GASTROINTESTINAL INFLUENCES ON POLIOVIRUS REPLICATION, DISSEMINATION AND PATHOGENESIS IN MICE

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DEDICATION

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Enteric viruses are transmitted between individuals by fecal-oral spread. After oral acquisition, enteric viruses encounter a complex environment within the gastrointestinal (GI) tract, including pH changes, mucus, resident bacteria and a variety of epithelial and immune cell types. Little is known about how factors within and comprising the GI tract influence viral replication, dissemination and pathogenesis.

In order to assess the influence of the intestinal environment on enteric viruses, poliovirus was used as a model enteric virus. Following infection within the GI tract, poliovirus has the capacity to spread to the central nervous system (CNS). Poliovirus infection of the CNS is uncommon, but it can result in acute flaccid paralysis known as poliomyelitis in humans. Poliomyelitis can be mimicked in mice susceptible to poliovirus. Initial studies were performed in mice to examine poliovirus infection within and dissemination from the GI tract to extra-intestinal tissues, including blood and the CNS. By monitoring spread of a marked poliovirus population in susceptible mice, many host barriers to intra-host viral trafficking were identified. Type I interferon responses and intestinal epithelial cell integrity are host barriers that were found to restrict poliovirus. Infecting cells within the GI tract was also difficult for poliovirus, which further limited dissemination from the intestine to the blood and CNS. Bottlenecks were imposed on poliovirus while trafficking through and disseminating from the GI tract, possibly providing an explanation for the low incidence of poliomyelitis disease onset in humans following poliovirus infection.

Because the GI tract was a substantial barrier to poliovirus, studies were undertaken to characterize factors that limit poliovirus dissemination from the GI tract. The naturally-residing microbiota are amongst many other factors present within the GI tract that may influence poliovirus infection. Although suspected to limit poliovirus, intestinal microbiota augmented poliovirus infection in mice and cell culture by enhancing viral infectivity. The studies described herein

demonstrate how host complexity imparts detrimental and beneficial influences on poliovirus acquired by the natural fecal-oral route.

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PRIOR PUBLICATIONS

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GI – gastrointestinal

CNS – central nervous system

WHO – World Health Organization

IPV – inactivated poliovirus vaccine

OPV – oral poliovirus vaccine

VAPP – vaccine-associated paralytic poliomyelitis

VDPV – vaccine-derived poliovirus

RdRp – RNA-dependent RNA polymerase

PVR/CD155 – poliovirus receptor

PVRtg – PVR transgenic

IC – intracranial

IS – intraspinal

IV – intravenous

IN – intranasal

IM – intramuscular

IP – intraperitoneal

IFNAR – interferon α/β receptor

IFN – interferon

M cell – microfold cell

AMP – antimicrobial peptide

MAMP – microbe-associated molecular pattern

PRR – pattern recognition receptor

TLR – Toll-like receptor

NLR – Nod-like receptor

LPS – lipopolysaccharide

MyD88 – myeloid differentiation primary response gene 88

NFκB – nuclear factor kappa B

HIV – human immunodeficiency virus

HCV – hepatitis C virus

PFU – plaque-forming unit

EB – Evan's blue dye

NR – neutral red

DSS – dextran sulfate sodium

FMDV – foot and mouth disease virus

MEF – mouse embryonic fibroblast

PBS – phosphate buffered saline

TC – tissue culture; cell culture

LTA – lipoteichoic acid

PG – peptidoglycan

CHAPTER ONE
Introduction

POLIOVIRUS INFECTION

History and Epidemiology

Poliovirus was first identified over 100 years ago as the etiologic agent for paralytic poliomyelitis in humans (Landsteiner and Popper, 1909); however, it remains a world health burden despite the introduction and continual worldwide use of two effective vaccines developed over 50 years ago. A series of poliomyelitis cases emerged in the late 19th century that eventually led to a poliomyelitis epidemic that sparked intense research efforts during the 1900s (Nathanson and Kew, 2010). Early research on poliomyelitis led to the discovery of the infectious agent (Landsteiner and Popper, 1909), animal pathology studies to determine tissue distribution and mode of transmission (Bodian, 1952b; Sabin, 1956), cultivation of the virus in cell culture (Enders et al., 1949) and eventually the development of two vaccines (Sabin, 1959; Salk et al., 1954).

Poliovirus is an enterovirus in the Picornaviridae family whose transmission occurs via fecal-oral route, and it has the propensity to invade the central nervous system (CNS). CNS infection culminates in acute flaccid paralysis in 0.5% of unvaccinated, infected individuals (Modlin, 1995; Pallansch and Roos, 2001). Three serotypes of poliovirus exist, type 1 (Mahoney), type 2 (Lansing) and type 3 (Leon). Poliovirus type 1 has been recognized as the most prevalent and virulent strain. Poliovirus type 1 and type 3 continue to circulate in

populations in Africa, Asia and the Middle East (CDC, 2010; Nathanson and Martin, 1979). Wild-type poliovirus type 2 has not been detected worldwide since 1999 (CDC, 2001), but vaccine-derived strains are occasionally detected in the environment (Wringe et al., 2008). Despite significant effort by the World Health Organization (WHO) to eradicate poliovirus, ~1600 reported cases of paralytic poliomyelitis currently exist and the disease remains endemic to four countries: Afghanistan, Pakistan, Nigeria and India (WHO, 2010). Localized outbreaks still commonly occur from wild-type poliovirus infections (CDC, 2010) as well as vaccine-derived polioviruses (discussed in "Vaccines and eradication") (Wringe et al., 2008).

Disease manifestations

Poliovirus disease manifestations can range from inapparent or low-grade throat infections to paralytic poliomyelitis (Nathanson and Kew, 2010). Paralytic poliomyelitis is defined as permanent acute flaccid paralysis characterized by poliovirus-induced destruction of lower motor neurons residing in the gray matter of the anterior horn of the spinal cord (Blondel et al., 2005). Although poliovirus has long been known to have neurotropic properties distinguished by the hallmark paralytic disease it is responsible for, the initial site of infection lies within the gastrointestinal (GI) tract. Poliovirus is acquired orally and excreted in feces. Astonishingly, the primary cell infected within the GI tract remains unidentified. Multiple studies have attempted to delineate the tissues

and specific cell type(s) that initiates poliovirus infection in humans, chimpanzees, monkeys and mice (Bodian, 1952b, 1955; Ouzilou et al., 2002; Sabin and Ward, 1941c; Sicinski et al., 1990; Takahashi et al., 2008), but it still remains unclear. A viremic stage is evident prior to CNS invasion (Bodian, 1952a; Horstmann et al., 1954), and poliovirus-specific antibodies with neutralizing capabilities are observed in human and primate sera after infection (Nathanson and Kew, 2010). If poliovirus reaches the CNS, robust replication occurs in neurons and can induce apoptosis directly and/or indirectly, via immune cells, or lysis causing irreparable damage to lower motor neurons. Destruction of neurons can result in paralysis that is usually manifested in the limbs of the infected individual. Disease severity can result in paralytic weakening of just one limb ranging to paralysis of all limbs and other musculature, such as the diaphragm, requiring respiratory assistance (Blondel et al., 2005).

Vaccines and Eradication

Children were among the most susceptible to poliovirus infection, and because of the potentially severe disease outcome, vaccine demand was immense. Jonas Salk and Albert Sabin played instrumental roles in poliovirus research by independently developing two very effective vaccines. Salk prepared a formalin-inactivated, trivalent injectable poliovirus vaccine (IPV), whereas Sabin generated a live-attenuated, trivalent oral poliovirus vaccine (OPV) (now available as monovalent and bivalent as well). The vaccines were

licensed and available in 1955 and 1961, respectively (Nathanson and Kew, 2010; Sabin, 1959; Salk et al., 1954). The advent of two efficient poliovirus vaccines was an extraordinary accomplishment at the time and led to worldwide vaccination programs. By 1973, the United States was declared polio-free, and by 1991, wild poliovirus had been eradicated from the Americas (Nathanson and Kew, 2010). Because of the large success of nearly complete poliovirus eradication in the Americas, in 1988, the WHO established the Global Poliovirus Eradication Initiative outlining the goal of poliovirus world eradication by the year 2000. Paralytic poliomyelitis cases were reduced 99% by 2000 (CDC, 2001); however, worldwide eradication of poliovirus has yet to be achieved. Poliovirus readily circulates in four countries and outbreaks still occur periodically in surrounding countries (CDC, 2010). Endemicity is mostly attributed to refusal to vaccinate, inefficient vaccine coverage, reduced vaccine efficacy in certain populations, virus importation into polio-free zones and fecal excretion of wild-type and vaccine-derived polioviruses that can be transmitted to unvaccinated individuals (Nathanson and Kew, 2010).

The oral live-attenuated poliovirus vaccine developed by Albert Sabin was more widely used upon its introduction because of ease of administration, cost, and the induction of immune protection at the initial site of infection. Soon after the introduction and use of OPV, cases of vaccine-associated paralytic poliomyelitis (VAPP) were reported (Sabin, 1969). Investigators discovered that viruses within the vaccine can revert attenuating mutations and are excreted in

feces of vaccinated individuals (Nathanson and Kew, 2010). The revertant viruses, referred to as vaccine-derived polioviruses (VDPV), also have the ability to infect the CNS and cause paralysis in vaccinees (Georgescu et al., 1994), a process known as VAPP. VAPP incidence is approximately 1 in 1,000,000 vaccinated individuals, and viruses isolated from the CNS of VAPP patients do occasionally, but not always, correlate with viruses isolated from feces of the same individual (Furione et al., 1993; Georgescu et al., 1997; Georgescu et al., 1994; Guillot et al., 2000; Kew and Nottay, 1984; Minor et al., 1986). The realization that VAPP can occur led to conversion of use from OPV to IPV in most developed countries, but under-developed countries still rely on administration of OPV for disease eradication (Nathanson and Kew, 2010).

An additional unfortunate outcome of OPV use was recognized in vaccinated individuals that were later diagnosed with hypogammaglobulinemia, characterized by inefficient antibody production due to B lymphocyte deficiency. Because circulating antibodies can protect from poliovirus dissemination and disease, in their absence, individuals become highly susceptible to disease induced by VDPV (Nathanson and Kew, 2010). Hypogammaglobulinemia patients develop severe VAPP and can excrete VDPV in feces for extended periods of time (Wringe et al., 2008). Continual VDPV excretion puts whole communities at risk, and indeed, multiple outbreaks are attributable to VDPV (Estivariz et al., 2008; Kew et al., 2002; Liang et al., 2006; Rakoto-Andrianarivelo et al., 2008; Wringe et al., 2008). Vaccination cannot be halted since

hypogammaglobulinemia patients can shed VDPV in their feces for a lifetime (MacLennan et al., 2004), potentially exposing communities to virulent poliovirus.

Additional factors confounding poliovirus eradication exist. Poliovirus, like other RNA viruses, mutates quickly, and under selective pressure it can acquire beneficial adaptive mutations that may aid in its transmission and infection cycles (Pfeiffer, 2010). Also, poliovirus genomes can recombine with other poliovirus or enterovirus genomes during a concomitant infection (Arita et al., 2005; Cuervo et al., 2001; Dahourou et al., 2002; Furione et al., 1993; Sergiescu et al., 1969), and the resulting viruses could have increased virulence. Both issues are exacerbated by use of OPV, rather than IPV, because OPV contains viable virus. IPV is now the more preferred vaccine due to the ability of the OPV to revert attenuating mutations and possibly cause paralysis (Nathanson and Kew, 2010). Multiple factors are recognized that impede poliovirus eradication, therefore, understanding fundamental aspects of poliovirus disease within a host will aid efforts to control poliovirus infection and spread within communities.

MOLECULAR BIOLOGY AND REPLICATION CYCLE OF POLIOVIRUS

Virion structure

Poliovirus is classified in the Family Picornaviridae and Genus *Enterovirus*. It is a non-enveloped, single-stranded RNA virus of positive polarity. Virion structure has proven to be an important feature of poliovirus regarding its

effects on infectivity. The poliovirus virion has an icosahedral arrangement composed of 60 copies of each of the four capsid proteins. VP1, VP2 and VP3 are exposed on the outer surface of the virion, while VP4 is internal and only exposed upon either engagement of the viral receptor or during viral “breathing”. The poliovirus virion is resistant to many harsh environments, including low pH, hyper- and hypotonic solutions, proteases, dessication and even organic solvents (Racaniello, 2001). Each capsid protein is tightly associated with the others in order to maintain the virion structure and protect the viral RNA. The poliovirus virion has been identified in several forms, which have densities of 160S, 135S (or A particle) and 80S in sucrose gradients. 160S is the full virion, 135S has VP4 and the amino-terminus of VP1 exposed and 80S represents an empty poliovirus capsid (Hogle, 2002).

The outer landscape of the poliovirus virion is composed of mesas and canyons. Five VP1 proteins interact to form a five-fold axis, termed the “mesa” that resembles a star-like structure. The poliovirus receptor, PVR, also known as CD155, interacts with the virion adjacent to the mesa in a crevice called the canyon. Below the canyon lies a small hydrophobic pocket, unique to enteroviruses (Pallansch and Roos, 2001). In cryo-electron microscopic reconstructions, this pocket commonly contains electron density predicted to be a single-chain fatty acid such as sphingosine or palmitate (Filman et al., 1989; Kim et al., 1993). It is unclear if this “pocket factor” is acquired during viral infection, or if it is an artifact of the processing prior to virion cryo-preservation.

Regardless, antiviral WIN compounds bind in the pocket and greatly stabilize enterovirus virions and inhibit uncoating (Grant et al., 1994; Smith et al., 1996), suggesting biological significance of the pocket. The VP2 and VP3 proteins comprise a three-fold axis resembling propellers opposing the five-fold axis across the canyon, and they contribute to virion stabilization (Hogle, 2002).

VP4 is located on the inner surface of the capsid and interacts with the amino-termini of VP1, VP2 and VP3 (Hogle, 2002). VP4 and VP2 are generated upon autocatalytic cleavage of VP0 (Hindiyeh et al., 1999). Enzymatic processing of VP0 is required for poliovirus infectivity (Fernandez-Tomas and Baltimore, 1973; Lee et al., 1993) and is consistent with RNA packaging (Hindiyeh et al., 1999; Vance et al., 1997). VP4 is occasionally surface-exposed since antibodies raised to a VP4 epitope can neutralize virus (Li et al., 1994).

Virion structure can change dynamically. Although the exact stimulus remains unknown, shifts in virion structure ("breathing") occur at physiological temperatures (Li et al., 1994). Viral breathing is a phenomenon that has been characterized for several animal and plant viruses (Bothner et al., 1998; Lewis et al., 1998; Li et al., 1994; Tama and Brooks, 2002; Zulauf, 1977). Essentially, breathing is expansion and collapsing of the virion in which some proteins, including poliovirus VP4 and the N-terminus of VP1, can be exposed outside the virion rather than internally contained (Li et al., 1994). The function of viral breathing is not fully understood. Dynamic virion rearrangements may be important for cell entry and nucleic acid release since antiviral WIN compounds,

which inhibit picornavirus breathing, stabilize virions and prevent enzymatic degradation and cell entry (Dove and Racaniello, 2000; Lewis et al., 1998).

Receptor binding and entry

Poliovirus requires its cognate receptor, PVR, for entry into cells and subsequent replication. Human PVR was initially identified and cloned in 1989 (Mendelsohn et al., 1989). PVR is a member of the immunoglobulin superfamily, like many other viral receptors (Dermody et al., 2009). The gene encoding PVR is differentially spliced such that four unique forms are expressed: α, β, δ and γ (Koike et al., 1990). PVRα and PVRδ are integral membrane glycoproteins, whereas PVRβ and PVRγ spliceoforms lack a transmembrane domain and are secreted. All PVR spliceoforms contain three variable loops, of which the outermost amino-terminal variable loop binds poliovirus to mediate cell entry (Koike et al., 1991a; Selinka et al., 1991). Eight glycosylation sites were predicted on the amino-terminal variable loop region for PVR (Mendelsohn et al., 1989), and the two sites residing on variable loop one are dispensable for poliovirus binding and entry in cell culture (Koike et al., 1992; Zibert and Wimmer, 1992). A cellular role for PVR was only hinted at in 2003 in which it was shown to play a role in cell-cell interactions (Mueller and Wimmer, 2003), and more recently in relation to monocyte migration and NK cell activation by target cells (Fuchs et al., 2004; Reymond et al., 2004; Tahara-Hanaoka et al., 2004).

PVR is expressed in humans and has homologs in apes and monkeys and orthologs in many other mammals (Ida-Hosonuma et al., 2003). Humans are the only natural host, while apes and old world monkeys can be infected experimentally (Nomoto et al., 1994). Animal models for poliovirus infections will be discussed later in the section “Poliovirus Animal Model Systems”.

Variable loop one of cell-surface exposed PVR engages the poliovirus virion on the canyon surface (Koike et al., 1991a; Selinka et al., 1991), after which major virion conformational rearrangements occur. It has been proposed that VP4, an alpha helical myristoylated protein, becomes surface exposed upon receptor binding and may be involved in membrane channel formation after receptor recognition (Tosteson et al., 2004). This interaction may guide the virion closer to the cell membrane by interaction of the myristate groups on VP4 with the lipid bilayer (Tosteson and Chow, 1997). Until recently, it was presumed that the virion five-fold axis widens releasing the VP3 protein “plug” at the base of the axis allowing viral RNA to pass from the virion into the cell cytoplasm through a channel formed by five copies of VP1 (Hogle, 2002). However, a recent study argues that viral RNA release from the virion occurs adjacent to the five-fold axis at a two-fold axis site (Bostina et al., 2010). Mechanistic details are unclear concerning viral RNA release in the host cytoplasm, but at least in fibroblast cells, it is clear that the poliovirus-receptor complex is endocytosed and viral RNA release into the cytoplasm occurs almost immediately following endocytosis (Brandenburg et al., 2007).

Replication initiation and translation

Once in the cell cytoplasm, poliovirus RNA is immediately translated as one polyprotein of 2209 amino acids. To initiate viral protein translation, the ribosomal translation machinery is recruited to the viral RNA via the internal ribosome entry site, a secondary RNA structure in the 5' untranslated region of the poliovirus genome (Pelletier et al., 1988). The resulting polyprotein is autocatalytically cleaved by $2A^{pro}$ and $3C^{pro}$ proteases into 12 distinct proteins, including non-structural proteins involved in replication and four structural capsid components (Pallansch and Roos, 2001).

Viral RNA translation and replication are unlinked and depend on the viral cloverleaf, which is a secondary RNA structure in the 5' untranslated region important for viral RNA replication (Andino et al., 1993). Like other RNA viruses, poliovirus replication occurs in a complex that is composed of viral and cellular proteins that are anchored on internal cellular membranes (i.e. autophagic vesicles of endoplasmic reticulum origin for poliovirus) to act as virus factories (Jackson et al., 2005; Salonen et al., 2005; Suhy et al., 2000). Of importance to studies outlined in this manuscript, the viral RNA-dependent RNA polymerase (RdRp), $3D^{pol}$, is an error-prone polymerase used to generate viral RNAs for further replication, packaging and release (Drake, 1993). The RdRp lacks proofreading capability, and therefore, many poliovirus genomes are generated that are similar, but differ by one to a few mutations. This swarm of genetically related yet distinct viral genomes is termed a viral quasispecies (Domingo et al.,

1985) (discussed in “Viral evolution: quasispecies and barriers”). Within the viral replication complex, negative-strand viral RNAs are transcribed to positive-strand RNAs by the RdRp that recognizes the protein primer VPg, a product of 3AB cleavage (Paul et al., 1998). Nascent viral RNAs are ~7440 nucleotides and complete with 5’ and 3’ untranslated regions. The 5’ region contains structures important for viral protein translation and viral RNA replication. The 3’ untranslated region contains the poly-A tail, an important genomic feature for multiple translation events to occur from one RNA and to circumvent host detection (Mueller et al., 2005). Multiple RdRps attach to and transcribe viral RNA, making further synthesis more efficient (Pata et al., 1995). Numerous viral RNAs are generated within a host cell to be packaged in viral capsids and released from cells.

Virion packaging and release

Positive polarity poliovirus RNAs are packaged within icosahedral viral capsids inside host cells. When a threshold number of virions are produced, the cell lyses and virions are released to infect naïve cells. Because of the abundant generation of poliovirus RNAs by the low fidelity RdRp and inefficient virion formation and maturation, only a fraction of the released virions (about 1 in 30 to 1000) are infectious, yielding a high particle to plaque forming unit (PFU) ratio (Racaniello, 2001).

POLIOVIRUS ANIMAL MODEL SYSTEMS

Host range and specificity

The only known natural hosts of poliovirus infections are humans; however, some apes and old world monkeys can be experimentally infected (Nomoto et al., 1994). The limited host range is attributed to expression of the viral receptor, PVR, which is expressed in humans, apes and monkeys. Although orthologs in other mammalian species exist, they are too divergent to function as proper receptors for poliovirus entry into cells (Aoki et al., 1994). This has posed a major challenge to the poliovirus research community since a small animal model was only developed 20 years ago (Ren et al., 1990).

Non-human primate models

Initial *in vivo* characterization of poliovirus pathogenesis was carried out in apes and monkeys (Bodian, 1952b; Sabin, 1956). Experimental infections were performed in a variety of primate species, including chimpanzees, macaques and African green monkeys (Racaniello, 2006). Investigators assessed host range within the primate order and determined that the majority of new world monkeys are not susceptible to poliovirus, and therefore, would not serve as a good model to study virus-host interactions (Hsiung et al., 1964). A general understanding of poliovirus tissue tropism, viremia and immunity was gained from ape and monkey studies (Racaniello, 2006). Poliovirus replication was restricted to the

oropharynx, GI tract and CNS neurons despite viremic seeding of additional organs, and just as in humans, paralysis was rare. Aspects of poliovirus disease manifestations in primate models strikingly mirrored human infections (Bodian, 1955; Sabin, 1956). However, primate models are intractable as they are costly and more difficult to maintain, therefore, a tractable small animal model was needed.

Mouse models

For several decades, attempts had been made to develop a small animal model to study poliovirus pathogenesis and spread within a host. Some strains of poliovirus were shown to infect and replicate in mice after intracranial inoculation, especially serotype 2 of poliovirus (Racaniello, 2006). Unfortunately, these mouse models were very limited in that they required or were adapted to use a different viral receptor than PVR, as PVR is not naturally expressed in mice. It was not until the mid-1980s, that investigators fully understood that rodents do not express a receptor sufficient for robust poliovirus infection. Mouse L cells transformed with a gene encoding human PVR demonstrated enhanced poliovirus susceptibility (Mendelsohn et al., 1986). Following the discovery of the receptor for poliovirus, human PVR (Mendelsohn et al., 1989), the first PVR-transgenic (PVRtg) mouse line was generated and successfully infected with poliovirus (Ren et al., 1990). This groundbreaking paper was the first of many reports on the generation of PVR-expressing mice (Crotty et al., 2002; Deatly et

al., 1998; Koike et al., 1994). PVRtg mice express the receptor nearly ubiquitously, however, not all cell types are permissive for poliovirus replication. For example, kidney is relatively resistant to poliovirus infection, suggesting that PVR is not the sole determinant of tissue tropism during poliovirus infection (Koike et al., 1991b; Ren and Racaniello, 1992a). None of the aforementioned PVRtg lines generated were orally-susceptible to poliovirus-induced disease, even despite over-expression of PVR in mice intestines under regulation of the fatty acid binding protein promoter (Zhang and Racaniello, 1997). Therefore, the natural route of infection remained unstudied.

Mechanisms of poliovirus infection and spread within mice following intracranial (IC), intraspinal (IS), intravenous (IV), intranasal (IN), intramuscular (IM) and intraperitoneal (IP) inoculation were becoming more well understood because PVRtg mice developed poliovirus symptoms following introduction via these routes. Poliovirus replicated within the CNS of PVRtg mice following IC, IS, IM and IN administration (Dragunsky et al., 1996; Nagata et al., 2004; Ren and Racaniello, 1992b; Ren et al., 1990). Following IM inoculation of poliovirus in the mouse hindlimb, viral trafficking in neurons is dependent on neuronal spread since transection of the sciatic nerve after inoculation limited limb paralysis (Ohka et al., 1998; Ren and Racaniello, 1992b). Poliovirus dissemination routes after IP infection are somewhat elusive, but this inoculation route is understudied compared to the former five. However, poliovirus has been isolated from lymphatic tissues following IP inoculation (Buisman et al., 2003), suggesting viral

spread through the lymphatic ducts that drain into the bloodstream. Although many laboratories elucidated fundamental factors involved in poliovirus infection in mice using many different routes of infection, the mystery remained as to why PVRtg mice that clearly expressed PVR within their intestinal tracts remained resistant to infection with poliovirus.

Almost two decades after the introduction of the first PVRtg mouse line, an orally-susceptible poliovirus mouse model was developed (Ida-Hosonuma et al., 2005; Ohka et al., 2007). Thorough investigations outlined the importance of innate immunity in controlling poliovirus infection following oral inoculation. PVRtg-interferon α/β receptor $(IFNAR)^{-/-}$ mice were generated in which PVR is expressed nearly ubiquitously, as in other PVRtg mice, but they also have IFNAR deleted such that cells cannot respond to induction of interferon (IFN) β (Ohka et al., 2007). Lack of an IFNα/β response abolishes a strong innate immune component known to aid in clearance of many viruses. Poliovirus sensitivity to an IFNα/β-induced antiviral cellular state is a determinant of tissue tropism (Ida-Hosonuma et al., 2005). PVRtg-$IFNAR^{-/-}$ mice were used extensively in the experiments addressed in this document to understand poliovirus spread from the gastrointestinal (GI) tract to peripheral tissues as well as the role of the GI tract and components within the GI tract during oral poliovirus infection.

Poliovirus trafficking within a host

Poliovirus is an enteric virus that has the ability to occasionally invade the CNS. The direct route of dissemination from the GI tract to the CNS is unknown. Observations from studies using the chimpanzee model in comparison to humans in the mid-20th century revealed poliovirus infection initiation in the GI tract. Viral replication occurred readily in oropharyngeal tissues of chimpanzees, probably in lymphatic tissues, but appeared limited in human oropharyngeal tissues despite replication within the intestine (Bodian, 1952b; Sabin, 1956; Sabin and Ward, 1941b). These studies also demonstrated virus within GI tissues, suggestive of productive infection. Virus was thought to translocate to the bloodstream via lymphatic drainage from GI tissues, and occasionally spread to the CNS by crossing the blood-brain barrier (Bodian, 1952b; Sabin, 1956). It is well documented that after oral inoculation in primates, viremia precedes CNS invasion (Bodian, 1952a; Horstmann et al., 1954), and serum antibodies to poliovirus are generated and can limit poliovirus spread to the CNS (Nathanson, 2008). If poliovirus invades the CNS, it transits in motor neurons via retrograde axonal transport, and subsequent replication and lysis or apoptosis of neurons can result in paralytic poliomyelitis (Nathanson and Kew, 2010). After many decades of poliovirus research, the current view of poliovirus trafficking within a host is viral translocation across the GI mucosal barrier, a primary viremia that seeds peripheral organs in which replication occurs, secondary viremia and potential CNS infection arising most likely from poliovirus infection of neurons

innervating blood-enriched tissues (Pfeiffer, 2010). This proposed view of poliovirus trafficking within a host has not changed substantially in comparison to that previously proposed from non-human primate studies over 60 years ago. A few reasons for the superficial understanding of poliovirus dissemination after oral acquisition is, in part, due to the long-term lack of an appropriate small animal model that mimics the natural oral route of infection, and the unknown identity of the primary infected cell within the GI tract. More thorough investigation is required to understand how poliovirus spreads from the GI tract to the CNS.

Poliovirus transit and infection in the gastrointestinal tract

Poliovirus can be acquired from direct contact with infected individuals or consumption of poliovirus-contaminated water (Pallansch and Roos, 2001). Once poliovirus is ingested, it is exposed to many diverse environments as it transits through the GI tract. Initially, poliovirus encounters the oral cavity lined with epithelial cells in close contact with lymphoid tissues in the oropharynx, such as the tonsils. The tonsils are important lymphoid structures that aid in detection and elimination of pathogens acquired orally and/or nasally. Foreign antigens are transported from the tonsils to the lymph nodes by dendritic cells in order to mount an immune response for pathogen clearance (KleinJan, 2011). Early studies demonstrated poliovirus replication in the oropharynx of chimpanzees

(Sabin, 1956), but replication in human oropharyngeal tissues appeared minimal (Bodian, 1952b; Sabin and Ward, 1941a).

It is likely that poliovirus infects epithelial or lymphoid cells of the GI tract, replicates and disseminates or is shed in feces (Racaniello, 2006). The initial cell(s) infected by poliovirus within the GI tract and responsible for viral shedding into the GI lumen following replication is unknown. Humans infected with poliovirus typically shed virus in feces for 2 to 8 weeks post-infection (Nathanson, 2008), contributing to viral transmission within unvaccinated communities.

Throughout the lower GI tract, poliovirus is exposed to a very complex, dynamic environment, such as the acidic environment of the stomach, digestive enzymes and bile salts, peristalsis, mucus and other microorganisms. Within the oral cavity, virus is exposed to digestive enzymes in saliva and commensal microorganisms. Additionally, the parasympathetic nervous system stimulates GI tract peristalsis for movement of food for proper nutrient acquisition, which may restrict poliovirus-receptor interactions. Peristaltic movement propels poliovirus to the stomach, which maintains a low pH of 1.5-2.0 for digestion of foodstuffs. Virus is then transported through the intestinal lumen and contacts abundant bile salts and digestive enzymes produced by the liver that drain to the GI lumen through the bile duct. The GI tract is layered with mucus to protect itself from harmful effects of enzymes, bile salts and microbes. Mucus is composed of several different types of mucins produced by goblet cells. The interweaving of mucin glycoproteins comprises a dual mucus layer that lubricates and protects

the GI tract from damage. Another major benefit of mucus within the intestines is to prohibit commensal microbial invasion of the mucosal barrier as the inner mucus layer is largely devoid of bacteria (Johansson et al., 2008).

Intestinal microorganisms have developed a symbiosis with their hosts and are often referred to as commensals since they contribute substantially to digestion of dietary polysaccharides, host nutrient acquisition and metabolism, maintenance and movement of the GI tract, and protection of the epithelium from enteric bacteria and protozoan parasites. The intestinal microbiota is composed of an estimated ~1000 species of bacteria that represents a beneficial, but sometimes threatening, community naturally residing within mammalian intestines (Duerkop et al., 2009). Aside from mucus, two key factors involved in bacterial restriction are secretory immunoglobulin A (sIgA) and antimicrobial peptides (AMP) (Hooper and Macpherson, 2010). Secretory IgA, produced by plasma cells residing in Peyer's patches, are induced by and neutralize intestinal microbes (Macpherson et al., 2000). In addition to IgA, AMPs, such as C-type lectins and defensins, are antagonistic to bacteria within the GI tract. C-type lectins, α-defensins and defensin-related cryptidins are induced by the natural flora following ligation of microbe-associated molecular patterns (MAMPs) to pattern recognition receptors (PRR) (Brandl et al., 2007; Kobayashi et al., 2005; Vaishnava et al., 2008). Many forms of PRRs exist, including Toll-like receptors (TLR) and Nod-like receptors (NLR). These receptors recognize different microbial components, such as lipopolysaccharide (LPS) from Gram-negative

bacteria. Upon activation of PRRs, a cytoplasmic adapter protein, myeloid differentiation primary response gene 88 (MyD88), activates the transcription factor nuclear factor kappa B (NFκB) through a signaling cascade involving multiple molecules. Activated NFκB translocates to nuclei of cells and initiates production of pro-inflammatory genes. In the intestine, pro-inflammatory cytokines, such as IL-6 and TNFα, induce immune cells, sIgA and AMPs that regulate bacteria to block infection (Artis, 2008). Symbiotic bacteria within the GI tract tremendously impact host health and contribute to maintenance of homeostasis within the intestine.

Much of the insight gained about microbiota-host interactions is attributable to use of microbiologically-sterile germ-free mice or antibiotic-treatment of mice to significantly reduce the bacterial load within the intestines. Whereas germ-free mice are extremely useful tools, they exhibit stark differences from mice raised in a conventional setting. Germ-free mice have under-developed immune systems and a variety of physiological deficiencies (Smith et al., 2007). Importantly, germ-free mice are expensive to maintain and there is limited availability of mouse strains. Therefore, a well-used alternative to germ-free mice is antibiotic treatment of conventional (harboring a natural microbiota) mice that can be maintained with relative ease in a specific pathogen-free facility. In addition, conventional mice are equipped with a fully-mature immune system and normal physiological functions.

Despite the recent abundance of knowledge gained about the intestinal microbiota, it is largely unknown how the natural flora affects viruses and whether they impact viral disease. Evidence from studies with germ-free mice suggests that the microbiota promote viral infections (Mirand and Grace, 1963; Schaffer et al., 1963). However, these experiments bypassed the intestine by IP injection of virus into mice, therefore, the experiments only addressed systemic effects of the microbiota. An additional study of multiple viruses in conventional and germ-free mice showed no change in viral load or susceptibility between the two mouse groups for all viruses tested (Tennant et al., 1965). Of the enteric viruses used in this study, rotavirus and reovirus type 3, only rotavirus was orally-inoculated into mice. Despite past conflicting reports on the effects of microbiota on influenza A virus infection (Dolowy and Muldoon, 1964; Tennant et al., 1965), a recent report underlines the importance of immune induction by the microbiota in regulating influenza A infection in mice lungs (Ichinohe et al., 2011). Although several of the above reports implicate commensal-derived protection against viral infections, others suggest the opposite. Stimulation of TLR4, which recognizes LPS on the surface of Gram-negative bacteria, is paramount in maintaining mouse mammary tumor virus transmission via interleukin-10 immunosuppressive activity (Jude et al., 2003). Additionally, Theiler's murine encephalitis virus and human immunodeficiency virus (HIV) infections are both exacerbated by systemic LPS (Brenchley et al., 2006; Pullen et al., 1995). A full understanding of how intestinal microbes influence enteric viral infections is lacking.

