mammalian intestine is colonized by a huge number of microorganisms of different kingdoms, including fungi and bacteria. These non-human cells have multiple functions. They can be protective as a natural defense barrier and by producing anti-microbial factors. They can also aid the host in the development of the immune system. Importantly, microbiota play a vital role in digestion and metabolism, for example, fermentation of non-digestible fiber, synthesis of vitamins and ion absorption (132).

The role of gut microbiota in both physiological and pathological conditions has been well studied. These microorganisms and their products not only exert their functions in the GI tract but also in adipose tissue, and immune and nervous systems (73, 119, 169). Researchers have explored ways to modify the composition of microorganisms in the gut both qualitatively and quantitatively. As a consequence, the concentrations of the compounds produced and released by microbiota in the lumen are also altered, which may play a role in the development of pathological conditions including inflammatory bowel disease (IBD), colon cancer, obesity and type 1 and 2 diabetes mellitus (42, 119, 166, 169).

Some of the microbiota-produced compounds have been well characterized and are implicated in various aspects of host development and health. One group of the compounds is microbial-derived ligands of toll like receptors (TLRs) including LPS and flagellin. LPS activates TLR-4 and flagellin activates TLR-5 and they can modulate distinct aspects of host metabolism and immune responses (24, 169). Another representative compound generated by the microbiota that also modulates the function of host tissues is ATP, which is involved in the differentiation of the Th17 cells, an important subset of CD4+ lymphocytes (8). Short chain fatty

acids (SCFAs) are another important subset of the products generated from anaerobic bacteria fermentation. Microbiota produced SCFAs affect host tissues. The concentration of these fatty acids in the GI tract and blood may affect pathological conditions such as IBD, cancer and diabetes. Previously, attempts have been made to modify the concentrations of SFCAs or alter the ability of host tissues to use SCFAs in these conditions (80, 120, 129, 167, 156).

SCFAs contain 2-6 carbon atoms and include acetate (C2), propionate (C3) and butyrate (C4). Their concentrations in the human intestine is approximately 13 mM in the terminal ileum, 130 mM in the caecum and 80 mM in the descending colon (38). The anaerobic fermentation of non-digestible dietary fiber and endogenous epithelial-derived mucus in the gut produces SCFAs. SCFAs released in the intestinal lumen are readily absorbed and used as energy source by colonocytes and also by other tissues including liver and muscle (121). SCFAs contribute to 5 to 10% of human basal energy requirements. In addition to energy supply, these fatty acids have other important functions, such as modulating cell proliferation and differentiation, regulating gene expression and hormones secretion (142, 182) and activation of immune/inflammatory responses (119, 170). Among all the SCFAs, butyrate is the most studied.

CHAPTER 2: Materials and Methods

Cells and virus

HeLa cells were propagated in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum. Chinese hamster ovary cells (CHO-K1) and CHO mutant cells (pgsA745, pgsB761 and pgsD677) were obtained from Dr. Jeffrey Esko (University of Califonia, San Diego) and were maintained in F12/DMEM medium supplemented with 5% fetal bovine serum. Human rhabdomyosarcoma (RD) cells, Huh7 cells, 293 cells, and murine L929 cells were maintained in DMEM supplemented with 5% fetal bovine serum.

The CVB3 Nancy infectious clone was obtained from Dr. Marco Vignuzzi (Pasteur Institute, Paris), who received it from Dr. Reinhard Kandolf. This version of CVB3 Nancy binds HS (87). Stocks of CVB3 were prepared in HeLa cells by co-transfection of the infectious clone plasmid and a plasmid expressing T7 RNA polymerase (139). Virus titers were determined using HeLa cells by plaque assay as previously described (139). Agar (1% final concentration) (Becton Dickinson) was used in plaque assay overlays unless otherwise indicated. Plaque size was quantified using Image J 1.47v software. To determine whether fecal viruses were input/inoculum virus or virus that had undergone replication, we used neutral red/light-sensitive CVB3 as described previously (104). To determine the percent of replicated virus, samples were processed in the dark, and a portion was light exposed. The "percent replicated virus" was calculated by dividing light-exposed PFU/mL by light unexposed PFU/mL and multiplying by 100. ^{35}S-labeled CVB3 was prepared as previously described (103). Briefly, after infection, cells were incubated in media lacking cysteine and methionine for 3 hours before adding [^{35}S] cysteine and [^{35}S] methionine. ^{35}S -labeled viruses were purified using cesium chloride density

gradient centrifugation. Specific activity of ^{35}S -CVB3 stocks was approximately 1000 plaque forming units (PFU)/counts per minute (CPM).

Sequencing of fecal isolates, cloning of N63Y CVB3, and structure/sequence alignments

Six individual plaques generated from fecal samples were plaque purified to generate independent "fecal isolates". Viral RNA was isolated and purified by using the RNeasy mini kit (Qiagen). RT-PCR was performed with SuperScript II RT (Invitrogen) by using primer (5′-CGCCACGGACGAGGTA -3) to generate cDNA. PCR was performed using primer pairs that cover most of the VP1-VP3 coding region: sense (5′-GCTGCCCGATGCTTTGT-3′), antisense (5′-GGCCGAACCACAGAACATAAAC-3′), sense (5′-GCAATATGACGTCACACCAGAGAT-3′), antisense (5′-AGGGCTGTTGAGTACTTGTTATGA-3′), sense (ACTACCGGTTTGTTGCTTCAGATC), and antisense (5′-CTACTAGACCTGGTCCTTCAAACGA-3'). PCR products were sequenced by the UT Southwestern sequencing core. All six isolates contained two amino acid changes, VP3-N63Y (mutation A to T at position 1956) and VP2-T151S (mutations C to G at position 1432 and G to C at position 1433), as well as other silent point mutations. Because position 151 of VP2 is highly variable in CVB3 sequences, we focused our efforts on VP3-N63Y. A 377 bp fragment containing the N63Y mutation was generated by BsiWI/BglII digestion of a RT-PCR product from one fecal isolate, and this fragment was cloned into a new CVB3-Nancy plasmid from nucleotide 1695 (BsiWI) to nucleotide 2072 (BglII). The new plasmid was then sequenced and N63Y was confirmed as the only mutation in the PCR-generated region. The large plaque phenotype of this new clone was confirmed by plaque assay. The virion structure was modified from a previous determined crystal structure (PDB accession number 1COV) (138). The

sequences of the region near 63 residue of VP3 from different viruses were aligned by PROMALS3D multiple sequence and structure alignment server (138).

Single-cycle growth curve assay

HeLa cells, Huh7 cells, RD cells, CHO cells, and L929 cells were seeded at 1×10^6 in 6 cm plates. Each plate was inoculated with WT CVB3 or N63Y CVB3 at a multiplicity of infection (MOI) of 0.1. After 30 min of incubation at 37°C, the inoculum was aspirated from cells. The cell monolayers were washed and 3 ml of fresh medium was added. At 2, 4, 6, 8, 10 and 12 h after infection, cells were typsinized and pelleted. To release intracellular virus, cell pellets were freeze-thawed three times before virus titer was determined by plaque assay using HeLa cells. All data were compared to input stock titers, which were set to 100%.

CVB3 cell attachment assay

^{35}S-labeled CVB3 (3 x 10^6 PFU/3,000 CPM) was incubated with 8.8×10^6 HeLa cells, Huh7 cells, RD cells, CHO cells, or murine L929 cells at 4°C for 40 min to facilitate viral binding. Cells were washed three times with cold PBS, trypsinized, and ^{35}S was quantified in a scintillation counter. For certain experiments, cells were treated with heparinase I (Sigma-Aldrich) diluted in 10 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, 3 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2, 0.1% glucose, 1% FCS, and 0.5% bovine serum albumin and were used at a final concentration of 10 U/ml (127) for 90 min prior to the viral attachment assay.

Heparin, CAR, and DAF binding assays

^{35}S-labeled CVB3 (5 x 10^6 PFU/5,000 CPM) was mixed with heparin agarose resin (Sigma-Aldrich) or streptavidin agarose resin (Sigma-Aldrich). Samples were incubated for 3 h at 37°C. Following incubation, resin was pelleted by centrifugation at 2,600 × g for 2 min. Resin was washed three times with PBS+, and resin-associated radioactivity was quantified by

scintillation counting. Data are expressed as percent of input CPM ([resin associated CPM/input CPM] × 100). For CAR and DAF binding assays, human (R&D systems) or murine CAR (USbiological) or human (R&D systems) or murine DAF (Sino Biological) were immobilized on nitrocellulose membranes and membranes were incubated with ^{35}S-labeled CVB3 (6 x 10^7 PFU/60,000 CPM) at 37°C for 12 hours (20). After washing, membrane-associated radioactivity was quantified by phosphorimager analysis.

Mouse experiments

All animals were handled according to the Guide for the Care of Laboratory Animals of the National Institutes of Health. All mouse studies were performed at UT Southwestern (Animal Welfare Assurance #A3472-01) using protocols approved by the local Institutional Animal Care and Use Committee in a manner designed to minimize pain, and any animals that exhibited severe disease were euthanized immediately using isoflurane. C57BL/6 PVR-IFNAR+/+ and C57BL/6 PVR-IFNAR−/− mice were obtained from S. Koike (Tokyo, Japan) (83) and all experimental mice were 4-10 week old males.

In oral infection experiments, mice were orally inoculated with 5×10^7 PFU of neutral red light sensitive CVB3 and feces were harvested in the dark. In other inoculation routes experiments, mice were orally, IP-inoculated with 5×10^7 PFU WT CVB3, or IM-inoculated with 2×10^6 PFU WT CVB3 and tissues (muscle, liver, heart, and/or spleen) were harvested at 48 hpi and feces were harvested from orally-inoculated mice at 72 hpi. In large plaque emergence from different inoculation routes experiments, mice were orally-inoculated with 5×10^7 PFU of CVB3 or inoculated IP or IM with 1×10^4 PFU and 1×10^2 PFU of CVB3, respectively. Tissue samples were processed as previously described (103). In ALT measurement experiments, mice were orally inoculated with 5×10^7 PFU of CVB3, blood was collected at 24 hpi by cheek bleed and at

72 hpi by heart puncture. Following heart puncture, mice were perfused with 4% paraformaldehyde, liver was collected and fixed in 4% paraformaldehyde for 48 h. H&E staining was performed by the UT Southwestern Molecular Pathology Core. For ALT quantification, serum was separated by centrifugation and ALT concentration was determined by ELISA (MyBiosource) according to manufacturer instructions. In virus homing experiments, mice were inoculated IP with 2×10^7 PFU/20,000 CPM of ^{35}S-labeled WT or N63Y CVB3 and tissues were collected 1 hpi for quantification in a scintillation counter. In all cases, feces and tissues were processed prior to viral titer assay or scintillation counting as previously described (103). In liver tissue binding experiments, fresh tissues were homogenized with a Bullet Blender homogenizer (Next Advanced Inc, Averill Park, NY) (109) prior to incubation with 3×10^6 PFU/3000 CPM of ^{35}S-labeled WT or N63Y CVB3. After 1h incubation at 37°C, tissues were washed with PBS+ and tissue-associated radioactivity was quantified by phosphorimager analysis.

In sex-difference mouse experiments, 10-12 week old male and female C57BL/6 PVR-IFNAR−/− mice were orally inoculated with 5×10^7 PFU of CVB3-nancy. Feces were harvested at 24, 48 and 72hpi and titers were quantified by plaque assay.

In butyrate mouse experiments, 8-12 week old male C57BL/6 PVR-IFNAR−/− mice were treated with 10mg Vancomycin and 10mg Ampicillin by gavage once a day for 4 days, followed by treatment with antibiotic drinking water containing 1mg/ml ampicillin and 0.5mg/ml vancomycin for 3-5 days. Mice were then inoculated with 5×10^7 PFU of CVB3-Nancy. On the day of infection before inoculation, 1 dpi and 2 dpi, mice were gavaged with 50mg/kg mouse weight tributyrin (Sigma-Aldrich). Feces were harvested at 24, 48 and 72hpi and titers were quantified by plaque assay. In figure 15 experiment, mice received antibiotic treatment as described above. SAHA was given by gavage at the same time points as tributyrin in butyrate

mouse experiments. SAHA (Sigma-Aldrich) solution was prepared in HOP-β-CD (2-hydroxypropyl-β-cyclodextrin) as already described by Hockly *et al* (76). Briefly, SAHA was dissolved in 5 molar equivalents of HOP-β-CD in water, it was heated until fully dissolved, rapidly cooled on ice to room temperature and stored at -20°C.

