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Identification of Viral Determinants of Murine Norovirus Pathogenesis

David Strong
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Immunology

Dissertation Examination Committee:
Herbert W. Virgin, IV, Chairperson
John P. Atkinson
Michael S. Diamond
Mark J. Miller
Thaddeus S. Stappenbeck
Emil R. Unanue

IDENTIFICATION OF VIRAL DETERMINANTS OF MURINE NOROVIRUS PATHOGENESIS

by

David Wesley Strong

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ABSTRACT OF THE DISSERTATION

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Professor Herbert W. Virgin IV, Chairperson

Human noroviruses are responsible for the majority of cases of epidemic non-bacterial gastroenteritis. Despite their importance as human pathogens, knowledge of the viral life cycle and host pathogenesis is limited due to the lack of practical models to study. In contrast, murine norovirus (MNV) has been a critical surrogate for the study of human noroviruses as it is the only member of this genus that establishes infection in a small animal model, can be grown in cell culture, and has a reverse genetics system. Noroviruses are divided into genogroups which can contain many viral strains. These viral strains can display dramatic variation in their virulence and ability to persist, but the factors responsible for these differences are poorly defined. The focus of this work is to describe biological differences between two MNV strains, CW3 and CR6, and to use molecular genetics tools to identify the viral determinants that contribute to these differences.

Here, we have cloned CW3 and CR6 into a reverse genetics system for production of virus and genetic analysis. We show virus production by this method limits the emergence of viral quasispecies. This makes the analysis of biological phenotypes less complex. We identify four phenotypes in which our cloned viruses behave differently from one another and have chosen two, lethality in STAT1-/- mice and persistent infection in wild type mice, for further study.

We show that the sequence of the protruding (P) domain of the viral capsid protein determines whether MNV infection is lethal in mice lacking STAT1, a regulator of innate immunity. The P domain is also a determinant of viral growth and dissemination in these mice. Further, we have identified the N-terminal non-structural protein (NTerm) as the viral determinant of persistent infection in wild type mice. Remarkably, a single amino acid in NTerm dictates the persistence phenotype of these two viral strains. This is the first demonstration of a function for the norovirus NTerm protein during infection of a host. We believe the system and reagents we have developed will be invaluable tools for associating norovirus genes and protein domains with specific pathogenic properties.

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LIST OF ABBREVIATIONS

BMM	bone-marrow derived macrophage
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FCV	feline calicivirus
LCMed	L929 cell conditioned media
MNV	murine norovirus
MOI	multiplicity of infection
PCR	polymerase chain reaction
PFU	plaque forming unit
SMV	Snow Mountain virus
STAT	signal transducer and activator of transcription
VLP	virus like particle

MATERIALS AND METHODS

Cloning and production of recombinant MNV. CW3 and CR6 were cloned into the pMNV* vector as previously described {10571}. Briefly, each strain was amplified by PCR (using the primers AACTTGGGATCCACCGGTGTGAAATGAGGATGGCAACGC and ATGCGGCCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAATGC ATCTAA) and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA). The sequence was then moved into the pMNV* vector in fragments (CW3: AgeI-SacII and SacII-NsiI; CR6: AgeI-DraIII, DraIII-NsiI and NsiI-NsiI). The coding sequence of each clone was verified by sequencing. Deviations from the consensus sequence of CW3 were repaired using the Quick Change II XL mutagenesis kit per manufacturer's protocol (Agilent Technologies, Santa Clara, CA) to reflect the previously published sequence (GenBank accession # EF014462.1). A single non-coding T→C substitution at nucleotide 5530 was identified in the CR6 clone as compared to the cDNA sequence published in GenBank (accession # JQ237823). A non-coding single nucleotide mutation was made in each strain, introducing a restriction enzyme site to facilitate identification of engineered virus (CW3 nucleotide 1001 C→A; creates an EcoRV site; CR6 nucleotide 512 C→G; creates a SacII site).

For production of recombinant virus, 293T cells were seeded at $5x10^5$ cells per well in a 6 well culture dish. Cells were incubated 16-24hr and transfected with virus encoding plasmid using FugeneHD according to the manufacturer's protocol using a 5:2 ratio. 48hr after transfection, cells were lysed by freeze-thaw at -80°C and the supernatant was clarified by centrifugation. Virus was passed in RAW 264.7 cells 1-2 times at multiplicity of infection (MOI) <0.05. Using this protocol, three independently generate virus stocks were sequenced and observed to have no deviations from the input sequence (data not shown). Viral RNA was isolated from infected cells using trizol according to the manufacturer's protocol, or from clarified viral supernatant using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For cDNA synthesis, 1µg of total RNA from cell lysate or 7 µL of RNA purified using the

RNeasy Kit was combined with 2.5 μM of the MNV specific primer used for whole genome amplification, 100μM (each) deoxynucleoside triphosphates, and incubated at 65°C for 5 minutes and on ice for 1 minute. 1x first strand buffer (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol, 40 U RNasin (Promega), and 200 U of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) were added to a final volume of 20 μL. The reaction was then heated at 50°C for 60 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. 2μL of cDNA was PCR amplified using the primers for whole gene amplification listed above. The PCR product was sequenced using the primers in table 2-1, which give roughly two fold coverage of the genome. At least two independently generated viral stocks were used for each experimental group.

Cell culture, viral growth and quantification. RAW 264.7 cells (ATCC# TIB-71) were cultured in DMEM supplemented with 10% fetal calf serum, glutamine, HEPES, and penicillin-streptomycin. Bone-marrow derived macrophages (BMMs) were harvested from the femur and tibia of C57BL/6 mice. LCMed differentiated cells were produced by culturing as described previously {1677}. CMG14-12 differentiated cells were produced with the following modifications: media used for the harvest and culture of bone marrow was DMEM supplemented with 10% fetal calf serum, 5% horse serum, 2mM L-glutamine, 1mM sodium pyruvate, 1x non-essential amino acids (Mediatech, Manassas, VA), and 10% CMG14-12 supernatant {13284} was substituted for LCMed.

For virus growth curves $4x10^5$ RAW 264.7 cells or $2x10^5$ BMMs were seeded in 24-well culture dishes and MOI 5 or 0.05 growth curves were completed as previously described {6091} with the following modifications: viral inoculum was diluted in 0.2 mL per well; samples underwent one freeze thaw cycle before plaque assay titration. Viral titer of each sample was determined by plaque assay on an indicator layer of RAW 264.7 cells as previously described {6091}.

Western blots. Viral supernatants were mixed with Laemmli sample buffer and boiled for 5 minutes prior to western blot analysis. Samples were resolved on a 10% polyacrylamide

SDS gel and transferred to PVDF membrane. The membrane was probed with a polyclonal rabbit antibody generated against MNV-1 virus-like particles {6091}.

Quantitative PCR. Viral RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturers protocol. For cDNA synthesis, 5 µL of purified RNA was combined with 0.5 µg of random hexamers, incubated at 70°C for 5 minutes and on ice for 5 minutes. 1x ImProm-II reaction buffer (Promega, Madison, WI), 3 mM $MgCl_2$, 0.5 mM (each) deoxynucleoside triphosphates, 40 U RNasin (Promega), and 1 µL of ImProm-II reverse transcriptase (Promega) were added to a final volume of 20 µL. The reaction was then heated at 25°C for 5 minutes and 42°C for 60 minutes. The reaction was inactivated by heating at 70°C for 15 minutes. PCR amplification was done using primers and a probe targeting the conserved junction between open reading frames 1 and 2 (sense: CACGCCACCGATCTGTTCTG; antisense: GCGCTGCGCCATCACTC; probe (Applied Biosystems, Foster City, CA): CGCTTTGGAACAATG). 2 µL of cDNA from each sample was PCR amplified in triplicate using the Step One Plus Real-Time PCR System (Applied Biosystems). Reaction conditions were as follows: 0.2 mM (each) deoxynucleoside triphosphates, 1x AmpliTaq buffer (Applied Biosystems), 2 mM $MgCl_2$, 0.2 µM sense primer, 0.2 µM antisense primer, 0.2 µM probe, 0.5 U AmpliTaq (Applied Biosystems) in a final volume of 20 µL. Genome copies were quantified by comparison against a standard curve of viral plasmid.

Mutation analysis. A segment of the MNV genome encompassing the P2 region was amplified by PCR using primers AAGGGTGCACGTTGATGGGAC and CTGACGCATGTAGGTCCGGAAC. The PCR product was cloned into the pCR4 TOPO vector (Invitrogen, Carlsbad, CA). Individual clones were isolated and sequenced by the Washington University Genome Sequencing Center using the T7 sequencing primer. Sequences were aligned and the number of mutations per sequence read was quantified using Sequencher (Gene Codes Corporation, Ann Arbor, MI).

Mouse infection and sample collection. All mice were bred and housed at Washington University in accordance with the rules and policies of the university animal protocol review

board. For infection of mice, virus was diluted in PBS and delivered per os using a pipette. Fecal pellets were harvested from live mice three or thirty-five days post-infection. Alternatively, mice were sacrificed three days post infection and tissues harvested as previously described {11271}.

Mouse immunization with cholera toxin and serum harvest. For immunization with cholera toxin, 10 µg of toxin was diluted in PBS and delivered per os using a pipette. Fourteen days after immunization, mice were anaesthetized by intra-peritoneal injection of 0.1 mL of a ketamine and xylazine solution, and approximately 0.05 mL of blood was collected from the retro-orbital sinus using a micro-capillary tube. Samples were spun at 6,000 x g for five minutes in a serum separator tube (BD Franklin Lakes, NJ, USA). Serum samples were stored at -20°C.

Cholera toxin antibody ELISA. ELISA buffers were made up of the following: coating buffer: 15 mM Na_2CO_3 , 3.5 mM $NaHCO_3$ pH 9.6; ELISA III buffer: 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl, 0.05% Tween 20, pH 7.4; wash buffer: 150 mM NaCl, 0.05% Tween 20. Each well of a high binding microtiter plate (Thermo Fisher Scientific, Waltham, MA) was coated with 0.25 µg of cholera toxin (List Biological Laboratories, Campbell, CA) diluted in coating buffer over night at 4°C or for 1 hour at 37°C. Plates were blocked with 1% BSA in coating buffer for 1 hour at 37°C followed by incubation with a 1:1 dilution (ELISA III buffer) series of sample serum from an original dilution of 1:100 for 1 hour at 37°C. Plates were then incubated with a horseradish peroxidase conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:1000 in ELISA III buffer for 1 hour at 37°C. Between each step plates were washed twice with ELISA wash buffer. The colorimetric assay was developed using 1-Step Ultra TMB-ELISA substrate (Thermo Fisher Scientific, Waltham, MA) and stopped using 2 molar sulfuric acid. Absorbance was measured at 450 nanometers.

CHAPTER 1

Introduction

Noroviruses as Human Disease Agents

Noroviruses are increasingly being recognized as a major cause of human disease. Studies have shown noroviruses to be the causative agent in between 80% and 95% of outbreaks of non-bacterial gastroenteritis, and approximately half of all outbreaks (2, 8, 28, 84). Further, the number of reports describing the role of noroviruses in cases of community acquired gastroenteritis is growing (10, 22, 33, 41, 51, 58, 60, 76). These studies report that between 5% and 20% of individuals seeking medical care for sporadic symptoms of gastroenteritis are positive for norovirus. Together, these studies implicate noroviruses as the single biggest cause of epidemic gastroenteritis and a major contributor to endemic gastroenteritis.

Norovirus infection is prevalent in all age groups (71). A seasonal association has been observed, with many more infections occurring in winter months (54). During norovirus outbreaks, symptomatic infection usually lasts 48 to 72 hours and is self-limiting, while community acquired infection may be indolent with symptoms lasting longer than one week (71). Symptoms of infection can include nausea, vomiting, diarrhea, and abdominal cramping and discomfort.

In 1999, the Centers for Disease Control and Prevention estimated that there are 23 million cases of norovirus gastroenteritis each year in the United States (53). Norovirus outbreaks occur most frequently in settings where a large number of people are housed in close quarters such as on cruise ships or in the military, with the largest percentage of outbreaks occurring in nursing homes, retirement centers, and hospitals (8). Norovirus infection in the latter settings is particularly significant, as infection in elderly and immuno-compromised individuals carries increased risk of morbidity and mortality (30, 40, 48, 49, 52, 57, 93). In immuno-suppressed individuals, complications such as viral shedding and diarrhea for months to years, symptoms resembling allograft rejection in intestinal transplant recipients, and necrotizing enterocolitis in neonatal intensive care unit patients can occur (30, 40, 57, 75, 85).

The source of many norovirus outbreaks is unknown. To date no animal reservoir has been identified for human noroviruses, although a porcine reservoir has been proposed (15, 16, 83, 86, 87). Person-to-person spread along with prolonged stability of virus in the environment

could account for outbreaks. Another possibility is that asymptomatic shedders of virus seed epidemic outbreaks. Several studies have documented prolonged shedding of norovirus in the feces of post-symptomatic individuals (25, 26, 32, 59, 62, 71). Strikingly, one study reported norovirus shedding in the stool of children under the age of six months for at least six weeks after initial symptoms (56). Other studies have reported the frequency of asymptomatic norovirus shedders to be between 4% and 13% of the populations surveyed (4, 17, 67). One study found that the frequency of asymptomatic shedders is age dependant, with the highest prevalence in children under five years of age (67). This phenomenon of asymptomatic shedding, along with the very low infectious dose of norovirus (80) may account for outbreaks with an unidentified source. This indicates the importance of understanding the molecular basis of persistence.

Susceptibility and Immunity to Noroviruses

The gene FUT2 has been identified as a susceptibility factor to infection with the Norwalk strain of human noroviruses (43). FUT2 catalyzes and enzymatic reaction which adds a fucosyl residue to the H type 1 precursor to generate the H type 1 carbohydrate antigen. This carbohydrate is found on epithelial cells of the intestine and in mucosal secretions of individuals with a functional FUT2 enzyme. Recombinant Norwalk virus like particles (VLPs) have been demonstrated to bind the H type 1 antigen (50). Binding to this carbohydrate is believed to facilitate viral entry and infection of cells, but this remains unproven, as human noroviruses have not been cultured to date. Individuals homozygous for an inactivating mutation in FUT2 are resistant to infection with Norwalk (82). However, resistant persons are still susceptible to other norovirus strains (42). Norovirus binding to carbohydrate antigens is strain specific, with each virus having its own binding profile (35, 44). Thus, an individual's susceptibility to infection is dependent on which carbohydrate antigens they produce as well as the carbohydrate binding profile of the virus they come in contact with.

Human challenge studies demonstrate that, in limited circumstances, immunity to norovirus infection does exist. After a first viral challenge, volunteers showed immunity for at

least six months if challenged with the same viral strain (24, 37, 61, 94). However, volunteers exhibit no immunity if given an antigenically distinct norovirus strain during the second viral challenge. Additionally, no long term immunity to re-challenge with the same or a different norovirus strain was observed. If 27 months or more passed after the first norovirus challenge, volunteers were no longer protected from a second challenge. These studies show that there is short term immunity to homologous but not heterologous norovirus challenge and no long term immunity.

Norovirus Structure and Function

Noroviruses are a member of the viral family *Caliciviradae*. They are small, non-enveloped single strand positive-sense RNA viruses with an approximately 7.5 kilobase genome. Within this viral family are four genera of which *Norovirus* is one. Noroviruses are further divided into five genogroups, each of which has many strains. The genome structure of Caliciviruses is generally conserved across this family of viruses. Noroviruses are composed of three open reading frames (ORF's). ORF one encodes the viral non-structural proteins. The transcript is translated to a poly-protein, which is then processed by the viral proteinase into the six non-structural proteins. From N-terminus to C-terminus, they are arranged in the following order: NTerm, NTPase, p22, VPg, Protease (Pro), Polymerase (Pol). The gene order of ORF1 and sequence preference for cleavage sites is also highly conserved across noroviruses. Much of what is known of the function of norovirus proteins is derived from expression studies of individual genes in recombinant expression systems. One problem with this approach is that protein functions important for the viral life cycle may be dependent on the expression of other viral proteins. Also, viral protein function may be affected by the cell type the protein is expressed in. Since the cell population targeted by human noroviruses *in vivo* is unknown, these over expression studies are fundamentally limited in their applicability to understanding pathogenesis. The function of some norovirus proteins has been speculated based on the function of similar proteins found in other viruses. Most frequently, norovirus proteins are compared to those of

poliovirus, as poliovirus has been studied in detail and these two viruses share an enteric tropism and have a similar genomic organization. While these types of comparisons are often useful as a starting point for the investigation of norovirus protein function, they have limited applicability as a basis to propose any model of viral pathogenesis in the absence of experimental data.

NTerm. The NTerm protein is of variable size dependent on norovirus strain. This protein may have two or more functional domains as additional proteolytic processing following cleavage by the viral protease has been reported (72). Cleavage of the NTerm protein by caspase 3 has been reported for murine norovirus (MNV) (74). When expressed in mammalian cells, the NTerm protein of the human norovirus strain Norwalk produces a peri-Golgi localization pattern, and has been shown to interact directly with VAP-A, a protein that plays a role in vesicle fusion (90). Finally, Golgi disassembly and inhibition of transport to the cell surface has been observed after expression of NTerm (27, 29). Whether these effects are mediated by specific interactions of the viral protein with host factors, or represent an artifact of overexpression is not known.

NTPase. Little is known regarding the function of the norovirus NTPase protein. Using *in vitro* assays, the NTPase from the human norovirus strain Shouthampton virus (SHV) was shown to bind and hydrolyze NTPs (66). One potential function for an NTP hydrolyzing protein is as a helicase, however the SHV NTPase failed to demonstrate helicase activity when tested *in vitro* (66).

p22. Stable expression of the p22 from human norovirus genotype II.4 variant 2002, in a cell line resulted in a slight increase in the frequency of apoptotic cells (34). Further, p22 expression resulted in impaired actin rearrangement and monolayer repair following single cell lesions and a decrease in trans-epithelial resistance over time (34). The authors speculate that these impairments in maintenance of monolayer integrity contribute to the development of diarrhea following norovirus infection.

VPg. The VPg protein is covalently linked to the genomic and sub-genomic RNA. Calicivirus RNA lacking the VPg is not infectious (11). The VPg interacts with proteins of the eIF3

translation initiation complex in infected cells (21). These interactions recapitulate those observed *in vitro* using human norovirus VPg (20). These data suggest a role for VPg in the translation of viral RNA.

Pro. Pro is the viral protease responsible for cleavage of the ORF 1 poly-protein into individual proteins. The catalytic site and substrate specificity of norovirus protienases have been defined (45, 73). Identification of the cleavage map for MNV shows that this process is conserved between human and mouse noroviruses (74). Detailed studies of protease processing have also shown the presence of stable precursors with protease activity (5). Similar protease precursors have been observed in MNV infected cells, although function of these precursors was not demonstrated (74).

Pol. The RNA-dependant RNA polymerase or Pol is responsible for synthesizing viral RNA. As described above, a Pro-Pol fusion precursor has been documented for both *in vitro* synthesis assays, using the ORF1 sequence of the human norovirus strain MD145 (5), and after infection of cells with MNV (74). A similar precursor protein has been observed while studying poliovirus, where the presence of the polymerase alters the specificity of the protease (95).

VP1. ORF two encodes the capsid protein, which aggregates into ninety dimers to form the norovirus capsid (68, 69). The capsid protein is composed of two domains. The S or shell domain forms the inner shell of the viral capsid and encloses the viral genome. This region is highly conserved between norovirus strains. The P or protruding domain extends from the capsid surface and is composed of two sub-domains, the proximal P1 domain and the more distal P2 domain. The P2 domain of the capsid protein is highly variable and is proposed to encode binding epitopes for anti-viral antibodies and a putative virus receptor (77, 78, 91). Studies of Human noroviruses, as well as FCV, have shown that the P2 domain mutates during persistent infection (1, 19, 38, 57, 63, 70). In the case of FCV, these mutations allow viral escape from immune pressure by changing antibody binding epitopes.

Using the MNV model, the binding epitope for a virus neutralizing antibody was mapped to the P2 domain of the viral capsid (46). Neutralizing antibody escape mutants were isolated

and the amino acid substitution responsible was identified as a leucine to phenylalanine change at position three-hundred-eighty-six in the P2 domain of the capsid. The antibody binding epitope overlaps with an amino acid motif that is conserved across all noroviruses (47). Further, when the MNV capsid sequence is aligned with the human norovirus capsid sequence of Snow Mountain virus (SMV), the MNV epitope is adjacent to a SMV epitope responsible for the binding of an antibody that inhibits SMV virus-like particle (VLP) induced haemagglutination (47). This data confirms that the norovirus P2 domain can encode binding domains important for norovirus attachment and growth. As the SMV and MNV binding domains overlap in a region that is conserved in all noroviruses, this may represent a conserved P2 motif that is important for norovirus receptor binding.

