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Mechanisms of Reovirus Bloodstream Dissemination

Karl W. Boehme^{*,†,1}, Caroline M. Lai^{§,†}, and Terence S. Dermody^{*,§,†,2}

^{*}Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[†]Elizabeth B. Lamb Center for Pediatric Research, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[§]Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Abstract

Many viruses cause disease within an infected host after spread from an initial portal of entry to secondary sites of replication. Viruses can disseminate via the bloodstream or through nerves. Mammalian orthoreoviruses (reoviruses) are neurotropic viruses that use both bloodborne and neural pathways to spread systemically within their hosts to cause disease. Using a robust mouse model and a dynamic reverse genetics system, we have identified a viral receptor and a viral nonstructural protein that are essential for hematogenous reovirus dissemination. Junctional adhesion molecule-A (JAM-A) is a member of the immunoglobulin superfamily expressed in tight junctions and on hematopoietic cells that serves as a receptor for all reovirus serotypes. Expression of JAM-A is required for infection of endothelial cells and development of viremia in mice, suggesting that release of virus into the bloodstream from infected endothelial cells requires JAM-A. Nonstructural protein $\sigma 1s$ is implicated in cell cycle arrest and apoptosis in reovirus-infected cells but is completely dispensable for reovirus replication in cultured cells. Surprisingly, a recombinant $\sigma 1s$ -null reovirus strain fails to spread hematogenously in infected mice, suggesting that $\sigma 1s$ facilitates apoptosis of reovirus-infected intestinal epithelial cells. It is possible that apoptotic bodies formed as a consequence of $\sigma 1s$ expression lead to reovirus uptake by dendritic cells for subsequent delivery to the mesenteric lymph node and the blood. Thus, both host and viral factors are required for efficient hematogenous dissemination of reovirus. Understanding mechanisms of reovirus bloodborne spread may shed light on how microbial pathogens invade the bloodstream to disseminate and cause disease in infected hosts.

I. Introduction

Many pathogenic human and animal viruses disseminate from mucosal sites to peripheral tissues where they cause organ-specific disease (Nathanson and Tyler, 1997). The capacity of a virus to spread systemically can correlate with increased virulence (de Jong et al., 2006; Gu et al., 2007; Kuiken et al., 2003; Pallansch and Roos, 2001). Systemic dissemination

²Corresponding author. Mailing address: Lamb Center for Pediatric Research, D7235 MCN, Vanderbilt University School of Medicine, Nashville, TN 37232. Phone: (615) 343-9943. Fax: (615) 343-9723. terry.dermody@vanderbilt.edu..

¹Current address: Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA

requires that the virus effectively navigate diverse intracellular and extracellular environments to infect, replicate, and evade immune detection in multiple cell types and tissues (Adair et al., 2012; Antar et al., 2009; Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009). Although some general principles of virus dissemination are understood, little is known about the precise viral and cellular determinants that govern virus spread. Defining mechanisms by which viruses disseminate within their hosts is of fundamental importance to an understanding of viral pathogenesis.

Mammalian orthoreoviruses (reoviruses) are highly tractable models for studies of viral pathogenesis. Studies of reovirus neural spread have provided important information about mechanisms by which neurotropic viruses cause disease in the central nervous system (CNS). The recent identification of new viral and host determinants that govern reovirus spread by the blood provides new insights into how hematogenous dissemination contributes to viral disease.

A. Reoviruses

Viruses of the *Reoviridae* family infect a wide range of host organisms, including mammals, birds, insects, and plants (Dermody, In Press). The *Reoviridae* includes rotaviruses, the most common diarrheal pathogen among children (Parashar et al., 1998), orbiviruses, which are economically important pathogens of sheep, cattle, and horses (Coetzee et al., 2012), and reoviruses. Three reovirus serotypes (T1, T2, and T3) currently circulate in humans and other mammals. The serotypes are distinguished on the basis of antibody-mediated neutralization of infectivity and inhibition of hemagglutination. Each serotype is represented by a prototype strain isolated from a human host: type 1 Lang (T1L), type 2 Jones (T2J), and type 3 Dearing (T3D). These strains differ dramatically in host cell tropism, mechanisms of cell killing, modes of dissemination, and CNS disease. In particular, studies of T1 and T3 reoviruses have generated foundational knowledge about strategies used by viruses to replicate and cause neural injury. Development of a plasmid-based reverse genetics system allows introduction of mutations into the viral genome to test specific hypotheses about the structure and function of viral proteins and RNAs (Kobayashi et al., 2007; Kobayashi et al., 2010). In concert with an experimentally facile mouse model of infection (Fields, 1992; Parashar, Tarlow and McCrae, 1992), reovirus is an ideal experimental platform for studies of virus-host interactions.

Reoviruses are nonenveloped, icosahedral viruses that contain a genome consisting of 10 segments of double-stranded (ds) RNA (Fig. 1) (Dermody, In Press). There are three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) dsRNA segments that are packaged in an equimolar stoichiometric relationship with one copy of each per virion. With the exception of the M3 and S1 gene segments, each of the reovirus gene segments is monocistronic. Reovirus virions are composed of two concentric protein shells, the outer capsid and core (Fig. 1) (Dryden et al., 1993). The outer capsid consists of heterohexameric complexes of the $\mu 1$ (encoded by M2) and $\sigma 3$ (encoded by S4) proteins. At each of the icosahedral five-fold symmetry axes, the attachment protein $\sigma 1$ (encoded by S1) extends from turret-like structures formed by pentamers of $\lambda 2$ (encoded by L2) protein. The inner core shell is formed by parallel asymmetric dimers of $\lambda 1$ (encoded by L3) protein that

are stabilized by $\sigma 2$ (encoded by S2) protein. The $\lambda 3$ (encoded by L1) and $\mu 2$ (encoded by M1) proteins are anchored to the inner surface of the core via interactions with $\lambda 1$. Lastly, the M3 gene segment encodes nonstructural proteins μNS and μNSC , the S3 gene segment encodes nonstructural protein σNS , and the S1 gene segment encodes nonstructural protein $\sigma 1s$.

Viral attachment protein $\sigma 1$ is a long filamentous molecule with head-and-tail morphology (Fig. 2A) (Chappell et al., 2002; Fraser et al., 1990; Mercier et al., 2004; Reiter et al., 2011). The $\sigma 1$ protein is comprised of three distinct structural domains: an N-terminal α -helical coiled-coil tail, a central β -spiral body, and a C-terminal globular head (Chappell et al., 2002; Reiter et al., 2011). Short regions of undefined structure separate each domain and are hypothesized to permit molecular flexibility required to engage cellular receptors during viral entry (Bokiej et al., 2012; (Chappell et al., 2002; Fraser et al., 1990; Reiter et al., 2011). Attachment of the $\sigma 1$ protein to cell-surface receptors initiates reovirus infection of susceptible host cells (Lee, Hayes and Joklik, 1981; Weiner, Ault and Fields, 1980). The $\sigma 1$ protein of T3 reovirus targets two different receptors, α -linked sialic acid (SA) (Armstrong, Paul and Lee, 1984; Dermody et al., 1990a; Pacitti and Gentsch, 1987; Paul, Choi and Lee, 1989; Paul and Lee, 1987) and junctional adhesion molecule-A (JAM-A) (Barton et al., 2001b; Campbell et al., 2005; Protá et al., 2003). Residues in the T3 $\sigma 1$ β -spiral body domain bind SA (Chappell et al., 2000; Reiter et al., 2011), whereas sequences in the $\sigma 1$ globular head domain engage JAM-A (Barton et al., 2001b; Kirchner et al., 2008).