Not only does poliovirus have to contend with GI factors within the lumen, but somehow the virus must locate, bind and enter cells for propagation and further spread. The cellular architecture of the GI tract is composed of a single layer of epithelial cells atop a layer of connective tissue, the lamina propria, which together comprise the GI mucosa. However, the composition of the mucosa is much more complicated. Within the single enterocyte layer are several specialized epithelial cells: microfold (M), goblet and Paneth cells (Hooper and Macpherson, 2010). M cells function as sentinels located throughout the GI tract that sample and survey lumenal contents for potential pathogens and toxic substances, and they are intimately linked to the underlying GI immune system via Peyer's patches. Peyer's patches house monocytes and lymphocytes that can respond quickly to harmful foreign substances. Secretory IgA is produced by B lymphocytes within Peyer's patches, and is an important host immune defense mechanism to maintain homeostasis with commensal microorganisms (Macpherson et al., 2000). Goblet cells are responsible for the production of mucins that help minimize damage to the mucosal barrier. The outermost mucus layer is loosely arranged and easily penetrable by bacteria, whereas the mucus aligning the apical surface of the epithelial layer is compact and limits bacterial invasion (Johansson et al., 2008). Paneth cells reside in the crypts of Lieberkühn in the small intestine and colon and are the main source of AMPs that are crucial for controlling the vast numbers of bacteria in the GI lumen (Bevins and Salzman, 2011). Aside from these specialized cells that help limit bacterial translocation

across the mucosa, immune cells and lymphocytes are interspersed throughout the lamina propria and in epithelial cell junctions to aid in rapid detection of foreign antigens that may require assistance with clearance via recruitment of other, activated immune cells. The architecture and composition of the GI tract is important to allow nutrient acquisition while protecting itself from possible insults from the bacterial community harbored in the lumen. How most of these factors affect viral infections is largely unstudied.

Cell entry and infection in the GI tract has been delineated for the enteric virus reovirus and may provide insight for poliovirus infections in the GI tract. Reovirus transcytoses M cells to infect Peyer's patches (Wolf et al., 1981). Intestinal epithelial cells are directly infected with reovirus from both basolateral and apical surfaces and shed virus into the GI lumen (Bass et al., 1988; Rubin et al., 1985; Wolf et al., 1987). Additionally, proteases in the GI tract activate reovirus by cleaving the outer capsid, which is pertinent for infection to ensue (Amerongen et al., 1994). Polarized Madine-Darby Canine kidney cells transduced with PVR revealed basolateral sorting of the receptor (Ohka et al., 2001). The results from this study suggest that poliovirus may infect polarized cells similarly as reovirus, from the basal surface, and then be shed into the GI lumen. It is plausible that poliovirus entry and infection in the intestine is similar to that of reovirus, but it is unclear which cells poliovirus infects and replicates in within the GI tract. Poliovirus has been shown associated with M-like cells in rhesus macaques (Takahashi et al., 2008), human Peyer's patches and M cells

ex vivo (Sicinski et al., 1990) and M-like cells associated with mouse Peyer's patches co-cultured with epithelial cells (Ouzilou et al., 2002). These observations together with the isolation of poliovirus from chimpanzee and occasionally human lymphatic tissues (Bodian, 1952b; Sabin, 1956; Sabin and Ward, 1941a, b), as well as poliovirus replication in lymphocytes (Eberle et al., 1995; Freistadt and Eberle, 1996; Wahid et al., 2005a; Willems et al., 1969) implicates lymphoid cells as a prime candidate for supporting poliovirus replication in the GI tract. Conversely, a study in humans with attenuated type 3 poliovirus revealed no viral replication in lymphatic tissues despite productive infection in the GI tract (Sabin, 1955; Sabin, 1956), suggesting an epithelial cell origin for viral replication. Additional observations in poliovirus-infected humans post-mortem and chimpanzees at day four post-inoculation showed little virus recovery in lymph nodes, whereas abundant virus was recovered from pharyngeal and intestinal mucosal washings (Sabin and Ward, 1941a). In addition, poliovirus was visualized only in epithelial cells in microvilli that were not of M-like or goblet cell origins in PVRtg-IFNAR$^{-/-}$ mice (Ohka et al., 2007). Aside from these limited data, virtually nothing is known about specific poliovirus interactions within the GI tract *in vivo*.

Cells responsible for release of poliovirus into the GI lumen remain unidentified. After transit through to GI tract, poliovirus is shed in feces (Pallansch and Roos, 2001), even following IP inoculation (Boot et al., 2003), indicating infection in the GI tract after introduction via this route. Epithelial cells

lining the mucosa have been proposed as virus shedding cells since Caco-2 cells can be infected basolaterally and release poliovirus virions from the apical surface (Tucker et al., 1993). Caco-2 cells are polarized human colonic epithelial cells that model the epithelial layer *in vivo*, and they primarily express PVR on the basolateral surface. Poliovirus is commonly shed in feces from people administered OPV or naturally infected, but poliovirus also has the ability to disseminate from the GI tract.

Dissemination from the gastrointestinal tract

The mode of poliovirus dissemination from the GI tract is unknown, owing partially to the difficulty in identifying the initial cells infected within the GI mucosa. Viremia precedes CNS invasion in non-human primates (Bodian, 1952b; Horstmann et al., 1954) and likely humans as well (Melnick et al., 1961), but remains unclear in mice. Primary and secondary viremic stages can occur. Secondary viremia can arise after poliovirus spreads hematogenously to non-neuronal tissues, undergoes replication, and again reaches the bloodstream. Once viremia onset is reached, poliovirus either invades neurons that innervate tissues throughout the body, or it translocates across the blood-brain barrier (Pfeiffer, 2010). Poliovirus replicates robustly in CNS neurons, but despite this predilection for the CNS, transmission from this site is probably rare and is a dead end for poliovirus.

Infection and transit in motor neurons

Poliovirus enters motor neurons via receptor-mediated endocytosis at the neuromuscular junction. It then traffics within an endosome through neuronal axons. The cytoplasmic portion of PVR binds Tctex-1, which interacts with the microtubule light-chain motor protein, dynein, to ride along this filament to the cell body (Mueller et al., 2002). Viral replication ensues in the cell body of neurons where all the appropriate host cell machinery is available to the virus. Poliovirus replication induces apoptosis or lysis for progeny virion release and infection in naïve neurons (Girard et al., 1999). Neuronal damage, especially in the spinal cord, can result in paralytic poliomyelitis that is usually manifested in limbs.

Poliovirus as a model system

Poliovirus is a tractable virus to use as a model system for understanding other enteric viruses (norovirus, reovirus, rotavirus, enterovirus 71, etc.) as well as neurotropic viruses (West Nile virus, rabiesvirus, measles, etc.). One main reason is availability of reagents and knowledge gained from over 100 years of research, as well as effective, approved vaccines for poliovirus that minimize safety concerns. Poliovirus undergoes robust replication in cell culture unlike some enteric viruses, and can be easily grown and quantified. Multiple tools, like susceptible mouse models and virus-specific antibodies, are available. Together, these aspects demonstrate how poliovirus is an attractive system to study viruses.

In addition, insight gained from poliovirus studies might aid eradication efforts. For instance, new vaccines and strategies to disrupt poliovirus infection and transmission are being developed by multiple laboratories. It is also important to learn what aspects of the two vaccines facilitate elicitation of efficient protection, whether it involves viral and/or host factors, and how we can apply this to other viruses for vaccine development. Studies to understand poliovirus pathogenesis could prove beneficial for these endeavors.

Although poliovirus is beneficial as a model for enteric and neurotropic viruses, focus will be on poliovirus used as a model system for enteric viruses. Developing mouse models to study enteric viruses has proved challenging. Mice are inherently more resistant to enteric infections than humans. Viruses from the Reoviridae Family, e.g. reovirus and rotavirus, require neonatal mice that are typically infected within days of birth for *in vivo* pathogenesis studies (Guglielmi et al., 2006). Because of this limitation, gestating mice must be monitored frequently for parturition, and only a limited time frame is available to inoculate mice and follow the course of disease. In addition, the number of pups produced is also unpredictable, and handling of neonatal mice is more difficult that adult mice. Aside from reoviruses, mouse models are also limited for enterovirus 71, the etiologic agent of hand, foot, and mouth disease. To study enterovirus 71 pathogenesis in mice, a neonatal mouse model and mouse-adapted virus model are used (Chen et al., 2004; Wang et al., 2004b). Mouse models are lacking for other important human viruses, such as norovirus that commonly causes

gastroenteritis. Therefore, murine models are based on related mouse viruses, such as murine norovirus (Wobus et al., 2006). The enteric model for poliovirus is still not ideal in that the mice are immunodeficient, as are many virus infection mouse models. However, the oral poliovirus model uses adult mice and the natural human virus, making virus-host pathogenesis studies more tractable and applicable to human infections.

VIRAL EVOLUTION: QUASISPECIES AND BARRIERS

RNA viruses and quasispecies theory

Poliovirus is often used as a model virus to investigate RNA viral population dynamics. In studies included herein, I used viral diversity as a tool to study host barriers encountered by a poliovirus population. RNA viruses likely have the highest mutation rates in nature, and therefore, have presented a considerable problem in the control of infectious viral diseases (Domingo and Holland, 1997; Drake, 1993). Many RNA viruses circulate in human populations and cause significant disease, such as poliovirus, hepatitis C virus (HCV) and HIV. It is partly because of the genetic variability imparted by error-prone viral replication that developing vaccines and treatments for the former two has been an extreme challenge (Margeridon-Thermet and Shafer, 2010). Mutations frequently arise within RNA viral populations that confer protection from host

defenses and/or resistance to drug therapies, and therefore, resistant viral variants expand within the population and are difficult to control.

RNA viruses are prone to high mutation frequencies by nature of their genetic composition. RNA viruses rely on their own RNA polymerases for replication and subsequent packaging. However, viral RdRp and reverse transcriptase lack proofreading capabilities and frequently generate mutations typically resulting in one mutation per 10,000 nucleotides (Domingo and Holland, 1997; Domingo et al., 1978; Drake et al., 1998; Mansky and Temin, 1995). The majority of misincorporated mutations are detrimental and result in non-viable viral RNAs that are often packaged into virions. Non-viable, packaged RNAs are partially responsible for the hallmark high particle to PFU ratio of RNA viruses, which ranges from 30:1 to 1000:1 (Racaniello, 2001). For example, if a monolayer of cells is infected with 5000 virions, only 5-167 plaques will form. Seldom, a mutation will arise to benefit the virus typically resulting in increased fitness, the ability of an organism to reproduce or replicate within a specific niche (Domingo and Holland, 1997). Due to the high error frequency of the RdRp, the resulting swarm of genetically similar genomes that can differ by one to a few nucleotides is often referred to as a “viral quasispecies”.

Quasispecies theory

The theory of viral quasispecies originated from a mathematical model developed by Eigen and Schuster in the 1970s in reference to the origin and

evolution of macromolecules in a pre-cellular world (Eigen and Schuster, 1977). The quasispecies theory conceptualizes that simple entities composed of large, self-replicating populations capable of high reproducibility, exist in nature as a heterogeneous mixture of molecules that are all related to a master, or consensus, sequence. Most sequences are "neutral mutants" and have no advantageous or disadvantageous trait, but excess mutation accumulation throughout the population can result in error catastrophe, an event in which a population cannot recover from the incorporation of too many mutations. Eigen and Schuster also postulated that incorporation of too few mutations would interfere with the ability to overcome environmental pressures. In the quasispecies theory, a true "wild-type" does not exist; rather, it is upheld as the master sequence within a population from which other sequences are derived. Virologists quickly adopted this population genetics theory and applied it to viral population dynamics.

Viral quasispecies

The existence of a large population of genetically-conserved, but distinct, viruses was first shown experimentally in the 1970s using Qβ RNA bacteriophage (Batschelet et al., 1976). Since then, many virologists have been intrigued by viral quasispecies and have tried to understand this viral heterogeneity. In consideration of RNA viruses, they are thought to have evolved the perfect error rate in which they replicate at the error threshold, meaning that they maintain a

manageable amount of mutations to cope with selective pressures, but uphold the basic genetic components required for viability (Domingo and Holland, 1997).

Despite the enormous potential for RNA viruses to inactivate or over-mutate their genomes, the low fidelity polymerase is paramount to afford them the mutation capacity to overcome pressures within a host that limit viral replication and transmission. Diversity of RNA viral populations is biologically significant for viral infectivity in whole organisms, as has been shown with poliovirus in mice (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). Polioviruses with a high fidelity polymerase were attenuated in mice compared to polioviruses harboring the low fidelity polymerase, indicating that increased viral diversity correlates with greater pathogenesis. Importantly, follow-up studies suggest diminution of viral quasispecies as an effective vaccine strategy (Vignuzzi et al., 2008). RNA viruses are constantly at battle with their hosts, and it is clear that they require continual but steady misincorporation of nucleotides in order to generate mutations that allow them to overcome bottleneck events within a host that limit viral diversity.

Bottleneck events and host barriers to viruses

Genetic bottlenecks can occur within virtually any population facing environmental pressures that impact survival and reproduction. Organisms may encounter barriers that impart a limitation on survival and reproduction that sometimes results in a bottleneck event. Bottlenecks can lead to a loss of

diversity and occasionally inactivation or death of an organism or species. Many pathogens are susceptible to bottleneck events, including plant RNA viruses (Li and Roossinck, 2004), plant DNA viruses (Ge et al., 2007; Isnard et al., 1998), fungi (Keely et al., 2003), bacteria (Barnes et al., 2006; Mecsas et al., 2001) and mammalian RNA viruses (Carrillo et al., 1998). Bottlenecks to viral populations have been observed *in vitro* by serial plaque-to-plaque transfers of clonal viral populations, which resulted in viral fitness loss (Elena et al., 1996; Escarmis et al., 1996; Lazaro et al., 2003). *In vivo* bottlenecks to viral populations were observed by monitoring viral diversity and/or fitness following infection within a host or transmission between hosts (Carrillo et al., 1998; Carrillo et al., 2007; de la Iglesia and Elena, 2007; Hughes et al., 2002; Li and Roossinck, 2004; Pfeiffer and Kirkegaard, 2006).

Intra-host barriers to enteric viruses

Intra-host barriers have been implicated in viral diversity loss (Carrillo et al., 2007; Gratton et al., 2000; Li and Roossinck, 2004; Pfeiffer and Kirkegaard, 2006). Potential barriers include environmental factors, such as pH changes, cellular/receptor access and availability, and host innate and adaptive immunity. Using experimental quasispecies, barriers to viruses within hosts have been observed (Li and Roossinck, 2004; Pfeiffer and Kirkegaard, 2006). However, the specific barriers limiting viruses within hosts have not been identified.

Poliovirus faces many challenges during infection within a host. Known hurdles poliovirus must overcome as it transits the GI tract are intestinal movement, the extremely low pH of the stomach and digestive enzyme and bile salt exposure. Using a ligated stomach model, one study has demonstrated that poliovirus fares better when stomach acid is neutralized by administration of sodium bicarbonate coincident with poliovirus inoculation (Ohka et al., 2007). Enteroviruses can withstand short exposure to acidic and proteolytic environments (Piirainen et al., 1998), but these factors may limit long-term poliovirus survival. However, poliovirus is probably not exposed to acidic and proteolytic environments for long periods, and this may present a minor barrier. Concerning Reoviridae members, intestinal proteases cleave virion proteins to aid cell entry (Amerongen et al., 1994; Bass et al., 1990; Clark et al., 1981; Estes et al., 1981). Therefore, exposure to harsh intestinal environments can sometimes benefit rather than limit viral infectivity.

Potential factors within the intestinal lumen that can act as barriers to enteric viruses are sIgA, lactoferrin, AMPs, mucus and commensal bacteria. Production of poliovirus-specific IgA in feces from children vaccinated with IPV and OPV occurred concurrently with the reduction of virus in feces (Valtanen et al., 2000), suggesting that intestinal sIgA accelerates viral clearance. Secretory IgA within the GI tract is known to efficiently prevent reovirus infection of Peyer's patches *in vivo* (Helander et al., 2004; Hutchings et al., 2004; Silvey et al., 2001), and perhaps provides similar protection against poliovirus. Lactoferrin is a

secreted glycoprotein that exhibits antibacterial and antiviral activity (Ochoa and Cleary, 2009) and may also limit poliovirus (Marchetti et al., 1999). Lactoferrin blocks enteric virus entry into cells such that infection is not initiated (Seganti et al., 2004). Other host-derived factors that have activity against viruses *in vitro* are AMPs (Buck et al., 2006; Daher et al., 1986; Gropp et al., 1999; Wang et al., 2004a), which are induced by and limit bacterial invasion of the intestinal mucosa (Hooper and Macpherson, 2010). Unfortunately, no *in vivo* studies have addressed AMP-mediated protection against viruses to prove biological significance, but they could restrict viral infections. Scaling the two layers of mucus lining the GI tract may also pose a challenge to poliovirus as it does for rotavirus infections *in vitro* (Chen et al., 1993). The mucus layer is 150 μm thick, with a 50 μm firm inner layer below a loosely-associated 100 μm thick layer (Atuma et al., 2001). Poliovirus must wade through layers of mucus 5000 times its size (30 nm) to contact the GI mucosa. Lastly, enteric viruses encounter a plethora of bacteria naturally residing in intestinal tracts of many species. These commensal microorganisms may act as a physical blockade to virus-cell interactions. The role of all of these factors during viral infection lacks thorough, if any, investigation.

Once enteric viruses reach host cells, there are another set of barriers to overcome, such as receptor access and availability, epithelial cell turnover and cellular control of viral infection. Amidst the dynamic environment of the GI tract, enteric viruses must reach their viral receptors for successful propagation.

Reovirus has successfully navigated this problem by use of the glycosyl hydrolase activity encoded by the sigma 1 protein that allows it to break down mucus *in vitro* (Bisaillon et al., 1999). Receptor access may be limited by other GI-intrinsic factors such as peristalsis and the microbiota but remains unknown. Another possible inhibitory factor to poliovirus infection in the GI tract is constant renewal of the epithelial layer. The intestinal epithelial layer completely renews itself every 3-5 days (van der Flier and Clevers, 2009) with recurrent maturation and cell sloughing, potentially creating difficulty for viruses to undergo a full replicative cycle and disseminate. In addition, once a virus has entered a cell, it encounters cellular armamentaria that limit viral replication and spread by antiviral responses and apoptosis. Innate immune signaling can lead to recruitment of immune cells for more thorough viral clearance. Many mechanisms exist to potentially limit enteric viruses within the GI lumen and mucosa.

Some enteric viruses, including poliovirus and reovirus, can successfully breach the GI mucosa. These viruses can enter the bloodstream where new barriers are faced, such as neutralizing antibodies. Once adaptive immunity is induced, poliovirus antibodies are generated with neutralizing capabilities (Nathanson, 2008). Interestingly, serum antibodies cannot protect the GI tract from poliovirus replication as seen when humans were first injected with IPV and later administered OPV (Horstmann, 1955; Sabin, 1956). Viral replication in peripheral tissues presents an additional barrier to viral spread within a host. In

PVRtg mice, tissue tropism of poliovirus is limited unless the mice also lack IFNα/β signaling, which confers enhanced viral replication in extra-intestinal cells (Ida-Hosonuma et al., 2005; Ohka et al., 2007). These results highlight the importance of adaptive and innate immunity in controlling poliovirus infections after dissemination from the GI tract.

Poliovirus and reovirus also have potential to invade the CNS if they can overcome barriers that allow them to infect neurons. Neuronal access barriers include entry into neurons from other tissue types such as blood and muscle. Poliovirus may breach the blood-brain barrier and/or gain access to neurons via neuromuscular junctions. Once in neurons, neurotropic viruses face many challenges. First, they must have the capacity to traffic long distances within neurons. Second, they must be able to replicate in order to productively infect new neurons. Third, viruses in the CNS are susceptible to innate immune effectors (Pfeiffer, 2010).

Collectively, poliovirus encounters numerous intra-host barriers that limit its ability to cause significant disease. It is astonishing to envision how many mechanisms hosts have evolved to subvert viruses, but even more amazing is the ability of viruses to overcome so many challenges within a host while further propagating themselves for transmission to a new host.

Inter-host transmission barriers to viruses

Inter-host barriers can also limit viral populations in several ways. Transmission of a viral quasispecies from one individual to the next while maintaining viability and diversity is a problem for viruses. They must survive on surfaces, in saliva, blood, semen, water, etc; otherwise, further propagation is impeded. Transmission bottlenecks occur frequently for viruses, especially arboviruses (Coffey et al., 2008; Coffey and Vignuzzi, 2011; Smith et al., 2006), HIV (Delwart et al., 2002; McNearney et al., 1992; Wolfs et al., 1992) and foot-and-mouth disease virus (FMDV) (Carrillo et al., 1998; Carrillo et al., 2007). Viruses transmitted from one host to the next tend to lose members of the population culminating in a dose with lower diversity than the original population. In regards to FMDV, serial transmission in pigs was abruptly interrupted after passage 14, but FMDV was recoverable from nasal and throat swabs of the passage 15 pigs (Carrillo et al., 2007). This highly virulent pig-adapted FMDV strain had undergone multiple transmission bottlenecks leading to a loss of viral virulence. Diminishment of viral virulence is possible if a viral population experiences multiple barriers, or one very strong barrier, when being transmitted between hosts. Barrier restriction can result in a genetic bottleneck that ultimately leads to viral fitness loss.

Poliovirus encounters many factors during transit within and between hosts that may restrict the diversity and fitness of the viral population. Investigation of intestinal barriers that inhibit viral infections is limited. I

addressed the hypothesis that factors within the gastrointestinal tract act as barriers to poliovirus and restrict dissemination within a host. This dissertation outlines the identification of barriers poliovirus encounters within a host and how factors within the GI tract can influence poliovirus replication, dissemination and pathogenesis.

CHAPTER TWO
Multiple host barriers restrict poliovirus trafficking in mice

Introduction

RNA viruses undergo error-prone replication and exist as quasispecies due to the high error rate of RdRp. Within these complex viral populations, genomes can differ by one to many nucleotides resulting from approximately one mutation incorporated per 10,000 nucleotides (Crotty et al., 2000; Domingo and Holland, 1997; Drake et al., 1998). For poliovirus, a mutant virus with a high fidelity RdRp attenuated the virus in mice suggesting that a diverse quasispecies is required for full virulence (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006). Genetic recombination also contributes to quasispecies diversity, and has been detected in poliovirus isolated from patients with paralytic poliomyelitis (Dahourou et al., 2002). Mutation and genetic recombination may contribute to greater viral population diversity leading to increased virulence (Dahourou et al., 2002; Domingo and Holland, 1997; Domingo et al., 1997).

Poliovirus is an enterovirus spread by fecal-oral transmission and can cause poliomyelitis in humans. Only ~1% of people infected with poliovirus develop paralytic poliomyelitis from viral invasion of the CNS (Gromeier and Wimmer, 1998; Modlin, 1995; Pallansch and Roos, 2001). Reversion of the live-attenuated Sabin OPV by mutation or recombination occurs rather frequently, but only causes VAPP in a very small percentage (0.000001%) of people that receive OPV (Furione et al., 1993; Guillot et al., 2000; Kew and Nottay, 1984; Minor et

al., 1986). The reason for such a low incidence of paralytic poliomyelitis and VAPP remains unclear. Interestingly, in human VAPP patients, viral isolates found in the CNS are a minor subset of those found in feces, suggesting viral transit from the gut to the CNS may be difficult in humans (Georgescu et al., 1994).

PVR-expressing mice are susceptible to poliovirus via intravenous (IV), intraperitoneal (IP), intracranial (IC) and intramuscular (IM) routes (Crotty et al., 2002; Koike et al., 1991b; Ren et al., 1990). Following IM injection, poliovirus traffics to the CNS by retrograde neuronal transport (Ohka et al., 1998; Ren and Racaniello, 1992b). IV injected poliovirus is thought to reach the CNS by the blood route, independent of the presence of PVR (Yang et al., 1997). IP injected poliovirus may reach the CNS by blood or neural routes. However, these injection models may not mimic the natural fecal-oral route of infection since PVRtg mice are not orally susceptible. Recently, PVRtg-IFNAR$^{-/-}$ mice, which lack a major component of innate immunity, demonstrated oral susceptibility to poliovirus (Ida-Hosonuma et al., 2005; Ohka et al., 2007). Oral poliovirus infection in PVRtg-IFNAR$^{-/-}$ mice resulted in dissemination of virus to many tissues such as esophagus, nasopharynx-associated lymphoid tissue, small intestine, spinal cord and plasma, as measured by viral titer assay (Ohka et al., 2007). Viral titers in PVRtg-IFNAR$^{-/-}$ mice were typically 100 to 10,000-fold higher than titers in PVRtg mice expressing IFNAR. Here, we use PVRtg-IFNAR$^{-/-}$

mice to measure bottlenecks of the viral population during trafficking inside a host.

Previously, we observed bottlenecks in PVRtg mice that resulted in poliovirus population diversity loss after peripheral injection by IV, IP and IM routes. An artificial quasispecies of four viruses with distinct genomic restriction enzyme site tags were injected, and upon disease onset, brains contained an average of 1.7 input viruses suggesting that an intra-host barrier was encountered during trafficking to the CNS (Pfeiffer and Kirkegaard, 2006). Barriers encountered during spread of microbes are common for many pathogens and can lead to bottlenecks. Bottlenecks have been described for plant RNA viruses (Li and Roossinck, 2004), fungi (Keely et al., 2003), and bacteria such as *Salmonella* and *Yersinia* (Barnes et al., 2006; Mecsas et al., 2001; Meynell and Stocker, 1957). Interestingly, the picornavirus foot-and-mouth disease virus, may encounter inter-host and intra-host barriers (Carrillo et al., 1998; Carrillo et al., 2007; Hughes et al., 2002).

In this study, we introduce a new system for monitoring viral quasispecies trafficking in a murine host orally susceptible to poliovirus. We developed a hybridization-based assay for detection of a population consisting of ten marked viruses. To corroborate our previous work, we examined viral trafficking following peripheral injection of PVRtg mice *vs.* PVRtg-IFNAR$^{-/-}$ mice. In addition, we orally inoculated PVRtg-IFNAR$^{-/-}$ mice to follow viral trafficking from the initial inoculation site, the oral cavity, to the gastrointestinal (GI) tract, blood, and brain.

We identified several bottlenecks to poliovirus spread following oral inoculation, and found means of overcoming some of these barriers by use of a colon-damaging agent.

Materials and Methods

Plasmid construction

The ten viral plasmids (2 through 11) were made using silent site-directed mutagenesis of the Mahoney serotype 1 viral cDNA clone beginning with nucleotide 2425 and ending at 2443 (Figure 2-1A) (Racaniello and Baltimore, 1981). Two unique silent restriction sites were added, Bgl II at nucleotide 5601 and Mlu I at nucleotide 7550, in order to facilitate cloning. Each PCR-generated region was confirmed by sequencing (Sequencing Core, UT Southwestern Medical Center, Dallas, TX).

Viruses and cell culture infections

All poliovirus work was done in WHO-approved elevated BSL2/poliovirus conditions. Cell culture infections and propagation of virus was performed from a single poliovirus plaque using HeLa cells grown in Dulbecco's modified Eagle's medium with 10% calf serum as previously described (Pfeiffer and Kirkegaard, 2003). For the viral serial passage experiment (Figure 2-1D), the ten viruses were combined at equivalent amounts and single-cycle infections beginning with a MOI of 0.1, were performed as described (Pfeiffer and Kirkegaard, 2005).

Virus stocks were titered using plaque assays in HeLa cells as previously described (Pfeiffer and Kirkegaard, 2003). A neutral red (NR)-poliovirus stock was prepared by infecting HeLa cells with wild-type poliovirus in the presence of 10 ug/ml neutral red (Sigma) in the dark, using a red safety light (Huang et al., 2000) (Kirkegaard, 1990) (Mandel, 1967). NR-poliovirus stocks were light inactivated by exposure to a fluorescent light bulb at a distance of 3 inches for 10 minutes. The ratio of light-insensitive to light-sensitive PFU in the NR-poliovirus stock was 1 to 1.27 x 10^6.

Mice, treatments and inoculations

All animal work was performed according to protocols approved by the UT Southwestern Medical Center IACUC. C57/BL6 PVR-Tg21 (PVRtg) mice and C57/BL6 PVRtg-IFNAR$^{-/-}$ (PVRtg-IFNAR$^{-/-}$) mice were obtained from S. Koike (Tokyo, Japan), and maintained in specific pathogen free conditions (Ida-Hosonuma et al., 2005). Intramuscular (50μl volume) and intracerebral (15μl volume) injections were done as previously described (Pfeiffer and Kirkegaard, 2005) using 2x10^7 PFU total (2x10^6 PFU of each of the 10 viruses), or 2 x 10^3 PFU total for low-dose IC injections. For intraperitoneal injections, 1 x 10^8 PFU total of the 10 viruses were injected in a volume of 50μl. It should be noted that inocula for all experiments in this study were based on viral titers obtained using HeLa cells. We have shown previously that poliovirus titers in PVRtg-derived mouse embryo fibroblasts (PVRtg-MEFs) are approximately 300-fold lower than

those obtained in HeLa cells (Pfeiffer and Kirkegaard, 2005). Therefore, in terms of poliovirus titers in mouse cells, mice were actually inoculated with 6.67 x 10^4 PFU for the "2 x 10^7 PFU" inoculations. Oral inoculations were performed by dispensing 15μl of virus, by pipette tip, in the mouth. Each mouse was euthanized at first signs of disease, which included encephalitis, ruffled fur, lethargy, and paralysis. In our experience, once symptoms develop, the mice die within a day. For DSS treatments, mice were pre-treated with DSS (molecular weight 36,000-50,000; MP Biomedicals LLC, Solon, OH) in their drinking water prior to oral inoculation (Rakoff-Nahoum et al., 2004). Mice receiving 3% DSS were pre-treated for three days, and mice receiving 5% DSS were pre-treated for five days. Once infections were performed, the mice were provided with regular drinking water for the course of the experiment. Sodium bicarbonate was added to virus to make 5% mixtures immediately prior to oral infections (Ohka et al., 2007). Mice were housed in individual cages and feces were collected at 24-hour intervals with subsequent bedding changes. A combination of moist, freshly acquired feces and dry feces were combined to generate the fecal samples for the population diversity assay. For kinetics of viral shedding experiments (Figure 2-7), fresh feces were harvested from each mouse. For Evan's Blue dye transit experiments (Figure 2-7A), feces were weighed, resuspended in 6 volumes of PBS, freeze-thawed three times, and samples were centrifuged at 13,000rpm for 1 minute. "Evan's Blue Score" was determined by assessing the level of blue color in the feces: slightly blue=1, light blue=2, moderate blue=3, dark blue=4,

intense blue=5. Upon euthanasia, blood, stomach, small intestine, colon, and brain were harvested and stored at -80°C prior to use. During tissue harvests, lumenal contents were removed from gut tissues.

Sample processing and hybridization-based viral diversity assay

Tissues (brain, stomach, small intestine, colon) were homogenized under liquid nitrogen using a mortar and pestle. For brain RNA extractions, 1 ml of TRIZOL (Invitrogen, Carlsbad, CA) was added to approximately 300mg of tissue, and extractions and RT-PCR were performed as previously described (Pfeiffer and Kirkegaard, 2005). BN2 antisense primer 5' ATGCTTTCAAGCATCTGACCTAACC 3' and NdeI sense primer 5' AAACTGTTGGTGTCATATGCGCCTCCTGGAG 3' were used for RT-PCR and PCR. To amplify virus from tissues, homogenized tissues were weighed and resuspended in 3 volumes of PBS+ (1X PBS supplemented with 100μg/ml $MgCl_2$ and $CaCl_2$), and freeze-thawed 3 times. Feces were weighed, resuspended in PBS, and freeze-thawed three times. Each tissue slurry was dounce homogenized and centrifuged at 13,000 rpm for 1 minute, and supernatants were kept as virus stocks. To limit microbial contamination, virus from gut samples (stomach, small intestine, colon, and feces) were chloroform extracted by adding 1/10 volume of chloroform, centrifuged at 13,000rpm for 2 minutes, and the supernatant was kept as the virus stock. Virus was amplified for 2-3 rounds of replication (12-16 hours) at 37°C in HeLa cells and the cells were harvested,

resuspended in 50-100μl of PBS+, freeze-thawed, and kept as amplified virus stock. Half of the amplified virus stock was added to 1ml of TRIZOL for RNA extractions and RT-PCR. PCR was performed in quadruplicate and products were combined before they were run on an agarose gel and quantitated by standards of known concentrations. These concentrations were used to normalize the amount of PCR product blotted to 50-100ng of PCR product for each sample. DNA was blotted onto Hybond N+ membranes (GE Healthcare, Buckinghamshire, UK) using a 96-well vacuum manifold, and membranes were pre-hybridized and hybridized following standard procedures (Brown, 1993). Optimal hybridization annealing temperature was empirically determined to be 59°C (data not shown). Probes were made by kinase treatment of specific primers (see Figure 2-2) with [γ-^{32}P] ATP and excess nucleotides were removed with the Qiagen Nucleotide Removal kit (Qiagen, Valencia, CA) (Pfeiffer and Kirkegaard, 2005). Membranes were exposed to PhosphorImager screens and scanned by Stormscan. Scanned blots were normalized by comparison of equivalently loaded products of perfectly matched PCR product to probe or mismatched PCR products to probe. Blot image intensities were adjusted such that any apparent mismatch dot was no longer visible, thus eliminating the minimal level of cross-reactivity of the probes with non-matched PCR products (Figure 2-1E).

