THE ROLE OF AFFINITY AND MECHANISM OF VIRUS ATTACHMENT TO CELLS IN ANTIBODY-DEPENDENT ENHANCEMENT AND NEUTRALIZATION OF FLAVIVIRUSES

A Dissertation
submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and Immunology

By

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Washington, DC
June 13, 2014
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ABSTRACT

Flaviviruses are RNA viruses that cause significant morbidity and mortality worldwide. There are currently no specific therapeutics or clinically licensed vaccines available for a number of flaviviruses, including dengue virus (DENV) and West Nile virus (WNV). Protection against flaviviruses is most closely correlated with a robust neutralizing antibody response. Neutralization is governed by a stoichiometric threshold. Virus particles engaged by a number of antibody molecules that exceeds this threshold are neutralized, while those engaged by fewer remain infectious. If these infectious particles have enough antibody bound, they may paradoxically show enhanced binding and infectivity on cells expressing Fc-γ receptors (FcRs), which bind to antibody. This phenomenon is known as antibody-dependent enhancement (ADE), and it has been implicated in more severe forms of disease during DENV infection.

The number of antibodies required per virion for both neutralization and enhanced binding have been estimated using model cell lines, but the role of the target cell in modulating these numbers is unknown. In this dissertation, we examine the contribution of variables that affect the functional affinity of virus or virus-bound antibody for cellular attachment factors. Any parameters tested that were predicted to increase the efficiency of FcR-mediated virus binding to cells decreased the number of antibodies required to mediate ADE. The neutralizing potency of antibodies was increased only in situations
where high affinity binding occurred between virus bound antibodies and FcRs. In contrast, changes in cellular expression of either FcRs or antibody-independent attachment factors had no effect on neutralization. This discrepancy can partially be explained by the clustered nature of virus attachment factors, suggesting that the function of antibodies may be dependent on the location of virus-cell contact.
DEDICATION

This work is dedicated to all those who teach, directly or otherwise.

Especially, this is for those teachers who go above and beyond. The ones who may not even know in the moment that they are delivering a life-altering, career-redefining lesson. These are the people at the sharp turns on the path of life, and for me they are: Susan Jacobs, Terry Stanley, Caryn McDermott, Faith Oi, Pauline Lawrence, Carl Barfield, and Jean Lim—each of whom played an important role in redirecting my path.

It is also for the most important teachers of all: those who taught me to love to learn almost as quickly as they helped me learn to love: Marla and John Obara, Phyllis and Lamar Miller, Betty Obara, and Marcell and Mia Newman.
ACKNOWLEDGEMENTS

This work would not have been possible without the guidance and mentorship of many people. I’m deeply thankful for the countless hours, emotional upheavals, and sleepless nights many had to contribute to help me grow into a scientist:

My advisor, Ted Pierson.

The Faculty of the Department of Microbiology and Immunology at Georgetown University, particularly R. Padmanabhan, John Casey, and Brent Korba.

The Faculty of the National Institute of Allergy and Infectious Diseases, particularly Philip Murphy, Alison McBride, and the members of the Laboratory of Viral Diseases.

The Office of Intramural Training and Education of the National Institutes of Health.

My colleagues and classmates, especially Swati Mukherjee, Margery Smelkinson, Wesley Stepp, Brittany Griffin, Jorge Mendez-Rios, Kevin Tosh, and Abhi Kole.

My larger support network, which includes far too many friends and family to name here, especially Frank Kraemer, Mike Jones, Brent Finklea, Chase Wiley, Kate Hardeman, and Ryan Merkel.

Without any of these people, this work would not have been possible—every person on this list is individually responsible at some point for keeping me from giving up.

Many thanks,

Christopher J. Obara
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CHAPTER 1

INTRODUCTION

1.1 Flavivirus Biology

1.1.1 Phylogeny and Epidemiology

The genus flavivirus is a geographically widespread group of genetically related viruses, many of which are capable of causing severe disease in humans (1). The viruses are divided into phylogenetic groups based on the genetic sequence of individual proteins or full-length genomes (2, 3). Flaviviruses are generally transmitted through the bite of an infected arthropod. The earliest division in the phylogenetic tree generally separates the viruses by their mode of transmission (Fig. 1.1) (4). Tick-borne flaviviruses are mostly associated with infection of rodents and primarily are found within the old world (5). In contrast, mosquito-borne flaviviruses (MBFVs) are prevalent across six continents and will be the focus of the majority of this dissertation (6). There are also a number of viruses within each lineage for which no known vector exists, but little is known of these viruses, and they are not associated with widespread disease in humans (1).

The MBFVs contain a number of rapidly emerging threats to public health, including West Nile virus (WNV), dengue virus (DENV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) (3). The rapid expansion or reemergence of these viruses may partially be the result of use of a mosquito as the vector, as there are endemic mosquito populations over much of the populated world that can serve as areas of easy expansion (6). Additionally, many species of mosquitoes that vector flaviviruses are closely tied to urbanization and human development. The increased transportation and
mobility associated with human life in the modern era may therefore contribute to the rapid expansion of flaviviruses (6, 7).

An example of rapid flavivirus expansion is the introduction of West Nile virus (WNV) into the Western Hemisphere in 1999. While the specific mechanism of introduction remains unknown, the virus caused a severe outbreak in New York City and the surrounding areas, apparently becoming entrenched in the local bird populations (8, 9). From there, it rapidly spread across the United States and established itself as an endemic pathogen within four years (10). It now maintains a stable infection cycle within North American birds, punctuated by periodic outbreaks in the human population (11). WNV is currently the primary cause of epidemic encephalitis in the United States (12).

Effective diagnosis of MBFV infections through serology is made difficult by the frequent induction of weakly cross-reactive antibodies, which can confound analysis (13-17). Despite the frequent nature of cross-reactivity, infection with a single MBFV does not ensure long-term protection against other MBFVs. This is clearly illustrated in the context of DENV infection. DENV exists as four antigenically distinct serotypes, of which multiple may simultaneously circulate within a geographical area. Infection with any of the four serotypes generally provides life-long, serotype-specific immunity; but usually does not provide long-lasting protection against the other serotypes (18). This is probably due at least in part to the relatively low conservation of sequence between serotypes in the proteins that make up the viral particle, and has served as a significant hurdle for the development of a tetravalent vaccine formulation (reviewed by (19)).

With DENV alone infecting nearly 400 million people annually worldwide, the impact of MBFVs on public health and world economic capacity has become quite severe.
(20, 21). Despite this, there are still no clinically licensed specific antiviral therapies for MBFVs, and there are a number of MBFVs for which vaccines are still not available for human use (22-24). The remainder of this dissertation will focus on two such MBFVs as models, WNV and DENV, though much of the information is more broadly applicable to flaviviruses in general.

1.1.2 Particle structure

Flaviviruses are small, spherical particles approximately 50 nm in diameter. The particle is a lipoprotein complex that is formed from the viral structural proteins, an endoplasmic reticulum (ER)-derived lipid bilayer, and a single, positive-sense RNA genome. Three structural proteins are associated with the viral particle, the capsid (C), pre-membrane/membrane (prM), and envelope (E) proteins. The C protein is located inside the lipid bilayer with the genome, while the prM and E proteins are located on the outside of the particle (25). The E protein is believed to be the primary target for neutralizing antibodies against flaviviruses (26).

The E protein is an elongated, three domain structure that orchestrates the assembly of virions and their entry into cells (Fig. 1.2) (27-29). Flaviviruses assemble at the ER membrane as immature virions that incorporate sixty heterotrimeric spikes of E proteins in complex with the premembrane protein (prM) (25, 30). During egress though the trans-Golgi network, pH-dependent changes in the conformation of prM and E allow for cleavage of prM by a cellular furin-like protease (31-33). Cleavage of prM is required for infectivity of the virion and defines the maturation step in the virus lifecycle (34). E proteins on the resulting mature virus particles exist as anti-parallel dimers organized into a dense herringbone arrangement (35).
The three domains of each E protein are connected by flexible hinge regions (reviewed by (25)). Studies with a variety of computational approaches to molecular chemistry have strongly implicated that at room temperature and above molecules are subject to substantial molecular breathing (36). In the context of proteins designed with highly flexible hinges, this effect is amplified. Consequently, the potential binding sites for antibodies or receptors on the virus particle may be initially obscured and become accessible with a frequency that is both time and temperature dependent (37, 38).

As an added level of complexity, flavivirus maturation is an inefficient process (39-42). Consequently, the heterogeneity of prM cleavage can have a substantial effect on both the antigenic surface exposed on the virion and the ability of the virion to bind to different cells. The arrangement of the domains I (DI) and II (DII) is most strongly affected (Fig. 1.2), and consequently most antibodies identified to date which bind to DI or DII show a maturation state-sensitive phenotype (41, 43). In contrast, the accessibility of domain III (DIII) to the solvent is mostly unaffected by the presence or absence of prM (44). A number of antibodies against the DIII lateral ridge (DIII-LR) have been described for both WNV and DENV, and some of these neutralize infection in a maturation-state independent manner (45, 46). These antibodies are also less sensitive to structural dynamics, probably due to the high accessibility of these epitopes even without dynamic motion (37). For this reason, most of the studies performed throughout this dissertation will be executed using these anti-DIII-LR antibodies to simplify analysis.

For many flaviviruses, the prM and E proteins are also glycoproteins. The exact locations and numbers of N-linked glycosylation sites vary from strain to strain. This is of particular importance with flaviviruses, as most of the attachment and entry factors
that have been described to date are lectins (see Section 1.1.3). They bind to the N-linked glycans on the virion, and binding is at least in part controlled by the way the sugar chains are arrayed in space (47). Many of these lectins are highly dependent on the processing state of the sugar, which can be affected by the structural arrangement of the protein during trafficking through the Golgi. Insects lack the enzymes that mediate many complex sugar modifications, suggesting that this component of the viral particle structure may vary when derived from an insect cell as compared to a mammalian cell. N-linked glycans on the E protein have been implicated in affecting the infectious properties of the virus (48).

1.1.3 Attachment and Entry

Flavivirus infection of a target cell begins with the mediation of stable attachment (Fig. 1.3). In many situations this is the primary block to flavivirus infection (see Chapter 2). Attachment factors promote the infection of flaviviruses by increasing the average duration of contact between the cell and the virion. This increases the number of opportunities for the subsequent steps of the viral entry pathway to occur. In contrast, virus receptors are generally implicated as promoting required steps in the entry pathway, but for flaviviruses this distinction is not well characterized. This is in part due to the fact that the cell biology of flavivirus entry is poorly understood (reviewed by (49, 50)).

