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FACTORS INFLUENCING RNA VIRUS TROPISM

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Abstract

Human norovirus (HNV) is a positive sense RNA virus that is the most common cause of gastroenteritis worldwide. Despite active interest in pursuing vaccination and therapies, there are currently no well-established cell culture or animal models for studying this virus or other well-characterized noroviruses. Murine norovirus (MNV) is a distinct yet closely related norovirus that replicates and can be grown in well-characterized cell culture systems and can cause measurable morbidity and mortality in mice, and is thus a useful proxy for HNV study. Recent work with MNV identified a proteinaceous receptor, murine CD300LF, as a major determinant of susceptibility to MNV. Depletion of CD300LF from an otherwise susceptible host cell results in resistance to the virus and expression of the virus on many common otherwise MNV-resistant immortalized cell lines results in susceptibility. Importantly, initial studies into CD300LF focused on the C57BL/6J-like variant of the protein found in a variety of inbred mouse lines, though other variants exist. Inoculation of bone marrow-derived macrophages (BMDMs) taken from I/LnJ mice yielded no detectable virus, suggesting resistance to the virus. After expression of the C57BL/6J variant of CD300LF on the otherwise-resistant I/LnJ BMDMs resulted in detectable viral replication, we hypothesized that endogenous I/LnJ CD300LF was nonfunctional as an MNV receptor. Expression of C57BL/6J and I/LnJ variants of CD300LF on the macrophage-like cell line BV2 yielded the same infection phenotypes for each variant. Comparison of these variants with a third variant, CAST/EiJ, led to the identification of a four amino acid cluster that differs between the original two variants that underlies their phenotype. Surprisingly, expression of either of these variants on other immortalized cell lines results in susceptibility to MNV, suggesting the existence of other determinants of CD300LF-mediated susceptibility to MNV.

Influenza A virus (IAV) is a negative sense RNA virus that results in significant mortality worldwide each year from seasonal epidemics. Several known strains of IAV also pose the risk of becoming a pandemic threat via genomic reassortment in nonhuman hosts, often with significantly increased rates of mortality in exposed individuals. As attempts to create universal IAV vaccines have been unsuccessful, it becomes critical to understand the pathogenesis of pandemic strains to inform potential therapies to ensure widespread availability before such a pandemic. To this end, studies into H5N1, a strain of highly pathogenic avian influenza (HPAI) considered to be among the most dangerous have identified several key factors, including endothelial cell tropism as critical for pathogenesis. We attempted to expand the understanding of HPAI pathogenesis by probing the contribution of endothelial cell tropism to the virulence and pathogenesis of H7N7, another virulent HPAI strain with the capacity to infect humans. Introduction of sequences complementary to tissue-specific microRNA (miRNA) into H7N7 mRNA restricting replication in endothelial cells and monocyte derived cells do not indicate a major role for such tissue tropism, indicating distinct pathogenesis from H5N1. These investigations into the role of host factors for RNA viruses underscore the notion that viral replication cannot take place without contributions from a host and indicate the importance of understanding the interplay between host and pathogen.

Chapter I: Introduction

Overview

Viruses are causative agents of some of the most catastrophic life loss in human history. Worldwide pandemics including the past 1918 Spanish flu up to and including the current SARS-COV-2 pandemics have fundamentally altered the manner in which many consider the value of human life. As a result, the discovery and subsequent study of viruses as both unique forms of life and causative agents of disease has led not only to the preservation of human life, but also to the prevention of suffering and easing of symptoms for countless individuals. Diseases caused by viral infection can vary as much as the viruses themselves and as a result, their impact on the hosts they infect can also vary; some infections may be entirely asymptomatic with no detectible effect on the host, some infections may lead to mild disease, and some may seriously threaten the life of the host. The study of viruses with more serious potential impact on humans has the capacity to immediately prevent the most suffering.

A major determining factor governing the ability of a virus to cause such suffering is its ability to infect various host cells. Defined by the combination of permissiveness, or the capacity of a host cell to support the replication cycle of a virus completely, and susceptibility, the state of expressing necessary receptors for viral entry, viral tropism can underlie the most critical facets of infection. As the expression of the factors required for viral entry into host cells and/or replication within those host cells can be restricted at any level including species and host tissue, understanding the cells a virus could potentially infect can inform understanding of pathogenesis on both a micro and macro scale. The understanding that host factors required to support viral replication exist in other nonhuman animals can be used for any number of purposes, including

developing laboratory *in vitro* systems to study viruses in a controlled setting to quickly identifying diseases afflicting susceptible animals and controlling outbreak. Such understanding has a continuous impact on worldwide economies, with a common example being influenza virus infection in domestic poultry, which are regularly checked for influenza virus infection due to their capacity to support replication of the virus absent symptoms or human hosts. Protection of workers and other poultry farms by culling thousands of animals due to the detection of influenza infection is not uncommon, with predictably severe economic impact (1, 2).

Viral pathogenesis within a host is also broadly determined by tissue tropism within the host for many viruses. Indeed, the disease state inflicted upon a host due to viral replication is often easily understood upon consideration of susceptible host cells. An example of this include Human Immunodeficiency Virus (HIV) resulting in acquired immunodeficiency syndrome (AIDS) caused in part by the requirement of HIV to infect cells expressing CD4, a glycoprotein whose expression is restricted to tissue types involved in the immune response (3-5). Herein, I will probe the role of two unique host factors in the replication and virulence of two unique viruses, seeking to understand the role of each of them in conferring susceptibility and contributing to virulence of their respective hosts. Specifically, I will examine the role of CD300LF in conferring susceptibility to murine norovirus in a variety of host cells and potential molecular determinants of such susceptibility. I will also examine the role of endothelial cell tropism in the pathogenesis and virulence of H7N7, a highly pathogenic strain of avian influenza virus.

Introduction to human norovirus

Human norovirus (HNV), originally known as the "Norwalk agent" due to an outbreak in Norwalk, Ohio is currently the leading cause of gastroenteritis worldwide (6, 7). HNV is a member of the *Calciviridae* family and encodes a single-stranded, positive sense RNA genome (8, 9). Translation of the HNV genome yields a large polyprotein that is subsequently cleaved into two structural proteins and six nonstructural proteins (8, 9). While the basic virology of HNV has been characterized, further in-depth study of the virus has historically been challenging due to the lack of robust, adaptable model systems both *in vitro* and *in vivo*. Despite early evidence that HNV RNA is infectious in mammalian cell culture, subsequent steps to simplify research including growing significant quantities of virus and measuring multicycle replication have proven challenging (10). Challenges in growth of the virus have included low infectious titer and interference in titer confirmation from helper viruses (10-14). More recent attempts using plasmid and replicon-based approaches have been nominally more successful in stable production of viral RNA and capsid protein, though the infectivity of potential progeny virions is at issue due to the lack of susceptible mammalian cells (9, 11). Similarly, attempts have been made to establish a small animal model for HNV infection, which have resulted in varying degrees of success. A model employing an immunocompromised BALB/c line has shown an ability to support HNV replication, but has yet to be adopted by many groups (15). Despite the clear and present concern caused by HNV, the laboratory models required for efficient testing *in vitro* and *in vivo* do not yet exist.

Introduction to murine norovirus and CD300LF

Despite these challenges in studying HNV and other noroviruses directly, useful surrogates have allowed for the advancement of norovirus research. One such surrogate is murine norovirus (MNV) a strain of norovirus closely related to HNV first discovered in 2003 as a pervasive, lethal agent in immunocompromised ($Rag2^{-/-}$, $STAT1^{-/-}$) mice (16). Isolation and sequencing of MNV, followed by maximum parsimony analysis of the major viral capsid protein (VP1) of MNV has identified it as a member of the norovirus genus, but genetically distinct from other, non-murine noroviruses (16, 17). Importantly, MNV is unique among noroviruses in that it is the only member currently known to infect and replicate in model *in vitro* systems, making rapid growth and study of the virus possible (16, 17). Not only can MNV replicate in easily manipulated cell culture systems, but initial isolation of MNV from laboratory mice suggests that the virus can be studied in well-characterized small animal models (16). Indeed, MNV proteins and RNA can be detected in WT animals post-inoculation and lethal infection is easily achieved in immunocompromised animals (16). The importance of these observations cannot be overstated, especially when compared to the tools that currently exist for other noroviruses. Models of infection for porcine and bovine noroviruses in their respective hosts exist, but are currently limited by a lack of a tissue culture model and are thus restricted in application in a manner similar to HNV (16-18). Further, advances in manipulation of the murine genome itself allow for sophisticated analyses of the role of host factors in viral replication. The lack of such tractability in tissue culture and animal models for other noroviruses limits their study in ways that do not inhibit MNV. Initial work into the establishment of an *in vitro* system for MNV showed strong, multicycle replication in macrophage and macrophage-like systems (16). Bone marrow derived macrophages (BMDMs), bone marrow derived stem cells that are differentiated

into macrophages via exposure to growth factors, are a common host for several other viruses. As BMDMs can be harvested from murine models of any genetic background, they are a useful tool for studying the role of host factors without having to infect whole animals.

Both BMDMs and well-characterized immortalized macrophage-like systems including RAW 264.7 and BV2 cells support replication of MNV and recent studies employing these systems has contributed to the rapid understanding of the MNV life cycle (16, 19). Among the most significant contributions to the understanding of MNV was the discovery of CD300LF as the major receptor for and determinant of susceptibility to MNV (20, 21). Also known as CLM-1, CMRF35, MAIR-V, and LMIR3, CD300LF was first characterized as a member of the multigene CD300 family as an inhibitory factor of osteoclast differentiation (22). Two independent, unbiased studies seeking to identify host factors that contribute to MNV infection identified murine CD300LF (henceforth CD300LF) as indispensable for MNV infection (20, 21). These and following studies have since shown that CD300LF contributes to attachment and internalization of the virus into host cells and that loss of CD300LF expression is sufficient to block infection of these cells by MNV (20, 21, 23). CD300LF expression is most abundant in cells of the myeloid lineage (22). CD300LF expression is not limited to mice and expression of human CD300LF is detectable in human myeloid cells (22). Despite their similarities, studies into human CD300LF (hCD300LF) have shown that it cannot serve as an entry factor for MNV, making human cells immune to MNV infection *in vitro (20, 24)*. This is likely the primary reason that humans are not susceptible to MNV infection. Surprisingly, lack of expression of murine CD300LF seems to be the only barrier preventing cells that are not susceptible to MNV infection from becoming so. Indeed, cells that are genetically distinct from murine myeloid cells can be made susceptible to MNV infection through stable expression of some alleles of

CD300LF (20). The ability to confer susceptibility to otherwise resistant cells in a CD300LF expression-dependent manner is not limited to other murine-derived cells, as human epithelial cells expressing murine CD300LF are also susceptible, suggesting that intracellular factors required to support MNV replication other than CD300LF are likely not unique to individual cell types (20).

The replication cycle of MNV is reminiscent of other *Calciviridae* and other small, positive sense, single stranded RNA viruses. After internalization of the virion via interaction with CD300LF, early proteins packaged within the virion and early products of translation of the positive sense genome result in reshaping of the host cell's internal membrane structure and the formation of replication complexes to house continued genomic and subgenomic RNA products (16, 17). Continued translation of the first, second, and third open reading frames of the MNV genome results in the production of a large polyprotein comprising six nonstructural proteins (NS1/2-NS7), the structure viral capsid protein VP1, and a smaller structural protein VP2 (16, 17). Recent work has identified a fourth open reading frame, which produces a small, mitochondria-localized protein that confers a fitness advantage but is dispensable for MNV replication, VF1 (25). Following membrane remodeling and compartmentalization within a replication complex, the genome is replicated and the virus lyses the host cell (18).

MNV host cell tropism is an area of active study due in part to its ability to inform understanding of the pathogenesis of HNV. Initial characterization of MNV infection in immunocompromised ($STAT1^{-/-}$) yielded detectable virus in a wide variety of tissues, including lung, liver, spleen, proximal intestine, and brain (16). Staining of mice infected with MNV-containing brain homogenates shows virus-specific staining in liver and spleen-adjacent cells, with resident macrophage and dendritic cells showing clear staining (18). Despite this, staining

of inoculated murine hepatocytes *in vitro* shows no obvious staining, suggesting that macrophages and dendritic cells themselves may be among the most susceptible to MNV infection (17, 18). Further study into MNV replication *in vitro* reveals that many cells of hematopoietic lineage support MNV replication (18). This is especially interesting given the capacity for both MNV and HNV to infect the proximal colon of their respective hosts, suggesting that while colonic epithelial cells may be susceptible to norovirus infection, they may not be the only or indeed primary site of replication. Subsequent *in vivo* studies of MNV have suggested that Tuft cells, a unique cell type known to populate the intestinal epithelium in mice may contribute to the overall viral load of infected hosts, further complicating understanding of viral pathogenesis (26). Infections in immunocompetent neonates also show susceptibility in a range of different cell types and produces gastrointestinal pathology similar to HNV (Karst, unpublished). While it is clear that intestinal epithelial cells and macrophages both support MNV replication *in vitro* and *in vivo*, the understanding of MNV viral tropism and its contribution to pathogenesis remains poorly understood.

Introduction to influenza virus

Influenza virus is the causative agent for the disease influenza – a common respiratory illness worldwide. Influenza viruses themselves are members of the *Orthomyxoviridae* family, lipidated virions encoding a negative sense RNA genome comprised of 8 segments encoding 10 viral proteins. Replication of the virus begins with the viral glycoprotein hemagglutinin (HA) engaging sialic acid residues on the host cell membrane, triggering attachment of the virion and eventual clathrin-mediated endocytosis or macropinocytosis of the entire lipidated virion (27-29). The lipidated-virion containing endosome is then acidified, resulting in a conformation

change in the virion and eventual fusion of the viral membrane with the endosome, cleavage of the HA protein, and release of the vRNP into the cytoplasm of the host cell, where the viral genome is imported into the host nucleus (30-34). From here, the genome is replicated and the mRNA is transcribed, primed by hijacking the host 5' cap machinery, increasing efficiency (35, 36). Subsequent translation of the viral mRNA and packaging of an individual copy of each segment of the genome aided by the packaging signal in each segment of the genome results in new vRNPs which are enveloped and cleaved from the host membrane via the viral neuraminidase (NA) glycoprotein, releasing infectious virions (37-39).

Influenza viruses constitute four of the seven genera that comprise the *Orthomyxoviridae* family. These genera, known as influenza A virus, influenza B virus, influenza C virus, and influenza D virus are divided principally on their genome structure and primary host species. Of these four genera, influenza D virus is not known to infect humans (40, 41). Influenza A, B, and occasionally C can infect humans, though epidemics are most often caused by influenza A viruses, influenza B viruses to a lesser extent, and rarely influenza C viruses (42). Seasonal epidemics of influenza results in millions of hospitalizations per year, with an estimated 389,000 deaths on average per year between 2002 and 2011 worldwide, the majority of which are caused by influenza A virus (IAV) infection (43). In addition to seasonal epidemics, pandemic strains including the 1918 Spanish flu and the 2009 Swine flu present an unpredictable threat to human health. Despite this, efforts at long-term, universal vaccination against IAV have heretofore been unsuccessful, due in part to the constantly changing nature of the virus. As with other RNA viruses, the IAV polymerase imperfectly replicates the genome, resulting in accumulation of non-deleterious mutations over time in a process known as antigenic drift (44). Additionally, segmentation of the genome affords influenza the unique ability to exchange similar segments of

its genome with other IAV strains in the case of coinfection. This process, known as antigenic shift, can result in novel IAV subtypes to which the majority of the population is immunologically naïve (45). Together, these processes make development of effective prophylactics for IAV challenging.

Discussion of individual strains and resistance to IAV typically consider two of the influenza glycoproteins, which have historically been the major target for neutralizing antibodies. These proteins, hemagglutinin (HA, H) and neuraminidase (NA, N) are primarily responsible for entry and egress from host cells respectively and tend to be associated with the virulence of an individual strain (46). As such, certain HA and NA types are associated with more virulent strains of the virus. To date, there are 18 known HA subtypes and 11 known NA subtypes (47). Of these HA subtypes, humans are known to be susceptible to viruses with at least eight different segments while others circulate within other animal hosts (48). Indeed, influenza viruses are capable of infecting a wide range of different host types, with several subtypes known to be restricted to such nonhuman hosts. Water fowl serve as a reservoir for the majority of these subtypes, with evidence of subtypes H1-H16 all found within them while H17 and H18 are currently thought to be restricted to bats (48-50). An unfortunate consequence of water fowl and other animals supporting replication of such a wide array of different influenza subtypes is the ability for different viruses to coinfect an individual and produce new strains of virus via antigenic shift undetected. From here, interaction with domestic poultry can result in widespread disease, resulting in the culling of thousands of animals and severe economic loss (1).

Continued passage of IAV in non-human hosts can result in strains that pose significant danger to humans. Occasionally, novel viruses passed from water fowl to domestic poultry can be passed to nearby humans. These viruses, known as avian influenza viruses after their original

hosts, tend to be highly virulent in humans hosts, have high mortality rates, and are among those considered to be the most potent potential pandemic threats (51, 52). One such avian virus, H7N9, is currently spreading rapidly and poses significant threat to human life in China and is currently considered to have the greatest potential to cause a pandemic, lacking only the capacity for efficient airborne transmission among potential hosts, including humans (53, 54). Other avian influenza viruses including H5N1 and H7N7 have demonstrated the ability to move from domestic poultry to humans and threaten to become the next pandemic strain (52, 55-57). Influenza virus is among the most potential infectious diseases currently known and although studies thus far have greatly enhanced our understanding of the virus, we lack the ability to reliably protect the human population at large from predictable seasonal epidemics and are relatively unprepared for a coming pandemic.