Results

A novel viral population diversity assay

Bottlenecks were previously studied using restriction enzyme site markers in the genomes of four distinct viruses; however, this assay was labor intensive and only included four pool members (Pfeiffer and Kirkegaard, 2006). To overcome these drawbacks, we developed a more streamlined assay based on signature-tagged mutagenesis technology used in bacterial pathogenesis studies (Hensel et al., 1995). Hybridization-based detection, 96-well format, and an increased number of pool members are advantages of the new assay.

The artificial quasispecies pool of ten members was engineered by incorporating silent mutations into the VP3 capsid-coding region of the genome, and oligonucleotide probes were designed for specific recognition of each variant (**Figure 2-1A, Figure 2-2**). To determine the specificity of the new assay, HeLa cells were infected with individual viruses or a pool of all ten viruses, RNA was isolated after one replication cycle and RT-PCR products derived from the RNA were blotted on a nylon membrane using a 96-well vacuum manifold. Oligonucleotide probes were ^{32}P-labeled and hybridized to each blotted membrane individually (**Figure 2-1B, 2-1C, 2-2**). Each blot was hybridized with only one labeled probe; therefore, ten blots were performed for each sample. Figure 2-1C displays the probe hybridization specificity following infection of HeLa cells and probing all samples with each probe. All oligonucleotide probes proved specific for their cognate virus. To ensure the viruses had no detectable

growth defects, single-cycle growth curves were performed for each virus and no differences in growth were observed (**Figure 2-3**). Additionally, a serial passage competition experiment was performed by infecting HeLa cells with a mixture of the ten viruses and then passaging the virus mixture five times, followed by assessment of input virus loss over time. All ten viruses were maintained throughout the passages, and therefore, no major growth defects of the marked viruses were detected *in vitro* (**Figure 2-1D**). For each hybridization assay, normalization was performed to eliminate cross-reactivity of nonspecific probes (**Figure 2-1E**). Perfectly matched product (PCR product specific for the probe) and mismatched products (all PCR products except for the one specific for the probe) were loaded on each membrane as controls. The image intensity level of the blots was adjusted until the mismatched product signal became undetectable, revealing only legitimate signals.

The bottleneck between the periphery and brain following injection is reduced in PVRtg-IFNAR$^{-/-}$ mice

Validation of the new hybridization assay confirmed the bottleneck effect observed in previous experiments (Pfeiffer and Kirkegaard, 2006). PVRtg mice were inoculated with $2x10^7$ PFU of a pool of all ten viruses ($2x10^6$ PFU each; viruses 2 through 11) by IM or IC injection. Brains of mice inoculated with $2x10^7$ PFU by the IC route contained most, if not all, input viruses upon disease onset; however, the brains of IM-injected mice contained 10% to 30% of the input

viruses (**Figure 2-4A, 2-4B**). For IM-injected mice, all ten viruses were present at the inoculation site, muscle. Brains of PVRtg mice inoculated by the IP route with $1x10^8$ PFU of the ten-virus pool contained only 10% of the input viruses. These experiments validated the new assay and confirmed our previous results (Pfeiffer and Kirkegaard, 2006).

Next, we measured viral population diversity in PVRtg-IFNAR$^{-/-}$ mice, which are hyper-susceptible to poliovirus (Ida-Hosonuma et al., 2005; Ohka et al., 2007). We hypothesized that innate immunity may contribute to the bottleneck, and therefore, we predicted increased population diversity in the brains of PVRtg-IFNAR$^{-/-}$ mice. PVRtg-IFNAR$^{-/-}$ mice were injected intramuscularly with $2x10^7$ PFU of the ten-member pool. As shown in **Figure 2-4A and 2-4B**, the brain bottleneck was greatly diminished in PVRtg-IFNAR$^{-/-}$ mice, with 40% to 100% of the input viruses detectable in the brain. In fact, the brains of IM-injected PVRtg-IFNAR$^{-/-}$ mice contain an average of 70% of the input viruses, a result comparable to PVRtg mice injected IC with $2x10^7$ PFU. Similarly, brains of IP-inoculated PVRtg-IFNAR$^{-/-}$ mice contained 80% of the input viruses. The diminished bottleneck in PVRtg-IFNAR$^{-/-}$ mice may be the result of increased peripheral titers in PVRtg-IFNAR$^{-/-}$ mice, essentially increasing the viral dose, physical barrier differences caused by the lack of IFNAR, such as alteration of neurons or the blood-brain barrier that affect viral trafficking, or, perhaps, a brain-specific IFNα/β response established by the first virus(es) to enter the brain contributes to the bottleneck observed in PVRtg mice. To determine whether the

amount of virus entering the brain influences viral diversity, PVRtg and PVRtg-IFNAR$^{-/-}$ mice were inoculated by the IC route with a low dose of the ten virus mixture, 2x10^3 PFU, which corresponds to 200 PFU of each pool member. Using this low input dose, viral diversity was low in the brains of both PVRtg and PVRtg-IFNAR$^{-/-}$ mice (13% and 24% of input viruses present, respectively) (**Figure 2-4B**). These results suggest that the bottleneck we observe is affected by the quantity of virus entering the brain.

The viral bottlenecks we observe are independent of selective advantages possessed by a particular marked virus. Based on a compilation of 479 hybridization signals, all ten viruses were approximately equally represented in a variety of tissues from over 25 mice, although virus 3 showed reduced representation, possibly indicating a slight growth defect (**Figure 2-1D; Figure 2-6A**). However, statistical analysis revealed that none of the viruses, including virus 3, were significantly under- or over-represented in mouse tissues (p=0.07 to p=1, Student's *t* test). This apparent random sampling of population members was also observed in our previous study (Pfeiffer and Kirkegaard, 2006).

Bottlenecks exist following oral inoculation of PVRtg-IFNAR$^{-/-}$ mice

Unlike PVRtg mice, PVRtg-IFNAR$^{-/-}$ mice are orally susceptible to poliovirus disease (Ida-Hosonuma et al., 2005; Ohka et al., 2007). Although the peripheral site-to-brain bottleneck was reduced in PVRtg-IFNAR$^{-/-}$ mice (**Figure 2-4A, 2-4B**), we sought to determine whether bottlenecks exist following oral

inoculation. Because the gut is a complex environment composed of many unique cell types and processes, barriers to viral spread may be encountered in PVRtg-IFNAR$^{-/-}$ mice despite the hyper-susceptibility of these animals to poliovirus. We orally inoculated PVRtg-IFNAR$^{-/-}$ mice with 2x10^{7} PFU of a mixture of the ten-member virus pool. Following oral inoculation, PVRtg-IFNAR$^{-/-}$ mice developed encephalitis rather than paralysis observed in injected mice, and disease onset was delayed, with symptoms developing on days five through ten or later, in agreement with published data (Ohka et al., 2007). Feces were harvested daily from individual mice, and tissues were collected upon disease onset. Viruses isolated from stomach, small intestine, colon, feces, and blood were amplified for approximately three replication cycles in HeLa cells to increase detection, as the detection limit of the hybridization assay is ~5,000 PFU (data not shown). *In vitro* amplification does not significantly affect diversity of virus extracted from tissues. For example, in the brain, where viral titers were high enough to perform the hybridization assay with or without amplification, viral diversity was equivalent in amplified and unamplified viral stocks (data not shown). Therefore, *in vitro* amplification allows detection without significantly altering the composition of the viral population.

Three major poliovirus bottlenecks were observed in orally inoculated PVRtg-IFNAR$^{-/-}$ mice. First, a major bottleneck occurred between the inoculation site (mouth) and gut tissues (**Figure 2-5**). Gut tissues were harvested upon disease onset, and lumenal contents were removed. An average of

approximately 20% of input viruses were present in the stomach, small intestine, and colon (**Figures 2-5B, 2-9**). Notably, virus was detectable in the stomach late in infection upon disease onset, suggesting that non-input replicating virus was present. These results support the notion that poliovirus is resistant to stomach acid and digestive enzymes, although it is possible that viruses entered the bloodstream and re-seeded organs later in the disease course. Interestingly, viruses found in one GI tract tissue did not always correlate with those detected in other GI tract tissues within the same animal (e.g. mouse 9-1, **Figure 2-5**).

Second, a major bottleneck occurred between the mouth and blood (**Figures 2-5, 2-9**). It is unclear how poliovirus enters the bloodstream, with evidence supporting upper GI and lower GI routes (Bodian, 1952a; Horstmann et al., 1954; Sabin, 1956). We found that less than 50% of mice had detectable virus in blood harvested at disease onset, with an average of 9% of input viruses present (**Figures 2-5B, 2-8C, 2-9**). Because it is likely that viremia occurred earlier in the disease course, we assessed viral population diversity in blood from a separate set of animals bled at several time points. Similar to the results obtained by sampling blood at disease onset, less than 60% of day three blood samples contained detectable virus, with an average of 17% of input viruses present (data not shown).

Third, a major bottleneck occurred between inoculation site and brain, with an average of 21% of input viruses detected in the brain, harvested upon

disease onset (**Figure 2-5**). Surprisingly, viruses found in the brain did not always correlate with those detected in other tissues within the same animal.

Interestingly, the timing of disease onset and viral population diversity were associated, such that earlier disease onset correlated with higher diversity. Mice developing symptoms prior to day seven had 3.3-fold ($p=0.025$) more input viruses in the brain than those developing symptoms after day seven, according to mean viral diversity comparison (**Figure 2-5B**). Higher diversity was also observed in blood and gut tissues of the early onset mice, with 9-fold higher diversity in blood ($p=0.042$), and 2.1 to 3.3-fold higher diversity in gut tissues (stomach, small intestine, and colon; $p<0.05$).

Passage through the GI tract is not difficult for poliovirus

With the finding that major bottlenecks occurred during viral trafficking from the mouth to other mouse tissues, it became important to determine whether transit through the gut environment is difficult for poliovirus populations. Interestingly, only a minimal bottleneck occurred between inoculation site (mouth) and feces (**Figure 2-5, 2-9**). For the population diversity assay, we analyzed fecal samples collected at 24 hours post-inoculation because relatively high viral titers were detected at this time. On average, more than 80% of input viruses were detected in feces (**Figure 2-5B, 2-9**). Many of the mice (5/13) shed all ten input viruses in feces.

Because viral diversity was high in feces, we sought to determine whether the 24-hour fecal samples contained replicated virus, non-replicated/input virus, or both. First, we monitored viral transit time through the GI tract by measuring fecal titers at several time points, and transit time of a dye. Mice were orally inoculated with $2x10^7$ PFU of poliovirus or Evan's Blue dye as a tracer. Fresh feces were harvested at regular intervals. Viral titers were determined by standard plaque assay using HeLa cells, and transit time of Evan's Blue was determined by scoring the relative dye intensity of fecal samples. As shown in **Figure 2-7A**, very high fecal titers were present at 2 hours post-inoculation for some animals. Since this time point is within the eclipse period of the viral replication cycle (**Figure 2-3**), we presumed that virus shed at 2 hours post-inoculation was input/non-replicated virus. Viral titers remained relatively high from 5-12 hours post-inoculation, and then declined at later time points. This rise and decline of viral titers correlated well with the transit time of Evan's Blue dye through the mouse GI tract (**Figure 2-7A**).

Although the results from the fecal virus kinetics study suggested that virus shed at early time points is input/non-replicated virus, the presence of replicated virus could not be excluded; therefore, we monitored the transit of light-sensitive poliovirus to directly measure the amount of replicated *vs.* non-replicated virus present in feces. Poliovirus grown in the presence of neutral red (NR) is sensitive to inactivation by light exposure due to dye incorporation and concentration in virions (Huang et al., 2000; Kirkegaard, 1990; Mandel, 1967);

hence, these viruses must be handled in the dark, using a red safety light. Upon replication in the absence of NR, viruses lose this light sensitivity. Therefore, the presence or absence of light-sensitive poliovirus in feces was utilized to monitor whether replication had occurred in the GI tract of orally inoculated mice. In the dark, mice were orally inoculated with $2x10^7$ PFU of light-sensitive NR-poliovirus, and feces were harvested in the light or in the dark. As a control, six hour feces harvested in the dark were subjected to titer analysis in light *vs.* dark conditions. The non-light exposed samples demonstrated high titers: viable virus titers were ~40% of non-NR poliovirus titers harvested at the six hour time point in **Figure 2-7A**. We presume that these NR-virus titers were not 100% of the non-NR titers due to intrinsic variability in the animal experiments and/or subtle defects in NR-containing virions. Upon exposure to light, <0.1% of the non-NR poliovirus titer was obtained, indicating a very low level of light-insensitive viruses in the population (**Figure 2-7B, right**). Fecal samples exposed to light contained negligible viral titers until after 10 hours post-inoculation, suggesting that prior to 10 hours, feces contain input/non-replicated virus (**Figure 2-7B, left**). However, at 24 hours post-inoculation, feces contained light-insensitive/replicated virus, although only ~14% of the non-NR poliovirus titer was obtained. Therefore, the 24-hour fecal samples used for our population diversity analysis contained a mixture of replicated and non-replicated/input virus.

Effect of colonic mucosal damage and antacid administration on viral titers

We hypothesized that the colonic mucosal epithelium and/or stomach acidity may create barriers that contribute to viral bottlenecks. Therefore, we treated mice with agents that damage the colonic mucosa or neutralize stomach acid and determined the effects on poliovirus titer and diversity. Damage to the colonic mucosa was induced by treating mice with dextran sulfate sodium (DSS) in drinking water. DSS directly damages colonic epithelia resulting in ulceration, immune infiltration and bloody feces (Cooper et al., 1993; Kitajima et al., 1999; Okayasu et al., 1990; Rakoff-Nahoum et al., 2004). We measured viral titers in feces (**Figure 2-8A, 2-8B**), blood (**Figure 2-8C**), and brain (**Figure 2-8D**) following oral inoculation performed +/- DSS pre-treatment. High-dose (5%) DSS treatment increased 72-hour fecal titers 56-fold ($p=0.000163$). Day one fecal titers were 16-fold higher in 5% DSS-treated mice compared to untreated mice. Blood titers for 5% DSS-treated mice were increased 66-fold, and virus was detected in the blood of all 5% DSS-treated mice (**Figure 2-8C**) compared to untreated mice, where less than 50% of animals had detectable virus in blood. Treatment with 3% DSS did not have an effect on viral titers suggesting that 3% DSS may not induce sufficient damage.

We next assessed the role of stomach acid in establishing the poliovirus bottleneck. The mouth-to-feces bottleneck is minor since the majority of the ten input viruses were detected in feces. However, Ohka and colleagues showed that sodium bicarbonate, an acid-neutralizing agent, increased poliovirus titers in

a ligated stomach model following oral inoculation of PVRtg-IFNAR$^{-/-}$ mice (Ohka et al., 2007). We orally inoculated PVRtg-IFNAR$^{-/-}$ mice with a virus/5% sodium bicarbonate mixture. Our results revealed no titer differences between sodium bicarbonate-treated and untreated animals (**Figure 2-8A-D**).

Colonic mucosal damage increases population diversity in GI tract and blood, but not brain

Since 5% DSS-treated poliovirus-infected mice demonstrated increased viral titers, we reasoned that viral population diversity may be increased in these mice. Therefore, we performed the viral population diversity assay for samples from 5% DSS-treated, orally inoculated PVR-IFNAR$^{-/-}$ mice. As expected, viral diversity in feces was high for all mice, regardless of treatment (**Figure 2-9**). Viral diversity in the stomach of 5% DSS-treated mice increased 1.8-fold ($p=0.0218$), diversity in the small intestine increased 2.2-fold ($p=0.00865$), and diversity in the colon increased 2.8-fold ($p=0.0000497$) compared to untreated controls (**Figure 2-9**). Additionally, viral diversity in blood increased 3.5-fold ($p=0.0101$). Interestingly, viral diversity in the brain was unaffected by DSS treatment (**Figure 2-9A**). Again, we found that viruses present in the brain do not necessarily correlate with those present in blood or gut tissues (**Figure 2-9B**). Viral diversity in tissues of mice treated with 3% DSS or sodium bicarbonate did not differ from untreated mice (**Figure 2-9A**).

Comparison of viral titer vs. viral population diversity unmasks bottlenecks following oral inoculation

Initially, one might assume that viral titer and viral diversity are linked, with high titer sites containing high population diversity, and vice versa. However, this is not the case, especially when bottlenecks are present (Duarte et al., 1992; Li and Roossinck, 2004). **Figure 2-10** compares viral titer *vs.* diversity for feces, blood and brain viruses from untreated mice orally inoculated with the ten-virus mixture. Fecal samples contained low to moderate titers of ~5-300 PFU/mg, but contained moderate to high population diversity. Titer and diversity may be linked before a major bottleneck is encountered, as in feces, in which higher titers correlate with higher diversity. These results confirmed that the bottleneck between mouth and feces is minor. Brain samples had the highest titers (~2,000-100,000 PFU/mg), but contained low diversity, which is characteristic of a major bottleneck. We propose that entry into the brain is difficult, but once in the brain, founder viruses undergo robust replication. Blood samples had low to moderate titers (~1-700 PFU/mg) and low diversity. Therefore, our data confirm that titer and diversity are not linked following bottlenecks.

Discussion

We have developed a new diversity assay that has allowed us to uncover barriers to viral trafficking that would be missed by standard viral titer assays.

Using our hybridization-based assay, we demonstrated bottleneck barriers by monitoring marked polioviruses.

We confirmed a previously observed bottleneck between peripheral injection sites and brain (**Figure 2-4**). As before, random sampling was revealed, in which no pool member had an apparent selective advantage over the others (**Figure 2-1D; Figure 2-3, 2-6**). The previous assay employed four viruses, and one to three were found in the brain (average ~50%) following IM injection (Pfeiffer and Kirkegaard, 2006). Here we found that, on average, ~20% of our ten marked viruses reached the brain, suggesting that this bottleneck was more severe than that previously observed. One possible explanation is that the previous study was performed using ICR-PVRtg mice (Crotty et al., 2002), while this study was performed using C57/BL6-PVRtg mice (Ida-Hosonuma et al., 2002). Additionally, the observed increase in bottleneck severity could be a result of our increased artificial quasispecies sample size.

Because interferons (IFN) play an important role in controlling viral infections, prior to this study, we proposed that the IFNα/β response may contribute to viral bottlenecks. In PVRtg-IFNAR$^{-/-}$ mice, the bottleneck following IM or IP injection was largely absent with an average of 70% or 80% of input viruses detected in the brain, respectively. In fact, direct injection of a large inoculum ($2x10^7$ PFU) of the virus pool into the brains of PVR mice resulted in an average of 76% of input viruses detected in the brain, confirming the absence of a major bottleneck in peripherally-injected PVRtg-IFNAR$^{-/-}$ mice. We propose

several possible reasons for the diminished bottleneck in peripherally-injected PVRtg-IFNAR$^{-/-}$ mice: 1) The first viruses to enter the brain in PVRtg mice established an anti-viral state which limited the spread of later viruses, resulting in a bottleneck effect. The lack of IFNα/β response in PVRtg-IFNAR$^{-/-}$ mice, therefore, facilitated higher brain diversity. 2) Increased peripheral titers in hyper-susceptible PVRtg-IFNAR$^{-/-}$ mice may have essentially increased the poliovirus dose. This effect could be unique to PVRtg-IFNAR$^{-/-}$ mice since our previous work determined it was very difficult to overcome the bottleneck by increased dose in PVRtg mice (Pfeiffer and Kirkegaard, 2006). 3) Physical barriers in PVRtg-IFNAR$^{-/-}$ mice may have been altered due to lack of the type I IFN environment. Perhaps lack of IFNAR created differences in neurons or the blood-brain barrier that may have contributed to higher viral brain diversity in PVRtg-IFNAR$^{-/-}$ mice. Importantly, data from our oral inoculation studies demonstrated a bottleneck exists between mouth and brain in PVRtg-IFNAR$^{-/-}$ mice (**Figure 2-5**). Therefore, the lack of the IFNα/β response in the brain was not the sole cause for the diminished bottleneck in peripherally-injected PVRtg-IFNAR$^{-/-}$ brains. Additionally, PVRtg and PVRtg-IFNAR$^{-/-}$ mice injected by the IC route with a low dose of the virus pool ($2x10^3$ PFU) demonstrated comparable low levels of viral diversity in the brain (13% and 24% of input viruses, respectively). These results suggest that viral diversity in the brain is governed by the amount of virus that enters the brain, and that elevated peripheral titers in

injected PVRtg-IFNAR$^{-/-}$ mice contribute to the elevated viral diversity in the brains of these animals.

Following oral inoculation of PVRtg-IFNAR$^{-/-}$ mice, poliovirus moves through the GI tract without much difficulty. Relatively high amounts of virus were shed in feces, including input/non-replicated viruses and replicated viruses, depending on the sampling time (**Figure 2-7**). Population diversity in feces was relatively high with an overall average of 81% of input viruses present (**Figures 2-5 and 2-9**), suggesting only a minor bottleneck was encountered during GI lumenal passage. Although we consider this bottleneck minor, it could actually represent the successful passage of just 0.025% ($5x10^3$ PFU) of the input virus, which would still allow detection of all pool members in our system. Regardless, this mouth-to-feces bottleneck was minor in comparison to other bottlenecks we observed.

Our experiments identified three major bottlenecks following oral inoculation of PVRtg-IFNAR$^{-/-}$ mice: mouth-to-gut tissues, mouth-to-blood and mouth-to-brain. First, a major bottleneck existed between the mouth and gut tissues. Of the ten viruses, an average of 16% of input viruses were present in the stomach, and 19% of input viruses were present in the small intestine and the colon (**Figures 2-5, 2-9**). We presume that virus must be replicating in these tissues to be detected late in infection when the tissues were harvested (day 5-10). However, gut tissues could have been re-seeded by virus in the blood.

We identified a second bottleneck between mouth and blood. Blood titers were moderate, but diversity was very low (avg.=9% of input viruses) (**Figures 2-8C, 2-5, 2-9**). We are uncertain how the virus is traveling from the inoculation site into the blood, but possibilities include drainage from lymph, mucosal passage to the blood and entrance into the bloodstream at sites of mucosal micro-damage. Viruses may have entered the bloodstream early in infection (Bodian, 1955; Melnick, 1996; Sabin, 1956).

Third, a prominent bottleneck existed between the mouth and the brain. Viral trafficking between the mouth and brain could have occurred through blood or neural routes. Historically, poliovirus invasion of the brain has been presumed to occur through the blood route because neutralizing antibodies are protective and IV-injected radiolabeled virions readily enter the murine brain (Bodian, 1954; Bodian and Paffenbarger, 1954; Yang et al., 1997). However, viral trafficking in neurons may also occur and contribute to pathogenesis (Gromeier and Wimmer, 1998; Mueller et al., 2002; Ohka et al., 1998; Ren and Racaniello, 1992b).

Surprisingly, 93% of viruses found in the brain were present in gut tissues of a given mouse, but only 35% were detected in blood (**Figure 2-5B**). This suggests that a gut tissue-to-brain pathway was involved in viral spread. Virus may have entered the blood from gut tissues and trafficked to the brain, or virus may have infected neurons associated with the GI tract and reached the brain by retrograde transport. Trafficking via neurons has been demonstrated by sciatic nerve transection experiments following poliovirus infection (Ohka et al., 1998;

Ren and Racaniello, 1992b). Although our data did not definitively discriminate between blood and neural routes, our data did show that absolute match of blood and brain viruses was rare. In some instances, there was no overlap between viruses present in the brain and blood (**Figure 2-5B**, mouse 9-6; **Figure 2-9**, mouse 9-1). These results indicated that virus may enter the brain by a non-hematogenous route, such as neurons, that low-abundance viruses in blood seeded the brain or that virus found in the blood at disease onset differed from the virus in the blood at earlier time-points. Interestingly, the blood/brain virus mismatch was confirmed in an experiment where blood was collected at day three post-inoculation and upon disease onset, and then blood diversity was compared with brain diversity. This experiment revealed that only 44% of viruses found in the brain are present in blood at day three post-infection, suggesting that not all viruses may spread to the brain via a blood route (data not shown). Aside from possible GI neuronal trafficking, it is likely that virus moves into and out of blood throughout infection by seeding other tissues with subsequent re-seeding of the blood. In humans, it is thought that a primary asymptomatic viremia may seed tissues, with a subsequent secondary viremia contributing to minor or major illness, which can lead to CNS invasion and paralytic poliomyelitis (Bodian, 1952a; Horstmann et al., 1954; Sabin, 1956).

Our results suggested that disease onset and viral diversity are linked. Earlier disease onset correlated with greater viral diversity in the gut tissues, blood, and brain. Mice that developed symptoms before day seven had 3.3-fold

(p=0.025) higher brain diversity than those that developed symptoms later (**Figure 2-5B**). These early onset mice also had higher blood diversity (9-fold, p=0.042) and gut diversity (2.1-3.3 fold, stomach: $p<0.05$). Greater diversity upon earlier onset was not simply due to a tissue sampling time bias, because a separate study demonstrated very low population diversity in tissues harvested on days one and three post-inoculation (**Figure 2-6B**). There are several possible explanations for the correlation of disease onset and viral diversity. First, since high population diversity and virulence are linked (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006), higher viral diversity may contribute to faster disease progression. Second, some component of host immunity may have developed later in infection, which limited viral replication, and ultimately, viral diversity.

Interestingly, two of the three major bottlenecks could be overcome by pre-treating the mice with a colonic epithelial-damaging agent, DSS. The first (mouth-to-gut tissues) and second bottlenecks (mouth-to-blood) were affected by DSS treatment: gut tissue and blood diversity increased ~2-3-fold and 3.5-fold, respectively. Additionally, blood titers increased 66-fold in the presence of colonic damage. Importantly, the mouth-to-brain bottleneck was unchanged in DSS-treated mice compared to untreated mice. These results suggest that either virus trafficked to the brain via a non-blood route, which was unaffected by DSS treatment, or virus trafficked to the brain via a blood route, but spread to the brain was limited by another barrier, such as the blood-brain barrier.

Viral titer and diversity were not linked after a bottleneck was encountered (**Figure 2-10**). By monitoring diversity, we uncovered limitations on viral trafficking that would be missed by viral titer analysis. For example, blood and fecal titers were similar; therefore, one might conclude that transit from the gut to blood was not difficult. Our assay allowed us to conclude that a major bottleneck exists since blood diversity was low. We presume that virus was replicating in blood and/or other tissues that seed blood, thus increasing the blood titer post-bottleneck encounter, resulting in founder effects.

We found that viral population trafficking was a very dynamic, stochastic process. Using virus 2 as an example, a given virus might be present in all tissues (**Figure 2-5**, mouse 9-1), in colon and feces only (**Figure 2-5**, mouse 9-2), in brain and feces only (**Figure 2-5**, mouse 9-6), or in other differing combinations. Similar random trafficking patterns have been observed in several microbial systems, including animal and plant viruses, bacteria, and fungi (Barnes et al., 2006; Carrillo et al., 1998; Carrillo et al., 2007; Hughes et al., 2002; Keely et al., 2003; Li and Roossinck, 2004; Mecsas et al., 2001; Meynell and Stocker, 1957). For highly mutable RNA viruses, host barriers likely play an important role in shaping viral populations and determining virulence (Clarke et al., 1993).

The random distribution of viral populations makes predicting VAPP impossible, because a viral isolate from the CNS of one person may not invade the CNS of another due to bottleneck effects and stochastic trafficking. Notably,

in human VAPP patients, fecal virus does not always correlate with virus found in the CNS (Georgescu et al., 1994). Perhaps physical barrier disruption and/or a defective innate immune response increased susceptibility to inadvertent poliovirus CNS invasion in individuals afflicted with paralytic poliomyelitis. We have shown that this artificial quasispecies system mimics the stochastic poliovirus trafficking observed in humans, and can be used to understand RNA virus population dynamics in an infected host.

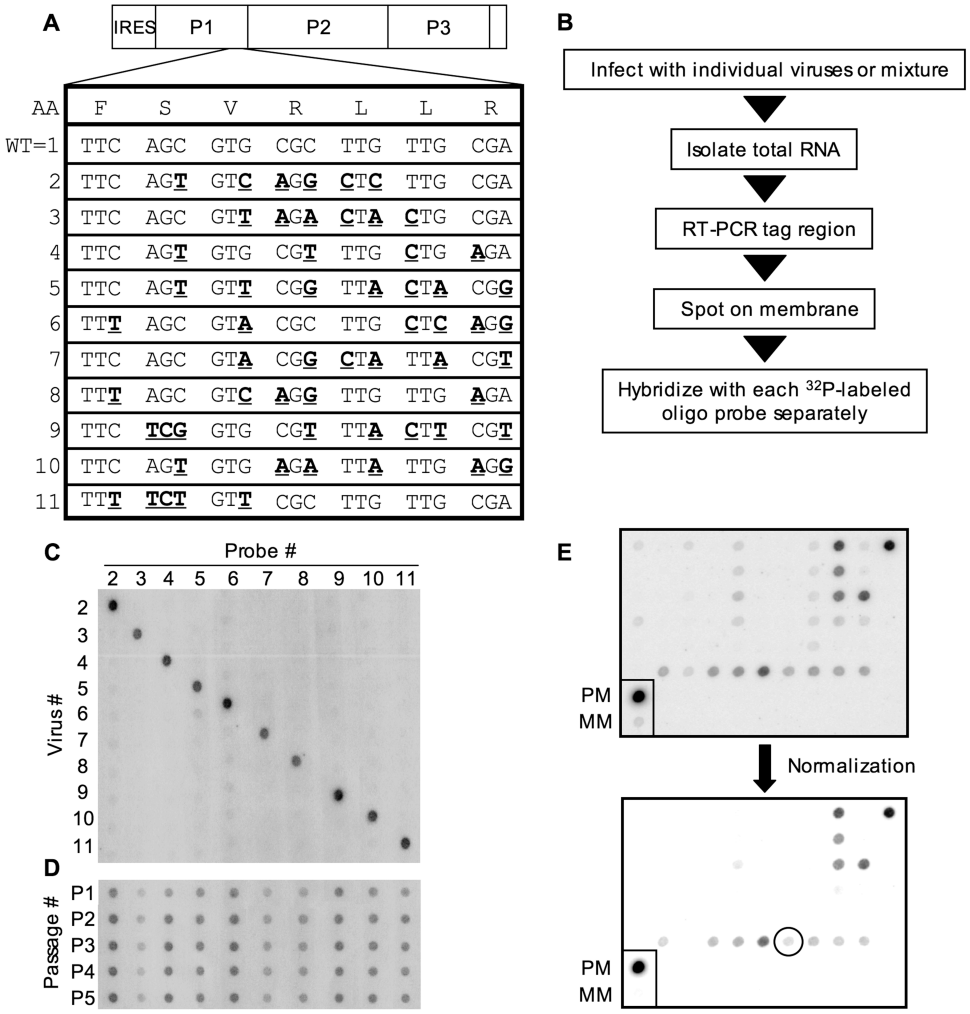

Figure 2-1. A novel hybridization-based viral diversity detection assay. (A) Poliovirus genome highlighting VP3 region of P1 where point mutations were made by site-directed mutagenesis (bold, underlined) to distinguish each of the ten viruses from one another. The amino acid (AA) sequence and the wild-type poliovirus genome sequence of that region are shown. Note: wild-type is virus #1 and was not included in studies due to cross-hybridization. (B) Strategy for the assay. (C) Blot showing the specificity of each [^{32}P]-γ-ATP-labeled probe for its respective viral RT-PCR product. (D) Serial passage competition experiment. HeLa cells were infected with equal amounts of each of the ten viruses, and amplified virus was harvested from cells to infect naïve HeLa cells for five total passages. (E) Normalization process to eliminate probe cross-hybridization. Signal intensity was adjusted such that mismatch (MM=all PCR products except for the one specific for the probe) was no longer detectable, and perfect match (PM: specific PCR product) was the positive control. C.A. Etheredge generated viruses and probes and performed the competition experiment.

Primer	Sequence
CEBN2anti	5' TCAGTGTCAGGCTCTTGCGAG 3'
CEBN3anti	5' TCAGCGTTAGACTACTGCGAG 3'
CEBN4anti	5' TCAGTGTGCGTTTGCTGAGAG 3'
CEBN5anti	5' TCAGTGTTCGGTTACTACGGG 3'
CEBN6anti	5' TTAGCGTACGCTTGCTCAGGG 3'
CEBN7anti	5' TCAGCGTACGGCTATTACGTG 3'
CEBN8anti	5' TTAGCGTCAGGTTGTTGAGAG 3'
CEBN9anti	5' TCTCGGTGCGTTTACTTCGTG 3'
CEBN10anti	5' TCAGTGTGAGATTATTGAGGG 3'
CEBN11anti	5' TTTCTGTTCGCTTGTTGCGAG 3'

Figure 2-2. Oligonucleotide probe sequences for detection of individual pool members. Probes designed by C.A. Etheredge.