After receptor binding, virions are internalized into endosomes via a process dependent on $\beta 1$ integrin (Maginnis et al., 2006) and distributed to organelles marked by Rab7 and Rab9 where viral disassembly takes place (Mainou and Dermody, 2012). During viral disassembly, outer-capsid protein $\sigma 3$ is degraded by cathepsin proteases, attachment protein $\sigma 1$ undergoes a conformational change, and outer-capsid protein $\mu 1$ is cleaved to form infectious subviral particles (ISVPs) (Danthi et al., 2010). The $\mu 1$ cleavage fragments undergo conformational rearrangement to facilitate endosome penetration and delivery of transcriptionally active core particles into the cytoplasm (Nibert et al., 2005; Odegard et al., 2004). Primary transcription occurs within the viral core, and nascent RNAs are translated or encapsidated into new viral cores, where they serve as templates for negative-strand synthesis. Within new viral cores, secondary rounds of transcription occur. Outer-capsid proteins are added to nascent cores, which silences viral transcription and yields progeny viral particles. Reovirus release from host cells is hypothesized to occur via a lytic mechanism, but the egress pathway is not understood (Dermody, In Press).

B. Junctional adhesion molecule-A

JAM-A is the only known proteinaceous receptor for reovirus. It mediates entry of prototype and field-isolate strains of all three reovirus serotypes (Barton et al., 2001b; Campbell et al., 2005). JAM-A is a member of the immunoglobulin (Ig) superfamily of proteins that functions in cell-cell adhesion (Bazzoni, 2003). It is expressed on the surface of endothelial and epithelial cells as a component of tight junctions that maintain the integrity of barriers formed between polarized cells (Martin-Padura et al., 1998; Woodfin et al., 2007). JAM-A also is expressed on hematopoietic cells, where it mediates leukocyte extravasation (Corada

et al., 2005; Ghislin et al., 2011), and on platelets, where it functions in platelet activation during blood clot formation (Bazzoni, 2003; Sobocka et al., 2004). JAM-A contains three distinct structural domains: an N-terminal ectodomain, a single-span transmembrane anchor, and a C-terminal cytoplasmic tail (Fig. 2B) (Prota et al., 2003). The ectodomain consists of two Ig-like domains, a membrane-distal D1 domain and a membrane-proximal D2 domain. The cytoplasmic tail terminates in a PDZ-binding domain that interacts with intracellular tight junction components (Bazzoni et al., 2005; Nomme et al., 2011). JAM-A participates in homotypic interactions between D1 domains on opposing monomers (Prota et al., 2003). An interaction between two JAM-A monomers on adjacent cells promotes cell adhesion (Iden et al., 2012; Mandell et al., 2005; Ostermann et al., 2005).

The $\sigma 1$ protein interacts with the JAM-A D1 domain to adhere reovirus virions to the surface of target cells (Kirchner et al., 2008). Interestingly, the $\sigma 1$ -JAM-A interaction is substantially stronger (approximately 1000-fold) than the interaction between JAM-A monomers (Kirchner et al., 2008). Consequently, $\sigma 1$ binding to JAM-A likely disrupts JAM-A homodimers. Studies using JAM-A-deficient mice indicate that JAM-A is required for the establishment of viremia, which is essential for dissemination and disease in newborn mice following peroral inoculation of reovirus (Antar et al., 2009). JAM-A is not required for reovirus replication in the murine CNS or development of encephalitis (Antar et al., 2009). These findings suggest that reovirus utilizes other cell-surface receptors to mediate entry into specific cell types.

C. Reovirus pathogenesis

Reoviruses are highly virulent in newborn mice and cause injury to a variety of host organs, including the CNS, heart, and liver (Dermody, In Press). T1 and T3 reovirus strains invade the CNS but use different routes and produce distinct pathologic consequences following peroral or intramuscular inoculation. T1 reoviruses spread by hematogenous routes and infect ependymal cells, causing nonlethal hydrocephalus (Tyler, McPhee and Fields, 1986; Weiner et al., 1977; Weiner, Powers and Fields, 1980). T3 reoviruses spread to the CNS by both hematogenous and neural routes and infect neurons (Antar et al., 2009; Boehme et al., 2011; Tyler, McPhee and Fields, 1986). In the brain, T3 reoviruses induce neuronal apoptosis, which results in fatal encephalitis (Morrison, Sidman and Fields, 1991a; Tyler, McPhee and Fields, 1986; Weiner et al., 1977; Weiner, Powers and Fields, 1980). Studies using T1L $\times$ T3D reassortant viruses mapped the major determinant of CNS pathology to the viral S1 gene (Dichter and Weiner, 1984; Tardieu and Weiner, 1982), which encodes attachment protein $\sigma 1$ and nonstructural protein $\sigma 1s$ (Sarkar et al., 1985; Weiner, Ault and Fields, 1980). Because of its role in viral attachment and entry, these serotype-specific differences in dissemination and disease have largely been ascribed to the $\sigma 1$ protein. However, $\sigma 1s$ plays a critical role in promoting reovirus spread by the bloodstream (Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009).

D. Nonstructural protein $\sigma 1s$

Protein $\sigma 1s$ is a 14 kDa nonstructural protein encoded by the viral S1 gene segment (Cashdollar et al., 1985; Ernst and Shatkin, 1985; Sarkar et al., 1985). The $\sigma 1s$ open-reading frame (ORF) completely overlaps the $\sigma 1$ coding sequence; however, $\sigma 1s$ lies in a different

reading frame (Cashdollar et al., 1985; Cenatiempo et al., 1984; Dermody et al., 1990b; Ernst and Shatkin, 1985; Sarkar et al., 1985). Although every reovirus strain sequenced to date contains a $\sigma 1s$ ORF, little amino acid sequence identity exists between the $\sigma 1s$ proteins from the different reovirus serotypes (Cashdollar et al., 1985; Dermody et al., 1990b). The only conserved sequence among $\sigma 1s$ proteins is a cluster of basic amino acids near the amino terminus (Cashdollar et al., 1985; Dermody et al., 1990b). The basic cluster from T3 $\sigma 1s$ functions as an autonomous nuclear localization signal that can redirect an appended heterologous protein to the nucleus (Hoyt, Bouchard and Tyler, 2004). While the majority of native $\sigma 1s$ localizes to the nucleus (Rodgers et al., 1998), it is not known whether the basic cluster mediates nuclear translocation in the context of reovirus infection. Functionally, the $\sigma 1s$ protein is implicated in reovirus-induced cell cycle arrest at the G₂/M boundary (Poggioli, Dermody and Tyler, 2001; Poggioli et al., 2000) and may influence reovirus neurovirulence by promoting reovirus-induced apoptosis in the murine CNS (Hoyt et al., 2005). Initial studies to define the function of $\sigma 1s$ in reovirus pathogenesis were complicated by the use of non-isogenic $\sigma 1s$ -null mutant and parental virus strains (Rodgers et al., 1998).