VP2. ORF three encodes a small basic protein. VP2 is a minor component of the capsid which plays a role in virion assembly and stability (6, 7, 31). Expression in insect cells of VP2 with VP1 resulted in increased VP1 protein half life as compared to expression of VP1 alone (6). Additionally, expression of VP2 with VP1 increased resistance of VP1 to degradation by the protease pancreatin (6). Because of the positively charged amino acids in this protein, VP2 has been proposed to play a role in packaging of the viral RNA.

Norovirus Persistence

There are two categories of persistence regarding noroviruses. One type is persistence of viral infection at the population level. This is characterized by the continual circulation of norovirus strains within the community. Another type of persistence occurs at the level of infection of individuals (25, 26, 32, 56, 59, 62, 71). This type of persistence is characterized by the continual shedding of virus in stool following the resolution of symptomatic infection. These two categories of persistence are likely inter-related, with persistent shedding at the level of the individual helping to facilitate viral spread and persistence in the community. This model of persistence has been described for the genetically related feline calicivirus (FCV).

A study investigating the mechanism of persistence at the population level found that the capsid protein evolves over time by antigenic drift and likely under immune system selective pressure. The capsid protein from epidemic noroviruses isolated over a twenty year period were sequenced (44). Over time, mutations accumulated in the P2 domain of the capsid and clustered around the carbohydrate binding domain (12, 44). Mutations in this location were shown to alter the structure of the P domain and binding of carbohydrate ligands. The authors propose a model explaining how these findings could contribute to viral persistence at the population level. First, the altered carbohydrate binding could facilitate infection in new groups of people, previously immune by virtue of the particular carbohydrate antigens which they express. Second, antigen drift functions as an immune-evasion strategy allowing repeat infection of previously exposed individuals.

This model of persistence has been described in detail for FCV, and its relation to persistent shedding in individual cats has been studied. Shedding cats can be divided into three categories; those that consistently shed virus at high titer, those that shed intermittently, and those that appear resistant to infection (18). In continuously shedding cats, the immuno-dominant variable regions of the capsid protein mutate to evade the immune response (18, 19, 38, 63, 70). This leads to the introduction of variant FCV strains into the population. The re-infection of animals by consistent shedders further contributes to the persistence of FCV within a cat population (19). FCV can be shed from the oropharynx of cats for as long as two years after resolution of acute infection (89). In persistently shedding cats, virus was found in superficial epithelial cells of the tonsil and in the stratum germinativum of the adjacent fossa mucosa (23). In comparison, human noroviruses are shed in the stool of infected individuals and the site of persistent viral replication has not been identified.

Murine Norovirus

The murine norovirus (MNV) model system is a valuable model for the study of noroviruses and host-norovirus interactions. MNVs are genogroup V noroviruses that, like human

noroviruses have many genotype strains (81). Unlike human noroviruses, MNV grows in cell culture (92) and can readily be studied in its native host. For these reasons, the MNV model has been used to study details of viral replication and *in vivo* pathogenesis, as well as mechanisms involved in the host immune response.

To better understand norovirus pathogenesis, the MNV model has been used to study the host immune response to infection. Studies show that components of both the adaptive and innate immune system contribute to host defense against MNV infection (13, 14, 39, 55). RAG1 deficient mice, which lack T and B cells, fail to clear virus (39). Furthermore, both CD4 and CD8 T cell subsets, as well as antibody produced by B cells contribute to viral clearance (13, 14). The innate immune system is also critical for resistance to MNV. Signal transducer and activator of transcription 1 (STAT1) is downstream of both type I and II interferon receptor signaling, and is critical for host defense against MNV (39, 55). Infection of mice lacking STAT1 with the first identified strain of MNV (MNV-1) results in lethality (39). STAT1 regulates viral growth *in vitro* and *in vivo*, with cells and animals lacking STAT1 growing virus to higher titer (39, 55, 92). These data demonstrate that both adaptive and innate immune mechanisms play an essential role in host defense to MNV infection, and that STAT1 is a key factor in the host response to infection.

In cell culture MNV grows in dendritic cells and macrophages (92). Consistent with the ability to bind carbohydrates observed for human noroviruses, sialic acid linked carbohydrates play a role in the binding of MNV (79). MNV infection of cultured cells is pH independent, as chloroquine treatment does not affect viral entry or growth (64). Further, MNV entry into permissive cells is partially dependent on cholesterol and dynamin II requiring processes (65). These reports have provided valuable insight to the early steps of norovirus entry and infection.

The MNV cell culture system has been used to identify and characterize host and viral factors important for viral replication. Similar to observations *in vivo*, STAT1 and type I interferon restrict MNV growth in cell culture (92). Microarray of infected cells was used to identify genes that are differentially regulated during infection. Survivin is an anti-apoptotic gene and was confirmed to be down regulated during MNV infection. This is potentially a mechanism for the

induction of apoptosis observed following MNV infection in cultured cells (9). The cellular distribution of MNV non-structural proteins following infection has also been investigated (36). All non-structural proteins had a similar staining pattern in infected cells and co-localized with double stranded RNA to peri-Golgi vesicular structures identified as replication complexes (36). Despite these advances in our understanding of noroviruses, significant questions remain. The functions of most viral proteins remain unknown. Even proteins with a known function may have additional unknown roles in the viral life cycle. Another critical task will be to identify and characterize interactions between viral and host proteins.

Another benefit of studying MNV is the availability of a reverse genetics system (88). Using this system it is possible to functionally investigate viral factors important to MNV pathogenesis. Previously, we identified a lysine to glutamic acid mutation at amino acid position 296 of the MNV-1 capsid protein capable of attenuating STAT1 mediated lethality (3). In addition to identifying host factors important in norovirus pathogenesis, the MNV model is a valuable tool for identifying and characterizing viral factors critical to pathogenesis. The goal of this thesis work is to identify viral determinants that play a role in MNV infection and pathogenesis.

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CHAPTER 2

Cloning and Characterization of Two MNV Strains: CW3 and CR6

Introduction

Human noroviruses are the number one cause of epidemic non-bacterial gastroenteritis worldwide (1). Despite their importance as human pathogens, progress in our understanding of these viruses has been limited. The lack of a small animal model and the inability to grow virus in cell culture has made the study of human noroviruses difficult. Enteric murine noroviruses are closely genetically related to human noroviruses (10). They infect mice, can be grown in cell culture, and a reverse genetics system is available (9, 10, 13, 14). These tools make MNV amenable to descriptive and mechanistic studies which may provide valuable insight into the pathogenesis of noroviruses.

The MNV model system has been used to study factors important to host defense against MNV, the immune response to MNV infection, viral attachment, entry and replication. Mice lacking STAT1 succumb to lethality following MNV infection (10). The adaptive immune system also contributes to host defense against MNV infection (3, 4). RAG1 deficient mice, which lack T and B cells, fail to clear virus (10). CD4 and CD8 T cell subsets, as well as antibody produced by B cells contribute to viral clearance (3, 4).

Despite these advances in our understanding of norovirus biology, our understanding of the functions of norovirus proteins is incomplete. One goal of the work described here is the development of reagents that can be used for the study of individual MNV proteins in the context of viral infection. Here, we clone two MNV strains and show that this system of viral production is superior to viral propagation by serial passage in limiting heterogeneity in the viral quasispecies. Further, we compare the virus clones with their parental strain in the standard virus assays of *in vitro* growth and particle to plaque forming unit (PFU) ratio. Finally, we construct a panel of single gene viral chimeras that will be used to identify the viral determinants of lethality and persistent infection, to be discussed in more detail later. This system provides an opportunity to gain insight into the functions of norovirus proteins during infection.

Results

In order to confirm that production of virus using the reverse genetics protocol is a suitable surrogate for the parental MNV strains, we generated plasmid derived virus stocks (pCW3 and pCR6) and compared them to their parent virus in two standard virus measures: *in vitro* growth and particle to PFU ratio.

Parental and cloned MNV grow similarly *in vitro*. To determine whether parental MNV strains and their cloned derivatives grow with similar kinetics *in vitro*, growth curves at multiplicity of infection (MOI) 5 and 0.05 were done in bone marrow derived macrophages (BMMs) and RAW 264.7 (RAW) cells. The serially passaged CW3 (14) parent strain was compared to clone derived virus (pCW3) (Figure 2-1). There was no statistical difference in growth between the two viruses at MOI 5 and 0.05 in RAW cells. In BMMs the mean titer of CW3 was higher than that of pCW3 at later time points for both MOIs. This difference was not statistically significant. Serially passaged CR6 (11) was compared to its cloned derivative (pCR6) (Figure 2-1). In RAW cells the growth of the two viruses was indistinguishable at MOI 5 and 0.05. In BMMs, CR6 and pCR6 grew similarly at both MOIs. This data confirms that the clonal MNV derivatives behave similar to their parent virus *in vitro* in primary and transformed macrophages. While there was no difference in the growth of plasmid derived viruses compared to their parent strain, we did observe a statistically significant difference between the growth of CW3 and CR6 at early times after infection of RAW 264.7 cells. This observation will be discussed in chapter 5.

Parental and cloned MNV strains have similar particle to PFU ratio. Another important characteristic to evaluate when comparing the cloned and parental viral strains is the particle to PFU ratio. Two assays were used for this measure (Figure 2-2). First, an equal number of PFU of the parental strain or recombinant viral supernatant were probed by western blot using polyclonal antisera generated against the MNV capsid protein. A single band migrating at the expected size of approximately 58kD was observed. For each comparison between the parent and clone viral strain, bands of similar intensity were observed, indicating that cloned CW3 and CR6 produce similar amounts of capsid antigen per PFU as their parent virus (Figure 2-2).

Second, we used quantitative PCR to measure the number of genome copies per PFU for each parent virus and its clone. There was no statistical difference in the genome to PFU ratio between the viral pairs calculated at two dilutions of virus (Figure 2-2B). Together, these data suggest that there is no difference in particle to PFU ratio between CW3 and pCW3 or CR6 and pCR6. This analysis was repeated four times using at least two independently generated plasmid derived virus stocks.

Analysis of quasispecies in plasmid derived and parental virus stocks. As viral RNA polymerases have poor fidelity in comparison to DNA polymerases, repeated replication cycles result in the introduction of mutations in the viral genome and the generation of a viral quasispecies (5). In order to generate the MNV stocks used for study, RAW 264.7 cells are infected at MOI 0.05 and allowed to grow virus for 36-48 hours. This results in multiple rounds of viral replication, where mutations can be introduced into the viral genome. Each time a new virus stock is generated, the mutations introduced from the previous rounds of growth are still present, and there is another opportunity for mutations to be introduced. This method of repeatedly expanding a virus stocks will subsequently be referred to as serial passage. In contrast, when virus is produced using the reverse genetics system, all viruses come from the defined sequence of the plasmid. While two rounds of passage in RAW 264.7 cells are required to produce a large volume of virus, the total number of passages, and thus opportunities for the introduction of new mutations is limited. As discussed in the materials and methods section, after two tissue culture passages, the consensus sequence of the viral stock is still identical to the plasmid sequence from which it is derived. Virus produced using the reverse genetics system is hereafter referred to as plasmid derived virus.

Previous work has shown that as few as three tissue culture passages can change the consensus sequence of a virus stock resulting in a new virus phenotype (2, 14). Thus, serial passage of virus stocks is potentially a complicating factor for studies of viral phenotype. Production of virus using reverse genetics is advantageous in that all viruses originate from a

single sequence and the number of tissue culture passages can be limited. In order to analyze the quasispecies diversity in serially passaged and plasmid derived virus stocks, virus from these two sources was analyzed using the procedures described in the mutation analysis section of materials and methods. We examined a segment of the MNV genome encoding the P2 region of the capsid protein as this is a highly variable region of the norovirus genome (6, 11, 12). Figure 2-3 shows the frequency of mutations observed in a 414 base pair segment of the MNV genome in a serially passaged viral stock of CW3 or a twice passaged viral stock of pCW3 generated according to the protocol described in materials and methods. The CW3 encoding plasmid was used as a control for the fidelity of PCR amplification, cloning, and sequencing. In this control group 85% of sequences were identical to the plasmid sequence from which they originated. 4% had one mutation and <3% of the sequences had three, four, or five mutations. 8% of the sequences had greater than five mutations. For the clone derived virus, ~60% of the sequences were identical to the original plasmid sequence. 10% of sequences had a single mutation, 5% had two mutations, and <6% had three, four, or five mutations. 17% of the sequences had more than 5 mutations. In comparison, only 3% of sequences from the serially passaged virus were identical to the known consensus sequence. 19% of the sequences had one mutation, 33% had two, 4% had three, 5% had four, and 2% had five mutations. 34% of the sequences from this group had more than five mutations. This data shows that viral stocks from serially passaged MNV have a more diverse quasispecies than virus produced using the reverse genetics protocol. Use of virus generated through reverse genetics will reduce the potential for MNV phenotypes to be confounded by the heterogeneity of the viral quasispecies. Stocks derived from pCW3 or pCR6 were used for all subsequent experiments unless otherwise noted.

MNV chimeras as a tool for the identification of viral determinants of phenotype.

Our hypothesis is that individual viral genes can be identified as determinants of phenotypic differences between CW3 and CR6 such as the previously reported difference in establishment of persistent infection (11), or the difference in ability to cause lethality in STAT1-/- mice (described

in chapter 3). To test this hypothesis, a panel of virus chimeras was generated. Individual genes from one viral strain were substituted to replace the same gene in the other MNV strain. Table 2-2 lists all of the single gene chimeras that we were able to construct and recover virus from using the reverse genetics protocol. Using these viruses we can determine whether individual viral genes are a necessary and sufficient determinant of the phenotypes described following infection.

Discussion

The ability to generate recombinant MNV strains provides an opportunity to study norovirus pathogenesis in a manipulable system. Here we have cloned and characterized two strains of MNV with distinct differences in biological behavior. We report that the clonal MNV strains behave similar to their parental virus in standard measures of viral growth and particle to PFU ratio. Further, we report the construction of a panel of virus chimeras, where individual MNV genes have been exchanged between CW3 and CR6. Using these virus chimeras, we propose to identify viral genes and protein domains that contribute to aspects of MNV pathogenesis as described in chapters three and four.

One important advantage we intend to gain by producing virus through reverse genetics is the limitation of the emergence of viral quasispecies in our viral stocks. We have shown that MNV stocks produced using the protocol described here result in a viral stock expressing fewer mutations from a consensus sequence than serially passaged virus. In our control population of sequences derived from plasmid, 15% of the sequences had one or more mutations. We believe this represents the baseline frequency of mutations and incorrect base calls as a result of the PCR and sequencing steps in the analysis. Roughly 60% of the sequences in the clonal virus stock had no mutations from the input sequence. As this is different from our baseline observation, it is clear that some amount of quasispecies variation does develop in virus stocks derived from plasmids using this protocol. However, we believe this system is preferable to the alternative of serial passaged virus, as the quasispecies development can be limited by limiting the number of passages. Further, the quasispecies variation and biological behavior of

independent plasmid derived virus stocks can easily be compared to one another. This is important because as discussed previously, very few tissue culture passages can alter a virus phenotype (14). If independent plasmid derived virus stocks were to display inconsistent biological behavior, the limited quasispecies variation would make sequencing and identification of the responsible mutation more practical. In comparison to the sequence variation observed in plasmid derived stocks, only 3% of sequences from serially passaged virus were free of mutations. The numbers quoted here are likely an underestimate of the frequency of mutations in each sample as only a 414 base pair segment of genome was analyzed. This segment of genome was chosen because it encodes the P2 region of the capsid protein, a highly variable region of noroviruses (6, 11, 12). We reasoned that analysis of this region would allow detection of the maximum amount of quasispecies variation. Nonetheless, this data makes it clear that significant viral quasispecies exist in MNV stocks and there is the potential for them to confound analysis of viral phenotypes. Using the serial passage method for producing virus, a new passage must be generated each time a new viral stock is needed. Producing virus in this manner will maintain and likely expand the viral quasispecies over time. Production of virus with the reverse genetics system is preferable for generating consistent and defined virus stocks, as tissue culture passage can be limited and virus stocks can more easily be compared to one another for consistency in sequence and biological behavior.

Like human noroviruses, murine noroviruses have many strains. Previous reports that identified and characterized multiple MNV strains, report that despite high sequence similarity, MNV strains can have remarkably divergent phenotypes (7, 8, 11). In order to study these phenotypes, we cloned CW3 and CR6 into a reverse genetics system whereby virus is produced from a DNA plasmid encoding the viral genome. We then generated a panel of single gene viral chimeras to use in the identification of viral determinants for each phenotype. Interestingly, we were unable to recover virus from three of the chimera constructs: CW3 Pro ^{CR6}, CR6 NTerm ^{CW3}, and CR6 Pol ^{CW3}. This may indicate that the protease of CR6 is not compatible with the other genes of CW3, and that the NTerm and polymerase of CW3 are incompatible with the genes of

CR6. An alternate explanation is that these chimeras do produce virus, but do so with such low efficiency that it cannot be detected with the methods used here. The reverse genetics protocol produces only a small amount of virus following transfection of 293T cells. An ideal transfection produces roughly five-thousand PFU of virus (unpublished observation). If 100% of cells produce virus, this translates to an average production of 0.01 PFU per cell. Thus, even a small decrease in efficiency caused by viral proteins that do not work well together could result in failure to recover virus using this system. Nonetheless, the panel of chimeras that do produce virus will be sufficient for phenotype mapping studies. We have at least one chimera for each viral gene, thus we can determine whether a gene is necessary and or sufficient for each phenotype. It is possible that one or all of these phenotypes is determined by multiple viral genes. In this case it will not be possible to identify a single gene chimera that results in a gain of function. However, we would expect to see multiple chimeras that result in loss of function. If this does occur, we will attempt to characterize the group of genes responsible for the phenotype by making viruses chimeric for multiple genes.

Here, we have described the development of a system to study aspects of MNV biology. Due to the inability to grow human noroviruses *in vitro* and the lack of a tractable model for the study of host infection, our understanding of this genus of viruses has expanded very slowly. With the development of the MNV model, we now have tools that allow us to study norovirus biology in its native host and at the level of cellular replication. Previous publications have described a difference in the ability to establish persistent infection for distinct MNV strains (8, 11). Here, we have identified additional differences in phenotype between CW3 and CR6, which will be detailed in subsequent chapters. We have developed reagents that should facilitate the identification of viral determinants for each phenotype. This has the potential to greatly expand our knowledge of norovirus pathogenesis and gene function.

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Table 2-1. MNV Sequencing Primers

Primer Name	CW3 Primer Sequences	CR6 Primer Sequences	Primer Direction
F149	GACCCCTCCTGAGCAGGAAGC	GACCCCTCCTGAGCAGGAAGC	Sense
F1197	ATGGCATGGATCTGGCC	ATGGCATGGATCTGGCC	Sense
F2156	GCCTTCAAAGCCATGGCGGC	TGCCTTCAAGGCTATGGCAG	Sense
F3726	CAGGTCATGCGAGATCAGC	CAGGTCATGCGAGATCAGC	Sense
F4631	GAGGCGGTACGGTCTCCTC	GAAGCGGTATGGTCTTCTCC	Sense
F5618	CACTCCGCACAAACAGCCC	CACTCCGCACAAACAGCCC	Sense
F6698	TTGGAGCGATTGGAGGTGGCC	TTGGAGCGATTGGAGGTGGCC	Sense
F6928	AAGCCCAGGCGCAGGCCC	AAGCCCAGGCGCAGGCCC	Sense
R604	CAGTCATGCCCACGCACTTCC	CAGTCATGCCCACGCACTTCC	Antisense
R1082	CCCGATCCCGCCCAACAGG	GCCAATCCCACCAAGAAGA	Antisense
R1652	TCTGGCTCCCTTGTAAGCATC	TCTGGCGCCCTTGTAGGCGTC	Antisense
R2563	GGTCCGTCACGGTAGGTGTA	GGTCCGTCACGGTAGGTGTA	Antisense
R3863	GAACGTCCAGGGCTTCTGTGGC	GAACGTCCAGGGCTTCTGTGGC	Antisense
R4929	CCCGGGAAGCCACAGTCC	CCCGGGAAGCCACAGTCC	Antisense
R5791	GCCAACGTGCGTGCGTGCAC	GCCAACGTGCGTGCGTGCAC	Antisense
R6363	CATGTAGGTCCGGAACCTCA	CATGTAGGTCCGGAACCTCA	Antisense

Table 2-2. Single Gene Virus Chimeras

MNV Strain	Protein Substituted	Chimera Name
CW3	NTerm	CW3 NTerm^{CR6}
CW3	NTPase	CW3 NTPase^{CR6}
CW3	p18	CW3 p18^{CR6}
CW3	VPg	CW3 VPg^{CR6}
CW3	Pol	CW3 Pol^{CR6}
CW3	VP1	CW3 VP1^{CR6}
CW3	VP2	CW3 VP2^{CR6}
CR6	NTPase	CR6 NTPase^{CW3}
CR6	p18	CR6 p18^{CW3}
CR6	VPg	CR6 VPg^{CW3}
CR6	Pro	CR6 Pro^{CW3}
CR6	VP1	CR6 VP1^{CW3}
CR6	VP2	CR6 VP2^{CW3}

Fig.2-1 Cloned and parental viruses grow similarly *in vitro*. Growth of CW3, CR6, and their clone derivatives was evaluated in (A-D) RAW cells and (E-H) bone marrow derived macrophages (BMM). Cells were inoculated at MOI 0.05 or 5. Error bars represent standard error of the mean from three independent experiments.