The pathogenesis of influenza virus depends heavily on both viral virulence factors as well as host factors and thus can differ between hosts and strains of the virus. A key factor in viral tropism of potential hosts is the availability of host sialic acid moieties with specific linkage patterns. Current seasonal epidemic strains of IAV nearly exclusively bind to cells expressing α2,6 sialic acid linkages, which are most commonly found on epithelial cells in the upper respiratory tract of humans, informing the potential disease state of the virus (58, 59). The requirement of α2,6 linked sialic acids restricts epidemic IAV strains to replication in the upper respiratory tract of humans, thus restricting spread of the virus and ultimately resulting in a mild disease. Importantly, these viruses replicate to high titers in the upper respiratory tract, contributing to the ease of spread of the virus. To facilitate viral membrane fusion with the host endosomal membrane and begin translocation of the IAV genome to the host nucleus for transcription and replication, the viral HA protein must be cleaved at a specific site by a host

protease (31, 32). Seasonal epidemic strains of IAV encode a single basic amino acid at this HA cleavage site, resulting in a motif that can only be recognized by trypsin-like proteases in humans (60, 61). These proteases are largely restricted to the respiratory tract of humans, further informing the tropism of the virus and the relatively mild disease state as a result. While it has been shown that mutations in many different IAV proteins can result in a change in virulence, restriction to the upper respiratory tract through the requirement of α2, 6 sialic acid linkages for attachment and trypsin-like proteases for vRNP release separate epidemic and pandemic IAV pathogenesis (62-68).

Pathogenesis of pandemic and pandemic-potential IAV differs from that of non-pandemic strains due in part to slightly altered host cell tropism. While seasonal epidemic strains of IAV require α2,6 sialic acid linkages, more virulent strains of the virus can bind either preferentially or entirely to α2, 3 linkages, which are expressed primarily on the epithelial cells of the alveoli in the lower respiratory tract in humans (58, 59, 69). As cells expressing α2, 3 linkages are fewer in number and deeper within the human respiratory tract compared to those expressing α2, 6 linkages, viruses with a strong preference for binding α2,3 linked sialic acid moieties have differing pathogenesis and virulence (46, 69). This phenomenon is likely due in part to a combination of the requirement to translocate to the lower respiratory tract of a new potential host and the lack of active replication in the upper respiratory tract resulting in fewer aerosolized viral particles being spread via nasal discharge. Indeed, H5N1, an avian IAV strain with a strong preference for α2, 3 sialic acid moieties, is known to spread poorly among humans (69). Mutations have also been identified in the H1N1 strain causative of the 1918 Spanish flu pandemic in which a preference for α2, 6 binding becomes a preference for α2, 3, resulting in a loss in transmissibility (70). Similarly, the required cleavage of HA to achieve uncoating is

common to both seasonal and pandemic strains, though the host protease capable of cleavage differs. Specifically, HPAI strains have a sequence of multiple basic amino acids at the cleavage site, which can be recognized by furin-like proteases (71). Unlike trypsin-like proteases which are restricted to the respiratory tract of humans, furin-like proteases are found in the trans-Golgi network and are thus ubiquitous in humans.

The differences in host tropism determined by HA cleavage and host cell binding inform the pathogenesis and virulence of IAV strains. The restriction of seasonal epidemic strains to the upper respiratory tract and requirement for trypsin-like proteases precludes infection of non-pulmonary cells. Additionally, preference for α2, 6 sialic acid moieties results in spatial separation from the lower respiratory tract of humans, often resulting in comparatively less damage to the lower lung that can result in pneumonia. HPAI and other more virulent strains of IAV differ in these aspects, with predictable implications for the disease they cause. H5N1 and other HPAI replication in the lower respiratory tract due to a preference for α2, 3 sialic acid moieties are associated with more damage to the lower lung and a corresponding increase in pneumonia (72). The ability to uncoat without the requirement for trypsin-like proteases affords HPAI strains the ability to replicate in different cell types, potentially resulting in a devastating systemic infection, neurological symptoms, multiple organ failure, and death (73). Mutations within several IAV proteins have been associated with differential pathogenesis and virulence in some capacity, but the expression of these host factors and resulting tropism are major determinants of the virulence of IAV.

miRNA and RISC

As both norovirus and influenza represent major threats to human health, it is important to consider potential mechanisms by which cells may combat infection. Among the most potent mediators of infection is a group of protein-RNA complexes known as RNA-induced silencing complexes (RISC) - a family of "programmable" complexes that is capable of repressing expression of a wide variety of nucleic acids, including viral transcripts. While the compositions of all complexes designated as "RISC" are not identical, each is comprised of at least a member of the Argonaute protein family and a small guide RNA (74). Importantly, an Argonaute protein and a small RNA alone has been shown to be a functional RISC for repression of gene expression, though the Argonaute protein in the complex may have other molecular binding partners, resulting in a larger protein complex unit of RISC (74-79). The general function of eukaryotic RISC is to find and identify RNA that is complementary to the small guide RNA incorporated into the complex and restrict expression of the encoded protein through several different means, including mRNA degradation and translational repression (80). Eukaryotic Argonaute proteins are divided into three distinct groups based on amino acid sequence (81). Only two of these groups, designated Argonaute (AGO) and Piwi, are found in humans and differ based on the manner in which they acquire small guide RNA and to a lesser extent the manner in which they repress gene expression (80, 82). The simplest result of RISC interacting with RNA with which its guide RNA has near-perfect sequence complementarity is mRNA degradation via hydrolysis. Such degradation requires nuclease activity from the Argonaute protein in the complex, which is restricted to the AGO2 protein of the Argonaute subfamily of Argonaute proteins (83). As such, mRNA degradation is a rarer form of gene repression mediated by RISC, especially compared to translational repression and mRNA decay, which is most commonly guided by a specific subfamily of small guide RNAs known as microRNAs

(miRNAs) (80). miRNAs are short, noncoding RNAs about 22 nucleotides in length generated through processing of pri-miRNAs that exhibit cell type- specific expression (84). Repression of translation requires comparatively less sequence complementarity between small guide RNAs and the targets than does mRNA hydrolysis and is estimated to account for between 66-90% of all gene silencing (84-86). Between mRNA hydrolysis, translational repression, and mRNA decay, RISC-induced gene repression is thought to control the expression of approximately one-third of all human genes.

In addition to its endogenous use of controlling expression of host genes, the RISC system can also be exploited to control expression of exogenous nucleic acids. Cytoplasmic dsRNA from a variety of different pathogens, including viruses, can be cleaved by the host-encoded Dicer protein, resulting in RNA fragments of suitable length for incorporation into RISC (87, 88). Harvesting of pathogen-specific RNA in this manner allows host RISC to cleave any remaining cytoplasmic RNA, thus contributing to the restriction of replication of the pathogen. Importantly, while miRNA target sequences are typically located within the UTR of target mRNA, RISC containing guide RNAs are able to inhibit translation of any sequence with which they are complementary. A result of this relative disregard for RNA source is the ability for cells to inhibit translation of recombinant RNA engineered to include sequences of RNA that are complementary to miRNA known to be expressed in those cells, with little to no effect on replication within cells that do not express a complementary miRNA. As miRNA expression can vary based on a number of factors including tissue type, host species, and period of development, significant insight can be gained via insertion of such target sites into otherwise isogenic pathogens. Indeed, this exploitation of cellular control of gene expression at the level of mRNA has been used to deepen understanding about the nature of host-viral interactions for several

important human pathogens, including polio, IAV, and several flaviviruses (89-93). Despite only being characterized within the past 15 years, the use of cellular RNA-silencing machinery to uncover meaningful relationships between host and pathogen has increased considerably and has proven to be an invaluable resource in such studies.

For these reasons, I have employed the use of miRNA-targeting to probe potential requirements for host cell tropism for H7N7 replication in a mouse model. Work into particularly virulent strains of IAV have indicated that the ability to infect and replicate within certain cell types may contribute to the pathogenesis of the virus (94). The loss of replication in monocyte-derived cells and endothelial cells corresponding with decreased inflammatory cytokine production and reduced virulence respectively give insight into the proximal and ultimate results of H5N1 infection (94). In order to understand the factors that may underlie the increased virulence of all HPAI rather than just individual ones, I have restricted the replication of H7N7, a highly virulent HPAI, in both monocyte-derived and endothelial cells. The advantages in this approach lie in the ability to work with a WT host and isogenic strains of the H7N7 virus, differing only in their inclusion of miRNA target sites. The ability to work with the same host and an isogenic virus can allow for a clearer picture of the direct effects of viral replication within a single cell type without the need to consider the side effects of other approaches, including creating knockout mice to use as models.

Host factors and RNA viruses

Since the inception of germ theory and the eventual characterization of viruses as agents that have been causative of some of the most well-studied diseases in human history, the study of viral infection and pathogenesis has been evolving. Early studies of infectious bacteria and

eventually viruses illustrate the basic capacity of these pathogens to infect and cause disease, while further work would more explicitly explore the mechanisms by which these diseases progress. As work continues into the exact causes of disease resulting from infection continues, it becomes clear that the nature of the host and its response to infection contribute significantly to the development of resulting disease. Thus, it becomes necessary to consider infectious diseases not only as a result of exposure to a pathogen, but as a result of the interaction between an infectious agent and the response of the host. Through this lens, the role of the host as a mechanism for the progression of disease can be considered. Viruses are able to utilize a variety of different factors in order to replicate and disseminate throughout a host. While some are virally encoded virulence factors, others are exclusively host-encoded. This relationship between pathogen, immune system capable of detecting and controlling the pathogen, and evasion of the immune system by the pathogen indicates a degree of coevolution between the two. As such, the study of expression and function of host factors contributes significantly to the understanding of disease progression. The potential for host factors to play indispensable roles in viral replication is well established, perhaps most easily illustrated examples in the requirement of many viruses to engage a host receptor in order to enter a host cell. For many viruses, the removal of a receptor may result in an otherwise susceptible host becoming completely immune to infection. Conversely, the addition of some entry factors to resistant cells may render them susceptible to infection. Viruses may also exploit a host's circulatory system as a means of establishing infection in several different locations or as a means to translocate to lymph nodes and combat its own detection. The reliance of a virus on its host for a range of different effects is well-characterized and illustrates the need for a complex understanding of the interplay between pathogen and host.

The relationship between host factors, host cell tropism, and viral pathogenesis is complex. Thorough understanding of the relationship is critical for gaining a clear picture of the viral replication cycle and thus understanding potential targets for therapeutics. The studies presented here are an effort to further understand the relationship between host factors and viral pathogenesis for two important human pathogen types: HPAI by way of H7N7 and noroviruses by way of MNV. I will probe the role of different variants of CD300LF in conferring susceptibility to MNV in a range of different cell types to understand potential host cell types. I will demonstrate that CD300LF from two distinct inbred mouse strains function differently in their ability to confer MNV susceptibility. I will show that this difference in conference of susceptibility in macrophage-like cells is due to a four amino acid polymorphism in the CC' loop region of CD300LF and that in contexts where one variant of the protein is nonfunctional, the I/LnJ encoded variant, functionality can be restored upon mutation of this region. Further, I will show that both variants of CD300LF can confer susceptibility to different cell types, suggesting the existence of cell type-specific modifiers of CD300LF function. I will also show that restriction of H7N7 replication in endothelial cells and monocytes has little effect on virulence. This lack of requirement for endothelial cell tropism is at odds with published results for H5N1 pathogenesis, suggesting divergent pathogenesis between the two closely-related HPAI strains.

Chapter II: CD300LF polymorphisms of inbred mouse strains confer resistance to murine norovirus infection in a cell type-dependent manner

The following chapter was submitted for publication to the Journal of Virology, excluding discussion of *in vivo* MNV infections in I/LnJ mice.

The authors were: Kevin Furlong, Scott B. Biering, Jayoung Choi, Craig B. Wilen, Robert C. Orchard, Christiane E. Wobus, Christopher A. Nelson, Daved H. Fremont, Megan T. Baldridge, Glenn Randall, and Seungmin Hwang.

Scott B. Biering conducted initial growth curve analysis of MNV, EMCV, MHV-68 in BMDMs. Scott B. Biering also transduced I/LnJ BMDMs with C57BL/6J CD300LF and produced the associated Western blot. Jayoung Choi conducted growth curve analysis of CW3, CW1, CR3, and RVSS in BMDMs. All other experiments were conducted by Kevin Furlong.

Abstract

Human norovirus is the leading cause of gastroenteritis worldwide, yet basic questions about its life cycle remain unanswered due to an historical lack of robust experimental systems. Recent studies on the closely related murine norovirus (MNV) have identified CD300LF as an indispensable entry factor for MNV. We compared MNV susceptibility of cells from different mouse strains and identified polymorphisms in murine CD300LF, which are critical for its function as an MNV receptor. Bone marrow-derived macrophages (BMDMs) from

I/LnJ mice were resistant to infection from multiple MNV strains, which readily infect BMDMs from C57BL/6J mice. The resistance of I/LnJ BMDMs was specific to MNV, since the cells supported infection of other viruses comparably to C57BL/6J BMDMs. Transduction of I/LnJ BMDMs with C57BL/6J CD300LF made the cells permissible to MNV infection, suggesting that the cause of resistance lies in the entry step of MNV infection. In fact, we mapped this phenotype to a 4 amino acid difference at the CC' loop of CD300LF; swapping of these amino acids between C57BL/6J and I/LnJ CD300LF proteins made the mutant C57BL/6J CD300LF functionally impaired and the corresponding mutant of I/LnJ CD300LF functional as an MNV entry factor. Surprisingly, expression of the I/LnJ CD300LF in other cell types made the cells infectible by MNV, even though the I/LnJ allele did not function as an MNV receptor in macrophage-like cells. Correspondingly, I/LnJ CD300LF bound MNV virions significantly in immortalized human B cells but not in macrophage-like cells. Collectively, our data suggest the existence of a cell-type specific modifier of MNV entry.

Importance

MNV is a prevalent model system for studying human norovirus–the leading cause of gastroenteritis worldwide and thus a sizeable public health burden. Elucidating mechanisms underlying susceptibility of host cells to MNV infection can lead to insights on the roles that specific cell types play during norovirus pathogenesis. Here, we show that different alleles of the proteinaceous receptor for MNV, CD300LF, function in a cell type-dependent manner. In contrast to the C57BL/6J allele that functions as an MNV entry factor in all tested cell types, including human cells, I/LnJ CD300LF does not function as an MNV entry factor in macrophage-like cells

but does allow MNV entry in other cell types. Together, these observations indicate the existence of cell-type specific modifiers of CD300LF-dependent MNV entry.

Introduction

Human norovirus (HNV) is a non-enveloped, positive-sense RNA virus of the *Caliciviridae* family, which is the leading cause of acute gastroenteritis worldwide (95-97). Despite its significant public health burden, a complete understanding of the host factors controlling the life cycle of HNV is still lacking. Currently, there are few *in vitro* models that support replication and detection of HNV, making it a difficult pathogen to study directly, though these systems are rapidly improving (18, 95, 98-100). Murine norovirus (MNV) is a genetically similar virus discovered in 2003 as a lethal agent in *Rag2*$^{-/-}$ *Stat1*$^{-/-}$ mice, which has since been used as a model virus to study HNV biology (16). Unlike HNV, MNV replicates robustly in several macrophage-like cell lines, including BV2 and RAW264.7 (20, 21).

Several studies have identified a wide range of host factors that modulate norovirus attachment and entry, including histo-blood group antigens (HBGAs), bile acids, sialic acid, and divalent cations (20, 23, 101-103). While these attachment factors have been shown to enhance attachment for several different noroviruses, none of them are required for MNV infection. In contrast, recent clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 screens uncovered CD300LF, a type I integral membrane protein containing a single immunoglobulin-like domain, as an indispensable host factor for MNV attachment and entry (20, 21, 24). In fact, expression of murine CD300LF alone was sufficient to confer MNV susceptibility to otherwise resistant host cells, including those from other species, like human 293T and HeLa cells (20, 21). Additional receptor molecules and attachment factors have been identified for closely related

members of the *Caliciviridae* family, including Feline Junctional Adhesion Molecule A (fJAM-A) as the receptor for Feline Calicivirus, which has been used historically as a surrogate for HNV (104-111). As understanding the mechanisms by which viruses enter susceptible host cells is integral to understanding the viral life cycle, recent studies on MNV entry have significantly advanced our understanding of norovirus biology (20, 21, 24, 26, 112, 113). Nevertheless, the modulation of norovirus entry factors and their mode of interaction with the viruses are still unclear, and it remains to be determined how these factors underlie norovirus host cell tropism.

The study of how genetically divergent hosts respond to viral infections can reveal the importance of host genetic factors, which may not be evident when using a single strain (114). With many cellular factors influencing norovirus infection, we asked if hosts from different genetic backgrounds might have different susceptibility to MNV. Variation exists in the protein sequences of different mouse strains, and these polymorphisms can help elucidate the functions of certain proteins. Here, we show that bone marrow-derived macrophages (BMDMs) from two different mouse strains have dramatically different susceptibilities to MNV infection. We found that these different susceptibilities are primarily due to divergence in the CC'-loop domain of CD300LF, which is essential for its function as an MNV receptor (20). Surprisingly, the CD300LF variant that cannot function as an MNV receptor in macrophage-like cells is able to bind MNV virions and is functional as an MNV receptor in different cell types. These data suggest the existence of cell type-specific modifiers of CD300LF-MNV interactions during viral entry.

Materials and Methods

Cloning

CD300LF cDNAs of C57BL/6J and I/LnJ mouse strains were synthesized based on public sequence database (http://www.ensembl.org and http://www.informatics.jax.org) and cloned into pCDH-MCS-T2A-copGFP-MSCV (System Biosciences CD523A-1) and pLenti-CMV-Puro-DEST (w118-1, Addgene plasmid #17452). To add HA-epitope at the N-term of CD300LF protein after cleavage of signal peptide, each *Cd300lf* sequences were modified as follows: C57BL/6J, …ACGGCTtacccatacgatgttccagattacgctGAGGAT… (…TAypydvpdyaED…); I/LnJ, …ACGGCTtacccatacgatgttccagattacgctCAGGAT… (…TAypydvpdyaQD…). Lowercase letters indicate the nucleotide and amino acid sequences of inserted HA-tag, respectively. Specific amino acids swapping mutants (B6/CD300LF - 7aa, IL/CD300LF - 7aa, B6/CD300LF - 4aa, and IL/CD300LF - 4aa) were also synthesized and cloned into pLenti-CMV-Puro-DEST (w118-1). The swapping mutants of potential glycosylation sites (B6/CD300LF S174G, B6/CD300LF T131A, IL/CD300LF G174S, and IL/CD300LF A131T) were generated using QuikChange XL Site-Directed Mutagenesis kit (Agilent) according to the manufacturer's instruction. The mutated sequences are as follows: C57BL/6J CD300LF 7aa mutant, …CAAGGAGTTCCTCAGAGATCATGT… > …CAAGGAGcTtaTtgGAaATCATGT… (…QGVPQRSC… > …QGaywkSC…), …AAAGTTACTGTGAAC… > …AAAGTTgCTGTGAAC… (…KVTVN… > …KVaVN…), …CTGACTAGCTACTAC… > …CTGACTgGCTACTAC… (…LTSYY… > …LTgYY…), …GCCATGCCT > …GCCATGcaT (…AMP > …AMh); I/LnJ C300LF 7aa mutant, …CGAGGAGCTTATTGGAAATCATGT… > …CGAGGAGtTccTcaGAgATCATGT… (…RGAYWKSC… > …RGvpqrSC…), …AAAGTTGCTGTGAAC… > …AAAGTTaCTGTGAAC… (…KVAVN… > …KVtVN…), …CTGACCGGCTACTAC… >

…CTGACCtcCTACTAC… (…LTGYY… > …LTsYY…), …GCCATGCAT > …GCCATGCcT (…AMH > …AMp). Lowercase letters indicate the nucleotide and amino acid sequences of the mutated, respectively; The sequence of all constructs was checked and confirmed upon cloning via sequencing.