Development of a plasmid-based reverse genetics system for mammalian reovirus (Kobayashi et al., 2007; Kobayashi et al., 2010) made it possible to elucidate the function of $\sigma 1s$ in reovirus replication and pathogenesis. Recombinant reoviruses deficient in $\sigma 1s$ expression were engineered by incorporating a single nucleotide change (AUG to ACG) to disrupt the $\sigma 1s$ translational start site into the plasmid containing the cDNA encoding the S1 gene segment. Importantly, the mutation does not affect the coding sequence of the overlapping $\sigma 1$ ORF. Thus, except for $\sigma 1s$ expression, the resultant viruses are isogenic with the parental strain. Viruses deficient in $\sigma 1s$ expression have been generated in the T1 and T3 S1 gene backgrounds. In both cases, the $\sigma 1s$ -null viruses are viable and replicate with equivalent kinetics and produce yields of progeny virus comparable to the corresponding wild-type viruses, indicating that the $\sigma 1s$ protein is dispensable for reovirus replication in cultured cells (Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009). These viruses were used to uncover a role for $\sigma 1s$ in promoting hematogenous reovirus dissemination.

II. Dynamics of reovirus infection in the intestine and lung

Reoviruses infect their hosts by the fecal-oral and respiratory routes. Virus enters the host by ingestion of contaminated food or inhalation of virus-containing aerosols. At both portals of entry, reoviruses infect epithelial cells and disseminate to peripheral sites where they cause disease.

A. Infection via the gastrointestinal tract

Reoviruses have been isolated from the stools of healthy (Ramos-Alvarez, 1956; Ramos-Alvarez and Sabin, 1954) and ill (Ramos-Alvarez and Sabin, 1958) children as well as a variety of animals (Ramos-Alvarez and Sabin, 1958). These findings suggest that reovirus is ingested into and shed from the gastrointestinal tract. The dynamics of reovirus infection *in vivo* have largely been elucidated using experimental mouse and rat model systems. Following entry into the gastrointestinal tract, intestinal proteases rapidly convert reovirus virions to ISVPs, suggesting that the form of the reovirus particle that initiates infection in the intestine is the ISVP (Bass et al., 1990; Bodkin, Nibert and Fields, 1989; Chappell et al.,

1998). In newborn mice, cells at the tips of microvilli are readily infected, whereas cells in the intestinal crypts are spared (Antar et al., 2009; Boehme, Guglielmi and Dermody, 2009). In contrast, intestinal crypt cells are infected in adult mice, and cells at the villus tips are uninfected (Rubin, Kornstein and Anderson, 1985). Infectious reovirus can be recovered following peroral inoculation from the duodenum, jejunum, ileum, and colon (Rubin, Eaton and Anderson, 1986; Rubin, Kornstein and Anderson, 1985). However, the vast majority of virus is produced in the ileum. This differential production of virus may be due to the capacity of reovirus to infect Peyer patches. Reoviruses are thought to penetrate the intestinal barrier via transport across microfold (M) cells, which are specialized cells of the follicle-associated epithelium (FAE) that overlay the Peyer patches (Amerongen et al., 1994; Wolf et al., 1987; Wolf et al., 1983; Wolf et al., 1981). M cells transfer antigens from the intestinal lumen to lymphoid cells of the gut-associated lymphoid tissue (GALT) (van de Pavert and Mebius, 2010) and serve to monitor luminal contents by exposing Peyer patch lymphoid cells to food antigens, the intestinal microbiota, and intestinal microbial pathogens. This process is essential for induction of oral tolerance and activation of immune responses to pathogenic microorganisms (van de Pavert and Mebius, 2010). The preferential targeting of crypt cells observed in adult mice is hypothesized to result from transcytosis of virus across M cells and subsequent infection of crypt cells via the basolateral surface (Rubin, 1987). However, M cells also take up reovirus in neonatal mice (Antar et al., 2009; Boehme, Guglielmi and Dermody, 2009; Wolf et al., 1981), suggesting that viral transcytosis across M cells is unlikely to explain the difference in intestinal cell tropism observed in adult and newborn mice. It is possible that the proliferative status of stem cells in the crypts of adult mice may recapitulate the cellular environment of neonatal intestinal cells, thereby facilitating reovirus infection of intestinal crypt cells.

B. Infection via the respiratory tract

Reovirus also infects the respiratory tract (Sabin, 1959). In rats, both T1 and T3 reovirus strains cause a pneumonia that is characterized by destruction of type I alveolar epithelial cells and infiltration of leukocytes into the alveolar spaces (Morin, Warner and Fields, 1996). The pathology associated with reovirus infection closely mimics disease progression in bronchiolitis obliterans organizing pneumonia, which is notable for fibrous extensions into alveolar spaces in the context of an organizing pneumonia (Bellum et al., 1997). Following inoculation into the respiratory tract, lung proteases convert reovirus virions to ISVPs (Golden and Schiff, 2005; Nygaard, Golden and Schiff, 2012). Similar to infection in the intestine, reovirus infects the lung by transcytosis through M cells that overlie the bronchus-associated lymphoid tissue (BALT) (Morin, Warner and Fields, 1994; Morin, Warner and Fields, 1996).

III. Hematogenous spread of reovirus

A. Transport of reovirus from the intestine to the bloodstream

Systemic reovirus infection is thought to originate from infected lymphoid cells in the Peyer patch. From the Peyer patch, reovirus transits intestinal lymphatics to the mesenteric lymph node (MLN) and ultimately enters the bloodstream via the thoracic duct (Antar et al., 2009; Boehme, Guglielmi and Dermody, 2009; Wolf et al., 1981). Many pathogens that cause

systemic disease, including poliovirus (Bodian, 1955; Sabin, 1956) and *Salmonella* (Carter and Collins, 1974; Galan and Curtiss, 1989; Jones, Ghori and Falkow, 1994), initiate extraintestinal infection and access the bloodstream via this route.

Reovirus reaches the Peyer patches early after infection; viral antigen is detected in Peyer patches within 24 hours after peroral inoculation (Antar et al., 2009; Bass et al., 1988; Boehme, Guglielmi and Dermody, 2009; Wolf et al., 1987; Wolf et al., 1983; Wolf et al., 1981). However, the mechanism by which reovirus infects Peyer patch cells is not known. It is possible that dendritic cells in the Peyer patches take up reovirus virions immediately following viral transcytosis across M cells. This is the most direct route from the intestinal lumen to the Peyer patch and the primary pathway used for processing of intestinal antigens for immune surveillance. A second possibility is that progeny virions released from the basolateral surface of infected FAE cells are taken up by lymphoid cells in Peyer patches. Both viral structural and nonstructural proteins are detected in FAE cells (Fleeton et al., 2004), indicating that active viral replication occurs in these cells. However, it is not known whether FAE cells produce virus. A third possibility is that dendritic cells in Peyer patches take up apoptotic fragments from infected FAE cells, which undergo apoptosis following reovirus infection (Fleeton et al., 2004). Dendritic cells in the underlying Peyer patches immediately adjacent to apoptotic FAE cells contain both active caspase-3 and reovirus structural proteins (Fleeton et al., 2004). These observations suggest that Peyer patch dendritic cells take up apoptotic bodies from infected FAE cells. Additionally, apoptosis induction in the FAE may signal Peyer patch cells to phagocytose the apoptotic remnants, along with reovirus particles.