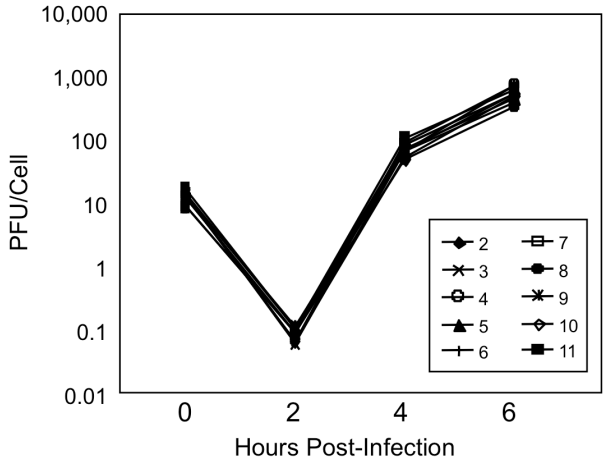

Figure 2-3. Single-cycle growth curves for each pool member. HeLa cells were infected using an MOI of 10 PFU/cell. Cell-associated virus was harvested at the indicated time points and titered on naïve HeLa cells to quantify recovered virus. Work performed by C.A. Etheredge.

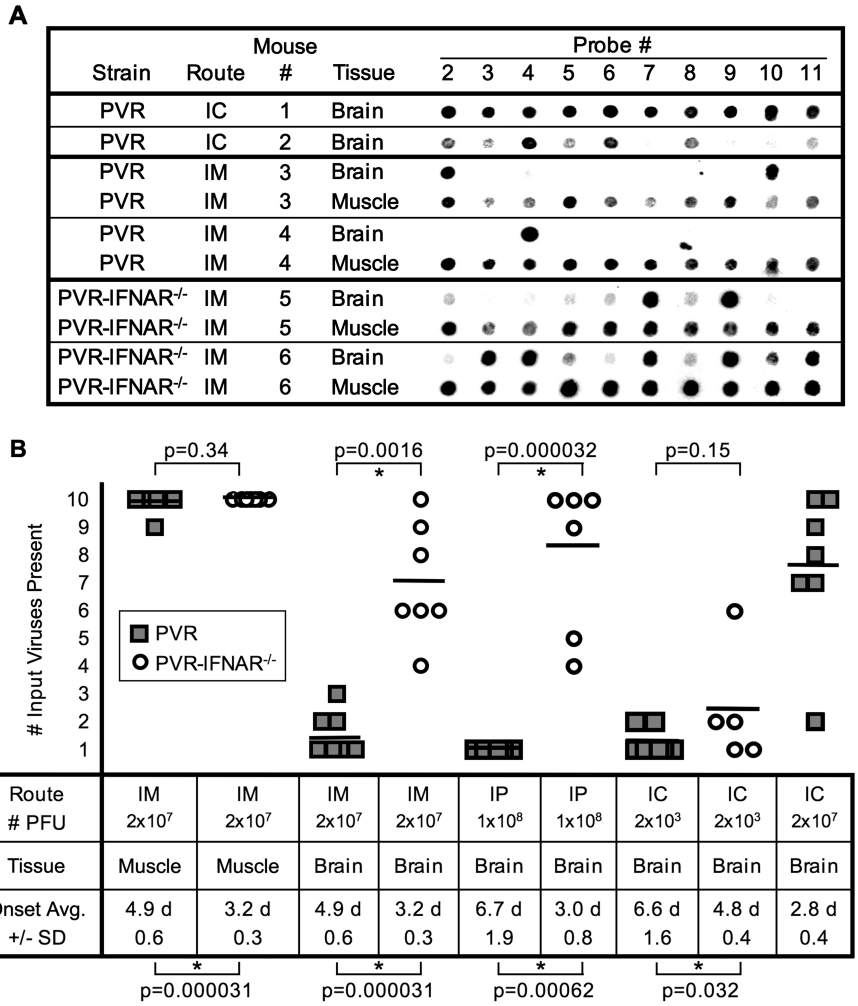

Figure 2-4. Viral diversity in injected PVRtg and PVRtg-IFNAR$^{-/-}$ mice. (A) PVRtg mice were injected intracerebrally (IC) and intramuscularly (IM) and with 2x10^7 PFU of the ten virus pool. Tissues were harvested upon disease onset, and viral diversity in brain and muscle was determined and compared to PVRtg-IFNAR$^{-/-}$ mice injected IC and IM. Blots results of viral-derived RT-PCR products are shown for two representative mice injected IC and IM, with specific probes numbered along the top. (B) Number of viral pool members detected in tissues from IM, intraperitoneally (IP), or IC-injected PVRtg mice and PVRtg-IFNAR$^{-/-}$ mice. Data from A were compiled with additional diversity assay, and at least five mice are shown per group. PVRtg mice: gray square; PVRtg-IFNAR$^{-/-}$ mice: open circles. Horizontal bars denote the average for each group. The average time of symptom onset (day post-inoculation), +/- standard deviation, is shown at the bottom. The p values from Student's t-tests are shown: values above the graph compare viral diversity, and values below the graph compare disease onset. Asterisks denote statistical significance ($p<0.05$). Work done in collaboration with C.A. Etheredge.

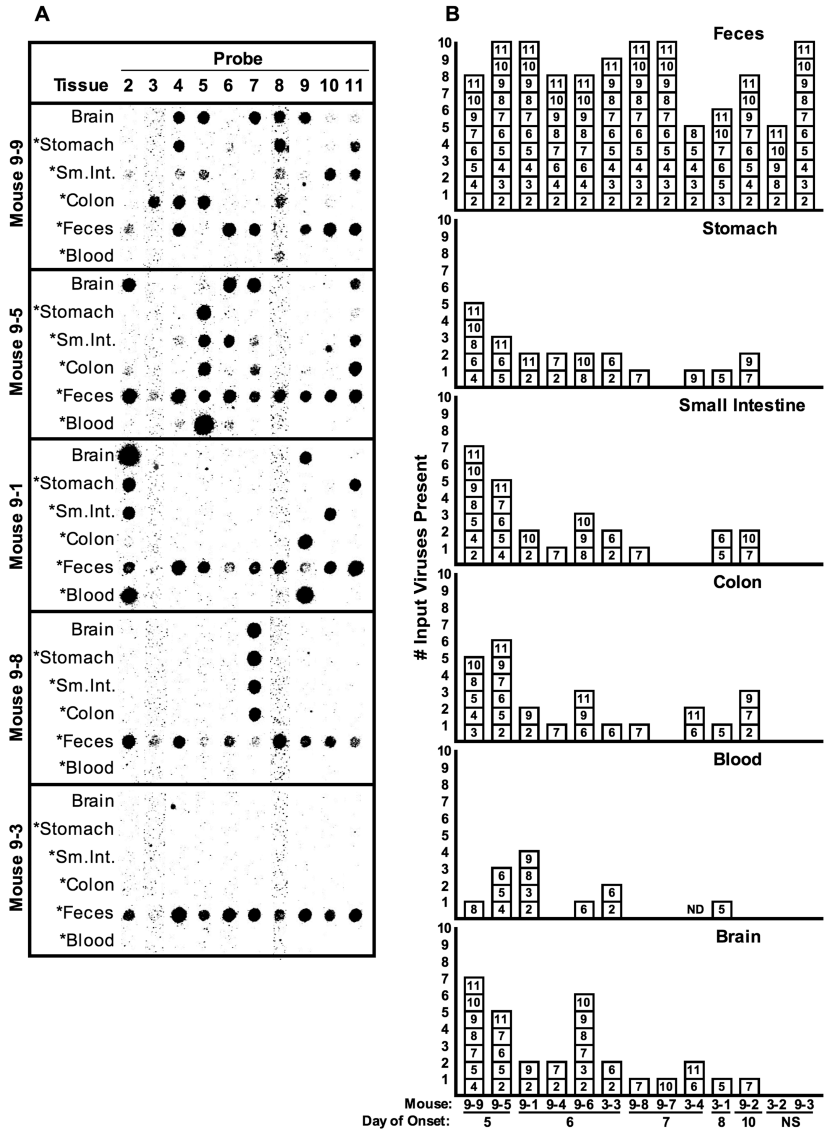

Figure 2-5: Viral diversity in tissues of orally-inoculated PVRtg-IFNAR$^{-/-}$ mice. Mice were orally inoculated with 2x10^{7} PFU of the ten virus pool, tissues were harvested at disease onset and analyzed for detection of pool members. (A) Representative blot results for five orally inoculated PVRtg-IFNAR$^{-/-}$ mice. Sm.Int.=small intestine. Mouse 9-3 did not develop disease during the 10 day post-inoculation time course. (B) Individual viruses found in feces collected on day one post-inoculation and tissues at disease onset. Each box depicts a particular virus based on blot results. Mice are arranged in order of earliest to latest disease onset (NS = no symptoms at day 10). ND = not determined. Work done in collaboration with C.A. Etheredge.

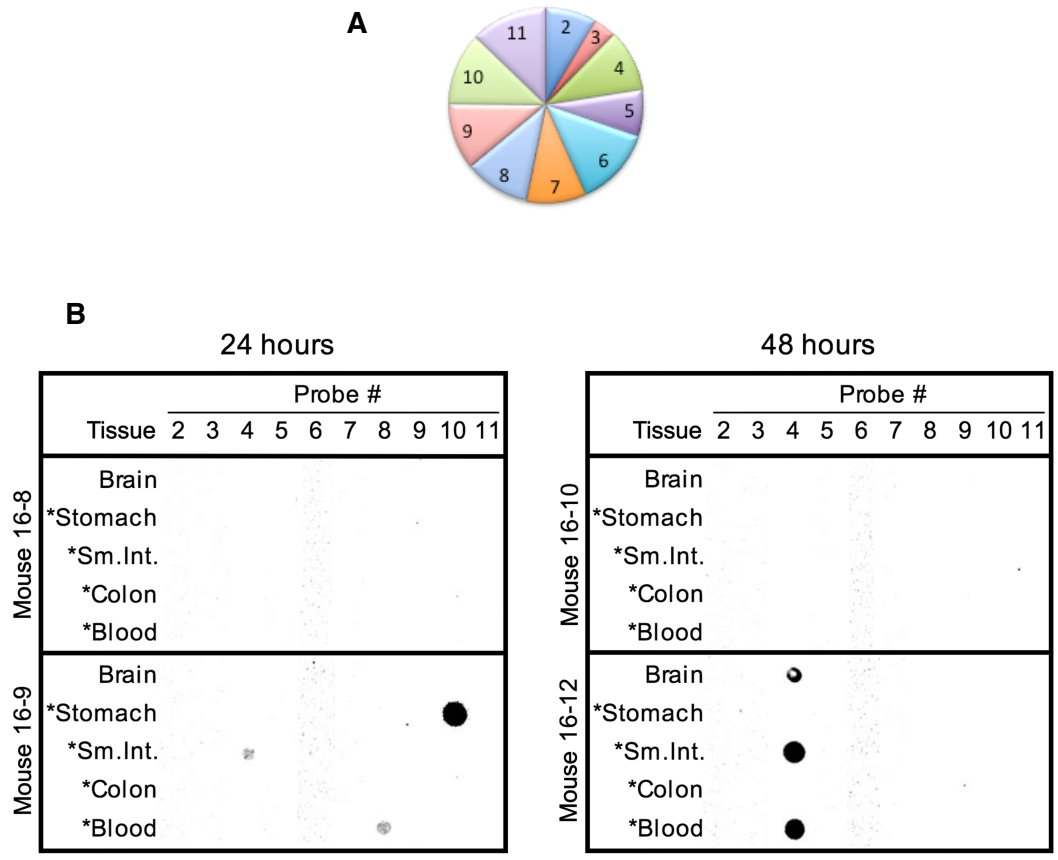

Figure 2-6. Pool member distribution in PVRtg-IFNAR$^{-/-}$ mouse tissues after oral inoculation. (A) Compiled data from 479 hybridization dots representing total tissue distribution of the ten member pool from over 25 mice. Numbers in each section indicate the virus number. (B) Mice were orally inoculated with 2x10^{7} PFU of the ten virus pool and tissues were harvested at 24 and 48 hours post-inoculation. Blots are shown from two representative mice per group. Hybridization assay done in collaboration with K.Z. Lancaster.

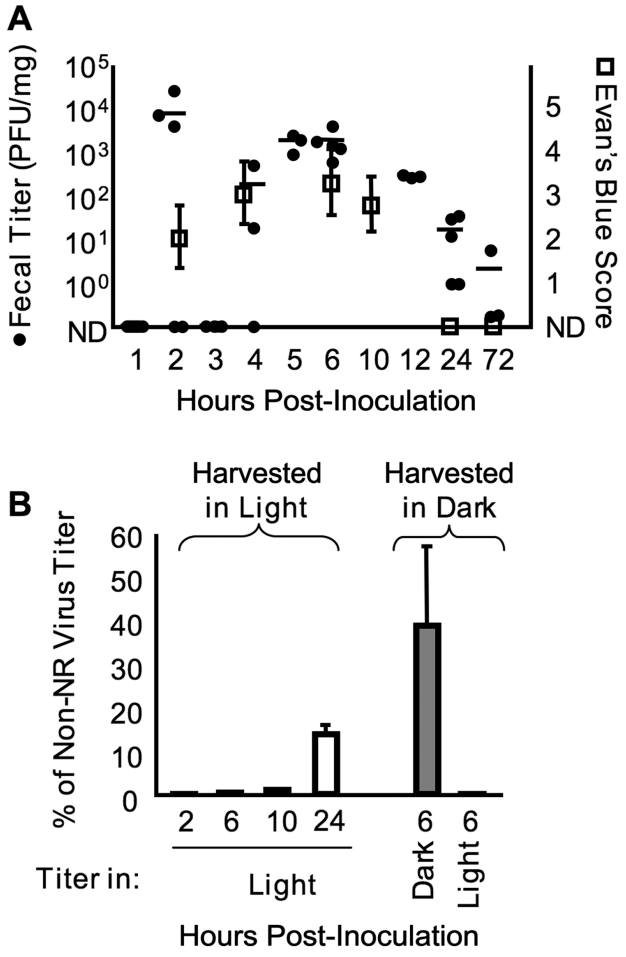

Figure 2-7. Kinetics of poliovirus shedding in feces. (A) Mice were orally inoculated with $2x10^7$ PFU of poliovirus or a solution of 8% Evan's Blue dye to trace transit through the GI tract. Fresh feces were harvested from individual mice at the indicated times, and viral titers (filled circles) or Evan's Blue score (open squares) were determined for 2-5 mice per time point. Titer averages are indicated by the horizontal lines. Evan's Blue score was determined by assessing the level of blue dye in fecal samples (see Materials and Methods). ND = not detected (below the detection limit). (B) Neutral red (NR), light-sensitive poliovirus was used to measure input/non-replicated *vs*. replicated virus in feces. Mice were orally inoculated with $2x10^7$ PFU of NR-poliovirus in the dark, and feces were harvested in the dark (right side, gray bars) or in the light (left side, white bars) at the indicated times. Fecal virus titers were determined in HeLa cells under light or dark conditions, and non-NR virus titers were divided by NR virus titers from the same time points (in panel A) to generate normalized titer values, to yield non-NR (or replicated) viral amounts. Work done in collaboration with C.A. Etheredge.

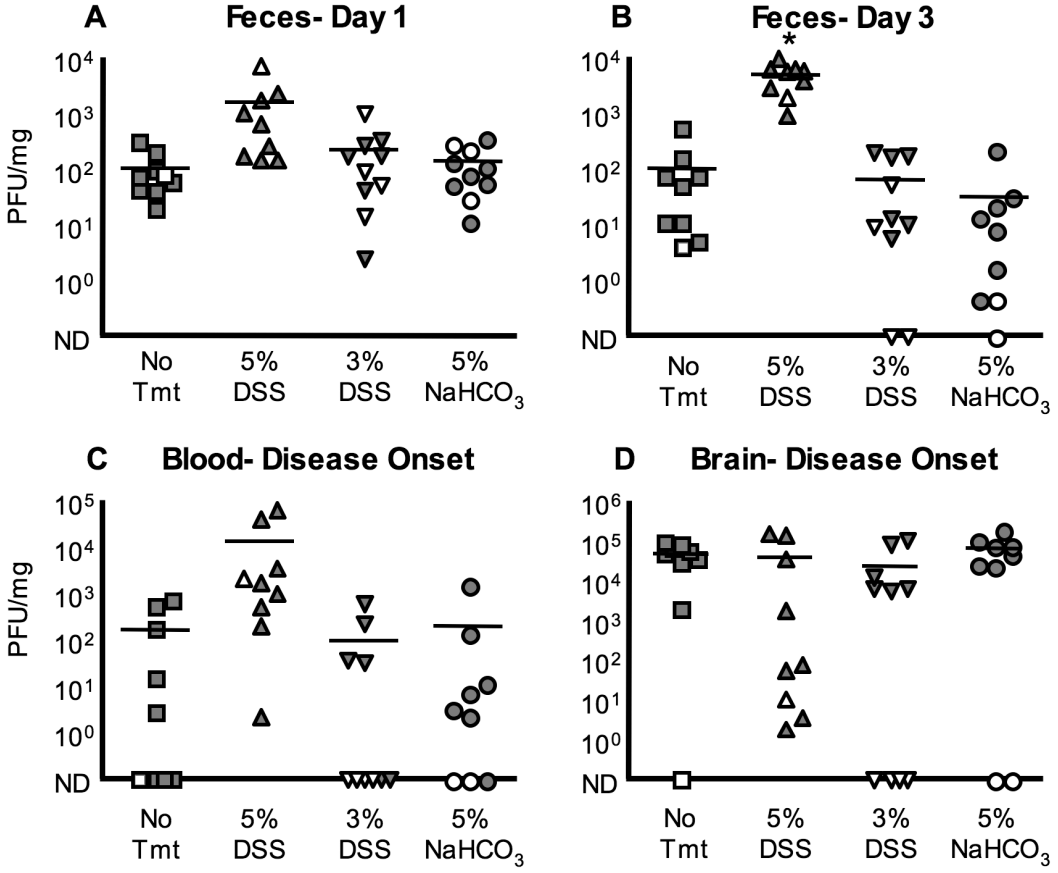

Figure 2-8. Poliovirus titers from orally-inoculated untreated, DSS-treated and antacid-treated PVRtg-IFNAR$^{-/-}$ mice. Mice were untreated or pre-treated with 3% or 5% dextran sulfate sodium (DSS) in drinking water for 3 or 5 days, respectively, after which mice orally received 2x10^7 PFU of the ten member pool. One group of mice was administered virus in the presence of 5% $NaHCO_3$. (A and B) Poliovirus titers, expressed as plaque-forming units (PFU) per mg of feces harvested on day 1 (A) or day 3 (B) from untreated, 5 or 3% DSS-treated and 5% $NaHCO_3$-treated mice. Asterisk indicates statistical significance between untreated and 5% DSS-treated mice ($p<0.0002$, Student's t-test). (C) Poliovirus titers from blood collected at disease onset. (D) Poliovirus titers from brain tissue collected at disease onset. Gray symbols: mice that developed disease; white symbols: mice that did not develop disease by day 10 post-inoculation. ND: not detected. Titer assay detection limit: ~1 PFU/mg. Horizontal lines indicate averages.

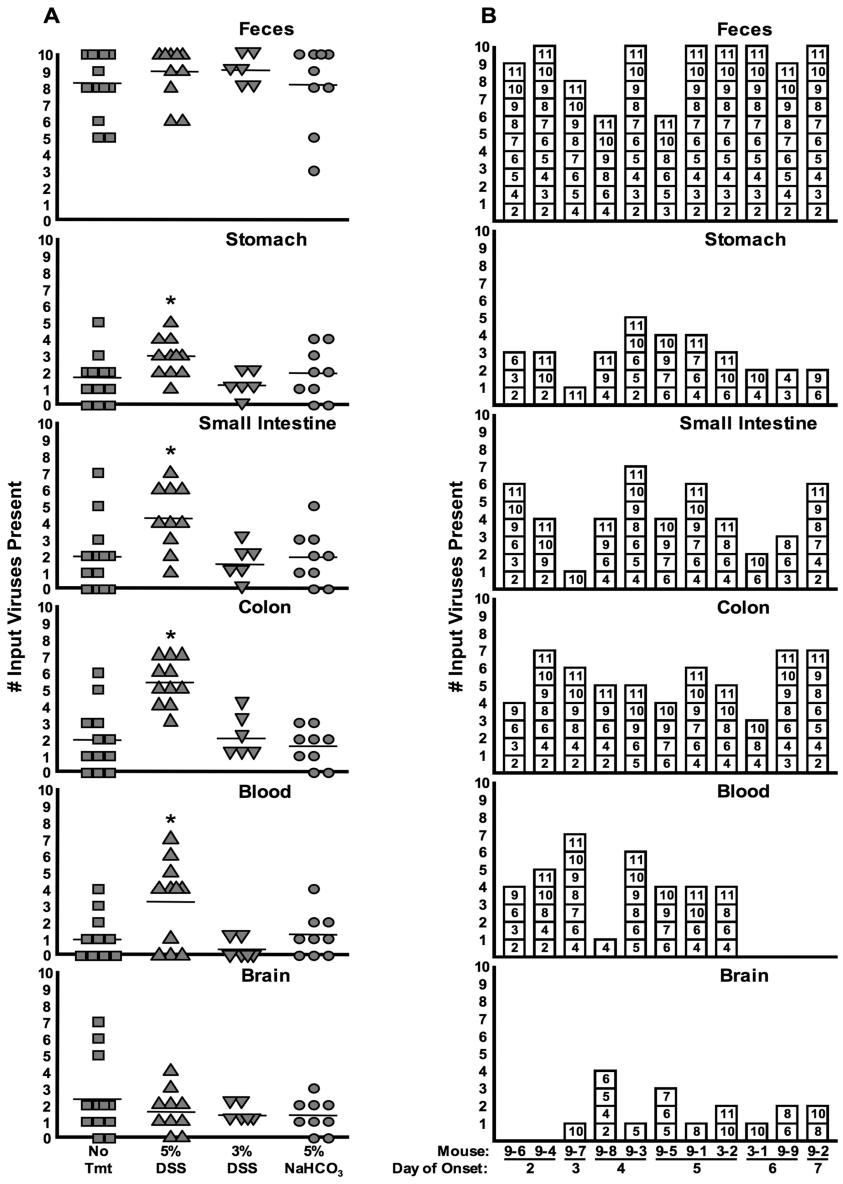

Figure 2-9. Viral diversity in tissues from orally-inoculated untreated, DSS-treated and antacid treated PVRtg-IFNAR$^{-/-}$ mice. Samples from figure 2-8 were subjected to the hybridization-based diversity assay. (A) Number of input viruses present, compiled from tissues and feces from untreated, 5% or 3% DSS-treated or 5% $NaHCO_3$-treated mice. Horizontal lines represent averages. Astericks indicate statistically significant differences between untreated and 5% DSS-treated mice ($p<0.05$, Student's t-test; see text for exact p values). (B) Viral diversity in tissues of 5% DSS-treated mice. Each box represent individual viruses from the ten member pool detected in tissues at disease onset or feces at day one post-inoculation by hybridization-based assay results. It is likely that mice 9-6 and 9-4 succumbed to DSS treatment rather than poliovirus since no virus was detected in the brain of each. Work done in collaboration with C.A. Etheredge.

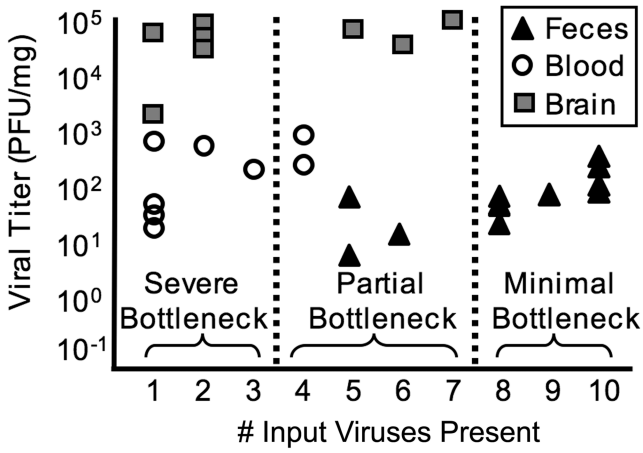

Figure 2-10. Bottlenecks revealed by comparing viral titer *vs*. diversity. Samples from untreated, orally-inoculated PVRtg-IFNAR$^{-/-}$ mice were compared by graphing viral titer (Figure 2-8) *vs*. number of input viruses present (see Figure 2-9). Samples with 1-3 input viruses present experienced a severe bottleneck, samples with 4-7 input viruses present experienced a partial bottleneck, and samples with 8-10 input viruses present experienced a minimal bottleneck.

CHAPTER THREE
Intestinal microbiota promote enteric virus infections

Introduction

Enteric viruses encounter a complex environment within the intestine, including about 10^{14} bacteria that inhabit the mammalian intestine and contribute to GI development, immune system development, digestion, and protection from pathogenic bacteria (Garrett et al., 2010). It is unknown whether these commensal microorganisms defend against mammalian enteric viruses. Several studies were performed comparing conventional to germ-free mice in the 1960s using viruses that induce tumorigenesis: friend and polyoma virus (Mirand and Grace, 1963; Tennant et al., 1965), respiratory viruses: influenza A and pneumonia virus (Dolowy and Muldoon, 1964; Tennant et al., 1965), and enteric viruses: coxsackie B, reovirus type 3 and rotavirus (or EDIM=epizootic diarrhea in infant mice) (Schaffer et al., 1963; Tennant et al., 1965). Collectively, these reports are conflicting in that some report that the microbiota protects the host from viral infections (Dolowy and Muldoon, 1964; Mirand and Grace, 1963; Schaffer et al., 1963), yet others reveal no difference in viral infectivity and pathogenesis when comparing conventional to germ-free mice (Tennant et al., 1965). Notably, many of these studies addressed enteric viral infections only after peripheral injections of the virus (Schaffer et al., 1963; Tennant et al., 1965) except for rotavirus, which was intragastrically introduced to mice but showed no difference in viral titers or susceptibility. IP inoculation of enteric viruses does not

mimic the natural infection route, and additionally, this bypasses the GI lumen containing intestinal bacteria. Therefore, most of the previous studies only addressed systemic effects of the microbiota on enteric viruses. Previous reports on influenza A virus in germ-free mice were conflicting (Dolowy and Muldoon, 1964; Tennant et al., 1965). However, thorough analysis recently revealed that the microbiota is important to induce immunity that limits influenza A virus replication in the lung, but viral pathogenesis was not examined (Ichinohe et al., 2011). The previous literature did not fully assess enteric virus infection via natural transmission routes in germ-free and conventional mice. Because we found that the gut poses a major barrier to poliovirus within mice, it was of interest to determine if intestinal microbes contribute to this barrier that restricts poliovirus in the lumen of the GI tract and beyond.

The intestinal microbiota are composed of an estimated 1000 different species of bacteria, of which Firmicutes and Bacteroidetes are the dominant phyla represented in nearly all mammalian intestinal tracts accounting for ~90% of the biodiversity (Ley et al., 2008). The majority of GI bacteria are not culturable using standard laboratory techniques, as revealed by high-throughput 16S rDNA sequencing methods to assess bacterial diversity (Eckburg et al., 2005). Eckburg et al. discovered that *Clostridia* species account for 95% of Firmicutes in the GI tract. The Bacteroidetes phylotypes in the GI tract were more diverse, but all samples included *Bacteroides thetaiotaomicron* (Eckburg et al., 2005), a well-established commensal bacterium that contributes to many

aspects of host health (Hooper et al., 2001). Intestinal microbes are restricted from the host epithelial layer by mucins (Johansson et al., 2008), limiting bacterial invasion of host tissues. Naturally-residing bacteria within the GI tract stimulate the mucosal immune system, protecting the GI mucosa from bacterial invasion by commensal or pathogenic counterparts (Brandl et al., 2007; Kelsall, 2008; Kobayashi et al., 2005; Macpherson et al., 2000; Suzuki et al., 2004; Vaishnava et al., 2008).

The vast diversity and abundance of microorganisms residing within the GI tract prompted me to examine the potential of this community to act as a physical barrier to poliovirus infection in orally-inoculated mice. I hypothesized that in their absence, poliovirus would be able to penetrate the epithelial barrier, replicate and disseminate to a greater degree than when the full microbiota was present. However, I learned that poliovirus and another unrelated enteric virus, reovirus, underwent greater replication and induced more severe pathology in the presence of the microbiota. Poliovirus infectivity was enhanced by intestinal microbes *in vivo* and *ex vivo*. In addition, several representative strains of bacteria found in the GI tract increased poliovirus infectivity *in vitro*. Additional *in vitro* experiments suggest that components of bacterial cell walls may help promote poliovirus infectivity.

Material and methods

Viruses and Cells

Virus work was performed in WHO-approved elevated BSL2+ areas. Poliovirus (serotype 1, Mahoney) cell culture infections and plaque assays were performed using HeLa cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Hyclone) and antibiotics (10,000U/ml penicillin and 10,000μg/ml streptomycin; Thermo Scientific). Poliovirus infections were also performed in PVRtg mouse embryonic fibroblasts (MEF) grown in DMEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and antibiotics (10,000U/ml penicillin and 10,000μg/ml streptomycin). PVRtg MEF were generated from day 13-16 embryos and isolated as previously described (Pfeiffer and Kirkegaard, 2005). PVRtg MEF cells were maintained for less than four weeks post-isolation to maintain a primary state.

Reovirus T3SA+ was amplified and purified as described previously (Barton et al., 2003) and quantified by plaque assay using murine L929 cells maintained in DMEM supplemented with 10% FBS and antibiotics (10,000U/ml penicillin and 10,000μg/ml streptomycin) (Virgin et al., 1988). All cells were incubated at 37°C with 5% CO_2.

Mice, treatments and inoculations

All animal work was performed in accordance with University of Texas Southwestern Medical Center IACUC approved protocols. C57BL/6 PVR-Tg21

(PVRtg) and C57BL/6 PVRtg-IFNAR$^{-/-}$ (PVRtg-IFNAR$^{-/-}$) mice, obtained from S. Koike (Tokyo Metropolitan Institute for Neuroscience), were maintained in specific pathogen-free conditions at University of Texas Southwestern Medical Center. Microbiologically-sterile germ-free C57BL/6 mice were maintained in gnotobiotic chambers (Cash et al., 2006) until the point of poliovirus infection. For poliovirus infection, germ-free mice were housed in the BSL2+ facility in sterile cages with autoclaved bedding, food and water. Feces were plated (see below) to monitor intestinal bacterial colonization, and fecal bacterial counts were undetectable through 48 hours post-infection. After 48 hours, mice became colonized with bacteria. Six week old C57BL/6 (non-PVR transgenic; obtained from Jackson Laboratory), PVRtg or PVRtg-IFNAR$^{-/-}$ mice were orally-administered a combination of four antibiotics: ampicillin, neomycin, metronidazole and vancomycin (Sigma-Aldrich, Research Products International) via oral gavage for 5 days (10 mg of each antibiotic per mouse per day) followed by ad libitum administration in drinking water (ampicillin, neomycin and metronidazole: 1g/L; vancomycin: 500mg/L) (Rakoff-Nahoum et al., 2004) for the duration of the experiment. Mice were antibiotic-treated for 10 days prior to peroral inoculation with poliovirus or reovirus, or were antibiotic-treated for 8 days prior to recolonization with a fecal suspension. Antibiotic treatment was carried out for over seven days to ensure that bacterial detritus was cleared from the intestinal tract of mice. Recolonized mice were perorally-administered 20-25 ul of untreated mouse feces resuspended in PBS (2-4 pellets resuspended in 500ul of

PBS), and 48 hours was allowed for colonization to occur. Mouse feces were plated on brain heart infusion agar (BHI, Sigma-Aldrich) plates supplemented with 10% calf blood (Colorado Serum Company) to assess bacterial loads. Plates were incubated for 48 hours in anaerobic chambers with oxygen-reducing, carbon dioxide-generating sachets and anaerobic indicator strips (BD). Untreated mice were either mock treated by oral gavage with sterile water or left completely untreated, since both methods yielded similar results.

For all poliovirus infections (untreated, antibiotic-treated or recolonized mice), 7-9 week old mice were perorally inoculated with 2 x 10^7 PFU of poliovirus as described in Chapter 2 Materials and Methods. For survival assays, PVRtg-IFNAR$^{-/-}$ mice were orally infected with 2x10^7 PFU poliovirus or IP inoculated with 2x10^7 PFU (PVRtg-IFNAR$^{-/-}$) or 1x10^8 PFU (PVRtg) after treatments, and disease was monitored until day 12 post-inoculation. In all cases, mice were euthanized upon severe disease onset, manifested as paralysis, encephalitis, and/or severe lethargy. Once mice reach this stage of severe disease onset, they do not survive more than 1 to 12 hours. Therefore, throughout this study, mice were examined twice daily and were euthanized upon the onset of severe disease for humanitarian reasons. Gastrointestinal motility was measured in PVRtg-IFNAR$^{-/-}$ mice by monitoring Evan's blue dye (MP Biomedicals, LLC) transit as previously described (Chapter 2 Materials and Methods). For shedding and replication experiments, PVRtg mice were orally inoculated with 2x10^7 PFU of standard

poliovirus or light-sensitive poliovirus (to measure replication), and feces were collected at multiple times post-inoculation from each mouse.