Cytokine Expression

Blood was collected by heart puncture from male and female IFNAR-/- mice either prior to infection (uninfected) or 72 hpi with CVB3-Nancy. Blood samples were incubated at room temperature for 30 min to induce coagulation. Serum was collected from each sample following centrifugation for 10 min at 1000 x g. Serum samples were stored at -20°C until analysis. Cytokine levels were measured using the Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) and the assay was carried out by the UTSW Metabolic Phenotyping Core.

Statistical analysis

Data throughout are shown as mean ± SEM. The differences between groups were examined by unpaired two-tailed Student's t tests. $P < 0.05$ was considered statistically significant: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. All analyses were performed using Graph Pad Prism 6.03 software (GraphPad Software, La Jolla, CA).

CHAPTER 3: Characterization of a large plaque variant of coxsackievirus B3

INTRODUCTION

CVB3 capsids are formed by viral proteins VP1, VP2, VP3, and VP4, and binding to the viral receptor, coxsackievirus adenovirus receptor (CAR), is required for infection (13, 128, 133). In polarized epithelial cells, CVB3 infection requires an additional attachment receptor, decay-accelerating factor (DAF), because CAR is sequestered in tight junctions (36, 37). If CAR expression is low, as in some non-polarized cell lines such as RD and CHO, CVB3 strains that do not bind DAF can evolve to use additional factors such as DAF and heparin sulfate (HS) to aid attachment efficiency (14, 25, 26, 126, 127, 143, 157, 183).

RNA virus replication is error prone, giving rise to genetically diverse viral populations and facilitating emergence of variants with altered properties including cell attachment potential. An appropriate amount of viral-population diversity is thought to aid viral adaptation in changing host environments and is required for full virulence *in vivo* (34, 43, 65, 66, 140, 168). When viral populations encounter selective pressure, distinct viral variants can emerge. For example, many viruses used in laboratories today are cell-culture adapted variants. Cell culture adaptation reduces virulence for many viruses and is frequently the basis of live-attenuated vaccine development. Some of these culture-adapted variants show changes in affinity for glycosaminoglycans (GAGs), which are sulfated polysaccharides including heparan sulfate and heparin. GAGs are present ubiquitously on cell surfaces (113). It has been shown that viruses from many different families interact with GAGs, which aids viral attachment. Viral affinity for GAGs is an important determinant of tissue tropism and pathogenicity (16, 96, 153).

To examine CVB3 replication and pathogenesis in mice, many studies use peripheral injection as a delivery method, which bypasses the normal oral route of infection. CVB3 does

not replicate efficiently in orally-inoculated C57BL/6 mice and orally-inoculated immune-competent C57BL/6 mice do not develop disease. While CVB3 replicates more efficiently in mice lacking the interferon alpha/beta receptor (IFNAR), the immune deficiency of these mice is not ideal for studies seeking to understand innate and adaptive responses to viral infection. Therefore, a better model system for CVB3 oral infections is needed since some effects, such as those imparted by intestinal microbiota, can only be uncovered using the natural oral infection route (11, 85, 88, 91, 103).

In this study, we identified a large plaque variant of CVB3 that emerged after oral inoculation of mice. We found that a single nucleotide change in the VP3 protein, N63Y, was sufficient for formation of large plaques. We characterized this variant both *in vitro* and *in vivo* and found that N63Y CVB3 has a growth defect in tissue culture, but is more pathogenic than WT CVB3 in mice. We also explored what factors promote the emergence of the large plaque variant, the basis for large plaque formation, and mechanisms underlying differences in viral replication and pathogenesis.

RESULTS

Emergence of a large plaque variant of CVB3 following inoculation of mice

To examine replication of CVB3 in the gastrointestinal tract we orally inoculated immune-deficient C57BL/6 interferon α/β receptor knockout mice (called IFNAR-/- throughout) with 5×10^7 PFU of neutral red labeled WT CVB3. Because mice shed unreplicated inoculum virus in feces, it is difficult to quantify viral replication in the intestine in the first few days after oral inoculation. To avoid this problem, we examined viral replication using light sensitive neutral red virus. Virus propagated in the presence of neutral red dye is sensitive to light-induced inactivation by RNA cross-linking but loses light sensitivity upon replication in the dark inside

mice, facilitating assessment of replication and differentiating inoculum virus from replicated virus in feces. Feces were harvested at 24, 48, 72 hours post infection (hpi) and virus titer was determined by plaque assay using agar overlays (Fig 1A). At 24 hpi, only two mice shed replicated virus while most mice shed replicated virus at 72 hpi (Fig 1B). Surprisingly, plaques with morphology different from inoculum CVB3 were observed on 72 hpi fecal sample titer plates (Fig 1C, 1D), suggesting that a variant emerged following viral replication in mice. Indeed, 0.67% of fecal virus plaques were large at 48 hpi while 11% were large at 72 hpi. We then examined the frequency of large plaque viruses in our inoculum to determine whether the large plaque variant was present prior to inoculation of mice. We found that 0.26% of inoculum CVB3 plaques had the large plaque phenotype, suggesting that the large plaque variant existed at a low level in the inoculum and was enriched following replication in the gut.

To determine what factors contribute to the emergence of the large plaque variant, we used different inoculation routes and examined the plaque morphology of virus from different tissues of immune-competent or immune-deficient mice. IFNAR-/- mice and immune-competent IFNAR+/+ mice were orally, intraperitoneally (IP), or intramuscularly (IM) inoculated with 5×10^7, 5×10^7, and 2×10^6 PFU of CVB3, respectively. Feces were harvested from orally-inoculated mice at 72 hpi; liver, heart, and spleen were harvested from IP-inoculated mice at 48 hpi; and muscle and liver were harvested from IM-inoculated mice at 48 hpi. We measured the virus plaque sizes and quantified the percentage of large plaques in different tissues (Fig 2). First, we found that there were more large plaque variants in IFNAR+/+ mice than in IFNAR-/- mice. The increased emergence of the large plaque virus in immune-competent mice correlates with reduced viral replication, indicating that increased selective pressure may drive emergence of the large plaque variant. Second, large plaque variants were present in feces from orally-inoculated

mice and visceral tissues from IP- or IM-inoculated mice, but not in muscle from IM-inoculated IFNAR-/- mice. We hypothesize that increased selective pressure in the intestine and peritoneal cavity facilitated emergence of large plaque variants and/or the large plaque variant has a fitness advantage in select tissues. Overall, these results suggest that the large plaque variant existed at a low level in our inoculum, but was enriched following viral replication in several tissues of infected mice.

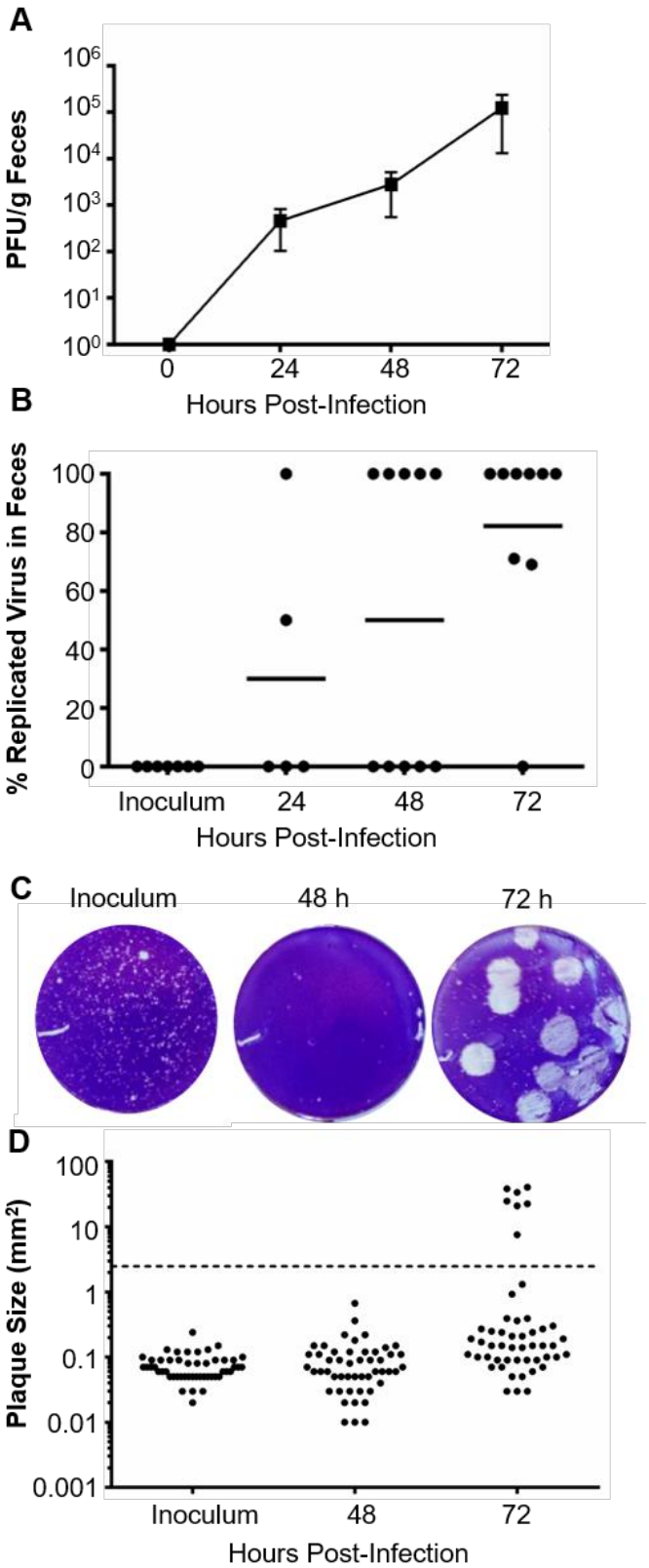

Figure 1. *(legend on following page)*

Figure 1. Emergence of a CVB3 large plaque variant following oral inoculation. 4-6 week old male IFNAR-/- mice were orally inoculated with 5×10^7 PFU of light-sensitive/neutral red WT CVB3 in the dark, and feces were harvested at 24, 48, and 72 hpi in the dark. (A) Total viral titers determined by HeLa cell plaque assays using agar overlays. (B) The percent of replicated (non-inoculum) virus in feces. Flow-through inoculum viruses are light sensitive whereas viruses that replicated in mice are light insensitive. Aliquots of each processed fecal sample were exposed to light or dark and replication status was determined by dividing the PFU/ml of light exposed samples by the PFU/ml of dark exposed samples and multiplying by 100%. Each symbol represents one mouse. (C) Representative plaque morphologies of inoculum and 48 and 72 hpi fecal samples. (D) Plaque size quantification. Each symbol represents a plaque. Large plaques are defined as the average inoculum plaque size multiplied by a factor of 10 (2.473 mm2 or larger; indicated by dashed line). For panel A and B, n=5-10.