Regardless of the mechanism by which reovirus accesses Peyer patches, reovirus antigen is detected in the MLN 24 hours after peroral inoculation. Little is known about the cell types that support reovirus growth within the intestine and dissemination to the MLN. In adult mice, CD11c⁺ dendritic cells harbor reovirus antigen, but these cells are not thought to be actively infected (Fleeton et al., 2004). Viral nonstructural proteins are not present in these cells (Fleeton et al., 2004), suggesting that active replication does not occur. CD11c⁺ dendritic cells are present in neonatal animals (Muthukkumar, Goldstein and Stein, 2000), but it is not known whether these cells internalize reovirus following peroral inoculation of newborn mice. Reovirus productively infects bulk splenocytes isolated from newborn mice (Tardieu, Powers and Weiner, 1983), suggesting that reovirus can replicate in primary lymphoid cells. Reovirus cannot productively infect splenocytes explanted after the mouse reaches 7 days of age (Tardieu, Powers and Weiner, 1983). Thus, the lack of viral replication in Peyer patch cells in older animals may contribute to the age restriction to reovirus infection.

From Peyer patches, reovirus is hypothesized to traffic via afferent lymphatics to the MLN, then through efferent lymphatics to the blood. It is possible that infected lymphoid cells or lymphoid cells harboring virus mediate transport from the Peyer patches to the bloodstream. However, migrating dendritic cells rarely exit lymph nodes once they enter and present antigen to B and T cells (Iwasaki, 2007). Thus, the cells responsible for transport of reovirus from the Peyer patch are likely retained in the MLN. Reovirus titers in the MLN increase rapidly after peroral inoculation (Antar et al., 2009; Boehme, Guglielmi and Dermody,

2009), suggesting that active viral replication occurs in the MLN. However, it also is possible that the increase in viral load in the MLN represents migration of infected lymphoid cells from the Peyer patches. Dissemination from the MLN to the bloodstream may occur as free virus or within another lymphoid cell subset.

An alternative mechanism for accessing the blood is direct uptake of viral particles from the gut. CD18⁺ phagocytes extend cellular processes between enterocytes to directly sample luminal contents. Dendritic cells also extend processes through the epithelial monolayer while maintaining barrier integrity to sample gut pathogens (Rescigno et al., 2001). A number of pathogens, including *Salmonella* (Vazquez-Torres et al., 1999) and *Yersinia* (Isberg and Barnes, 2001), use macrophages or dendritic cells to invade the bloodstream and cause extraintestinal infection. Following uptake of luminal pathogens, CD18⁺ phagocytes traffic across the lamina propria and directly into the blood allowing for rapid entry of the pathogen into the bloodstream.

B. Reovirus viremia

Although virus is detected in the blood of infected animals, it is not known whether reovirus virions within the blood are free in the plasma or associated with hematopoietic cells. Other *Reoviridae* family members, including bluetongue virus (BTV) and Colorado tick fever virus, produce cell-associated viremia during infection. BTV infects and replicates in mononuclear cells, lymphocytes, and endothelial cells (Barratt-Boyes and MacLachlan, 1994; Ellis et al., 1993; MacLachlan et al., 1990; Mahrt and Osburn, 1986; Veronesi et al., 2009). Colorado tick fever virus is detected in mature erythrocytes (Oshiro et al., 1978). However, arthropod vectors transmit BTV and Colorado tick fever virus, making viremia a necessary part of the viral infectious cycle in nature. Mammalian reoviruses are not transmitted by arthropod vectors and may produce a distinctly different type of viremia. Studies in which oncolytic reovirus was delivered intravenously to persons with cancer revealed that virus is largely found in hematopoietic cells, specifically mononuclear cells, granulocytes, and platelets (Adair et al., 2012). Each of these cell types express JAM-A (Martin-Padura et al., 1998; Naik et al., 2001; Sobocka et al., 2000), suggesting that reovirus associates with or infects blood cells to disseminate through the blood to target organs. However, in these studies, virus was delivered directly into the bloodstream by intravenous inoculation. It is not known how reovirus spreads systemically following infection from a natural portal, such as the intestine or lung.

C. Role of receptors in reovirus dissemination

Interactions between viral attachment proteins and host cell receptors play a pivotal role in viral pathogenesis. Receptor engagement is a primary mechanism to define cells targeted by viruses. Therefore, patterns of receptor expression are a key determinant of viral disease. Reoviruses engage two types of cellular receptors: cell-surface carbohydrate (Paul, Choi and Lee, 1989) and JAM-A (Barton et al., 2001b; Paul, Choi and Lee, 1989). Both T1 and T3 (Dermody et al., 1990a; Pacitti and Gentsch, 1987; Paul, Choi and Lee, 1989) reoviruses bind cell-surface SA (Armstrong, Paul and Lee, 1984; Dermody et al., 1990a; Pacitti and Gentsch, 1987; Paul, Choi and Lee, 1989; Paul and Lee, 1987). However, the domains of $\sigma 1$

that engage glycans differ between the serotypes (Dermody et al., 1990a), as do the specific glycans bound.

SA engagement enhances reovirus infection through an adhesion-strengthening mechanism in which viral particles are tethered to the cell surface via a low-affinity interaction with the carbohydrate (Barton et al., 2001a). This interaction maintains the virus on the cell surface and increases the opportunity to engage JAM-A. SA-binding reovirus strains have an increased capacity to infect cells compared with non-SA-binding viruses; pre-treatment of cells with neuraminidase to remove cell-surface SA eliminates this advantage (Barton et al., 2001a). SA engagement also enhances reovirus tropism for bile duct epithelial cells in mice following peroral inoculation (Barton et al., 2003). The resulting disease closely mimics biliary atresia in human infants (Barton et al., 2003), an illness epidemiologically associated with reovirus (Richardson, Bishop and Smith, 1994; Tyler et al., 1998).

Reovirus strains circulating in nature vary in the capacity to bind SA (Dermody et al., 1990a; Dermody et al., 1990b). This finding suggests that SA binding comes with a fitness cost. Accordingly, SA binding appears to inhibit the capacity of reovirus to establish infection at mucosal portals of entry. Non-SA-binding viruses infect primary human airway epithelial cells substantially more efficiently than SA-binding strains (Excoffon et al., 2008). Moreover, infection of primary human airway epithelial cells by SA-binding viruses is enhanced by removal of cell-surface SA with neuraminidase. Mucosal surfaces are covered with a glycocalyx consisting of polysaccharides and glycoproteins that are rich in SA (Excoffon et al., 2008). SA-binding viruses may be trapped by SA within the glycocalyx and incapable of reaching the underlying epithelium (Excoffon et al., 2008). However, once infection is established, SA binding may enhance the capacity of reovirus to cause disease. In addition to the capacity to target bile duct epithelium, SA-binding strains are more neurovirulent than non-SA-binding viruses following intracranial inoculation (Barton et al., 2003). This increase in virulence is likely due to more efficient infection of neurons, which results in neuronal apoptosis and encephalitis. The function of SA binding in reovirus hematogenous spread remains to be determined.