To isolate poliovirus-containing lower small intestine lumenal contents, C57BL/6 or PVRtg mice were orally inoculated with $2x10^7$ PFU poliovirus, and mice were euthanized at 1.5-2 hours post-infection and lumenal materials from the lower half of the small intestine were extracted from each mouse and processed as described below.

To assess *in vivo* effects of antibiotics on poliovirus, light-sensitive poliovirus was mixed with the four antibiotics (ampicillin, neomycin and metronidazole: 1g/L; vancomycin: 500mg/L) prior to oral inoculation of untreated PVRtg-IFNAR$^{-/-}$ mice. Feces were collected from each mouse at multiple times post-inoculation and survival was monitored to day 12 post-inoculation. Pathogenesis of poliovirus in antibiotic-treated mice harboring antibiotic-resistant bacteria was examined in PVRtg-IFNAR$^{-/-}$ mice after selection of antibiotic-resistant bacteria. Four-week old PVRtg-IFNAR$^{-/-}$ mice were administered low-dose antibiotics in drinking water (ampicillin, neomycin and metronidazole: 500mg/L; vancomycin: 250mg/L) for two weeks, and depletion of gastrointestinal microbes was confirmed by plating feces. The concentration of antibiotics in water was increased to the standard treatment regimen (ampicillin, neomycin and metronidazole: 1g/L; vancomycin: 500mg/L), and feces were collected and plated periodically on BHI/blood agar plates without or with antibiotics (ampicillin: 100μg/ml, neomycin: 50μg/ml, metronidazole: 25μg/ml, vancomycin: 5μg/ml) to

assess bacterial loads (**Figure 3-7B**). When antibiotic-resistant fecal bacteria were present at similar abundance as bacteria in untreated mouse feces, mice were orally inoculated with light-sensitive poliovirus, feces were collected for viral replication assessment and survival was monitored.

Reovirus infections were performed in 8-week old untreated or antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice by peroral inoculation with 1x10^{8} PFU of the T3SA+ strain (Barton et al., 2003). Mouse feces were monitored up to day four post-inoculation for fecal pathology (see **Table 3-1** for scoring system). At four days post-infection, mice were euthanized and tissues collected for viral titer analysis (see below). Intestines were flushed with cold PBS for gross analysis, Peyer's patch measurements, and viral titer assays.

Sample processing and titer analyses

Feces and lower small intestine lumenal contents from infected mice were resuspended in 1-5 volumes of PBS+ (1X PBS supplemented with 100ug/ml $MgCl_2$ and $CaCl_2$) and freeze-thawed 3 times. The suspension was clarified by centrifugation at 13,000 rpm for 5 minutes, supernatants were extracted with 1/10 volume of chloroform to eliminate bacteria and samples were subjected to centrifugation for 3.5 minutes. Virus in supernatants was quantified by plaque assay using HeLa cells (Pfeiffer and Kirkegaard, 2005). Feces containing neutral red/light-sensitive poliovirus were processed as above, but in the dark under a red safety light, and a portion of each sample was light exposed. Light-exposed

and unexposed viruses were quantified by plaque assay to determine the amount of replicated virus by dividing light-exposed PFU/ml by light unexposed PFU/ml and multiplying by 100.

Visualization of intestinal architecture and immune cell infiltration 48 hours post-inoculation with 2 x 10^7 PFU of light-sensitive poliovirus was performed as follows. Whole intestines were isolated and flushed well with cold PBS from three untreated and three antibiotic-treated PVRtg mice. Intestines were transected, opened with the villi facing upward, pinned every ~1-1.5 inches and washed well with cold PBS to remove all lumenal contents. Tissues were submerged in 10% neutral-buffered formalin overnight at 4°C. The next day, using a wooden probe, intestines were rolled concentrically with the villi outwardly exposed. Tissue rolls were stabilized in a biopsy mega-cassette (Tissue Tek) using formalin-soaked biopsy sponges with holes cut in which to place each tissue. All tissue-containing cassettes were soaked in 10% neutral-buffered formalin at 4°C for at least 48 hours. Tissue rolls were paraffin-embedded, cut into 6μm sections and stained with hemotoxylin and eosin (UT Southwestern Histology Core). Images were taken on a Zeiss Axio Imager.M1 microscope using Axio Vision Release 4.8.2 software.

Tissues collected from mice infected with reovirus were suspended in PBS, freeze-thawed and homogenized using a Bullet Blender Tissue Homogenizer (Next Advanced Inc) or sonication. Viruses in supernatants were

quantified by plaque assay using L929 cells as described previously (Virgin et al., 1988).

Poliovirus infectivity assays

Infectious center assays, modified from (Brandenburg et al., 2007), were performed by comparing HeLa cell-derived poliovirus (TC) to lower small intestine lumenal content poliovirus isolated from untreated, antibiotic-treated and germ-free mice. HeLa and PVRtg MEF cells ($5x10^5$ cells/well) were infected with each virus (3000-5000 PFU) for 10 minutes at 37°C, after which virus was removed and cells were washed thoroughly with PBS. Cells were trypsinized in 500μl and added to 500μl of DMEM with 10% FBS, and 300μl of undiluted, 1:10 or 1:100 cells in media were plated on a monolayer of HeLa cells, which were incubated 3.5 hours at 37°C for cellular attachment to occur. Agar overlays were then added, and plates were incubated at 37°C for two days for plaque generation. The PVRtg MEF viral titers were expressed as a percentage of the HeLa cell viral titers to normalize for different amounts of virus in individual mice, and to reflect relative infectivity.

Poliovirus infectivity after exposure to PBS or suspensions of untreated, antibiotic-treated or germ-free mouse feces in PBS were performed as follows. Feces were collected from untreated, antibiotic-treated or germ-free mice, resuspended in 5-6 wt/vol of PBS and solutions were aliquoted into two tubes. Tissue culture-derived poliovirus ($2x10^3$ - $2x10^4$ PFU) was added to PBS alone or

each fecal suspension in glass tubes and incubated at 37°C for six hours. Samples were clarified by centrifugation, chloroform-extracted (10%), and viral titers were quantified by plaque assay in HeLa cells as described above to yield virus recovery.

Poliovirus infectivity was measured after exposure to *Escherechia coli* K12, *Ochrobactrum intermedium* (see below), *Bacillus cereus* Abx UK-1 or *Enterococcus faecalis* V583 (*E. coli*, *B. cereus* and *E. faecalis* strains were kindly provided by L. Hooper). Bacteria were grown to stationary phase overnight shaking at 37°C. Bacteria were pelleted by centrifugation at 3750rpm for 30 minutes, washed with PBS and repelleted at 3750rpm for 30 minutes. Bacteria were resuspended in minimal media (DMEM, no serum). Poliovirus ($2x10^4$ PFU) was added to $5x10^6$ - $5x10^7$ CFU of each bacterial strain per glass tube and incubated at 37°C for six hours and processed as outlined in the previous paragraph. Similar protocols were followed for LPS and PG experiments. Poliovirus ($1x10^5$ PFU) was added to suspensions of LPS (Sigma-Aldrich) or PG (L. Hooper) and incubated at 37°C for six hours, after which samples were diluted and quantified by plaque assay. Experiments were performed to eliminate cellular effects induced by diluted LPS and PG (data not shown).

In vitro viral replication kinetics

Poliovirus growth curves were performed in HeLa and PVRtg MEF cells in the absence and presence of antibiotics (ampicillin, neomycin and metronidazole:

1g/L; vancomycin: 500mg/L). Poliovirus ($1x10^6$ PFU) was suspended in PBS or PBS supplemented with all four antibiotics, and cells were infected and harvested at multiple times post-infection, and viral yields were quantified by plaque assay as previously described (Pfeiffer and Kirkegaard, 2005).

Identification of antibiotic-resistant bacteria

Feces were collected from antibiotic-treated mice that maintained bacterial colonization, due to antibiotic-resistant bacteria. Feces were stored at -20°C prior to isolation of bacterial DNA via QIAamp DNA Stool Mini Kit (Qiagen). Genomic DNA from fecal bacteria and bacterial colonies from mouse feces plated on BHI-blood agar plates were PCR amplified using universal 16S rDNA primers (forward: 5'-AGAGTTTGATYMTGGCTCAG-3', reverse: 5'-ACGGYTACCTTGTTACGACTT-3'). PCR products were purified using the QIAquick Nucleotide Removal Kit (Qiagen) and then cloned into a TOPO vector following vendor instructions (TOPO TA Cloning Kit, Invitrogen). Plasmids were heat-shock transformed into *E. coli* DH5α competent cells and plated on LB agar plates supplemented with 10 μg/ml of ampicillin (Research Products International) and 40 μg/ml of X-Gal (Fisher Scientific). *E. coli* colonies were amplified to purify plasmid DNA using the QIAprep Spin Miniprep Kit (Qiagen) and plasmid DNA was sequenced (McDermott Sequencing Core, UT Southwestern Medical Center, Dallas, TX). Sequences of seven feces-derived clones and two colony-derived clones were queried via BLAST (NCBI) for

identification of the antibiotic-resistant bacterium. In all nine clones, sequences aligned with the 16S rDNA region of *Ochrobactrum intermedium* (strain CCUG 43465, AM490610, NCBI database by Clustal W method).

Results

Intestinal microbiota enhance poliovirus pathogenesis in mice after oral inoculation

To investigate how intestinal microbiota influence poliovirus infection, mice susceptible to poliovirus were treated with a combination of four antibiotics (ampicillin, neomycin, metronidazole and vancomycin) to deplete the intestinal microbes prior to poliovirus inoculation (**Figure 3-1A**). PVRtg-IFNAR$^{-/-}$ mice develop poliovirus symptoms after oral inoculation (Ida-Hosonuma et al., 2005; Ohka et al., 2007), so I followed disease progression in these mice that were untreated, antibiotic-treated or antibiotic-treated and recolonized with fecal bacteria. Prior to poliovirus infection, depletion of intestinal microbes was confirmed by plating fecal suspensions on brain-heart infusion agar supplemented with blood and grown anaerobically to assess the number of culturable bacteria present in feces. Intestinal microbiota were reduced by 99.99% in antibiotic-treated mice (**Figure 3-1B**). Recolonization of antibiotic-treated mice with fecal suspensions from untreated mice fully restored bacterial loads in the intestine. Untreated, antibiotic-treated and antibiotic-treated/recolonized mice were orally inoculated with $2x10^7$ PFU of poliovirus and

monitored for poliovirus symptoms for 12 days post-infection. Surprisingly, I observed that mice harboring intestinal microbes were more susceptible to poliovirus than mice with reduced flora (**Figure 3-2A**). To ensure that enhanced poliovirus dissemination in orally inoculated, untreated PVRtg-IFNAR$^{-/-}$ mice was dependent on poliovirus encountering intestinal microbes within the GI tract, untreated and antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice were infected with 2x10^{7} PFU of poliovirus by IP injection. This inoculation route bypasses the GI tract, so virus traffics to the CNS without encountering the GI lumen. IP inoculated untreated and antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice succumbed to poliovirus infection with the same kinetics (**Figure 3-2B**). Because PVRtg-IFNAR$^{-/-}$ mice mortality occurred so quickly potentially masking any small differences in poliovirus pathogenesis via IP route, untreated and antibiotic-treated PVRtg mice were IP inoculated with 1x10^{8} PFU of poliovirus. Disease progression occurred similarly in IP inoculated untreated and antibiotic-treated PVRtg mice (**Figure 3-2C**), as in PVRtg-IFNAR$^{-/-}$ mice, suggesting that enhanced poliovirus pathogenesis in orally-inoculated, untreated mice was dependent on poliovirus encountering intestinal microbes within the GI tract.

Intestinal microbiota alter fecal shedding of poliovirus

To determine if microbiota-containing mice support more poliovirus replication within their GI tracts than mice with a depleted flora, virus was quantified from feces of infected PVRtg-IFNAR$^{-/-}$ (**Figure 3-3A**) and PVRtg

(**Figure 3-3B**) mice. Kinetics of poliovirus shedding in feces differed between untreated and antibiotic-treated mice. Antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice displayed an initial delay in poliovirus excretion followed by prolonged, elevated excretion until five days post-infection (**Figure 3-3A**). Conversely, mice with microbes shed the majority of poliovirus within the first six hours post-infection, and shedding quickly declined thereafter. Similar fecal shedding differences were observed from untreated and antibiotic-treated PVRtg mice (**Figure 3-3B**). Poliovirus was quickly cleared from the GI tracts of antibiotic-treated recolonized PVRtg mice. Short-term recolonization of antibiotic-treated PVRtg mice may induce type I IFN signaling that aids clearance of poliovirus, since I did not observe rapid clearance of poliovirus in feces from antibiotic-treated recolonized PVRtg-IFNAR$^{-/-}$ mice (**Figure 3-3A, 3-3B**).

In order to determine if the observed differential fecal shedding kinetics of poliovirus is a result of general GI differences between microbiota-harboring and antibiotic-treated mice independent of virus cell entry and replication, poliovirus excretion was monitored in non-PVR C57BL/6 mice. As with PVR mice, antibiotic-treated C57BL/6 mice had an initial shedding delay following by prolonged, elevated shedding of poliovirus in feces (**Figure 3-3C**). C57BL/6 mice harboring a microflora exhibited similar shedding kinetics as seen for PVR mice with a microflora, but poliovirus shedding ceased much sooner in C57BL/6 mice which was probably due to lack of replication since the viral receptor is absent. In addition, C57BL/6 microbiologically-sterile germ-free mice displayed similar

poliovirus shedding in feces compared to antibiotic-treated C57BL/6 mice indicating that antibiotic treatment alone does not confer this altered shedding phenotype (**Figure 3-3C**). Poliovirus excretion from germ-free mice could only be followed until 48 hours post-inoculation since the mice became colonized with bacteria after 48 hours. Together, these data suggest that intestinal microbes stimulate peristalsis, and therefore, promote early virus shedding.

Because a delay in poliovirus excretion was detected in all strains of antibiotic-treated mice tested, I performed fecal dye analysis after oral inoculation of mice with Evan's blue dye to evaluate intestinal motility. PVRtg-IFNAR$^{-/-}$ mice were untreated or antibiotic-treated, orally administered Evan's blue dye and monitored for blue coloration in feces to determine dye transit through the GI tract (see Chapter 2). A lag was evident in fecal excretions from antibiotic-treated mice (**Figure 3-3D**), suggesting that intestinal motility is slower in mice with reduced flora. These results are consistent with a previous report showing that GI peristalsis is delayed in germ-free mice compared to conventional mice (Abrams and Bishop, 1967).

Intestinal microbiota augment poliovirus replication in mice following oral inoculation

Because I observed differences in pathogenesis and fecal shedding of poliovirus between antibiotic-treated and untreated mice, I decided to directly quantify poliovirus replication within mouse GI tracts. I performed experiments

using light-sensitive poliovirus to distinguish input virus (from the inoculum) from replicated virus excreted in feces. Poliovirus virions containing neutral red (NR) are sensitive to light-induced cross-linking of NR to viral RNA, thereby inactivating virus (Huang et al., 2000; Kirkegaard, 1990; Mandel, 1967). Upon replication within a mouse, light-sensitive poliovirus releases NR and new viral genomes are packaged in the absence of the dye conferring light-insensitivity. This allows us to differentiate the light-insensitive replicated virus from light-sensitive input virus via virus quantification from sample aliquots that are either exposed to light or not. Untreated, antibiotic-treated and antibiotic-treated/recolonized PVRtg-IFNAR$^{-/-}$ mice were orally inoculated with NR poliovirus in the dark. Feces were collected and processed in the dark. I titered extracted virus that was unexposed or light-exposed and compared the values to determine the amount of replicated virus at multiple intervals post-infection. Mice harboring intestinal microbes supported poliovirus replication in their GI tracts, whereas antibiotic-treated mice did not (**Figure 3-4A**). Poliovirus replication was also assessed in untreated and antibiotic-treated PVRtg mice, and similar results were obtained (**Figure 3-4B**). Notably, this is the first report of poliovirus replication taking place in the GI tracts of PVRtg mice. These data suggest that type I IFN antiviral responses do not limit poliovirus replication within the intestines, rather, it is more likely that IFNα/β restricts poliovirus replication in extra-intestinal sites inhibiting spread to the CNS.

To determine whether poliovirus infection altered intestinal pathology, intestines from untreated and antibiotic-treated mice were collected 48 hours post-inoculation, fixed and stained by hemotoxylin and eosin to visualize intestinal architecture and immune cell infiltration. Epithelial cell disruption and infiltration of immune cells was evident in only one of three untreated PVRtg intestinal sections, but no abnormalities were detected in intestines from three antibiotic-treated PVRtg mice (**Figure 3-5A**). Additionally, it was difficult to detect poliovirus infection within intestinal sections by plaque assay (**Figure 3-5B**), indicating that detection of poliovirus and poliovirus-induced pathology in intestinal tissues is challenging. Nonetheless, these results reveal that poliovirus replication is enhanced in mice with a microbiota, which may contribute to more severe pathogenesis in mice.

Enhanced poliovirus pathogenesis and replication are dependent on intestinal microbes and are not due to direct effects of antibiotic treatment

Poliovirus pathogenesis and replication are promoted in mice containing a microbiota, so to ensure that the observed phenotypes were a result of microbial presence within the intestine and not the antibiotic regimen, several experiments were performed. First, shedding, replication and pathogenesis phenotypes were recapitulated when antibiotic-treated mice were recolonized with fecal bacteria indicating that antibiotics do not permanently alter the host (**Figure 3-2A, 3-3A, 3-4A**). Second, poliovirus exposed to antibiotics replicated as well as poliovirus

alone in HeLa and mouse embryonic fibroblast (MEF) cells (**Figure 3-6**). Third, poliovirus shedding in feces from germ-free C57BL/6 and antibiotic-treated C57BL/6 closely mirrored each other (**Figure 3-3C**). Fourth, poliovirus pre-mixed with antibiotics prior to oral inoculation demonstrated similar levels of replication and pathogenesis as poliovirus alone (**Figure 3-7C, 3-7D**). Fifth, mice harboring antibiotic-resistant bacteria within their GI tracts supported poliovirus replication and succumbed to poliovirus disease similarly to untreated PVRtg-IFNAR$^{-/-}$ mice (**Figure 3-7C, 3-7D**). These experiments were performed to examine poliovirus replication and pathogenesis in the presence of a microbiota as well as antibiotics to affirm that antibiotics were not responsible for inhibition of poliovirus replication and dissemination in mice. PVRtg-IFNAR$^{-/-}$ mice were administered antibiotics in drinking water to select for antibiotic-resistant bacteria within the GI tract (**Figure 3-7A**). Microbiota depletion was confirmed, and feces were monitored frequently to assess outgrowth of bacteria. After a few weeks, culturable bacteria from feces of antibiotic-treated mice exhibited antibiotic-resistance and isomorphic colony formation, whereas fecal bacteria from untreated mice were completely sensitive to the combination of four antibiotics *in vitro* (**Figure 3-7B**). The predominant bacterial strain isolated from mice harboring the antibiotic-resistant bacteria was identified as *Ochrobactrum intermedium* by 16S rDNA sequencing of subclones (**Figure 3-7A, Appendix A**). *O. intermedium* is a Gram-negative aerobe that is commonly found in soil, but can be an opportunistic human pathogen (Apisarnthanarak et al., 2005; Lebuhn

et al., 2000; Moller et al., 1999; Velasco et al., 1998). Because *O. intermedium* can support poliovirus replication and pathogenesis in mice in the presence of antibiotics, I concluded that the antibiotic regimen is not directly responsible for diminished poliovirus replication and pathogenesis in antibiotic-treated mice. Rather, the significantly reduced microbiota, by antibiotic treatment, limited poliovirus replication and pathogenesis.

Poliovirus infectivity is enhanced by intestinal microbiota

The microbiota may promote poliovirus replication and pathogenesis by influencing the host, poliovirus or a combination of both. Initially, I examined poliovirus infectivity following exposure to intestinal microbes *in vivo*. I orally administered poliovirus to untreated, antibiotic-treated or germ-free mice and isolated lumenal contents from the lower small intestine of each at two hours post-inoculation. Lower small intestine contents were chosen since bacteria accumulate at more distal sites within the GI tract. Samples were collected at two hours post-inoculation because maximal amounts of virus can be isolated at this time and it is prior to a cycle of viral replication. Therefore, any potential infectivity differences are attributable to poliovirus exposure to lumenal contents. To evaluate differences between tissue culture-derived stock virus (TC) and viruses isolated from untreated, antibiotic-treated and germ-free mice, minimally-susceptible primary MEF cells (Pfeiffer and Kirkegaard, 2005) were infected in comparison to highly-susceptible transformed HeLa cells. I reasoned that

poliovirus infectivity differences would be more distinguishable in minimally-susceptible cells. Because different amounts of virus were isolated from each mouse, both MEF and HeLa cells were infected and resulting MEF plaque numbers were compared as a percent of HeLa cell titer. Lower small intestine virus isolated from untreated mice was twice as infectious in MEFs than TC-derived virus or virus isolated from antibiotic-treated and germ-free mice (**Figure 3-8A**). These data are consistent with the magnitude of poliovirus pathogenesis observed in orally infected mice (**Figure 3-2A**).

To determine if lumenal contents alone could alter poliovirus infectivity *ex vivo*, TC-derived virus was incubated with PBS or feces from untreated, antibiotic-treated or germ-free mice, followed by viral titer assay using HeLa cells. Infectivity of microbe-exposed poliovirus was significantly enhanced in comparison to poliovirus infectivity following exposure to solutions lacking microbes (**Figure 3-8B**). These results strongly suggest that exposure to microbes enhances poliovirus infectivity. Therefore, I performed similar poliovirus infectivity experiments using defined bacterial species present within the GI tract. Exposure to *Escherechia coli* maintained poliovirus infectivity compared to buffer controls, whereas *Bacillus cereus* and *Enterococcus faecalis* each significantly enhanced poliovirus infectivity after a six-hour exposure (**Figure 3-8C**). Surprisingly, *O. intermedium* minimally affected poliovirus infectivity *in vitro*, despite its ability to support poliovirus replication and pathogenesis *in vivo*. These results revealed that both Gram-negative and

Gram-positive bacteria are capable of enhancing poliovirus infectivity *in vitro*. Therefore, we set out to test specific bacterial cell wall components since they may come into direct contact with poliovirus *in vivo*. LPS and PG promoted poliovirus infectivity at concentrations of 10 μg/ml or more (**Figure 3-8D**). Thus, LPS and PG macromolecules decorating bacterial cell walls are sufficient to enhance poliovirus infectivity *in vitro*. Bacteria within the GI tract may be responsible for altering poliovirus infectivity, thereby promoting poliovirus infection.

Reovirus infection is enhanced by intestinal microbes

All enteric viruses encounter intestinal bacteria, which prompted me to investigate the specificity of the microbiota-dependent effects observed for poliovirus by using another enteric virus, reovirus. Reovirus is unrelated to poliovirus, but it is also transmitted by a fecal-oral route and has the propensity to invade the CNS (Tyler, 2001). Reovirus infection and replication within the GI tract has been well characterized. Reovirus is processed into its infectious form by proteases in the GI lumen, which facilitates uptake by M cells, infection in Peyer's patches and replication and release from enterocytes (Amerongen et al., 1994; Bass et al., 1990; Bass et al., 1988; Rubin et al., 1985; Wolf et al., 1987; Wolf et al., 1981). Unfortunately, the widely used reovirus mouse model employs neonatal mice that are difficult to treat with antibiotics and would also be too mature to follow reovirus pathogenesis after the full course of antibiotic treatment.

To circumvent this drawback, adult immunocompromised PVRtg-IFNAR$^{-/-}$ mice were used for reovirus infections. In my studies, PVRtg-IFNAR$^{-/-}$ mice survived reovirus infections, but disease symptoms were observed after mice were perorally administered 1x10^{8} PFU of the reovirus strain T3SA+. Feces from untreated, reovirus-infected mice appeared yellow with a hardened, oily consistency, whereas feces from uninfected mice and antibiotic-treated mice displayed normal coloration and consistency (**Table 3-1, Figure 3-9A**). Fecal pathology was scored (**Table 3-1**), and antibiotic-treated mice maintained normal fecal appearance and consistency throughout the infection, unlike feces from untreated mice (**Figure 3-9B**).

The observed fecal pathology is likely a result of biliary obstruction resulting from T3SA+ reovirus infection in the liver and bile duct (Barton et al., 2003), so I quantified reovirus from select tissues four days post-inoculation. Upon tissue collection, I noticed that Peyer's patches in the small intestine were severely enlarged in untreated, reovirus-infected mice, but not antibiotic-treated, reovirus-infected mice (**Figure 3-9C, 3-9D**), suggesting reovirus replication and/or a strong immune response to reovirus in untreated mice only. Quantification of reovirus from tissues collected on day four post-inoculation revealed that reovirus titers were higher in untreated mouse tissues (**Figure 3-9E**). However, reovirus was still present in antibiotic-treated tissues indicating that poliovirus may rely on intestinal microbes more than reovirus. Together,

these results indicate that the intestinal microbes can promote pathogenesis of at least two unrelated enteric viruses.

Discussion

Although intestinal microbes are beneficial for the host they naturally reside in, we present a case in which they augment pathogenic potential of enteric viruses by enhancing viral infectivity. We observed that if intestinal microbes were depeleted, mice were more protected from poliovirus pathogenicity, and viral replication was more robust within the GI tract (**Figure 3-2A, 3-4A, 3-4B)**. Polioviruses isolated from the GI tracts of antibiotic-treated mice were less infectious in a mouse primary cell line than polioviruses isolated from mice harboring a natural flora (**Figure 3-8A**). Similarly, infectivity of tissue culture-derived poliovirus was enhanced when exposed to feces containing intestinal microbes, not feces from antibiotic-treated or germ-free mice (**Figure 3-8B**). Data generated in these studies suggest that multiple bacterial strains are capable of enhancing poliovirus infectivity *in vitro*, and this may be imparted by cell wall components, such as LPS and PG (**Figure 3-8C, 3-8D**). Also, another enteric virus, reovirus, was less pathogenic and had reduced viral titers in antibiotic-treated mice (**Figure 3-9**). It would be interesting to examine how intestinal microbes influence additional enteric viruses. Comprehensively, our results show that commensal microbes within a host promote enteric viral infections. Interestingly, it was recently shown that GI microbes induce egg-

hatching of an intestinal nematode parasite in mice contributing to proliferation of this enteric pathogen (Hayes et al., 2010). In conjunction with my studies, these are examples implicating the intestinal microbes in propagation and transmission of enteric pathogens.

The microbiota may produce a factor or induce a host factor that alters poliovirus virions thereby enhancing poliovirus infectivity. Constituents of bacterial cell walls could be responsible for this phenotype, but the overall effects observed may be multifactorial. From here, several hypotheses arise concerning the action of the microbiota on poliovirus. (1) Poliovirus may interact with LPS and PG such that virions are stabilized. In the GI lumen, possible interactions with bacteria may help poliovirus navigate the thick mucus layers or enhance infection through other means. Binding experiments with poliovirus and cell wall components or whole bacteria will help elucidate this idea further. (2) Bacteria may produce a molecule that alters the conformation of the poliovirus virion, thereby enhancing infectivity. In fact, *Staphylococcus aureus* V8 protease cleaves VP1 on the surface of the poliovirus capsid yielding an altered conformation, as shown by cryo-electron microscopic reconstructions (Bubeck et al., 2005; Fricks and Hogle, 1990). Perhaps a bacterial protease activates poliovirus. (3) Bacteria may modify a host factor that then promotes infectivity of poliovirus, possibly by altering the virion to make it more infectious. If this were the case, this event would not occur in antibiotic-treated and germ-free mice potentially explaining reduced viral pathogenesis in antibiotic-treated mice. (4)

Bacterial presence within the intestine alters the overall environment possibly making it more conducive to poliovirus infection. Intestinal factors that are known to differ between germ-free and conventional mice that could be involved include altered osmolarity, mucin concentration and host enzyme concentrations (Smith et al., 2007).

In reference to hypotheses 2 and 3, reovirus infection in untreated mice is also enhanced, and reovirus virions require proteolytic processing within the host lumen for formation of the infectious unit, the intermediate subvirion particle (ISVP) (Amerongen et al., 1994; Bass et al., 1990; Bodkin et al., 1989). Many viruses that infect hosts at mucosal surfaces, including influenza and astroviruses, require proteolytic processing for *in vitro* cultivation (Lee and Kurtz, 1981; Tobita et al., 1975). I imagine a scenario in which intestinal bacteria may produce or induce an enzyme that processes virions to enhance infectivity, and therefore, act as a trigger for virion binding and entry into cells.

Notably, a single bacterial species, *O. intermedium*, was sufficient for poliovirus disease progression in mice, but only partially supported poliovirus replication (**Figure 3-7**). This phenotype suggests that bacterial components or bacterially-induced factors may stimulate virion changes that facilitate binding and entry into host cells, and *O. intermedium* only partially provides the factor. Additional investigations are underway to determine if this factor(s) is bacterially-derived or host-derived, and if the host mucosal immune response is altered in virally-infected untreated or antibiotic-treated mice.

Identification of a microbiota produced or induced factor that has the ability to enhance enteric viral infection would be a prime antiviral target. In addition, these results imply that antibiotics have antiviral potential, although I do not recommend this approach as a viral therapeutic. However, this application of my work might prove beneficial in very extreme cases to halt poliovirus replication and shedding in hypogammaglobulinemia individuals that received the OPV. These individuals can develop severe paralytic poliomyelitis and can excrete poliovirus in their feces for prolonged periods (Nathanson and Kew, 2010). Combination antibiotic treatment in these people may limit poliovirus disease and aid poliovirus eradication efforts. From these studies, I conclude that poliovirus may have the ability to "sense" the intestinal environment that is most conducive for its replication and persistence, potentially using the resident microbiota as a trigger for replication.

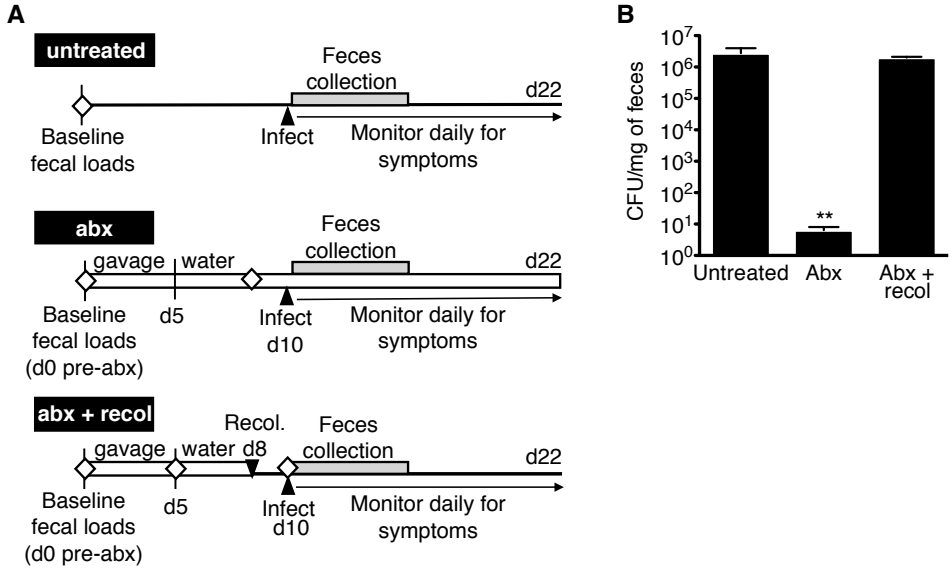

Figure 3-1. Microbe depletion and recolonization. (**A**) Strategy for treatments prior to poliovirus infections. ◇: fecal bacteria were plated on BHI-blood agar plates and grown anaerobically for enumeration; white box: duration of antibiotic (abx) treatment; gray box: duration of feces collection; recol = recolonized. (**B**) Culturable bacterial loads in feces. PVRtg-IFNAR$^{-/-}$ mice (n=4-7) were untreated, antibiotic-treated (abx) for 10 days, or antibiotic-treated for 8 days and recolonized (recol) for 2 days with fecal bacteria. Feces were plated and grown anaerobically, yielding colony-forming units (CFU) per milligram of feces. Bars represent mean + SEM, $^{**}p<0.01$, Student's t-test.