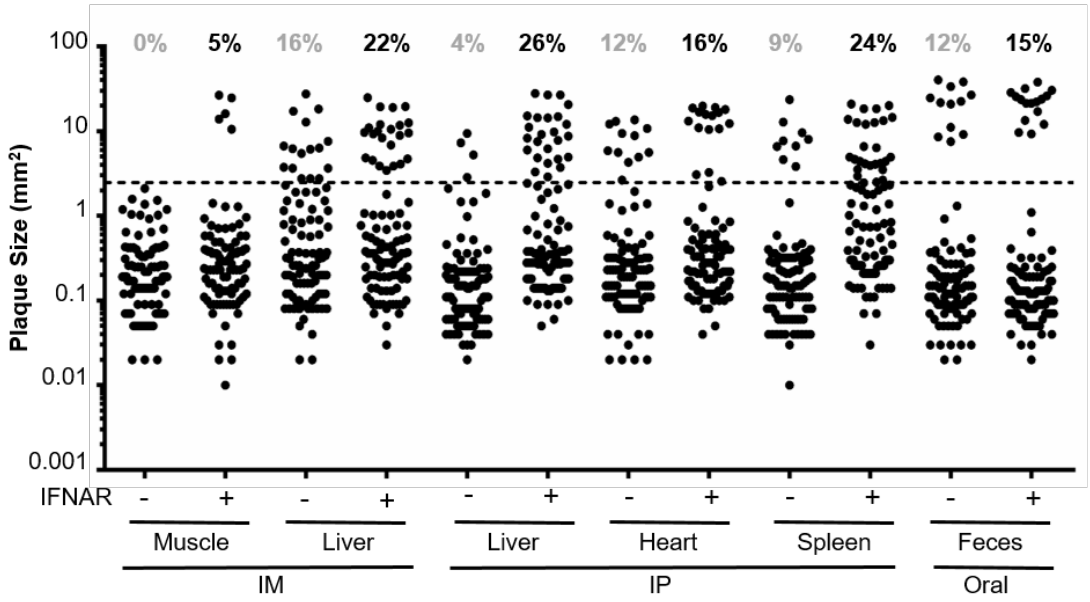

Figure 2. Emergence of large plaque variant differs in various tissues. 4-6 week old male IFNAR-/- or IFNAR+/+ mice were orally, inoculated intraperitoneally (IP) or intramuscularly (IM) with 5×10^7 PFU of WT CVB3. Feces were harvested from orally inoculated mice at 72 hpi. Liver, heart, and spleen were harvested from IP-inoculated mice at 48 hpi. Muscle and liver were harvested from IM-inoculated mice at 48 hpi. Following processing, viruses were plated on HeLa cells using agar overlays. The sizes of a hundred randomly picked plaques were quantified using Image J. The total percentages of large plaques among hundreds of plaques per condition are indicated above. Large plaques are defined as the average inoculum plaque size multiplied by a factor of 10 (2.473 mm2 or larger; indicated by dashed line).

A single amino acid change, VP3-N63Y, is sufficient to confer the large plaque phenotype

To identify the mutation(s) responsible for the large plaque phenotype, we isolated 6 individual large plaque viruses from mouse feces, performed plaque purification to generate large plaque "fecal isolate" samples, extracted RNA, performed RT-PCR, and sequenced products by consensus sequencing. We sequenced the capsid-coding region with the rationale that major changes in plaque size frequently map to capsid proteins (87, 89). Sequence alignment revealed several silent point mutations and two amino acid changes in the capsid-coding region of large plaques: T151S in VP2 and N63Y in VP3. Sequence alignment revealed that amino acid 151 of VP2 is a variable position, with T or S present in many NCBI samples. However, N63 is highly conserved among coxsackieviruses and echoviruses (Fig 3A) and is located on the virion surface near the DAF binding site (Fig 3B) (183). Using reverse genetics, we cloned N63Y into the CVB3 infectious clone, and we found that N63Y was sufficient for the large plaque phenotype (Fig 3C). Recently, Borderia *et al*. identified polymorphisms at position 63 of VP3, including N63Y, N63D, and N63H, which emerged following viral passage in A549 human lung cells (20). Therefore, VP3-63 may affect viral replication in different cell types.

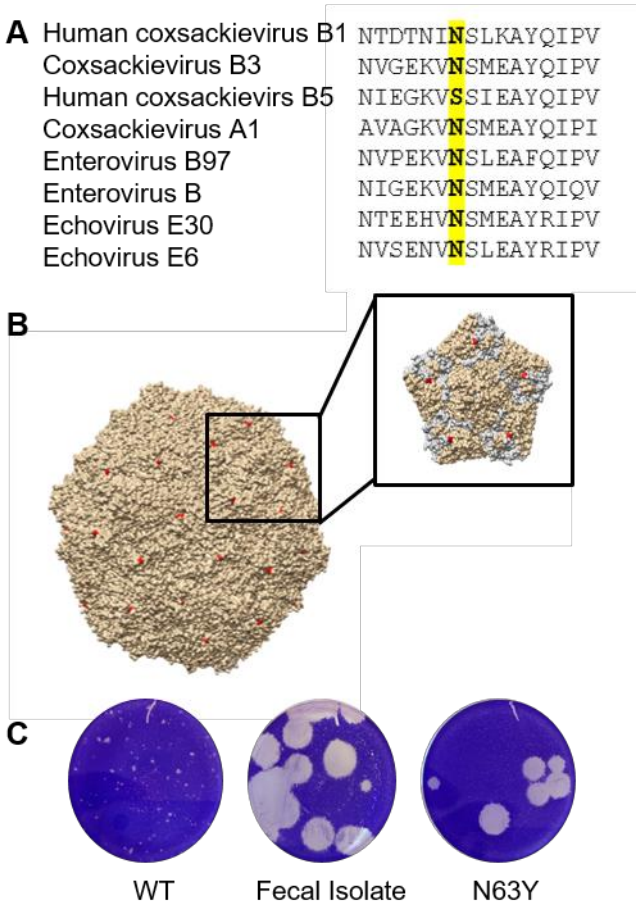

Figure 3. VP3-N63Y is sufficient for the large plaque phenotype. RT-PCR products from several large plaques were sequenced and contained the VP3-N63Y mutation. N63Y was cloned into a new infectious clone and virus was generated. (A) Sequence alignment of the VP3-63 region of various viruses. The highlighted residue is the 63 position. (B) CVB3 structure with the location of VP3-63 highlighted in red. The inset shows one 5-fold symmetry axis, with the VP3 proteins labeled in grey. (C) Plaque phenotypes of WT CVB3, a plaque purified fecal isolate with the large plaque phenotype, and N63Y CVB3.

N63Y CVB3 has a growth defect in cell culture

To characterize N63Y CVB3, we first compared the growth of N63Y and WT CVB3 in cell culture using single cycle growth curve assays. Four human cell lines, 293, HeLa, Huh7, RD cells, and two rodent cell lines, CHO-K1 and L929 cells, were infected at an MOI of 0.1 and viral titers were determined over time. N63Y CVB3 demonstrated a growth defect in most of the cell lines (Fig 4A-C and Fig 5). Furthermore, both WT and N63Y CVB3 replicated better in human cell lines than in rodent cell lines. These cell lines have different expression levels of the CVB3 receptors. 293, HeLa, and Huh7 have high levels of CAR, DAF and HS; RD and CHO cells have low levels of CAR, moderate levels of DAF and high levels of HS (51, 52, 159); and L929 cells have unknown levels of CAR and DAF, but are likely to have high levels of HS. Therefore, we hypothesized that the replication differences between WT and N63Y CVB3 may be due to altered cell attachment and receptor binding.

N63Y CVB3 has reduced binding to cells and GAGs

To determine why N63Y CVB3 has a growth defect in cell culture we first examined cell attachment. For the cell attachment assay, ^{35}S-labeled CVB3 was incubated with HeLa, Huh7, RD, L929, and CHO-K1 cell layers at 4°C to facilitate binding but not internalization. After washing, we quantified cell-associated ^{35}S. N63Y CVB3 had significantly reduced cell attachment to HeLa and Huh7 cells (Fig 4D), and both N63Y and WT CVB3 had limited attachment to RD, L929 and CHO-K1 cells. Therefore, the efficiency of viral attachment correlates with growth in cell culture.

We next examined whether N63Y CVB3 has altered binding to CAR or DAF compared to WT CVB3. ^{35}S-labeled WT or N63Y CVB3 was incubated with nitrocellulose membranes containing immobilized human or murine CAR (hCAR or mCAR) or human or murine DAF

(hDAF or mDAF). After washing, the amount of virus bound was quantified by phosphorimager analysis (149). We found that WT and N63Y CVB3 have equivalent binding to both hCAR and mCAR (Fig 6) and neither virus had detectable binding to hDAF or mDAF (data not shown).

Because altered binding to known CVB3 proteinaceous receptors could not explain the attachment defect of N63Y CVB3, we quantified viral binding to GAGs. First, we performed the cell attachment assay in three cell lines derived from CHO-K1 that have different levels of HS on cell surface due to knockout of host proteins involved in GAG synthesis (51, 52): GAG-negative pgsA745, HS-negative pgsD677, and 5% HS pgsB761. While N63Y CVB3 had significantly reduced attachment to CHO-K1cells compared to WT CVB3, it had comparable attachment to WT CVB3 in the other three cell lines (Fig 4E). These data indicate that N63Y CVB3 may have altered binding to GAGs. Second, we quantified CVB3 binding to GAGs using a direct method, heparin-agarose pull-down assays. ^{35}S-labeled CVB3 was incubated with heparin-agarose resin or control streptavidin-agarose resin at 37°C for 3 hours. After washing, resin-associated ^{35}S was quantified. We found that N63Y CVB3 had significantly reduced binding to heparin-agarose compared to WT CVB3 (Fig 4F).

Based on the reduced binding of N63Y CVB3 to heparin-agarose and previous studies showing that GAGs in agar can inhibit plaque formation of some viruses (164), we hypothesized that WT CVB3 binds GAGs in agar overlays, which reduces plaque size. Conversely, we hypothesized that N63Y CVB3 plaque formation would not be inhibited by GAGs in agar. To test this, we examined plaque sizes of WT and N63Y CVB3 using agar vs. agarose overlays and in the presence of added HS or heparin. Agarose contains low levels of GAGs, and WT and N63Y CVB3 generated similar sized, large plaques under agarose overlay. Addition of HS or heparin to the overlay reduced WT plaque size but not N63Y plaque size (Fig 7). Taken together,

these data suggest that N63Y has reduced binding to GAGs, which facilitates formation of large plaques in the presence of GAGs but reduces its attachment to cells and growth in cell culture.