Although all reoviruses bind JAM-A, T1 and T3 reoviruses infect distinct cells and cause serotype-specific patterns of pathologic injury within the CNS. These observations suggest that JAM-A binding does not influence serotype-specific differences in reovirus neural tropism and CNS disease. Following peroral inoculation, reovirus produces similar viral titers in the intestine of wild-type and JAM-A-deficient mice, suggesting that JAM-A is not required for reovirus replication in the mouse gastrointestinal tract (Antar et al., 2009). In sharp contrast, viral titers at all sites of secondary replication are significantly lower in JAM-A-deficient animals compared with wild-type controls (Fig. 3). Viral loads are comparable within the brains of wild-type and JAM-A-deficient animals after intracranial inoculation, suggesting that JAM-A is not required for viral replication at this site of secondary replication (Antar et al., 2009). These results suggest that JAM-A is required for dissemination of the virus from the intestine to replication sites in target organs.

How might JAM-A promote hematogenous dissemination? Substantially lower reovirus titers are detected in the blood of JAM-A-deficient mice compared with wild-type mice (Fig.

4), suggesting that JAM-A is involved in the establishment of viremia (Antar et al., 2009). Diminished viremia is detected in mice inoculated with either T1 or T3 reovirus, indicating that JAM-A functions in promoting bloodborne spread of T1 viruses that disseminate by strictly hematogenous mechanisms as well as neurotropic T3 reoviruses. Primary pulmonary endothelial cells isolated from JAM-A-deficient mice are refractory to reovirus infection compared with those harvested from wild-type mice (Fig. 5). These data suggest that reovirus engages JAM-A to infect endothelial cells following infection, likely in the lymphatics or vasculature of the gastrointestinal tract. It is possible that virus released from endothelial cells invades the bloodstream to disseminate to peripheral target organs either free in the plasma or associated with hematopoietic cells.

D. Function of nonstructural protein $\sigma 1$ s in reovirus dissemination

Studies using T1 $\sigma 1$ s-null virus uncovered a role for $\sigma 1$ s in promoting bloodborne reovirus spread (Boehme, Guglielmi and Dermody, 2009). The $\sigma 1$ s protein is not required for the initial establishment of reovirus infection in the gut. Wild-type and $\sigma 1$ s-null viruses replicate to comparable levels in the gastrointestinal tract following peroral inoculation (Fig. 6) (Boehme, Guglielmi and Dermody, 2009). Reovirus antigen is evident in the intestinal epithelium and Peyer patches of mice inoculated with wild-type or $\sigma 1$ s-null virus, indicating that $\sigma 1$ s does not influence reovirus tropism in the intestine. In contrast to wild-type virus, the $\sigma 1$ s-null mutant fails to produce substantial titers in the MLN. The $\sigma 1$ s-null virus is detected at low titer in the MLN, but viral titers do not increase over the course of infection. These findings indicate that $\sigma 1$ s either is essential for transit through lymphatic channels to the MLN or serves to promote replication in MLN cells. Wild-type virus is detected in the blood and sites of secondary viral replication, including the brain, heart, liver, and spleen (Fig. 6). The $\sigma 1$ s-null virus is not detected in the blood or any of the target organs examined. This difference is probably not due to a requirement for $\sigma 1$ s in mediating replication at these sites, as wild-type and $\sigma 1$ s-null viruses produce comparable titers in the brain following intracranial inoculation. Together, these findings suggest that the $\sigma 1$ s protein performs a function that is essential for reovirus to spread from the gut through intestinal lymphatics and ultimately to the blood where it gains access peripheral organs.

In contrast to T1 reoviruses that spread by strictly hematogenous mechanisms, T3 reoviruses disseminate by both hematogenous and neural pathways. The amino acid sequences of the $\sigma 1$ s proteins from the different reovirus serotypes differ substantially (Dermody et al., 1990b). Therefore, it is possible that the $\sigma 1$ s proteins perform serotype-specific functions. In nature, reovirus infects by the peroral route and spreads to the CNS in infant animals resulting in neuropathology. However, infectivity of T3 prototype strain T3D is diminished significantly within the gastrointestinal tract (Bodkin and Fields, 1989; Bodkin, Nibert and Fields, 1989) due to cleavage of its $\sigma 1$ protein by intestinal proteases (Bodkin and Fields, 1989; Bodkin, Nibert and Fields, 1989; Nibert, Chappell and Dermody, 1995). Consequently, intramuscular inoculation into the hindlimb is used to assess mechanisms of T3D dissemination. Inoculation of type 3 reoviruses intramuscularly leads to invasion of the brain by neural routes (Tyler, McPhee and Fields, 1986). Following intramuscular inoculation, wild-type T3D produces substantially higher titers than the $\sigma 1$ s-null virus in peripheral organs including the heart, liver, intestine, and spleen, similar to results obtained

using wild-type and $\sigma 1s$ -null T1 viruses (Boehme et al., 2011). Moreover, wild-type T3D but not the T3D $\sigma 1s$ -null mutant virus is detected in the blood. Together, these data suggest that $\sigma 1s$ functions to promote the establishment of reovirus viremia in a serotype-independent manner, which ultimately leads to infection of peripheral target tissues.

In contrast to its function in hematogenous spread, the $\sigma 1s$ protein is dispensable for reovirus spread to the CNS by neural routes. Both wild-type and $\sigma 1s$ -null viruses produce comparable titers in the spinal cord following inoculation into the hindlimb muscle (Boehme, 2011). Both viruses also produce comparable titers in the brain following direct intracranial inoculation and in cultured primary neurons. Together, these findings indicate that $\sigma 1s$ is not required for reovirus neural spread or replication in the murine CNS. Thus, although T3 viruses spread via neural and hematogenous mechanisms, the T3 $\sigma 1s$ protein only influences the efficiency of hematogenous dissemination.

IV. Neural dissemination of reovirus

In addition to bloodborne spread, T3 reoviruses use neural circuits to disseminate to the CNS (Boehme et al., 2011; Tyler, McPhee and Fields, 1986). Spread via neural routes is a fundamental mechanism of reovirus pathogenesis that is essential for development of reovirus-induced encephalitis (Boehme et al., 2011; Tyler, McPhee and Fields, 1986). Direct infection of neurons at peripheral sites provides the virus with access to the CNS and serves as a conduit to the brain. Although the importance of neural spread to reovirus pathogenesis is well-appreciated, the cellular and molecular mechanisms that underlie neuronal reovirus trafficking are not well understood.

In contrast to hematogenous spread, JAM-A is dispensable for neural dissemination. Although JAM-A is expressed in the brain, the cell types on which it is present have not been defined. JAM-A is found on NG2-glia cells, which are a subset of stem cells that give rise to oligodendrocytes (Nomme et al., 2011). It is unclear whether JAM-A is expressed on peripheral or CNS neurons. Viral titers in the brains of wild-type and JAM-A-deficient mice are comparable after intracranial inoculation (Antar et al., 2009). Viral tropism in the brain for hippocampal, thalamic, and cortical regions also does not differ between wild-type and JAM-A-deficient mice. Concordantly, primary cortical neurons isolated from wild-type and JAM-A-deficient mice are equally susceptible to reovirus infection and produce equivalent yields of viral progeny (Antar et al., 2009). Together, these data indicate JAM-A is not required for reovirus infection of neural tissue and suggest that JAM-A is dispensable for reovirus spread by neural routes. These findings further suggest that a cellular receptor distinct from JAM-A mediates reovirus infection of neurons.