Figure 2-1

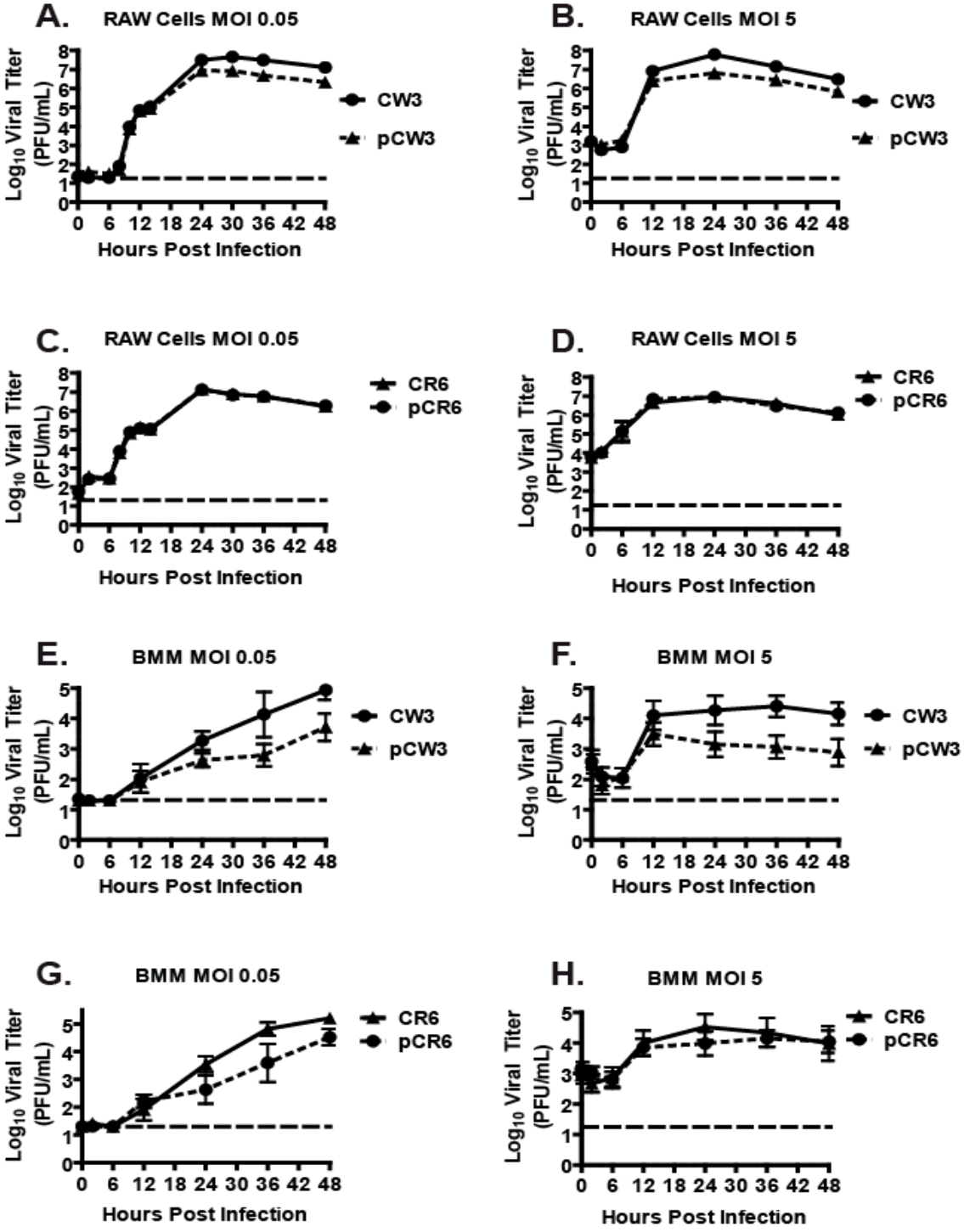

A.
RAW Cells MOI 0.05
B.
RAW Cells MOI 5
C.
RAW Cells MOI 0.05
D.
RAW Cells MOI 5
E.
BMM MOI 0.05
F.
BMM MOI 5
G.
BMM MOI 0.05
H.
BMM MOI 5
Log10 Viral Titer (PFU/mL)
Hours Post Infection
0 6 12 18 24 30 36 42 48
CW3
pCW3
CR6
pCR6

Fig.2-2 CW3 and CR6 have similar particle to PFU ratio. (A) Western blot of viral supernatant probed using polyclonal anti-MNV serum. A representative blot from three experiments is shown. (B) Genome copies for the indicated number of PFUs were quantified by Taqman. Data is from two independently generated stocks and represents three experimental replicates.

Figure 2-2

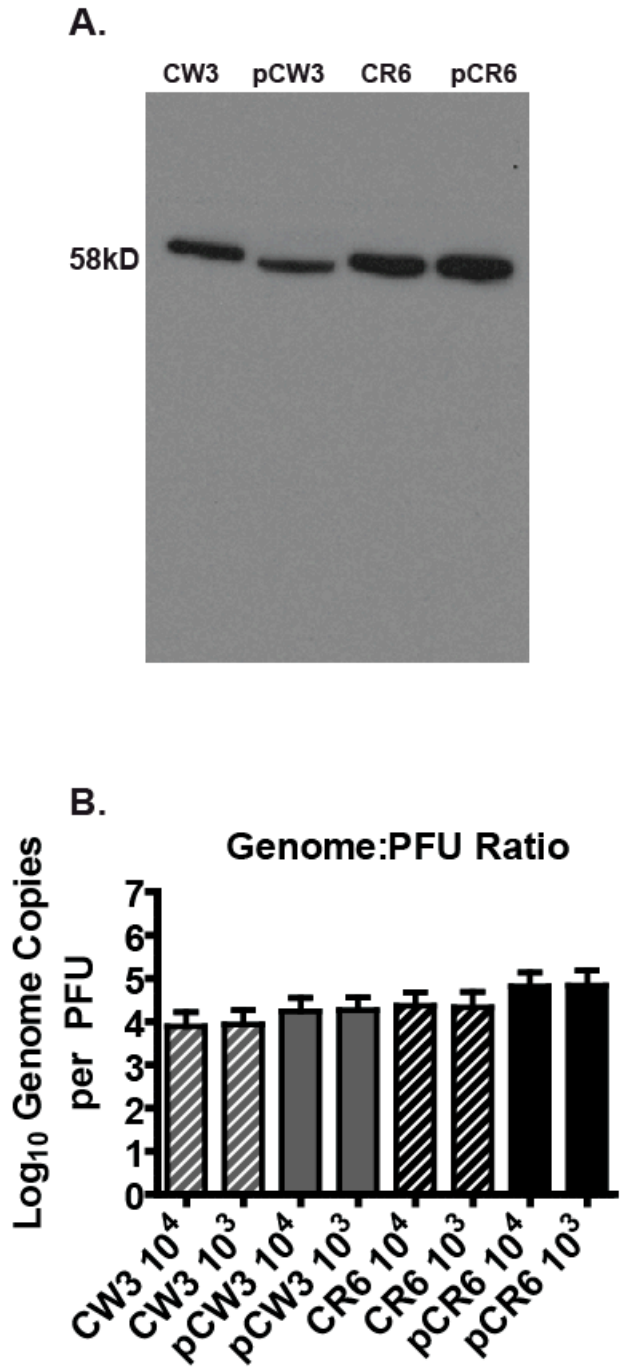
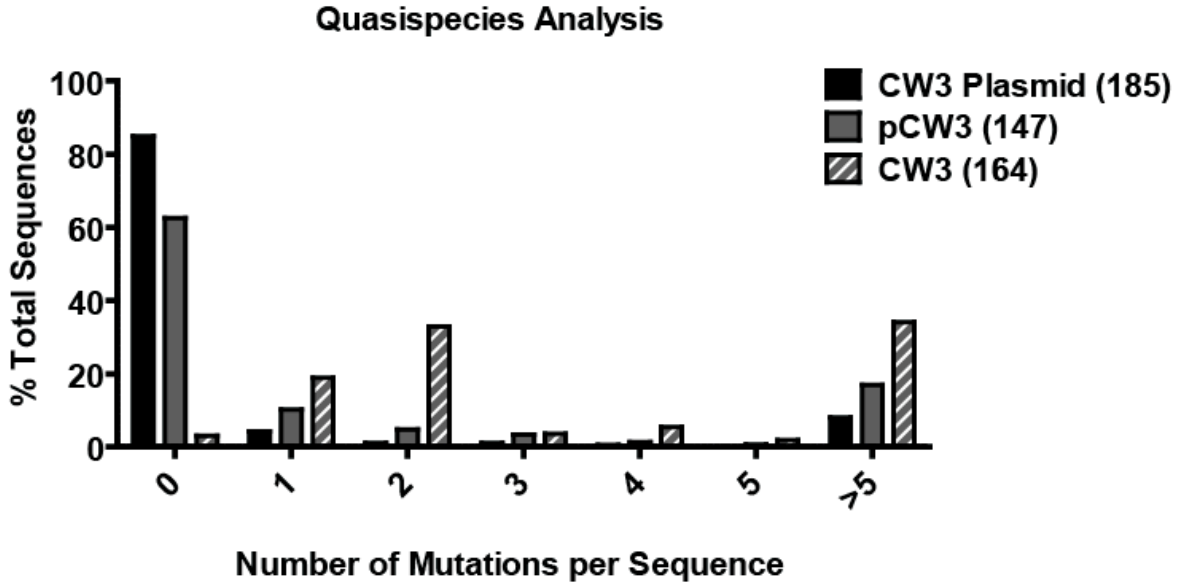

A.
CW3
pCW3
CR6
pCR6
58kD
B.
Genome:PFU Ratio
Log10 Genome Copies per PFU
0
1
2
3
4
5
6
7
CW3 10^4
CW3 10^3
pCW3 10^4
pCW3 10^3
CR6 10^4
CR6 10^3
pCR6 10^4
pCR6 10^3

Fig.2-3 Clonal virus stock has reduced quasispecies. RNA from serially passaged CW3 or clone derived CW3 (pCW3) viral supernatant was reverse transcribed and amplified by PCR. The CW3 encoding plasmid was PCR amplified as a control. Data is presented as percentage of the total number of sequence reads analyzed with the indicated number of mutations per read. Total number of sequences analyzed for each group is shown in parentheses.

Figure 2-3


Quasispecies Analysis
CW3 Plasmid (185)
pCW3 (147)
CW3 (164)
% Total Sequences
100
80
60
40
20
0
0
1
2
3
4
5
>5
Number of Mutations per Sequence

CHAPTER 3

Protruding Domain of the MNV Capsid Protein is a Determinant of Lethal Infection in STAT1-/- Mice

Introduction

Norovirus infection is the primary cause of epidemic non-bacterial gastroenteritis worldwide (6-8). While typically a self limiting infection, severe complication can arise in elderly or immuno-compromised individuals (2, 10, 11, 14). Noroviruses are divided into five genogroups, with those infecting humans falling into genogroups I, II, and IV. Due to the inability to grow human noroviruses in cell culture, and the lack of a small animal model, it has not been possible to study the details of viral replication and *in vivo* pathogenesis. Murine noroviruses (MNVs) are genogroup V noroviruses that infect mice and can be grown in cell culture. Thus, the identification of these viruses affords researchers the ability to complete in depth studies of norovirus infection in its native host.

To better understand norovirus pathogenesis, the MNV model is used to study the host immune response to infection. Studies show that components of both the adaptive and innate immune system contribute to host defense against MNV infection (3, 4, 9, 13). RAG1 deficient mice, which lack T and B cells, fail to clear virus (9). Furthermore, both CD4 and CD8 T cell subsets, as well as antibody produced by B cells contribute to viral clearance (3, 4). The innate immune system is also critical for resistance to MNV. Signal transducer and activator of transcription 1 (STAT1) is downstream of both type I and II interferon receptor signaling, and is critical for host defense against MNV (9, 13). Infection of mice lacking STAT1 with the first identified strain of MNV (MNV-1) results in lethality (9). STAT1 regulates viral growth *in vitro* and *in vivo*, with cells and animals lacking STAT1 growing virus to higher titer (9, 13, 18). These data demonstrate that both adaptive and innate immune mechanisms play an essential role in host defense to MNV, and that STAT1 is a key factor in the host response to infection.

In addition to identifying host factors important in norovirus pathogenesis, the MNV model is a valuable tool for identifying and characterizing viral factors critical to pathogenesis. Previously, we identified a lysine to glutamic acid mutation at amino acid position 296 of the MNV-1 capsid protein capable of attenuating STAT1 mediated lethality (1). This mutation arose

after serially passaging virus in tissue culture. Many additional strains of MNV have been identified and sequenced. As some of these strains have the attenuating glutamic acid mutation, we sought to determine whether this mutation contributes to attenuated virulence in a naturally occurring strain. Further, we sought to better define the pathogenesis following lethal norovirus infection and the role of viral determinants in this process.

Here we report the cloning and characterization of two previously isolated strains of MNV. Our prototype strain of MNV-1 hereafter referred to as CW3, and a recently isolated strain, CR6. Our current study reports the contribution of the MNV capsid protruding (P) domain to viral pathogenesis. We found that the capsid P domain is a necessary and sufficient determinant of MNV induced lethality, spleen and liver pathology, and viral growth in STAT1 deficient mice.

Results

Two strains of MNV, CW3 and CR6, differ in STAT1 mediated virulence. With the identification of new naturally occurring strains of MNV (16), we sought to determine whether any display a virulence phenotype different from that reported for CW3. STAT1 knockout mice on B6 background were infected with $3x10^4$ PFU of CW3 or CR6. As expected, infection with CW3 was lethal, with all mice dying by 8 days post infection. Lethality following CR6 infection was greatly attenuated with all mice surviving until the experimental endpoint of 30 days post infection (Figure 3-1).

Cloned MNVs recapitulate lethality phenotype of parental strain. In order to manipulate the viral genomic sequence and identify the determinant(s) responsible for lethality, each strain was cloned into the previously published reverse genetics system for MNV using the strategy outlined in materials and methods (17). Each cloned virus phenocopied the lethality of its parental virus. Infection with plasmid derived CW3 (pCW3) resulted in an average time to death of ~5 days, while pCR6 was attenuated with only one death over the 30 day period (Figure 3-1). As there was no statistical difference between the parental viral strains and their plasmid derived counterparts, all subsequent studies were done using cloned viruses.

We next sought to determine the magnitude of the difference in virulence between the two viruses. CW3 was extremely virulent, with 30 PFU killing all mice by 11 days post infection (Figure 3-1C). At this dose the average time to death was 7.5 days, slightly delayed as compared to infection with 1000 fold more virus. In contrast, 75% of mice infected with $3x10^7$ PFU of CR6 survived to the experimental endpoint. This data demonstrates a greater than 100,000 fold difference in virulence between these two strains of MNV.

As we have previously identified a determinant of MNV lethality in STAT1 deficient mice, we tested whether it was responsible for the difference observed here. Mice infected with CW3 containing the attenuating lysine to glutamic acid mutation at amino acid position 296 of the capsid protein exhibit a slight reduction in lethality, with 2 of 12 mice surviving to the 30 day endpoint. This lack of complete attenuation is contrary to our previous report. However, in the first report the STAT1 deficient mice used were on the 129S6/SvEv background. In the experiments reported here, the mice are on a C57BL/6. Additionally, the STAT1 gene in the mice used in the previous experiments was targeted by replacing the first three exons with a neo cassette, resulting in an N-terminal truncation of the STAT1 protein (12). In the mice used here, the STAT1 gene was targeted by deleting exons encoding amino acids 221-365 of the STAT1 gene, which resulted in complete loss of STAT1 protein expression (5). We believe the modest phenotype of the capsid 296 mutation reported here is most likely due to these differences. We attempted to introduce the corresponding E296K mutation into CR6, but were unable to recover virus after transfection of this DNA construct into 293T cells. To ensure that this lack of viral production was not due to a plasmid defect introduced during mutagenesis, we removed a portion of the plasmid containing the desired mutation using restriction enzymes, and ligated it into the parental CR6 plasmid. As we were still unable to recover virus, we believe this represents a true incompatibility of a lysine residue at this position of the CR6 capsid protein. Together, these data show that the lysine to glutamic acid mutation previously identified is not the lethality determinant in our current system.

***In* vitro viral growth is not a determinant of lethality.** One potential explanation for the difference in lethality between CW3 and CR6 is that they differ in their ability to replicate in cells. To test this hypothesis, growth curves at MOI 5 and MOI 0.05 were completed in RAW cells (Figure 3-2). Recombinant CW3 and CR6 grew similarly at MOI 5 and 0.05. While there was no statistical difference in overall growth, a reproducible difference in viral titer during the early phase of the growth curve was observed (discussed in Chapter 5). This data shows that an *in vitro* growth defect as measured in RAW cells does not account for the difference in lethality between these two strains. However, a contribution of the viral titer difference observed during the early phase of growth to the lethality difference remains a formal possibility.

Another potential explanation for the difference in lethality is that there is a difference in the particle to PFU ratio of the viruses. We tested this possibility using two assays (Figure 3-2). First, serial dilutions of each recombinant viral supernatant were probed by western blot using polyclonal antisera generated against the MNV capsid protein. A single band migrating at the expected size of approximately 58kD was observed. At each dilution the CW3 and CR6 bands were of similar intensity, indicating that these viruses produce similar amounts of capsid antigen per PFU (Figure 3-2C). Second, we measured the number of genome copies per PFU for the two viruses using quantitative PCR. There was no statistical difference in the genome to PFU ratio calculated at each dilution of virus or between the two strains (Figure 3-2D). Together, these data suggest that there is no difference in particle to PFU ratio that could explain the lethality difference between these viral strains.

MNV capsid P domain is the determinant of lethality in STAT1 deficient mice. To identify the viral element responsible for lethal infection, STAT1-/- mice were infected with chimeric viruses constructed as described in Chapter 2. All of the CW3 chimeric viruses, encoding a single CR6 gene substitution, were tested (Figure 3-3). The capsid gene chimera was the only virus attenuated in lethality, indicating that the VP1 of CW3 is necessary for expression of the lethality phenotype. On the basis of this data, we generated additional virus chimeras, exchanging domains of the capsid protein between CW3 and CR6, and tested their

lethality in order to more precisely identify the domain of the capsid responsible for the lethality phenotype.

A model of the capsid protein dimer shows that the capsid protein is composed of a P domain and a shell domain (Figure 3-4A). The P domain can be further subdivided into a P1 domain and a P2 domain, which is the most distal and exposed surface of the capsid protein. Figure 3-4B shows that the MNV capsid is both necessary and sufficient to confer the lethality phenotype in the context of viral infection. Substituting the VP1 of the lethal CW3 strain into the non-lethal CR6 strain resulted in a lethal virus. Conversely, replacing the CW3 capsid protein with that of CR6 produced a non-lethal virus. In order to identify the domain of that capsid protein that is responsible for the lethality phenotype, virus chimeras for the shell or P domain of the capsid were constructed and evaluated for their ability to induce lethality in STAT1-/- mice. Substituting the shell domain of the capsid protein (amino acids 49-218) had no effect on the lethality of either viral strain (Figure 3-4E). Further, exchanging the P domain of the capsid, defined as amino acids 229-537, was enough to change the phenotype of the virus (Figure 4C). Interestingly, swapping the P domain also changed the time until death of infected mice as compared to infection with CW3 ($p<0.001$). All mice infected with CW3 were dead by 5 days post infection. In mice infected with CR6 P^{CW3} 40% died by 5 days post infection with the remainder dying between days 5 and 14. In an effort to further characterize the amino acids contributing to the lethality phenotype, virus chimeras of the P1 (amino acids 229-277 and 416-537) and P2 (amino acids 278-415) sub-domains were generated. Introduction of the CR6 P2 domain into the CW3 strain resulted in an attenuated virus, indicating that the lethality determinant is likely in the P2 domain of CW3. Additionally, the P1 domain of CW3 was expressed in the CR6 background. This virus was also non-lethal, further supporting the conclusion that the lethality determinant resides in the P2 domain of the capsid protein. For further confirmation of this, we attempted to generate chimeras expressing the P2 domain of CW3 with the P1 domain of CR6. We were unable to recover CW3 and CR6 chimeras expressing this P domain construct. This is further evidence of an incompatibility between elements of the capsid from distinct strains that prevents

production of live virus. Taken with the inability to introduce the previously identified virulence mutation into CR6, this suggests an interaction between amino acid position 296 of the capsid protein and an as yet identified site in the P1 domain.