A number of cellular factors have been suggested as either attachment factors or receptors for flaviviruses, but none of these has been clearly demonstrated to be both necessary and sufficient for infection (reviewed by (51)). The signaling components of several of those implicated as receptors seem to be dispensable (e.g., (52, 53)).
Attachment factors or receptors for flaviviruses can be roughly divided into three categories by the mechanisms that they use to mediate attachment.

Glycosaminoglycans (GAGs) are extracellular proteins that may concentrate virus close to the surfaces of cells. Binding depends upon positively charged residues in the E protein (54), and passaging virus in cell culture enhances this property (55). The role for these proteins in mediating attachment to cells in vivo may be tissue specific, as GAGs are most highly expressed in tissues, though many known targets of flavivirus infection are blood cells. However, pathways of frequent immune cell migration in tissues are enriched in GAGs, and it has been suggested that flaviviruses may bind preferentially to these areas to increase the frequency of interaction with these cells (56).

Direct attachment factors are cell surface proteins that bind directly to the virus particle. Most of the attachment factors described for flaviviruses to date are direct attachment factors. As discussed above, there is an important role for cellular lectins in mediating attachment of some flaviviruses via N-linked glycans on the E protein. The c-type lectins DC-SIGN (CD209) and DC-SIGNR (CD209L) have been implicated in mediating attachment of a number of virus families, including flaviviruses (see Chapter 2); and the related human mannose receptor (CD206) has been implicated in affecting DENV infection of macrophages (57). A more recently described group of direct attachment factors that have been described for flaviviruses are the TIM family of phosphotidylserine receptors. These bind directly to lipids incorporated into the membrane of flavivirus particles, and may also possess a signaling contribution to virus entry (58).
Attachment can also be mediated by use of indirect virus attachment factors. These cell surface proteins do not bind directly to the virus particle, but rather bind to the particle via a molecular intermediate. The most highly cited example of this is antibody-dependent binding of virus to cells via Fc-γ receptors (FcRs), which is discussed in more detail in Section 1.4. *In vitro* experiments show that human complement receptors can mediate attachment of virus to cells in a similar manner (59, 60), and the recently described TAM family of phosphotidylserine receptors binds to the virus membrane by use of a bridging molecule (ProS or Gas6) (reviewed in (50)). Additionally, a number of studies have shown that mosquito saliva enhances the infectivity of a variety of flaviviruses (56, 61, 62). The mechanisms for this are still not well characterized, but one possible explanation is an increase in the efficiency of attachment due to the bridging effects of some component in mosquito saliva.

Additionally, analysis of entry factors is complicated by variation from cell to cell or virus to virus. For example, the \( \alpha V \beta 3 \) integrin has been shown to be a receptor for both JEV and a lineage II strain of WNV in Vero cells (63). In contrast, a lineage I strain of WNV infects mouse embryonic fibroblasts in an \( \alpha V \beta 3 \) integrin-independent manner (64). It has been suggested that virus attachment may be a relatively nonspecific process, and individual virus particles can find their way into preformed pits for rapid internalization (65). This would be consistent with the observation that some poorly permissive cells can become susceptible to infection if a means of stable attachment is provided (see Chapters 2 and 3).

It is clear, however, that after attachment flavivirus entry is dependent upon low pH and occurs in the endosome. In most situations, this process is mediated by clathrin
(66-68); though clathrin-independent pathways have been described in some specific cases (69, 70). For DENV, at least, this process also requires the specific anionic lipid content of the late endosome in mammalian cells (71). The combination of low pH and anionic lipids induce a series of well-characterized structural rearrangements in the E protein (reviewed by (25, 72)). These mediate fusion of the viral and endosomal membranes, releasing the C protein and the genome into the cytoplasm.

1.1.4 Genome structure and replication

The genome alone is sufficient to mediate infection. Flaviviruses possess a non-segmented RNA genome of approximately 10.8 kb. The genome is capped at the 5’ end with a type I m\(^7\)G(5’)pppA cap, though it lacks a polyA tail at the 3’ end. Despite this, the genome is packaged as a positive-sense molecule, and can be translated immediately upon contact with the host cell cytoplasm. It encodes a single open reading frame, which is translated to produce a polyprotein that is cleaved by both host-derived and self-contained proteases. This yields 10 distinct proteins, three structural (C, prM, and E, described above) from the amino terminus and seven nonstructural (NS) from the carboxy terminal end of the genome (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Fig. 1.4). The structural proteins are used to assemble new virus particles (see above), and may also play a role in immunosuppression if the innate immune response (73). The NS proteins with the exception of NS1 are generally believed to stay in the infected cell, carrying out cellular rearrangements, immunosuppression, and replication of the genome. NS1 is required for virus replication, but is also released from cells as a secreted hexamer. Several functions including immunomodulatory function have been implicated for this secreted NS1, though the specifics remain poorly understood (reviewed in (74)).
1.1.5 Immunology and protection

All arms of the immune response are implicated in protection against flaviviruses, and susceptibility factors for severe flavivirus induced disease have been discovered in all branches of the immune system (reviewed in (1)). Despite this, studies suggest that the humoral immune response is both required and likely to be sufficient for protection against flavivirus infection. (46, 75-80). There are clinically licensed vaccines available for two MBFVs (YFV and JEV), and studies using these vaccines show that protection correlates most strongly with a robust neutralizing antibody response (81, 82). Additionally, antibodies provided by passive transfer or IVIG are protective for either WNV or DENV (46, 83-85).

1.2 Antibodies and Flavivirus-Induced Disease Pathogenesis

1.2.1 Exacerbation of disease by antibodies

Despite their clear role in protection against flaviviruses, the role of antibodies in flavivirus disease pathogenesis is complex. In some situations, weak or poorly neutralizing antibody responses are associated with more severe forms of disease. The clearest example of this is seen in the context of DENV infection. Infection with any of the four serotypes of DENV can manifest in a variety of clinical outcomes ranging from asymptomatic infection to severe hemorrhagic forms of disease (DHF/DSS) (86). Severe disease, while rare, is most strongly correlated with preexisting, poorly neutralizing antibody responses (reviewed by (87)). This correlation is supported by the fact that poorly neutralizing sera or preparations of monoclonal antibodies can enhance virus infection of FcR-bearing cells in vitro, a phenomenon known as antibody-dependent
enhancement (ADE). ADE has been implicated in severe disease manifestations of several other pathogens, as well; this is discussed in more detail below (section 1.4).

DENV infected individuals who develop DHF can be generally divided into two groups. The first group is comprised of people who are suffering from a secondary DENV infection by a heterologous serotype. In this situation, the preexisting antibody response is targeted to the serotype of the first infection, and as such is poorly neutralizing. The second group is comprised of the infants of DENV-immune mothers who are experiencing their first DENV infection. For these patients, the susceptibility to severe disease is likely a result of the decaying levels of maternally transferred anti-DENV antibodies. There may also be many factors that can affect the propensity of poorly neutralizing antibody responses to result in severe disease, which are discussed below in more detail.

1.2.2 Secondary infection as a risk factor for DHF/DSS

As early as the late 1960s, it was identified that patients presenting with DENV infection for the second time were at elevated risk of disease. In an initial report, it was found that of 528 patients admitted to a special treatment ward of the Bangkok Children’s Hospital between 1962-1964, 41% of those with a secondary infection and only 14% of those with a primary infection developed DSS (88). More detailed analysis later revised these numbers slightly and revealed that nearly 40% of the DSS cases in primary infections were infants less than a year old, where maternally transferred antibody could be present (89).

In the intervening years, a number of studies have been set up that allowed dengue cases to be matched with a blood sample taken before the transmission season. A
summary of five such studies performed in Southeast Asia is shown in Figure 1.5 (data from (87)). In all of them, secondary infection was strongly correlated with DHF/DSS. Additionally, most of them do not observe DHF/DSS in patients with a primary infection.

In the new world, the isolation of Cuba has provided an interesting experiment of nature to clarify this point. Three separate DENV epidemics have swept the island since the 1960s. In 1977-78, a strain of DENV1 was introduced into Cuba, resulting in a sweeping epidemic with more than 400,000 cases of disease reported. Later serological studies suggested that nearly 50% of the Cuban population had been exposed (90), but there were no cases of DHF or DSS reported at any point during the epidemic (reviewed in (87)). In 1981, a strain of DENV2 was introduced to the island, resulting in 334,000 cases (91). In contrast to the previous outbreak, this time there were at least 10,000 severe cases, and both children and adults presented with DHF/DSS (92-94). Of note, no children between the ages of 1-2 presented with severe disease, as they were not yet born when the last epidemic had occurred (93, 94). Two subsequent outbreaks occurred in Cuba in 1997 (DENV2) and in 2001-2002 (DENV3), though they were constrained to much smaller (non-overlapping) geographic areas. Unlike in the 1982 outbreak, in the later outbreaks no severe disease cases were reported in children. DHF/DSS was only reported for individuals who had been alive during the earlier outbreaks (95-98).

These cohort-based studies are supported by the observation that subneutralizing antibody responses can enhance virus replication \textit{in vitro}. Early work on DHF patients identified that there was a relationship between weak antibody responses and disease (89), and that antibody targeted the infection to monocytes and macrophages (99, 100). Kliks et al. unified the two observations in an elegant study comparing the neutralizing
potency of serum collected from patients before the DENV season and correlating it to disease outcome. Almost uniformly, patients who had asymptomatic DENV infections later in the season had donated serum that was robustly neutralizing of monocyte infections \textit{ex vivo}. In contrast, patients who were hospitalized with severe disease exhibited poor or no neutralizing potency in the serum (101).

\textbf{1.2.3 Factors affecting severity of secondary DENV infections}

Despite the clear relationship between secondary infection and severe disease, DHF/DSS are fairly rare events across the human population. Studies from many geographically distinct cohorts show similar distributions, with approximately 1\% of secondary infections presenting as severe hemorrhagic forms of disease (reviewed by (87)). There are likely to be a number of complex variables that affect disease outcome in humans, including host susceptibility factors, which are discussed in more detail later. A few additional factors are briefly discussed here.