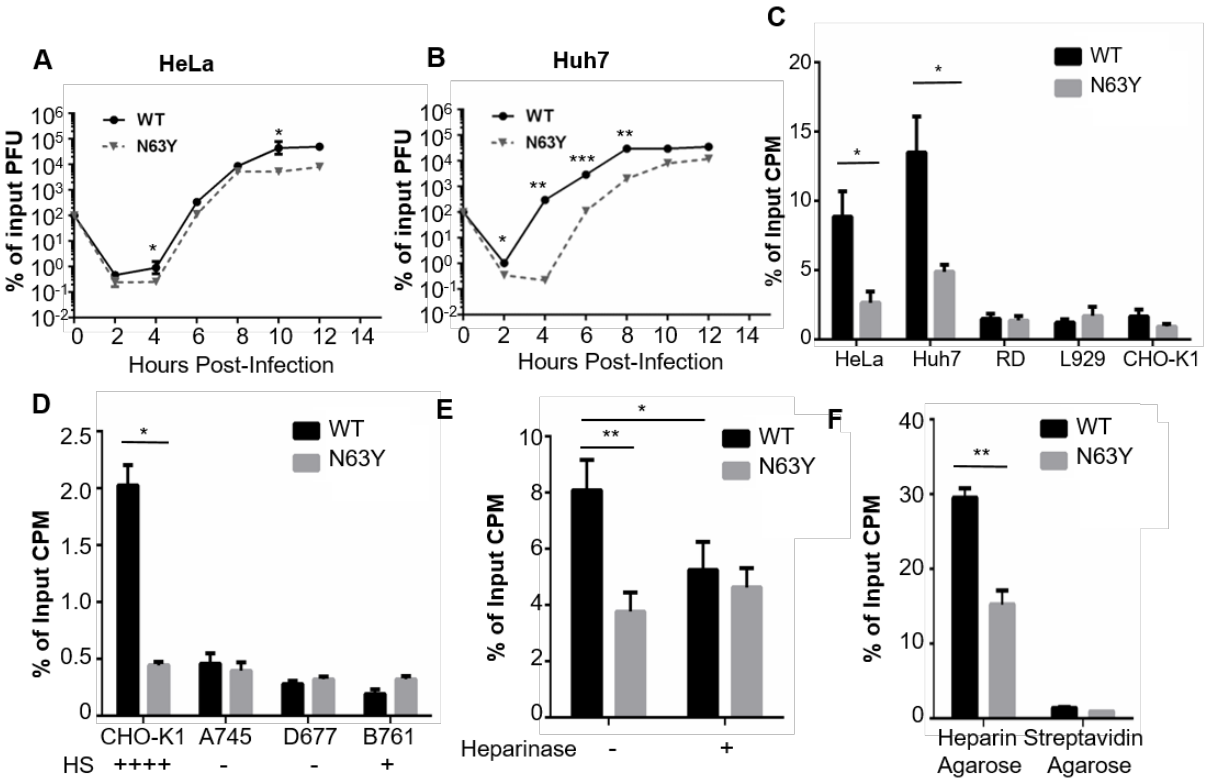

Figure 4. N63Y CVB3 has a growth defect in cell culture and reduced glycan-mediated cell attachment. Single cycle viral replication assays in (A) HeLa cells, and (B) Huh7 cells. Infections with WT CVB3 or N63Y CVB3 were performed at a multiplicity of infection of 0.1. Viral titers were determined by plaque assay using HeLa cells. n=3. (C) Cell attachment of ^{35}S-labeled WT or N63Y CVB3. Virus was incubated with cells at 4°C for 40 min. Cells were washed, trypsinized, and cell-associated ^{35}S was quantified. (D) ^{35}S-labeled WT or N63Y CVB3 was incubated with CHO cells (CHO-K1, pgsA745, pgsD677 and pgsB761) that vary in GAG expression. + and - indicate the relative level of GAGs on the cell surface. (E) Effect of heparinase treatment on CVB3 cell attachment. Cells were treated +/- heparinase I for 90 min prior to quantification of ^{35}S CVB3 attachment. n=7. (F) Heparin–agarose pull-down assay. ^{35}S-labeled WT or N63Y CVB3 was incubated with heparin-agarose resin or streptavidin-agarose resin (control). Resin was washed and bound ^{35}S CVB3 was quantified. n=3. *p<0.05. **P<0.01. ***P<0.001

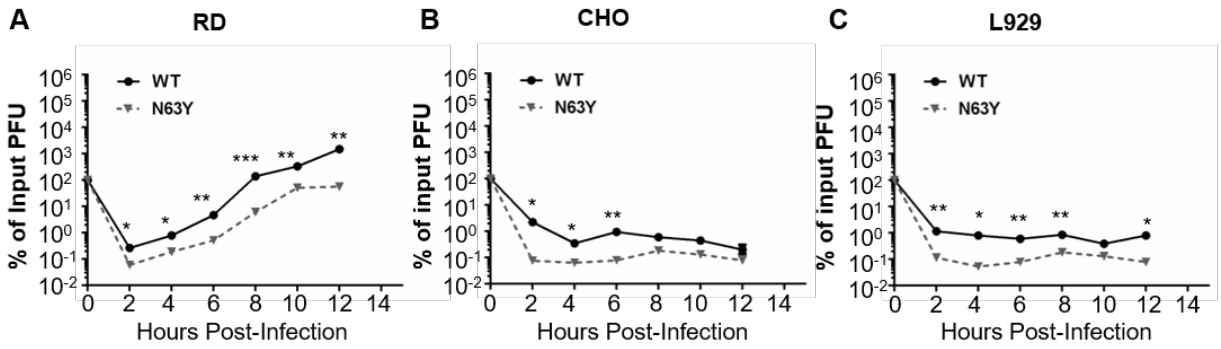

Figure 5. N63Y CVB3 has a growth defect in several cell lines. Single cycle viral replication assays in (A) RD cells, (B) CHO cells, and (C) L929 cells. Infections with WT CVB3 or N63Y CVB3 were performed at a multiplicity of infection of 0.1. Viral titers were determined by plaque assay using HeLa cells. *p<0.05. **P<0.01. ***P<0.001

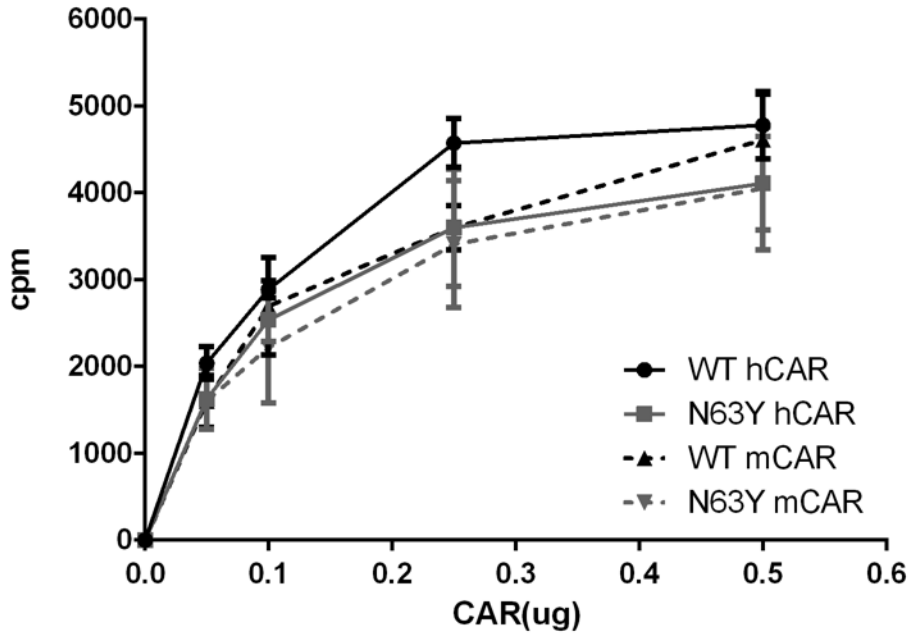

Figure 6. N63Y CVB3 does not have altered binding to human or murine CAR. ^{35}S-labeled WT or N63Y CVB3 was incubated with nitrocellulose membranes containing immobilized human CAR (hCAR) or murine CAR (mCAR) protein. After washing, bound viral CPM was used to quantify binding. n=3.

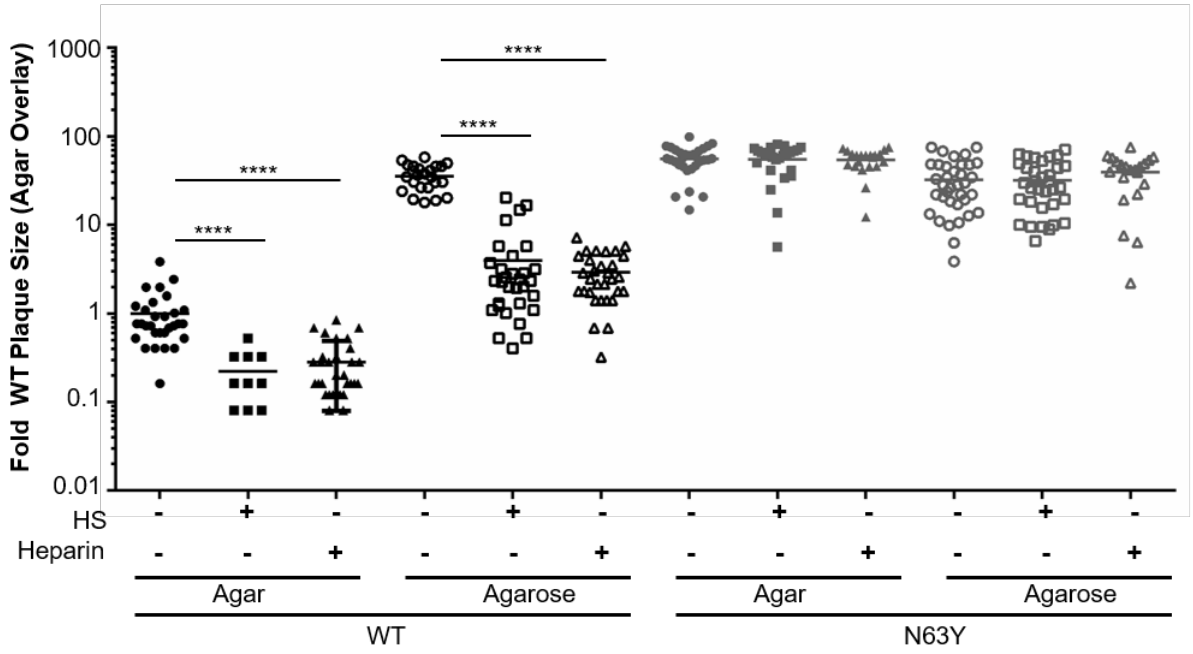

Figure 7. Sulfated glycans/GAGs inhibit WT CVB3 plaque formation. Plaque assays were performed with WT or N63Y CVB3 using agar vs. agarose overlays in the presence or absence of 0.1 mg/mL HS or heparin. Plaque sizes were quantified using Image J. For ease of comparison, data are displayed as "Fold WT Plaque Size", which is the size of each plaque divided by the average size of all WT plaques from plates with agar overlays (average=0.2473 mm2). ****P<0.001

N63Y CVB3 has increased replication and pathogenesis in mice

Given that N63Y CVB3 emerged following replication in mice, we hypothesized that N63Y CVB3 may have a fitness advantage in vivo. To test this hypothesis, we first examined viral replication in IFNAR-/- mice orally inoculated with 5×10^7 PFU WT or N63Y CVB3. Feces were harvested at 24, 48, or 72 hpi and titers were determined by plaque assay. N63Y CVB3 had higher viral titer in feces than WT CVB3, especially at early time points (Fig 8A). Since it was shown previously that WT CVB3 has limited replication in immune-competent mice, we wondered whether N63Y CVB3 would have enhanced replication in IFNAR+/+ mice. To test this, we orally inoculated IFNAR+/+ mice, and we observed that N63Y replicates more efficiently than WT CVB3 (Fig 8B). We also examined viral titers in various tissues from orally-inoculated IFNAR+/+ or IFNAR-/- mice at 72 hpi. In IFNAR-/- mice, N63Y CVB3 titers were significantly higher than WT CVB3 titers in all tissues examined (Fig 8C). While IFNAR+/+ mice had low viral titers in most tissues, N63Y CVB3 titers were significantly higher than WT CVB3 titers in leg muscle, indicating that N63Y was more capable of dissemination to body sites distant from the gut in immunocompetent animals (Fig 8D). These results indicate that N63Y CVB3 has enhanced replication and systemic dissemination in orally inoculated mice, including immune competent mice.

To examine virulence of WT vs. N63Y CVB3, we monitored the survival of mice following infection via different inoculation routes. We infected IFNAR-/- or IFNAR+/+ mice orally with 5×10^7 PFU, IP with 1×10^4 PFU, or IM with 1×10^2 PFU CVB3 (Fig 8E). First, we found that IFNAR+/+ mice did not succumb to CVB3 infection regardless of the virus or inoculation route. Second, we found that IFNAR-/- mice are most susceptible to IM infection (100 PFU) and least susceptible to oral infection (5×10^7 PFU). Third, we found that N63Y

CVB3-infected mice had a lower survival rate than WT CVB3-infected mice. In summary, N63Y CVB3 replicates more efficiently in both IFNAR+/+ and IFNAR-/- mice and is more pathogenic than WT CVB3 in IFNAR-/- mice.