MNV capsid P domain is a determinant of *in vivo* growth and morbidity in STAT1 deficient mice. To further investigate the differences between lethal CW3 infection and non-lethal CR6 infection, spleen, liver, lung, brain, distal ileum, and mesenteric lymph node (MLN) were collected three days post infection and viral titer quantified by plaque assay. All pairwise comparisons were statistically analyzed. Infection with CW3 produced significantly higher viral titer, as compared to CR6, in all organs assayed with the exception of distal ileum (Figure 3-5). As with lethality, the P domain of the capsid protein was a necessary and sufficient determinant of the *in vivo* growth phenotype. Expression of the CR6 P domain in the CW3 background resulted in statistically significant reduction in viral titer for every tissue examined. Conversely, expression of the CW3 P domain on the CR6 background resulted in greater titer in the spleen, liver, lung, and MLN. Finally, titer from CW3 P^{CR6} infected mice was statistically decreased compared to CR6 in the distal ileum and MLN. No significant change was observed in the spleen, liver, lung, and brain. Generally, infection with a lethal virus was associated with higher viral titers in the organs assayed as compared to infection with a non-lethal strain.

To characterize the pathology associated with CW3 or CR6 infection, hematoxylin and eosin stained sections of spleen and liver from day 3 infected STAT1-/- mice were evaluated. Spleens of CW3 infected mice exhibited marked cell death and loss of architecture in the red and white pulp (Figure 3-6B). High magnification images of the white pulp show dying cells with condensed nuclei and ubiquitous cellular debris. In contrast, the only consistent finding in the spleen of CR6 infected mice was a mild expansion of the marginal zone (Figure 3-6D). To determine whether the P domain of the capsid protein is the determinant of the pathology observed in the spleen, mice were infected with the P domain chimeric viruses. Mice infected with CR6 P^{CW3} displayed a range in severity of pathology which we believe is reflective of the extended time to death after infection with this virus (Figure 3-3C). Roughly 50% of mice had a

splenic lesion as severe as that observed in CW3 infected mice. In the remainder of mice we observed cell death radiating from the T cell zone of the white pulp (Figure 3-6E). We also observed expansion of the marginal zone like that described for CR6 infected mice. We believe this represents the early stage of the spleen lesion observed in CW3 infected mice. Spleens of mice infected with CW3 P^{CR6} displayed mild marginal zone expansion but lacked the cell death and loss of splenic architecture characteristic of infection with the lethal strains (Figure 3-6C).

In the liver of CW3 infected mice we observed focal areas of cell death and inflammation (Figure 3-7A). In order to measure the extent of liver damage and determine whether the P domain was the determinant of this phenotype, we counted the number of these foci per square millimeter of tissue in livers from mice infected with our MNV strains and their P domain chimeras (Figure 3-7B). CW3 infected mice developed foci at a rate of approximately 1 per 2 mm^2 of liver tissue. None of the CR6 infected mice developed liver foci. The capsid P domain was the determinant of liver pathology. STAT1-/- mice infected with CR6 P^{CW3} developed liver lesions at a frequency similar to that observed in CW3 infected mice. Mice infected with the virus CW3 P^{CR6} failed to exhibit this liver pathology.

Discussion

The pathogenesis of and host response to Norovirus infection are still not completely understood. The discovery of MNV, along with its capacity to grow in a small animal model, provides an opportunity to investigate these questions in greater detail. Previous work details the role of host genes such as RAG1 and STAT1 in the outcome of MNV infection (9, 13). In these studies STAT1 was identified as a determinant of pathology, mortality, and viral spread following MNV infection. Here, we show that these outcomes can also be dependent on viral factors. We identified the P domain of the capsid protein as being a necessary and sufficient determinant of lethal MNV infection. We showed that it is also the determinant of high titer replication and spread from the primary site of infection *in vivo*. Finally, we have shown that the P domain is the viral determinant of pathology in the spleen and liver following MNV infection.

While expression of the P domain of a lethal virus in an attenuated strain produced a lethal virus, the average time until death was increased as compared to the parent strain. This may represent an additional contribution from the remainder of the capsid protein, as virus chimeras of the full capsid protein completely reconstituted the phenotype of the parent strain. Our data show that the P2 domain of the CW3 capsid is necessary for MNV induced lethality. We attempted to show that this P2 domain was also sufficient for the lethality phenotype by expressing a P domain composed of the CW3 P2 and CR6 P1 domains in both the CW3 and CR6 background. We were unable to recover virus from either of these constructs. This indicates that domains from distinct viral strains are not always compatible. This is a second piece of data suggestive of an interaction between domains of the capsid protein. We showed that the previously identified lethality attenuating mutation E296K may account for a portion of the lethality difference between CW3 and CR6, but clearly does not explain the entire phenotype. We were unable to generate a CR6 strain with the K296E, pro-lethality, mutation. This is a third piece of evidence for a critical interaction between viral elements. It is possible that this incompatibility of a glutamic acid residue at position 296 of the CR6 capsid protein accounts for our inability to generate virus chimeras expressing the P2 domain of CW3 with the P1 domain of CR6. As amino acid 296 lies in the P2 domain, this would be consistent with a hypothesis for a direct or indirect interaction between the P1 and P2 capsid domains that is necessary for production of live virus.

Our data on viral titer in various tissues following infection demonstrates a role for the MNV P domain in viral spread and growth *in vivo*. Three days after oral inoculation with virus, CW3 and CR6 titers were statistically equivalent in a primary site of infection, the distal ileum. At this same time point, there was a three log difference in the amount of virus that spread to the MLN, which is the draining lymph node for this site. In all other tissues examined, CW3 infection produced a statistically higher viral titer than CR6 infection. This is indicative of a difference in an intrinsic capacity to either spread systemically or grow to high titer in the tissues examined. The capsid P domain is a determinant of this phenotype, as introduction of the CW3 P domain into the

CR6 genome was able to increase viral titer in the spleen, liver, lung, and MLN while the CR6 P domain decreased viral titer in these same organs. Further examination of this data reveals that the P domain is clearly not the only determinant of *in vivo* growth and spread. In the spleen and liver we observed significantly higher titer of CR6 P^{CW3} as compared to CW3. Were the P domain the sole determinant of these phenotypes, we would expect these titers to be statistically equivalent. Also, in the distal ileum and MLN, animals infected with CR6 had higher titer than those infected with CW3 P^{CR6}. This again indicates the presence of additional determinants of viral growth and spread in these tissues. The alternate determinant(s) may interact directly or indirectly with the P domain and could be an intragenic interaction between domains of the capsid protein, or an intergenic interaction between the P domain and another viral gene.

We believe that the cooperative interactions between viral elements observed here is an important consideration for future studies of norovirus genetics. The relatively poor fidelity of viral RNA polymerases results in significant diversity between virus strains. This is true for murine as well as human noroviruses. Previously, in studying two closely related MNV strains which differ at only 2 amino acid positions, we identified a single amino acid determinant of lethality in STAT1 deficient mice (1, 18). Here we show that this site plays a minor role in determining the virulence of a more distantly related strain of MNV. Our data demonstrate that the isolated study of a viral determinant can be predictive for the behavior of a given viral strain but irrelevant to the biological behavior of a more distantly related strain.

The mechanism by which the MNV capsid protein determines lethality following infection of STAT1-/- mice is incompletely understood. The data presented here characterizing viral titer in mouse tissues following infection with CW3, CR6, and their P domain virus chimeras is suggestive of a role for viral spread and high titer virus growth *in vivo* in producing the lethality phenotype. These differences could result from a difference in the cell populations targeted by the viruses *in vivo* or the efficiency with which each strain spreads from cell to cell. This hypothesis is consistent with the identification of the capsid protein, and specifically the P domain, as the determinant of the lethality phenotype since the P domain plays a role in MNV binding and

entry to permissive cells (15). While the data from growth curves in RAW 264.7 cells shows that there is no difference between CW3 and CR6 in growth or cell to cell spread *in vitro*, this does not rule out the possibility of an *in vivo* difference that occurs in a specific cell population. Further, it is possible that the difference in viral titer observed at early time points after infection of RAW 264.7 cells may play a role in the different lethality phenotypes of CW3 and CR6.

Finally, we demonstrated the utility of the MNV reverse genetic system for elucidating aspects of MNV biology. We were able to clone two viral strains into this system and identify a viral determinant of pathogenesis by transferring whole viral proteins or protein sub-domains between the two strains. This system will be a powerful tool for investigating *in vitro* and *in vivo* elements of MNV infection and will provide insight into unique functions of the proteins of this family of viruses.

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Fig.3-1 MNV strain CR6 is attenuated in lethality. STAT1-/- mice were inoculated with the indicated dose and strain of virus. (A) Parental MNV strains, (B) Clone derived parental strains and CW3 with K296E attenuating mutation, (C) Dose dependence of viral lethality.

Figure 3-1

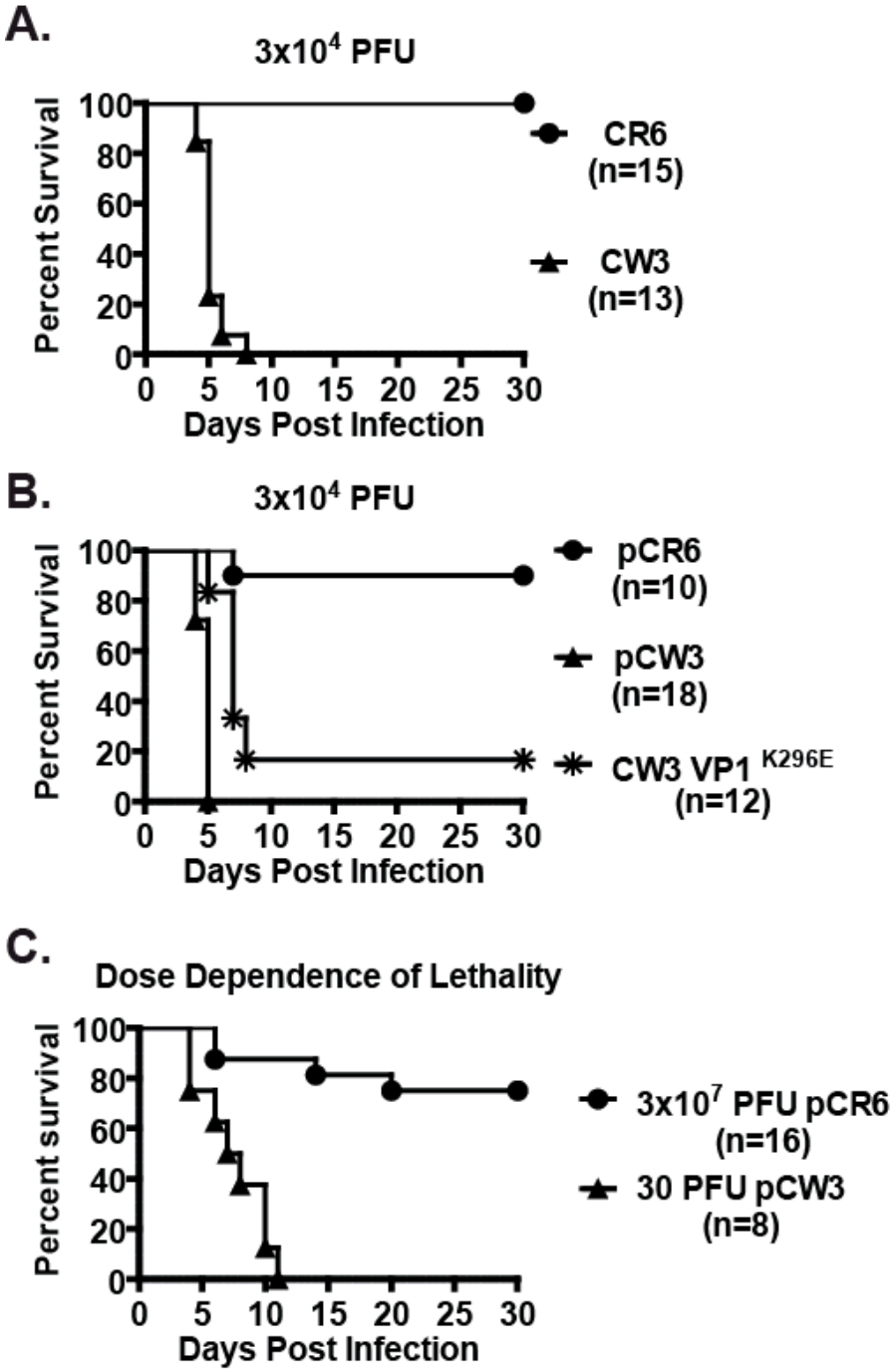

A.
3x10^4 PFU
Percent Survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
CR6
(n=15)
CW3
(n=13)
B.
3x10^4 PFU
Percent Survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
pCR6
(n=10)
pCW3
(n=18)
CW3 VP1 K296E
(n=12)
C.
Dose Dependence of Lethality
Percent survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
3x10^7 PFU pCR6
(n=16)
30 PFU pCW3
(n=8)

Fig.3-2 CW3 and CR6 have similar *in vitro* characteristics. Growth of clone derived CW3 and CR6 was evaluated in RAW cells. Cells were inoculated at MOI 0.05 or 5. Error bars represent standard error of the mean from 3 independent experiments. (A) RAW cells MOI 0.05, (B) RAW cells MOI 5. (E) Western blot of viral supernatant probed using polyclonal anti-MNV serum. A representative blot from three experiments is shown. (F) Genome copies for the indicated number of PFUs were quantified by Taqman. Data is from two independently generated stocks and represents three experimental replicates.

Figure 3-2

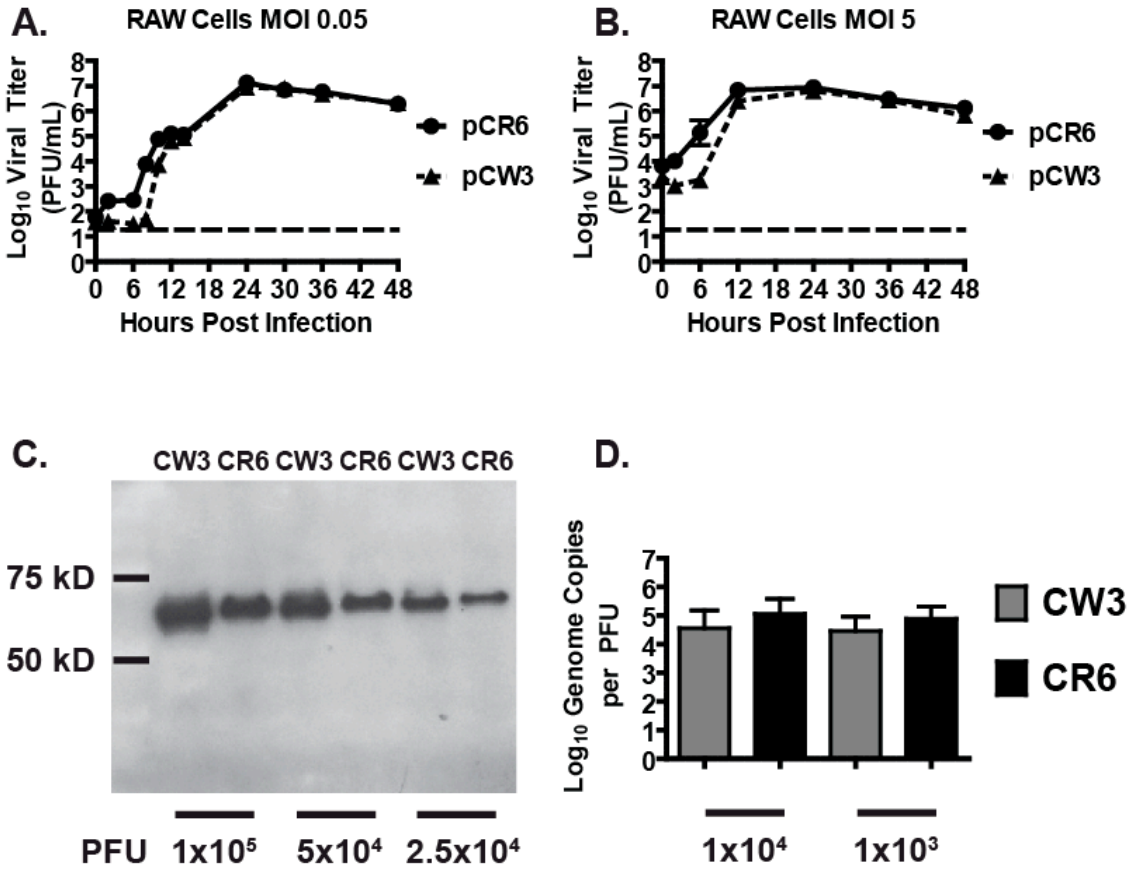

A.
RAW Cells MOI 0.05
Log10 Viral Titer (PFU/mL)
Hours Post Infection
pCR6
pCW3
B.
RAW Cells MOI 5
Log10 Viral Titer (PFU/mL)
Hours Post Infection
pCR6
pCW3
C.
CW3 CR6 CW3 CR6 CW3 CR6
75 kD
50 kD
PFU 1x10^5 5x10^4 2.5x10^4
D.
Log10 Genome Copies per PFU
CW3
CR6
1x10^4 1x10^3

Fig.3-3 MNV capsid gene is a lethality determinant. CW3 chimera viruses with a single gene from CR6 were evaluated for ability to induce lethality. STAT1-/- mice were inoculated with the indicated dose and strain of virus. Mice infected with CW3 or CR6 are shown on each graph for comparison. (A) NTerm and NTPase chimeras, (B) p18 and VPg chimeras, (C) VP1 and VP2 chimeras.

Figure 3-3

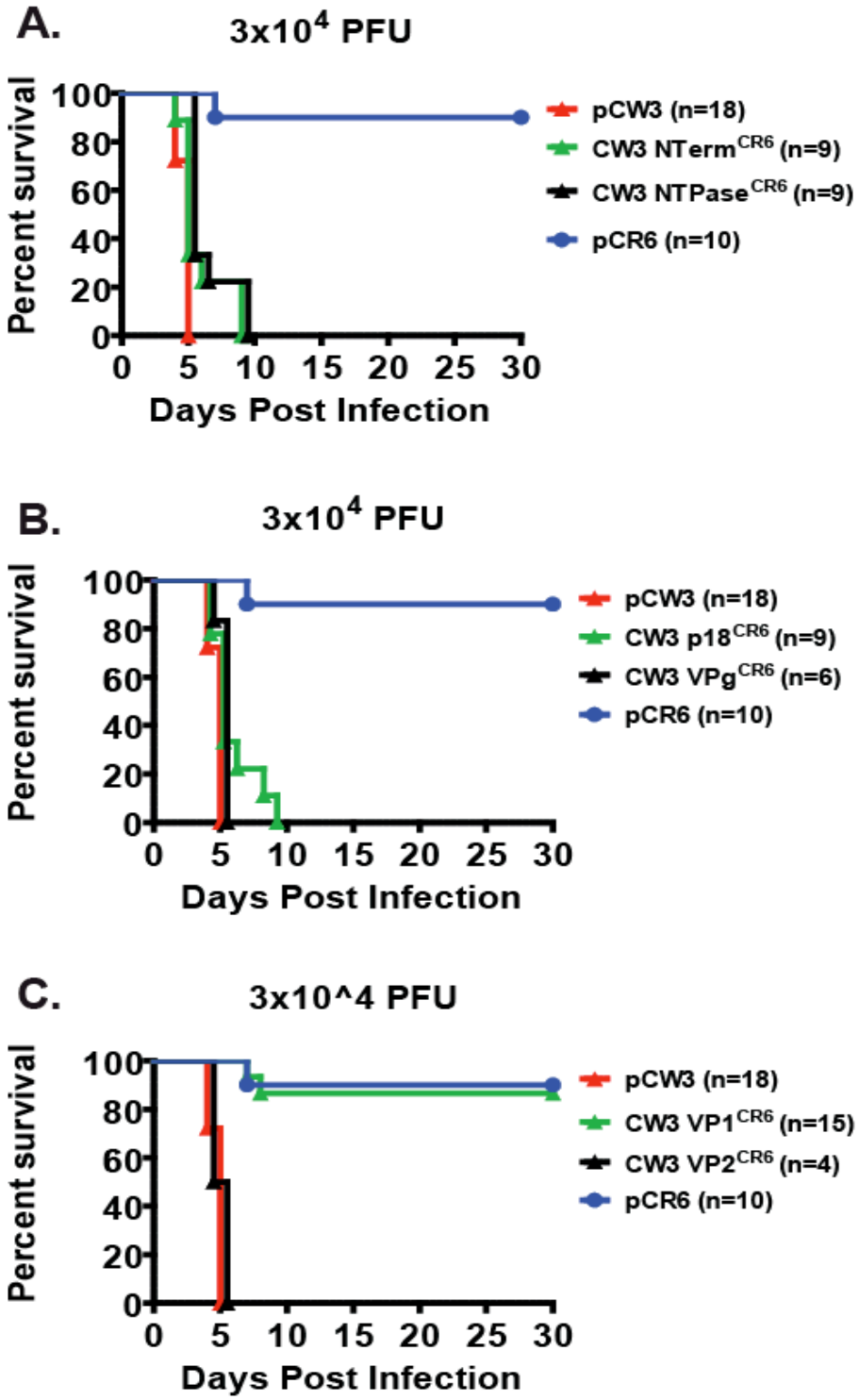

A.
3x10^4 PFU
Percent survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
pCW3 (n=18)
CW3 NTerm^CR6 (n=9)
CW3 NTPase^CR6 (n=9)
pCR6 (n=10)
B.
3x10^4 PFU
Percent survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
pCW3 (n=18)
CW3 p18^CR6 (n=9)
CW3 VPg^CR6 (n=6)
pCR6 (n=10)
C.
3x10^4 PFU
Percent survival
100
80
60
40
20
0
0 5 10 15 20 25 30
Days Post Infection
pCW3 (n=18)
CW3 VP1^CR6 (n=15)
CW3 VP2^CR6 (n=4)
pCR6 (n=10)

Fig.3-4 MNV P domain is necessary and sufficient determinant of lethality. STAT1-/- mice were inoculated with the indicated dose and strain of virus. (A) Model of MNV capsid protein (VP1) (reproduced courtesy of Dr. Tom Smith PMID: 20335264), (B) Capsid Protein virus chimeras, (C) P domain virus chimeras, (D) P sub-domain virus chimeras, (E) Shell domain virus chimeras.