\textbf{Sequence of DENV infections.} There are many studies that have been published drawing conclusions about the importance of the order in which an individual is exposed to specific serotypes of DENV (reviewed by (87)). In general, it has been suggested that secondary infections by DENV3 are more likely to result in DHF/DSS than infections with the other serotypes (e.g. (96, 102-104)), but experiments of this nature are complicated in areas where multiple DENV serotypes (and other flaviviruses) are cocirculating. In Cuba, where the order of infection is easier to ascertain, secondary infections with DENV3 were associated with more severe disease if the primary infection was DENV1 but not when the primary infection was DENV2 (97, 105, 106). In are more recent retrospective study in Thailand, secondary infection with DENV2 was implicated
in being more severe (107); and a number of studies in the Americas find low incidence of severe disease specifically with DENV2 and DENV3 secondary infections (e.g., (108)). The conclusion that is reached when comparing these large and disparate datasets is that the order of acquisition may play a role in disease severity, but it either is not a defining factor or may contribute to a varying extent based upon a number of other factors, such as host genetics.

**Interval between infections.** The amount of time between two infections also seems to correlate with the severity of secondary infections. Early mathematical predictions estimated that the risk of DHF should be only in the 5 years following the primary infection (89, 109), but a number of studies have shown that DHF can be acquired many years after the primary infection (96, 110). There is also some evidence that the time between the outbreaks may correlate positively with the disease severity, as patients in Cuba infected with a secondary infection 20 years after their primary exposure had more severe disease than those infected in the epidemic 4 years after the primary exposure (105). This study is complicated, however; many factors in Cuba may have changed over 16 years, and the role of the aging of the patients was not accounted for. Further studies are needed to look at a full time course of the antibody responses to DENV in humans, and to correlate these to the risk of disease.

**Age when infected.** Historically, DENV has been treated as primarily a pediatric disease. Presumably, this reflects the fact that in endemic areas most individuals are exposed within the first few years of life; certainly the virus is perfectly capable of infecting older individuals when exposure occurs later in life. In several places in the world, there has been a substantial increase in the number of severe disease cases in
adults in recent years (reviewed by (87)). Some potential explanations have been proposed from mathematical modeling studies, but interpretation of these results is difficult in the complex, multivariate systems where the data is collected (111).

1.2.4 Enhancement of maternally transferred antibodies

Perhaps the most clear piece of evidence tying antibodies and severe disease manifestations during flavivirus infection is that infants of DENV-immune mothers show high susceptibility to DHF, but only during a time window when the maternally transferred antibody is dwindling. Immediately following birth, the passively transferred antibodies show robust neutralizing activity and infants have low probability of developing hemorrhagic disease (112). As the infant develops, the maternal antibodies are slowly catabolized until the remaining anti-DENV antibodies are no longer present in sufficient levels to neutralize infection (Figure 1.6). In this period, the infants are susceptible to DHF/DSS, and the sera of these infants enhance DENV infection on monocytes \textit{ex vivo} (113). A number of studies have examined this phenomenon in larger, cohort-based settings in many geographical locations. Essentially without exception, the same results are observed (114-117).

1.2.4 Host genetic factors in severe flavivirus-induced disease

Despite a clear role for host genetics in susceptibility to severe disease during DENV infection, the factors involved have not been clearly identified. A number of immune factors such as complement and other antiviral factors have been implicated, but the studies have not been performed on a large scale (118-120). Interestingly, the majority of the host factors implicated by analysis of single nucleotide polymorphisms (SNPs) in humans are known attachment factors for the virus like DC-SIGN (121-124) or
FcRs (125, 126). This suggests an important role for flavivirus attachment and entry in defining disease pathogenesis, and the finding of a SNP in FcRs supports the idea that ADE is an important factor in defining susceptibility to DHF/DSS.

1.3 Antibody Neutralization of Flaviviruses

1.3.1 Antibody structure

The primary anti-DENV antibody component in the blood after flavivirus infection is immunoglobulin G (IgG) (127), and passive transfer of monoclonal IgG is sufficient for protection against WNV or DENV (46, 83-85). IgG antibodies make up the majority of antibodies in the blood under both resting and inflammatory conditions. They are almost always present in humans as monomeric complexes, comprised of two heavy and two light chains (Figure 1.7). The resulting “Y” shaped structure provides two Fab arms that bind to specific antigen with high affinity, and the crystallizable fragment (Fc) that mediates effector functions (reviewed in (128)).

1.3.2 Mechanisms of antibody-mediated neutralization

Antibodies are glycoproteins that can exhibit antiviral activity both by direct neutralization of virus attachment or entry and through Fc-mediated effector functions (129). Antibodies are crucial component of the human immune response to most viruses, and with many viruses (including flaviviruses) they are sufficient to confer protection (reviewed by (130)). Consequently, the generation of potent neutralizing antibody responses is one of the primary goals for the development of antiviral vaccines.

In the context of flavivirus infection, the neutralizing potency of antibodies in vitro correlates well with their protective capacity in vivo (46, 75-80). This indicates a clear role for direct virus neutralization in antibody-mediated protection against
flaviviruses, and direct neutralization can occur by blocking either attachment or a postattachment step during virus entry (131, 132). In addition, a number of studies have described a protective capacity for antibodies against the NS1 protein, which is not included in the virion (133, 134), implicating a role for indirect methods of virus neutralization as well. Other Fc-mediated effector functions have been shown to be important for protection as well, including complement fixation and FcR engagement for ADCC (46, 135).

1.3.3 FcR biology and Fc-dependent protection

One of the mechanisms of Fc-mediated antiviral activities is via engagement of FcRs. FcRs are potent immunomodulatory proteins that balance the levels of inflammation during an immune response. They bind directly to the Fc region of antibody, and can deliver either inflammatory or anti-inflammatory signals (reviewed by (136)). They also facilitate phagocytosis of immune complexes by macrophages and monocytes. In humans, there are six distinct subtypes of FcR (Figure 1.8). These vary substantially in their affinity for FcR (reviewed by (137)), and in the cellular expression of the receptor. Additionally, there are a number of SNPs that have been described that affect either binding affinity for immune complexes or other aspects of receptor biology (138, 139).

Besides binding to IgG-containing immune complexes and mediating phagocytosis and clearance of complexes from the blood, the FcRs are also potent immune signaling molecules. With the exception of a single member of the family (FcRIIB, only expressed on neutrophils), all of them possess either an immunoreceptor tyrosine-based activation motif (ITAM) or an immunoreceptor tyrosine-based inhibitory
motif (ITIM). These motifs mediate potent propagation of inflammatory (ITAM) or inhibitory (ITIM) signals when the receptors are cross-linked, and may be either contained within the chain of the receptor itself (e.g. FcRIIA, FcRIIB, FcRIIC) or by means of an accessory signaling chain (FcRIA, FcRIIIA) that may vary as function of cell type and immune status (reviewed by (139)). FcRIIIA expressed on NK cells is believed to be the primary mediator of antibody-dependent cytotoxicity (ADCC), an important aspect of viral clearance in many systems.

Since ITIM signaling blocks signals through cross-linked ITAM receptors, FcRIIB (termed the “inhibitory FcR”) is often associated with blocking the phagocytic or immune signaling capacity of the other FcRs. This is believed to be an important aspect of maintaining immunological tolerance. Mice deficient in the receptor develop lupus (140), and the anti-inflammatory effects of intravenous immunoglobulin treatment are thought to be mediated by FcRIIB (141, 142). Interestingly, there is a SNP in the transmembrane domain of the receptor that causes failure to associate with lipid rafts (143, 144). The SNP is correlated with susceptibility to lupus and resistance to malaria (145).

1.3.4 Stoichiometric models of neutralization

Two separate models have been proposed for the stoichiometry of antibody neutralization of viruses. The “single-hit” concept states that neutralization occurs when the virion is bound by antibody at a critical site. First developed extensively with the advent of animal cell culture techniques (146), this model built on the previous work studying antibody neutralization of phages. It postulates that antibody must bind to critical sites on the virus particle to functionally inhibit entry, and early models suggested
that this irreversibly inactivated the virion (147). Therefore, the epitope targeted is critical, and antibodies that bind to unimportant epitopes do not contribute to virion neutralization.

The “multiple-hit” model suggests that neutralization of animal viruses is governed instead by a stoichiometric threshold, with a single virus particle becoming neutralized when it has sufficient antibody bound to block the infectious process. This was first proposed early in the study of viral immune responses as result of the ability of serum to block infection of eggs by animal viruses, and was proposed to be reversible (148). In the late 1970s, a complete review of the data for flaviviruses established that flaviviruses were likely neutralized by the multiple-hit mechanism (149). This is thought to reflect a process by which a virus particle is coated by antibody at a sufficient density to inhibit the necessary interactions for either attachment or entry (reviewed in (150)).

There are a number of situations with other viruses which are harder to explain with the multiple-hit model. These have been reviewed in detail elsewhere (130), but one is briefly mentioned here as they are addressed later in the dissertation. In a number of experiments with Influenza A, it has been reported that mutations that result in increased binding affinity for cells can confer protection against neutralizing antibodies that target distant epitopes (151, 152). In a potentially analogous situation, the expression level of CCR5, but not CD4, can affect the neutralizing potency of some antibodies to HIV. This role for cellular control of the neutralizing potency of antibodies is challenging to marry with the idea that neutralization is defined by a critical density of antibody interference. This is examined more carefully in Chapter 2 and Chapter 4.

1.3.5 Flaviviruses are complex immunogens
Given that flavivirus neutralization is governed by a threshold, the potency of an antibody response is evaluated only by its ability to bind as much of the virus population as possible with an occupancy higher than the threshold for neutralization. Therefore, any factor that adjusts the ability of a monoclonal antibody or a polyclonal antibody response to reach this threshold will affect the neutralizing potency of the antibody. As flaviviruses are complex, heterogeneous, and highly dynamic antigens (see section 1.1.2), the final result of neutralizing potency can be difficult to interpret without considering all of the contributing pieces (reviewed in (38)). A number of such factors that are known to be important in flavivirus neutralization are discussed below.

1.3.6 The role of antibody affinity

Antibody affinity is a chemical property of the glycoprotein surfaces that make up the epitope and the portion of the Fab that binds it (referred to as the paratope) (reviewed by (153)). It defines the fraction of places an antibody is capable of binding that will be bound at any given concentration of antibody. The affinity is generally reported in terms of a coefficient known as the $K_D$, which is defined to be the concentration of the antibody at which half of the potential binding sites are occupied. Dowd et al. have illustrated this nicely as shown in Figure 1.9 (38).

In this figure, a schematic is shown for a theoretical anti-flavivirus antibody that binds to the virion a maximum of 180 times. When incubated with the virion at a concentration equal to its $K_D$, half of these epitopes are expected to be occupied. Therefore, virus particles in this reaction will be bound by an average of 90 antibodies, and adjusting the concentration will change the number of antibodies bound as shown in the figure (reproduced from (38)). It is then evident that in general, high affinity
antibodies are desirable for generation of neutralizing responses, as they bind to a higher fraction of their target sites at any given concentration than their lower affinity counterparts. However, other factors can drive this as well.