Cells

BMDMs were prepared as described previously (115) from mice provided by Dr. Tatyana Golovkina at The University of Chicago. Briefly, bone marrows were isolated from femurs and tibias of 6-8-week-old mice, plated in non-tissue culture treated dishes in 10 mL of BMDM media. Cells were supplemented with fresh media on day 4 and seeded for experiments on day 7. Wildtype (WT) and *Cd300lf*$^{-/-}$ BV2 cells, mouse embryonic fibroblasts (MEFs), and 293T cells were provided by Dr. Herbert "Skip" Virgin (Washington University in St. Louis). HeLa cells were purchased from ATCC. Jurkat (JRT3.5) and BJAB cells were provided by Dr. Erin Adams (The University of Chicago) and Dr. Stephanie Karst (University of Florida), respectively.

Viruses

MNV-1.CW3 (herein referred to as CW3) was produced by transfection of $1x10^6$ 293T cells with 2.5 µg of a cDNA clone containing the genome of CW3 (116). Cells were then incubated at 37 °C for 48 hours, frozen at -80 °C, thawed, and passed through a 0.45 µm filter. 500 µl of the resulting filtered virus was used to infect $1x10^6$ WT BV2 cells. Inoculated cells are incubated until ~90 % cytopathic effect (CPE) was observed. Two 15-cm tissue culture treated plates are then seeded with $1.5x10^7$ BV2 cells and then inoculated with 50 µl of virus and incubated at 37 °C until ~90 % CPE was observed. These cells were frozen at -80 °C and thawed. Cell lysates were pooled

and centrifuged at 3,000 rpm for 20 minutes to remove cell debris. The supernatants were ultracentrifuged at 123918.9 x g for 3 hours in order to concentrate virus. The resulting pellet was resuspended in DMEM supplemented to contain 10 mM HEPES (Mediatech, 25-060-CI), 1X MEM nonessential amino acids (Mediatech, 25-025-CI), 100 U/ml each of penicillin and streptomycin (Mediatech, 30-002-CI) and 10 % fetal bovine serum (Biowest, US1520) and frozen at -80 °C until use. Passage 2 stocks of MNV-1.RVSS (RVSS) and MNV-1.CW1 (CW1), and passage 5 stocks of GV/CR3/2005/USA (CR3) strains of MNV were used for infection as described in Figure 2.2 (117, 118). For each passage, inoculated cells were incubated at 37°C for near complete cell death and frozen at -80°C. Thawed cell lysates were clarified by centrifugation and supernatant was used to inoculate new cells for amplification. Encephalomyocarditis virus (EMCV) and murine gamma-herpesvirus 68 (MHV-68) were provided by Drs. Marco Colonna and Herbert "Skip" Virgin at Washington University in St. Louis, respectively. EMCV and MHV-68 viral stocks were further passaged and amplified in L929 and MEF cells, respectively.

Viral infection

All infections were performed as described previously (119). Briefly, $1x10^5$ cells were seeded per well in 24-well tissue culture treated plates. 24 hours after seeding, the media was either replaced with virus-containing inoculum (adherent cell lines) or a concentrated sample of virus was added to the media (non-adherent cells) at the indicated MOI. Adherent cells were incubated in virus-containing inoculum for 30 minutes, washed twice with PBS, and replenished with fresh media. Nonadherent cells were inoculated with 50 µl of concentrated viral stock and mixed by gentle pipetting to achieve the indicated MOI. Cells were then washed and pelleted twice to remove

inoculum, and infected cells were harvested at the indicated time points by freezing at -80 °C for median tissue culture infectious dose ($TCID_{50}$) analysis.

$TCID_{50}$ assay

$TCID_{50}$ assays were performed as reported previously (120). Briefly, inoculated cells were frozen at -80 °C to lyse the cells via a cycle of freeze and thaw. Lysates were serially diluted 10-fold in cell growth media. The samples were then added to BV2 cells seeded in a 96-well plate. 8 wells of cells were inoculated per dilution and incubated for 5 days. $TCID_{50}$ was calculated by determining the dilution required to show cytopathic effect in 4 out of 8 wells, according to a standard protocol (120). Briefly, the following formula was used to calculate Proportionate Distance (-PD): ([%Positive at or above 50%] – 50%) / ([%Positive at or above 50%] – [%Positive below 50%]). The PD is then used to calculate log $TCID_{50}$ using the formula: (log dilution at or above 50%) + (−PD) = log$TCID_{50}$. This log value was then used to express the virus titer as $TCID_{50}$/unit volume, e.g., $TCID_{50}$/ml.

Western blot

Western blots were performed as described previously (115). In short, total cell lysates were harvested in protein sample buffer (0.1 M Tris [pH 6.8], 4 % SDS, 4 mM EDTA, 286 mM 2-mercaptoethanol, 3.2 M glycerol, 0.05 % bromophenol blue) and proteins were resolved by SDS-PAGE. Proteins were then transferred onto PVDF membranes, probed with primary antibody diluted in PBS/0.1 % TWEEN 20 (PBST) containing 5 % skim milk for overnight at 4 °C, and then stained with secondary antibody diluted in PBST with 5% skim milk for 1 hour at room temperature (RT). Probed proteins were detected using ECL reagents on a ChemiDoc system with

Image Lab software (Bio-Rad). The following antibodies were used: mouse anti-HA (Frank W. Fitch Monoclonal Antibody Facility, The University of Chicago Clone 12CA5), goat anti-mouse CD300LF (R&D systems, AF2788), mouse anti beta-actin HRP conjugated (Santa Cruz Biotechnology, sc-47778), and goat anti-mouse HRP conjugated (BioLegend, #405306).

Flow cytometry

To examine the cell surface expression of HA-tagged CD300LF mutants, cells ($1x10^6$ cells/well in 6-well plates) were gently detached by 5-minute incubation with 600 µl of 0.5 mM ethylenediaminetetraacetic acid (EDTA) and gentle scraping. At this point, cells were centrifuged for 5 minutes at 1,000 rpm. Cells were then washed in PBS containing 2% FBS and 1 mM EDTA. Cells were resuspended in wash buffer containing 1% FcR blocker, stained with mouse anti-HA antibody on ice for 30 minutes, washed 5 times, stained with donkey anti-mouse antibody conjugated with Alexa Fluor 647 for 30 minutes at RT, and washed again 5 times. Cells were then immediately analyzed with a BD LSRFortessa cell analyzer. Untransduced, unstained, samples stained only with primary antibody, samples stained only with secondary antibody, and samples incubated in primary isotype-control (Mouse IgG2b k isotype, Biolegend) antibodies were used as controls.

Lentiviral transduction

A modified version of lentiviral vector pCDH-MCS-T2A-copGFP-MSCV was used to express CD300LF alleles in C57BL/6J and I/LnJ BMDMs, as described previously (115, 119). Lentivirus was generated by transfecting lentiviral vectors with a packaging vector (psPAX2) and a pseudo-typing vector (pCMV-MD2.G) into 293T cells via a standard calcium-phosphate method.

A third-generation lentiviral vector pLenti-CMV-Puro-DEST (w118-1) was used to express HA-tagged CD300LF alleles in all immortalized cell lines used. Lentivirus was generated by transfecting lentiviral vectors with HIV gag/pol (pMDLg/pRRE), rev (pRSV-rev), and pseudotyping vector (pCMV-MD2.G). Supernatants were collected 24- and 48-hours post-transfection, filtered through a 0.45 μm filter (Millipore) and added to the indicated cells. After 48 hours, cells were selected with puromycin (2 μg/ml for HeLa cells and 3 μg/ml for all non-HeLa cells).

Binding assays

Binding experiments were performed in accordance with previously published assays (101). Briefly, $1x10^5$ cells were seeded in 12-well plates. 24 hours later, the cells were inoculated with MNV (CW3) at an MOI of 2 $TCID_{50}$/cell and incubated at 4 °C for 1 hour with gentle rocking. Cells were then washed three times with ice-cold PBS to remove unbound virus. BJAB cells were pelleted between washes via centrifugation. Cells were then lysed with 1mL TRI-Reagent (Sigma) and RNA was extracted according to the manufacturer's instructions. Genome equivalents were determined via qPCR as described previously (115).

Mouse experiments

Mice used in this study were of C57BL/6, I/LnJ, or C3H/HeJ background. C57BL/6 mice were provided by the laboratory of Dr. Seungmin Hwang, while I/LnJ mice mice were provided by Dr. Tatyana Golovkina. Control and experimental mice were 6-8-week-old littermates of both genders. On the day of infection, mice were given a per-oral, 15uL dose of 1×10^4 $TCID_{50}$ units of MNV (CW3). Three days post-inoculation, mice were sacrificed and their proximal colon, mesenteric

lymph nodes, and feces were harvested and flash frozen. Organs were subsequently homogenized and viral titer was measured via $TCID_{50}$ assay.

Statistical Analysis

All statistical analyses were performed in Graphpad Prism using two-tailed, unpaired t-tests. All differences not specifically indicated as significant were not significant ($p > 0.05$). Significant values were indicated as; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Results

I/LnJ BMDMs resist MNV infection.

Inbred mouse strains show differences in innate susceptibility to viral infections (121). While examining the susceptibility of BMDMs from different mouse strains to MNV inoculation, we found that I/LnJ BMDMs were completely resistant to MNV infection. Such resistance is in strong contrast to C57BL/6J BMDMs, which support robust MNV infection (Fig. 2.1A). To investigate the specificity of the resistance of I/LnJ BMDMs to MNV infection, we examined the replication of EMCV as another virus with a positive-sense RNA genome, and MHV-68 as a virus with a DNA genome. In contrast to MNV, the replication of both EMCV and MHV-68 was supported in C57BL/6J and I/LnJ BMDMs with similar growth kinetics (Fig. 2.1B, C). We also performed high MOI (5) infection and did not detect any sign of MNV replication in I/LnJ

BMDMs, other than remaining input virus (Fig. 2.1D). These results suggest that the inability of I/LnJ BMDMs to support viral replication is specific to MNV.

Different strains of MNV are known to bind glycoproteins differentially, which can lead to different levels of infection *in vivo* (118). To examine whether I/LnJ BMDMs are resistant to a broad range of MNV strains, we inoculated C57BL/6J and I/LnJ BMDMs with several strains of MNV and measured viral replication over 48 hours. Specifically, in addition to the CW3 strain, we chose CW1, RVSS, and CR3. CW1 is a plaque isolate of MNV-1; RVSS is a mutant strain of CW1 with an altered glycoprotein binding profile; CR3 is a field isolate with over 95% sequence identity with the capsid region of CW1 and CW3 (117, 118). Similar to the result with CW3 strain, I/LnJ BMDMs were resistant to infection with all MNV strains tested (Fig. 2A, B). These data demonstrated that I/LnJ BMDMs are resistant to multiple MNV isolates, suggesting a general resistance of I/LnJ BMDMs to MNV infection.

I/LnJ BMDMs expressing C57BL/6J CD300LF are susceptible to MNV infection.

Recently, unbiased CRISPR/Cas9 screenings of host factors for MNV infection identified CD300LF as a host factor to mediate MNV entry into cells (20, 21). Given the requirement of CD300LF for MNV entry coupled with the complete resistance of I/LnJ BMDMs to MNV infection, we speculated that I/LnJ BMDMs might fail to express a functional CD300LF receptor required for MNV to infect cells. To this end, we transduced BMDMs from C57BL/6J and I/LnJ mice with lentiviruses expressing either the C57BL/6J allele of CD300LF (B6/CD300LF) that was shown to mediate MNV entry (20) or enhanced green fluorescent protein (EGFP) as a control. Expression of B6/CD300LF in C57BL/6J BMDMs did not significantly affect susceptibility of the cells to MNV infection. In contrast, I/LnJ BMDMs became susceptible to MNV infection upon

expression of B6/CD300LF (Fig. 2.3A, B). These data clearly demonstrated that the resistance of I/LnJ BMDMs to MNV infection was largely, if not completely, at the step of viral entry, suggesting a defect of I/LnJ BMDMs in expressing functional MNV receptor CD300LF.

A cluster of four consecutive amino acids determines the functionality of CD300LF to support MNV infection.

The different susceptibility to MNV infection between I/LnJ and C57BL/6J BMDMs could reflect a simple difference in the cell surface expression of CD300LF or genetic variations within the protein itself. Unfortunately, none of the currently available antibodies for CD300LF could detect I/LnJ CD300LF, and we have been unable to detect surface expression of endogenous CD300LF in I/LnJ BMDMs. Thus, we examined publicly available sequences of murine CD300LF proteins to determine how the different alleles of CD300LF vary from each other and whether these differences could contribute to the functionality of the proteins. When we compared the sequences of CD300LF proteins from C57BL/6J and I/LnJ mouse strains, we found that the C57BL/6J and I/LnJ variants of CD300LF differ by 14 amino acids, 9 of them in the regions important to function as a receptor for MNV (20). We hypothesized that the sequence difference in CD300LF proteins could underlie the inability of I/LnJ CD300LF to function as an MNV receptor in BMDMs.

To determine the amino acids that could potentially be responsible for this MNV-resistant phenotype of I/LnJ CD300LF, we sought a mouse strain that could serve as a "genetic intermediate" between C57BL/6J and I/LnJ. The CD300LF sequences of the CAST/EiJ and I/LnJ mouse strains differ by 11 amino acids; 7 of these 11 sites are identical between CAST/EiJ and C57BL/6J CD300LF proteins, but the other 4 amino acids are not identical between the two strains

(Fig. 2.4A). To test whether the CD300LF allele of CAST/EiJ mice could serve as a receptor for MNV, we assessed whether BMDMs from the CAST/EiJ mouse are susceptible to MNV infection. In contrast to the I/LnJ strain, BMDMs from the CAST/EiJ strain supported MNV replication (Fig. 2.4B). These data suggest that the seven amino acids, in which CAST/EiJ CD300LF is identical to C57BL/6J but different from I/LnJ CD300LF proteins, might be responsible for the MNV-resistant phenotype of I/LnJ BMDMs.

To expedite the mapping process, we also examined whether the highly tractable macrophage-like BV2 cell line could mimic the phenotypic difference between C57BL/6J and I/LnJ BMDMs (20). We transduced *Cd300lf*$^{-/-}$ BV2 (BV2-KO) cells with lentiviruses expressing the HA-tagged CD300LF protein of either C57BL/6J or I/LnJ. While C57BL/6J CD300LF (B6/CD300LF) expression confers MNV infection capacity to BV2-KO cells, expression of I/LnJ CD300LF (IL/CD300LF) is insufficient to permit infection of BV-2KO cells (Fig. 2.4C), similar to the results obtained using BMDMs (Figs. 2.1, 2.2). To determine the role of the CAST/EiJ-identified seven amino acids in MNV infection, we expressed chimeric alleles of CD300LF: an otherwise WT C57BL/6J CD300LF with the seven amino acids of I/LnJ CD300LF at the CAST/EiJ-identified loci (B6/CD300LF-7aa) and the converse (IL/CD300LF-7aa). BV2-KO cells expressing the B6/CD300LF-7aa had a significant reduction in their ability to support MNV replication (Fig. 2.4D). In contrast, BV2-KO cells expressing IL/CD300LF-7aa supported MNV replication like the BV2-KO cells with B6/CD300LF. All CD300LF proteins were expressed similarly on the cell surface (Fig. 2.4E). Collectively, these data suggested that the seven amino acids difference of the I/LnJ CD300LF from that of CD57BL/6J is responsible for the majority of the resistance of I/LnJ BMDMs to MNV infection.

We next set out to determine which combination of these seven amino acids is responsible for the resistance phenotype; we chose to prioritize six of the seven as they are in the extracellular domain of CD300LF. According to software predictive of O-glycosylation NetOGlyc (DTU Bioinformatics), two of the seven amino acid variations in B6/CD300LF had high probability to be O-glycosylated. Since differential glycosylation status has been linked to the susceptibility of hosts to norovirus infection (101, 122), we examined these loci first. Expression of CD300LF mutants swapped at these potential glycosylation sites did not switch the phenotype of B6/CD300LF and IL/CD300LF in BV2-KO (Fig. 2.5A); that is, B6/CD300LF mutants with I/LnJ amino acid sequence at the potential glycosylation sites (B6/CD300LF S174G and T131A) still made BV2-KO cells infectible by MNV, and expression of the corresponding I/LnJ CD300LF mutants (IL/CD300LF G174S and A131T) did not, despite similar levels of cell surface expression (Fig. 2.5B). The remaining difference in the extracellular domain of the CD300LF alleles was four consecutive amino acids at the CC'-loop, which flanks a phospholipid binding pocket along with the CDR3 domain and is critical for MNV infection (20, 112). When we swapped these four amino acids between C57BL/6J and I/LnJ CD300LF, the respective mutants, B6/CD300LF-4aa and IL/CD300LF-4aa, recapitulated the phenotype of the seven amino acids-swapped mutants in BV2-KO cells (Fig. 2.5C, phenotypes summarized in Table 1). Again, a similar level of cell surface expression was observed for all constructs (Fig. 2.5D). Taken together, these data demonstrated that the four amino acid polymorphisms at the CC'-loop of I/LnJ CD300LF is primarily responsible for its non-functionality as an MNV receptor in macrophage-like cells (Fig. 2.6).

I/LnJ CD300LF can function as an MNV receptor in a cell type-dependent manner.