Some evidence exists about the means by which reovirus traverses neural circuits. Treatment of animals with colchicine to inhibit fast axonal transport impairs reovirus spread to the spinal cord following hindlimb inoculation (Sjostrand and Karlsson, 1969; Tyler, McPhee and Fields, 1986). However, treatment with β - β' -iminodipropionitrile to inhibit slow axonal transport does not affect reovirus dissemination to the spinal cord (Hansson et al., 1971; Tyler, McPhee and Fields, 1986). These findings suggest that reovirus traffics in neurons along fast axonal transport pathways. However, these inhibitors may act non-specifically to

impair other aspects of viral replication. It also is not known whether reovirus uses afferent or efferent neurons to traffic to the CNS or whether virions can travel using both retrograde and anterograde pathways within neurons. Finally, it is not known where or how progeny virions exit neurons. Much work is required to fully elucidate how reoviruses replicate and traffic in neurons.

V. Function of hematogenous and neural spread in reovirus pathogenesis

T1 reoviruses disseminate to sites of secondary viral replication solely by hematogenous pathways. Following peroral inoculation of JAM-A-deficient mice, T1 reovirus does not reach the blood or peripheral organs (Antar et al., 2009). Similarly, T1 $\sigma 1s$ -null virus fails to disseminate from the intestine to sites of secondary replication (Boehme, Guglielmi and Dermody, 2009). Because T1 reoviruses utilize a single mechanism to spread within its host, inhibiting that mode of dissemination prevents virus-induced systemic disease.

T3 reoviruses, in contrast, disseminate to peripheral organs using a combination of hematogenous and neural mechanisms. Neural spread is essential for maximal neural injury induced by T3 reovirus (Tyler, McPhee and Fields, 1986). Following peroral inoculation or inoculation into the hindlimb muscle, T3 reovirus infects peripheral neurons and travels along nerve fibers to infect the CNS and cause disease (Morrison, Sidman and Fields, 1991b; Tyler, McPhee and Fields, 1986). Inhibiting neural spread by sectioning the sciatic nerve prior to hindlimb inoculation prevents virus spread to the spinal cord (Tyler, McPhee and Fields, 1986). This finding indicates that T3 reovirus spreads along neural routes to the CNS and suggests that neural dissemination is essential for reovirus neuropathogenesis.

The importance of hematogenous spread in reovirus neuropathogenesis is evident from studies that identified host and viral factors that mediate reovirus transport through the blood. JAM-A-deficient mice are completely resistant to reovirus-induced disease following peroral inoculation with T3 reovirus, whereas wild-type mice succumb to infection (Antar et al., 2009). Viral titers in the brains of JAM-A-deficient mice are substantially reduced in comparison to those in wild-type controls. Concordantly, viral loads in the blood of JAM-A-deficient mice are lower than those detected in wild-type mice (Antar et al., 2009). However, following intracranial inoculation, wild-type and JAMA-deficient mice are equally susceptible to reovirus disease, and equivalent viral yields are produced in the brains of wild-type and JAM-A-deficient mice (Antar et al., 2009). These results indicate that reduced reovirus virulence in JAM-A-deficient mice following peroral inoculation is not the result of differences in reovirus replication in the brain.

Studies of T3 $\sigma 1s$ -null viruses also highlight the requirement of hematogenous dissemination for reovirus neuropathogenesis. Wild-type T3 reovirus is substantially more virulent than the T3 $\sigma 1s$ -null virus following hindlimb inoculation (Fig. 7) (Boehme et al., 2011). Approximately 75% of animals inoculated with wild-type virus succumb to infection compared with 25% of mice inoculated with the $\sigma 1s$ -null virus (Boehme et al., 2011). Wild-type and $\sigma 1s$ -null T3 reoviruses induced 100% mortality following intracranial inoculation, although animals inoculated with wild-type virus succumbed to CNS disease with slightly faster kinetics than those inoculated with the $\sigma 1s$ -null virus. Wild-type and $\sigma 1s$ -null viruses

also produce equivalent titers in the brain following intracranial inoculation, indicating that $\sigma 1s$ is dispensable for viral replication in the murine CNS. Thus, the disparity in virulence between wild-type and $\sigma 1s$ -null viruses following intramuscular inoculation does not result from differences in replication in the CNS between the two viral strains.

Following hindlimb inoculation, wild-type virus is detected in the brain 1 day after infection (Figure 7). In contrast, the $\sigma 1s$ -null virus is not found in the brain until 2 days after inoculation. At days 2 and 4 post-inoculation, viral titers in the brains of animals inoculated with wild-type virus are markedly higher than those observed in mice inoculated with the $\sigma 1s$ -null mutant. This finding correlates with significantly higher loads of wild-type virus in the blood of infected animals at early times post-inoculation compared with the $\sigma 1s$ -null virus (Boehme et al., 2011). Comparable titers of wild-type and $\sigma 1s$ -null viruses are found in the spinal cord at days 1, 2, and 4 post-inoculation. This observation suggests that transport of the $\sigma 1s$ -null virus to the CNS by neural pathways is not impaired. At day 8 post-inoculation, peak titers of wild-type and $\sigma 1s$ -null viruses in the brain are equivalent, possibly reflecting delivery of virus to the brain via neural routes. Collectively, these findings suggest that hematogenous spread is required for reovirus transport to the brain at early times after infection. These results also suggest that the timing of reovirus delivery to the brain is critical for neuropathogenesis. Viral transport by neural routes does not differ between wild-type and $\sigma 1s$ -null viruses, and both virus strains produce equivalent peak titers in the brain. However, peak titers of the $\sigma 1s$ -null virus appear to be achieved after the mice reach the age-imposed limit to reovirus infection, and these animals are no longer susceptible to reovirus-induced CNS disease (Mann et al., 2002). Thus, reovirus transport to the brain by the blood at early times after infection is critical for neuropathogenesis.

Reovirus spreads to the spinal cord via the sciatic nerve following intramuscular inoculation into the hindlimb (Tyler, McPhee and Fields, 1986). Transection of the sciatic nerve prior to inoculation inhibits neural transmission of the virus to the spinal cord; however, viral dissemination by the blood is unaffected (Tyler, McPhee and Fields, 1986). T3 reovirus retains the capacity to spread to the brain after sciatic nerve section (Boehme et al., 2011), suggesting that reovirus can access the brain even in the absence of neural spread, likely via the bloodstream (Fig. 8). In addition, almost no virus is detected in the brain following hindlimb inoculation with the $\sigma 1s$ -null virus when the sciatic nerve is sectioned. Thus, virus cannot access the brain when both hematogenous and neural pathways of spread are inhibited.

Together, these findings suggest that (i) spread by neural routes alone is not sufficient to cause reovirus CNS disease, (ii) bloodborne spread is required for delivery of reovirus to the brain at early times post-infection, (iii) hematogenous viral dissemination to the brain is an essential mechanism of reovirus neuropathogenesis, and (iv) virus must be delivered to the brain by the blood early after inoculation for full reovirus neurovirulence.

VI. Unanswered questions and future directions

We have identified host and viral factors essential for the hematogenous dissemination of reovirus. However, many unanswered questions remain. Because viruses capable of

bloodstream spread may share similar mechanisms of dissemination, understanding how reovirus spreads in the infected host may aid in the development of therapeutics that target this critical step in viral pathogenesis.