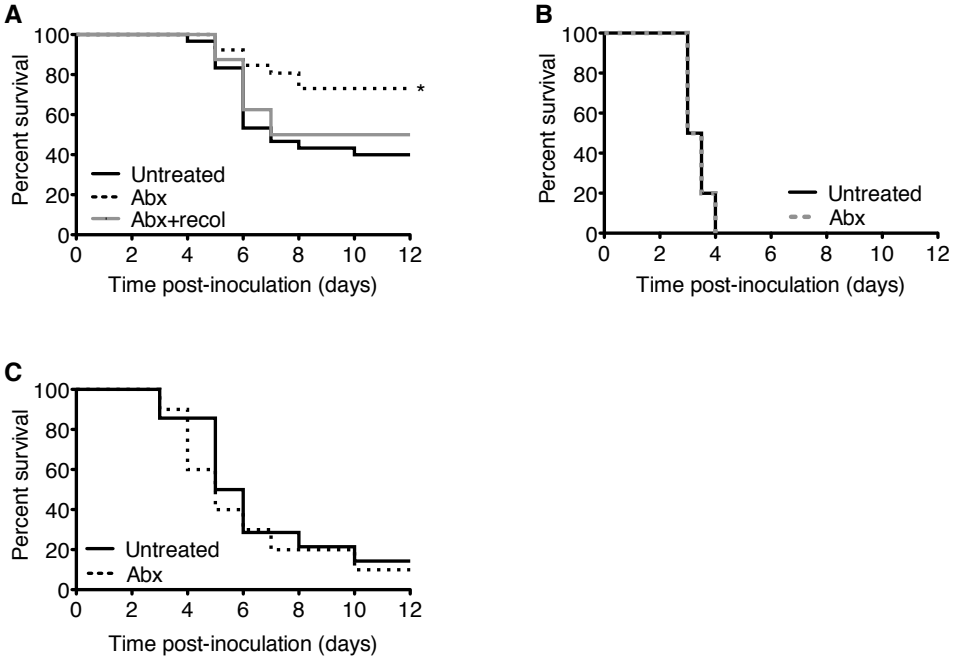

Figure 3-2. Poliovirus pathogenesis in untreated, antibiotic-treated (abx) and antibiotic-treated recolonized (abx+recol) mice. (**A**) Survival of PVRtg-IFNAR$^{-/-}$ mice orally infected with 2 x 10^7 plaque-forming units (PFU) poliovirus (untreated: n=30, abx: n=26, abx+recol: n=8). *p=0.012, Log-rank (Mantel-Cox) test. (**B**) Survival of PVRtg-IFNAR$^{-/-}$ mice intraperitoneally infected with 2 x 10^7 PFU poliovirus (untreated: n=14 , abx: n=10). (**C**) Survival of PVRtg mice intraperitoneally infected with 1 x 10^8 PFU poliovirus (untreated: n=10 , abx: n=10).

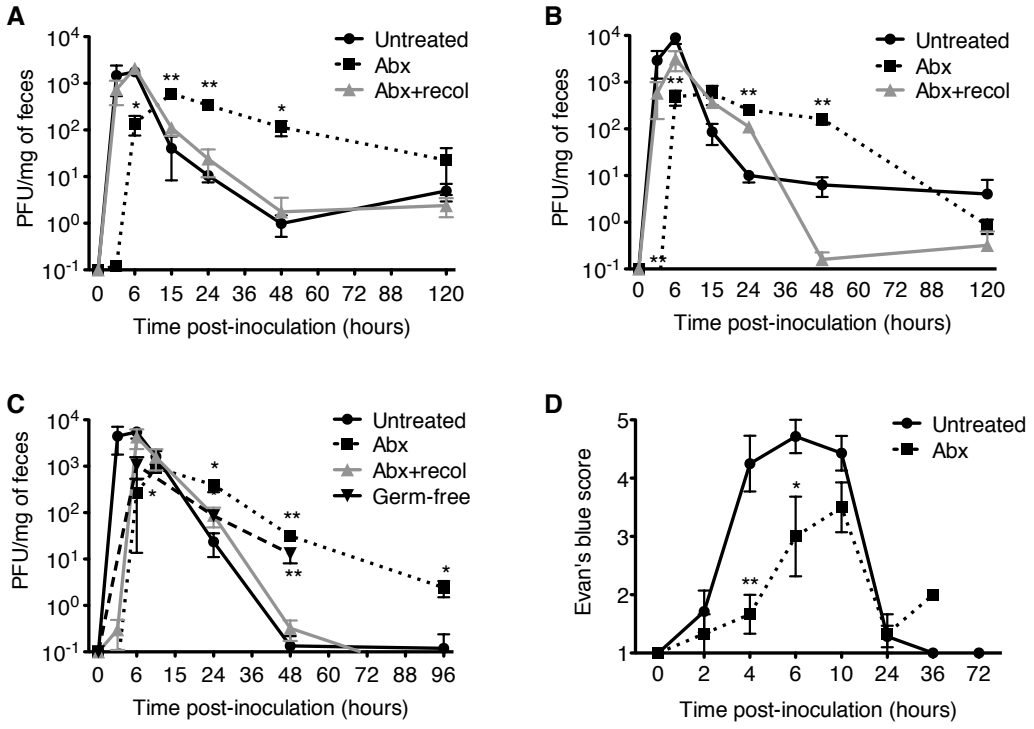

Figure 3-3. Poliovirus shedding in mice feces. Feces were collected from untreated, abx and abx+recol mice orally inoculated with $2x10^7$ PFU poliovirus. Poliovirus was isolated from feces and quantified by plaque assay, yielding plaque-forming units (PFU) per milligram (mg) of feces. (**A**) Poliovirus shedding in PVRtg-IFNAR$^{-/-}$ mice (n=2-26 per treatment group for each time point). (**B**) Poliovirus shedding in feces from PVRtg mice (n=2-20 per treatment group for each time point). (**C**) Poliovirus shedding in C57BL/6 mice (n=5 per treatment group for each time point) and germ free C57BL/6 mice (n=3 for each time point). (**D**) Intestinal transit time was measured in untreated or abx-treated PVRtg-IFNAR$^{-/-}$ mice. Mice (n=2-7) were orally administered Evan's blue dye and feces were collected at multiple times post-inoculation. Feces were suspended in PBS and the amount of dye excreted was scored. Symbols represent the mean $\pm$ SEM. *p<0.05, **p<0.01, Student's t-test.

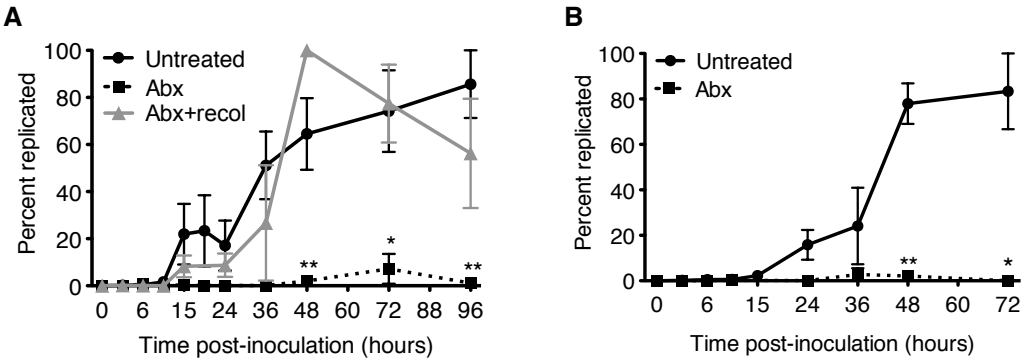

Figure 3-4. Poliovirus replication in the GI tracts of mice. (**A**) Replication of poliovirus in the intestinal tract of PVRtg-IFNAR$^{-/-}$ mice (n=2-12 per treatment for each time point). Untreated, abx or abx+recol were orally inoculated with 2 x 10^7 PFU of light-sensitive poliovirus, feces were harvested, and virus was extracted and quantified after light or dark exposure to determine % replication. (**B**) Replication of poliovirus in the intestinal tract of PVRtg mice (n=2-11 per treatment for each time point) (done as in A). Symbols represent the mean $\pm$ SEM. *p<0.05, **p<0.01, Student's t-test.

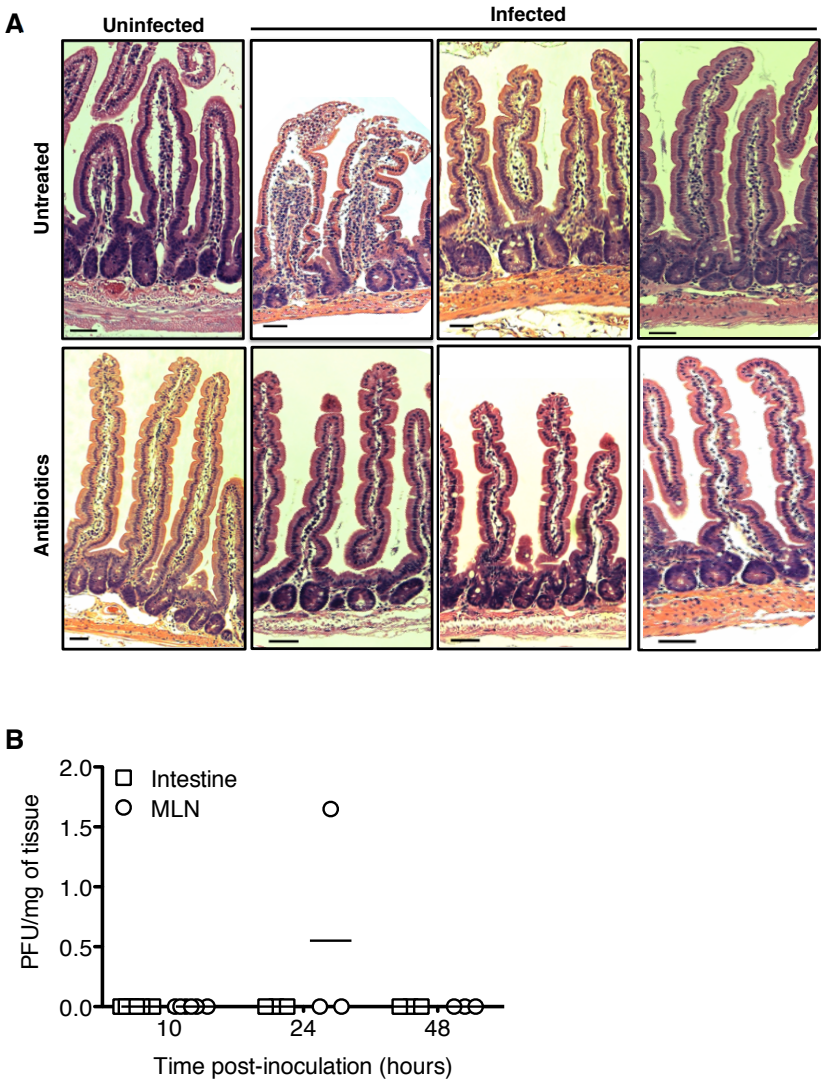

Figure 3-5. Intestinal architecture and poliovirus infection in the GI tracts of mice. (**A**) Intestinal architecture 48 hours post-inoculation. PVRtg mice (n=3) were orally inoculated with 2 x 10^7 PFU of poliovirus. After 48hpi, whole small intestine was isolated, washed, fixed and stained with hemotoxylin and eosin for microscopic analysis. Scale bars = 50μm. (**B**) Quantification of poliovirus isolated from intestine and mesenteric lymph nodes (MLN). PVRtg mice (n=3-6) were orally inoculated with 2 x 10^7 PFU, and at different intervals post-inoculation, tissues were collected and intestines were flushed with cold PBS. Poliovirus was isolated from tissues and quantified by plaque assay using HeLa cells. Each symbol represents one mouse and bars indicated the mean.

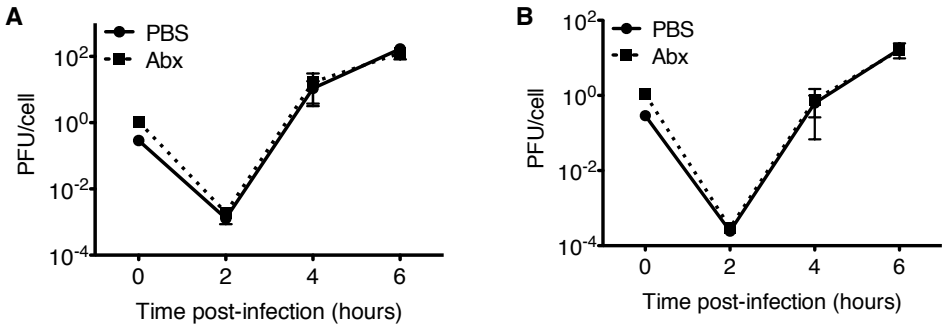

Figure 3-6. Poliovirus replication in the presence and absence of the four antibiotics *in vitro*. Growth curve analysis of poliovirus (1 x 10^6 PFU) mixed with PBS or PBS and abx prior to infection of (**A**) HeLa cells and (**B**) Mouse embryonic fibroblasts (MEF) (n=2-6 for each cell line). Work done in collaboration with G.T. Best.

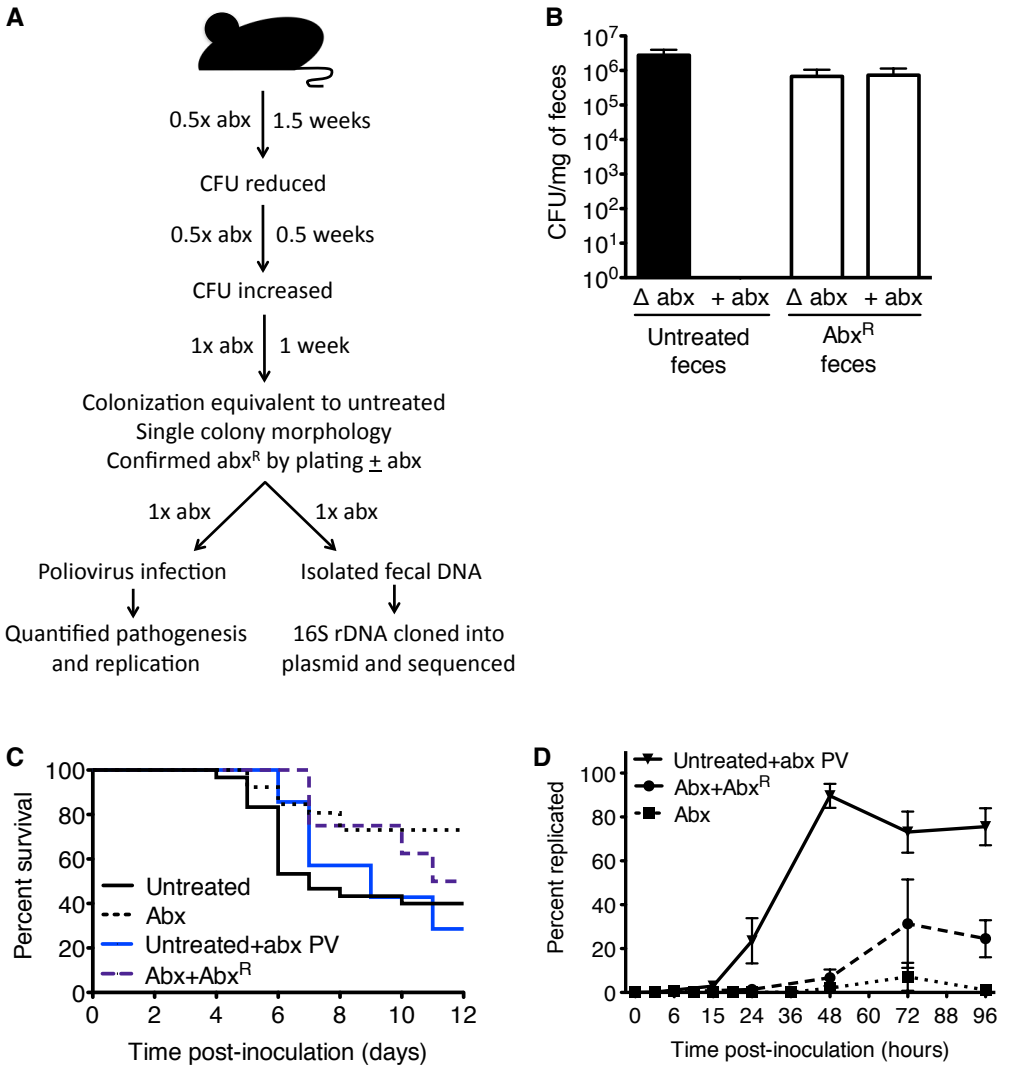

Figure 3-7. Effects of antibiotic treatment on poliovirus replication and pathogenesis. (**A**) Strategy for isolating and identifying antibiotic-resistant (abx^R) bacteria. (**B**) Bacterial loads in feces from untreated mice and abx mice harboring abx^R bacteria. Feces were plated on rich media with or without the four antibiotics and grown anaerobically. (**C**) Survival of PVRtg-IFNAR$^{-/-}$ mice orally inoculated with 2 x 10^7 PFU of poliovirus pre-mixed with the four antibiotics (untreated+abx PV, n=9) or poliovirus alone in abx mice harboring abx^R bacteria (abx+abx^R, n=8). Untreated and abx survival are as shown in Figure 3-2A. (**D**) Replication of light-sensitive poliovirus in untreated mice receiving the poliovirus+abx inoculum and abx mice harboring abx^R bacteria in comparison to abx mice (abx results are from Figure 3-4). Each symbol represents mean ± SEM.

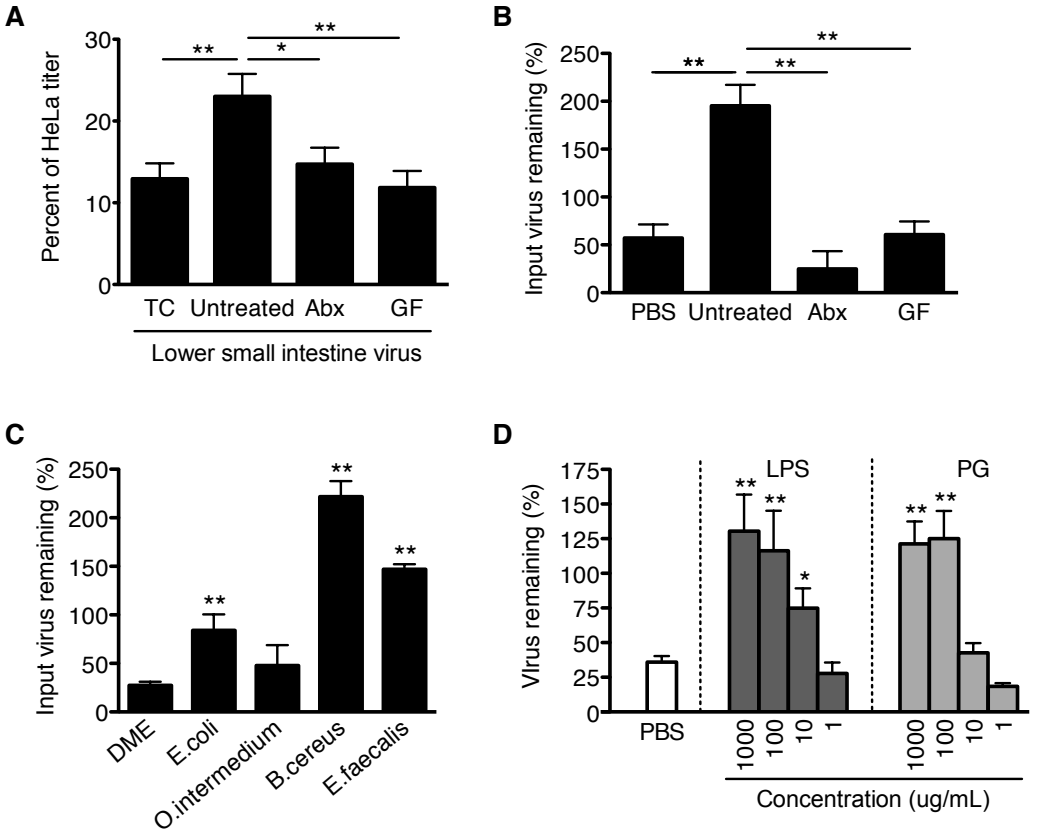

Figure 3-8. Poliovirus infectivity after exposure to microbes and microbial components. (**A**) Infectivity of *in vivo* isolated poliovirus. Infectivity of tissue culture-derived (TC) or mouse intestine lumenal content-derived poliovirus in PVRtg mouse embryonic fibroblasts (MEF) *vs.* HeLa cells (untreated: n=5, abx-treated: n=4, germ-free (GF): n=3). Seven infectious center assays were performed and data are displayed as MEF titers as a percentage of HeLa titers. (**B**) Infectivity of poliovirus exposed to feces *ex vivo*. Tissue culture-derived poliovirus was mixed with PBS, untreated feces, abx feces or GF feces and incubated at 37°C for six hours. Virus was isolated from samples and quantified by plaque assay. (**C**) Infectivity of poliovirus exposed to pure bacterial cultures. Poliovirus was mixed with Dulbecco's Modified Eagle Medium (DME), *E. coli*, *O. intermedium*, *B. cereus* or *E. faecalis* (n=3-5). Bacteria were washed in PBS and suspended in DME prior to virus addition. The infectivity assay was performed as in (C). (**D**) Infectivity of poliovirus exposed to PBS only or purified bacterial components: lipopolysaccharide (LPS) or peptidoglycan (PG) (n=2-6). Assay performed as described in (C). For all figures, each symbol represents mean + SEM. *p<0.05, **p<0.01 compared to controls unless indicated otherwise, Student's t-test. Data in D generated by G.T. Best.

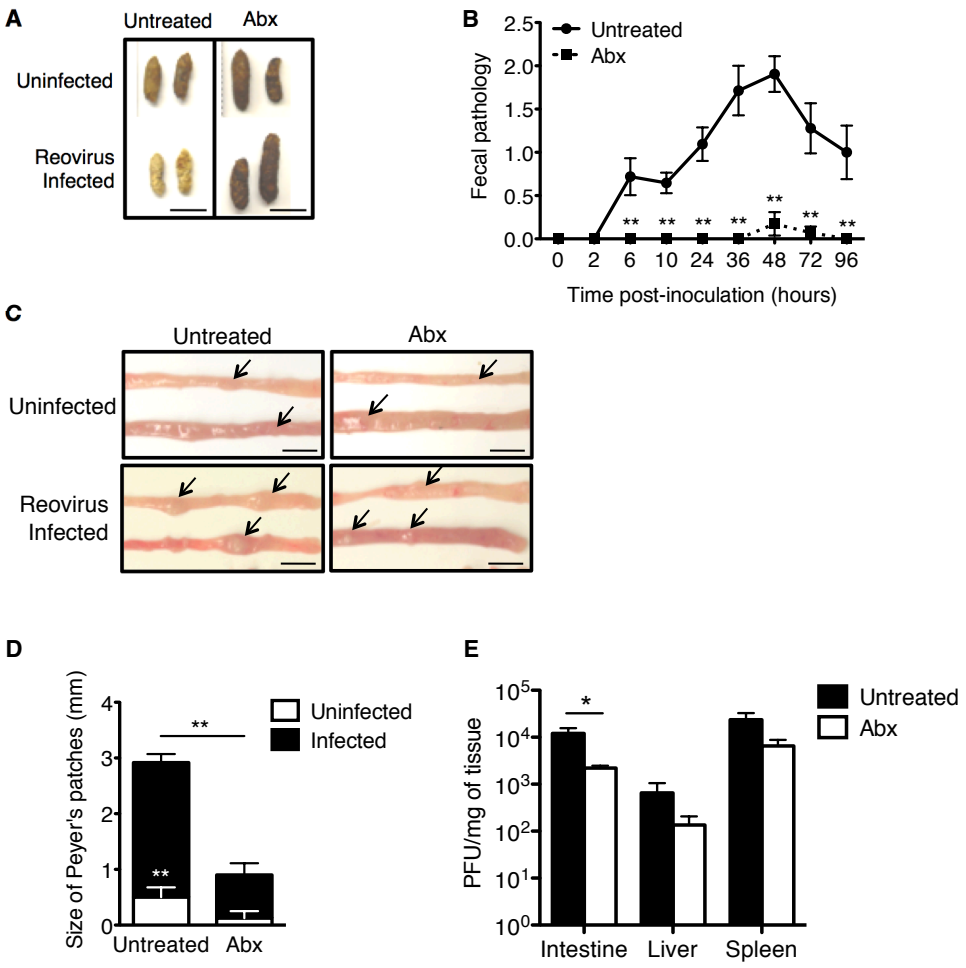

Figure 3-9. Effects of microbiota depletion on reovirus pathogenesis. (**A**) PVRtg-IFNAR$^{-/-}$ mice were orally inoculated with 1x10^{8} PFU of T3SA+ reovirus, and feces were collected 24 hours post-inoculation from untreated (n=13) or abx (n=15) mice, as well as from uninfected/untreated (n=5) and uninfected/abx (n=5) mice. (**B**) Fecal pathology was scored at multiple times post-inoculation (See Table 3-1). (**C**) Upper (top) and lower (bottom) small intestines were harvested from untreated (n=10) or abx (n=12) PVRtg-IFNAR$^{-/-}$ mice on day 4 post-infection. Arrows indicate Peyer's patches. (**D**) Quantification of Peyer's patch size (from C) from uninfected mice (untreated, n=4 or abx-treated, n=2) and infected mice (untreated, n=9 or abx-treated n=9). (**E**) Reovirus titer analysis from PVRtg-IFNAR$^{-/-}$ mouse tissues harvested at day 4 post-infection. Plaque assays were performed using murine L929 cells, yielding PFU per milligram of tissue. For all data sets, each symbol or bar denotes the mean ± SEM. ** $p<0.01$ as compared to untreated, Student's t-test. Scale bars = 5mm.

Table 3-1. Fecal pathology scoring schema

	Color		Consistency	Example
0	normal		normal	brown, firm
1	slight discoloration	or	slight alteration	brown, soft
2	discoloration	and	alteration	tan, hard
3	extreme discoloration	and	extreme alteration	yellow, hard

CHAPTER FOUR
The influence of intestinal microbiota-mediated induction of host mucosal immunity on poliovirus shedding and infection

Introduction

Maintenance of symbiosis between the GI microbiota and host GI mucosa has prompted the evolution of many unique host immune functions specific to the microbe-laden GI tract. Aside from bacterial restriction to the outermost layer of mucus in the GI lumen (Johansson et al., 2008), several immune mechanisms exist to limit bacterial translocation from the GI lumen (Hooper and Macpherson, 2010). Innate and adaptive immune effectors induced by and/or responding to microbes in the GI lumen include AMPs and sIgA. Bacterial-induced host mucosal immune response may affect poliovirus dissemination and disease.

Evidence exists demonstrating that AMPs can be antiviral. *In vivo* significance of virus inactivation by AMPs has not been demonstrated, but human defensins display activity against papillomavirus (Buck et al., 2006), herpes simplex virus 1 and 2, cytomegalovirus, vesicular stomatitis virus, influenza A virus (Daher et al., 1986), adenovirus (Gropp et al., 1999) and HIV-1 (Wang et al., 2004a) *in vitro.* Interestingly, of the viruses listed, adenovirus is the only non-enveloped virus. It was recently established that human α-defensins inhibit adenoviral disassembly and endosome penetration in cell culture, thereby restricting nucleic acid release (Smith and Nemerow, 2008). It is plausible that bacterial-induced defensins, or other AMPs, may bind and limit poliovirus

infection. Another microbiota-induced host factor that may influence poliovirus in the GI tract is sIgA. Production of IgA in the intestinal tract correlated with reduction in poliovirus titers recovered from feces in humans (Valtanen et al., 2000), suggesting that IgA neutralizes poliovirus. Intestinal microbes may aid induction of sIgA after poliovirus administration since probiotic *Bifidobacterium* species may increase poliovirus-specific mucosal IgA following vaccination with IPV (Mullie et al., 2004). Conversely, poliovirus may usurp AMPs and/or sIgA for its benefit. Perhaps coupling of poliovirus to an AMP or sIgA promotes enhanced uptake of the virus by immune cells that support viral replication, such as dendritic cells or macrophages (Eberle et al., 1995; Freistadt and Eberle, 1996; Wahid et al., 2005a). In fact, antigen conjugation to sIgA may enhance sIgA stability and mediate uptake via FcαRI receptors (Duc et al., 2010), which are commonly expressed by phagocytic cells at mucosal surfaces.

AMPs are induced by commensal microbes in the intestinal tract. AMPs are amongst the most ancient form of innate immune effectors known (Zasloff, 2002), and 1746 have been identified and confirmed to date (The Antimicrobial Peptide Database: http://aps.unmc.edu/AP/about.php). AMPs exhibit potent bactericidal activity by direct disruption of cell walls or membranes. Enterocytes and goblet cells can generate AMPs, but Paneth cells are the main source of AMPs within the GI tract (Bevins and Salzman, 2011). AMP production within the GI tract can occur independently of bacterial stimuli since some α-defensins are constitutively produced (Putsep et al., 2000). Alternatively, bacterial recognition

governs the expression of other AMPs such as defensin-related cryptidins, RegIIIγ (a C-type lectin) and a specific subset of α-defensins in mice (Brandl et al., 2007; Kobayashi et al., 2005; Vaishnava et al., 2008). Induction of these AMPs relies upon recognition of MAMPs, expressed by intestinal microbes.

Non-self antigenic patterns, including MAMPs, are sensed by PRRs whereby signals are conveyed to the nucleus to initiate inflammatory responses. MAMPs are recognized by PRRs, such as TLR5 recognition of flagellin and NOD2 recognition of muramyl dipeptide. Most TLR and NLR stimulated signaling converges with NFκB activation that occurs through a distinct signaling network induced by variable stimuli (Hill and Artis, 2010). Of importance to studies outlined in this dissertation, upstream signaling leading to NFκB activation is mediated through the adaptor protein MyD88 for TLR1, 2, 4, 5, 6, 7, 8 and 9 (Kelly and Conway, 2005). All of these TLRs are MyD88-dependent, but TLR4 can activate a MyD88-independent pathway through TRIF and TRAM (Yamamoto et al., 2003a; Yamamoto et al., 2003b). TLR1 and 6 dimerize with TLR2 and recognize bacterial lipoproteins, peptidoglycan (PG) and lipoteichoic acid (LTA). TLR4 mediates signaling with the aid of co-stimulatory CD14 and MD2 upon LPS recognition. TLR7 and TLR8 are specific for single-stranded RNA, and TLR9 recognizes CpG DNA.

Once constituents of bacteria are sensed by TLRs (or NLRs), signaling cascades lead to activation of NFκB, which induces pro-inflammatory cytokines that stimulate production of AMPs (Hill and Artis, 2010). Intestinal epithelial cell

recognition of bacteria is extremely important for controlling invasion (Brandl et al., 2007; Kobayashi et al., 2005; Vaishnava et al., 2008). In fact, truncation of the NFκB signaling cascade in intestinal epithelial cells leads to spontaneous intestinal inflammation (Gong et al., 2010; Nenci et al., 2007; Zaph et al., 2007). These studies highlight the importance of MyD88-dependent control of GI microbes.

Another method used by the host to restrict bacterial translocation across the GI epithelial barrier is sIgA. Secretory IgA is specifically induced by dendritic cells containing bacteria, independent of T cells (Macpherson et al., 2000; Macpherson and Uhr, 2004). Bacteria-containing dendritic cells are confined to the mesenteric lymph nodes to limit inflammation within the GI tract so as not to induce a systemic response and to maintain homeostasis. Germ-free mice exhibit decreased intestinal IgA production (Tezuka et al., 2007), implicating the microbiota as a key component to induce sIgA within the GI tract. Additionally, MyD88$^{-/-}$ mice exhibit diminished levels of mucosal sIgA compared to wild-type mice (Suzuki et al., 2010; Tezuka et al., 2007), suggesting that MyD88-induced effectors and NFκB signaling affect sIgA production. In IgA-deficient mice, overgrowth of the microbiota occurs (Suzuki et al., 2004), and therefore, sIgA significantly restricts bacterial spread from the GI tract.

Because GI microbes activate mucosal immunity, I was interested in examining the effects of the mucosal immune response to bacteria on poliovirus infection following oral inoculation. To assess potential effects of immune

effectors such as AMPs and sIgA on poliovirus following oral inoculation, I evaluated shedding and replication of poliovirus after induction of mucosal immunity by administering a bacterial-derived component to susceptible mice prior to poliovirus infection. In addition, the effects of microbial-induced host immunity on poliovirus shedding in feces were examined using mice lacking MyD88 or sIgA.

Materials and methods

Viruses and cells

Virus work was performed in WHO-approved BSL2+ safety areas. Poliovirus type I (Mahoney) was used for all infections. Virus stock preparations, infections and plaque assays were done as previously described (Pfeiffer and Kirkegaard, 2005) in HeLa cells. HeLa cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Hyclone) and antibiotics (10,000U/ml penicillin and 10,000μg/ml streptomycin; Thermo Scientific).

Mice, treatments and inoculations

Animal work was performed according to UT Southwestern Medical Center IACUC approved protocols. Mice were housed in specific pathogen-free conditions at University of Texas Southwestern Medical Center. S. Koike (Tokyo Metropolitan Institute for Neuroscience) kindly provided C57BL/6 PVR-Tg21

(PVRtg) and C57BL/6 PVRtg-IFNAR$^{-/-}$ (PVRtg-IFNAR$^{-/-}$) mice. C57BL/6 MyD88$^{-/-}$ (MyD88$^{-/-}$) and C57BL/6 J_H $^{-/-}$ mice were graciously provided by L. Hooper. Wild-type C57BL/6 mice were obtained from Jackson Laboratory.

MyD88$^{-/-}$ mice breeding was performed by crossing a MyD88$^{-/-}$ male or female with a MyD88$^{+/-}$ female or male. Genotyping for the MyD88 transgene was performed by PCR analysis of tail snip DNA. Tail snips were collected from 2 week old pups and subjected to DNA extraction via REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), following manufacturer's protocol. Primers specific for MyD88 (MyD88 sense: 5'-TGGCATGCCTCCATCATAGTTAACC-3'; MyD88 anti: 5'-GTCAGAAACAACCACCACCATGC-3') or the endogenous neomycin-resistant cassette (MyD88 sense in combination with Neo anti: 5'-ATCGCCTTCTATCGCCTTCTTGACG-3') from the MyD88 deletion were used. PCR was performed with an annealing temperature of 58°C. Agarose gel electropheresis was performed to determine correct band sizes corresponding to the presence or absence of MyD88 (wild-type: 550bp band, knock-out: 625bp band, heterozygous: 550bp band + 625bp band).