Expression of C57BL/6J CD300LF in cells non-infectible by MNV makes the cells permissive to MNV infection, demonstrating the entry step as a major barrier for MNV infection (20). We examined whether I/LnJ CD300LF functions similarly in cell lines other than macrophage-like cells. Thus, we stably expressed C57BL/6J or I/LnJ CD300LF via lentiviral transduction in a variety of cell types that do not express murine CD300LF and thus are not susceptible to MNV infection, including mouse embryonic fibroblasts (MEFs) and human cell lines 293T, HeLa, BJAB and Jurkat. CD300LF was expressed on the cell surface of all transduced cells (Fig. 2.7). Strikingly, while I/LnJ CD300LF did not function as an entry factor for MNV infection in BMDMs and BV2 cells, its expression in MEFs, 293T, HeLa, and BJAB cells made the cells permissive for MNV replication (Fig. 2.7A-D). In human Jurkat T cells, however, only the expression of C57BL/6J CD300LF, but not I/LnJ CD300LF, made the cells susceptible to MNV infection, reminiscent of the macrophage-like phenotype (Fig. 2.7E). Taken together, while C57BL/6J CD300LF expression is necessary and sufficient for MNV infection in all contexts tested, the cell-type dependent functionality of I/LnJ CD300LF suggests the necessity of additional cell-type specific modifiers mediating MNV entry.

Binding of CD300LF to MNV capsid proteins and its role in MNV attachment to host cells have been established (102, 103). To investigate the cell-type dependent functionality of I/LnJ CD300LF, we performed binding assays to determine how different CD300LF expression affects the attachment of MNV to cells, as described previously (118). BV2 and BJAB cells were chosen for this assay, as they produced the highest viral titer in infection experiments yet showed varied ability to support replication when expressing IL/CD300LF (Fig. 2.7). MNV bound to BV2-KO cells expressing B6/CD300LF significantly more than the BV2-KO cells; in contrast, MNV binding to BV2-KO cells expressing IL/CD300LF was comparable to its binding to BV2-KO cells

(Fig. 2.8A). These data suggest that B6/CD300LF mediated the binding between MNV and the BV2-KO cells but IL/CD300LF could not, which is consistent with the non-functionality of IL/CD300LF as an MNV receptor in BV2-KO cells (Fig. 2.4). On the contrary, MNV binding to BJAB cells expressing IL/CD300LF was significantly greater than its binding to WT BJABs, although less than its binding to BJAB cells expressing B6/CD300LF (Fig. 2.8B). The result was in line with the MNV replication of the BJAB cells expressing CD300LF proteins (Fig. 2.7D). Collectively, these data suggest that different binding of IL/CD300LF to MNV virions in different cell types might be responsible for the cell-type dependent functionality of I/LnJ CD300LF.

I/LnJ mice support MNV replication

As a major advantage of MNV compared to other noroviruses is its ability to replicate in a small animal model, we were interested in the ability of I/LnJ mice to support MNV replication *in vivo.* As BMDMs from I/LnJ mice are resistant to MNV infection, we hypothesized that all macrophages in I/LnJ mice would be resistant to the virus. Expression of I/LnJ CD300LF on non-macrophage, non-T cells renders them susceptible to MNV infection, suggesting that some cells may support replication of the virus *in vivo*. To address this question, we infected C57BL/6J and I/LnJ mice with MNV and measured viral replication in several organs known to support MNV replication. Though levels of replication were low, likely due to low initial dosage, we detected infectious virus in the proximal colon, mesenteric lymph node, and feces of infected animals, suggesting that although macrophages themselves may be resistant, I/LnJ mice are still susceptible to the virus (Fig 2.9).

Discussion

Previous studies have demonstrated that expression of murine CD300LF is necessary and sufficient for MNV infection; indeed, deletion of *Cd300lf* in C57BL/6J mice or in BV2 cells, a microglial cell line derived from C57BL/6 mouse, makes them resistant to MNV infection; expression of CD300LF in HeLa cells confers MNV susceptibility to human cells (20). Here we show that BMDMs from the I/LnJ mouse strain are resistant to MNV infection while susceptible to a range of other viruses, and that this resistance is due to polymorphisms in the extracellular domain of CD300LF in the I/LnJ mouse strain. Published amino acid sequences of CD300LF indicate significant variation among different mouse strains, including in regions important for MNV attachment and entry (20, 23). We identified a series of four amino acids at the CC' loop, which swapped the MNV susceptibility phenotype when exchanged between C57BL/6J and I/LnJ CD300LF. Surprisingly, I/LnJ CD300LF could function as an MNV entry factor when expressed in MEFs, 293T, HeLa, and BJAB, although it could not do so when expressed in BV2 CD300lf-KO or Jurkat T cell lines. Collectively, our data suggest that cell-type specific factors can modulate utilization of I/LnJ CD300LF in MNV entry.

Public sequence data of various mouse strains indicate that CD300LF proteins broadly segregate into two distinct groups: C57BL/6J-like, such as BALB/cJ and FVB/NJ, and I/LnJ-like, such as C3H/HeJ and CBA/J (aligned in Table 2.2). Comparatively fewer strains express an intermediate CD300LF like CAST/EiJ. Since the origin and lineage history of inbred mouse strains are not well documented (123), it is difficult to speculate on whether this clustering traces back to an evolutionary pressure in nature or a single divergence point. Evasion of MNV infection might be a selective pressure to drive the divergence of CD300LF proteins. However, as MNV infection in immune-competent mice is not lethal (95), it is possible that MNV infection would not have

exerted a selective pressure strong enough to cause this kind of divergence. Furthermore, considering that there are many cell types known to be infected by MNV *in vivo* (e.g. macrophages, dendritic cells, tuft cells, B cells, and T cells) (26) and that I/LnJ CD300LF could function as an MNV receptor in a cell-type specific manner, it is not surprising that even the mouse strains containing an I/LnJ-like *Cd300lf* allele are susceptible to MNV infection *in vivo*. Indeed, the C3H/HeJ mouse strain has an identical CD300LF to the I/LnJ strain and it was previously shown to be infected with MNV (124). Our preliminary data also suggest that I/LnJ mice are infectible with MNV, even though I/LnJ BMDMs are not infectible with MNV (Figs. 1, 2), potentially corresponding with a change in tissue tropism (118, 125). Based on the data shown in Figure 7, we would predict that I/LnJ B cells are susceptible to MNV infection. Therefore, substantive discussion on the natural variation of CD300LF, including its potential causes and consequences, would require a robust understanding of its function outside the context of infection.

Although a significant amount of work has been done tracing the evolutionary lineage of the CD300 family, the exact ligands, specific function, and redundancy of function between members is not well understood (126). It is also unclear whether the 4 amino acid difference in the CC' loop between mouse strains impact the cellular functions of CD300LF. It is useful to note that in each of our experiments in which the "4aa" exchange was made (i.e. 7aa and 4aa experiments), the C57BL/6J background-containing allele showed a severe attenuation in ability to confer infectivity, rather than a complete loss of function. This suggests that while the CC' loop contribute substantially to conference of MNV susceptibility, it is likely that other sections of the CD300LF protein that differ between C57BL/6J and I/LnJ alleles also contribute. Further study on the structure and function of CD300LF and CD300 family members in general is warranted.

The most surprising finding in our study is the functionality of I/LnJ CD300LF as an MNV entry factor in a cell type-dependent manner. We consider the following three possibilities for this cell type-specific functionality. First, there may be differential binding of ligands. Other groups have posited that the binding pocket flanked by the CC' and CDR3 loop may function to augment MNV attachment to CD300LF through the binding of a soluble factor(s), including ceramide (23, 127). Given the proximity of the 4 amino acids to the lipid-binding CC'-loop, the polymorphism could impact the ligand-binding capacity of CD300LF, such that the CD300LF-localized concentration of that ligand or local cell-surface density could differ between cell types and consequently affect functionality for MNV entry. Second, there may be cell-type specific modulators of MNV entry. Positive modifiers of MNV entry would likely be cell-type specific coreceptors. A classic example is HIV entry, in which the virus requires both its receptor, CD4, and a coreceptor in order to successfully enter a host cell (5). These coreceptors, CXCR4 and CCR5, contribute substantially to the host cell tropism of different strains of HIV (128-134). For MNV, the I/LnJ CD300LF might be unable to interact with the macrophage-/T-cell specific cofactor, while maintaining its interaction with alternative coreceptors expressed in other cell types. Lastly, there may be cell type-specific inhibitors of MNV entry, which can interact with I/LnJ CD300LF but not with C57BL/6J CD300LF. A recent study into critical entry factors for MNV into macrophages and dendritic cells identified several proteins, including CD98 and CD36 that influence attachment and could potentially fulfill such a role (135). All these three possibilities are plausible, and future studies will investigate these possibilities and attempt to identify these modulatory factor(s).

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Chapter III: Endothelial cell tropism is not a major determinant of H7N7 pathogenesis

All experiments were conducted by Kevin Furlong.

Abstract

Influenza A viruses infect millions of people worldwide each year. While seasonal epidemics cause the majority of illnesses, pandemics have caused a significant amount of morbidity and mortality. A major putative source of potential pandemic strains is reassortant viruses caused by co-infection of nonhuman hosts, often water fowl and domestic poultry. A subset of these reassortant viruses known as highly pathogenic avian influenza (HPAI) viruses are of particular interest due to their known capacity to infect humans and consumable domestic poultry. Such instances of human infection resulting from HPAI exposure have unusually high rates of mortality and are associated with more severe symptoms compared to seasonal strains. While individual HPAI strains have been studied, little is known about general patterns of pathogenesis that are common to multiple strains. Previous studies have indicated that endothelial tropism is a major contributor to virulence of H5N1, the most well-studied HPAI strain. In the current study, we investigate the importance of endothelial cell tropism in the pathogenesis of another highly virulent HPAI – A/Netherlands/219/2003 (H7N7). Using miRNA-mediated gene silencing to restrict viral replication within endothelial cells and monocyte-derived cells, we observed no relationship between endothelial cell tropism and lethality of H7N7. To our surprise, we also did not observe extravasation of the virus to non-pulmonary organs, despite robust replication in the lungs of infected mice. Our study indicates that the pathogenesis of two highly virulent strains of HPAI are significantly different,

highlighting the need for future work to elucidate mechanisms of virus-mediated death in the search for prophylactic treatment to prevent future HPAI pandemics.

Importance

Seasonal epidemics of IAV are responsible for millions of hospitalizations and hundreds of thousands of deaths worldwide. Avian strains of IAV have significantly higher mortality rates and are considered to be among the most potent threats of a worldwide pandemic. Understanding the mechanisms that contribute to the unusually high virulence and mortality rates of all HPAI rather than individual ones will help to develop critical therapeutics that could be applicable to any potential pandemic, reducing mortality and burden on the healthcare industry that could otherwise become overwhelmed by another pandemic.

Introduction

Influenza A virus (IAV) is a persistent, changing threat to human health worldwide. As the largest subfamily of the causative agent of the respiratory disease influenza, exposure leading to seasonal epidemics worldwide leads to millions of cases per year, with an estimated 389,000 deaths on average per year between 2002 and 2011 (43). Current seasonal infections are most often caused by H1N1 and H3N2 – the two most prevalent circulating strains (43). Exposure to these common, circulating strains generally results in a self-limiting infection, which is cleared by the majority of immunocompetent patients within 6 to 8 days, with elderly patients, young children, and otherwise immunocompromised most at risk (42). The segmented nature of the IAV genome affords different strains of the virus the capacity to reassort functionally identical segments, which can result in a genetically distinct virus to which the majority of people will be

immunologically naïve. In addition to infecting humans, IAV is known to replicate in a wide variety of different animal hosts, with water fowl serving as the most common reservoir for the virus. Water fowl or other hosts coming into contact with domestic poultry can bring such recombinant strains of IAV into close contact with humans, potentially precipitating an outbreak. Occasionally, exposure to a virus generated by such recombination within animal hosts results in a pandemic, a distinct threat to worldwide health compared to more common seasonal strains. Within the past century, there have been four instances of such a pandemic resulting in widespread morbidity and mortality: 1918 Spanish flu, the Asian flu (1957), the Hong Kong flu (1968), and the Swine flu (2009) (136). Deaths from these diseases and resulting complications ranges from 500,000 individuals to several million (136). Understanding the manners in which these viruses cause such mortality is paramount to future prevention.

Among the putative strains for future pandemics, those classified as HPAI viruses due to their pathogenicity in intravenously-injected young chickens are considered to be most threatening. Bearing the hemagglutinin H5 or H7 almost exclusively, HPAI viruses have been responsible for significant loss in poultry farming and, rarely, have infected humans (57, 137, 138). Asian lineage H5N1 (henceforth, H5N1) is a strain of HPAI that has been detected often in domestic poultry has a wide range of potential species compared to other known IAV strains (57). Humans infected with H5N1 have had severe clinical outcomes accompanied by a mortality rate of approximately 60% (57). Infections often do not present with the expected respiratory distress associated with seasonal influenza and thus can be difficult to detect, making thorough understanding and prevention important.

As H5N1 infections have been the most common HPAI strain detected in humans, significant work has been done to advance understanding of the virus. One such study identified

endothelial cell tropism a major determinant of H5N1 virulence using miRNA-mediated silencing of the virus (94). Through the inclusion of miRNA-target sequences complementary to miRNA exclusive to the endothelium or monocytes in the nucleoprotein (NP) of H5N1, the study shows that endothelial and hematopoietic-cell tropism contribute to hypercytokinemia during H5N1 infection, but only endothelial cell tropism contributes to lethality of the virus. Importantly, the inclusion of miRNA target sites themselves had no effect on total viral titer in the whole lung – the primary site of viral replication (94).

As the ability to develop a functional vaccine in advance of a potential HPAI pandemic is contingent upon knowing which strains will circulate, we were interested in determining potential characteristics of a wide range of HPAI viruses. Specifically, we were interested in whether endothelial cell tropism was a requirement for the virulence seen in other HPAI strains known to be able to be transmitted to humans. A/Netherlands/219/2003 (H7N7) is one such virus that has already had a significant impact on domestic poultry that has caused a minor epidemic and shown a significant capacity for spread among humans (55, 56). Thus, we sought to determine if endothelial cell tropism contributed significantly to H7N7 pathogenesis in order to gain insight into potential host requirements that span viruses containing the two most common hemagglutinin subtypes among HPAI viruses. We employed miRNA-mediated silencing to restrict viral replication within cell types of interest and measured the effects on virulence and mortality on C57BL/6 mice.

Materials and Methods

Cells

Madin-Darby Canine Kidney (MDCK) cells maintained in Minimal Essential Media (MEM, Lonza) supplemented with 10% FBS, 1% Penn/Strep, 1% MEM Nonessential Amino Acids, and 1% L-glutamine. Human lung epithelial cells (A549), 293T cells, mouse lung epithelial cells (LA-4), ferret lung epithelial cells, and mouse endothelial cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% Penn/Strep. THP-1 cells were maintained in RPMI media (Gibco) supplemented with 10% FBS. All cell lines were acquired from ATCC.

Cloning

Generation of targeted viruses was performed as described previously. Briefly, four copies of the indicated target site were incorporated into a plasmid expressing the mRNA of the NP segment of the A/Netherlands/219/2003 (H7N7) virus (ScrbT: GAGAATCTAAACGACTCAATACA, 126T: CGCATTATTACTCACGGTACGA, 142T: TCCATAAAGTAGGAAACACTACA). Upon confirmation of incorporation of the target sites via sequencing, rescue transfections were performed with DNA plasmids incorporating the remaining seven segments of the H7N7 genome via Lipofectamine 2000 transfection into 293T cells according to the manufacturer's instructions. Transfected 293T cells were then cocultured with MDCK cells and monitored for cytopathic effect. Viruses were subsequently amplified twice via inoculation of fresh MDCK cells with supernatant of infected cultures, plaque purified, and confirmed not to have any other detrimental mutations via sequencing of the viruses.

Western Blot

293T cells were co-transfected with DNA plasmids expressing the targeted NP indicated and/or a separate plasmid encoding the miRNA indicated at a ratio of 8:1 via Lipofectamine transfection

according to the manufacturer's instructions. 48 hours post transfection, cells were harvested, lysed with 1% NP-40 lysis buffer and probed for NP expression with NP-specific antibody #4554 (BEI Resources). Ku80 (Ku) levels are included as loading controls.

Growth Curve Analysis

The indicated cell lines were seeded in tissue-culture treated 12-well plates one day prior to infection. Immediately before infection, cells were washed three times with room temperature PBS to remove FBS-containing media. Cells were infected at the indicated MOI in DMEM supplemented with 0.2% BSA and incubated at 37^{o}C for one hour with gentle rocking. After one hour, cells were washed three times with room temperature PBS to remove virus-containing inoculum and maintained in the indicated media for 72 hours. At each of the indicated time points, ~500uL of supernatant of infected cells was harvested and used for plaque assay to determine viral titer.

Plaque Assay

Plaque assays were used to determine viral titer from supernatants harvested from tissue culture as well as homogenates from infected animals. Animal tissue was harvested on the indicated day and flash frozen. Whole organs were then homogenized using a tissue homogenizer and homogenization beads according to the manufacturer's instructions. From here, organ homogenates or tissue culture supernatant were serially diluted at a ratio of 1:10 and used to inoculate MDCK cells seeded as a monolayer in 12-well, tissue culture-treated plates, which were then covered with an overlay media (1x MEM with 0.2% BSA, Avicel (FMC Biopolymers), 0.1% NaHCO3 (Sigma), 0.01% DEAE-dextran (MP Biomedicals)) and incubated

at 37C for 48 hours. Cells were then fixed with 4% formaldehyde and stained with a 0.1% crystal violet solution to visualize plaques.

Mouse studies

Eight-week old C57BL/6J mice were purchased from Jackson Laboratories and were housed in the Animal Biosafety Level 3 facility at the Howard T. Ricketts research facility (University of Chicago.) On the day of infection, animals were anaesthetized via intraperitoneal injection of ketamine and xylazine, followed by intranasal infection with a dilution of the indicated virus in 25uL PBS. Infected animals were then monitored for symptoms and measured for weight change. Animals displaying neurological symptoms and animals who lost >25% of their initial body weight were euthanized.