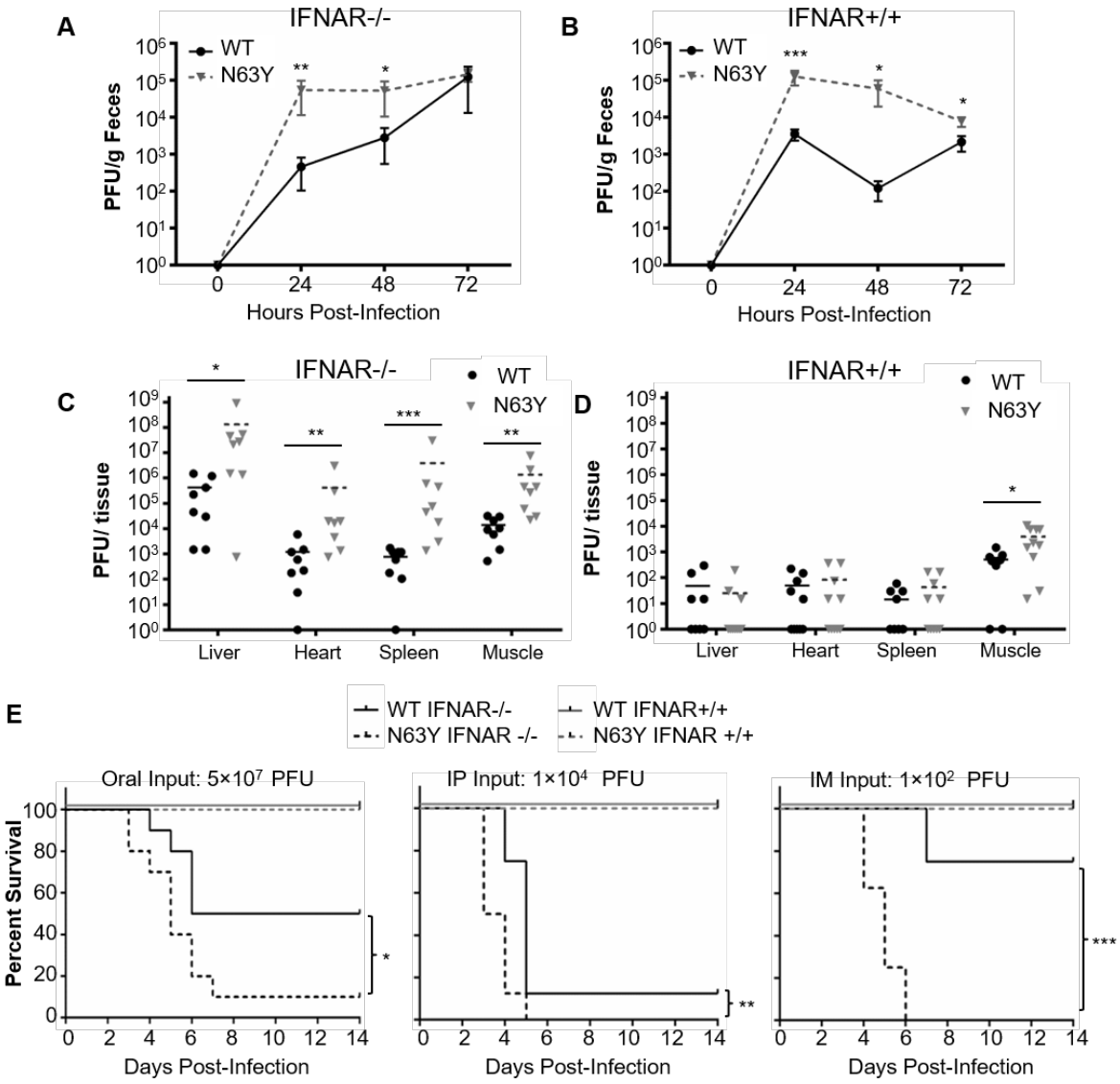

Figure 8. N63Y CVB3 has enhanced replication and virulence in mice. Viral fecal shedding profiles in (A) IFNAR-/- or (B) IFNAR+/+ mice orally inoculated with 5×10^7 PFU of WT or N63Y CVB3. Virus titer was determined by plaque assay. Viral tissue titers at 72 hpi in (C) IFNAR-/- or (D) IFNAR+/+ mice orally inoculated with 5×10^7 PFU of WT or N63Y CVB3. (E) Survival curves of CVB3-infected mice. From left to right, IFNAR-/- (black lines) or IFNAR+/+ (gray lines) mice were inoculated orally with 5×10^7 PFU, IP inoculated with 1×10^4 PFU, or IM inoculated with 1×10^2 PFU of WT (solid line) or N63Y (dashed line) CVB3. For all panels, n=5-8. *p<0.05. **P<0.01. ***P<0.001

N63Y CVB3 has enhanced dissemination to liver and induces more liver damage than WT CVB3

To determine the cause of death in CVB3-infected IFNAR-/- mice and understand why N63Y CVB3 is more pathogenic than WT CVB3, we examined liver pathology. Wessely *et al*. recently demonstrated that CVB3-infected IFNAR-/- mice develop liver pathology (174). We orally-inoculated IFNAR-/- mice with 5×10^7 PFU WT or N63Y CVB3. Blood was collected at 24 and 72 hpi to measure serum protein levels and liver was harvested at 72 hpi for histological analysis. First, we measured serum levels of serum alanine aminotransferase (ALT), a marker of liver damage. ALT levels were low at 24 hpi in all mice (Fig 9A). However, ALT levels were significantly elevated at 72 hpi in mice infected with N63Y CVB3 compared with uninfected controls. While 33% of mice infected with WT CVB3 had elevated ALT levels compared with uninfected mice, 66% of mice infected with N63Y CVB3 had elevated ALT levels. Second, we performed histological analysis on liver tissue harvested from mice at 72 hpi. As shown in Figure 10, mice infected with N63Y CVB3 had more severe liver pathology compared with uninfected mice or WT CVB3 infected mice. Histological examinations revealed hepatic cell necrosis with extensive polymorphonuclear cells infiltration at 72 hpi in N63Y CVB3 infected mice. Moderate polymorphonuclear cells infiltration was observed in WT CVB3 infected mice. In agreement with Wessely *et al*., our data suggest that CVB3-infected IFNAR-/- mice develop liver damage (174). Furthermore, our data suggest that mice infected with N63Y CVB3 have enhanced liver damage compared to WT CVB3 infected animals.

Due to the enhanced liver damage observed in N63Y CVB3 infected mice, we hypothesized that N63Y CVB3 may have increased homing to the liver. To test this, we IP-injected IFNAR-/- mice with ^{35}S-labeled WT or N63Y CVB3, collected heart, liver, and spleen

at 1 hpi, and quantified the amount of tissue-associated virus by scintillation counting. While WT and N63Y CVB3 were present at equivalent levels in heart and spleen, there was 8-fold more N63Y CVB3 present in liver (Fig 9B). In fact, on average, half of the injected N63Y CVB3 was found in the liver. These effects were observed in liver harvested at 1 hpi, hours before a single cycle of viral replication can occur. The elevated amount of N63Y CVB3 found in the liver could be due to increased binding to liver tissue and/or increased dissemination to the liver. To distinguish between these possibilities, we quantified biding of ^{35}S-labeled WT vs. N63Y CVB3 to liver tissue homogenates from IFNAR-/- mice. We found equivalent binding of WT and N63Y CVB3 to liver tissue homogenates (Fig 9C). Therefore, these data suggest that N63Y virions disseminate to the liver more efficiently than WT virions.

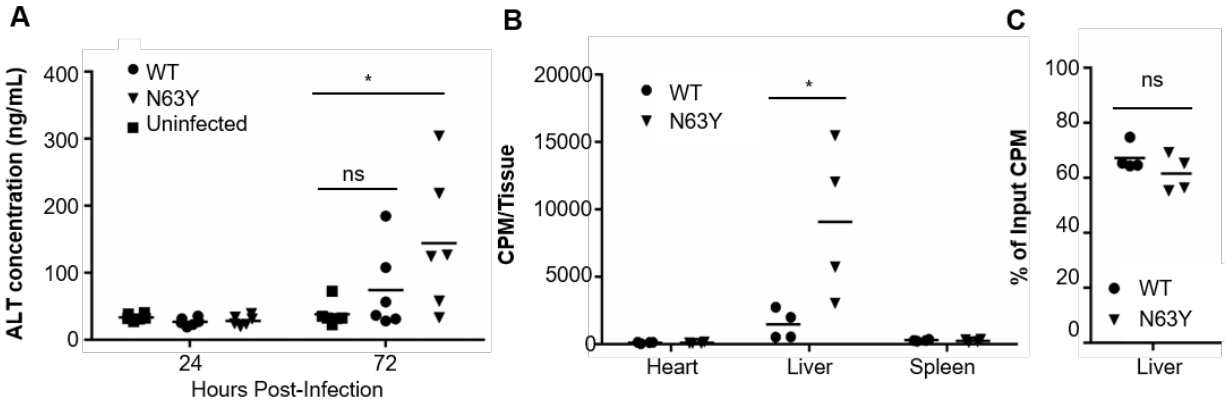

Figure 9. N63Y CVB3 has enhanced dissemination to liver and induces more liver damage than WT CVB3. IFNAR-/- mice were orally infected with 5×10^7 PFU of WT or N63Y CVB3. (A) Blood was harvested at 24 and 72 hpi and ALT levels were quantified by ELISA. n=6. (B) IFNAR-/- mice were inoculated IP with 2×10^7 PFU/20,000 CPM ^{35}S labeled WT or N63Y CVB3. Heart, liver and spleen were harvested 1 hpi, and tissue-associated CPM was quantified. (C) Binding of CVB3 to homogenized tissues. 3×10^6 PFU/3000 CPM ^{35}S labeled WT or N63Y CVB3 was incubated with homogenized liver tissue from IFNAR-/- mice for 60 min, followed by washing and quantification of tissue-associated CPM. *$p<0.05$.

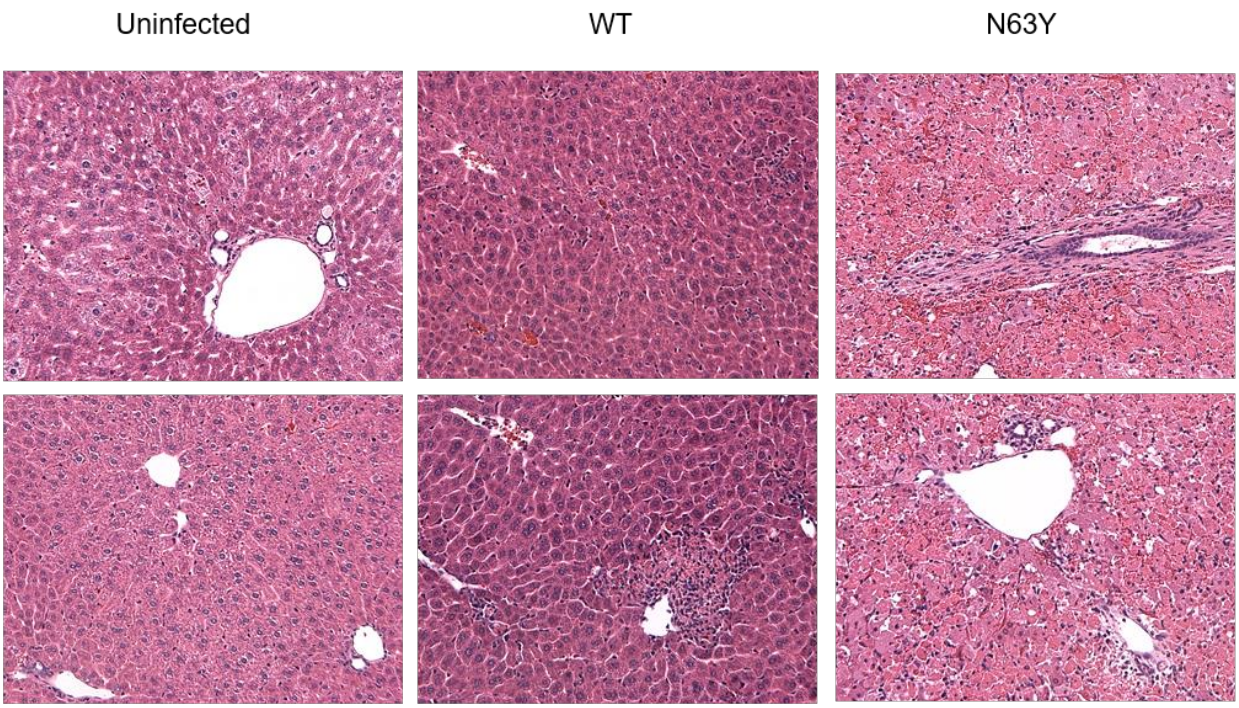

Figure 10. Histopathology of liver. IFNAR-/- mice were orally infected with 5×10^7 PFU of WT or N63Y CVB3. At 72 hpi, liver tissue was collected and hematoxylin and eosin staining was performed. Tissues from two representative mice per condition are shown.