Figure 3-4

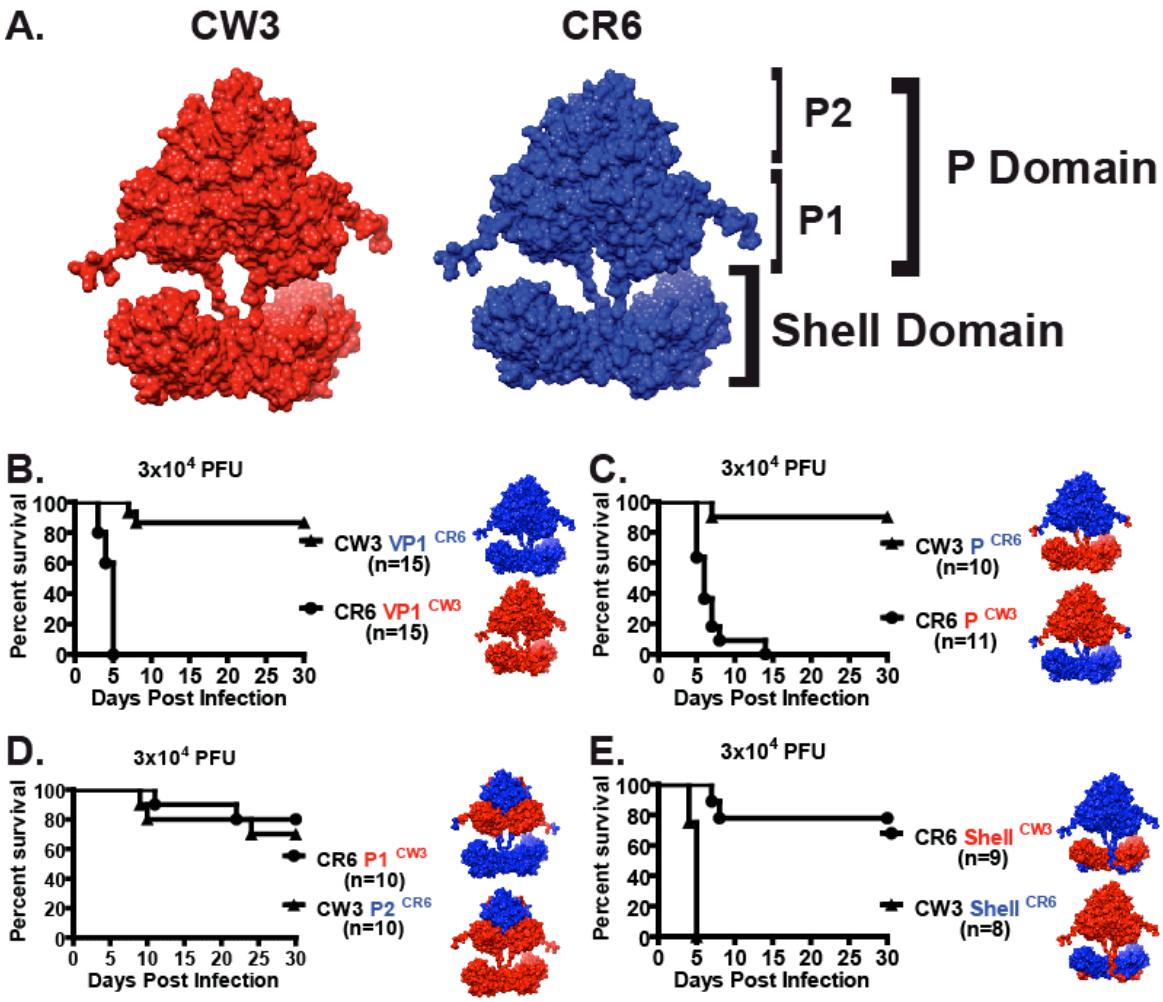

A.
CW3
CR6
P2
P1
P Domain
Shell Domain
B.
3x10^4 PFU
Percent survival
Days Post Infection
CW3 VP1 CR6 (n=15)
CR6 VP1 CW3 (n=15)
C.
3x10^4 PFU
Percent survival
Days Post Infection
CW3 P CR6 (n=10)
CR6 P CW3 (n=11)
D.
3x10^4 PFU
Percent survival
Days Post Infection
CR6 P1 CW3 (n=10)
CW3 P2 CR6 (n=10)
E.
3x10^4 PFU
Percent survival
Days Post Infection
CR6 Shell CW3 (n=9)
CW3 Shell CR6 (n=8)

Fig.3-5 MNV P domain is a determinant of *in vivo* growth. STAT1-/- mice were infected with $3x10^4$ PFU of the indicated virus. 3 days after infection mice were sacrificed and tissues harvested. The amount of virus in each sample was measured by plaque assay and is reported as LOG_{10} PFU/mL. n.s. not significant, *p<0.05, **p<0.01, ***p<0.001.

Figure 3-5

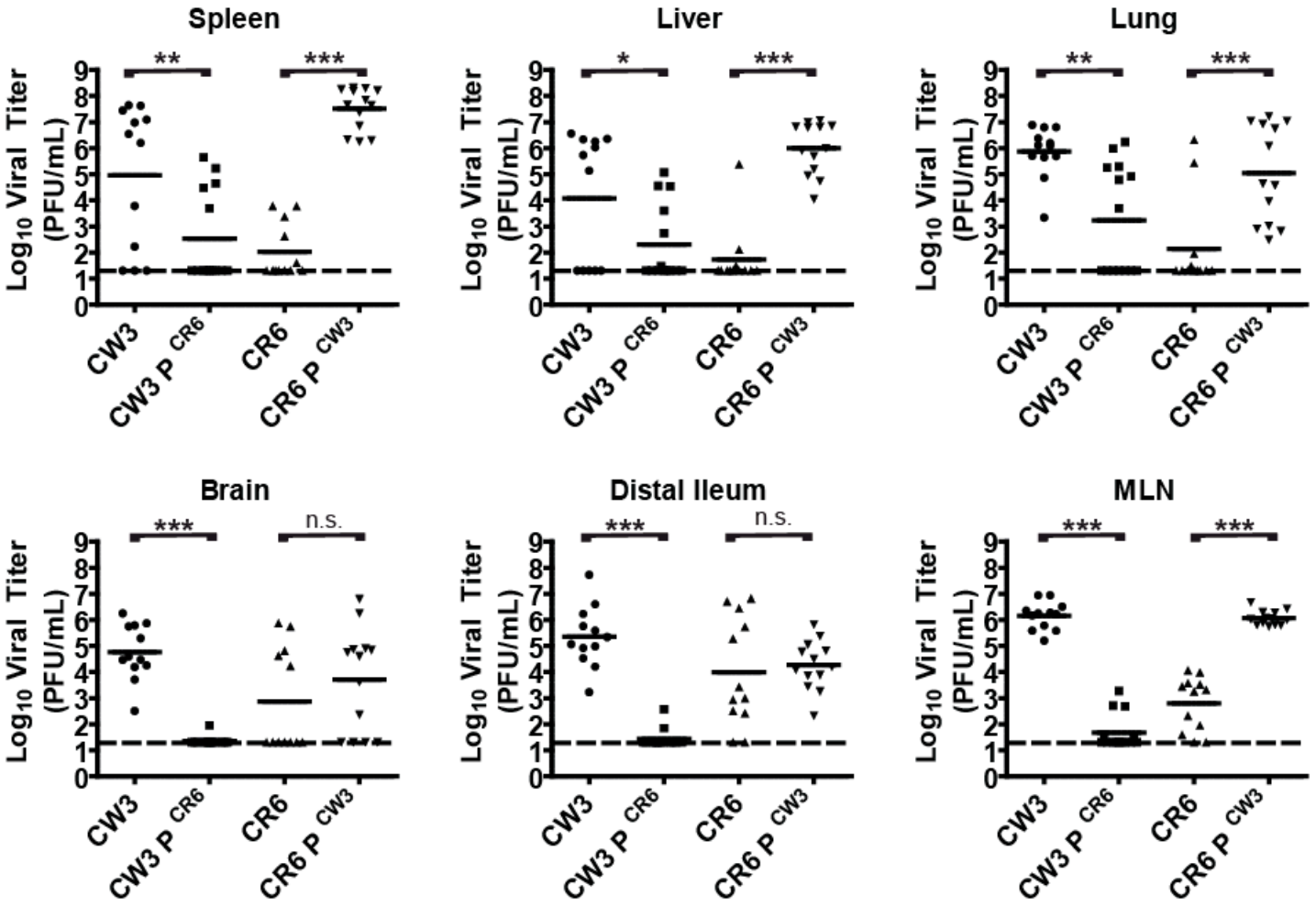

Spleen
Liver
Lung
Brain
Distal Ileum
MLN
Log10 Viral Titer (PFU/mL)
**
***
*
n.s.
CW3
CW3 P CR6
CR6
CR6 P CW3

Fig.3-6 MNV P domain is a determinant of cell death in the spleen. STAT1-/- mice were infected with $3x10^4$ PFU of the indicated virus. 3 days after infection mice were sacrificed and the spleen was fixed in 10% buffered formalin. H&E sections were examined by light microscope and photographs were taken at 2x, 20x and 100x magnification. (A) Mock, (B) CW3, (C) CW3 P^{CR6}, (D) CR6, (E) CR6 P^{CW3}.

Figure 3-6

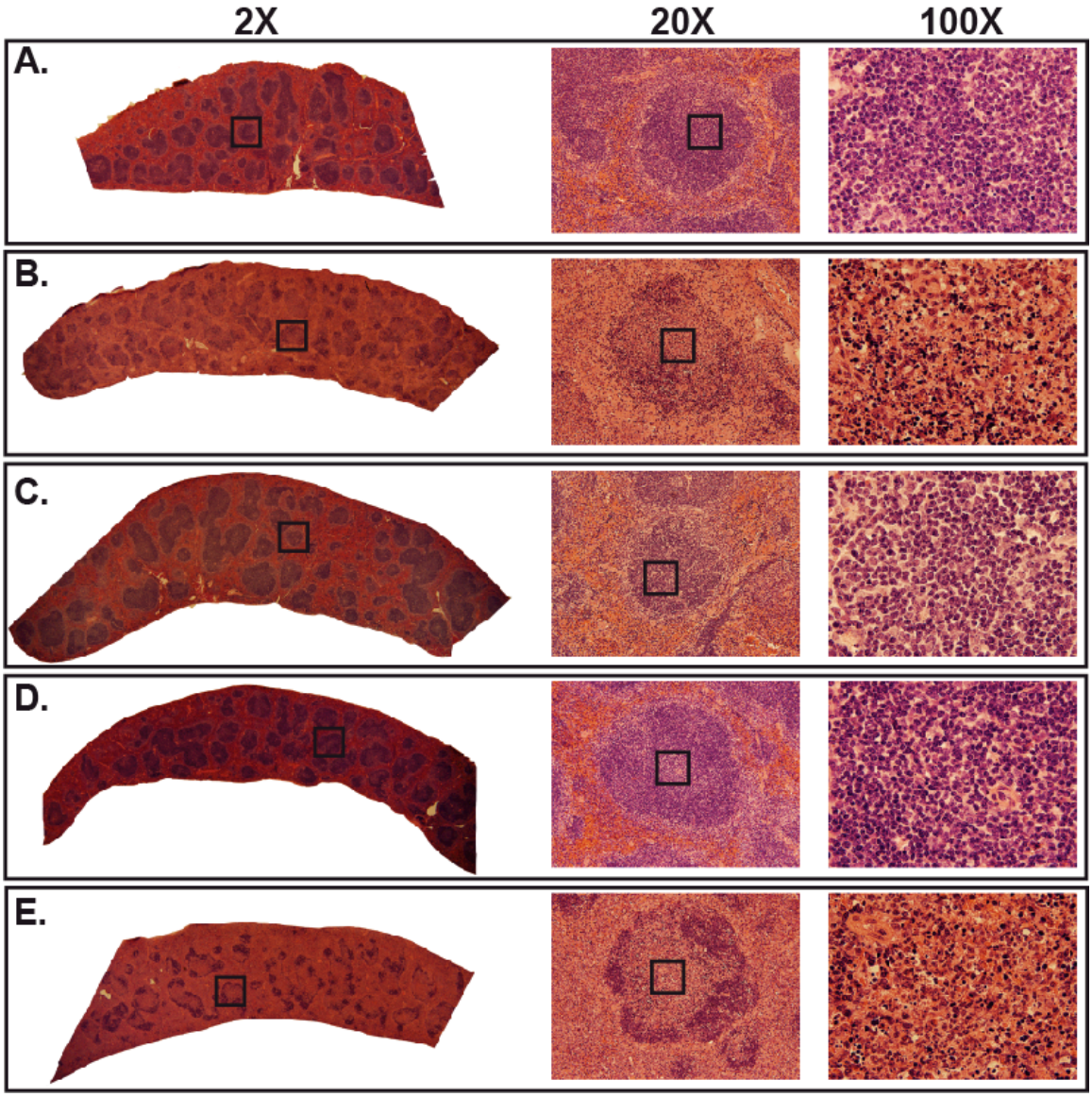
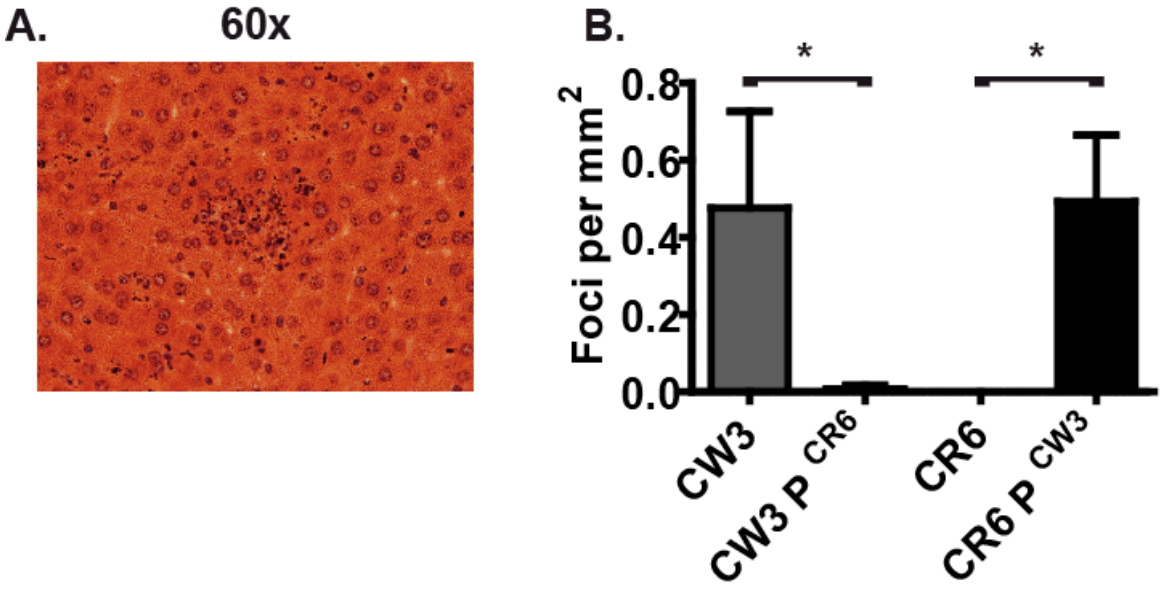


Fig.3-7 MNV P domain is a determinant of cell death in the liver. STAT1-/- mice were infected with $3x10^4$ PFU of the indicated virus. 3 days after infection mice were sacrificed and the liver was fixed in 10% buffered formalin. H&E sections were examined by light microscope. (A) A representative focus of dead and dying cells from CW3 infected mouse, (B) the number of foci observed at 4x magnification were counted and normalized per unit area. * $p<0.05$

Figure 3-7



CHAPTER 4

N-Terminal Non-Structural Protein is a Determinant of Persistent MNV Infection

Introduction

The phenomenon of persistent viral infection and the understanding of strategies for viral persistence are of major import to human health. Viruses capable of establishing persistent infection must have a strategy to maintain infection of the host while overcoming the ability of the immune response to recognize and clear virus. Several mechanisms by which different viruses accomplish these goals have been described. Studies done using models of persistent infection show that persistent viruses can disrupt specific cellular processes in specialized cells (reviewed in (15)). As the pathways involved in the persistence of viruses are so diverse, the study of new models of persistent infection is likely to uncover novel models of viral persistence.

Noroviruses are increasingly being recognized for their ability to establish persistent infection, with virus being shed in the feces of post-symptomatic individuals for prolonged periods (4, 5, 7, 13, 14, 16, 18). Studies have identified the frequency of asymptomatic shedders in community populations at between 4% and 13% of individuals (2, 3, 17). Despite this increasing awareness, the consequences of persistent norovirus infection to human health are unknown. One possibility is that the persistent shedders serve as a reservoir for epidemic outbreaks of norovirus infection. However, the mechanisms governing establishment of persistent infection and the lack of an effective immune response are unknown.

Here, we use infection of mice with murine norovirus (MNV) as a surrogate for the study of human noroviruses. MNV is closely genetically related to human noroviruses and is the only norovirus with a small animal model, reverse genetics system, and can be grown in cell culture (11, 22, 23). Like human noroviruses, MNV is shed in the stool of its host for prolonged periods following initial infection (20). In this study, we use the aforementioned tools to identify a viral protein responsible for the persistence phenotype. Remarkably, a single amino acid allele in the non-structural N-Terminal protein (NTerm) determines whether the virus establishes persistent infection. Additional studies must be done to identify the interactions between host factors and NTerm that result in persistent infection.

Results

CR6 establishes persistent infection. We previously reported that infection of wild type mice with CR6 results in the establishment of a persistent infection, while virus is cleared following infection with CW3 (20). Subsequently, we cloned these two MNV strains into a reverse genetics system. Figure 4-1A-B shows that as reported for the parental strains, CR6 establishes persistent infection while mice infected with CW3 are able to clear the virus. Thirty-five days after infection with $3x10^4$ PFU of CR6, mice shed 10^7 genome copies per fecal pellet. In contrast, all mice infected with CW3 are at or near the limit of detection for this assay. Clearly there is also a difference in infectivity between CW3 and CR6. At three days post infection there is already a statistically significant difference in viral shedding between the two strains, with CR6 shedding more viral RNA ($p<0.001$). CW3 infected mice shed at or near the level of detection for this assay. There is significant variability in the amount of virus shed by CR6 infected mice at this early time point. Interestingly, the level of viral shedding on day three is not a predictor of persistence or the level of shedding at day thirty-five. CR6 infected mice shedding at a level comparable to that of CW3 infected mice on day three still establish persistent infection and are not distinguishable from other CR6 infected mice with regard to the level of viral shedding on day thirty-five post-infection.

In order to determine whether CR6 infection is eventually cleared by an immune response, we measured viral shedding at a much later time point (Figure 4-1C). Even six months after initial infection, CR6 infected mice are shedding more than 10^7 genome copies per fecal pellet. As there is no decrease in viral load between thirty-five days and six months post-infection, we believe CR6 infection does not get cleared in these mice.