Results

H7N7 NP-miRT recombinant strains are designed for restriction of viral replication upon expression of complementary miRNA

In order to ultimately determine whether cell type-specific tropism is a requirement for H7N7 pathogenesis, we first sought to create recombinant strains of H7N7 with restricted replication within cell types of interest. As previously published results have indicated the importance of endothelial cell tropism for H5N1 virulence, we focused on measuring a potentially similar contribution to H7N7 virulence. We employed a method in which a sequence of bases complementary to a cell type-specific miRNA is inserted into the viral genome as previously described (94). MicroRNA-126 (miR-126) is an endothelium-specific miRNA that

has previously been used for IAV replication restriction (93, 94, 139). Previously published work has suggested that another major contributor to IAV pathogenesis is the spike in production of various cytokines and chemokines from monocyte-derived cells that results from infection (140). Therefore, in addition to miR-126 and endothelium, we chose to include monocytes and microRNA-142 (miR-142), a well-characterized miRNA with expression restricted to monocyte-derived cells (141-143). We chose also to include a randomized sequence of the nucleotides contained within miR-126 (miR-Scrb) as a control for expression of miRNA target sites in recombinant viruses.

A major strength of IAV as a model system is the robust reverse genetics system that allows for relatively simple creation of recombinant viral strains. Due to the segmented nature of the IAV genome, such viruses can be created from plasmids encoding each of the viral genomic segments, allowing for the manipulation of the genome in discreet genetic elements (144). As our goal was to restrict viral replication completely in cells expressing the miRNA of interest, we sought to insert target sequences to these miRNAs into a gene expressing a protein that is indispensable for IAV replication. We chose to insert four consecutive miRNA target sites into the mRNA of the nucleoprotein (NP) of H7N7, as NP serves both a structural role in maintaining virion stability as well as a transcriptional role in viral replication (145, 146). Thus, replication in which NP production is disrupted is heavily impaired.

NP expression from NP-miRT plasmids is restricted upon co-expression of complementary miRNA

Upon successful insertion of the four consecutive target sites into cDNA plasmids encoding otherwise WT H7N7 NP mRNA, we sought to determine whether expression of NP

protein from these plasmids could be restricted in a virus-free system. Understanding whether NP expression is impaired upon co-expression of complementary miRNA would help interpretation of potential restriction in viral replication in downstream applications. To determine this, we co-transfected 293T cells with a plasmid encoding mRNA for a NP with one of the designated miRNA target sites (NP-miRT, collectively) and a plasmid encoding a miRNA of interest. Importantly, 293T cells do not express either of the miRNA sequences of interest. After 48 hours, we harvested whole cell lysates from each co-transfection and probed them for NP expression. As we expected, co-transfections in which NP-miRT target sequences were not complementary to the expressed miRNA or in which no miRNA was expressed showed robust protein expression. However, when an NP-miRT sequence was co-expressed with a complementary miRNA sequence, NP expression was heavily restricted (Figure 3.1). Taken together, these results suggest that NP-miRT expression is limited by the expression of complementary miRNA.

H7N7-miRT replication is restricted only in cells expressing complementary miRNA

After confirming that plasmid-based expression of NP-miRT protein is restricted in the presence of complementary miRNA, we sought to create recombinant H7N7 viruses encoding the targeted NP. We co-transfected 293T cells with the NP-miRT-containing plasmids discussed previously alongside 7 other plasmids encoding the mRNA for the remaining 7 segments of the WT H7N7 genome. Importantly, rescue of the virus in this manner results in an otherwise isogenic genetic background allowing us to associate any defects in viral replication with the inserted miRT target sites. Upon confirmation of target site insertion and otherwise isogenic background via sequencing, we sought to determine if miRT sites would affect viral replication

in vitro. Specifically, we were interested in the potential ability for endogenous expression of miRNA complementary to target sites to inhibit viral replication. Thus, in order to determine the range of the effects of miRT insertion on viral replication in relevant systems in addition to potential effect of endogenous expression of relevant miRNA, we analyzed multi-stage replication kinetics via growth curve over a 72-hour period. We measured replication in a variety of cell types including human lung epithelial cells (A549, Figure 3.2A), ferret lung epithelial cells (Figure 3.2B), mouse epithelial cells (LA4, Figure 3.2C), human monocytes (THP-1, Figure 3.2D), mouse endothelial cells (MS1, Figure 3.2E), and human endothelial cells (teloHAEC, Figure 3.2F). Of these, the mouse system remains among the most well-characterized animal model for IAV infection. Despite this, due to an extended esophageal tract and development of human-like symptoms, ferrets are recognized as a closer proxy to humans for IAV infection (147, 148). As miR-126 is an endothelial specific miRNA and miR-142 is a monocyte-specific miRNA, the mouse endothelial cell line endogenously expresses miR-126 and both the mouse and human immune cells express miR-142, while lung epithelial cells are the most commonly infected cell type *in vivo* (149).

As endothelial cells express miR-126 and monocyte-derived cells express miR-142, we expected viruses expressing the complementary miRNA in NP mRNA to be heavily inhibited in translation, thus resulting in a severe limitation in viral replication. Indeed, replication of H7N7-miR-142 was restricted in human monocyte cell lines (Figure 3.2D), while H7N7-miR-126 replication was restricted in both human and mouse endothelial cells (Figures 3.2E, F). Importantly, while virus replication was restricted in cells expressing the complementary miRNA, viruses with miRT sites replicate to WT-like titers in the absence of such restriction. Taken together, these data suggest that viral replication for H7N7-miRT viruses is restricted in

miRNA-dependent manner. Additionally, insertion of miRNA target sites into H7N7 NP has no detectible effect on replication in the absence of corresponding miRNAs.

Restriction of H7N7 replication in endothelial and monocyte-derived cells does not affect viral lethality

After confirming restriction of replication in a miRNA-dependent manner *in vitro*, we sought to determine whether restriction of replication in this manner affects viral pathogenesis and lethality *in vivo*. As such *in vivo* experiments with H7N7 are challenging to perform due to their BSL3-level pathogen status, few experiments have been done to determine effective dosing for pathogenesis studies. Initially, we inoculated WT C57BL/6 mice with a range of doses: 250, 50, 10, and 2 plaque-forming units (pfu) each of each virus and observed them for mortality and weight loss once a day each day for 20 days. Continuous weight loss and mortality continued from day 2 to day 12 for animals infected with each of the viruses. Surprisingly, we did not detect a significant increase in survival of mice infected with either H7N7-142T or H7N7-126T compared to the control H7N7-ScrbT, suggesting that any role endothelial cell tropism may play in H7N7 virulence is minor (Figure 3.3).

In addition to monitoring infected mice for weight loss and mortality, we harvested the lungs, kidneys, spleens, and brains of infected mice. Extravasation from the respiratory tract into the blood resulting in detectable viral replication in these organs has been reported following exposure to H5N1, in which lower viral titers in extrapulmonary organs correlated with virulence (94). As pulmonary epithelial cells serve as the primary target for IAV replication and express neither miRNA-126 nor miRNA-142, we expected to detect robust replication of each virus within the lungs. As we expected, high titers of the virus were readily detected in homogenized

lungs at each time point tested with no significant difference between any of the viruses at any time point, corresponding well with our *in vitro* data suggesting insertion of target sites do not affect viral replication in the absence of complementary miRNA (Figure 3.4). Surprisingly, however, we detected very few indications of viral replication in any organ other than the lung. The majority of organs at each time point had no detectible virus and there did not seem to be a pattern among organs that became infected (Figure 3.5). Taken together, these results suggest that viral replication in monocyte-derived cells and endothelial cells contributes primarily to virulence and unlike H5N1, extravasation of the virus from host lungs is not associated with lethality.

Discussion

In this study, we demonstrate a method for measuring the contribution to pathogenesis and lethality of the HPAI strain H7N7. We have shown a process by which a recombinant component of the flu genome can be made with inserted target sites complementary to a miRNA of interest. Correspondingly, we have shown that co-expression of a viral protein in this manner with a complementary miRNA can result in restricted protein expression in a virus-free system. Subsequent co-transfection of such a recombinant segment of the genome with cDNA plasmids encoding the remaining segments of the genome results in rescue of a virus with an otherwise isogenic H7N7 background, differing only in the inclusion of complementary miRNA target sites. Measurement of replication kinetics via growth curve show that replication of the virus is largely unaffected by the inclusion of such target sites with the exception of replication of cell lines encoding a complementary miRNA. In such systems, insertion of target sites that are not known to be complementary to any miRNA expressed has no effect on viral replication.

However, viral replication is restricted when a complementary miRNA is expressed. Specifically, we have shown that replication of H7N7 with target sites complementary to miRNA-126 results in restriction of replication in a mouse endothelial cell line while replication of the virus in non-endothelial cell lines is unaffected. Similarly, H7N7 with target sites complementary to miRNA-142 is restricted in monocyte-derived cells but replicates to WT-like levels in other cell lines.

Clear restriction of protein production in a plasmid-based transfection system along with restriction of replication with complementary miRNA expression suggests that viral replication is indeed restricted in a miRNA dependent manner. Thus, as miRNA-126 and miRNA 142 are known to be highly expressed in endothelial cells and monocyte cells respectively, we hypothesize that *in vivo* replication is likewise restricted in a cell-type dependent manner. Previous studies using a similar method of restriction of viral replication based on cell type-specific miRNA expression have demonstrated the importance of viral replication within endothelial cells as a contributing factor for virulence in both mouse and ferret systems. Thus, we hypothesized that H7N7 restricted in replication might have a similar phenotype with respect to endothelial cell tropism and virulence. Surprisingly, mice inoculated with a virus restricted in endothelial cell replication did not show increased survival rates compared to mice infected with unrestricted viruses or viruses restricted in replication in monocyte cells. This suggests that unlike H5N1, endothelial cell tropism is not a major determinant of H7N7 virulence and likely does not substantially contribute to pathogenesis. Additionally, we could not detect evidence of extravasation of the virus from the lungs via plaque assay of organ homogenates. Together, these data suggest that while H7N7 is virulent in small doses, its pathogenesis may differ from H5N1.

A complete understanding of such differences warrants future investigations into the pathogenesis of HPAI including H5N1 and H7N7. Several important observations were made in the study measuring the effect of endothelial cell tropism on H5N1 virulence that would clarify potential differences in pathogenesis between the two viruses. High levels of inflammatory cytokines and chemokines have been associated with HPAI infections and measurement of levels of these cytokines in response to H5N1 infection indicate correlation between them and virulence (94, 140). Specifically, restriction of endothelial cell tropism via insertion of miRNA-126 target sites results in a significant decrease in every measured cytokine compared to mice infected with a control virus in addition to being associated with increased mortality. These results suggest that hypercytokinemia may be associated with H5N1-related mortality, though similar experiments conducted with monocyte-restricted viruses were associated with a similar decrease in local cytokine levels, but not mortality, making a causal relationship between these unlikely. A similar investigation into the cytokine levels in response to H7N7 infection could reveal whether such decreases are still present in response to restricted H7N7 infection. It is possible that infection with H5N1 results in activation of endothelial and monocyte-derived immune cells that results in production of such cytokines that contribute to the loss of integrity of the endothelial barrier, resulting in vascular leakage and ultimately death. Similarly, a decrease in vascular leakage was observed in response to H5N1-126T – a phenotype that was not measured in response to H7N7 infection.

Perhaps the most surprising observation of the H7N7 study was the lack of detectable virus in non-pulmonary tissue without a correlating decrease in mortality. It is important to note that another study into the pathogenesis of H7N7 in animal models observed some extrapulmonary replication of the virus in BALB/C mice, particularly in the brain (150). As their

inoculation dose was 3.5 orders of magnitude greater than their measured LD_{50}, it is likely that extrapulmonary replication is possible at a high enough dose, but is not necessary for lethality. The contention that extrapulmonary replication of H7N7 is rare is supported by the observation that virtually no extrapulmonary replication was detected in ferret experiments in the same study. It is difficult to identify exactly how these observations inform our understanding of H7N7 pathogenesis, as less is known about H7N7 pathogenesis compared to H5N1 overall. A common interpretation of the severe lung damage, hypercytokinemia, and pneumonia that can be associated with H5N1 infection is that active replication in the lower respiratory tract of an infected individual results in local inflammatory cytokine and chemokine production. Immune cells likely including macrophages are recruited to and enter the lower lungs via the endothelium, resulting in vascular damage and pneumonia. It is important to note that H5N1 infection in humans and other animals results in active replication in non-pulmonary tissue and that death is not always associated with pneumonia (57). Thus, the understanding of the exact mechanisms of H5N1-induced lethality are not understood. Reduction in cytokine production via restriction of H5N1 replication in hematopoietic cells does not reduce virulence (94). This suggests that the production of inflammatory cytokines is not a major contributor to H5N1 pathogenesis. Increased vascular leakage in the lungs likely due to damage resulting from H5N1 infection is associated with increased lethality, however. Together, these data suggest that damage to the host lung following H5N1 infection that is independent of cytokine production contributes to H5N1 virulence, and the same may be true for H7N7. That is, damage to the lung that is independent of replication in non-pulmonary cell types may be the primary cause of death. As H7N7 is lethal at even lower doses than H5N1, we hypothesize that lung damage resulting from infection may be more severe. This may inform interpretation of the lack of detectible virus

in non-pulmonary tissue. The lack of requirement of endothelial cell tropism, hematopoietic cell tropism, and extra-pulmonary replication suggests that H7N7 pathogenesis simply does not involve replication anywhere but the host lung epithelium. Given the relationship between extrapulmonary spread and mortality in relation to H5N1, future studies will be necessary to determine the cause of mortality in response to H7N7 infection.

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Chapter IV: Conclusions

The work presented herein describes two studies that expand our appreciation of the complexities of virus-host interactions in viral tropism and pathogenesis. Although presented from the perspective of two distinct RNA viruses, the reliance on host machinery to achieve replication and pathogenesis is common and strategies are often shared. In both studies we relied on genetics to probe questions of tropism and pathogenesis. For HPAI H7N7, the power to manipulate the genetics of the virus was essential to the experimental design. The ability to rescue recombinant influenza viruses via transfection of cDNA clones encoding miRNA-sensitive mRNA allowed us to probe the importance of endothelial and hematopoietic cells in H7N7 pathology. In the MNV study, host genetic differences was essential to uncover the existence of cell type-specific modifiers of viral entry.

Despite showing a clear lack of function for MNV entry of I/LnJ CD300LF in I/LnJ BMDMs and BV2-KO cells, we observed that transduction of the I/LnJ variant of CD300LF into some cell lines, including immortalized human B cells, resulted in susceptibility to viral infection. These results suggested that functionality of I/LnJ CD300LF was likely affected by other cellular cofactors whose presence or absence in different cell types modulated MNV entry. The classical example of this is cell-type specific coreceptors, such as CCR5 and CXCR4 I HIV entry. MNV has been shown to infect resident immune cells in the gastrointestinal tract, including macrophages, dendritic cells, and other hematopoietic cells C57BL/6J mice (16, 26). Given the ability of MNV to infect I/LnJ CD300LF expressing B cells, but not T cells or macrophages, we would predict that I/LnJ mice are infected by MNV, but with an altered tropism. This hypothesis is supported greatly by our observations that I/LnJ mice are infected by

MNV. In conjunction with our own *in vitro* analysis, we believe that intestinal resident B cells are a likely candidate for supporting MNV infection of I/LnJ mice, and further study will be needed to explore this possibility.

Currently, we believe that there are three likely possibilities underlying this phenotype. In one case, it is possible that there are positive regulators (coreceptors) for MNV entry. One coreceptor would be expressed in macrophages and dendritic cells, while another coreceptor would be expressed in B cells. CD300LF from C57BL/6J is able to bind both coreceptors. But I/LnJ CD300LF can only functionally interact with the B cell coreceptor, but not the macrophage/dendritic cell coreceptor. This would implicate the 4 amino acids near the CC' loop as part of a CD300LF-macrophage/dendritic cell coreceptor interaction domain.

The second possibility underlying the differential infection phenotype is a negative regulator of CD300LF function that functions exclusively on I/LnJ CD300LF in cell types tested in which it does not function, i.e. macrophages and T-cells. The functionality of C57BL/6J CD300LF in all tested contexts suggests that the receptor would lack the ability to bind to the hypothetical negatively regulating cofactor. This negative regulator would have to either not be expressed in cell types in which I/LnJ CD300LF is functional, or be unable to bind the protein in these contexts, potentially due to competition with another binding factor or the assumption of a conformation by CD300LF that precludes such an interaction. Given the data presented, we believe a positive regulator to be the more likely case.

The third option we consider is the possibility that I/LnJ and C57BL/6J CD300LF have different capacities to bind potential ligands that influence their ability to serve as an MNV receptor. In our study of MNV and CD300LF, we identified a four amino acid sequence proximal to the CC' loop that itself may have binding capacity for other ligands, including

ceramide. Such a differential ability to interact with non-MNV ligands could affect the local structure of the receptor and prevent binding to MNV

A useful first step in testing these hypotheses would be approaches to identify cell type-specific binding partners for CD300LF, including both potential molecular cofactors and ligands. For molecular binding partners, we believe a proteinaceous binding partner to be both most likely and most straightforward to determine via mass spectrometric analysis of proteins found associated with membrane-bound CD300LF in B cells versus macrophages. As the commercial antibodies against CD300LF are neither strong enough to readily detect endogenous CD300LF nor specific enough to readily bind CD300LF, but not other structurally similar members of the CD300 family via Western Blot, a pulldown of endogenous protein is unlikely to be simple or sufficient. Therefore, immunoprecipitation of membrane-localized, overexpressed CD300LF with a detectable protein tag is likely to be more effective. Subsequent analysis of proteins either bound directly to or in complex with either C57BL/6J or I/LnJ CD300LF could help form a clearer picture of other factors underlying its function. This would be performed +/- MNV infection, as MNV itself might coordinate the interaction between CD300LF and its coreceptors. As we consider both a positively regulating and negatively regulating cofactor to be legitimate possibilities, a series of analyses of binding partners in different cell types would be necessary to clarify this. In the simplest case in which a single cofactor determines CD300LF function as an MNV receptor in every cell type, we would predict different binding profiles based on allele and cell type. As C57BL/6J is universally functional in cell types tested, it is most useful to consider variations in I/LnJ binding. A single, positively regulating cofactor would most likely be detected bound to I/LnJ CD300LF in the cells in which the receptor functions, being MEF, HeLa, 293T, and BJAB, and would be absent in BMDMs, Jurkat T-cells, and BV2s. In this case,

the identified cofactor would likely be bound to C57BL/6J CD300LF in all cases, suggesting a differential ability to bind the receptor between the two alleles, or in none of them, suggesting that C57BL/6J requires either a different coreceptor or lacks one altogether. In the case of a negatively regulating cofactor, we expect to find it only in cell types in which I/LnJ CD300LF doesn't function, which are macrophage-like cells and Jurkat T-cells and not bound to C57BL/6J CD300LF at all. In each case, identification of a binding partner followed by restricted expression via CRISPR or siRNA in the indicated cell type followed by attempted rescue via plasmid expression would clarify the role of such a factor.