A. How does reovirus enter and exit the bloodstream?

To spread to peripheral organs by hematogenous pathways, reovirus must first enter the bloodstream. Studies of reovirus pathogenesis suggest that following peroral inoculation, reovirus infects Peyer patch lymphoid cells that transport virus to the bloodstream (Figure 9). However, reovirus also disseminates hematogenously following intracranial inoculation (Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009). This observation suggests that reovirus has the capacity to cross endothelial barriers to enter the blood. Little is known about how reovirus infects polarized cells, such as those that constitute the endothelium. JAM-A localizes to tight junctions linking endothelial cells and functions in maintaining the barrier between the tissue and blood compartments. JAM-A is required for hematogenous spread of reovirus and infection of primary cultures of pulmonary vascular endothelial cells (Antar et al., 2009). We envision several possible mechanisms to explain how reovirus uses JAM-A to facilitate entry into the blood. First, JAM-A may function as a gatekeeper for reovirus entry into the bloodstream (Figure 9). Although reovirus infection does not change the barrier function of primary human airway epithelial cells (Excoffon et al., 2008), it is unclear whether the same phenomenon occurs during reovirus infection of the endothelium. Free reovirus virions may interact with JAM-A in endothelial cell tight junctions, transiently disrupt these structures, and cause focal breaches of the endothelial barrier to allow viral invasion of the bloodstream. Other viruses are known to disrupt polarized cell barriers during infection. Mouse adenovirus-1 infection of endothelial cells reduces tight junction protein expression and decreases barrier function in polarized endothelial cell monolayers (Gralinski et al., 2009). Coxsackieviruses engage decay-accelerating factor, an apically distributed protein of polarized epithelial cells, to disrupt tight junctions (Coyne et al., 2007). In doing so, coxsackieviruses gain access to the basolaterally located coxsackievirus and adenovirus receptor (CAR) (Coyne et al., 2007). HIV-1 gp120 diminishes expression of tight junction proteins and increases vascular permeability (Kanmogne et al., 2009).

Second, it is possible that reovirus infects endothelial cells to allow progeny virus to be released directly into the blood (Figure 9). Endothelial cells function as sites of amplification for many viruses that spread via the bloodstream. Murine cytomegalovirus dissemination occurs after an episode of secondary viremia that requires viral replication in endothelial cells (Sacher et al., 2008). It is possible that reovirus productively infects endothelium from the basolateral surface on the abluminal side of the endothelium and is released from the apical surface into the blood. Many viruses that infect polarized cells egress apically (Roberts 1995). This mechanism is common for respiratory viruses, in which release from the apical surface of infected respiratory epithelial cells ensures that the virus will be shed into the respiratory tract to facilitate transmission to susceptible hosts (Gerl et al., 2012; Brock et al., 2003; Rodriguez and Sabatini, 1978). Studies of reovirus infection of polarized endothelial cells will shed light on mechanisms used by the virus to traverse endothelial monolayers.

Third, reovirus spread may involve infection or association with hematopoietic cells (Figure 9). Hematopoietic cells express JAM-A as an adhesion molecule to allow monocyte extravasation across endothelial barriers (Martin-Padura et al., 1998; Williams et al., 1999). It is not known whether hematopoietic cells are infected or whether infected blood cells transport reovirus systemically following infection by a natural route of inoculation. However, in cancer patients treated with intravenous infusions of reovirus, virions associate with mononuclear cells, granulocytes, and platelets to allow dissemination to tumors localized in the viscera (Adair et al., 2012). If hematopoietic cells are responsible for hematogenous reovirus dissemination, age-dependent restriction of reovirus replication in these cells may be one mechanism to explain the limitation of reovirus disease to newborn animals (Tardieu, Powers and Weiner, 1983).

Reovirus exit from the bloodstream is required for infection and replication in target tissues and development of organ-specific disease. After peroral inoculation of reovirus, high viral titers are found in virtually all organs (Antar et al., 2009; Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009). Mechanisms similar to those that facilitate reovirus entry into the vasculature may mediate reovirus escape from the blood. Reovirus interactions with JAM-A may induce localized perturbations of tight junction integrity that permit virus escape into tissues. Reovirus virions in the blood may infect endothelial cells from the apical surface and progeny virions may be released basolaterally. Finally, infected hematopoietic cells may transport virus from the blood into target organs. None of these possibilities is mutually exclusive; reovirus may use multiple strategies to enter and exit the bloodstream. Studies using mice with tissue-specific expression of JAM-A may help to elucidate mechanisms by which JAM-A facilitates reovirus spread through the bloodstream.

B. How does $\sigma 1s$ promote hematogenous spread?

Mechanisms by which $\sigma 1s$ promotes dissemination have not been determined. The $\sigma 1s$ protein is required for reovirus-induced cell cycle arrest at the G₂/M boundary (Poggioli et al., 2002; Poggioli, Dermody and Tyler, 2001) and has been implicated in apoptosis *in vivo* (Hoyt et al., 2005). It is not known whether inhibition of cell cycle progression is related to the induction of apoptosis following reovirus infection. Cells respond to replication stress or DNA damage by activating checkpoints that arrest the cell cycle. For cells in which genomic damage cannot be repaired, apoptosis is induced to ensure that only faithfully replicated DNA is passed to daughter cells. The relationship between cell cycle arrest and apoptosis in the context of reovirus infection has not been examined. It is possible that $\sigma 1s$ -mediated cell cycle arrest contributes to reovirus-induced apoptosis. Interaction of $\sigma 1s$ with components of the host cell cycle machinery that inhibit normal cell cycle progression could cause the cell to undergo apoptosis.

It is not known whether $\sigma 1s$ -dependent cell cycle arrest and apoptosis are responsible for $\sigma 1s$ -mediated reovirus dissemination. It is possible that $\sigma 1s$ -dependent apoptosis in intestinal epithelial cells promotes reovirus uptake by phagocytic cells at the site of inoculation, and these cells in turn traffic virus to the bloodstream where the virus has access to JAM-A. Although $\sigma 1s$ is not required for reovirus growth in cultured cells (Boehme et al., 2011; Boehme, Guglielmi and Dermody, 2009; Rodgers et al., 1998), it is possible that $\sigma 1s$ is

necessary for efficient reovirus replication in specific cell types that are required for viral dissemination. Defining the cell types used by reovirus to spread through the blood may help uncover how σ 1s promotes hematogenous spread. Finally, σ 1s may mediate evasion of the host immune response, thereby allowing viral spread. Differences in viral dissemination between wild-type and σ 1s are evident at early times post-inoculation. This suggests that σ 1s would impact host innate immune mechanisms, as opposed to adaptive responses that develop at later times after infection. Determining how σ 1s promotes hematogenous reovirus spread is essential to understand how an enteric, neurotropic virus circuits from the intestine to the CNS.

C. Clinical implications

Defining factors that govern reovirus dissemination in the blood is essential for optimum use of reovirus in clinical applications. Reovirus efficiently replicates in and kills cancer cells (Adair et al., 2012; Karapanagiotou et al., 2012). Phase II and III clinical trials are underway to test the efficacy of reovirus as an adjunct to conventional cancer therapies (Adair et al., 2012; Karapanagiotou et al., 2012; Kottke et al., 2011). Following intravenous administration, reovirus must navigate and exit the bloodstream to infect solid organ tumors. Intratumoral injection of reovirus may allow for enhanced replication in tumor cells and subsequent spread through the blood to target metastatic tumor foci. Thus, determining viral and cellular determinants underlying how reoviruses gain access to the blood compartment, spread within the bloodstream, and exit from the circulation may aid in oncolytic design. Use of the reverse genetics system may allow engineering of reovirus therapeutics with mutations that increase vector potency or safety by manipulating dissemination determinants (Kobayashi et al., 2007).