For antibiotic treatments, six to seven week-old mice were orally administered a combination of four antibiotics: ampicillin, neomycin, metronidazole and vancomycin (Research Products International) for 10 days prior to inoculation with poliovirus. Depletion of intestinal microbes was confirmed by quantification of culturable bacterial loads in feces. Recolonized mice were first treated with antibiotics and then orally received fecal bacteria from

untreated mice (all as described in Chapter 3 Materials and Methods). For LPS-treated mice, a subset of antibiotic-treated mice were administered 1mg/mL of LPS (Sigma-Aldrich) in drinking water for two days prior to poliovirus inoculation. Untreated mice did not receive any treatments since oral gavage with sterile water did not alter the outcome of poliovirus infections (see Materials and Methods in Chapter 3).

For poliovirus infections, 7-9 week old untreated, antibiotic-treated, antibiotic + LPS-treated or antibiotic + recolonized mice were perorally inoculated with 2 x 10^7 PFU of poliovirus. Feces were collected from mice at multiple times post-inoculation to quantify poliovirus. To differentiate replicated from input virus in feces from PVRtg-IFNAR$^{-/-}$ mice, 2 x 10^7 PFU light-sensitive, NR-containing poliovirus was used. As previously described, poliovirus was grown in the presence of 1% NR-containing media for incorporation into viral capsids that confers light sensitivity.

Generation of PVRtg-MyD88$^{-/-}$ mice

For future assessment of poliovirus replication in the GI tracts of mice in the absence of MyD88 signaling, PVRtg-MyD88$^{-/-}$ mice were generated (**Figure 4-3**). Homozygous PVRtg MyD88$^{+/+}$ mice were crossed with non-PVR MyD88$^{-/-}$ mice to yield PVR$^{+/-}$ MyD88$^{+/-}$ F1 generation mice. To generate PVR$^{+/+}$ MyD88$^{-/-}$ F2 generation mice, PVR$^{+/-}$ MyD88$^{+/-}$ F1 generation mice were interbred. Resulting pups were initially genotyped for MyD88 presence as outlined above,

and MyD88$^{+/-}$ or MyD88$^{-/-}$ mice were used in subsequent steps. Confirmation of the MyD88$^{+/-}$ or MyD88$^{-/-}$ mice homozygous for PVR was performed as follows. F2 generation mice were back-crossed to wild-type C57BL/6 mice obtained from Jackson Laboratory. Tail snips were taken from resulting pups and DNA was extracted (REDExtract-N-Amp Tissue PCR Kit, Sigma-Aldrich). DNA was diluted 1:10 - 1:50 for PCR analysis with primers specific for the human PVR transgene (PVR sense: 5'-GTCATCCTCCCACCTCAGCC-3'; PVR anti: 5'-TCTGGTGGCCCACACCCTT-3'). Annealing temperature for the PVR primers was set at 60°C. DNA was resolved by agarose gel electrophoresis to confirm the presence of PVR. If all pups had positive PCR signal for PVR, the F2 generation mouse bred with non-PVR C57BL/6 was likely PVR$^{+/+}$. Four PVR$^{+/+}$ MyD88$^{+/-}$ were confirmed and further interbred to generate PVR$^{+/+}$ MyD88$^{-/-}$ mice, confirmed by DNA genotyping analysis.

Sample processing and titer analyses

Feces collected from infected mice were resuspended in 1-5 volumes of PBS+ (1X PBS supplemented with 100ug/ml $MgCl_2$ and $CaCl_2$) and processed as outlined in Chapter 3 "Materials and Methods". Virus isolated from feces was quantified by plaque assay using HeLa cells (Pfeiffer and Kirkegaard, 2005). Feces containing light-sensitive, NR poliovirus were processed in the dark using a red photography bulb, and a portion of each sample was light exposed to determine replication status. Quantification of unexposed and light-exposed virus

by plaque assay was done by dividing the amount of replicated virus by total virus and multiplying by 100 to yield percent replicated.

Results

LPS treatment is not sufficient to restore normal fecal shedding kinetics of poliovirus but may partially restore poliovirus replication within the GI tract of microbe-depleted PVRtg mice

I wanted to determine if treatment with lipopolysaccharide (LPS), and possible immune stimulation, in the GI tracts of mice with reduced flora would restore fecal shedding kinetics and replication of poliovirus similar to what was previously observed for conventional mice (**Figure 3-3A, 3-3B, 3-4A, 3-4B**). Gram-negative bacteria produce LPS on the surface of their cells, and conjugation of LPS to TLR4 activates the intestinal immune system to initiate protection via MyD88 signaling (Kawai et al., 1999). In addition, LPS enhances poliovirus infectivity *in vitro* (**Figure 3-8D**). To mimic the presence of Gram-negative bacteria within the GI tract of antibiotic-treated PVRtg mice, LPS was administered in drinking water *ad libitum* three days prior to oral inoculation with poliovirus. Examination of poliovirus fecal excretion from PVRtg antibiotic-treated mice receiving LPS demonstrated that early shedding kinetics (0-15hpi) were identical to previous results in antibiotic-treated mice (**Figure 4-1A**). However, I observed a modest reduction in the amount of virus shed from antibiotic/LPS-treated mice compared to antibiotic-treated mice at later times post-inoculation.

Next, I assessed fecal shedding of poliovirus from PVRtg-IFNAR$^{-/-}$ antibiotic-treated mice that were orally administered LPS. Using PVRtg-IFNAR$^{-/-}$ mice that cannot respond to IFNα or IFNβ eliminates MyD88-independent immune responses to LPS elicited through the TRIF/TRAM pathway (Yamamoto et al., 2003a; Yamamoto et al., 2003b). I monitored fecal excretion of poliovirus in untreated, antibiotic-treated and antibiotic/LPS-treated mice. No discernable difference in shedding occurred between antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice and antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice administered LPS (**Figure 4-1B**). These results suggest that LPS-induced effects on poliovirus shedding in PVRtg mice may be mediated through the MyD88-independent TLR4 pathway that stimulates IFNα/β production since no differences were observed in shedding between antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice and antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice administered LPS.

Poliovirus fecal shedding kinetics and viral replication within the GI tract are not necessarily linked (**Figure 3-3A, 3-3B, 3-4A and 3-4B**); therefore, I also evaluated poliovirus replication in antibiotic-treated mice in the presence or absence of LPS treatment. I orally inoculated PVRtg mice with light-sensitive poliovirus and quantified viral replication according to light sensitivity (see Chapter 3). I observed minimal poliovirus replication in antibiotic-treated mice, but LPS treatment partially restored poliovirus replication in antibiotic-treated PVRtg mice (**Figure 4-1C**). When examining similar treatments in PVRtg-IFNAR$^{-/-}$ mice, I noticed extremely limited poliovirus replication in mice treated

with antibiotics in the presence and absence of LPS (**Figure 4-1D**). Collectively, these preliminary data suggest that LPS treatment may be partially beneficial for poliovirus replication within the GI tract in IFN-sufficient mice. The LPS-mediated enhancement of viral replication could occur through stimulation of TLR4 pathways or effects on the virus itself. The fact that the restoration of LPS-mediated viral replication is not observed in PVRtg-IFNAR$^{-/-}$ mice suggests a potential role for LPS stimulation of IFNAR-dependent host pathways. More careful examination of exogenous LPS effects after oral administration in mice harboring or lacking GI microbiota are needed to corroborate these findings. It remains to be determined if *ad libitum* LPS induces an intestinal immune response. Additional analysis of other MAMPs is necessary to determine if poliovirus replication is enhanced by recognition of multiple Gram-positive and Gram-negative bacterial MAMPs and not just LPS from Gram-negative bacteria.

MyD88 signaling influences poliovirus shedding in feces

Although LPS administration did not rescue the fecal shedding kinetics of poliovirus observed in conventional mice, many additional TLR ligands are abundant within the GI tract. For example, TLR5 recognizes flagellin, the main component of flagella, TLR2 recognize PG and TLR6/TLR2 recognize LTA. To more globally investigate the role of bacterial-induced intestinal immunity, I assessed poliovirus shedding in the absence of the important TLR adaptor, MyD88 in the absence or presence of the microbiota. Unfortunately, PVR-

expressing MyD88$^{-/-}$ mice have not been derived, and therefore, I could only examine poliovirus excretion in feces. C57BL/6-MyD88$^{-/-}$ (MyD88$^{-/-}$) and C57BL/6-MyD88$^{+/+}$ (MyD88$^{+/+}$, wild-type) mice were untreated or antibiotic-treated and orally-inoculated with 2x10^{7} PFU of poliovirus. Fecal shedding kinetics of poliovirus were monitored. I observed microbiota-dependent and independent effects on fecal shedding of poliovirus from MyD88$^{-/-}$ mice. First, I found that poliovirus excretion in feces is altered in untreated MyD88$^{-/-}$ mice compared to untreated MyD88$^{+/+}$ mice (**Figure 4-2A**). Fecal shedding of poliovirus ceases as soon as 36hpi in MyD88$^{-/-}$ mice but persisted another 12hpi in MyD88$^{+/+}$ mice. Second, I also observed stunted shedding of poliovirus in feces from antibiotic-treated MyD88$^{-/-}$ mice compared to antibiotic-treated MyD88$^{+/+}$ mice (**Figure 4-2B**). Third, I observed altered poliovirus fecal shedding kinetics from untreated and antibiotic-treated MyD88$^{-/-}$ mice (**Figure 4-2C**), as also observed in PVR mice +/- antibiotics (**Figure 3-3A, 3-3B**). However, the shedding kinetics of poliovirus from MyD88$^{-/-}$ mice +/- antibiotics were drastically different from my previous observations in PVR mice +/- antibiotics. I found that excretion of poliovirus in antibiotic-treated MyD88$^{-/-}$ mice was slightly extended compared with untreated MyD88$^{-/-}$ mice (**Figure 4-2C**), which was previously observed in MyD88-competent mice but to a much greater extent (**Figure 3-3A, 3-3B**). In general, it appears that poliovirus shedding from MyD88$^{-/-}$ mice subsides much sooner compared with MyD88$^{+/+}$ mice (**Figure 4-2A, 4-2B**). I originally hypothesized that if fecal shedding of poliovirus was regulated by a microbe-

mediated MyD88-induced factor, such as an AMP or sIgA, that untreated and antibiotic treated MyD88$^{-/-}$ mice would have identical poliovirus shedding kinetics. However, the results differed drastically from this idea suggesting that another microbe-induced factor, independent of MyD88 signaling, is influencing fecal shedding of poliovirus. These data reveal that presence of MyD88 potentiates fecal shedding of poliovirus.

I want to assess poliovirus replication in the absence of MyD88, and because the previously used MyD88$^{-/-}$ mice do not express PVR, I am generating PVRtg MyD88$^{-/-}$ mice as outlined in "Material and Methods" (**Figure 4-3**). Once a colony of these mice is established, I will examine poliovirus replication in the GI tracts of these mice in comparison to PVRtg mice. The experiments will be performed in the presence and absence of antibiotic treatment to determine how the bacterially-induced host MyD88 response affects poliovirus infectivity. In addition, it will be interesting to examine the effects of MyD88-mediated responses on poliovirus within the GI tract and systemically, regardless of microbiota presence. To date, no one has investigated the role of MyD88 during oral poliovirus infection, and these experiments may reveal novel innate immune responses to poliovirus infection in mice.

Fecal poliovirus shedding is unaltered in the absence of secretory IgA

Secretory IgA is produced in the GI tract in response to oral administration of poliovirus (Faden et al., 1990; Mullie et al., 2004; Ogra et al.,

1968; Valtanen et al., 2000). Intestinal microbiota facilitate localized production of sIgA from the GI mucosa (Macpherson et al., 2000). Although this sIgA is selective for commensal microorganisms, I reasoned that the sIgA may be polyclonal and recognize poliovirus, or the microbiota may promote sIgA specific for poliovirus and aid in clearance. Interestingly, mice lacking sIgA within the GI tract are exquisitely sensitive to *Salmonella typhimurium* infection (Wijburg et al., 2006), suggesting that induction by commensal bacteria may help limit bacterial pathogens. In order to assess possible sIgA effects on poliovirus, I employed C57BL/6-$J_H^{-/-}$ ($J_H^{-/-}$, wild-type) mice that lack functional secretion of immunoglobulins A and M. As with MyD88$^{-/-}$ mice, $J_H^{-/-}$ mice do not express PVR, and therefore, I could only evaluate fecal shedding of poliovirus from mice. Presumably, if bacterial-induced sIgA affects poliovirus shedding, then lack of sIgA in the presence of the microbiota should alter poliovirus shedding kinetics, more closely mimicking shedding kinetics observed in antibiotic-treated mice.

I set out to assess fecal excretion of poliovirus in $J_H^{+/+}$ and $J_H^{-/-}$ mice that harbor intestinal microbes since I wanted to examine the potential effects of microbe-specific induction of sIgA on poliovirus. Feces were collected at multiple times after oral inoculation with $2x10^7$ PFU of poliovirus, processed and titered for poliovirus quantitation. I observed no significant difference in fecal shedding of poliovirus between $J_H^{+/+}$ and $J_H^{-/-}$ mice (**Figure 4-4**). These results indicated no major role of microbiota-induced sIgA during poliovirus GI transit and clearance in mice. Experiments to address the influence of microbiota-dependent induction

of sIgA in PVR-expressing $J_H^{-/-}$ mice, once generated, would be interesting to assess potential effects of microbe-induced sIgA on poliovirus replication and dissemination.

Discussion

Results from mouse experiments incorporating LPS indicate that Gram-negative intestinal bacteria, such as *Bacteroides thetaiotaomicron* and *E. coli*, may partially contribute to poliovirus replication within the GI tract in the presence of functional IFN responses (**Figure 4-1A, 4-1C**). LPS administration to antibiotic-treated PVRtg mice did not tremendously affect poliovirus shedding in feces. Modest LPS effects were observed in PVRtg mice (**Figure 4-1A, 4-1C**), but not PVRtg-IFNAR$^{-/-}$ mice (**Figure 4-1B, 4-1D**). Some evidence suggests that the IFNAR is required for proper LPS-induced host monocyte responses (Gautier et al., 2005; Vadiveloo et al., 2000a; Vadiveloo et al., 2000b; Wegenka et al., 2007), suggesting that LPS responses may not occur in the absence of IFNAR. The data presented here are preliminary and require additional experimentation. Similarly, confirmation of intestinal immune induction by oral administration of LPS is required. It would be interesting to examine the *in vivo* effects of Gram-positive bacterial MAMPs such as LTA and PG. Additionally, combinations of Gram-positive and Gram-negative MAMPs administered to antibiotic-treated mice may reveal a more striking phenotype, such as complete restoration of poliovirus replication in their GI tracts.

Previous evidence from Chapter three demonstrated that both Gram-negative and Gram-positive bacteria, as well as their components, promote poliovirus infectivity *in vitro* (**Figure 3-8C, 3-8D**). In fact, systemic LPS has been implicated in Theiler's murine encephalitis virus and HIV disease progression (Brenchley et al., 2006; Pullen et al., 1995). In addition, mouse mammary tumor virus is horizontally maintained in mice with sufficient TLR4 signaling, suggesting a possible role of microbe-induced immune stimulation in virus transmission (Jude et al., 2003). How might TLR4 induction via LPS and/or microbiota stimulation affect poliovirus infection within the GI tract? Perhaps TLR4 stimulation dampens immune responses to maintain homeostasis within the GI tract (Rakoff-Nahoum et al., 2004), thereby eliciting a weak immune response to incoming viral pathogens, such as poliovirus. Additionally, subsequent responses to MAMP recognition of bacterial products are regulated through NFκB, which promotes AMP and sIgA production (Hill and Artis, 2010), both of which have largely unknown functions during poliovirus infection. It remains to be determined whether AMPs have activity against poliovirus or whether sIgA has a role in viral neutralization or infection.

Since most bacterial ligands induce MyD88-dependent host signaling, I set out to address the effects of microbiota-stimulated mucosal immune responses to poliovirus. I have demonstrated that MyD88-induced immune responses affect fecal shedding of poliovirus after oral inoculation of mice, and therefore, may influence poliovirus infection. Potential effects on poliovirus

replication and pathogenesis remain to be determined in PVR-expressing MyD88$^{-/-}$. Untreated MyD88$^{+/+}$ mice displayed extended fecal shedding of poliovirus compared to untreated MyD88$^{-/-}$ mice (**Figure 4-2A**). Additionally, microbiota-independent MyD88 effects govern fecal shedding of poliovirus. I discovered differential poliovirus shedding kinetics in feces from antibiotic-treated MyD88$^{+/+}$ mice in comparison to antibiotic-treated MyD88$^{-/-}$ mice (**Figure 4-2B**). Interestingly, antibiotic-treated MyD88$^{-/-}$ mice shed significantly lower amounts of poliovirus for nearly a similar amount of time as untreated MyD88$^{-/-}$ mice (**Figure 4-2C**), whereas I previously observed that antibiotic-treated MyD88$^{+/+}$ (C57BL/6) mice shed poliovirus for greatly extended times post-inoculation compared to untreated MyD88$^{+/+}$ (C57BL/6) mice (**Figure 3-3C**). Unfortunately, I could only assess excretion of poliovirus in MyD88$^{-/-}$ mice since PVRtg-MyD88$^{-/-}$ mice have not been successfully generated. However, I am currently in the process of generating such a line that will allow us to examine MyD88 effects on poliovirus replication in mice in the presence and absence of the microbiota (**Figure 4-3**). MyD88 signaling during oral poliovirus infection has never been assessed, but MyD88 signaling impacts many other viral infections, especially those activating TLR7, 8 and 9 signaling (Kumar et al., 2009). Collectively, results from studies in MyD88$^{-/-}$ mice suggest that microbiota-induced MyD88 responses may enhance poliovirus stability or diminish clearance of poliovirus within the GI tract. It is unclear how MyD88-induced immune responses affect poliovirus. As mentioned above, immune tolerance to bacterial antigens may be beneficial for enteric

viruses to go unnoticed and establish productive infections; however, this will be best addressed using PVRtg MyD88$^{-/-}$ mice in the future to assess viral replication and virus-specific immune responses.

Lastly, I was interested in determining if bacterial-induced sIgA was cross-reactive and affected poliovirus shedding in feces, but I discovered that mucosal production of sIgA did not influence kinetics of poliovirus excretion in feces (**Figure 4-4**). It remains unclear whether sIgA impacts poliovirus replication and dissemination. In the future, a considerably important tool would be PVR-expressing $J_H^{-/-}$ or IgA$^{-/-}$ mice for *in vivo* evaluation. Reovirus studies in IgA$^{-/-}$ mice revealed a protective effect of sIgA only after re-challenge with reovirus (Silvey et al., 2001); however, it is unclear if commensal microbes affected either of the reovirus infections. Secretory IgA is produced in humans and mice following oral administration of poliovirus (Faden et al., 1990; Mullie et al., 2004; Ogra et al., 1968; Valtanen et al., 2000), and clearance of poliovirus excretion occurs coincidentally with the detection of sIgA in mouse feces (Valtanen et al., 2000). Notably, PVRtg mice orally-inoculated with poliovirus serotype 1 produce very little intestinal IgA unless they were previously IP-injected with poliovirus (Buisman et al., 2000). This group also found that intestinal IgA production is dependent on PVR following IP inoculation. My results may be consistent with these findings in that non-PVR-expressing mice may not produce poliovirus-specific sIgA, however it is possible that microbiota-induced sIgA that is secreted by the mucosa, may be multivalent and recognize

poliovirus, since it is the most abundant of all immunoglobulins (Cerutti and Rescigno, 2008; Macpherson et al., 2008).

Based on preliminary experimentation, I propose that the host mucosal immune response to naturally-residing microbes, utilizing the MyD88 pathway, influences fecal shedding of poliovirus. In addition, bacterial LPS may promote poliovirus infections in mice whether by directly influencing poliovirus or by induction of host mucosal immune responses.

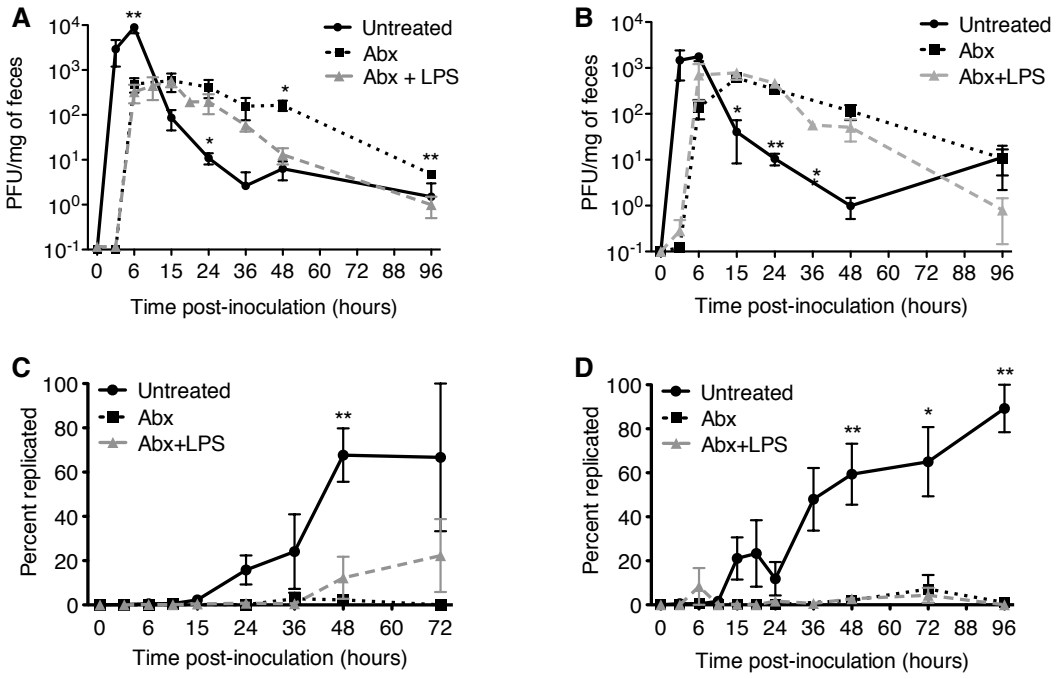

Figure 4-1. LPS-mediated effects on poliovirus shedding and replication. (**A**) Fecal shedding of poliovirus in PVRtg mice that were untreated (n=2-14 per time point), untreated + LPS-treated (n=3 per time point), abx (n=3-21 per time point) or abx + LPS-treated (n=3-6 per time point). Untreated and abx results are those shown in Figure 3-3B. (**B**) Fecal shedding of poliovirus in PVRtg-IFNAR$^{-/-}$ mice that were untreated (n=3-24 per time point), abx (n=4-21 per time point) or abx + LPS-treated (n=3-6 per time point). Untreated and abx results are those shown in Figure 3-3A. (**C**) Intestinal replication of poliovirus in untreated (n=1-11 per time point), abx (n=4-5 per time point) or abx + LPS-treated (n=3-6 per time point) PVRtg mice. (**D**) Intestinal replication of poliovirus in untreated (n=4-14 per time point), abx (n=3-6 per time point) or abx + LPS-treated (n=3-6 per time point) PVRtg-IFNAR$^{-/-}$ mice. Symbols indicate mean $\pm$ SEM. *p<0.05, **p<0.01, as compared to abx + LPS-treated, Student's t-test.

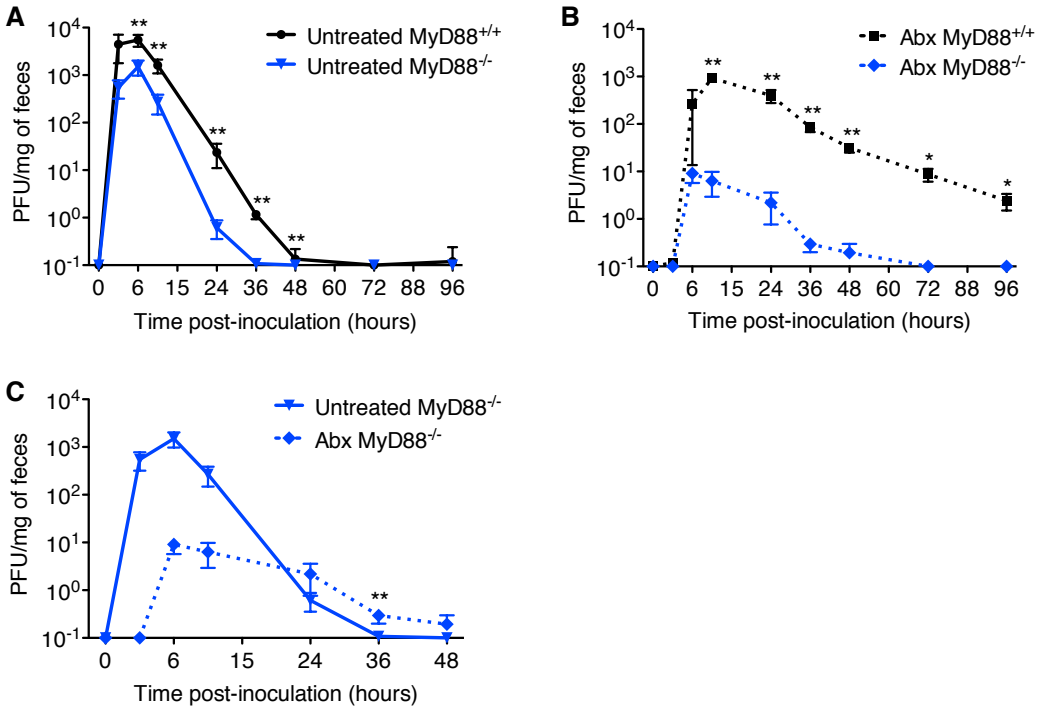

Figure 4-2. Poliovirus shedding in feces from C57BL/6-MyD88$^{+/+}$ (wild-type) and C57BL/6-MyD88$^{-/-}$ mice. (**A**) Fecal shedding of poliovirus from untreated C57BL/6-MyD88$^{+/+}$ (n=5) and C57BL/6-MyD88$^{-/-}$ (n=8-16) mice. Feces were collected at multiple times from mice receiving 2 x 10^7 PFU of poliovirus orally and processed. Poliovirus was quantified by standard plaque assay on HeLa cells. Untreated MyD88$^{+/+}$ are those shown in Figure 3-3C. (**B**) Fecal shedding of poliovirus from abx C57BL/6 wild-type (n=5) and C57BL/6 MyD88$^{-/-}$ (n=8) mice and poliovirus was isolated and quantified as in (A). Abx MyD88$^{+/+}$ are those shown in Figure 3-3C. (**C**) Comparison of poliovirus shed in feces from untreated (A) and abx-treated (B) C57BL/6 MyD88$^{-/-}$ mice. Symbols represent mean $\pm$ SEM. **p*<0/05, ***p*<0.01 by Student's t-test.

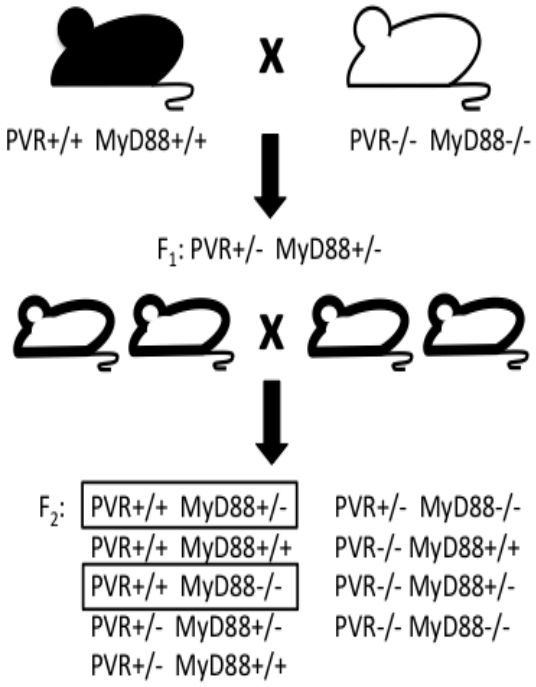

Figure 4-3. Strategy for generation of $PVR^{+/+}$ $MyD88^{-/-}$ mouse strain. Parental strains PVRtg ($PVR^{+/+}$ $MyD88^{+/+}$) C57BL/6 mice and C57BL/6 $MyD88^{-/-}$ ($PVR^{-/-}$ $MyD88^{-/-}$) mice were crossed to yield an F_1 generation of $PVR^{+/-}$ $MyD88^{+/-}$ mice. F_1 generation mice were interbred to yield an F_2 generation that would consist of the listed genotypes, by Mendelian genetics. Of the nine different possible genotypes, $PVR^{+/+}$ $MyD88^{+/-}$ mice and $PVR^{+/+}$ $MyD88^{-/-}$ mice were preferred for founding breeders of the new mouse strain. F_2 pups were screened first for MyD88, and all $MyD88^{+/-}$ and $MyD88^{-/-}$ mice were further screened for PVR status. F_2 crosses yielded $PVR^{+/+}$ $MyD88^{+/-}$, $PVR^{+/+}$ $MyD88^{-/-}$, $PVR^{+/-}$ $MyD88^{+/-}$ that were used as breeders to expand this colony to produce $PVR^{+/+}$ $MyD88^{-/-}$ mice for future experimentation.

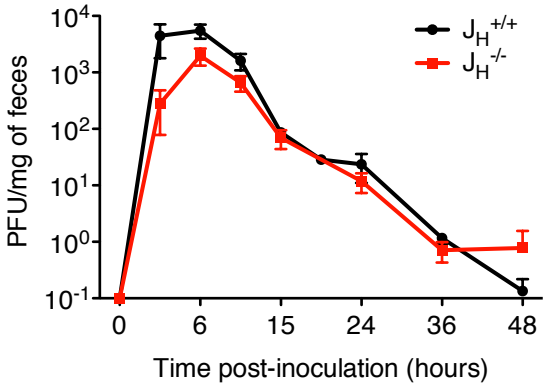

Figure 4-4. Poliovirus shedding in feces from C57BL/6-$J_H^{+/+}$ (wild-type) and C57BL/6 $J_H^{-/-}$ mice. C57BL/6-$J_H^{+/+}$ or C57BL/6 $J_H^{-/-}$ (lack sIgA) mice (n=5 mice per strain per time point) were orally inoculated with 2 x 10^7 PFU of poliovirus, and feces were collected from each mouse at multiple times post-inoculation. Feces were processed for virus isolation and poliovirus was quantified by plaque assay on HeLa cells. C57BL/6-$J_H^{+/+}$ are those shown in Figure 3-3C. Each symbol represents the mean titer $\pm$ SEM.

CHAPTER FIVE
Conclusions and Recommendations

DISCUSSION

Overview

Enteric viruses, such as poliovirus, rotavirus and reovirus, are a continual source of disease worldwide, mostly affecting people in under-developed countries and immunocompromised individuals. Insight into how they are influenced within the GI tract is pertinent to understand how viral replication and transmission can be interrupted. In addition, because a limited amount of vaccines and therapies are available that counteract RNA viruses, including poliovirus, it is of utmost importance to understand how they interact with their hosts and apply this knowledge to development of more effective preventative and prophylactic treatments. My projects focused on the use of poliovirus as a model enteric RNA virus to investigate viral population dynamics and virus-host pathogenesis.

The studies outlined in this dissertation initially set out to identify intra-host barriers to poliovirus after oral inoculation in mice. Results from these studies contribute to our understanding of RNA virus population dynamics and enteric virus pathogenesis. First, barriers to poliovirus were identified following oral inoculation of mice with a pool of marked polioviruses. One of the barriers could be partially overcome by disrupting the integrity of the colonic epithelium. These results indicate that host barriers can limit dissemination of enteric viruses,

and that the host mounts several barriers to viral populations. Second, as an extension to the initial observation of the GI tract posing a barrier to poliovirus infection, I investigated the role of the GI microbiota during poliovirus oral infection. These studies yielded surprising results in which I found that the natural GI flora promotes poliovirus infectivity, replication and pathogenesis. Third, additional experiments have been aimed at elucidating the mechanism involved in microbiota-dependent enhancement of poliovirus infection. I have approached this problem by examining virus-specific differences in the presence and absence of intestinal microbiota. I found that exposure to microbiota and MAMPs enhanced poliovirus infectivity. In addition, preliminary evidence in which I assessed poliovirus shedding and replication following LPS administration to antibiotic-treated mice and in which I compared poliovirus shedding in MyD88$^{+/+}$ and MyD88$^{-/-}$ mice suggests that bacterial-induced host mucosal immunity may be involved in poliovirus infection as well, but has not been as thoroughly investigated. The insight gained from my studies aids our understanding of enteric virus-host pathogenesis. Moreover, these studies illustrate that enteric viruses may have evolved to exploit naturally-residing intestinal microbes for productive infection.

Poliovirus encounters multiple barriers within hosts that alter viral population dynamics

Two previous reports used artificially-marked viruses to follow a viral population during spread within a plant and mammalian host, respectively (Ali et al., 2006; Pfeiffer and Kirkegaard, 2006). Pfeiffer and Kirkegaard (2006) examined host restriction of a marked viral population following peripheral injections of PVRtg mice using a marked poliovirus population. Of the four marked viruses used, they rarely observed all four in the brains of IM-injected mice and never witnessed all four in IV and IP-injected mice. However, if the four virus population was IC-injected, all four were recovered from mice brains 100% of the time. Further analysis showed that inoculum dose was partially responsible for the observed genetic bottleneck imparted on the viruses, but barriers still existed to restrict the artificial viral population. Poliovirus oral inoculations of PVRtg mice were not performed since these mice do not develop poliovirus symptoms by this route. In my studies, I employed PVRtg-IFNAR$^{-/-}$ mice, which do develop poliovirus symptoms and succumb to disease (Ohka et al., 2007), to investigate host barrier restriction on a larger marked population of polioviruses orally administered to mice. I wanted to determine if poliovirus spread was limited in these mice in the absence of a type I IFN response, and what host factors comprised barriers.