1.3.7 The role of epitope accessibility

One factor that has been shown to be of particular importance with flaviviruses is the total number of epitopes present on a virion. An extremely high affinity antibody that targets an epitope only present on the virion a few times is a poorly neutralizing antibody. Even though it binds to a high fraction of the potential binding sites, there may not be a sufficient number of binding sites to reach the required stoichiometric threshold. Two antibodies that have been well characterized for WNV which illustrate this nicely are E16 (anti-DIII-LR) and E53 (anti-DII-FL). These two antibodies bind with very similar affinity to their respective epitope, but they differ in their neutralizing potency by orders of magnitude (41). This can be explained by the number of epitopes available to binding on the average virion. E16 binds to a highly accessible epitope which is available independently of the cleavage of prM and as such is highly potent. E53 binds to an epitope which is poorly accessible on mature portions of the virion, and it therefore shows poor neutralizing potency. As proof of principle, E53 can become a highly potent antibody if virion maturation is made inefficient, increasing the average number of uncleaved prM molecules per virion and increasing the accessibility of the epitope targeted (40, 41).

With this variable in mind, the complexity of neutralization for flaviviruses becomes more clear. Cleavage of prM during maturation is one factor that controls the accessibility of some epitopes; it is certainly not the only one. A number of other factors
have been described for flaviviruses and are reviewed in detail elsewhere (38). As these do not directly affect the purpose of this dissertation, they are only briefly mentioned here. Steric constraints can control the accessibility of epitopes, rising either from the dense packing of the E protein in the virion structure or from interference of previously bound antibody. Additionally, the dynamic nature of the E proteins at nonzero temperatures creates a shifting target. Many antibodies for flaviviruses that have been described to have poor neutralizing potency can become highly efficient neutralizers when provided either time to wait for an epitope to expose or increased thermal energy to accelerate the rate of epitope exposure (37).

1.3.8 Estimation of the stoichiometric threshold for flavivirus neutralization

The stoichiometric threshold represents the number of antibodies required to coat the virion with a minimum density to inhibit some crucial function. Determination of this number is dependent on the removal of many of the confounding variables described above. Fortunately, the previously introduced antibody E16 and closely related E24 are unaffected by most of these factors. They bind to a discontinuous epitope on the WNV DIII-LR that is believed to be accessible on 120 of the 180 E proteins present on each virion (46, 154). The 60 inaccessible epitopes represent those clustered around the 5-fold axes of symmetry, where the DIII may be packed too tightly to allow the antibody to bind (155). Using these antibodies, the stoichiometric threshold for WNV was estimated to be 30 antibodies (156). This estimate fits predictions generated using a model where antibodies must be able to interfere with the entire surface area of a particle of the correct size (150).

1.4 Antibody dependent enhancement of flavivirus infection
As described above, antibodies may also play a role in enhancing virus infection and exacerbating disease (157). Antibody-dependent enhancement of infection (ADE) describes a dramatic increase in infection of FcR-bearing cells in the presence of sub-neutralizing concentrations of antibody or immune sera (99, 158). ADE is most closely correlated with an increase in the efficiency of virus attachment (67, 159-161), though it may also represent an alternative cell biology of virus entry (see section 1.4.3).

1.4.1 A stoichiometric perspective

Flavivirus particles bound by more than 30 antibody molecules are neutralized; those bound by less remain infectious (see section 1.3.8) (156). These particles may exhibit ADE if they are bound by sufficient antibody to mediate FcR-dependent attachment to cells (156, 162). This implies that an additional stoichiometric threshold governs ADE: the number of antibodies required to mediate stable attachment. This stoichiometric threshold is likely to be highly dependent on the target cell, and host factors that affect it could be potential risk factors for severe DENV-induced disease. Factors that govern the affinity of antibodies for FcRs are especially likely to contribute to this, since higher affinity interactions should require less physical interactions to mediate attachment (see Chapter 3).

1.4.2 The role of cellular receptors

Monocytes, macrophages and dendritic cells are believed to be the primary target of ADE during DENV infection (99, 163, 164). These cells express a complex battery of FcRs with varying affinities for antibody, which are known to interact in complex ways (reviewed by (139, 165)). A number of studies have examined the role of different FcRs in mediating ADE through siRNA- or blocking antibody-mediated loss of function
experiments in monocytes or monocyte-derived cell lines (166-168). These experiments agree that multiple FcR subtypes contribute to ADE, implicating FcRIA (high affinity binding) and FcRIIA (low affinity binding) in particular (138). A crucial observation of several of these studies is that either monoclonal antibodies (mAb) or DENV-reactive sera neutralize ADE more effectively when FcRIA is present than when cells only express FcRIIA. The mechanism for this enhanced neutralizing potency remains unknown, but it does not require signaling competency (53, 169). This implies that the presence of FcRIA on cells may decrease the stoichiometric threshold for neutralization, but the contribution of this receptor to the stoichiometric threshold for attachment remains unknown.

It has also been suggested that neutralization of ADE requires cross-linking of the phagocytosis-inhibiting FcRIIB receptor present on monocytes (168). This conclusion is primarily based upon the observation that the specific infectivity of immune complexes decreases as they become larger, presumably due to ability to cross-link the FcRIIB molecules which are present in lower levels on the cell surface than FcRIIA or FcRIA are. This could possibly be a contributing factor, but is difficult to interpret in the context of individuals who express functional FcRIIC, estimated to be close to 40 % of the human population (170). These individuals only rarely express FcRIIB on myeloid cells at all (reviewed by (137)), and FcRIIC contains an inflammatory ITAM instead of the ITIM that is thought to mediate the effect (171). Additionally, FcRIIB is alternatively spliced into two forms based on the cellular substrate. The form expressed in myeloid cells (FcRIIB2) is a potent inducer of clathrin-mediated endocytosis. Consequently, we find that neutralization of ADE is possible on FcRIIB-deficient cells, and that FcRIIB is
perfectly capable of mediating ADE on its own in the proper cellular context (unpublished data).

Since ADE is primarily defined by an antibody-dependent increase in the efficiency of virus attachment to cells, it is expected that other immune complex-binding receptors may be able to mediate similar phenotypes. Accordingly, ADE of flaviviruses has been described by complement receptors as well (59). The authors further showed that ADE in this system could be blocked using an anti-complement receptor 3 (CR3) antibody but not an anti-FcR antibody, indicating the specificity of the result (60). Accordingly, studies examining the binding of WNV immune complexes to cells showed a substantial increase in antibody-dependent binding in complement-sufficient systems (159). The role of complement-mediated ADE in vivo has not yet been established.

1.4.3 Mechanisms of enhanced disease

The specific mechanisms by which ADE cause severe disease in DENV infection are unknown. It is plausible that ADE simply increases the number of infected cells in an individual, the resulting increased viremia leads to more severe immune responses and eventual shock and death. It is known that severe disease patients show elevated levels of proinflammatory cytokines that are associated with monocytes and that ADE of monocytes can be robustly mediated by the serum of at risk individuals (99, 101, 112, 113).

Another hypothesis has been proposed suggesting that cells infected by ADE may bias the immune response in a different way than cells infected by another mechanism. This is supported by data suggesting that ADE infected cells may produce more virus per cell and that IL-10 levels are elevated as a function of ADE (172). This phenomenon is
termed intrinsic ADE, and has received some interest in the field (173), but the relevance of intrinsic ADE in primary cells or in vivo is less clear. Studies with primary monocytes find that IL-10 is secreted by ADE infected monocytes in an IL-10 promoter-dependent fashion, but since monocytes are poorly permissive to infection in the absence of ADE the specific contribution of ADE was not identified (166). Additional work suggests that ADE of primary monocytes enhances IL-10 and interferon secretion primarily by increasing the efficiency of infection, so the role of intrinsic ADE in monocytes remains unclear (174).

1.4.4 ADE in non-DENV infections

ADE can be clearly observed with most flaviviruses. In addition to DENV, it has been studied extensively with WNV (67, 156, 159, 161-163), TBEV (175, 176), YFV (177, 178), JEV (179), and Murray Valley encephalitis virus (MVEV) (180). Whether there is a role for ADE in disease manifestations of these viruses remains unclear, but ADE of poorly permissive cells is certainly possible with these viruses. Additionally, ADE of these other flaviviruses is mechanistically indistinguishable from what is observed with DENV(181), suggesting that the correlation between ADE and DHF/DSS may be unique to DENV.

ADE has also been hypothesized to play a role in more severe clinical manifestations of an array of infections including HIV in AIDS (182, 183), Streptococcus pneumoniae in meningitis (184), enteroviruses in type-I diabetes (185, 186), and feline infectious peritonitis virus (FIPV) in hemorrhagic disease in cats (187, 188), though many of these remain contentious.

1.5 Specific Aims
The development of antibodies is a critical aspect of the host immune response to flavivirus infection and is a goal of ongoing vaccine development efforts. A poorly understood aspect of this interaction is the contribution of the cellular target of the virus towards the potency of neutralizing antibodies. This is of particular importance in the context of the infection of FcR-expressing cells, since sub-neutralizing concentrations of antibody cause enhanced infection of these cells.

The primary barrier to flavivirus infection in most cells is the efficiency of virus attachment. Given that antibodies can both mediate attachment or block it, we are interested in how cellular factors that affect attachment influence the enhancing or neutralizing potency of antibodies. In particular, we are interested in the role of virus or immune complex affinity, as this may affect the likelihood of infection and could define the protective capacity of antibodies. We examine this in the following three specific aims:

In **Specific Aim 1**, we evaluate the role of viral attachment factor expression level in defining the neutralizing potency of antibodies specific for flaviviruses. This is addressed using the attachment factors DC-SIGN and DC-SIGNR with WNV and DENV in Chapter 2.