DISCUSSION

Due to error-prone RNA replication, RNA viruses generate mutations in every single cycle of propagation and selective pressure dictates which variants are present in the population. In this study, we discovered emergence of a CVB3 mutant *in vivo*, examined its properties *in vitro* and *in vivo*, and used it as a platform to understand viral adaptation and factors that influence the emergence of variants.

Many viruses attach to cells by binding HS or other GAGs (136). For some viruses, GAGs are *bone fide* receptors. Other viruses have non-glycan, proteinaceous receptors but binding to GAGs can enhance attachment. GAGs most commonly interact with viral particles via positively charged amino acids such as lysine or arginine (16, 75, 96, 159). Therefore, the N63Y amino acid change observed here is slightly unusual. It is possible that a change from asparagine to tyrosine disrupts GAG binding to a nearby site on the viral capsid or that non-canonical GAG-capsid interactions occur.

Acquisition of HS binding is a common cell culture adaptation for several viruses. In several cases, cell culture adaptation to bind HS attenuates viruses *in vivo*. For example, cell culture adaptation of foot-and-mouth disease virus selects viruses that bind HS, but these viruses are attenuated in cattle (153). A cell-culture adapted vaccine strain of chikungunya virus is attenuated due to increased GAG binding (180). A dengue virus variant with reduced HS binding caused severe disease in mice, with reduced clearance from serum (144). Similarly, Sindbis laboratory strains bind HS (96); however, HS-binding Sindbis strains are attenuated in mice. Interestingly, Byrnes *et al*. isolated a large plaque variant of Sindbis from mice inoculated with an HS-binding strain. The large plaque variant had reduced HS binding and was more virulent in mice (23). The non-HS binding variant had increased viremia and decreased clearance from

serum, perhaps due to reduced clearance by heparin-binding proteins in the host. These Sindbis results are very reminiscent of our CVB3 results.

Our results demonstrate that WT CVB3 plaque formation is inhibited by substances, likely GAGs, in agar whereas N63Y CVB3 is not. Importantly, if we had used agarose rather than agar for plaque assay overlays, we would not have observed emergence of N63Y CVB3 in mice. We only identified emergence of this mutant due to a very obvious phenotypic change in plaque size. It is possible, and even likely, that emergence of variants occurs frequently in animals, but may be missed due to lack of observable phenotypes.

The emergence of the large plaque variant varied when we used different inoculation routes. We observed large plaque variants emerge in fecal viruses from orally-inoculated mice and tissues from IP-injected mice, but in not muscle tissue from IM-injected IFNAR-/- mice. However, we did observe large plaques in liver from IM-injected IFNAR-/- mice. Additionally, more large plaque variants emerged from IFNAR+/+ mice than IFNAR-/- mice. These results suggest that increased selective pressure in the intestine and peritoneal cavity may have facilitated emergence of large plaque variants, particularly in immune-competent mice. Alternatively, it is possible that N63Y CVB3 has a fitness advantage only in certain tissues.

N63Y CVB3 had enhanced replication and pathogenesis in mice regardless of the inoculation route. We do not know whether the GAG-binding deficiency of N63Y CVB3 is sufficient for its enhanced replication and pathogenesis or if other effects contribute. In agreement with a previous study, we found that IFNAR-/- mice developed liver pathology upon CVB3 infection. Liver pathology was more severe in mice infected with N63Y CVB3, potentially because N63Y CVB3 had increased dissemination to the liver (Fig. 9). Whether the increased viral shedding in mice inoculated orally with N63Y CVB3 is due to altered viral

dissemination to the liver or other tissues is unknown. Regardless, because N63Y CVB3 is more pathogenic than WT CVB3 in orally-inoculated immune-deficient mice, and N63Y CVB3 replicates well in orally-inoculated immune-competent mice, it may be useful as a new model system for examining CVB3 replication and pathogenesis using the natural route of infection.

CHAPTER 4: Sex difference in CVB3 infection

INTRODUCTION

Sex contributes to the frequency and severity of many human diseases (94, 134, 171). Epidemiological studies show that men are more susceptible to bacterial, viral, and fungal infections (171). Women, while more resistant to pathogens, are at an increased risk for autoimmune disorders. Sexual dimorphism in disease has been attributed to differential gene expression, sex hormones, and variations in immune function (41). However, sex as a variable in disease models is understudied, limiting our understanding of the mechanism of sex bias in various diseases.

Coxsackievirus is implicated in a range of diseases (6, 7, 33, 71, 98, 155). In humans, males are twice as likely as females to develop sequela from coxsackievirus infection (158). Mouse models have served as an important tool in advancing our knowledge of CVB3-induced myocarditis. Following intraperitoneal (IP) injection, CVB3 replicates to high titers in tissues and causes disease similar to that observed in humans (55, 62, 176), but male mice are more likely to develop severe myocarditis than female mice. Experimental evidence suggests two mechanisms contribute to CVB3-induced myocarditis in these mouse models: direct viral lysis of infected cardiomyocytes and immune-mediated damage of cardiac tissue (61). The latter is hypothesized to be responsible for the sex bias in disease. This hypothesis is based on the observation that CVB3 replicates equally well in the hearts of male and female mice, yet only male mice develop increased cardiac damage due to sex-specific immune responses (59, 110, 111).

While previous mouse experiments have provided vital insights into CVB3 pathogenesis, they are based on systemic infection of CVB3 rather than infection by the natural fecal-oral

route. CVB3 is an enteric virus and IP injection models bypass the first stage of infection in the gastrointestinal tract. Relatively few studies have used oral inoculation of mice with coxsackievirus (18, 19, 69, 114). Therefore, CVB3 replication and factors that influence viral replication in the intestine remain poorly understood. Here we describe an oral inoculation mouse model of CVB3 to examine CVB3 replication and lethality following initial intestinal infection. We found that, similar to previous mouse models and human infection, only male mice succumbed to CVB3-induced disease. Contrary to results from previous IP injection mouse models, we found that CVB3 replicated in the intestine of male mice, but not female mice. Immune responses were distinct in male and female mice. These data highlight the importance of studying enteric viral replication in the intestine.

RESULTS

CVB3 replication is enhanced in the intestine of male mice

To investigate replication of CVB3 in the gastrointestinal tract we orally inoculated male and female C57BL/6 IFNAR-/- mice with $5x10^7$ PFU of CVB3-Nancy. We selected IFNAR-/- mice based on previous work showing that lack of the type 1 interferon (IFN) response enhances susceptibility of mice to human enteric viruses by the oral route (83, 135). Following oral inoculation, feces were collected 24, 48, and 72 hours post-inoculation (hpi) and CVB3 titers were quantified by plaque assay. At 48 and 72 hpi, we observed 100-1000 fold higher fecal titers in male mice compared to female mice (Fig. 11A). The fecal titers that we observed in male mice suggested viral replication had occurred, but due to potential "flow through" of inoculum virus, viral fecal shedding is not always indicative of viral replication in the intestine (104). Therefore, we differentiated inoculum vs. replicated CVB3 in feces of male mice using

light-sensitive CVB3. Virus propagated in the presence of neutral red dye is light-sensitive due to RNA cross-linking leading to inactivation. Upon viral replication in the dark (e.g., intestine), replicated virions lose light sensitivity, which facilitates the assessment of replication by differentiating inoculum virus from replicated virus (103, 149, 172). Following oral inoculation of mice with light-sensitive CVB3 in the dark, feces were collected at 24, 48, and 72 hpi in the dark. Following processing, CVB3 replication was quantified by determining the ratio of light- verses dark-exposed fecal titers. Similar to previous data from our laboratory (172), at 48 and 72 hpi, we found that most virus shed from male mice was replicated virus (Fig 11B). In contrast, undetectable or limited CVB3 replication was observed in feces collected from female mice (Fig 11B). Overall, these data suggest that CVB3 replicates more efficiently in the intestine of male IFNAR-/- mice compared to female IFNAR-/- mice.

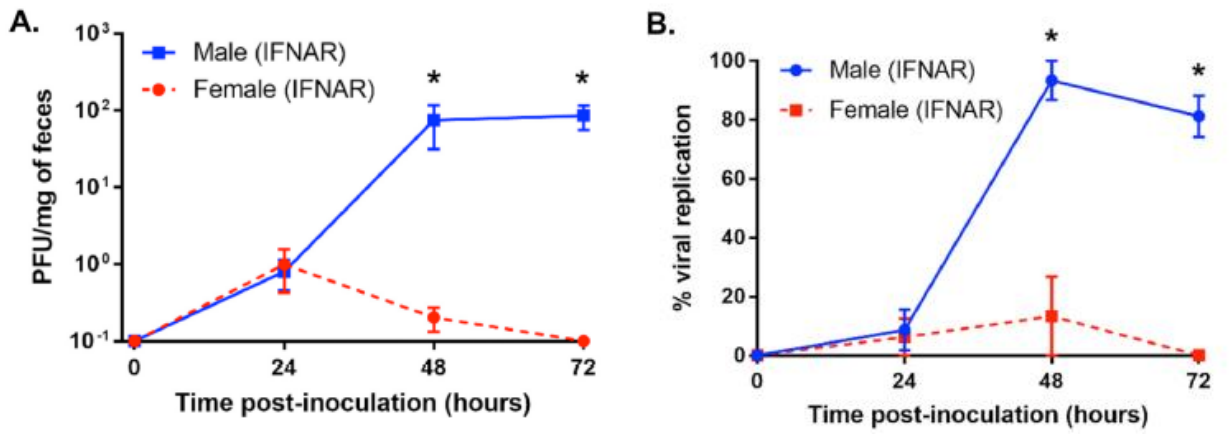

Figure 11 (generated by Christopher Robinson). Sex-dependent intestinal replication of CVB3. (A) CVB3-Nancy fecal titers in male and female IFNAR-/- mice orally inoculated with $5x10^7$ PFU of CVB3-Nancy. n = 14-15 per sex. (B) CVB3-Nancy replication in male and female IFNAR-/- mice using light sensitive, neutral-red labeled virus. Replication status was determined by dividing the number of PFU/ml of light exposed samples by the number of PFU/ml of dark-exposed samples and multiplying by 100%. n= 4-7 per sex. All data are mean ± SEM.*$p<0.05$. Mann-Whitney Test.

CVB3-induced lethality in IFNAR-/- mice is sex-dependent

As in humans, previous mouse models have demonstrated a sex bias in CVB3 pathogenesis (59, 110, 115, 116). To examine if orally infected mice display sex-dependent CVB3-induced lethality, we inoculated IFNAR-/- mice and examined survival following oral infection. We found that only male IFNAR-/- mice succumbed to disease following CVB3-Nancy infection (Fig 12). This data suggest that survival following oral inoculation of CVB3 is sex-dependent in IFNAR-/- mice.

CVB3-Nancy induces an immune response in orally inoculated male IFNAR-/- mice but not in female IFNAR-/- mice

Since CVB3-Nancy failed to replicate in orally-inoculated female IFNAR-/- mice, we hypothesized that a heightened immune response in female mice may limit CVB3 replication in the intestine. To investigate this hypothesis, we examined serum cytokine levels in male and female IFNAR-/- mice at 72 hpi since this time point correlated with peak viral shedding in males. Contrary to our hypothesis, we observed increased cytokine levels, as compared to uninfected control mice, in CVB3-infected male mice but not in female mice. Male IFNAR-/- mice had increased serum cytokine levels of IFN-γ, IL-1β, IL-6, IP-10, KC, MCP-1, RANTES, and TNF-α after CVB3 infection compared with uninfected control male IFNAR-/- mice (Fig 13). Furthermore, serum cytokine levels in CVB3-infected female IFNAR-/- mice were low and similar to uninfected female mice. These data indicate that orally inoculated female IFNAR-/- mice do not elicit a strong immune response at 72 hpi.