Finally, understanding the nature of the effect of ligand-binding on CD300LF could elucidate its contribution to MNV-CD300LF interactions. As previously stated, the binding pocket flanked by the CC' loop and CDR3 domain has been posited to bind ceramide and potentially other ligands. Initial experiments in which ceramide levels were altered in combination with MNV infection in BV2/IL-CD300LF and BV/B6-CD300LF showed no difference in viral output based on ceramide concentrations (data not shown). Perhaps more importantly, neither CD300LF nor the CD300 family of proteins have been well-studied up to this point and their ligands outside of a viral context are not known. A strong contributing experiment to the work presented would be an assay that measured CD300LF function in each cell type with either allele in the context of infection. However, due to the understudied nature of the protein, it is not known what ligands might be useful in measuring such function. Correspondingly, it is somewhat challenging to determine whether ligand binding contributes significantly to MNV-CD300LF interactions. It is possible that local binding of lipids or another molecule alters local surface structure in a manner that is detectable by very sensitive assays

such as those measuring membrane curvature or cryo-electron microscopy. Such future studies are necessary to understand the other factors underlying CD300LF function.

We described an unexpected observation for H7N7 pathogenesis: endothelial cell replication, which is essential for H5N1 virulence, is dispensable for H7N7 pathogenesis. Unlike H5N1 infection, very little extra-pulmonary H7N7 replication was detected, suggesting that localized lung pathology is solely responsible for H7N7 virulence. These key differences between two HPAI variants illustrate the complex relationships between viruses and their hosts.

In general, we believe the data suggest that both endothelial cell tropism as well as extravasation from the primary site of replication are not related to H7N7 virulence, despite being associated with virulence and lethality in H5N1 infection. This raises the question of the differences in replication and virulence between the two viruses, and investigation of this phenomenon could take several different forms, each of which could contribute significantly to future understanding. Historically, it has been the case that the HA and NA subunits of IAV have been determinants of virulence. Given the ease with which recombinant strains of IAV can be created via rescue transfection, a novel strain of H7N7 in which the HA and NA subunits of the virus are exchanged for the HA and NA subunits of H5N1 could help distinguish a causality for extravasation and requirement for endothelial cell tropism. As this would be a novel reassortant of two virulent BSL3-level pathogens, the knowledge gleaned from such a study would be invaluable provided proper precautions are taken. First, rescuing a novel pathogen created via recombination of two highly virulent strains of IAV would result in a new pathogen with unknown virulence. It is possible that the virulence of the resulting virus would be greater than the sum of its parts and would thus be neither easily comparable to its comprising strains nor suitable for study under the same biosafety level. Perhaps more likely, it is possible that H5

and/or H7 require specific residues or protein structures that may differ slightly between the viruses. Exchange of the HA and NA segments while excluding any other potentially compensatory mutations may result in a pathogen with attenuated replication or virulence. In either case, making direct connections to H5N1 or H7N7 pathogenesis could be challenging. The H7N7 work was undertaken with the hope of understanding factors that unite HPAI in their unusual virulence in humans, addition of more HPAI infection information to the study's repertoire would be useful. For example, study about the potential for extravasation and requirement for endothelial cell tropism in the emerging HPAI pathogen H5N6 could help point toward a role for the H5 HA subunit in H5N1-like pathogenesis (151). Likewise, H7N9, a virus that is technically a low pathogenic avian influenza virus (LPAI) by the conventional definition but has proven to be a serious threat to human health, could prove useful in defining a potential role for the H7 or N7 subunits. As H7N9 is a LPAI, it is also possible that virulence requirements differ greatly between this virus and HPAI. The use of a wider range of viruses in the studies like those presented in this work could contribute to a more complete understanding of the nature of virulence in all avian IAV subtypes.

In addition to using other viruses, finer characterization of the relationship between endothelial cell replication, extrapulmonary replication, and the direct causes of mortality could help construct a clearer image of HPAI pathogenesis in a manner that is conducive to developing treatment strategies. An example previously presented is follow-up of the H7N7 studies with those that more directly connect H5N1 virulence with endothelial cell tropism by way of connection to hypercytokinemia and endothelial barrier membrane integrity. That is, previous studies have shown that WT H5N1 infection results in loss of endothelial barrier integrity and increase in production of inflammatory cytokines in the lung, each of which is correlated with

increased mortality in a mouse model. However, a reduction in cytokine production was also seen in H5N1 viruses restricted in their capacity to replicate in monocytes, where mortality was not significantly reduced. Similar analysis of cytokine production and barrier integrity in response to H7N7 infection could help establish a role for either of these in virulence of the virus.

The data surrounding H7N7 pathogenesis in a mouse model strongly suggest that lung pathogenesis itself is a major determinant of H7N7 virulence, with extrapulmonary replication, endothelial cell tropism, and hematopoietic cell tropism playing little to no role. As such, experiments that address the effect of H7N7 replication on host lungs have the potential to contribute the most to our understanding of the virus. The ferret respiratory tract and lungs are considered to be better models for human infection compared to their murine counterparts. Probing the effect of H7N7 replication on ferret lungs is a necessary step in understanding pathogenesis. While such studies have been done, they have historically been at high titers for the purpose of comparing H7N7 isolates and could be improved with more variation in dose. Histological analysis of infected lungs with the targeted viruses to address the role of hematopoietic cells would demonstrate the extent of lung damage in infected individuals and would support these conclusions. Along the same lines, analysis of vascular leakage in response to infection would provide additional data about potential lung damage. Creation of a recombinant virus with a reporter tag could also confirm extrapulmonary and specifically endothelial cell replication using a sorting strategy to identify fluorescent virions in cells with endothelial cell specific markers or antibodies.

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Appendix I: Tables

Table 2.1. BV2 variant infection phenotypes.

Visual reference of all generated CD300LF variants and their ability to support MNV (CW3) replication when expressed on BV2-KO cells.

CD300LF Variant in BV2-KO	MNV Infection Phenotype	Description
(none)	Resistant	BV2 cells lacking CD300LF
B6/CD300LF	Susceptible	C57BL/6J WT CD300LF
IL/CD300LF	Resistant	I/LnJ WT CD300LF
B6/CD300LF – 7aa	Attenuated	B6/CD300LF mutant with I/LnJ 7aa
IL/CD300LF – 7aa	Susceptible	IL/CD300LF mutant with B6 7aa
B6/CD300LF S174G	Susceptible	Putative glycosylation mutant
IL/CD300LF G174S	Resistant	Putative glycosylation mutant
B6/CD300LF T131A	Susceptible	Putative glycosylation mutant
IL/CD300LF A131T	Resistant	Putative glycosylation mutant
B6/CD300LF – 4aa	Attenuated	B6/CD300LF mutant with I/LnJ 4aa
IL/CD300LF – 4aa	Susceptible	IL/CD300LF mutant with B6 4aa

Table 2.2. Alignment of CD300LF Coding Sequences.

Available protein sequences for CD300LF in the indicated mouse line, grouped based on their similarity to the sequence of C57BL/6J or I/LnJ CD300LF. Asterisks indicate an amino acid that is common among all sequences.

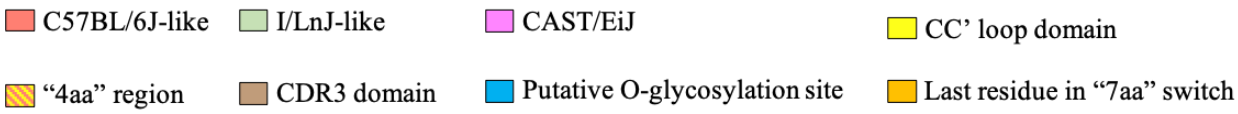

Table 2.2 Continued

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	**20**	21	22	23	24	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	33	34	**35**
C57BL/6J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
AKR/J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
BALB/cJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
C57BL/6NJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
DBA/2J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
FVB/NJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
NOD/ShiLtJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
NZO/HlLtJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
WSB/EiJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	E	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
I/LnJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
C3H/HEJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
CBA/J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
LP/J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
PWK/PhJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	V
A/J	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	G	P	E	E	D	P	V	T	G	P	E
CAST/EiJ	M	H	L	S	L	L	V	P	F	L	F	W	I	T	G	C	C	T	A	Q	D	P	V	T	-	-	-	-	-	-	-	-	G	P	E
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*									*	*	

Strain	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	**64**	**65**	**66**	**67**	**68**	**69**	70
C57BL/6J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
AKR/J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
BALB/cJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
C57BL/6NJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
DBA/2J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
FVB/NJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
NOD/ShiLtJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
NZO/HlLtJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
WSB/EiJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	Q	G	V	P	Q	R	S
I/LnJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
C3H/HEJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
CBA/J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
LP/J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
PWK/PhJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
A/J	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	A	Y	W	K	S
CAST/EiJ	E	V	S	G	Q	E	Q	G	S	L	T	V	Q	C	R	Y	T	S	G	W	K	D	Y	K	K	Y	W	C	R	G	V	P	Q	R	S
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*							*

Strain	71	**72**	**73**	74	75	76	77	78	**79**	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	**103**	104	105
C57BL/6J	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
AKR/J	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
BALB/cJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
C57BL/6NJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
DBA/2J	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
FVB/NJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
NOD/ShiLtJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
NZO/HlLtJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
WSB/EiJ	C	K	T	L	V	E	T	D	A	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
I/LnJ	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
C3H/HEJ	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
CBA/J	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
LP/J	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
PWK/PhJ	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
A/J	C	E	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	T	M	E
CAST/EiJ	C	D	I	L	V	E	T	D	K	S	E	Q	L	V	K	K	N	R	V	S	I	R	D	N	Q	R	D	F	I	F	T	V	I	M	E
	*			*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*

Strain	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	**122**	123	**124**	125	126	127	128	129	130	**131**	132	133	134	135	136	**137**	**138**	**139**	**140**
C57BL/6J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
AKR/J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
BALB/cJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
C57BL/6NJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
DBA/2J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
FVB/NJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
NOD/ShiLtJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
NZO/HlLtJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
WSB/EiJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	G	G	L	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
I/LnJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	A	V	N	I	G	P	A	I	Q	V
C3H/HEJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	A	V	N	I	G	P	A	I	Q	V
CBA/J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	A	V	N	I	G	P	A	I	Q	V
LP/J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	A	V	N	I	G	P	A	I	Q	V
PWK/PhJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	T	G	P	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
A/J	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	A	V	N	I	G	P	-	-	-	-
CAST/EiJ	D	L	R	M	S	D	A	G	I	Y	W	C	G	I	T	K	A	G	P	D	P	M	F	K	V	T	V	N	I	G	P	A	I	Q	V
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*		*	*	*	*	*	*		*	*	*	*	*				

Table 2.2 Continued

Strain	**141**	**142**	**143**	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	**168**	169	170	171	172	173	**174**	175
C57BL/6J	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
AKR/J	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
BALB/cJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
C57BL/6NJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
DBA/2J	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
FVB/NJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
NOD/ShiLtJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
NZO/HlLtJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
WSB/EiJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	S	Y
I/LnJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
C3H/HEJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
CBA/J	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
LP/J	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
PWK/PhJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
A/J	-	-	-	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	M	F	P	T	L	T	G	Y
CAST/EiJ	P	I	T	V	P	T	M	P	P	I	T	S	T	T	T	I	F	T	V	T	T	T	V	K	E	T	S	V	F	P	T	L	T	S	Y
				*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*		*

Strain	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	**193**	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	**210**
C57BL/6J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
AKR/J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
BALB/cJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
C57BL/6NJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
DBA/2J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
FVB/NJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
NOD/ShiLtJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
NZO/HlLtJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
WSB/EiJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
I/LnJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
C3H/HEJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
CBA/J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
LP/J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
PWK/PhJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	A
A/J	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	V	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
CAST/EiJ	Y	S	D	N	G	H	G	G	G	D	S	G	G	G	E	D	G	I	G	D	G	F	L	D	L	S	V	L	L	P	V	I	S	A	V
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	

Strain	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245
C57BL/6J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
AKR/J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
BALB/cJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
C57BL/6NJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
DBA/2J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
FVB/NJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
NOD/ShiLtJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
NZO/HlLtJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
WSB/EiJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
I/LnJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
C3H/HEJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
CBA/J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
LP/J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
PWK/PhJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
A/J	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
CAST/EiJ	L	L	L	L	L	L	V	A	S	L	F	A	W	R	M	V	R	R	Q	K	K	A	A	G	P	P	S	E	Q	A	Q	S	L	E	G
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Strain	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280
C57BL/6J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
AKR/J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
BALB/cJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
C57BL/6NJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
DBA/2J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
FVB/NJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
NOD/ShiLtJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
NZO/HlLtJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
WSB/EiJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
I/LnJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
C3H/HEJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
CBA/J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
LP/J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
PWK/PhJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
A/J	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
CAST/EiJ	D	L	C	Y	A	D	L	S	L	K	Q	P	R	T	S	P	G	S	S	W	K	K	G	S	S	M	S	S	S	G	K	D	H	Q	E
	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Table 2.2 Continued

Strain	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315
C57BL/6J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
AKR/J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
BALB/cJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
C57BL/6NJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
DBA/2J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
FVB/NJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
NOD/ShiLtJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
NZO/HlLtJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
WSB/EiJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
I/LnJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
C3H/HEJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
CBA/J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
LP/J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
PWK/PhJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
A/J	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
CAST/EiJ	E	V	E	Y	V	T	M	A	P	F	P	R	E	E	V	S	Y	A	A	L	T	L	A	G	L	G	Q	E	P	T	Y	G	N	T	G
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Strain	316	317	318	319	320	**321**	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	**345**
C57BL/6J	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
AKR/J	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
BALB/cJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
C57BL/6NJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
DBA/2J	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
FVB/NJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
NOD/ShiLtJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
NZO/HlLtJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
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I/LnJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	H
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A/J	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	H
CAST/EiJ	C	P	I	T	H	V	P	R	T	G	L	E	E	E	T	T	E	Y	S	S	I	R	R	P	L	P	A	A	M	P
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Appendix II: Figures

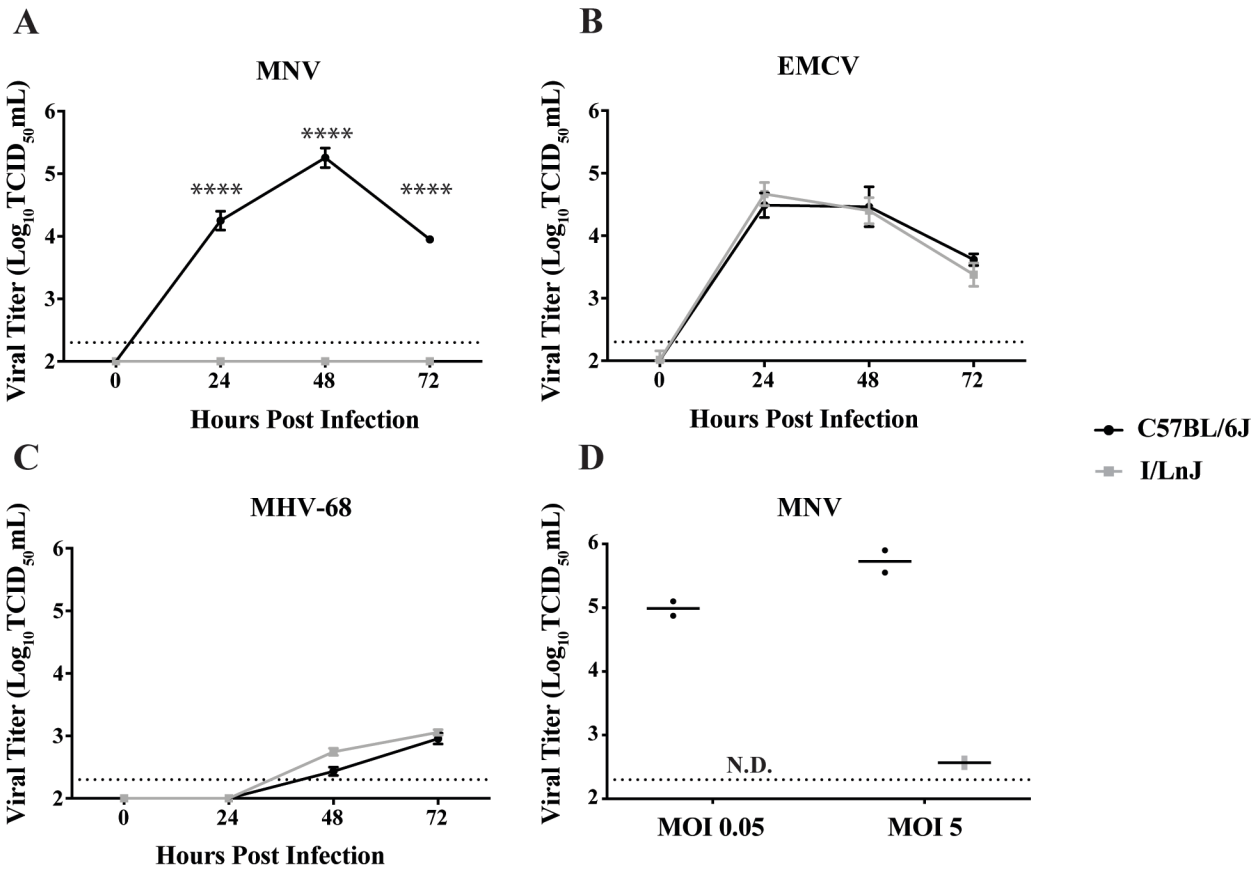

Figure 2.1. I/LnJ BMDMs resist MNV infection. Analysis of replication kinetics of MNV (A), EMCV (B), and MHV-68 (C) in C57BL/6J and I/LnJ BMDMs. Cells were inoculated at a multiplicity of infection (MOI) of 0.05 $TCID_{50}$/cell and harvested at the indicated time points to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM. (D) Analysis of MNV replication at 24 hours-post-infection in C57BL/6J and I/LnJ BMDMs after inoculation at an MOI of 0.05 and 5 TCID50/cell. The experiment was done twice, and data are presented as dots with mean as a bar. N.D., Not Detected.