We have uncovered a central role for hematogenous dissemination in reovirus neuropathogenesis and elucidated molecular mechanisms that govern reovirus spread by the blood. However, we have much more to learn. Understanding mechanisms of reovirus dissemination will provide broader insight into events at the pathogen-host interface that lead to systemic disease and may aid in the development of therapeutics that target this critical step in viral pathogenesis.

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Figure 1.

The reovirus virion. (A) Cryo-electron micrograph image reconstruction of a reovirus virion. Outer-capsid protein $\sigma 3$ (blue), is the initial target for virion disassembly in infected cells. Pentameric $\lambda 2$ protein (yellow) forms an insertion pedestal for $\sigma 1$, which is the viral attachment protein. Copyright © American Society for Microbiology, *Journal of Virology*, vol. 75, 2001, pages 146625–6634, doi: 10.1128/JVI.75.14.6625-6634.2001 (Nason et al., 2001). (B) Schematic of a reovirus virion. Reovirus particles are formed from two concentric protein shells, the outer capsid and core. The core contains the viral genome, which consists of ten dsRNA segments. Reovirus also encodes nonstructural proteins, σNS , μNS , μNSC , and $\sigma 1s$.

Figure 2. Structure of $\sigma 1$ and JAM-A

(A) Full-length model of attachment protein $\sigma 1$ bound to JAM-A. A model of full-length $\sigma 1$ extending from the virion is shown as a ribbon drawing, with the known structure of the C-terminus (Chappell et al., 2002) in tricolor and the predicted structure of the N-terminus in grey. Arrows indicate predicted regions of flexibility. A model of full-length JAM-A is shown in green as a ribbon drawing of the known structure of the extracellular domain (Prota et al., 2003) and a schematic representation of the transmembrane and intracellular domains. For clarity, only two JAM-A monomers are shown bound to $\sigma 1$. Adapted from Kirchner et al. (2008, Fig. 1). (B) Structure of human JAM-A D1 and D2 domains. Ribbon drawings of a JAM-A homodimer, with one monomer shown in yellow and the other in green. Two orthogonal views are displayed. Adapted from Prota *et al.* (2003). Copyright (2003) National Academy of Sciences, USA.

Figure 3. JAM-A is required for hematogenous reovirus dissemination

(A) Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁶ PFU of strain T1L. At days 4, 8, and 12 after inoculation, mice were euthanized, organs were resected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 6 animals for each time point. Error bars indicate SD. *, *P* < 0.005 by Student's *t* test. When all values are less than the limit of detection (spleen, liver, heart, and brain in *JAM-A*^{-/-} mice), a Student's *t* test *P* value cannot be calculated. (B) Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁴ PFU of strain T3SA-. At days 4, 8, and 12 after inoculation, mice were euthanized, organs were resected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 6–13 animals for each time point. Error bars indicate SD. *, *P* < 0.05 by Student's *t* test in comparison to *JAM-A*^{-/-} mice.¹

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Figure 4. JAM-A is required for reovirus viremia

Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁸ PFU of T1L. At days 1, 2, 4, and 6 after inoculation, mice were euthanized, mesenteric lymph node (MLN), blood, and spleen were collected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 3–8 animals for each time point. Error bars indicate SD.¹

Figure 5. JAM-A is required efficient reovirus infection of endothelial cells

JAMA^{+/+} and *JAM-A*^{-/-} primary endothelial cells were adsorbed with T1L or T3SA- at MOIs of 1, 10, and 100 PFU/cell and incubated for 20 h. The percentage of infected cells was quantified by dividing the number of cells exhibiting reovirus staining by the total number of cell nuclei exhibiting DAPI staining in whole 96 wells for triplicate experiments. Wells contained between 200 and 1600 nuclei. Error bars indicate SD. *, $P < 0.05$ as determined by Student's *t* test in comparison to *JAM-A*^{-/-} endothelial cells inoculated at the same MOI.¹

Figure 6. The σ 1s protein is required for systemic reovirus dissemination following peroral inoculation

Newborn C57/BL6 mice were inoculated perorally with 10^4 PFU of wild-type or σ 1s-null reovirus. At days 4, 8, and 12 post-inoculation, viral titers in the organs shown were determined by plaque assay. Error bars indicate SEM. *, $P < 0.05$ as determined by Mann-Whitney test in comparison to wild-type virus. When all values are less than the limit of detection, a Mann-Whitney test P value cannot be calculated. Adapted from Boehme *et al.* (2009). Copyright (2009) National Academy of Sciences, USA.

Figure 7.

The $\sigma 1s$ protein enhances reovirus virulence following intramuscular inoculation. (A) Newborn C57/BL6 mice were inoculated in the left hindlimb with 10^6 PFU of wild-type or $\sigma 1s$ -null T3 reovirus. Mice ($n = 19$ for each virus strain) were monitored for survival for 25 days. *, $P < 0.001$ as determined by log-rank test in comparison to wild-type T3 reovirus. (B) The $\sigma 1s$ protein is not required for reovirus spread by neural routes. Newborn C57/BL6 mice were inoculated in the left hindlimb with 10^6 PFU of wild-type or $\sigma 1s$ -null T3 reovirus. At days 1, 2, 4, 8, and 12 post-inoculation, mice were euthanized, hindlimb muscle, spinal cord, and brain were resected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 6–9 animals for each time point. Error bars indicate SEM. *, $P < 0.05$ as determined by Mann-Whitney test in comparison to wild-type T3 reovirus. Copyright © American Society for Microbiology, *Journal of Virology*, vol. 85, 2011, pages 11781–11790, doi: 10.1128/JVI.02289-10.

Figure 8. Reovirus disseminates to the CNS by hematogenous and neural routes

The left sciatic nerve of newborn C57/BL6 mice was sectioned prior to inoculation in the left hindlimb with 10^6 PFU of wild-type or $\sigma 1s$ -null T3 reovirus. In parallel, mice in which the left sciatic nerve was not sectioned were inoculated in the left hindlimb with 10^6 PFU of wild-type or $\sigma 1s$ -null T3 reovirus. At days 2 and 4 post-inoculation, mice were euthanized, (A) hindlimb muscle, spinal cord, and brain and (B) heart, intestine, liver, and spleen were resected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 6 animals for each time point. Error bars indicate SEM. *, $P < 0.05$ as determined by Mann-Whitney test in comparison to animals in which the sciatic nerve was not sectioned. Copyright © American Society for Microbiology, *Journal of Virology*, vol. 85, 2011, pages 11781–11790, doi: 10.1128/JVI.02289-10.

Figure 9. Model of reovirus hematogenous spread from the intestine

(1) Following peroral inoculation, reovirus infects intestinal epithelial cells (2) and is taken up by lymphoid cells in the Peyer patch. (3) Infected dendritic cells or lymphocytes carry reovirus from the Peyer patch through the lymphatics and finally to the blood. (4) Phagocytic cells that extend processes into the lumen of the intestine also might be infected for subsequent transport of virus through the lymphatics. (5) Reovirus may enter directly into the blood by passing between endothelial cells or via release into the bloodstream from infected cells.