In **Specific Aim 2**, we characterize the dependency of ADE on FcR subtype and expression level quantitatively, finally allowing evaluation of which human cells could be susceptible to ADE based on their *in vivo* expression of FcR. This is addressed using WNV and DENV when possible in Chapter 3.
In **Specific Aim 3**, we evaluate the contribution of the immune complex (stoichiometry and antibody isotype) to the susceptibility of cells to ADE. This will elucidate what kind of antibody responses are most likely to contribute to ADE *in vivo*. This is also addressed in Chapter 3, using WNV.
Figure 1.1. Phylogenetic tree of the genus *Flavivirus*. A maximum-likelihood tree was generated from the complete polyprotein sequence of the known flaviviruses by Kitchen et al. (4). Virus abbreviations are colored by arthropod vector as shown. The
definitive host is labeled in red. The abbreviations are detailed in (1). Figure is reproduced from (4) with permission.
Figure 1.2. Structure of the flavivirus envelope proteins and their organization on the virus particle. (A) The E protein is composed of three domains separated by flexible hinge regions and arranged as homodimers on the mature virion. The dimer is shown from above and from the side. The domains are colored as D1-red, DII-yellow, and DIII-blue. The fusion loop of DII is highlighted in green. (B) The arrangement of the E protein on the surface of the mature virion is shown. (C) The structure of the DENV E protein in complex with the cleaved portion of prM is shown. The coloring is identical to (A), but with the components of prM colored in purple. (D) The arrangement of the prM and E proteins on a full immature virion is shown. The proteins are arranged in trimeric spikes of heterodimers, one trimeric spike is shown enlarged in the right panel. Figure reproduced from (189) with permission.
Figure 1.3. The life cycle of flaviviruses. A schematic of the life cycle of flaviviruses. Reproduced from (189) with permission.
Figure 1.4. Flavivirus genome and polyprotein structure. (A) A schematic of the flavivirus genome is shown, with the structural genes encoded at the 5’ end of the genome. (B) The genome encodes a single polyprotein, which is cleaved into separate proteins by both self-contained and cell-derived proteases. (C) The structures and functions are known for some of these proteins, though many remain unclear. New virus particles only contain the structural proteins at significant levels. Picture reproduced from (190) with permission.
Figure 1.5. DHF/DSS are more frequent in secondary DENV infections. The percentage of cases resulting in DHF/DSS over 5 studies in Asia. The first study (Bangkok, 1962-1964) is a hospitalized cohort. The others are community-based cohorts. Data from (87).
Figure 1.6. The enhancing properties of passively transferred antibody correlate with risk of severe disease. The decay of maternally transferred anti-DENV antibodies is illustrated. After birth, the levels are sufficiently high to protect against enhancement in cell culture (green in right panel). As they decay to levels that enhance infection in cell culture (red in right panel), the risk of severe disease becomes elevated. After this window, the antibody responses are so low that they do not affect infection in cell culture (white in right panel), and there is no effect on the risk of DENV-induced disease. The right panel is reproduced from (191) with permission.
Figure 1.7. The structure of an antibody molecule. A schematic of an antibody molecule is shown. Heavy chains are shown in red or orange, light chains in tan. Domains are labeled, V indicates variable region and C indicates constant region. The blue lines indicate the location of an N-linked glycan. Adapted by permission from Macmillan Publishers Ltd: (128).
Figure 1.8. The FcR repertoire of mice and humans. The known FcRs of humans and mice are shown. Reprinted by permission from Macmillan Publishers Ltd: (139).
Figure 1.9. Flavivirus neutralization is governed by a stoichiometric threshold. Antibody affinity determines the percentage of epitopes that are bound by an antibody at a given concentration. For example, incubation of a theoretical virus displaying 180 E protein epitopes with an antibody at a concentration equal to its $K_D$ results in the engagement of the virion with an average of 90 antibody molecules. If the antibody concentration is reduced by two-fold (0.5 x $K_D$), the particle will only be engaged by 60 antibodies. Figure reproduced and modified from (38) with permission.
CHAPTER 2

THE IMPACT OF VIRAL ATTACHMENT FACTOR EXPRESSION LEVEL ON ANTIBODY-MEDIATED NEUTRALIZATION OF FLAVIVIRUSES

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This chapter has been published as:

2.1 Abstract

Neutralization of flaviviruses requires engagement of the virion by antibodies with a stoichiometry that exceeds a required threshold. Factors that modulate the number of antibodies bound to an individual virion when it contacts target cells impact neutralization potency. However, the contribution of cellular factors to the potency of neutralizing antibodies has not been explored systematically. Here we investigate the relationship between expression level of a viral attachment factor on cells and the neutralizing potency of antibodies. Analysis of the attachment factor DC-SIGNR on cells in neutralization studies failed to identify a correlation between DC-SIGNR expression and antibody-mediated protection. Furthermore, neutralization potency was equivalent on a novel Jurkat cell line induced to express DC-SIGNR at varying levels. Finally, blocking virus-attachment factor interactions had no impact on neutralization activity. Altogether, our studies suggest that cellular attachment factor expression is not a significant contributor to the potency of neutralizing antibodies to flaviviruses.

Keywords: West Nile virus, dengue, antibody neutralization, stoichiometric threshold, attachment factor

2.2 Introduction

Flaviviruses are small RNA viruses responsible for considerable morbidity and mortality worldwide for which vaccines and therapeutics are badly needed. Members of this genus that cause severe disease in humans include West Nile virus (WNV), dengue virus (DENV), yellow fever virus, tick-borne encephalitis virus, and Japanese
encephalitis virus. WNV is a mosquito-born flavivirus that circulates in an enzootic cycle between mosquitoes and birds; a variety of additional vertebrate species have been shown to represent dead-end hosts for WNV, including humans (10). WNV was first introduced into the Western Hemisphere in 1999 resulting in 59 hospitalizations with severe neurologic disease in the New York city area and seven fatalities (192). WNV has since spread across North America into Canada and Central America (11). It is estimated that more than three million human WNV infections have occurred in the United States since its introduction (193). The majority of human WNV infections do not cause overt disease. Clinically apparent infections range from a self-limiting febrile illness to more severe neurologic manifestations that include encephalitis, meningitis, and a polio-like paralytic syndrome (12). At present, treatment of WNV infection is limited to supportive measures. Despite significant progress, there is currently no WNV vaccine licensed for use in humans (reviewed by (194)).

Antibodies are a critical component of immunologic protection from flavivirus infection (reviewed by (38)). Passive transfer of antibody has been shown to confer protection in several animal models of flavivirus infection. Monoclonal antibodies (mAbs) may also be effective when administered therapeutically (reviewed by (195)). One such mAb, the WNV-specific neutralizing mAb E16, is currently being evaluated in phase I human trials (NCT ID: NCT00927953) (46, 84). Antibodies have the potential to contribute to protection from WNV via several mechanisms, including directly neutralizing virus infectivity (reviewed by (38)). This activity can be augmented in an antibody subclass-dependent manner through interactions between the complement component C1q and the constant region of the antibody heavy chain (Fc region) (159).
Other Fc-dependent effector functions may also contribute to protection by a variety of mechanisms, including facilitating complement deposition on infected cells and antibody-dependent cellular cytotoxicity (196-198).

The primary targets of neutralizing antibodies are the envelope (E) proteins incorporated into the virion (reviewed by (38, 199)). The E protein is an elongated three domain structure that orchestrates the assembly of virions and their entry into cells (reviewed by (25)). Flaviviruses assemble at membranes derived from the endoplasmic reticulum as immature virions that incorporate 60 heterotrimeric spikes of E proteins in complex with the premembrane protein (prM). During egress through the trans-Golgi network, pH-dependent changes in the arrangement of prM and E allow for cleavage of prM by a cellular furin-like protease (31-33). Cleavage of prM is required for infectivity of the virion and defines the maturation step in the virus lifecycle (34). E proteins on the resulting mature virus particle exist as anti-parallel dimers organized into a dense herringbone arrangement (25, 200, 201).

How antibodies neutralize virus infection has been studied extensively. In this regard, one informative perspective is to consider how the number of antibody molecules bound to the virion governs its infectivity; stoichiometric models of neutralization have been presented for several classes of viruses, including flaviviruses (reviewed by (130, 149). Neutralization of flaviviruses is a "multiple-hit" phenomenon that requires engagement of the virus particle by antibody with a stoichiometry that exceeds a required threshold (reviewed by (38)). Our estimate of this threshold is ~30 antibody molecules per virion (156). Two factors principally govern the stoichiometry of antibody engagement of the virion at any particular concentration of antibody: antibody affinity
and epitope accessibility (reviewed by (38)). Both have been shown to modulate the potency of neutralizing antibodies to flaviviruses (39, 41, 154, 156, 202-207). Because antibodies bind virions quite rapidly relative to the rate of virus attachment to cells, the number of antibodies bound to an individual virion at steady state is likely determined prior to contact with a target cell (37, 149, 208). A role for cellular factors in determining the neutralization potency of anti-flavivirus antibodies is not well established, and may not be predicted solely from this stoichiometric perspective. In this study, we explore whether differences in the expression level of a virus attachment factor modulate the neutralization potency of monoclonal and polyclonal antibodies.

2.3 Results and Discussion

Attachment factor expression correlates with the efficiency of WNV infection on Raji-DCSIGNR cells. The cell biology of flavivirus entry is poorly understood (reviewed by (49)). While several cellular proteins have been identified with a capacity to bind flaviviruses, few have been characterized in detail and shown to be sufficient for virus attachment and entry (51). The c-type lectins DC-SIGN and DC-SIGNR bind several viral pathogens and markedly increase the efficiency of viral infection (reviewed by (209)). These attachment proteins recognize the carbohydrate moieties of N-linked sugars arrayed on the surface of virions (210, 211); the specificity and affinity of these interactions is governed in part by geometrical orientation of the carbohydrate chains in space (reviewed by (47)). Both DC-SIGN and DC-SIGNR have been characterized as attachment factors for WNV and DENV (211-214). While a role for DC-SIGN or DC-SIGNR in the lifecycle of WNV in vivo has not been investigated, cells expressing these molecules provide a reductionist system in which to explore how antibody-mediated
neutralization of infection is modulated by the efficiency of virus-cell interactions. Furthermore, cells expressing c-type lectins are now commonly used in high-throughput assays of antibody-mediated neutralization (41, 156, 215-217).

WNV reporter virus particles (RVPs) are pseudo-infectious virions that allow virus infection to be scored as a function of reporter gene expression, and have been used extensively to study virus entry and its inhibition by antibodies (37, 156, 159, 218, 219). The introduction of DC-SIGNR into a cell line that is poorly permissive for WNV due to an inability to bind virions (e.g. Raji) markedly increases their permissiveness to infection (220). To quantify the DC-SIGNR expression level required for infection, Raji-DCSIGNR cells were incubated with WNV RVPs and analyzed for virus entry and DC-SIGNR expression two days post-infection. DC-SIGNR surface expression was quantified using a standard curve prepared using Quantum™ Simply Cellular beads with a known number of antibody binding sites (Bangs Laboratories, Inc.). A comparison of the DC-SIGNR expression level of the uninfected Raji cell population to those infected by WNV RVPs (Fig. 2.1A) revealed that infection was strongly correlated with high expression of DC-SIGNR. In agreement, increased expression of DC-SIGNR correlates with increased susceptibility to WNV infection (Fig. 2.1B). Roughly 20,000 DC-SIGNR molecules/cell are required to support detectable WNV RVP infection using this system.