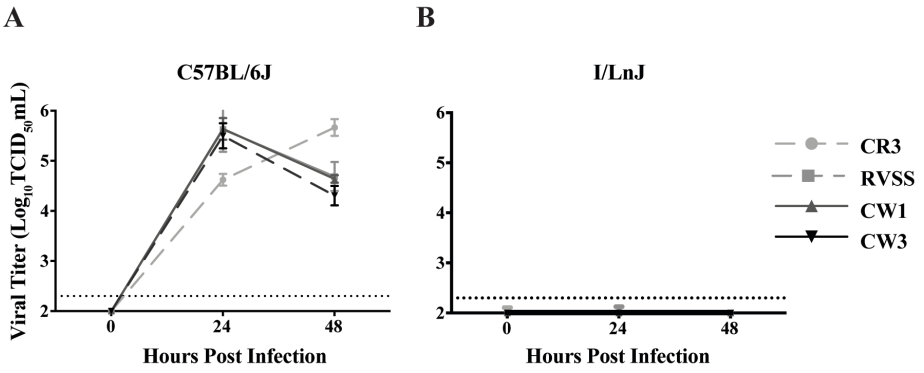

Figure 2.2. I/LnJ BMDMs are resistant to different strains of MNV. Analysis of replication kinetics of four different strains of MNV: CR3, RVSS, CW1, and CW3 in C57BL/6J (A) or I/LnJ (B) BMDMs. Cells were inoculated at an MOI of 0.05 $TCID_{50}$/cell and harvested at the indicated time points to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM.

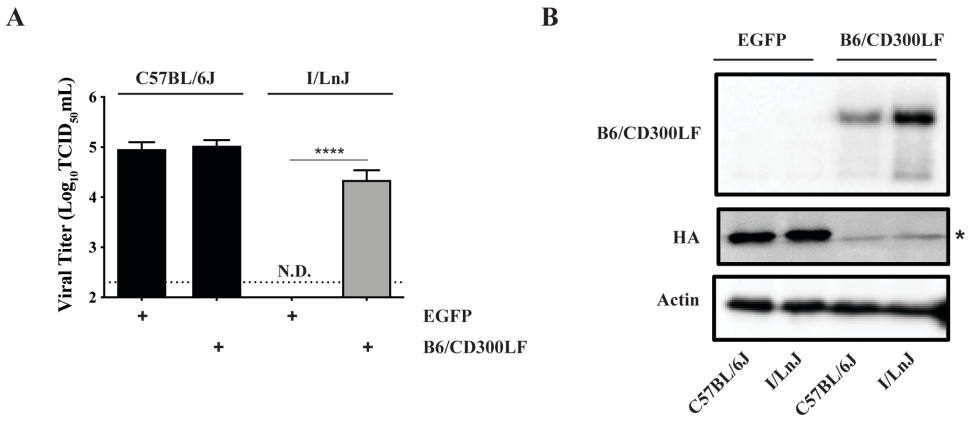

Figure 2.3. I/LnJ BMDMs expressing C57BL/6J CD300LF are susceptible to MNV infection. (A) Comparison of MNV replication in C57BL/6J and I/LnJ BMDMs transduced with lentivirus expressing EGFP (control) or the C57BL/6J allele of CD300LF. Transduced cells were inoculated with MNV at an MOI of 0.05 $TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM. N.D., Not Detected. (B) A representative Western blot of C57BL/6J and I/LnJ BMDMs transduced with lentivirus expressing EGFP (control) or the C57BL/6J allele of CD300LF as detected with goat anti-mouse CD300LF (R&D systems, AF2788). * indicates a non-specific signal in the blot. Actin is included as a loading control. N = 3 replicates.

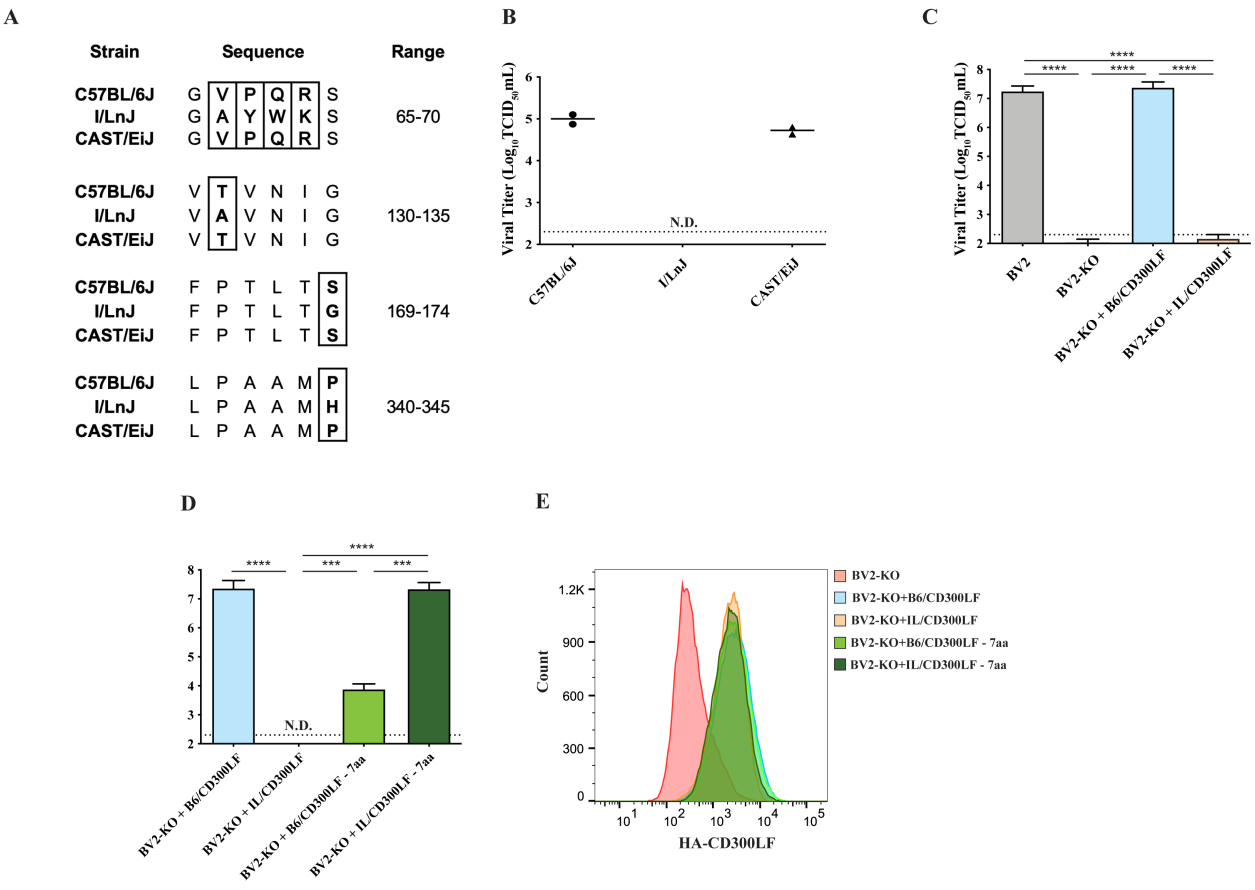

Figure 2.4. Identification of seven amino acids determining the functionality of CD300LF as an MNV receptor. (A) The amino acids sequence for the CD300LF of the indicated mouse strains at the indicated ranges. Boxed sequences denote the amino acid of CAST/EiJ CD300LF that is different from I/LnJ CD300LF but identical to C57BL6J CD300LF. (B) Comparison of MNV replication in C57BL/6J, I/LnJ, or Cast/EiJ BMDMS. Cells were inoculated with MNV at an MOI of 0.05 $TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. The experiment was done twice, and data are presented as dots with mean as a bar. N.D., Not Detected. (C) Comparison of MNV replication in BV2 cells. BV2 cells, BV2 cells with endogenous *Cd300lf* knocked out (BV2-KO) , or BV2-KO cells transduced with lentiviruses expressing either C57BL/6J or I/LnJ CD300LF were inoculated with MNV at an MOI of 5

Figure 2.4 Continued

$TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM. (D) Comparison of MNV replication in BV2-KO cells transduced with WT or 7aa swapped constructs. Cells were inoculated with MNV at an MOI of 5 $TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM. N.D., Not Detected. (E) Analysis of surface expression of transduced CD300LF of the indicated background in BV2-KO cells. A representative image from three independent experiments.

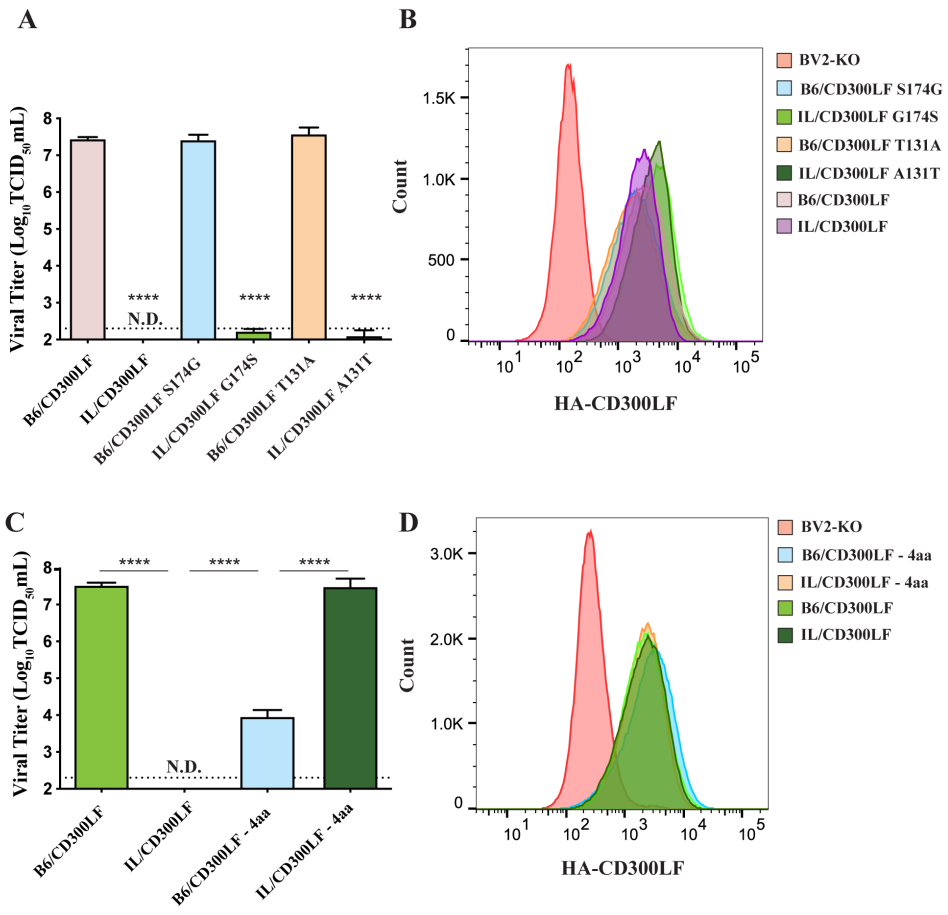

Figure 2.5. A cluster of four amino acids determines the functionality of CD300LF as an MNV receptor. (A and C) Analysis of MNV replication in BV2-KO cells transduced with the indicated mutant alleles of CD300LF. Cells were inoculated with MNV at an MOI of 5 $TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. All experiments were done in triplicates and data are presented as mean ± SEM. N.D., Not Detected. (B and D) Analysis of surface expression of HA-tagged CD300LF mutants in the indicated cells. Data shown are representative plots from three independent experiments. Asterisks indicate significance compared to "B6/CD300LF"; ns = not significant.

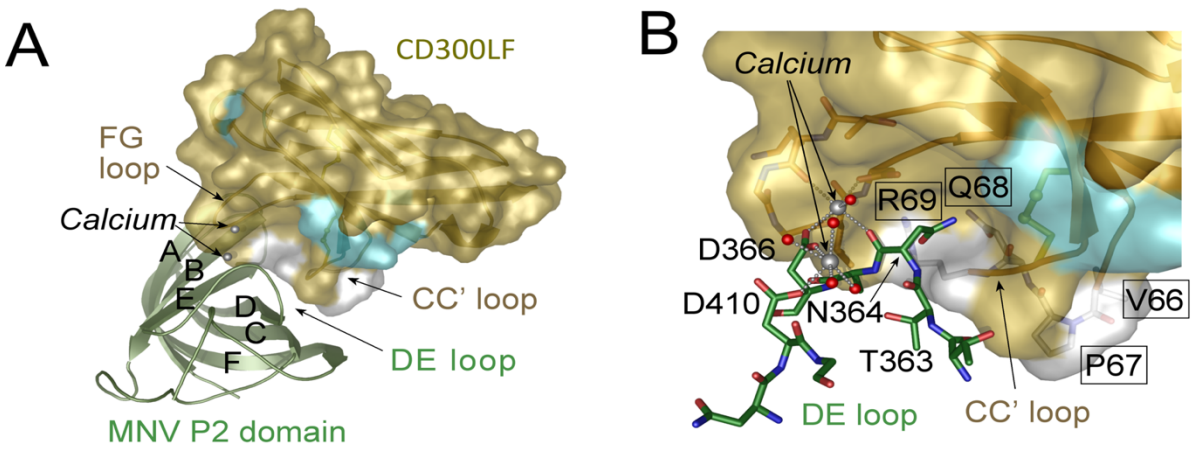

Figure 2.6. Residues critical for MNV infection occur in the CC'-loop flanking the phospholipid binding pocket of CD300LF. (A) Model showing the orientation of the MNV major capsid protein (VP1) P2 subdomain relative to CD300LF in complex with bound calcium ion. Positions that differ in sequence between B6/CD300LF and IL/CD300LF are colored white or cyan on the B6/CD300LF surface. (B) Close-up of the CD300LF–P domain complex showing the side chain of Asn364 from the P domain DE loop binding to CD300LF in a pocket made by the CC′ loop. The carbonyl oxygen of Asn364 coordinates with a calcium ion at the binding interface. A second calcium ion supports the P domain DE loop, holding it against the binding pocket, by bridging Asp410 and Asp366. The four residues in the CC' loop that differ between B6/CD300LF and IL/CD300LF have been labeled with residue numbers in black boxes and their side-chain carbon atoms have been colored in white. The area contributed by these residues is in white on the solvent accessible surface. Most of the hydrophobic pocket shown to bind PC choline is contributed by the CD300LF residues Q68 and R69. Binding of the viral P2 domain to CD300LF is metal ion dependent and requires the DE loop of P2 to associate with the FG and CC' loops of CD300LF through a network of metal ion coordination and hydrogen bonding centered on Asn364. Substitution of the four critical residues in the CC' loop (VPQR to AYWK) almost certainly

Figure 2.6 Continued

perturbs conformation of the CC' loop, disrupting the receptor binding interface. The four consecutive amino acids in the CC' loop correspond to positions 39-42 in the B6/CD300LF crystal structure (PDB accession 6E48).

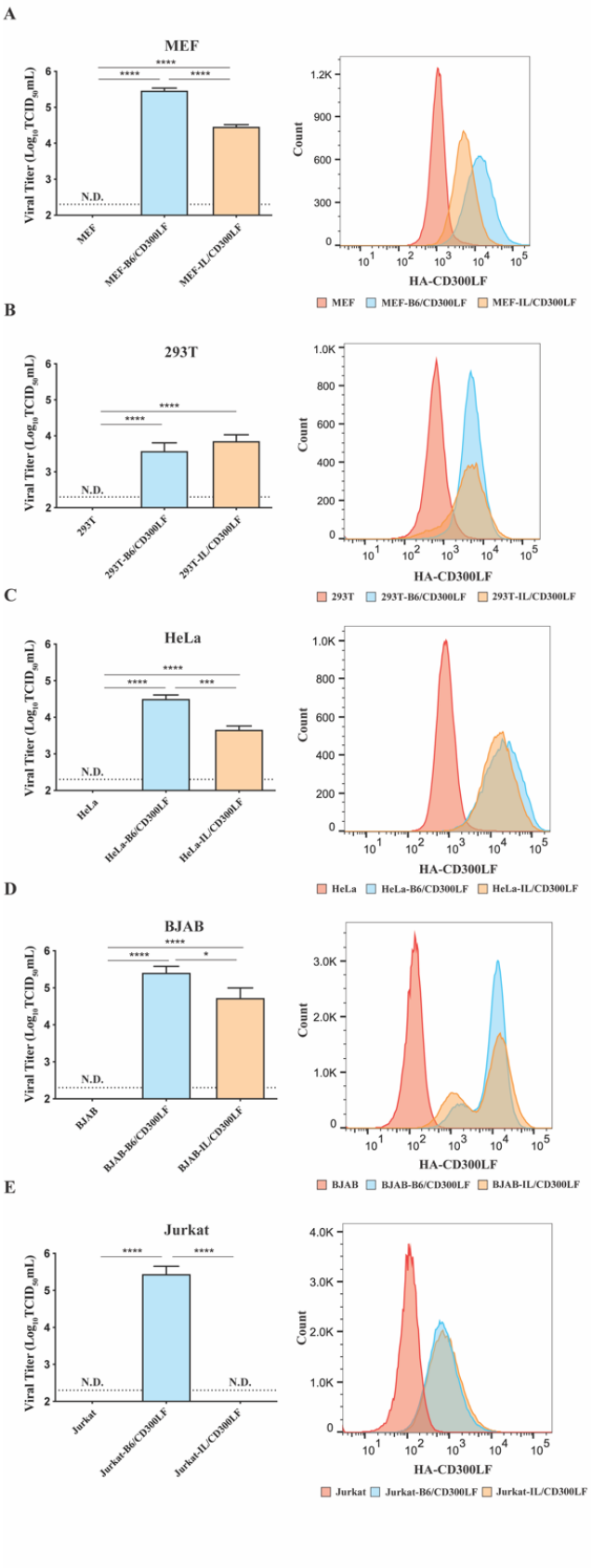

Figure 2.7. I/LnJ CD300LF can function as an MNV receptor in a cell type-dependent manner. (Left) Analysis of MNV replication in MEF (A), 293T (B), HeLa (C), BJAB (D), and Jurkat (E) cell lines transduced with the indicated allele of CD300LF. Cells were inoculated with MNV at an MOI of 5 (A, B, C) or 0.1 (D, E) $TCID_{50}$/cell and harvested at 24 hours-post-infection to titer infectious viruses via $TCID_{50}$ assay in BV2 cells. Untransduced cells were included as a control. All experiments were done in triplicates and data are presented as mean ± SEM. N.D., Not Detected. (Right) Analysis of surface expression of HA-tagged CD300LF alleles in the indicated cells. A representative plot from three independent experiments is shown.