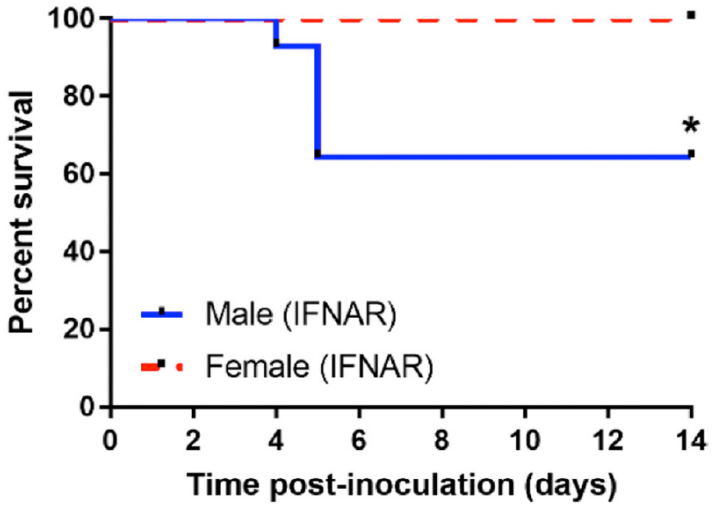

Figure 12 (generated by Christopher Robinson). Sex-dependent CVB3-induced lethality in orally inoculated mice. Survival of male and female C57BL/6 IFNAR-/- mice following oral inoculation with $5x10^7$ PFU of CVB3-Nancy. *$p<0.05$, Log-rank test. n = 14-15 per sex.

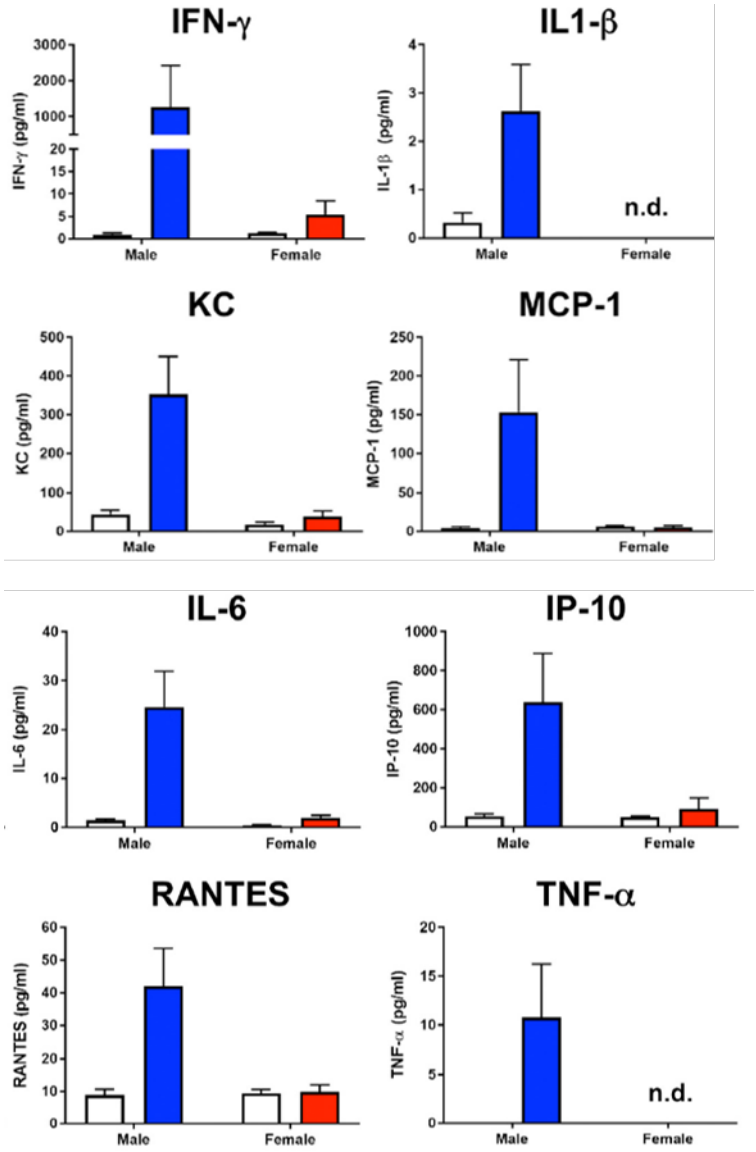

Figure 13. Sex-dependent immune response to intestinal CVB3-Nancy replication. Serum cytokine quantification in CVB3-Nancy infected male (blue) and female (red) IFNAR-/- mice 72 hpi or uninfected (white) mice. Infected mice were orally inoculated with $5x10^7$ PFUs of CVB3-Nancy. Serum cytokine levels for interferon-γ (IFN- γ), interleukin-1β (IL-1β), interleukin-6 (IL-6), interferon gamma-induced protein 10 (IP-10), mouse keratinocyte-derived cytokine (KC), monocyte chemoattractant protein-1 (MCP-1), RANTES, and tumor necrosis factor-α (TNF-α). n = 3-6 per sex. n.d. = not detectable.

DISCUSSION

Enteric viruses are spread through the fecal-oral route and initiate infection in the gastrointestinal tract. While previous mouse models for coxsackievirus recapitulate systemic viral infection and pathogenesis of the heart, factors that affect initial CVB3 replication in the intestine are unclear. Herein, we used an oral inoculation model for CVB3 to study viral replication in the intestine. Contrary to previous work in IP-injected mice, we observed sex-dependent replication of CVB3 within the intestine. CVB3 replicated efficiently and with enhanced lethality in male IFNAR-/- mice, but not female IFNAR-/- mice. To our knowledge, this represents the first time that sex has been demonstrated to influence the replication of an enteric virus in the intestine.

We also investigated the immune response to CVB3 in our oral inoculation IFNAR-/- mouse model. Previous studies suggest that women generate superior cell-mediated immune responses to viral pathogens than men (1). Therefore we hypothesized that the lack of viral replication in female mice was due to an increased antiviral cytokine response. However, we observed that male IFNAR-/- mice had increased serum cytokines 72 h following CVB3 infection, while the levels of serum cytokines in female IFNAR-/- mice were similar to untreated controls. These data indicate that female mice fail to elicit an immune response 72 h following oral infection of CVB3, however future experiments are required to determine if the kinetics of cytokine induction differ between the two sexes. Overall, these data suggest that female mice are resistant to CVB3 intestinal infection and that factors in the intestinal environment may play a key role in sex dependent CVB3 replication. Previously, our lab has shown that intestinal bacteria enhance poliovirus replication and pathogenesis (103, 149). Poliovirus is an enteric virus from the same viral family as coxsackievirus, therefore intestinal bacteria may enhance CVB3

replication as well. Interestingly, recent evidence suggests that sex influences the bacterial composition within the intestine (118, 181). Therefore, sex-specific bacteria may contribute to the replication of CVB3 within the intestine.

In conclusion, we found that CVB3 replication and lethality is higher in orally inoculated male mice compared with female mice due to differential viral replication in the intestine. Furthermore, immune response plays an important role in intestinal CVB3 replication and pathogenesis. Overall, these data suggest that sex and the natural oral route of infection should be considered when investigating enteric viruses.

CHAPTER 5: The effect of butyrate in CVB3 infection

INTRODUCTION

We have shown that intestinal microbiota promote replication and pathogenesis of several viruses, including poliovirus (PV), reovirus, and coxsackievirus B3 (CVB3). Although PV and CVB3 are two closely related viruses in the *Picornaviridae* family, our recent data suggest that microbiota enhance PV and CVB3 infection through distinct mechanisms. Bacterial surface polysaccharides enhance cell attachment of PV but not CVB3, which has led us to examine how microbiota enhance CVB3 infection.

Based on recent literature (148), removing butyrate-producing *Clostridia* from the mouse intestinal lumen by antibiotic treatment, leading to decreased levels of butyrate, increased epithelial oxygenation, and aerobic expansion of *Salmonella enterica* serovar Typhimurium. So we sought to examine if butyrate can alter CVB3 replication in the gut. Bacteria in the colon produce millimolar quantities of butyrate and other short-chain fatty acids (SCFAs) through fermentation of dietary fiber. SCFAs are among the most abundant molecules in the distal gastrointestinal tract. To determine whether bacterial-derived SCFAs such as butyrate impact CVB3 replication in the intestine, we antibiotic-treated mice to deplete bacteria and we supplied exogenous butyrate by gavage and through the diet via tributyrin, a butyrate analog that is not absorbed in the small intestine but is metabolized in the colon. We found that CVB3 replication and pathogenesis was restored in antibiotic-treated mice that received oral tributyrin treatment. These results suggest that bacteria are dispensable for CVB3 replication as long as butyrate is present.

Butyrate may enhance viral replication through several mechanisms. Beta-oxidation of butyrate is a major fuel source for colonocytes. In addition to its role in metabolism, butyrate

alters host gene expression by acting as a histone deacetylase (HDAC) inhibitor. Butyrate alters expression of host genes relevant to CVB3 infection, including up regulation of the coxsackievirus receptor and down regulation of innate immune genes. Our preliminary data demonstrate that oral delivery of an HDAC inhibitor, Vorinostat, is sufficient to restore CVB3 replication in antibiotic-treated mice, suggesting that the HDACi activity of butyrate may promote CVB3 infection.

RESULTS

Butyrate can restore CVB3 replication in the intestines of antibiotic-treated mice

To test the hypothesis that butyrate can promote CVB3 replication in mouse intestine, I used our antibiotic-treated mouse model. Four conditions were tested, antibiotic-treated mice with and without exogenous butyrate, and untreated mice with and without exogenous butyrate. Two cages of male IFNAR-/- mice were treated with Vancomycin and Ampicillin by gavage once per day for four days. The two cages of mice were then given antibiotic water for 3-5 more days. Mouse feces were collected before and after antibiotic treatment to confirm the knockdown of gut bacteria. Following antibiotic treatment, one cage of mice from antibiotic-treated groups and one from untreated groups were treated with tributyrin, a butyrate analog that is not absorbed in the small intestine but is metabolized in the colon, by gavage before oral inoculation of $5x10^7$ PFU of CVB3-Nancy. These mice were then gavaged with tributyrin 24, 48 hpi and supplied tributyrin-supplemented chow to ensure the mice have butyrate in the gut during infection. Feces were collected at 24, 48 and 72 hpi and titers were determined using plaque assay. First we confirmed that CVB3 replication is decreased in antibiotic-treated mice (Fig 14). The survival rate of antibiotic-treated mice (Fig 14A) was higher while the viral titers (Fig 14B) were significantly lower than untreated mice. However, for mice given exogenous butyrate, the

survival rate and viral titers were both restored to comparable levels of control mice. Overall, these data suggest that butyrate can restore CVB3 replication in orally infected antibiotic-treated mice.