Impact of attachment factor expression level on antibody-mediated neutralization of WNV. To investigate the impact of attachment factor expression on the potency of neutralizing anti-flavivirus antibodies, we pursued three complementary approaches. We first investigated whether differences in the expression of DC-SIGNR on target cells impact the concentration of antibody required to inhibit 50% of WNV
infection (EC\textsubscript{50}). WNV RVPs were incubated with the WNV domain III lateral-ridge (DIII-LR) specific mAb E24 for one hour at 37°C and then added to Raji-DCSIGNR cells (46, 156); this incubation has been shown previously to be sufficient to allow for steady state binding between antibody and the virion (37). Cells were harvested two days post-infection and analyzed for GFP and DC-SIGNR expression using flow cytometry. Analysis of the total cell population for GFP expression revealed the expected sigmoidal neutralization profile for mAb E24 characterized by an EC\textsubscript{50} of 0.035 nM (+/- 0.004 nM, n=9) (Fig. 2.2A) (156). To explore whether DC-SIGNR expression modulated the potency of E24, the data was re-analyzed by gating on cells expressing high or low levels of attachment factor as shown in Fig. 2.2B. No significant difference in the EC\textsubscript{50} was detected between DC-SIGNR high and low expressing cells (n=9, p=0.739) (Fig. 2.2C and 2.2D). Similar results were obtained with the mAb E53, which binds the structurally distinct domain II fusion loop (DII-FL) epitope and neutralizes infection by blocking attachment (data not shown, n=4, p=0.34) (154), as well as polyclonal antibody present in the sera of eight recipients of a candidate WNV vaccine (Fig. 2.2E and Fig. 2.S1) (221). Furthermore, neither DC-SIGNR nor DC-SIGN expression level significantly modulated the neutralization sensitivity of DENV1 RVPs to the type-specific DIII-reactive mAb E105 (p=0.8639, n=5; p=0.4938, n=3, respectively) (Fig. 2.2F) (85). Altogether, these data do not reveal a significant impact of DC-SIGNR expression level on the neutralizing potency (EC\textsubscript{50}) of antibodies to WNV or DENV.

WNV infection of Raji-DCSIGNR cells did not significantly impact DC-SIGNR expression as compared to uninfected cells (when assayed two days post-infection; n=9, p=0.15). However, we could not rule out the possibility that subtle changes in DC-
SIGNR expression might occur during the course of the neutralization assay and confound our interpretation of the experiments presented in Fig. 2.2. Therefore we next created a stable Jurkat cell line that expresses DC-SIGNR under the control of a tetracycline-inducible promoter. Jurkat-DC-SIGNR cells become susceptible to infection only in the presence of tetracycline (Fig. 2.3A); tetracycline dose-response studies revealed a large range of expression levels was achievable with saturation occurring at approximately 280,000 DCSIGNR molecules per cell. To investigate the impact of differences in DC-SIGNR expression on neutralization potency, cells were induced to express high, medium, or low levels of DC-SIGNR (Fig. 2.3B). Antibody dose response curves were generated on these populations using mAbs that bind epitopes on each of the three E protein domains (Fig. 2.3C-E) and polyclonal antibody from a recipient of a WNV DNA vaccine (Fig. 2.3F). In each case, neutralization activity was not markedly impacted by the level of DC-SIGNR expressed on the target cell.

**Analysis of the distribution of DC-SIGNR on cells infected in the presence of neutralizing antibodies.** To complement our analysis of neutralization potency on cells that differ with respect to DC-SIGNR expression, we next compared attachment factor distribution on cells at different points of an antibody neutralization profile. If the neutralizing potency of an antibody is truly independent of attachment factor expression on the target cell, one would expect the distribution of DC-SIGNR expression to be equivalent among populations infected by WNV RVPs with or without antibody present. In contrast, if antibody more effectively prevents infection of cells with low levels of attachment factor expression, the distribution of DC-SIGNR expression on cells infected in the presence of antibody would be skewed towards a higher average number of DC-
SIGNR molecules/cell as compared to cells infected without antibody present. This type of comparison can be made quantitatively using previously described chi-squared probability binning analysis described in detail in the materials and methods section (222).

WNV RVPs were incubated with E24 at the EC\textsubscript{50} (Fig. 2.4A, Box 2) or a non-neutralizing antibody concentration (Fig. 2.4A, Box 1) and added to Raji-DCSIGNR cells. On day two post-infection, cells were harvested and analyzed for GFP and DC-SIGNR expression. Histograms that describe the distribution of DC-SIGNR expression on cells infected (GFP+) in the presence of neutralizing and non-neutralizing concentrations of E24 were compared; no difference was observed (Fig. 2.4B). As a positive control for this method of analysis, we repeated this experiment using K562-DCSIGNR cells that express an activating Fc-receptor that provides a DC-SIGNR-independent entry pathway in the presence of antibody (156, 211). Antibody dependent enhancement (ADE) of infection describes a marked increase in the efficiency of virus infection in the presence of antibody that is mediated by Fc-receptors (Fig. 2.4C) (reviewed by (157)). Previous studies using cell lines and primary dendritic cells revealed the magnitude of ADE correlates inversely with the expression of viral attachment factors on target cells (156, 217). Analysis of the DC-SIGNR expression profile of K562-DCSIGNR cells infected in the presence of enhancing concentrations of antibody (Fig. 2.4C, Box 2) revealed a large shift towards lower DC-SIGNR expression (Fig. 2.4D), reflecting the susceptibility to infection of cells expressing low levels of DC-SIGNR due to Fc-receptor facilitated ADE.
Since this method was effective in analyzing the role of DC-SIGNR in neutralization, the data from each independent experiment described in Fig. 2.2 was reanalyzed using this method. At several points on each neutralization curve, the DC-SIGNR expression of infected cells in each sample was compared to the infected cells in the no-antibody control using chi-squared probability binning analysis. No significant differences were observed for WNV RVPs and E24 (n=25), E53 (n=33), or vaccine candidate recipient serum (n=14) on Raji-DCSIGNR cells, or for DENV1 RVPs and E105 (n=28) at any concentration tested in the experiment.

**Blocking DC-SIGNR does not change the neutralization potency of anti-WNV antibodies.** As a final approach, we studied the neutralizing potency of mAb E24 under conditions where the number of DC-SIGNR molecules available for WNV attachment was reduced using three different inhibitors. The mAb 120604 binds the carbohydrate recognition domain region of DC-SIGNR and has been shown to block interactions with WNV (211). Incubation of Raji-DCSIGNR cells with mAb 120604 resulted in a dose-dependent inhibition of WNV RVP infection (EC$_{50}$=2.3x10$^{-9}$ M, n=2). Likewise, pre-treatment of Raji-DCSIGNR cells with glucose or mannose blocks WNV RVP infection by competing for binding to the carbohydrate recognition domain (EC$_{50}$=8.1x10$^{-3}$ M, n=2 and EC$_{50}$=3.5x10$^{-3}$ M, n=3, respectively). Raji-DCSIGNR cells were incubated with each of these inhibitors at a concentration that corresponds roughly to the EC$_{50}$, followed by use in neutralization studies with mAb E24. As a control, cells were also incubated in the presence of each inhibitor at a level shown in our studies not to block WNV RVP entry. A comparison of the potency of E24 in the presence of inhibitory and non-inhibitory concentrations of mAb 120604, glucose, or mannose failed to identify
an impact of treatments that functionally reduce the number of DC-SIGNR on Raji cells (Fig. 2.5).

The efficiency of antibody-mediated neutralization has been shown in several contexts to vary when assayed using different cellular substrates (223, 224). For example, studies with a panel of mAbs to La Crosse virus show marked differences in the protective capacity of different antibodies based upon the cell type used in the assay (225). In addition, WNV-reactive antibodies have been described that neutralize infection in a cell type-dependent manner via unknown mechanisms (202). Several studies with influenza A suggest neutralization potency is modulated by the efficiency of virus attachment to cells (151, 152). This raises the intriguing possibility that the neutralization activity of anti-flavivirus antibodies might be impacted by factors that control the efficiency of virus infection of target cells. Evaluating this possibility is challenging in light of an incomplete understanding of factors involved in the attachment and entry of these viruses into cells. To date, only a small number of cellular factors that modulate the efficiency of flavivirus attachment and entry into cells have been characterized; none of these have been shown to be broadly necessary and sufficient for infection among permissive cell types (51).

DC-SIGN and DC-SIGNR are related c-type lectins which directly bind virus particles and promote more efficient infection (211). In this study, we investigated how changes in expression of these attachment factors (which in turn control the efficiency of virus binding and infection) modulate antibody-mediated neutralization. Using several complementary approaches and two different cell types, we found that changes in the expression of DC-SIGN or DC-SIGNR do not modulate detectable changes in the
neutralizing activities of flavivirus-reactive antibodies. These results suggest the fate of
the virion is determined by the number of antibodies bound to the virus particle before it
contacts the target cell. Thus, changes in the expression of cellular receptors are not likely
to be mechanistically responsible for the cell-type dependent neutralization observed for
some viruses.

The studies presented within were limited to cell types upon which the cellular
attachment structure was defined and could be manipulated; the cellular factors involved
in attachment to many cell substrates traditionally used in flavivirus neutralization studies
have not yet been established. The functional properties of flavivirus reactive antibodies
on cells expressing both DC-SIGNR (or DC-SIGN) and an Fc-receptor reflect an overlay
of neutralizing and enhancing activities in a manner which may be quite complex (156, 217). While extending our studies to primary cell types that are targets of virus infection
in vivo (e.g. DC-SIGN+ dendritic cells) (164, 214) would have been interesting, these
experiments are complicated by the presence of Fc-receptors capable of enhancing viral
infection, particularly when low levels of attachment factor are present. As the
requirements for flavivirus entry are better understood, the contribution of distinct
cellular factors to the effect of antibodies will undoubtedly become more clear.