As discussed in Chapter 2, I helped develop and optimize techniques for a novel hybridization-based viral diversity assay in collaboration with a former

labmate (**Figure 2-1**). This assay allowed us the ability to monitor spread of an artificial viral population, composed of ten distinct viruses, following oral inoculation of PVRtg-IFNAR$^{-/-}$ mice. First, we assessed viral trafficking in the presence and absence of type I IFN signaling, and found that type I IFN greatly contributes to viral restriction in trafficking to the brain after peripheral injection (**Figure 2-4**). However, type I IFN is not the only barrier encountered after peripheral injection of virus since all 10 viruses are not consistently observed in brain of PVRtg-IFNAR$^{-/-}$ mice. The type I IFN response plays a significant role in viral restriction from the GI tract since PVRtg-IFNAR$^{-/-}$ mice succumb to poliovirus disease if it is administered orally (Ida-Hosonuma et al., 2005; Ohka et al., 2007), whereas PVRtg do not. Secondly, we identified three major barriers within PVRtg-IFNAR$^{-/-}$ mice that limited trafficking of our engineered poliovirus population, in which the viral population was reduced by at least 80% from the oral cavity to three different sites (**Figure 2-5, 2-9**). The resulting viral bottleneck from mouth-to-gut tissues and mouth-to-blood was overcome by disrupting the GI epithelium highlighting the importance of this physical host barrier (**Figure 2-9**). Third, we discovered that viral diversity may contribute to disease onset since mice succumbing to poliovirus disease displayed more viruses in all tissues examined (**Figure 2-5**). Fourth, we observed that viral titer and diversity are not always linked (**Figure 2-10**). Several of the mice exhibited high titers in their brains, but the number of marked viruses detected was usually reduced 80-90%.

It is likely that the diminished viral population able to reach the brain underwent robust replication since poliovirus has the propensity to replicate well in neurons. The results from this study revealed general physical and immunity-based constraints to enteric virus trafficking that are not revealed by viral titer analysis. We quantified the difficulty in poliovirus trafficking from the GI tract. This difficulty in dissemination from the GI tract may explain the rare occurrence of VAPP cases after OPV administration when nearly everyone receiving OPV carries revertant, virulent polioviruses within their GI tracts (Nathanson, 2008). The GI epithelium presents a major challenge to poliovirus spread since overcoming it by DSS-induced damage enhanced the dissemination of more marked viruses to sites within the GI tract and beyond. However, damage in the GI tract did not correlate with greater viral diversity in the brain, and therefore, GI damage cannot fully explain the onset of VAPP.

Intestinal microbes promote poliovirus infectivity, replication and pathogenesis

Poliovirus populations are restricted from transit within and beyond the GI tract, which compelled me to more clearly define the barrier(s) involved. A variety of molecules and macromolecular structures are contained within the GI tract, making it an extremely dynamic, complex environment. In considering the environment of the intestinal lumen, I was curious to determine if the natural microbial inhabitants of the GI tract represented a barrier to poliovirus dissemination and disease.

The GI microbiota is implicated in numerous host physiological processes. GI microbes are highly regulated by the intestinal mucosa in order to maintain homeostasis, since some have pathogenic potential. Many cell-types composing the GI tract readily recognize bacteria to facilitate localized immune responses that limit bacterial invasion (Hooper and Macpherson, 2010).

Until I began investigating the GI microbiota influence on poliovirus, very little research was available on the subject of viruses and GI microbes. Contrary to my original hypothesis, I discovered that the intestinal bacteria augment poliovirus infection in mice, and this was recapitulated *in vitro* (**Figure 3-2, 3-4, 3-8**). I concluded from these studies that the microbiota does not present a barrier to poliovirus infection and dissemination. Rather, the microbiota enhances poliovirus infectivity, replication and pathogenesis. I also made similar observations using reovirus, another enteric virus. However, reovirus phenotypes were not as profound as those observed for poliovirus (**Figure 3-9**). The GI microbes may be more beneficial for poliovirus than other enteric viruses, but this requires future assessment with additional enteric viruses.

One issue I considered when addressing microbiota influences on poliovirus infection was whether similar replication phenotypes were observable in PVRtg mice. Recall that PVRtg mice do not develop poliovirus-associated disease following oral inoculation, but it has been unclear for decades if poliovirus replication occurs in PVR-expressing mice intestines with competent type I IFN signaling. Surprisingly, poliovirus replication was evident in untreated

PVRtg mice and comparable to that observed for untreated PVRtg-IFNAR$^{-/-}$ mice (**Figure 3-4A, 3-4B**). As observed with antibiotic-treated PVRtg-IFNAR$^{-/-}$ mice, antibiotic-treated PVRtg mice support little poliovirus replication within the GI tract. These data demonstrate that poliovirus replication takes place in the GI tract of microbiota-containing PVRtg mice regardless of type I IFN signaling. This suggests that the type I IFN response has minimal effects on poliovirus replication in the intestine but may be important in limiting poliovirus replication in extra-intestinal sites. Therefore, the type I IFN response probably dictates poliovirus tissue tropism within mice, consistent with a previous report (Ida-Hosonuma et al., 2005). Results from the two mouse strains further supports the idea that virion modifications in the presence of GI microbes could enhance cell binding and entry to achieve similar replication kinetics irrespective of the host response.

Because general infectivity of poliovirus virions appears altered by GI microbial presence independent of viral replication (**Figure 3-8**), the viral capsid stucture may be modified. Little is known about *in vivo* poliovirus capsid dynamics, but a few studies have characterized the effects of intestinal fluid and purified proteases on poliovirus *in vitro*. Upon exposure to human intestinal fluid, poliovirus serotypes 2 and 3 undergo capsid modifications as visualized by electropheretic gel, but this still remains unclear for poliovirus serotype 1 as protein bands were shifted but cleavage products were not visualized (Piirainen et al., 1998; Roivainen and Hovi, 1988; Roivainen et al., 1990). These groups

observed similar protein processing when poliovirus types 2 and 3 were exposed to trypsin, but this is absent or below the detection limit for poliovirus type 1 (Fricks et al., 1985; Roivainen and Hovi, 1988). I have attempted to investigate broad changes to poliovirus capsid proteins after passage of ^{35}S-labeled poliovirus through mice upper GI tracts. However, poliovirus recovery from intestinal contents is low, and protein bands corresponding to capsid proteins are only faintly visible by radioactive gel electropheresis. In the future, these studies will be important to follow up as they may explain the change in poliovirus infectivity in the presence of the microbiota.

Reovirus capsid conformations are well documented *in vitro* and *in vivo*, but poliovirus and reovirus capsids differ in their basic molecular biology. Reovirus is composed of two capsids, whereas poliovirus is only singly encapsidated. Reovirus virions are proteolytically processed within the intestinal tract to the infectious form known as the infectious subvirion particle (ISVP) (Bass et al., 1990). ISVPs display a semi-processed outer capsid required for M cell binding, evidenced by loss of σ3, μ1C cleavage and extension of σ1 (Amerongen et al., 1994). ISVPs are further processed to form core particles with a single capsid upon cell binding and internalization (Guglielmi et al., 2006). It is believed that the double-encapsidation scheme of reovirus provides more stability. Poliovirus, on the other hand, is naturally resistant to short exposures to low pH, organic solvents and intestinal contents (Racaniello, 2001). Despite these variations in capsid structure, poliovirus may undergo infectivity-enhancing

conformational changes within the GI tract that would require more intense research efforts to elucidate.

The host mucosal immune response mediates differential fecal shedding of poliovirus and may influence viral replication

Intestinal microbes constantly stimulate host mucosal immune responses to maintain symbiotic relations. Much of the immune reactions to these resident microbes are mediated through the TLR adaptor protein MyD88, which is upstream of the transcription factor NFκB. NFκB activates transcription of pro-inflammatory effectors to regulate bacteria within the GI lumen (Hill and Artis, 2010). Intestinal bacteria are restricted from breaching the epithelium by immune mechanisms such as AMPs and sIgA (Hooper and Macpherson, 2010). Pilot experiments were performed to initially address the influence of these factors on poliovirus shedding and replication in untreated and antibiotic-treated mice.

I assessed the function of bacterial-induced MyD88 signaling during poliovirus infection. LPS administration to antibiotic-treated PVRtg mice slightly restored intestinal poliovirus replication (**Figure 4-1C**). These preliminary results indicate that host responses to bacterial stimuli may alter the outcome of poliovirus infection in the GI tract. Therefore, I next investigated the role of innate immune signaling through most TLRs and NLRs by infecting MyD88$^{-/-}$ mice with poliovirus. Fecal shedding of poliovirus was the only method possible to follow infection in these non-PVR-expressing mice, as PVRtg-MyD88$^{-/-}$ mice had not

been generated. In comparison to wild-type MyD88$^{+/+}$ mice, MyD88$^{-/-}$ mice displayed stunted shedding kinetics of poliovirus in feces (**Figure 4-2A, 4-2B**). Surprisingly, this phenotype was evident in both untreated and antibiotic-treated MyD88$^{-/-}$ mice, whereas antibiotic-treated C57BL/6 (MyD88$^{+/+}$) mice exhibited elevated, extended shedding 48 hours after it had ceased in untreated C57BL/6 (MyD88$^{+/+}$) mice (**Figure 3-3C**). From experimentation in a limited mouse model, it appears that MyD88 may influence poliovirus in both microbiota-dependent and independent manners. As a consequence, I am generating PVRtg-MyD88$^{-/-}$ mice in order to fully evaluate the function of microbiota-induced MyD88-mediated effects during poliovirus infection in both untreated and antibiotic-treated mice (**Figure 4-3**). Currently, the colony is being expanded for future experimentation.

Because sIgA may affect poliovirus shedding, I examined fecal excretion of poliovirus in the absence of sIgA. I reasoned that microbiota-induced sIgA may aid in clearance of poliovirus from the GI tract, and therefore, may explain the differential fecal shedding kinetics observed between untreated and antibiotic-treated mice. Initial studies with $J_H^{-/-}$ mice that lack sIgA suggest that microbial-induced sIgA is not responsible for the early heightened, quickly diminished poliovirus excretion observed in untreated mice (**Figure 4-4**). In fact, lack of sIgA did not alter poliovirus shedding in $J_H^{-/-}$ mice feces compared to untreated wild-type $J_H^{+/+}$ mice, and therefore, sIgA may not influence poliovirus transit in mice. One caveat to these studies is that the sIgA-deficient mouse line that is not susceptible to poliovirus due to lack of PVR expression. It is possible

the sIgA influences poliovirus replication but not shedding kinetics. This could be addressed by generating PVRtg-$J_H^{-/-}$ mice to assess poliovirus replication and pathogenesis in the absence of sIgA.

Host recognition of resident bacteria within the GI tract is one way to promote immune tolerance to this mass of potentially threatening microorganisms. Immune tolerance is a localized mechanism to maintain homeostasis with the microbiota without eliciting systemic immune responses that may act to clear these beneficial organisms (Hill and Artis, 2010). An interesting hypothesis to investigate is whether the microbiota-induced immune tolerance allows poliovirus to remain relatively unnoticed with the GI tract for ease of infection and dissemination. How this would play out in relation to enhanced poliovirus infectivity may be additive, but future studies are needed to address this idea.

Future Perspectives

Viral epidemics present a constant threat to the human population, whether humans are directly infected (e.g. poliovirus, HIV) or if the food supply is at risk (e.g. FMDV). RNA virus infections impact world health and economics. Many recent viral epidemics were caused by RNA viruses, such as influenza H1N1. In addition, many emerging viruses are also of RNA origin, including West Nile virus and SARS-CoV. Currently, there is no effective cure for RNA viruses, only limited preventative and prophylactic treatments to aid host protection and

clearance of viral infections. It is imperative that rapid replication kinetics and mutability be taken in account when developing treatments for RNA viruses, and gaining a full understanding of RNA virus-host interactions can contribute to more adequate treatments of viral infections as well.

Viral trafficking and host barriers to RNA viruses

The ability of RNA viruses to constantly mutate and potentially escape host immune responses is an important confounding factor in controlling these infections. I have helped identify general limitations that a host imposes on a RNA virus population (Chapter 2); however, full characterization of these barriers is still lacking.

It has been clearly defined that the type I IFN response to poliovirus is paramount in restricting viral trafficking and tropism (Ida-Hosonuma et al., 2005; Kuss et al., 2008; Ohka et al., 2007), but the exact downstream innate and adaptive responses remain unclear *in vivo*. A plethora of research has established that viral-induced type I IFN induction results in cell autonomous and non-autonomous antiviral and immunoregulatory responses (Katze et al., 2002). How IFNα/β responses restrict poliovirus replication in certain tissues, such as the intestine and kidney, needs further clarification. Do epithelial cell or immune cell type I IFN responses to poliovirus govern recognition and clearance of poliovirus following oral infection? Adaptive immunity is linked to innate immunity and has antiviral potential. Serum antibodies can control poliovirus infections

(Nathanson and Kew, 2010), and CD4+ and CD8+ T cells are cytotoxic to poliovirus-infected cells (Wahid et al., 2005b). However, localized immune responses at the inoculation site are less clear. Because poliovirus can infect and replicate in macrophages and dendritic cells (Wahid et al., 2005a), it is enticing to hypothesize that poliovirus may enter these cells in the GI tract, replicate and spread to peripheral tissues despite stimulating an immune response. However, we found that poliovirus dissemination from the GI tract is still restrictive despite the lack of IFNα/β responses (**Figure 2-5, 2-9**). Viral dissemination from the GI tract is partially attributed to epithelial barrier integrity (**Figure 2-8, 2-9**). Further characterization of host barriers limiting poliovirus spread from the GI tract will be cumbersome unless the primary infected cell in the GI tract is identified.

Great interest lies in defining the route of poliovirus dissemination from the GI tract to the blood and/or CNS. We have concluded that poliovirus detection within the bloodstream is stochastic suggesting difficulty in reaching this tissue or very transient blood infection (Chapter 2). Once poliovirus productively infects cells within the GI tract, how does it reach the bloodstream? Reovirus uses junction adhesion molecule A (JAM-A) to invade the bloodstream (Antar et al., 2009), so perhaps poliovirus has adopted a similar mechanism of reaching the bloodstream. Relatively simple *in vitro* interaction studies can be undertaken to address this possibility. Additionally, Yang et al. has implicated PVR-independent poliovirus infection of CNS tissues following IV inoculation of

non-PVR mice, suggesting alternative uptake mechanisms independent of PVR (Yang et al., 1997). A positive correlation exists between viremia and CNS infection in primates (Bodian, 1952a; Horstmann et al., 1954), but it is unknown how poliovirus invades the CNS from the bloodstream. Popular opinion implicates either breachment of the blood-brain barrier or infection of neurons innervating muscles or other tissues (Pfeiffer, 2010). Teasing out exact routes of poliovirus dissemination in mice will require very rigorous time course studies to monitor step-by-step spread of the virus.

Many outstanding questions remain concerning exactly how poliovirus infects within the GI tract and leads to hematogenous spread and/or CNS invasion. Which cell within the GI tract is initially infected for virus propagation and spread? Besides type I IFN responses and mucosal epithelial cell integrity, what other factors restrict poliovirus spread from the GI tract? Does poliovirus enter blood vessels directly from GI tissues? Do immune cells contribute to poliovirus spread to, in and from the bloodstream? In contrast, can poliovirus infect peritoneal neurons innervating GI tissues and transit neuronal pathways to the CNS? How exactly does poliovirus reach the CNS? Initial studies aimed at defining poliovirus spread within mice, outlined in Chapter 2, help set the stage for future assessment of these questions.

Poliovirus infection within the GI tract

Poliovirus, like many other viruses, is transmitted by fecal-oral spread, therefore, understanding factors within the GI tract that contribute to transmission will aid in our ability to interrupt enteric virus infections. Because we found that poliovirus spread from the oral cavity to the GI tract and beyond is difficult (Chapter 2), I further assessed GI factors that may limit poliovirus infection.

I wanted to examine the intestinal microbiota as a potential barrier to poliovirus infection within the GI tract. Counterintuitive to my initial hypothesis, I discovered that GI microbes greatly enhance poliovirus infection (**Figure 3-2A, 3-4, 3-8**). I have not clearly defined the exact mechanism underlying the phenotypes I observed, but poliovirus infectivity was enhanced by untreated mouse feces but not antibiotic-treated or germ-free mouse feces (**Figure 3-8B**). It is likely that this enhancement is due to a microbial factor since pure cultures of particular bacterial strains can augment poliovirus infectivity (**Figure 3-8C**). These results strongly support the idea that virion modifications are made by a factor within untreated mouse feces. Additionally, this unidentified factor is either induced by or derived from the microbiota since this enhancement is not observed when poliovirus is exposed to feces from antibiotic-treated and germ-free mice. In considering reovirus, we know that virions are modified by proteases within the GI tracts of mice in order to form the ISVP responsible for intestinal infection (Amerongen et al., 1994; Bass et al., 1990). Poliovirus and reovirus have capsid dissimilarities, but this strategy would be advantageous for

virus propagation and transmission and may be conserved for many enteric viruses.

The results obtained from poliovirus pathogenesis, replication and infectivity studies in the presence and absence of intestinal microbes in addition to what is known for reovirus introduces several important hypotheses to consider: (1) Proteolytic processing of reovirus may be more efficiently carried out by bacterial proteases, and may be why I observed enhanced reovirus infection in microbiota-harboring mice (**Figure 3-9**). (2) Poliovirus may undergo similar processing within the GI tract for adequate receptor-binding and cell entry. (3) A specific bacterial commensal, or related bacterial species, might be responsible for production of such virion-modifying molecules. (4) GI bacteria may produce a factor that alters a host molecule that modifies poliovirus. Ideally, cryo-electron microscopic reconstructions of polioviruses isolated after exposure to untreated or antibiotic-treated mouse feces or intestines would prove most beneficial in visualizing potential modifications, but a major caveat is extracting the virus in a solution that is conducive to this method without comprimising the conformation of the isolated viruses. Several additional methods exist to help examine virion modifications. Using the same approach that was taken for reovirus, one could orally administer protease inhibitors to mice during poliovirus infection and monitor virus replication and disease. Also, poliovirus virion surface-specific antibodies that recognize particular epitopes can be useful in determining if those epitopes are still displayed on the virion surface, or if the

virus has an altered conformation after exposure to feces from untreated mice. Additionally, one could expose poliovirus to untreated mouse feces *in vitro* and use the isolated virus as inoculum in antibiotic-treated mice. If the fecal-exposed virus can replicate and is pathogenic in antibiotic-treated mice, it is likely that conformational changes induced by microbiota presence are involved in enhanced infectivity in mice. I have preliminary evidence to indicate that poliovirus isolated from antibiotic-treated mice is as infectious as TC-derived poliovirus when administered to untreated mice (data not shown). Additional experimentation is needed, but if conformational changes conferring greater infectivity are induced, then they may be reversible. Experiments evaluating binding partners to poliovirus isolated from GI tracts of mice might also reveal some interesting findings since bacterial proteins from a wastewater culture were found to associate with a peptide of poliovirus VP1 capsid protein (Sano et al., 2004).

What virion modifications could change poliovirus infectivity in mice? Poliovirus virion conformations have been well studied *in vitro*. Poliovirus can adopt many different conformations that act at different stages during infection, which has been extensively reviewed by J. Hogle (2002). Native poliovirus sediments at 160S in sucrose gradients. Upon receptor binding and occasionally at physiological temperatures, poliovirus externalizes VP4 and the N-terminus of VP1 forming a virion that sediments at 135S, also known as the “A particle”. The externalization of VP4 and VP1 is irreversible when poliovirus is receptor bound,

but at physiological temperatures, during the process of breathing, it is reversible (Li et al., 1994). I speculate that virion breathing helps prime poliovirus for receptor binding and to undergo more significant conformational changes once PVR is bound. Perhaps breathing is induced by intestinal bacteria, thereby enhancing poliovirus-receptor interactions.

It is also possible that the GI microbes may cleave viral capsid proteins to enhance infectivity. V8 protease from *S. aureus* cleaves the N-terminus of VP1 once it is externalized (Bubeck et al., 2005; Fricks and Hogle, 1990). This cleavage ablates direct interactions with lipid membranes but perhaps PVR is more accessible facilitating viral entry into cells. Conversely, germ-free mice have increased host protease activity within their intestines than their microbiota-harboring counterparts (Norin et al., 1991; Ramare et al., 1996), and because the 135S particle is protease-sensitive (Fricks and Hogle, 1990), perhaps many poliovirus virions are proteolytically processed to a form that is no longer infectious. This would suggest that any recoverable virus from antibiotic-treated or germ-free mice has not been cleaved to an inactivate form. Poliovirus conformations have not been characterized from an animal infection; therefore, it is difficult to conclude what structural changes may take place in this infection model. Perhaps one could use poliovirus-specific antibodies that recognize particular viral protein epitopes to investigate this further.

Another factor that may affect infectivity of poliovirus virions is pocket factor. Pocket factor is a molecule that has been visualized in cryoelectron

microscopic reconstructions of virions and resembles a single-chain fatty acid (Filman et al., 1989; Kim et al., 1993). Investigators have questioned the biological significance of this component and are unsure if it is an artifact of the cryoelectron microscopy virion processing. However, antiviral WIN compounds bind the pocket of the virion surface of enteroviruses resulting in hyper-stabilization that disallows virus uncoating (Grant et al., 1994; Smith et al., 1996), suggesting that pocket factor may contribute to viral stability. What remains unclear at the moment is whether viral stability within the GI tract can enhance poliovirus interactions with susceptible cells via reducing degradation potential of virions within the lumen, or whether viral stability in the GI tract decreases receptor interactions and inhibits viral uncoating. At this time, it is difficult to say what effects pocket factor virion incorporation may have on poliovirus infectivity within the GI tract, and especially if intestinal microbes are contributing to generation or degradation of pocket factor molecules. Feces from untreated mice enhance stability of poliovirus *ex vivo* (**Figure 3-8B**), suggesting the likelihood of viral stability contributing to poliovirus infectivity *in vivo*. Utilizing antiviral WIN compounds could help make distinctions *in vivo* by pre-stabilizing poliovirus with WIN compounds prior to oral inoculation and evaluating poliovirus stability via fecal excretion of radiolabeled poliovirus. A caveat to this approach is ensuring conjugation of WIN compounds to virions throughout GI tract transit. Another drawback is that poliovirus replication cannot be assessed since WIN compounds prevent replication, and that is why assessing fecal shedding of

radiolabeled poliovirus is necessary. Despite difficulty in experimentally addressing pocket factor *in vivo*, this provides an attractive alternative to mediate poliovirus virion modifications that may promote infectivity.

Lastly, in consideration of poliovirus virion alterations that may enhance infectivity, there is evidence that ions influence poliovirus. In one report, addition of calcium chloride to a Tris-HCl solution converted native poliovirions to 135S particles (Wetz and Kucinski, 1991). It has also been known for decades that divalent cations stabilize poliovirus (Wallis and Melnick, 1961). We reasoned that bacteria within the GI tract may contribute to increased concentrations of divalent cations in untreated mice GI tracts. To this end, I measured the amount calcium in feces from untreated and antibiotic-treated mice. I discovered that untreated mice tend to have higher concentrations of calcium in fecal suspensions (data not shown), probably corresponding to elevated divalent cations within intestines. *In vitro* experiments have confirmed that addition of calcium chloride or zinc chloride, but not magnesium chloride, to poliovirus can stabilize virions (data not shown). It remains to be determined exactly how these effects are manifested and whether there is *in vivo* relevance to these findings, but ionic concentrations may alter poliovirus virions and affect overall infectivity.

For microbiota-dependent enhancement of poliovirus infectivity, it will be important to confirm phenotypes from antibiotic-treated mice in germ-free PVR mice once they are available. Antibiotic treatment of mice does not completely eliminate microbes within the GI tract, but results shown in Chapter 3 argue that

my observations are not an effect of antibiotic treatment alone (**Figure 3-7**), and that the reduction of intestinal microbes confers the phenotypes observed. In addition, results from experiments done with germ-free mice feces correspond to results obtained from antibiotic-treated mice feces (**Figure 3-3C, 3-8B**). Regardless, it is critical to perform these experiments in PVRtg and PVRtg-IFNAR$^{-/-}$ mice that are generated under germ-free conditions.

Although I have more specifically explored the influence of the intestinal microbiota on poliovirus virions, it is still important to consider the effects of microbiota-induced host immunity on poliovirus. One strong limitation to undertaking these studies arises from the fact that mice must express PVR to assess poliovirus replication and disease progression. Currently, PVRtg and PVRtg-IFNAR$^{-/-}$ mice are the only available strains. Whereas much useful information regarding poliovirus-host interactions have been gleaned from these model mouse strains, it is difficult to study other innate and adaptive immune responses to poliovirus in genetically-modified mice. Flow cytometric analysis or antibody-mediated depletion of immune cell types are alternative methods to genetic mouse models to address immune regulation of poliovirus. Because intestinal microbes facilitate host mucosal immune responses mainly through the TLR adaptor MyD88 (Hill and Artis, 2010), I felt is was important to generate PVRtg-MyD88$^{-/-}$ mice to aid in studying such interactions. Once the colony of PVRtg-MyD88$^{-/-}$ mice is established (**Figure 4-3**), poliovirus replication and disease can be evaluated in these mice in the presence and absence of intestinal

microbes. These studies will investigate the role of bacterial-induced MyD88-dependent and independent effects on poliovirus. It will be interesting to determine if commensal microbiota mediate host mucosal immune clearance of poliovirus, or rather, induce immune tolerance to poliovirus such that infection is enhanced. Additional future studies should be aimed at dissecting the influence of specific immune cell types, such as T_H17, T_{reg} and CX3CR1 dendritic cells, during poliovirus oral infection in the presence and absence of intestinal microbes.

Of utmost importance to progress our understanding of poliovirus interactions within the GI tract is to identify the primary cell that poliovirus infects and replicates within in the GI tract. Data gleaned from such experiments will help us more closely define poliovirus infection within the GI tract, what role the intestinal microbiota plays to promote infection of such a cell type and how poliovirus might disseminate from this particular cell. Previous reported attempts to identify poliovirus-infected cells within the GI tract are conflicting and employ indirect approaches. A major limitation is inconsistent detection of poliovirus by plaque assay in isolated tissues from mice (**Figure 3-5B**). Plaque assays might not have the appropriate level of sensitivity to detect poliovirus within intestinal tissues. Additionally, the virus could be undergoing structural changes prior to replication, and therefore, may no longer be infectious during quantification in an infection-based cell culture assay. Methods to detect negative strand poliovirus genomes, such as microscopic analysis via *in situ* hybridization or more sensitive

techniques, such as laser-capture microdissection and RT-PCR might prove beneficial to this endeavor.

CONCLUDING REMARKS

I believe that the data presented in this document have progressed our understanding of RNA virus population dynamics within a host, poliovirus-host interactions and the influence of the intestinal microbiota on enteric virus infections. The most interesting finding was that the intestinal microbes promote poliovirus infection and this may be applicable to other enteric viruses as well, as I have seen similar effects with reovirus. Enhanced poliovirus replication and pathogenesis are most likely not attributable to one specific factor, rather, it is probably a multifactorial phenomenon that requires much more investigation. The work herein has implications for antiviral therapeutics in that if an “enhancing factor” can be isolated from intestinal tracts of untreated mice, then it is likely that it can be used as a drug target to limit enteric virus-mediated gastroenteritis and possible viral spread to extra-intestinal tissues. This potential antiviral could also be used in conjunction with OPV in problem vaccinees to limit possible progression to VAPP and VDPV excretion in feces. In addition, perhaps transient reduction of intestinal microbes via antibiotic treatment in hypogammaglobulinemia patients would lead to the cessation of poliovirus replication and fecal excretion, restricting the threat of virulent poliovirus on these individuals and their communities. This would likely aid poliovirus eradication

efforts. It will be incredibly interesting to determine if intestinal microbes promote infection of other enteric viruses and to assess virus-microbiota interactions at additional mucosal surfaces, such as the genitourinary tract and nasal and oral cavities.

My work demonstrates the complexities an enteric virus encounters within a host and how some of these factors impact the outcome of poliovirus infection. Poliovirus is a very efficient pathogen. First of all, it is well known that poliovirus evades host responses through its ability to mutate quickly during replication, and we found that is highly restricted from breaching the GI tract. Being limited to the GI tract is actually beneficial for poliovirus because invasion of the CNS would result in loss of transmission. In fact, paralytic poliomyelitis is rare in poliovirus-infected individuals. Secondly, poliovirus exploits the natural habitat of the GI tract for its own benefit of proliferation and transmission. From an evolutionary perspective, poliovirus has evolved clever methods to propagate and persist in the human population for thousands of years, and this contributes to its ability to exist despite major eradication efforts.

APPENDIX A

16S rDNA sequence from a representative antibiotic-resistant fecal bacterial clone aligned to *Ochrobactrum intermedium* strain CCUG 43465

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O.intermedium      ------------------------------------------------------------CAGG 4
AbxR_isolate       AGCGGCCGCGAATTCGCCCTTAGAGTTTGATCCTGGCTCAGAACGAACGCTGGCGGCAGG 60
                                                                            ****

O.intermedium      CTTAACACATGCAAGTCGAGCGCGTAGCAATACGAGCGGCAGACGGGTGAGTAACGCGTG 64
AbxR_isolate       CTTAACACATGCAAGTCGAGCGCGTAGCAATACGAGCGGCAGACGGGTGAGTAACGCGTG 120
                   ************************************************************

O.intermedium      GGAATCTACCCATCACTAGGGAATAACTCAGGGAAACTTGTGCTAATACCCTATACGACC 124
AbxR_isolate       GGAATCTACCCATCACTAGGGAATAACTCAGGGAAACTTGTGCTAATACCCTATACGACC 180
                   ************************************************************

O.intermedium      GAGAGGTGAAAGATTTATCGGTGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGG 184
AbxR_isolate       GAGAGGTGAAAGATTTATCGGTGATGGATGAGCCCGCGTTGGATTAGCTAGTTGGTGGGG 240
                   ************************************************************

O.intermedium      TAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGG 244
AbxR_isolate       TAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACACTGGG 300
                   ************************************************************

O.intermedium      ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG 304
AbxR_isolate       ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCG 360
                   ************************************************************

O.intermedium      CAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTC 364
AbxR_isolate       CAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTC 420
                   ************************************************************

O.intermedium      ACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGC 424
AbxR_isolate       ACCGGTGAAGATAATGACGGTAACCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGC 480
                   ************************************************************

O.intermedium      CGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGG 484
AbxR_isolate       CGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGATTTACTGGGCGTAAAGCGCACGTAGG 540
                   ************************************************************

O.intermedium      CGGGCTAATAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTG 544
AbxR_isolate       CGGGCTAATAAGTCAGGGGTGAAATCCCGGGGCTCAACCCCGGAACTGCCTTTGATACTG 600
                   ************************************************************

O.intermedium      TTAGTCTTGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGAT 604
AbxR_isolate       TTAGTCTTGAGTATGGAAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGAT 660
                   ************************************************************

O.intermedium      ATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCG 664
AbxR_isolate       ATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGTCCATTACTGACGCTGAGGTGCG 720
                   ************************************************************

O.intermedium      AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATG 724
AbxR_isolate       AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATG 780
                   ************************************************************

O.intermedium      TTAGCCGTTGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGG 784
AbxR_isolate       TTAGCCGTTGGGGAGTTTACTCTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGG 840
                   ************************************************************
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O.intermedium      GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA 844
AbxR_isolate       GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA 900
                   ************************************************************

O.intermedium      GCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGC 904
AbxR_isolate       GCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATCCCGATCGC 960
                   ************************************************************

O.intermedium      GGTTAGTGGAGACACTTTCCTTCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGT 964
AbxR_isolate       GGTTAGTGGAGACACTTTCCTTCAGTTCGGCTGGATCGGAGACAGGTGCTGCATGGCTGT 1020
                   ************************************************************

O.intermedium      CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTT 1024
AbxR_isolate       CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTT 1080
                   ************************************************************

O.intermedium      AGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGG 1084
AbxR_isolate       AGTTGCCAGCATTCAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGG 1140
                   ************************************************************

O.intermedium      TGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGG 1144
AbxR_isolate       TGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCTACACACGTGCTACAATGG 1200
                   ************************************************************

O.intermedium      TGGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTC 1204
AbxR_isolate       TGGTGACAGTGGGCAGCGAGCACGCGAGTGTGAGCTAATCTCCAAAAGCCATCTCAGTTC 1260
                   ************************************************************

O.intermedium      GGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCA 1264
AbxR_isolate       GGATTGCACTCTGCAACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGCA 1320
                   ************************************************************

O.intermedium      TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGG 1324
AbxR_isolate       TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGG 1380
                   ************************************************************

O.intermedium      TTTTACCCGAAGGCGCTGTGCTAACCGCAAGGAGGCAGGCGACCACGGTAGGGTCAGCGA 1384
AbxR_isolate       TTTTACCCGAAGGCGCTGTGCT---------------------------------------- 1402
                   **********************

O.intermedium      CTGGGG 1390
AbxR_isolate       ------
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