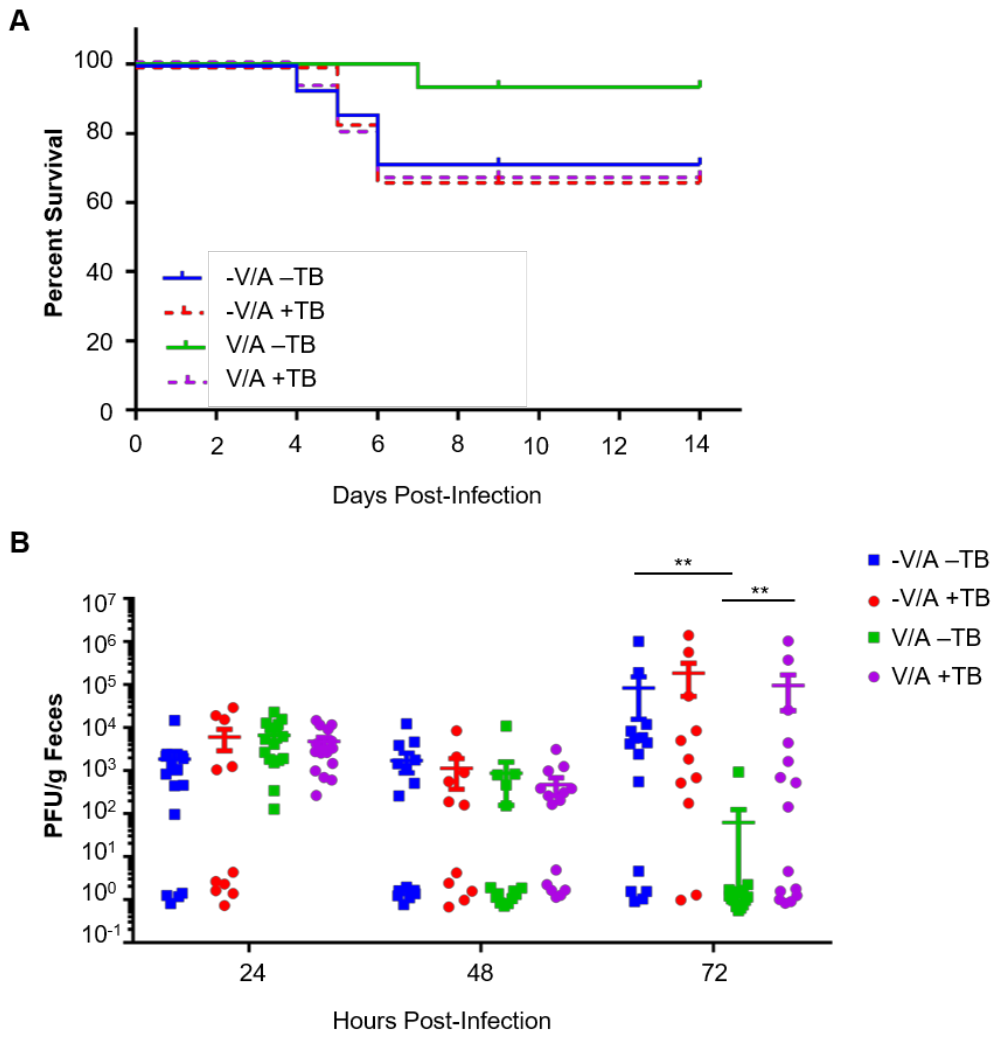

Figure 14. Butyrate can restore CVB3 infection in antibiotic-treated mouse intestines. (A) Survival curves of CVB3-infected IFNAR-/- male mice. No antibiotic treated without exogenous tributyrin (blue line), with exogenous tributyrin (red dash line), antibiotic treated without exogenous tributyrin (green line) and with exogenous tributyrin (purple dash line) were inoculated orally with 5×10^7 PFU CVB3. (B) Viral fecal shedding profiles in four conditions stated above. Virus titer was determined by plaque assay. n= 11-15 per condition. All data are mean ± SEM.*$p<0.05$. **$P<0.01$. TB=tributyrin. V/A=Vancomycin/Ampicillin antibiotic treatment.

Butyrate may promote CVB3 replication via HDACi activity

I next tried to determine by which mechanism butyrate promotes CVB3 replication. One of the functions of butyrate is acting as an HDAC inhibitor which can alter expression of host genes relevant to CVB3 infection, including up regulation of the coxsackievirus receptor and down regulation of innate immune genes (30, 93, 106, 154).

To test the hypothesis that butyrate promotes CVB3 replication via HDACi activity, I first used Vorinostat (also known as suberoylanilide hydroxamic acid (SAHA)), an HDAC inhibitor that can be given to mice orally (188). I tested three conditions: no antibiotic treatment, antibiotic treatment without SAHA and antibiotic treatment with SAHA. The antibiotic treatment is as described above with Vancomycin and Ampicillin. I gave the SAHA group mice 50mg SAHA/kg mouse weight by gavage before oral inoculation of $5x10^7$ PFU of CVB3-Nancy. These mice were then gavaged with same dose of SAHA at 24, 48 hpi. Feces were collected at 24, 48 and 72 hpi and titers were determined using plaque assay. Viral titer data indicated that treatment with SAHA restored the CVB3 replication in antibiotic-treated mice (Fig 15). Overall, these preliminary data suggest that butyrate may promote CVB3 replication via HDACi activity but further confirmation is needed.

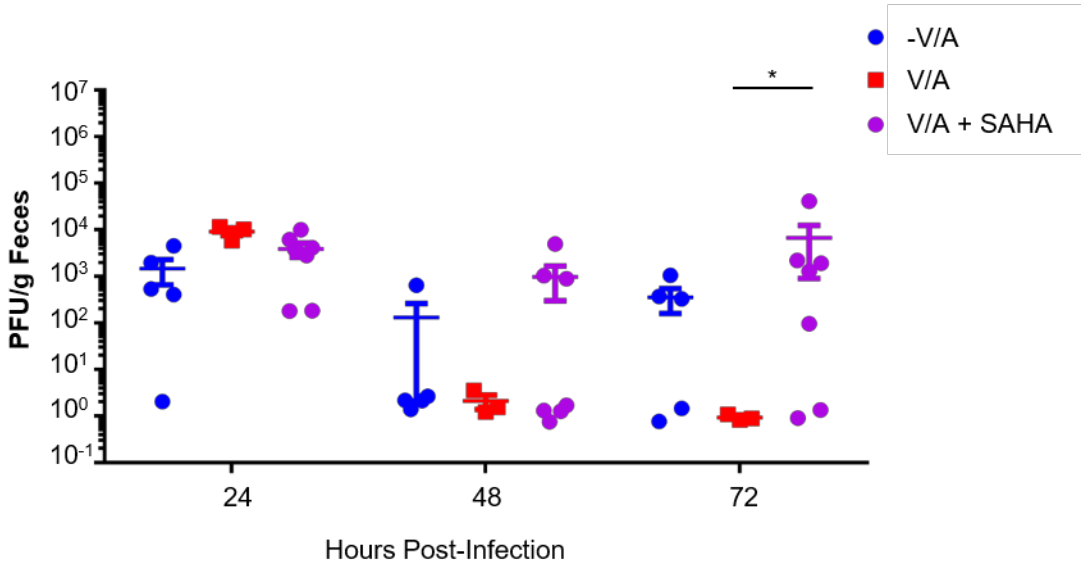

Figure 15. SAHA may restore CVB3 infection in antibiotic-treated mouse intestines. Viral fecal shedding profiles. Three conditions were tested: no antibiotic treated without SAHA (blue line), antibiotic treated without SAHA (red line) and with SAHA (purple dash line). Mice were inoculated orally with 5×10^7 PFU CVB3. Virus titer was determined by plaque assay. n= 3-7 per condition. All data are mean ± SEM.*$p<0.05$. V/A=Vancomycin/Ampicillin antibiotic treatment.

DISCUSSION

We are very curious about how microbiota enhance CVB3 infection. In this project, I examined the role of butyrate in CVB3 replication in vivo. I found that butyrate could restore CVB3 replication in antibiotic-treated mice and it may be via butyrate's HDACi activity and subsequent changes in host gene expression. Previous studies have shown that butyrate can upregulate the expression of CAR and downregulate genes including STAT6, IL-6, IL-12, IFNAR2 and TNFα. If we confirmed that SAHA could restore CVB3 replication as butyrate, we would further examine if butyrate alters the gene expression in mice and if those genes influence CVB3 infection.

Another mechanism by which butyrate may influence CVB3 infection is as an energy source. We would test this hypothesis using Acads-/- mice which are deficient in an important enzyme in the oxidation process of butyrate. If butyrate promotes CVB3 infection as an energy source, we would expect to see the effect disappear in Acads-/- mice.

To facilitate the testing of the mechanisms by which butyrate exerts its function and to make the manipulation of conditions easier, we want to set up an in vitro system using primary colonocytes isolated from mice. We will also test other SCFAs, acetate and propionate, to determine if promoting CVB3 infection is exclusive to butyrate.

CHAPTER 6: Discussion and future perspectives

It has been fascinating to identify factors that influence CVB3 pathogenesis. It was surprising to find emergence of the large plaque mutant in mice, even though we know that RNA viruses have high mutation rates. In the large plaque variant study, I discovered emergence of a CVB3 mutant *in vivo*, examined its properties *in vitro* and *in vivo*, and found that the N63Y mutant has diminished fitness *in vitro* but enhanced pathogenicity *in vivo*. It may be useful as a new model system for examining CVB3 replication and pathogenesis using the natural route of infection.

Interestingly, we found that the altered HS binding may also contribute to the plaque morphology. If we had used agarose rather than agar for plaque assay overlays, we would not have observed emergence of N63Y CVB3 in mice. We only identified emergence of this mutant due to a very obvious phenotypic change in plaque size. It is possible, and even likely, that emergence of variants occurs frequently in animals, but may be missed due to lack of observable phenotypes.

N63Y CVB3 had enhanced replication and pathogenesis in mice regardless of the inoculation route. It will be interesting to pursue whether the GAG-binding deficiency of N63Y CVB3 is sufficient for its enhanced replication and pathogenesis or if other effects contribute. In agreement with a previous study, we found that IFNAR-/- mice developed liver pathology upon CVB3 infection. Liver pathology was more severe in mice infected with N63Y CVB3, potentially because N63Y CVB3 had increased dissemination to the liver. Whether the increased viral shedding in mice inoculated orally with N63Y CVB3 is due to altered viral dissemination to the liver or other tissues remains unknown.

We observed large plaque variants emerge differently when we used different inoculation routes. Large plaques were seen in fecal viruses from orally-inoculated mice and tissues from IP-injected mice, but not in muscle tissue from IM-injected IFNAR-/- mice. However, we did observe large plaques in liver tissue from IM-injected IFNAR-/- mice. Additionally, more large plaque variants emerged from IFNAR+/+ mice than IFNAR-/- mice. These results suggest that increased selective pressure in the intestine and peritoneal cavity may have facilitated emergence of large plaque variants, particularly in immune-competent mice. Alternatively, it is possible that N63Y CVB3 has a fitness advantage only in certain tissues. These questions need to be answered in future study.

Previous studies have shown that, in humans, males are twice as likely as females to develop diseases from coxsackievirus infection. We observed sex-dependent replication of CVB3 within the intestine in contrast to previous work in IP-injected mice. To our knowledge, this represents the first time that sex has been demonstrated to influence the replication of an enteric virus in the intestine. We hypothesized the female mice induced stronger immune response to clear CVB3 infection because females were known to have a more robust immune system. However, we observed that male IFNAR-/- mice had increased serum cytokines 72 h following CVB3 infection but female mice maintain the cytokine levels similar to uninfected controls. Future experiments are required to determine if the kinetics of cytokine induction differ between the two sexes. Besides immune responses, recent evidence also suggests that sex influences the bacterial composition within the intestine (118, 181). Since we have shown that gut microbiota can enhance poliovirus and coxsackievirus pathogenicity, sex bias in microbiota composition may influence coxsackievirus infection. One of our next step will be to examine the role of microbiota in sex-bias of CVB3 infection.

To understand the interaction between gut microbiota and enteric viruses, we cannot ignore the various molecules that the microbiota produce. Here we examined an important subset of the products, SCFAs. Surprisingly, butyrate itself is sufficient to promote CVB3 infection in the gut. Based on the known functions of butyrate, we tested if butyrate promotes CVB3 infection via HDACi activity and the preliminary data supports this hypothesis. It will be interesting to further confirm these results and determine if butyrate altered the expression of host genes related to CVB3 pathogenesis. At the same time, we also want to test if butyrate functions as an energy source in this process by using knockout mouse strains and *in vitro* assays. Additionally, other SCFAs and other bacterial products should be examined to discover more factors that influence CVB3 pathogenesis.

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