2.4 Materials and Methods

Maintenance of cell lines. K562 cells were grown in RPMI-1640 medium
(Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS)
(HyClone) and 50 U/ml penicillin-streptomycin (PS) (complete RPMI). Media used for
the culture of a K562 line that stably expresses DC-SIGNR was supplemented with 10
µg/ml blasticidin (Invitrogen). Raji and Raji-DCSIGNR cell lines were cultured in
complete RPMI. The inducible Jurkat-DCSIGNR cell line was maintained in RPMI supplemented with 7% FBS, 50 U/ml PS, 5 µg/ml blasticidin, and 1000 µg/ml G418. HEK-293T cells were passaged in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS and 50 U/ml PS). All cell lines were maintained at 37°C in the presence of 7% CO₂.

**Human immune sera.** Serum from recipients of a candidate WNV DNA vaccine was obtained for use in neutralization studies. A phase I single-site, open-label clinical study (NCT ID: NCT00106769) to evaluate the safety and tolerability of a recombinant nucleic acid vaccine has been described (221). The protocol and conduct of the clinical research adhered to the experimental guidelines of the US Department of Health and Human Services and was approved by the NIAID Intramural Institutional Review Board. Sera from eight subjects was collected at 12 weeks post-final vaccination and studied for neutralizing activity.

**Production of a Jurkat cell line that can be induced to express DC-SIGNR.** A Jurkat T cell line was constructed to express DC-SIGNR under the control of a tetracycline inducible CMV promoter using the T-REx system (Invitrogen). Briefly, the coding sequence of human DC-SIGNR was cloned into the pT-REx-DEST30 vector by recombination using a previously described pDONR221 entry clone (220). The resulting expression clone, pDC-SIGNR-EXP30, was electroporated into 1.5 x 10⁶ T-REx Jurkat cells in 160 µl of Buffer SF (divided among eight wells) using the Amaxa 96-well shuttle nucleofection system (Lonza) and program CM-150. Transfected cells were selected by repeated passage in complete RPMI containing 5 µg/ml blasticidin and 1000 µg/ml geneticin (Invitrogen). Individual clones were isolated by limiting dilution.
Production of WNV and DENV reporter virus particles. Pseudo-infectious reporter virus particles (RVP) were produced by transfection of HEK-293T cells with DNA plasmids encoding the structural genes and a WNV sub-genomic replicon as described (156, 219). Briefly, a mixture (1:3 by mass) of two plasmids encoding a GFP-expressing WNV replicon (pWNIIrepGZ) and the WNV1 (NY99) or DENV1 (Western Pacific-74) structural genes were transfected into HEK-293T cells using Lipofectamine LTX (Invitrogen) according to the manufacturer’s specifications. The transfection media was replaced after four hours with a low glucose formulation of DMEM supplemented with 10% FBS and 50 U/ml PS. RVPs were collected at 48 hours, aliquoted, and stored at -80°C until use.

Neutralization of reporter virus particles. Neutralization of WNV and DENV RVPs was performed as previously described (37, 41, 156). Briefly, RVPs were incubated with serial dilutions of antibody or human serum for one hour at 37°C and then added to cells (total infection volume of 300µl in each well of a 96-well plate). Infected cells were incubated for 48 hours at 37°C in 7% CO₂. GFP-expressing infected cells were fixed with 2% paraformaldehyde (PFA) and then enumerated using a FACSCalibur flow cytometer (BD Biosciences). Neutralization potency (EC₅₀) was estimated by a least squared minimization non-linear regression analysis using Prism software (GraphPad).

Quantification of DC-SIGNR expression by flow cytometry. The expression of DC-SIGNR was measured by flow cytometry using phycoerythrin-conjugated mAbs (R&D Systems clone 120604). Cells were stained, washed once in cold Dulbecco’s phosphate-buffered saline (PBS), and fixed with 2% PFA in PBS. The number of DC-SIGNR molecules expressed on each cell was estimated by comparison to a standard
curve generated using Quantum Simply Cellular beads that have a known number of antibody binding sites (Bangs Laboratories, Inc.) processed in parallel. Use of this standard curve assumes each DC-SIGNR molecule is engaged by a single antibody molecule. Beads and cells were collected using a FACSCaliber cytometer (BD Biosciences).

**Chi-squared probability binning analysis.** DC-SIGNR expression profiles of infected cells (GFP positive cells) were compared between samples where antibody was present or absent during infection using the probability chi-squared binning analysis tool in the FlowJo software package (Tree Star, Inc.). This allows a comparison of two cell populations of different sizes without biasing towards effects at the tails of the distribution. Briefly, the control population (infected cells without antibody present) was divided into approximately 300 gates with roughly equal numbers of cells per gate. The total number of cells in each gate was divided by the total number of cells in the sample, and the resulting metric was recorded as the value for that gate. The same gates used in the control population were then applied to the experimental population (infected in the presence of antibody), and the process was repeated. For each gate, the two population test metrics were compared by traditional chi-squared analysis. The resulting statistic $T(\chi)$ is analogous to a t-score, and can be used to find the probability that two populations could be drawn from the same sample.

### 2.5 Acknowledgements

We would like to thank Dr. Michael S. Diamond for providing all the antibodies used in this study and for stimulating discussions, and Drs. Barney S. Graham and Heather D. Hickman for critical comments on the manuscript. This study was funded by
the intramural research program of the National Institute of Allergy and Infectious Diseases and the NIH Office of AIDS Research. The funding sources had no role in study design; collection, analysis, or interpretation of the data; writing the report; or the decision to publish.
Figure 2.1. Increased expression of viral attachment factor correlates with increasing probability of infection. (A) Shown is a contour plot of Raji-DCSIGNR cells infected with GFP-expressing WNV RVPs and stained with anti-DC-SIGNR antibody. The percentage of positive cells in each quadrant is indicated. (B) The Raji-DC-SIGNR cells shown in (A) were divided equally into eight gates based on DC-SIGNR expression and the percent of GFP positive cells within each gate was determined. These calculated values were plotted at the median DC-SIGNR expression level of the cells in each gate. Linear regression analysis of the logarithm of DC-SIGNR expression (molecules) revealed a strong positive correlation (p < 0.0001, $R^2 = 0.9834$, dashed grey line). The number of DC-SIGNR molecules was quantified from a standard curve generated using beads with defined numbers of antibody-binding sites. Dashed orange lines represent the 95 percent confidence interval of the linear regression.
Figure 2.2. Neutralizing potency of antibody is independent of target cell attachment factor expression level. Viral immune complexes were generated by pre-incubating WNV RVPs with serial dilutions of mAb E24, followed by infection of Raji-DCSIGNR cells. Infection was monitored as a function of GFP expression and cells were stained with anti-DC-SIGNR antibody to measure the surface expression of DC-SIGNR. (A) The neutralization curve for E24 and WNV RVPs on the total population of Raji-DCSIGNR cells is shown. (B) The expression level of the total cell population stained with anti-DCSIGNR (black) or an isotype control (yellow) antibody is shown. The subset of anti-DCSIGNR stained cells that were GFP positive are shown in green. Cells with levels of DC-SIGNR sufficient for infection were divided into categories for analysis based upon expression (Low, High) as shown by the dotted lines. (C) Dose response neutralization curves are shown for cells with high or low expression of DC-SIGNR. (D) The EC$_{50}$ value of the mAb E24 neutralization profile on high and low DC-SIGNR expressing cells was compared between nine independent experiments, and the difference was not statistically significant (p=0.739). (E and F) Additional neutralization profiles of immune complexes were generated by pre-incubating WNV RVPs with serial dilutions of serum from a vaccine trial volunteer, followed by infection of Raji-DCSIGNR cells (E), or DENV1 RVPs and serial dilutions of the DENV-specific mAb E105, followed by infection of Raji-DCSIGNR and Raji-DCSIGN cells (F). Results were analyzed based on
high versus low DC-SIGNR or DC-SIGN expression as described for mAb E24 in panels B and C. Volunteer I shown in (E) is one representative serum sample of eight volunteers tested (Fig. S1). All dose-response curves in Fig. 2 are representative of at least three independent experiments. Results are normalized to the infectivity obtained in the absence of antibody, and error bars show the standard error of the mean of triplicate wells.
Figure 2.3. Neutralizing potency of antibodies to all three domains of the E protein are unaffected by the cellular expression of attachment factor. An inducible DC-SIGNR-expressing cell line was generated using Jurkat T cells. The cells do not express DC-SIGNR and are not permissive to infection, but can become infected as a function of DC-SIGNR expression when tetracycline is added. (A) Cells were incubated with the indicated amount of tetracycline for 36 hours and either stained with anti-DCSIGNR (black) or infected with RVPs and monitored for infection (red) as described in the text. Axis scaling was selected to place maximum DC-SIGNR expression and maximum infection at approximately the same level. (B) Jurkat-DCSIGNR cells were incubated with tetracycline for 36 hours to generate high, medium, or low DC-SIGNR expression. Immune complexes were generated by pre-incubating WN V RVPs with mAbs specific for epitopes in domain III (C), domain II (D), and domain I (E) of the WNV E protein or serum from a vaccine trial volunteer (F), followed by infection of target cells. After 48 hours, cells were monitored for infection. Neutralization curves are normalized to the infectivity obtained in the absence of antibody and are representative of at least two independent experiments. Error bars show the standard error of the mean of triplicate wells.
Figure 2.4. Antibody protects cells from infection independently of their attachment factor expression level. Immune complexes were generated by pre-incubating WNV RVPs with serial dilutions of mAb E24, followed by infection of Raji-DCSIGNR cells (-FcR) (A and B) and K562-DCSIGNR cells (+FcR) (C and D). Infection was monitored after 48 hours as a function of GFP expression using flow cytometry. (A and C) Neutralization dose-response curves are shown normalized to the infectivity obtained in the absence of antibody. Non-neutralizing (Box 1, blue) and neutralizing (Box 2, orange) concentrations of E24 were used in (B) and (D). (B and D) Cells were infected with immune complexes generated at the E24 concentrations shown boxed in (A) and (C). Cells were stained with anti-DC-SIGNR antibody, and DC-SIGNR expression of the infected (GFP positive) cells is shown.
Figure 2.5. Neutralizing potency of antibody to WNV is not affected by blocking attachment factor on target cells. WNV infection of Raji-DCSIGNR cells can be inhibited by blocking virus engagement of DC-SIGNR with the mAb 120604 (A), glucose (B), or mannose (C). Cells were incubated in the presence of titrations of the indicated DC-SIGNR blocking agent, followed by infection with WNV RVPs (left panels). Non-inhibitory (Box 1, orange) and inhibitory (Box 2, green) concentrations correspond to the levels used in the panels to the right. Immune complexes were generated by pre-incubating WNV RVPs with serial dilutions of mAb E24, followed by infection of Raji-DCSIGNR cells that had been pre-incubated with the indicated DC-SIGNR blocking agent (right panels). Results are normalized to the infectivity obtained
in the absence of antibody or blocking agent and are representative of at least three independent experiments. Error bars show the standard error of the mean of triplicate wells.
Figure 2.S1. The neutralizing potency of human serum to WNV does not show dependence on the DC-SIGNR expression level of target cells. Neutralization profiles were generated by pre-incubating WNV RVPs with serial dilutions of serum from seven additional vaccine trial volunteers, followed by infection of Raji-DCSIGNR cells. Results are normalized to the infectivity obtained in the absence of serum and are representative of two independent experiments. Error bars show the standard error of the mean of triplicate wells.
CHAPTER 3