NTerm amino acid ninety-four is a persistence determinant. In order to identify a viral gene responsible for the persistence phenotype, we screened CW3 virus chimeras for the ability to establish persistent infection. We identified one gene of CR6, the N-Terminal non-

structural protein, which facilitated the establishment of persistent infection when substituted into the CW3 virus background (Figure 4-1D-E). There are twenty-four amino acid differences between the CW3 and CR6 NTerm proteins. We sought to define the minimal domain or a single amino acid allele of NTerm that could dictate the persistence phenotype. We took the approach of generating CW3 chimeras encoding the five prime (amino acids 1-136) or three prime (amino acids 137-341) portion of the CR6 NTerm in order to identify which half of the protein encodes the persistence determinant (Figure 4-1F-G). To facilitate rapid identification of the persistence determinant, we undertook a second strategy of generating targeted amino acid mutations, changing the individual residues encoded by CW3, to the CR6 allele (Figure 4-1H). We prioritized which targeted mutations to generate by identifying alleles where amino acid charge or reactivity was not conserved between CW3 and CR6. In some cases, where two polymorphic alleles were located in close proximity to one another, both were mutated simultaneously. While little is known regarding the function of MNV NTerm during virus replication, two functional canonical DXXD caspase 3 cleavage sites have been identified (19, 21). At the cleavage site encoded between amino acid positions 118 and 121, the variable amino acids differ between CW3 and CR6. Based on this, we also prioritized the targeting of this allele. We identified the five prime portion of the CR6 NTerm as encoding the persistence determinant. Thirty-five days after infection, mice infected with the CW3 5' $NTerm^{CR6}$ virus were still shedding an average of seven logs of viral genomes per fecal pellet (Figure 4-1G). In comparison, mice infected with CW3 3' $NTerm^{CR6}$ were all at the limit of detection of this assay. Of note, three days after infection with these two virus chimeras, there is also a large difference in virus shedding (Figure 4-1F). This is similar to the difference in CW3 and CR6 infectivity discussed above, and suggestive that the loci defining infectivity and persistence may be the same.

From analyzing the data from day thirty-five virus shedding in mice infected with a CW3 strain encoding specific amino acid mutations, it is apparent that amino acid position ninety-four is a major determinant of the persistent shedding phenotype. Mice infected with CW3 $NTerm^{D94E}$ shed an average of seven logs of genome copies per fecal pellet thirty-five days post infection

(Figure 4-1H). Interestingly, mice infected with two of the single gene CW3 chimeras (NTPase and VPg), and several of the single or double amino acid mutant viruses (amino acid 45, 119, 119 and 120, 136, 157 and 159, and 192) shed a low level of virus thirty-five days after infection (Figure 4-1E and 4-1H). While the mean level of virus shedding in these groups is more than two logs below what is observed in mice infected with CR6 or the CW3 NTerm^{D94E} mutant, this may indicate a small role for an additional viral locus or loci in the expression of the persistence phenotype. While this conclusion cannot be definitively made from the limited sample size here, future experiments should investigate this possibility.

In order to confirm the role of the NTerm protein and specifically amino acid ninety-four in the establishment of persistent infection, we evaluated the phenotype of the relevant viruses using independently generated virus stocks. Figure 4-1I-J shows the combined data for all experiments. We confirmed that mice infected with CW3 NTerm^{CR6} or CW3 NTerm^{D94E} persistently shed virus thirty-five days after infection (Figure 4-1J). Further, we again saw that CR6 infected mice shed significantly more virus three days post infection than do CW3 infected mice (Figure 4-1A and 4-1I). This phenotype correlated with the ability of CW3 NTerm^{D94E} to persist, as mice infected with this virus shed significantly more virus on day three than CW3 infected mice (Figure 4-1I). While the day three shedding phenotype did not map with CW3 NTerm^{CR6}, additional experiments may show that this virus sheds at high titer on day three as well, since the day three shedding phenotype can be extremely variable as demonstrated for infection with CR6 (Figure 4-1A) and discussed above.

We have identified a single amino acid position that controls the ability of CW3 to establish persistent infection. Mutation of the aspartic acid at position ninety-four of the CW3 NTerm protein to a glutamic acid, as encoded by CR6, allows the virus to establish persistent infection (Figure 4-1J). This same allele is responsible for the difference in viral shedding on day three post-infection (Figure 4-1I). Again, despite the correlation between high titer shedding three days post infection and long term viral persistence, there is no causal association.

CW3 and CR6 grow similarly in RAW 264.7 cells. We hypothesized that the differences in persistent infection and viral shedding could be explained by a difference in viral replication at the cellular level. We therefore evaluated growth of CW3, CR6, and CW3 $NTerm^{D94E}$ in RAW 264.7 cells. There was no statistically significant difference between the growth curves of these viruses at MOI 0.05 or 5 (Figure 4-2). However, the ability of CR6 to produce higher titer virus, as compared to CW3, at 6 hours after infection of RAW 264.7 cells was again evident. This phenotype will be discussed in greater detail in Chapter 5. As CW3 and the persistent CW3 mutant grow identically at early and late time points, the difference in ability to persist and the difference in viral shedding on day three do not correlate with a difference in *in vitro* growth as measured in RAW 264.7 cells.

Discussion

The issue of persistent viral infection is of general importance to human health and disease processes. Viruses such as HIV and Hepatitis B establish persistent infections and have adverse sequelae over the life of the host (1). The ability of noroviruses to establish persistent infection is increasingly recognized, with humans reportedly shedding virus asymptomatically for weeks after infection (4, 5, 7, 13, 14, 16, 18). However, the contribution of persistent norovirus infection to human disease is unknown. As MNV infection of mice is the only available small animal model of norovirus infection, we believe study of this system can provide valuable insight into the mechanism of persistent norovirus infection. Further, we believe these studies will be of general interest when considering mechanisms and pathways involved in establishment of persistent viral infections. As we have already identified a role for the non-structural viral protein NTerm, which previously had no known *in vivo* function, in the establishment of persistent infection, we believe it likely that further investigation will lead to the identification of a novel mechanism for viral persistence.

We show here that infection with the CR6 strain of MNV results in long term persistent infection which likely lasts for the life of the host. The ability to establish persistent infection was

mapped to a single amino acid in the non-structural NTerm protein. Encoding of the CR6 glutamic acid allele at position ninety-four of this protein facilitated persistence of a previously non-persistent virus. It will be important for future studies to determine whether mutation of the CR6 glutamic acid at NTerm position ninety-four to an aspartic acid results in a non-persistent virus. If it does, this would indicate that the amino acid encoded at position ninety-four is both necessary and sufficient to determine the persistence phenotype of these two MNV strains. Alternately, it is possible that one or more additional genetic loci play a role in this phenotype, which would be uncovered by performing the above mentioned experiment. Additionally, we observed that this same amino acid allele was responsible for the ability of MNV to shed at high titer in stool on day three post-infection. Interestingly, this high titer shedding was not responsible for the ability to persist, as the level of shedding on day three post-infection did not correlate with the amount of virus being shed thirty-five days after infection in persistently infected mice. Our data shows that persistent infection is not correlated with *in vitro* viral growth in RAW 264.7 cells. This finding does not preclude the possibility that differential growth in a specialized *in vivo* cell type results in persistent infection.

The function of the NTerm protein is poorly understood. Expression of a fluorescent fusion protein of the NTerm from human norovirus strain Norwalk results in co-localization with the Golgi apparatus marker golgin 97 in transformed cell lines (6). Expression of a hexa-histidine tagged NTerm protein of MNV-1 resulted in co-localization with the endoplasmic reticulum marker calnexin, but not the Golgi apparatus markers GM130 or giantin (10). However, this staining pattern was distinct from the peri-nuclear staining observed in infected RAW 264.7 cells, indicating that MNV NTerm has a different localization pattern in infected cells as compared to cells transfected with the individual protein. It is possible that this results in differential protein function of NTerm in infected cells versus cells transfected with the protein alone. This may be true for human norovirus NTerm as well, and should be considered when interpreting the results of exogenous expression studies.

The human norovirus and MNV NTerm proteins encode three recognized protein domains (6). A domain composed of hydrophobic amino acids is encoded near the carboxy terminus and is a putative trans-membrane domain. An H-box and NC domain are also encoded in NTerm and are conserved between human and MNV NTerm proteins (6). The function of H-box and NC domains in proteins is unknown. The H-rev107 family of proteins encodes these two domains as well as a carboxy terminus hydrophobic region (8). One member of the protein family, H-rev107-1, has been identified as a tumor suppressor, as protein expression is absent in tumor cell lines from many organ sources, and in primary squamous carcinoma cells (9). Although the similarities in protein domain encoding between norovirus NTerm proteins and the H-rev107 family of proteins does not indicate a mechanism for the function of NTerm or its role in MNV persistence, it does facilitate data driven hypothesis formulation that may lead to studies that answer these questions.

Here, we have identified a viral determinant of norovirus persistent infection. Surprisingly, a single amino acid in the non-structural protein NTerm dictates whether the CW3 strain of MNV is able to establish persistent infection. This finding necessitates consideration of a new model for persistence as previous hypothesis focused solely on the capsid protein as a mechanism for immune evasion and establishment of persistent infection. Future experiments should focus on identifying a mechanism for NTerm function in the establishment of persistence by identifying host molecules with which NTerm interacts. Further, the immune response or lack thereof to persistent infection must be investigated. CR6 and the persistent CW3 mutant could establish persistent infection by inhibiting the immune response against the virus. NTerm could play a role in such a mechanism by inhibiting early steps in viral recognition following cell entry. MDA5 has previously been identified as an intracellular sensor of MNV infection, so study of its role in infection with persistent and non-persistent strains is a logical follow up to this work (12). Alternately, the immune response may be ineffective in clearance of CR6 and the persistent CW3 mutant due to immune evasive strategies employed by the virus, such as the mutation of immuno-dominant epitopes. Again, NTerm could facilitate such a mechanism by increasing the

mutation rate of the viral polymerase, or targeting mutations to specific areas of the viral genome. Further studies are needed to identify a mechanism for this interesting phenomenon.

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Fig.4-1 Persistent MNV shedding and identification of viral determinant. (A-J) C57BL/6 mice were infected with $3x10^4$ PFU of the indicated virus per os. Fecal pellets were collected at the indicated times after infection for quantification of MNV genome copies. Dashed line indicates the assay limit of detection. (I-J) statistical comparison for each group is to the CW3 infected group. ***p<0.001

Figure 4-1

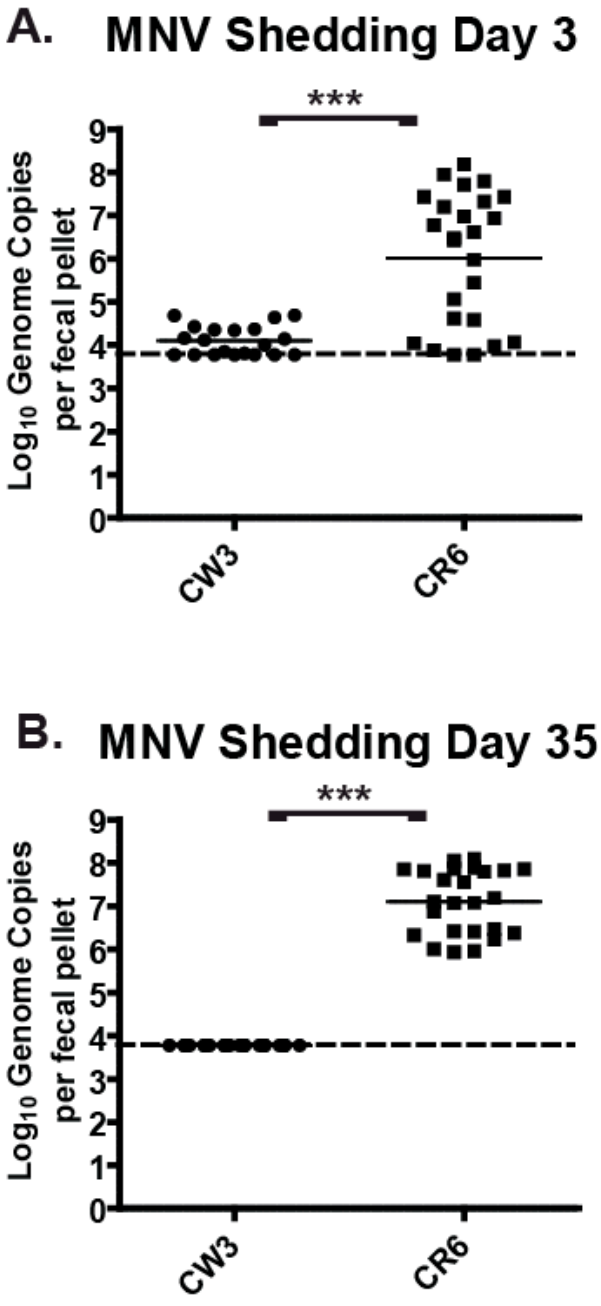

A.
MNV Shedding Day 3
***
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3
CR6
B.
MNV Shedding Day 35
***
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3
CR6

Figure 4-1

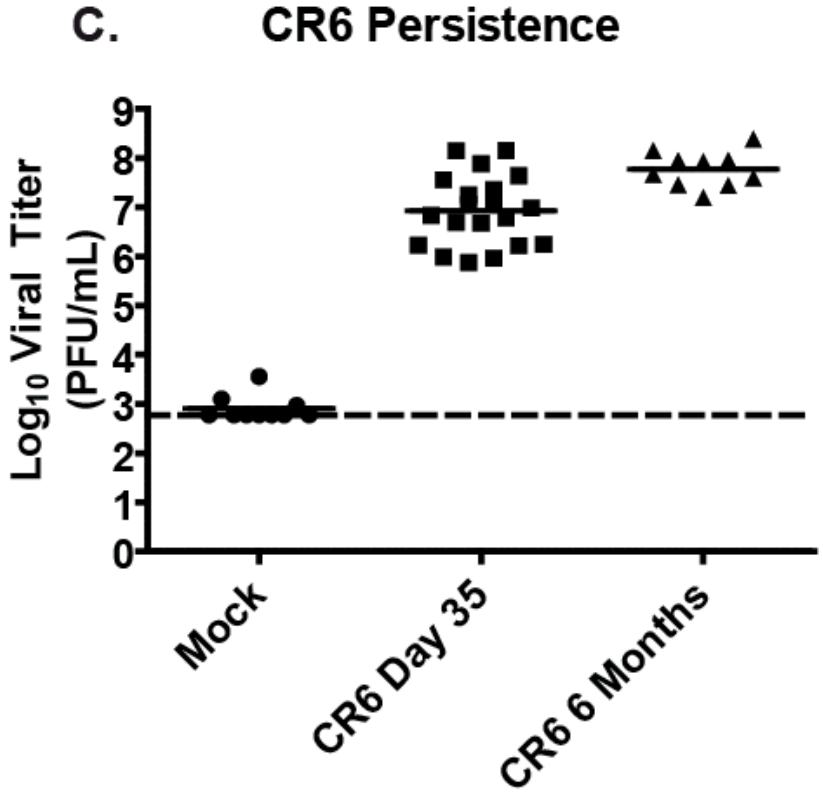
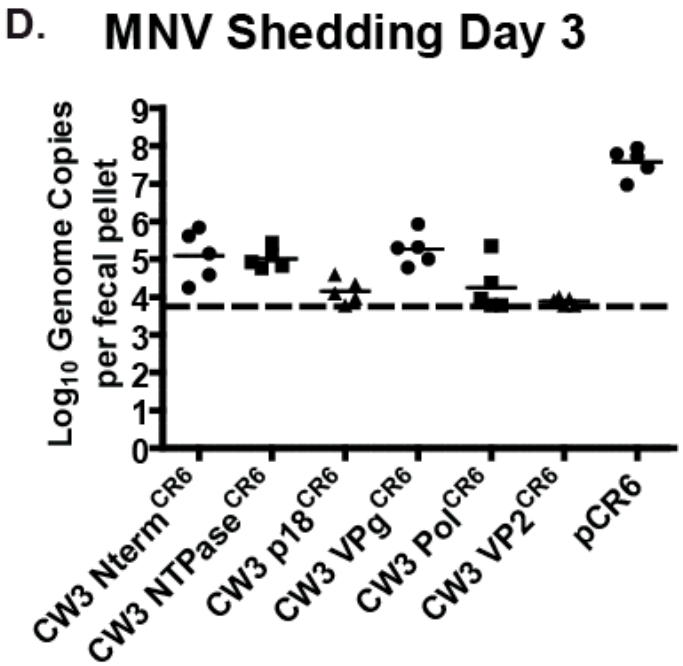

C.
CR6 Persistence
Log10 Viral Titer (PFU/mL)
9
8
7
6
5
4
3
2
1
0
Mock
CR6 Day 35
CR6 6 Months

Figure 4-1


D.
MNV Shedding Day 3
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3 Nterm CR6
CW3 NTPase CR6
CW3 p18 CR6
CW3 VPg CR6
CW3 Pol CR6
CW3 VP2 CR6
pCR6

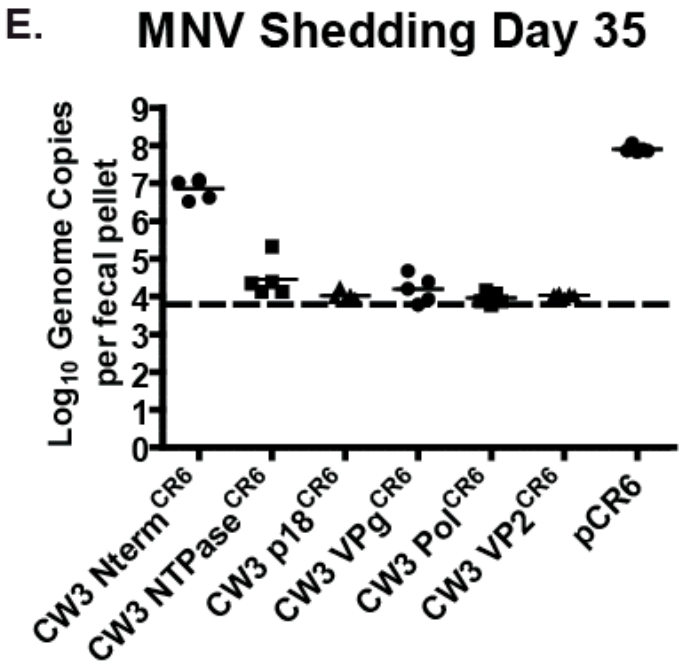

E.
MNV Shedding Day 35
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3 Nterm CR6
CW3 NTPase CR6
CW3 p18 CR6
CW3 VPg CR6
CW3 Pol CR6
CW3 VP2 CR6
pCR6

Figure 4-1

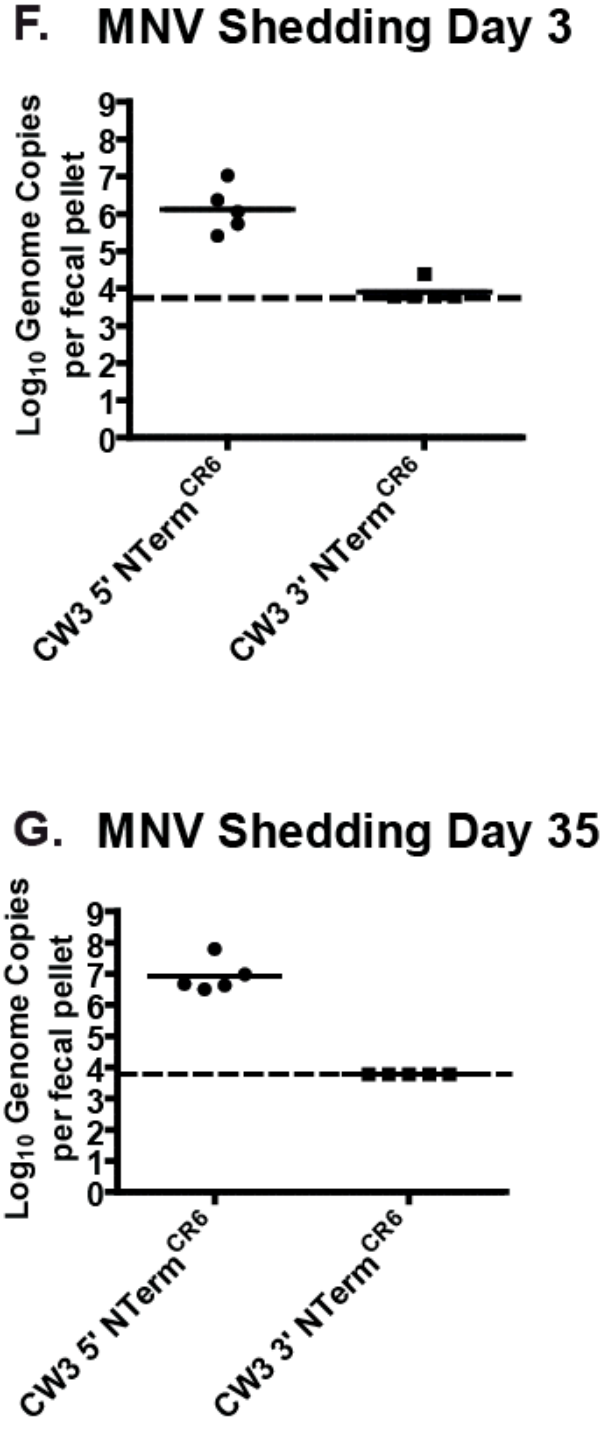

F. MNV Shedding Day 3
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3 5' NTerm CR6
CW3 3' NTerm CR6
G. MNV Shedding Day 35
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3 5' NTerm CR6
CW3 3' NTerm CR6