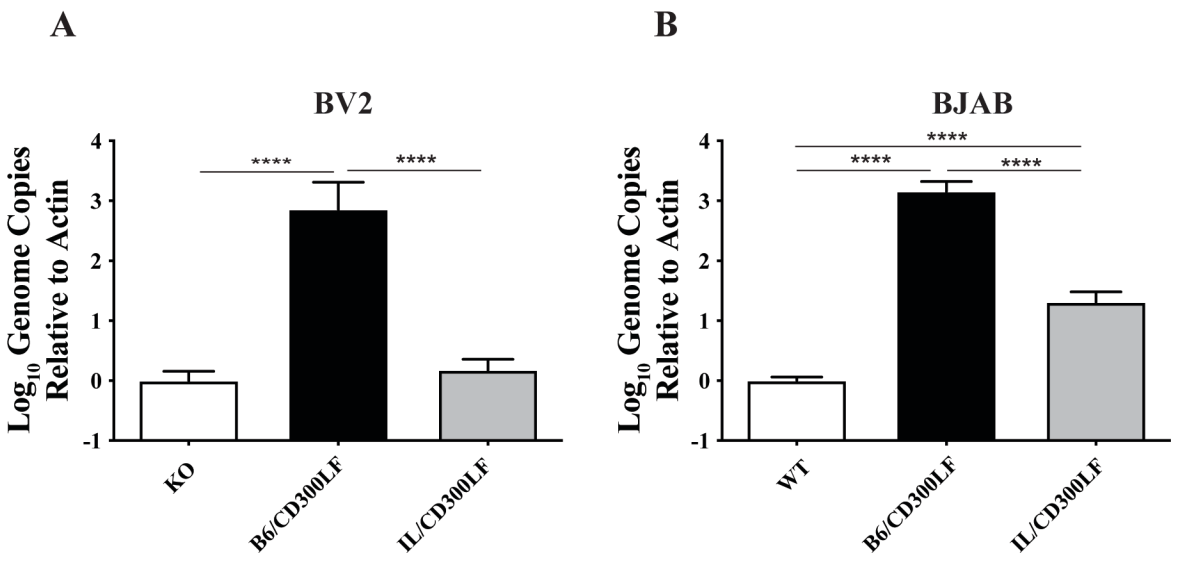

Figure 2.8. I/LnJ CD300LF supports MNV attachment in a cell-type dependent manner. Analysis of MNV binding to BV2 cells (A) or BJAB cells (B) expressing the indicated allele of CD300LF. Cells were inoculated at 4°C at an MOI of 2 $TCID_{50}$/cell. 1 hour-post-infection, cells were washed 3 times with ice-cold PBS to remove unbound virus. Cells were lysed with 1mL TRI-Reagent (Sigma) and genome equivalents were determined via qPCR. All experiments were done in triplicate and presented as mean ± SEM. Bars represent ratio of MNV genomes to actin compared to mean KO (BV2) or WT (BJAB).

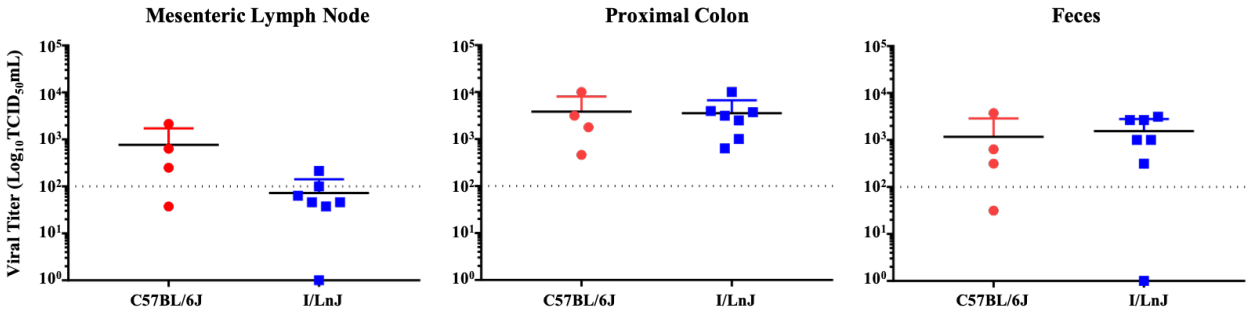

Figure 2.9. I/LnJ mice are susceptible to MNV infection Viral titer detected in homogenates of the indicated organ 3 days post per-oral inoculation of mice of the indicated genotype with 1 x 10^4 $TCID_{50}$ units. Organs were flash frozen upon harvesting and viral titer was determined by TCID50 analysis in BV2 cells. Each point represents a sample from an individual mouse.

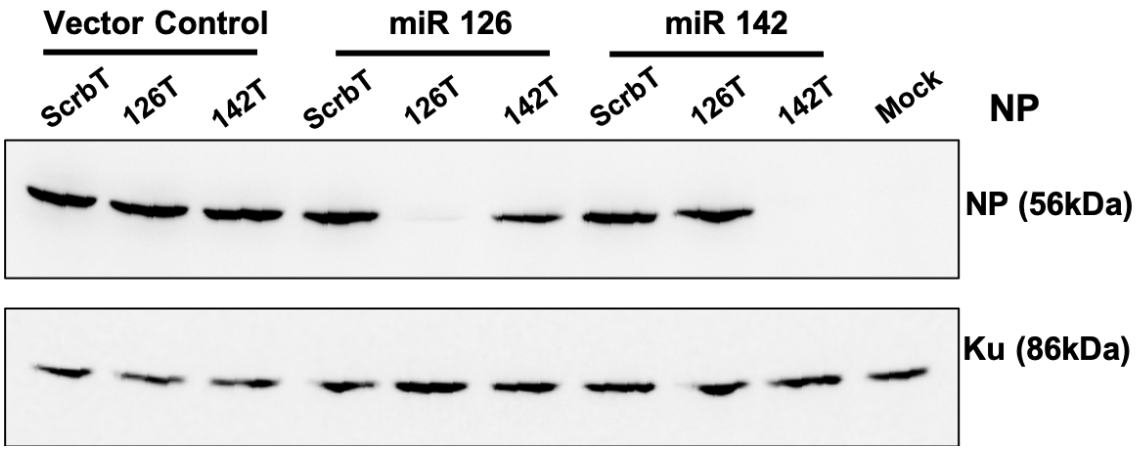

Figure 3.1. Targeted H7N7 NP production is restricted upon co-expression of complementary miRNA. A representative Western blot of H7N7 NP production in 293T cells transfected with plasmids expressing the indicated recombinant NP segment and the indicated miRNA. Cells were harvested 48-hours post transfection. Ku is included as a loading control. N = 3 replicates.

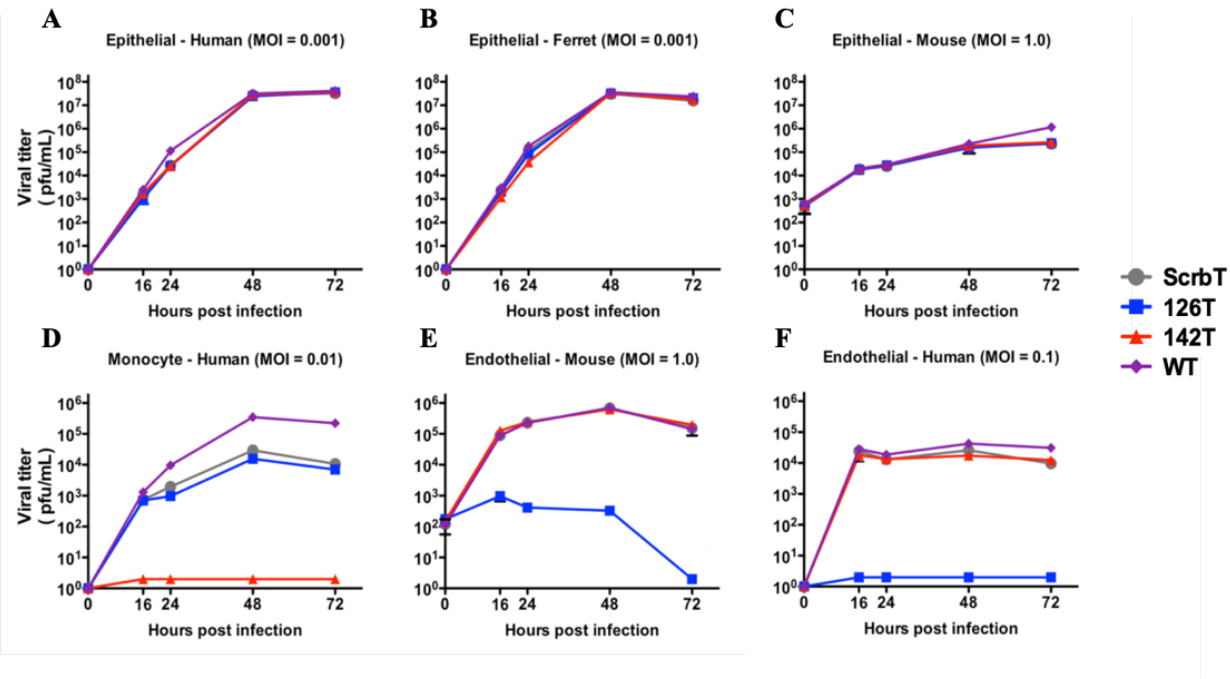

Figure 3.2. Targeted H7N7 replication is restricted *in vitro* in a cell type-specific manner. Analysis of replication kinetics of the indicated H7N7 variant in the indicated cell type. Cells were inoculated at the indicated multiplicity of infection (PFU). Samples of supernatant were harvested at the indicated time point to titer infectious viruses via plaque assay in MCDK cells. All experiments were done in triplicates and data are presented as mean ± SEM.

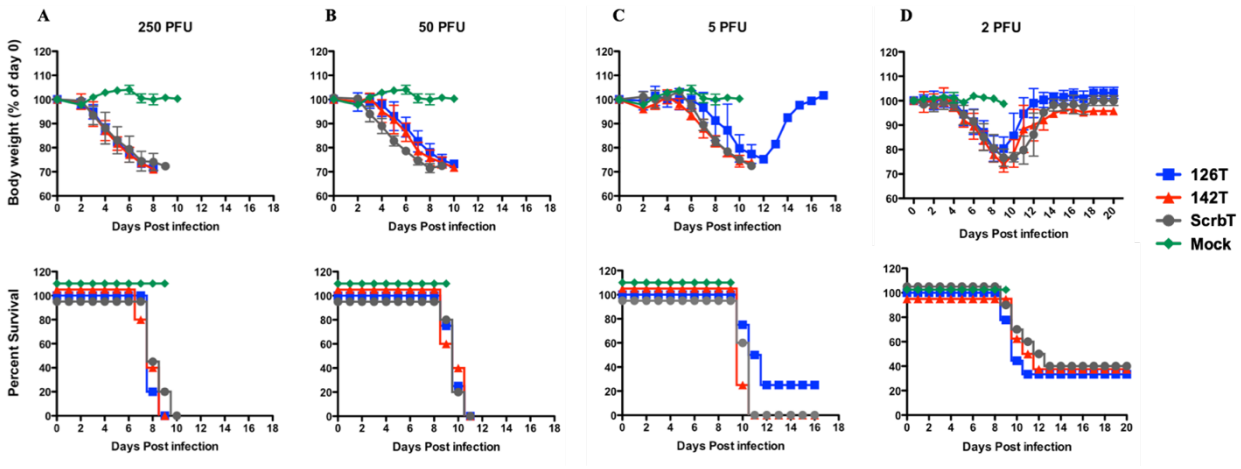

Figure 3.3. Infection with targeted H7N7 is comparable to infection with control virus. Weight change and survival of C57BL/6 mice infected with the indicated virus at the indicated dose. Ten mice per dose per virus were monitored over the course of 10 days. Mice who reached 70% of their starting body weight were sacrificed.

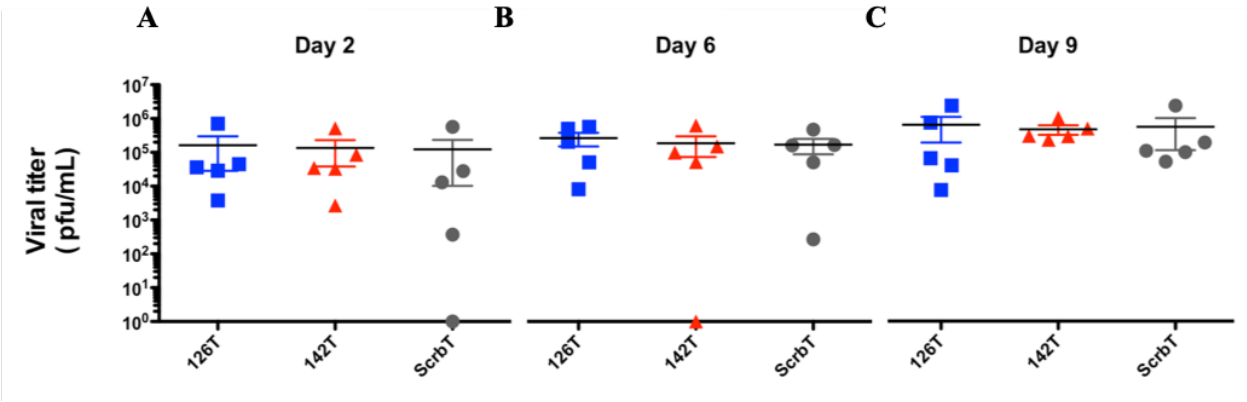

Figure 3.4. H7N7 variants replicate to similar titers in mouse lungs. Titers of the indicated virus in lung homogenates from C57BL/6 mice. On the indicated day, mice were sacrificed, and lungs were extracted and immediately flash frozen. Lungs were homogenized in 1mL sterile DMEM + 10% FBS and immediately tittered via plaque assay. Each data point represents a single animal.

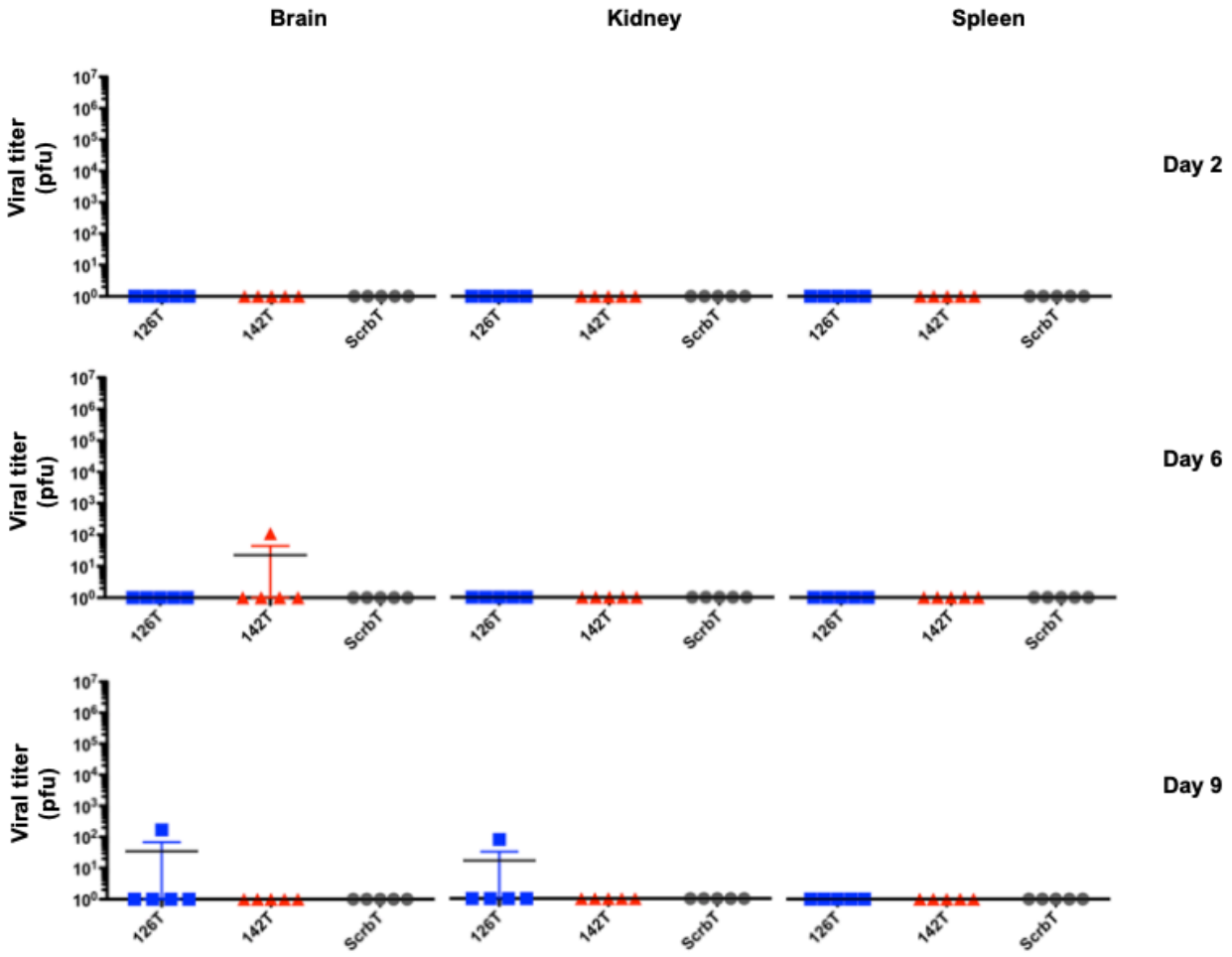

Figure 3.5. H7N7 extravasation to non-pulmonary tissue is rare. C57BL/6J mice infected with the indicated strain of targeted H7N7 were sacrificed on the indicated day. Organs were immediately extracted and flash frozen. To assess viral titer, organs were homogenized and supernatant was used for plaque assay. Individual data points represent organs extracted from a single mouse.