Figure 4-1

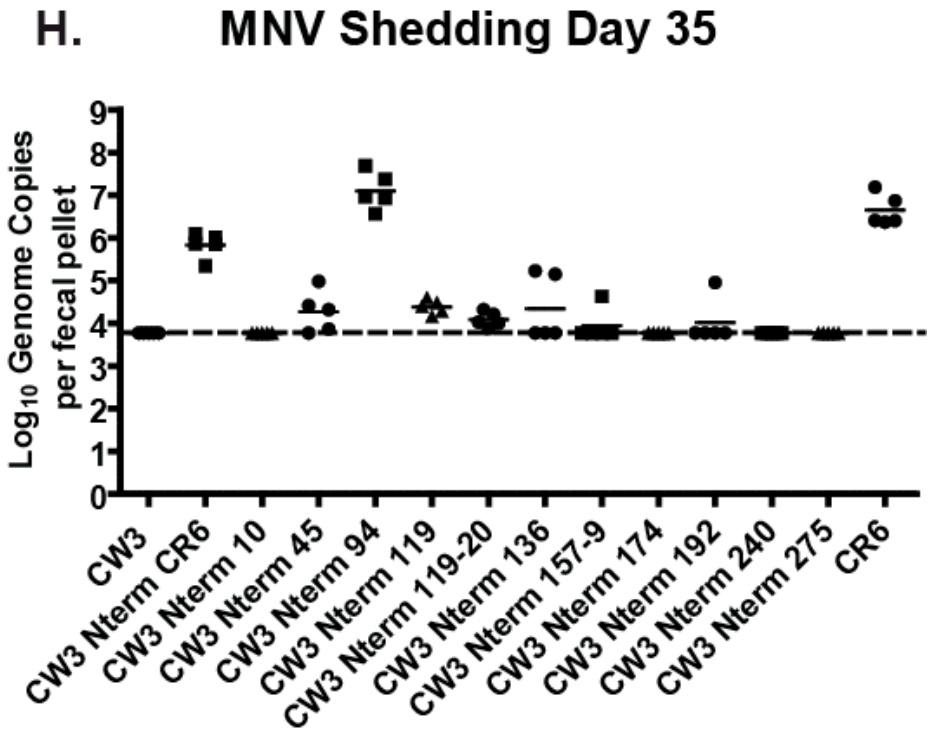
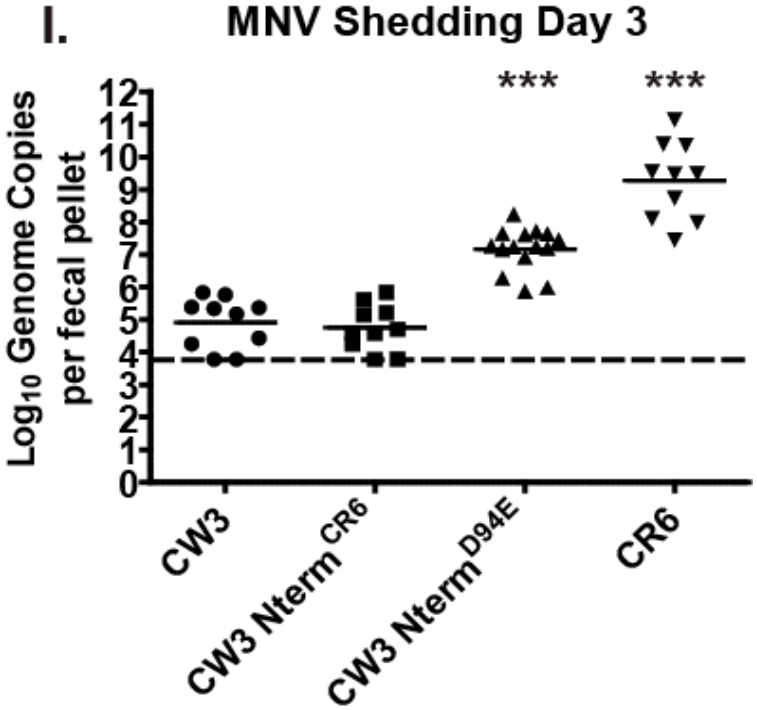

H.
MNV Shedding Day 35
Log10 Genome Copies per fecal pellet
0
1
2
3
4
5
6
7
8
9
CW3
CW3 Nterm CR6
CW3 Nterm 10
CW3 Nterm 45
CW3 Nterm 94
CW3 Nterm 119
CW3 Nterm 119-20
CW3 Nterm 136
CW3 Nterm 157-9
CW3 Nterm 174
CW3 Nterm 192
CW3 Nterm 240
CW3 Nterm 275
CR6

Figure 4-1


I.
MNV Shedding Day 3
Log10 Genome Copies per fecal pellet
***
***
CW3
CW3 NtermCR6
CW3 NtermD94E
CR6

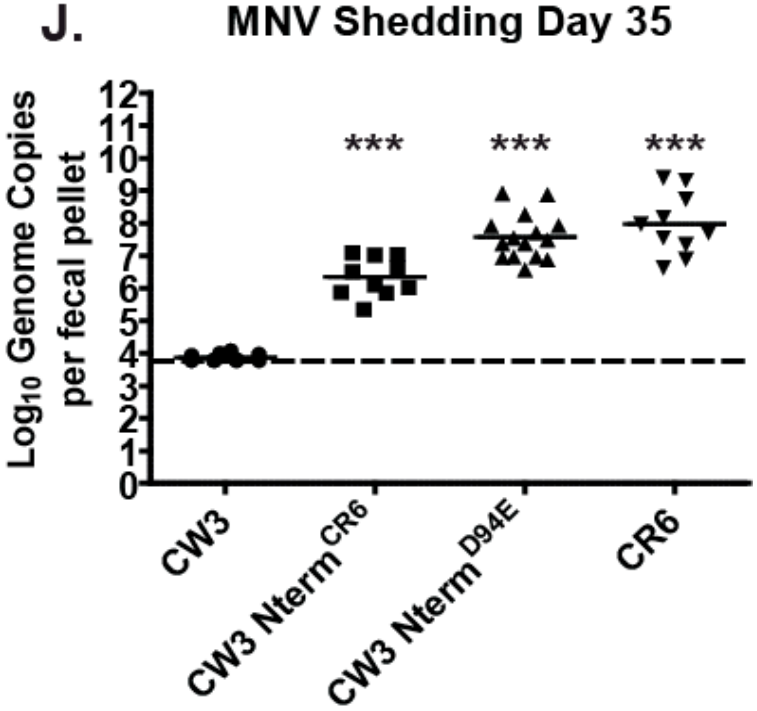
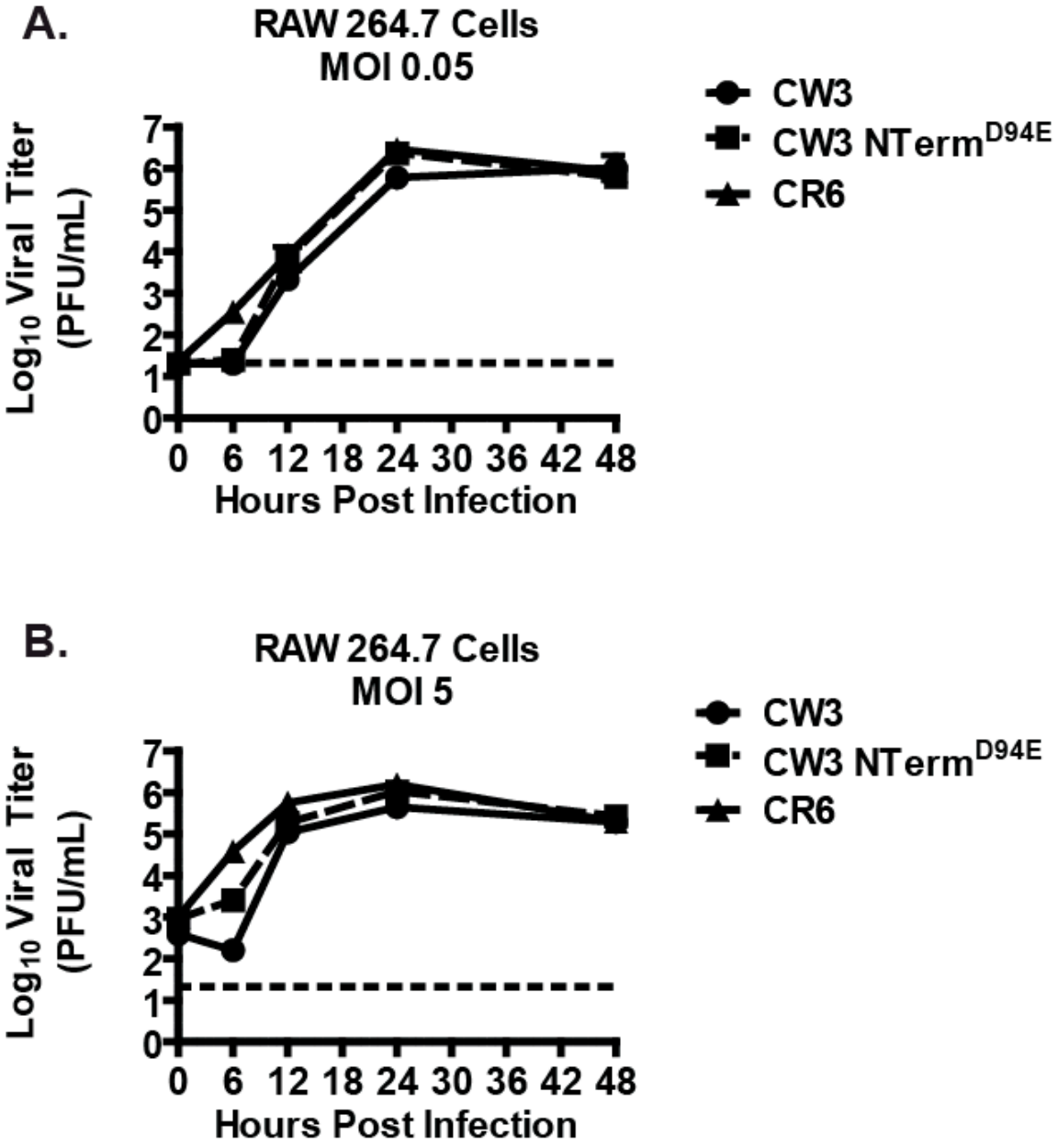

J.
MNV Shedding Day 35
Log10 Genome Copies per fecal pellet
***
***
***
CW3
CW3 NtermCR6
CW3 NtermD94E
CR6

Fig.4-2 Persistent and non-persistent strains grow similarly *in vitro*. Growth of CW3, CR6, and CW3 NTerm^{D94E} was evaluated in RAW 264.7 cells. Cells were inoculated at (A) MOI 0.05 or (B) MOI 5. Error bars represent standard error of the mean from 3 independent experiments.

Figure 4-2


A.
RAW 264.7 Cells
MOI 0.05
Log10 Viral Titer (PFU/mL)
0 1 2 3 4 5 6 7
0 6 12 18 24 30 36 42 48
Hours Post Infection
CW3
CW3 NTermD94E
CR6
B.
RAW 264.7 Cells
MOI 5
Log10 Viral Titer (PFU/mL)
0 1 2 3 4 5 6 7
0 6 12 18 24 30 36 42 48
Hours Post Infection
CW3
CW3 NTermD94E
CR6

CHAPTER 5

Summary and Future Directions

Summary and Future Directions

The goal of this work has been to create and use a novel set of molecular genetics reagents to identify viral determinants of murine norovirus (MNV) pathogenesis. We have cloned two strains of MNV into a previously published reverse genetics system (23). We show that production of virus using this system reduces the viral quasispecies diversity that is present in serially passaged virus, thereby decreasing the potential for ambiguities in the analysis of viral phenotypes. A second advantage of this system is the ability to manipulate the genome sequence. We have exploited this potential by constructing virus chimeras and point mutants for use in identifying viral determinants of pathogenesis.

Chapters three and four detailed the use of virus chimeras to identify the MNV genes and protein domains responsible for differences between CW3 and CR6 in lethality in STAT1-/- mice and the ability to establish persistent infection in wild type mice. These same methods and tools can be used to map the genes responsible for other differences between these two virus strains. We have identified two additional phenotypic differences between the cloned CW3 and CR6 strains. One additional phenotype that differentiates these viruses is a difference in how soon they begin to increase viral titer during growth in tissue culture. This is the difference observed while analyzing viral growth curves in chapters two, three, and four. After MOI 0.05 infection of RAW cells, CR6 infected cells have a higher mean viral titer at two, six, and twelve hours post-infection as compared to CW3 infected cells. This difference is between five and ten fold and is statistically significant (Figure 5-1A). By twenty-four hours post-infection, the mean viral titer of each strain is statistically equivalent. This early difference in viral titer was also observed at MOI 5 (Figure 5-1B). At two hours post-infection CR6 infected cells had tenfold more viral titer than CW3 infected cells. By six hours the difference was one-hundred fold. CW3 and CR6 viral titers were statistically equal by twelve hours post-infection. These data show that there is an early difference in viral titer between CW3 and CR6 after infection of cells *in vitro*, but this difference is absent at later time points.

We have identified a second novel phenotype that differentiates CW3 and CR6. Mice infected with CW3 or CR6 differ in their immune response to a cholera toxin challenge when compared to one another (Figure 5-2). CW3 infected mice were challenged with cholera toxin and the antibody response was measured by ELISA fourteen days later. These mice had a robust anti cholera antibody response. By comparison, mice infected with CR6 and challenged with cholera toxin had a less effective antibody response. In CR6 infected mice, the cholera toxin antibody binding, as measured by mean absorbance at 450nm, was only slightly above the background observed for cholera toxin naïve mice. Binding of antibody from CR6 infected mice was statistically lower than the binding response of antibody from CW3 or uninfected mice. This data indicates a role for CR6 infection in the suppression of the immune system antibody response.

MNV Persistence

We have definitively identified a viral determinant of norovirus persistence. Surprisingly, a single amino acid at position ninety-four of the non-structural protein NTerm determines whether the CW3 strain of MNV persists. Glutamic acid at this position results in a virus capable of establishing persistent infection. Aspartic acid at NTerm position ninety-four, as encoded in the wild type CW3 strain, facilitates clearance of virus. It will be important to determine whether it is true that a single amino acid at this position determines persistence in the CR6 background as well. It is possible that the encoding of glutamic acid at this allele is sufficient to result in CW3 persistent, but not necessary for the persistence of CR6. Such a result would indicate that additional virus determinants can play a role in persistent MNV infection. Interestingly, of the more than twenty MNV strains we have analyzed (22), all encode either glutamic acid or aspartic acid at this allele. Determination of the phenotype for other amino acids at this position may be helpful for future studies to determine the mechanism of persistence. Whether other amino acids result in a partial phenotype or are all together not tolerated by the virus at this position could provide insight into the protein interactions taking place. More important in this regard is the

identification of host proteins that interact with NTerm. Future studies should screen for these proteins using a high throughput method such as a yeast two-hybrid hybrid screen or immuno-precipitation followed by mass spectrometry, and then determine whether encoding aspartic acid or glutamic acid at NTerm position ninety-four results in different interactions with the identified target(s).

Little is known regarding the structure of the norovirus NTerm protein. The NTerm protein sequence varies markedly between norovirus strains, and is the most variant region of ORF1 (8, 19). The carboxy terminal portion of the protein is relatively conserved, and much of the variation occurs in the amino terminal portion of the protein (8, 19). This same pattern of sequence similarity is observed when comparing human to murine norovirus NTerm proteins, with the amino terminal portions of the protein being most divergent (unpublished observation). For this reason, it was not possible to identify an amino acid in human norovirus NTerm proteins that is analogous to position ninety-four of the murine proteins. Three protein sequence motifs have been identified that are conserved across strains of human and murine noroviruses (9, 13). The H-box and NC domains are purely descriptors of the conserved amino acid sequences encoded, with no known function. A hydrophobic domain, postulated to be a trans-membrane domain, is also conserved between these proteins. These same three sequence motifs are encoded in the H-rev107 family of proteins (13). H-rev107 has been identified as a tumor suppressor (10, 14, 20). H-rev107 protein expression is absent in tumor cell lines and in primary squamous carcinoma cells (10, 14, 20). Overexpression of H-rev107 inhibits the growth of RAS-transformed cells (20). MNV NTerm proteins, including the CW3 persistence enabling mutant, and human norovirus NTerm proteins should be tested for this same activity. The identification of a cellular pathway in which NTerm functions will be beneficial in elucidating the mechanisms resulting in persistent MNV infection. Further, determination of whether the human norovirus NTerm proteins can perform a function identical to their murine homologues will help in evaluating the validity of the MNV system as a surrogate for the study of human noroviruses. The fact that a non-structural viral protein regulates whether persistent infection is established suggests that the

ability to persist is the result of the activation or inhibition of a cellular process by NTerm in infected cells. Once identified, this process could be a new pathway to consider in the study of persistent infections.

One remaining question regarding the persistence phenotype is whether CW3 is completely cleared from mice at thirty-five days post-infection. The quantitative PCR assay described here is relatively insensitive, with a limit of detection on the order of one-thousand genome copies. At thirty-five days post-infection, some of the CW3 infected mice appear to be shedding a small amount of virus, slightly above the limit of detection. It is possible that this represents a technical problem due to variation in background or sensitivity at low numbers of genome copies. Alternatively, it could indicate that some mice do shed a small amount of CW3 persistently, and the phenotype we have described is actually a difference in the amount of virus shedding in infected mice rather than an absolute difference in the ability of each virus to persist. As only a single nucleotide mutation is required for CW3 to gain the persistent phenotype, it may be that a small number of viruses mutate and gain the ability to persistent after infection, and this virus is subsequently being detected in the day thirty-five samples. This should be resolved by sequencing virus from samples that are slightly above the limit of detection to determine whether viral RNA is present, and if so, whether it is a mutant of CW3 capable of establishing persistent infection. Another approach is to serially passage the stool filtrate from samples slightly above the limit of detection in RAW 264.7 cells. If a small amount of virus is present, cytopathic effect on cells should be observable after growing the virus in tissue culture. Alternately, these samples should be used to infect STAT1-/- mice, as they are sensitive to virus inoculums as low as 30 PFU (Chapter 3). Lethality in these mice would be confirmation that live CW3 is present in the samples in question.

Similar experiments will help to resolve the question of whether the virus shed from persistently infected mice is infectious. As CR6 is not lethal in STAT1-/- mice, mice should be infected and monitored for shedding of virus in stool. Alternately, virus in the stool of persistently shedding mice should be measured by plaque assay, or evaluated for the ability to cause

cytophatic effect when repeatedly passed in tissue culture. If these experiments show that virus from persistently shedding mice is infectious to mice and can replicate in cells, the infectious nature of persistently shed virus will be confirmed.

Immune response to persistent MNV infection

While the primary focus of the experiments presented here is the identification of viral factors that contribute to persistent infection, investigation of the immune response to persistent and non-persistent MNV strains will also be informative to the mechanisms involved in viral persistence. As CW3 is recognized and cleared by the immune system while CR6 can persist, there is likely a qualitative difference in the immune response to these viruses. To test this hypothesis, the innate and adaptive immune responses following infection with CW3 and CR6 should be characterized. Studies should focus on the characterization of T and B cell responses to MNV infection. Identification of the viral T cell epitopes will allow quantification of specific T cell responses following CW3 and CR6 infection. This is an important consideration, as impairment of the T cell response can lead to viral persistence (17). To evaluate the B cell response, convalescent serum from CW3 and CR6 infected mice should be tested for the ability to neutralize both viral strains in a plaque neutralization assay. This experiment will answer the question of whether CR6 persists due to failure to stimulate production of neutralizing antibodies.

As establishment of persistent infection requires subversion of both the innate and adaptive immune responses, it will also be informative to characterize the innate immune response to CW3 and CR6 infection. The innate immune response is known to be critical for host defense to MNV infection, as STAT1 has been identified as a necessary host gene to prevent lethality following MNV infection (15). MDA5 is an intracellular sensor of double stranded RNA and has been shown to initiate the innate immune response to CW3 (16). It is possible that a difference between the two viral strains in the initiation of the immune response by MDA5 or downstream events in this signaling pathway are responsible for one or more of the phenotype

differences we have observed here. Future studies should determine whether MDA5 is equally effective at sensing and initiating an immune response to infection with CW3 or CR6.

MNV Lethality

Previous work has shown that MNV infection is lethal in STAT1-/- mice (15). Serial passage of virus in RAW 264.7 cells resulted in a lethality attenuating lysine to glutamic acid substitution in the most distal P2 domain of the viral capsid (3, 24). Here, we identified a field strain of MNV that is attenuated in its ability to induce lethality in STAT1-/- mice. We show that the sequence of the viral capsid P domain is predictive of lethality. Notably, the previously identified lysine to glutamic acid mutation at position 296 of the capsid protein was not responsible for viral attenuation in this system, as CW3 encoding glutamic acid at this allele remained lethal (Chapter 3). This data confirms that the P domain is an important determinant of viral lethality and demonstrates that the attenuated phenotype can be encoded at separate sites within the protein. Further, we show that the P domain and lethality are correlated with viral spread from the primary site of infection, *in vivo* growth, and pathology in the spleen and liver.

The mechanism for the lethality difference between CW3 and CR6 is still unknown. As the capsid P domain sequence is an indicator of virulence, the data is suggestive of a role for viral binding and or entry in this phenotype. While the receptor for MNV binding is not known, carbohydrates that enhance binding have been identified (21). CW3 and CR6 should be tested for P domain dependant differential binding to these carbohydrate ligands. Differential binding may indicate that these viruses infect unique cell populations *in vivo*, and preliminary data supports the hypothesis for the existence of a cell population that is permissive for the growth of CW3 but not CR6 (Figure 5-3). CR6 displays a significant growth defect as compared to CW3 in bone marrow derived macrophages differentiated using cell supernatant from the monocyte colony stimulating factor (M-CSF) producing cell line CMG14-12. This conflicts with previous data showing that there is no growth difference between CW3 and CR6 in bone marrow derived macrophages differentiated using L929 cell supernatant following the protocol described by Heise

et al (11) (Chapter 2 Figure 2-1). The principal difference in the derivation of these two cell populations is the media used for their differentiation. This could result in a difference in a number of cellular parameters such as the rate of cellular division or the cellular differentiation state. Identification of the difference between these cell populations will provide a clue as to whether there is a difference in the requirement for a specific cell intrinsic differentiation state or protein expression profile for growth of these two viruses. As these are two cell populations which should be similar, but display dramatically different growth permissiveness for CR6, we believe this data supports a hypothesis for the existence of an *in vivo* cell population that is permissive for CW3 infection but not CR6. Again, this difference could be the basis for any of the phenotypes described in this work. Identification of the viral determinant for this growth difference will be important in determining whether it is correlated with and possibly causative to the difference in lethality between CW3 and CR6. Further, the two populations of bone marrow derived macrophages should be studied to identify the host factor responsible for the difference in permissiveness to CR6 infection. The two cell types should be tested for differential expression of glycosylceramide synthase, an enzyme necessary for production of the gangliosides known to play a role in attachment to and growth of CW3 in permissive cells. This may facilitate the determination a host cell mechanism for differential growth between CW3 and CR6.

Early Viral Titer Increase

Understanding of the early events in viral entry and replication is important for understanding how viruses establish infection and how the host recognizes the virus to initiate an immune response. We have observed an increase in CR6 viral titer as early as two hours after infection of cells *in vitro* (Figure 5-1). Such an increase in viral titer is not observed following infection of cells with CW3. This time point is well before a full round of viral replication can take place. At MOI 0.05, most of the increase in viral titer takes place between time zero and two hours post-infection. No further increase occurs between hours two and six after infection. Thus, we think it unlikely that this represents viral growth, rather an allosteric event that results in an

increase in viral infectivity. Such a phenomenon has been observed during reovirus infection, where a proteolysis event targeting the capsid protein occurs in the intestine, resulting in a conformational change in the capsid that increases infectivity (1, 4, 5). It is possible that a similar interaction occurs for MNV during infection of cells *in vitro*. Alternately, it is possible that a conformational change is induced by receptor binding, as has been demonstrated for FCV (18). For this hypothesis to be true, it would require the presence of non-infectious particles in the CR6 virus stock that can be converted to infectious particles. There is evidence for the existence of such particles, as the genome to PFU ratio of CR6 is approximately ten-thousand to one (Chapter 3). Thus, there may be viral particles in the stock that are not accounted for in the viral titer measured by plaque assay. In order to explain the difference in titer increase between the two strains, the proteolysis or conformational change would have to occur differentially between CW3 and CR6. The next step in studying this phenotype is to use virus chimeras to map the phenotype to a viral gene or domain. If the phenotype maps to the P domain of the capsid protein, it would further support the conformational change hypothesis and will indicate that the early difference in virus titer is possibly related to the lethality phenotype. Detailed structural binding studies of this even must await the identification of an MNV receptor.

Inhibition of Cholera Toxin Antibody Response

We show here that the antibody response to a cholera toxin challenge is suppressed in CR6 infected mice as compared to those infected with CW3. This is a long lasting effect of MNV infection, as the cholera toxin challenge was administered thirty-five days after initial infection. At the time of the cholera toxin challenge, the immune response has successfully cleared CW3 infection, while CR6 is still being shed in feces. We hypothesized that the suppression of anti-cholera toxin antibody response was due to the presence of high titer virus and not intrinsic to CR6 per se. To test this hypothesis, we measured the cholera toxin antibody response in mice that were infected with the persistent CW3 mutant. Surprisingly, the ability to persist does not correlate with the anti-cholera toxin response as both CW3 infected and CW3 $NTerm^{D94E}$ infected

mice produced a robust antibody response that was not statistically different from uninfected mice (Figure 5-4). For further confirmation that this phenotype is not related to persistent viral infection, the cholera toxin antibody response should be evaluated in mice infected with a non-persistent CR6 virus. Suppression of the antibody response in these mice would indicate that the phenotype is the result of a CR6 determinant unrelated to persistence.

This finding has implications for the potential of MNV to alter the immune response to, or infectious course of subsequent infections. Several studies have reported on the effect of MNV infection on other infectious model systems. MNV infection did not alter the course of infection of vaccinia virus, influenza A virus, or Friend virus (2, 12). However it did prolong viral shedding of mouse parvovirus and worsen pathology in a model of helicobacter induced inflammatory bowel disease (6, 7, 12). The conclusions regarding the efficacy of MNV to alter the immune response in these studies is complicated by the fact that most of the studies evaluated a single MNV strain at one dose of virus. Data presented here clearly show that MNV induced phenotypes can be strain dependent. Additionally, it is possible that the manifestation of phenotype is dependent of the amount of viral inoculum or length of time the animal has been infected. Interestingly, the studies that found an effect of MNV infection on a subsequent pathogen challenge used a pathogen inoculated via the oral route. It is possible that MNV infection has an effect specifically on immune responses initiated in the gut. This hypothesis can be tested using the assay described here. The anti-cholera toxin antibody response should be measured in CR6 infected mice following oral or peripheral cholera toxin challenge. Further, virus chimeras should be used to identify the gene that facilitates CR6 suppression of the antibody response. This series of experiments may lead to the identification of a pathway for the gut specific suppression of immune responses. This would represent an important advance in our understanding of gut immunobiology.

Overall, the work described here has resulted in the generation of novel reagents for the study of norovirus biology and the identification of specific viral elements that contribute to virus pathogenesis. We have used this system to identify a novel *in vivo* function for the non-structural

protein NTerm, which previously had no known function during viral infection. We have also identified a potential role for norovirus infection in the suppression of immune responses initiated in the gut. Importantly, we have generated new testable hypothesis regarding the consequences of norovirus infection and the function of norovirus proteins *in vivo*.

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Fig.5-1 Cloned CR6 displays rapid increase in titer during *in vitro* infection. RAW 264.7 cells were infected at (A) MOI 0.05 or (B) MOI 5. Virus titer was measured at the indicated times post-infection. Data is from two independently generated stocks and represents three experimental replicates. Dashed line indicates the assay limit of detection. **p<0.01, ***p<0.001.

Figure 5-1

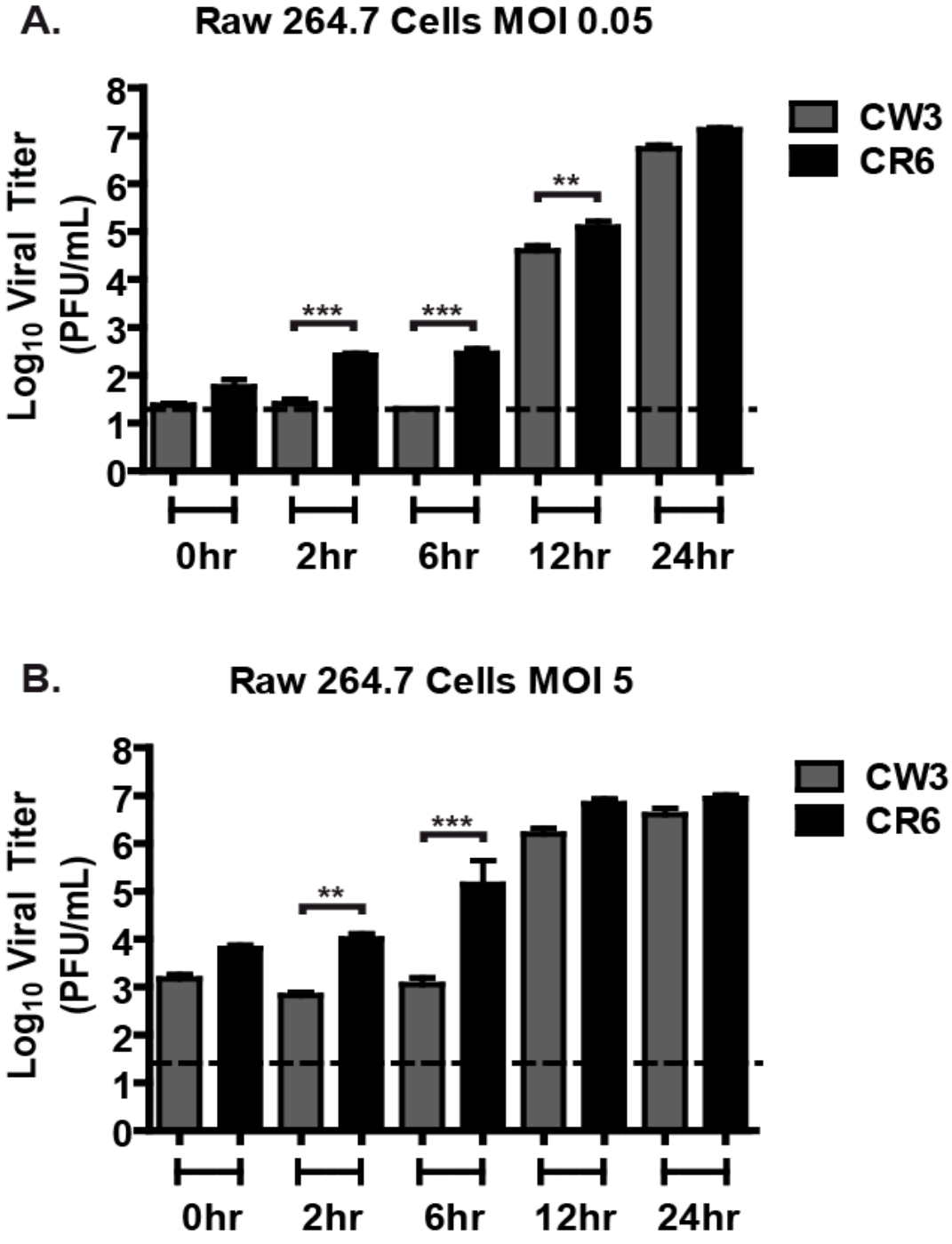

A.
Raw 264.7 Cells MOI 0.05
Log10 Viral Titer (PFU/mL)
CW3
CR6
***
***
**
0hr
2hr
6hr
12hr
24hr
B.
Raw 264.7 Cells MOI 5
Log10 Viral Titer (PFU/mL)
CW3
CR6
**
***
0hr
2hr
6hr
12hr
24hr

Fig.5-2 CR6 infection suppresses antibody response to cholera toxin immunization. Thirty-five days after infection with $3x10^4$ PFU of CW3 or CR6, mice were challenged with 10μg of cholera toxin per os. As a control, uninfected mice were also immunized with toxin. Serum antibody response to cholera toxin was measured by ELISA over the indicated dilution range. The plotted line represents the mean response, measured by absorbance, for the indicated number of mice in each group. The background response from naïve mice was subtracted from each group. Data is from two independent experiments. Statistical comparison is between CW3 and CR6 infected mice. Statistical comparison is between CW3 and CR6.

Figure 5-2

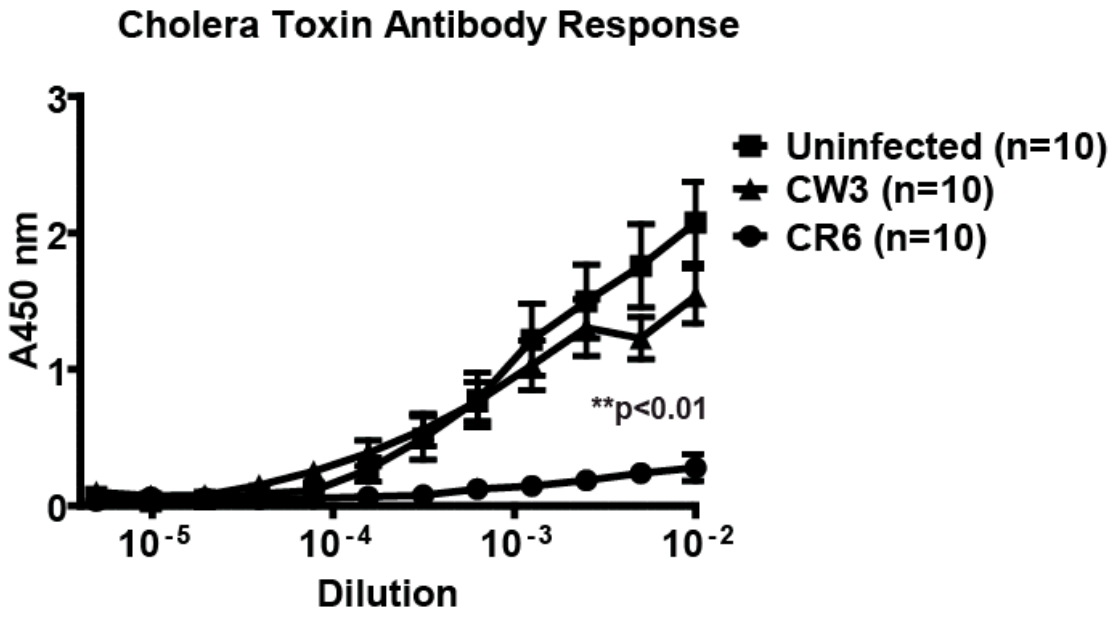

Cholera Toxin Antibody Response
A450 nm
3
2
1
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Dilution
**p<0.01
Uninfected (n=10)
CW3 (n=10)
CR6 (n=10)

Fig.5-3 CR6 has growth defect in CMG14-12 differentiated bone marrow derived macrophages. Bone marrow derived macrophages (BMM) differentiated using cell supernatant from the M-CSF producing cell line CMG14-12 were inoculated at MOI 0.05 with CW3 or CR6. Error bars represent standard error of the mean from 2 independent experiments. Dashed line indicates the assay limit of detection.

Figure 5-3

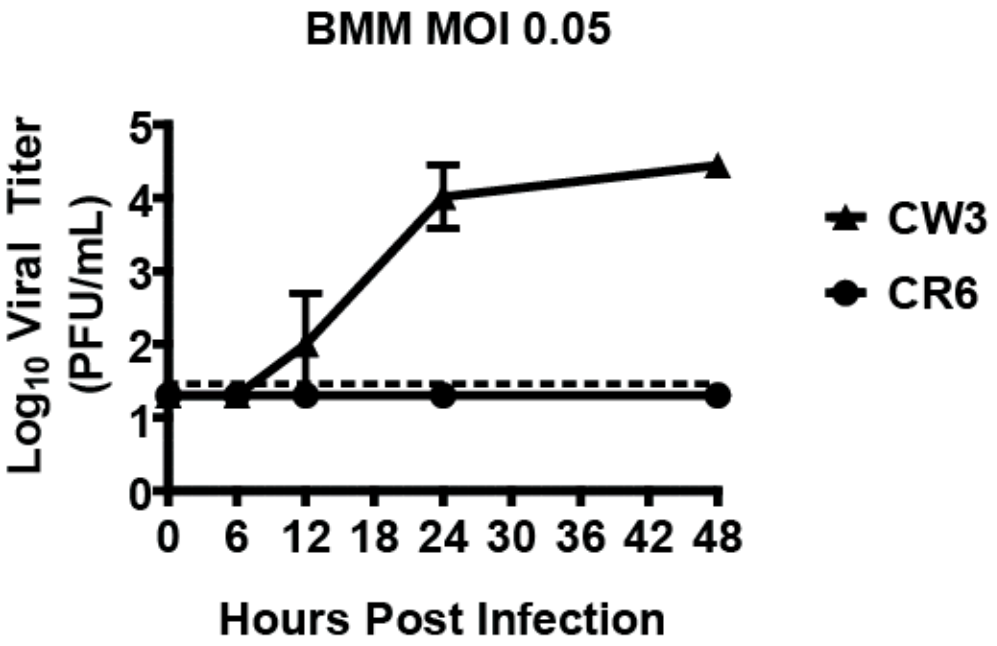
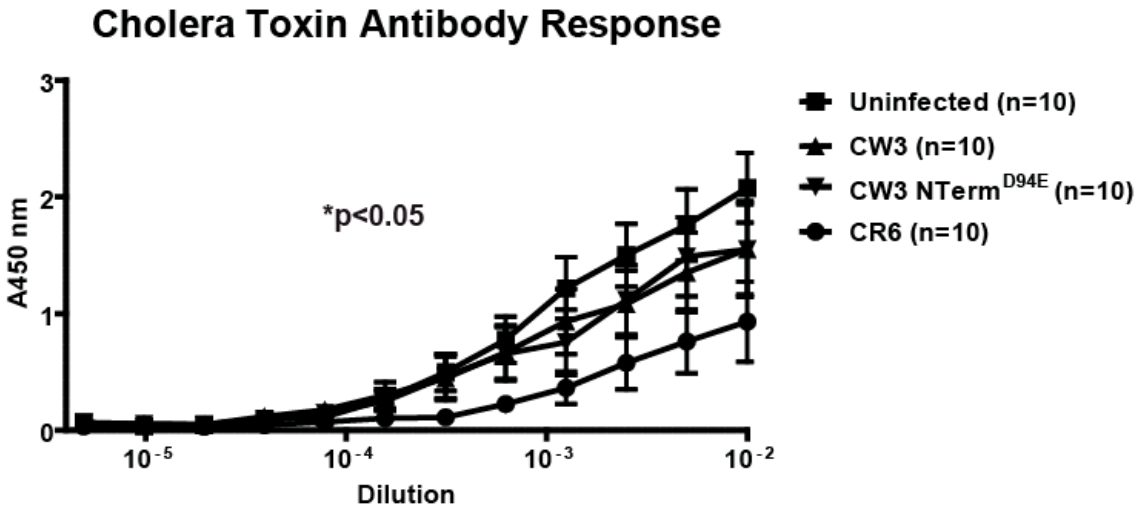

BMM MOI 0.05
Log10 Viral Titer (PFU/mL)
0
1
2
3
4
5
0 6 12 18 24 30 36 42 48
Hours Post Infection
CW3
CR6

Fig.5-4 Viral persistence does not suppress antibody response to cholera toxin immunization. Thirty-five days after infection with $3x10^4$ PFU of CW3, CR6, or CW3 $NTerm^{D94E}$, mice were challenged with 10μg of cholera toxin per os. As a control, uninfected mice were also immunized with toxin. Serum antibody response to cholera toxin was measured by ELISA over the indicated dilution range. The background response from naïve mice was subtracted from each group. The number of mice in each group is indicated in parentheses. Data is from two independent experiments. Statistical comparison is between CR6 and CW3 $NTerm^{D94E}$.

Figure 5-4


Cholera Toxin Antibody Response
A450 nm
3
2
1
0
*p<0.05
10-5
10-4
10-3
10-2
Dilution
Uninfected (n=10)
CW3 (n=10)
CW3 NTermD94E (n=10)
CR6 (n=10)