ANTIBODY AND FC-γ RECEPTOR MODULATION OF THE STOICHIOMETRIC THRESHOLDS FOR ANTIBODY DEPENDENT ENHANCEMENT AND NEUTRALIZATION OF FLAVIVIRUSES

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This chapter is based upon:

\textbf{Obara CJ et al., 2014. In preparation.}
3.1 Abstract

Flaviviruses are a group of positive-stranded RNA viruses that can cause potentially severe human disease manifestations including neurotropic or hemorrhagic disease. Neutralizing antibodies are the strongest correlate of protection for many flaviviruses, but poorly neutralizing antibody responses to dengue virus (DENV) have been implicated in more severe forms of disease. Severe disease is correlated with marked increases in the infectivity of immune complexes on cells bearing human Fc-\(\gamma\) receptors (FcRs), a phenomenon known as antibody-dependent enhancement (ADE). Prior studies demonstrate that ADE occurs at antibody concentrations corresponding to engagement of the virion with a stoichiometry below the neutralization threshold, yet sufficient to promote stable attachment to FcR-expressing target cells. In this study we investigate the role of antibody-FcR interactions in governing cellular susceptibility to ADE. Analysis of a panel of humanized antibodies and alternate FcRs suggests that the number of antibodies (per virion) required to support ADE varies as a function of the affinity of the Fc-FcR interaction. Additionally, experiments reveal a clear relationship between FcR expression level and the concentration of antibody required to mediate ADE. We further show that the cellular distribution of FcRs is not uniform in cell lines or primary cells, and that the number of FcRs available for engagement of an immune complex may vary greatly across the surface of a single cell or between cell types. Our results provide new insight into the cellular contexts in which ADE may be possible, and elucidate potential mechanisms for severe disease manifestations in humans.
3.2 Importance

Nearly a third of the world’s population is at risk of infection with dengue virus (DENV). Development of a protective vaccine has been complicated in part by the paradoxical observation that preexisting antibodies are both required for protection and can enhance disease in some situations. Antibody-enhanced disease is associated with an antibody-dependent increase in the efficiency of virus attachment to cells that express Fc-\(\gamma\) receptors (FcRs), though why this causes disease in some individuals and not others remains unclear. In this study, we examine three factors that control the affinity of antibody-bound virus for cells. We find a clear relationship between binding affinity and both the enhancing and neutralizing potency of antibodies. We also show that some cell surface proteins that affect antibody potency have distinct localization within the cell membrane, implying that antibody potency may depend upon the location where the virus and cell first interact.

3.3 Introduction

The genus *Flavivirus* contains a number of insect-borne viruses that are associated with severe disease in humans. A single member of the genus, dengue virus (DENV), is estimated to infect nearly 400 million people a year and cocirculates as four antigenically distinct strains (20). Other members of the genus include Yellow Fever (YFV), Japanese encephalitis (JEV), and West Nile (WNV) viruses (226). All of these flaviviruses share similar genomic organization (positive-sense RNA) (190, 227), cellular life cycles (189), and virion structures (spherical particles, \(~50\) nm in diameter) (25). For several clinically important flaviviruses including both DENV and WNV, there are still no specific therapeutics or clinically licensed vaccines (228).
A number of studies suggest that the humoral immune response is both necessary and likely to be sufficient for protection from flaviviruses (46, 75-80). Additionally, there are clinically licensed vaccines available for several members of the genus, and studies using these vaccines show that protection correlates most strongly with a robust neutralizing antibody response (81, 82). The primary target of neutralizing antibodies to flaviviruses is believed to be the envelope (E) protein (199, 229, 230). Antibodies mediate their protective capacity against flaviviruses both through direct binding and inhibition of attachment or entry (26, 78, 131, 176) and through crystallizable fragment (Fc)-dependent effector functions such as engagement of Fc-γ receptors (FcR) or induction of the complement cascade (46, 135).

Despite the clear role of antibodies in protection against flaviviruses, the full contribution of anti-flavivirus antibodies to disease pathogenesis is complex. During a recent phase 2B trial of a tetravalent DENV vaccine, neutralizing antibodies were observed, but the vaccine did not induce protection (231). Furthermore, it has been known for some time that weakly neutralizing antisera are implicated in more severe forms of disease during DENV infection (reviewed by (87)). There are several lines of evidence converging on antibodies as the susceptibility factor. Severe DENV-induced disease in adults, while rare, is most strongly associated with secondary infections by heterologous serotypes where preexisting, cross-reactive antibody responses would be expected (89). There is also a second group of patients who have elevated risk of severe disease—infants of dengue-immune mothers (113). During the time window when these infants are at highest risk, their sera enhances virus replication in cell culture (101); a phenomenon known as antibody-dependent enhancement (ADE). ADE has been
described for a number of flaviviruses (156, 157, 162, 175, 181), and is related to neutralization by the amount of antibody present (38). ADE is most easily observed in poorly permissive cells that express human FcRs such as monocytes and macrophages (181). FcRs bind to the Fc region of antibody, and ADE correlates most directly to an increase in the efficiency of virus attachment to these cells (159, 232). Some studies have suggested that ADE may also represent an alternate virus entry pathway leading to repressed interferon responses, though whether this is the case in all cellular contexts remains unclear (172, 233).

The mechanisms of antibody neutralization and enhancement remain a field of active inquiry, but studies with many animal viruses suggest that antibody neutralization is governed by a stoichiometric threshold (reviewed by (38, 130)). For flaviviruses, this threshold has been estimated to be approximately thirty antibodies per virion (156); virus particles engaged with occupancies that do not reach this number remain infectious. These particles may exhibit ADE if they are bound by sufficient antibody to mediate FcR-dependent attachment to cells (156, 162). This implies that an additional stoichiometric threshold governs ADE: the number of antibodies required to mediate stable attachment. This stoichiometric threshold is likely to be highly dependent on the target cell, and host factors that affect it could be potential risk factors for severe DENV-induced disease.

In this study, we examine three aspects of the antibody-FcR interaction (FcR subtype, antibody isotype, and FcR expression level) that are known to affect the affinity of antibody-dependent binding to cells. We analyze their contribution to both the neutralizing and enhancing potency of antibodies to flaviviruses. We show that these
three factors can exert a substantial influence over whether ADE is possible. Furthermore, FcR expression is not uniform over the surface of cells, so the neutralizing and enhancing potency of antibodies are properties of the local environment where an immune complex and a cell interact.

### 3.4 Results and Discussion

Since the lower stoichiometric threshold for ADE is defined by the stability of antibody-mediated attachment to FcRs, factors that are known to adjust antibody affinity for FcRs are likely contributors to ADE. One such factor that has been studied in some detail in the context of ADE is the FcR subtypes present on a cell. Monocytes, macrophages and dendritic cells are believed to be the primary target of ADE during DENV infection (99, 163, 164). These cells express a complex battery of FcRs with varying affinities for antibody, which are known to interact in complex ways (reviewed by (139, 165)). A number of studies have examined the role of different FcRs in mediating ADE through siRNA- or blocking antibody-mediated loss of function experiments in monocytes or monocyte-derived cell lines (166-168). These experiments agree that multiple FcR subtypes contribute to ADE, implicating FcRIA (high affinity binding) and FcRIIA (low affinity binding) in particular (138). A crucial observation of several of these studies is that either monoclonal antibodies (mAb) or DENV-reactive sera neutralize infection more effectively when FcRIA is present than when cells only express FcRIIA. The mechanism for this enhanced neutralizing potency remains unknown, but it does not require signaling competency (53, 169). This implies that the presence of FcRIA on cells may decrease the stoichiometric threshold for neutralization, but the contribution of this receptor to the stoichiometric threshold for attachment remains
unknown. In order to simultaneously study the relative contribution of FcRIA and FcRIIA to both stoichiometric thresholds, the ADE experiments must be performed using a cell that is non-permissive to infection by flaviviruses due to a failure in attachment.

We have previously shown that this is true of Jurkat T cells (234, 235). Additionally, Jurkat cells do not express any FcRs (139, 236). Despite this, they do possess the correct downstream signaling molecules for several FcR subtypes in an inactive form, which can be made active by simply stimulating the cells (237, 238) (data not shown). Accordingly, transfection of cDNA encoding an FcR has been previously shown to result in a functional form of the receptor (239). Therefore, we generated stable, transgenic lines expressing either FcRIA or FcRIIA from a tetracycline-inducible promoter. The two resulting cell lines (Jurkat-TREx-FcRIA and Jurkat-TREx-FcRIIA.LR) express the appropriate receptor as a function of tetracycline induction (Fig. 1A and 1D), and if the cells are activated (to release the signaling pathways) they become highly phagocytic (Fig. 1B and 1E).

WNV and DENV reporter virus particles (RVPs) are pseudo-infectious virions produced by packaging a reporter gene (GFP)-bearing replicon in place of the infectious genome during virus assembly (219, 234). These particles undergo a single round of replication and allow infection to be easily quantified using flow cytometry; consequently, they have been extensively used to characterize antibody neutralization and ADE (37, 40, 41, 156, 240). The ability of the cells to support ADE was measured by incubating murine antibody E16 (mE16), a well-characterized anti-WNV Domain III-lateral ridge (WNV DIII-LR) monoclonal antibody, with RVPs and adding them to activated cells. In the absence of tetracycline, no infection is observed (Fig. 1C and 1F,
black dots). In contrast, when cells are induced with tetracycline to express FcRs, a characteristic ADE curve is observed with poor infection at both high concentrations where mE16 is neutralizing and at vanishingly low concentrations where there is insufficient antibody to enhance infection (Fig. 1C and 1F, red dots).

**Both enhancement and neutralization occur with fewer antibodies present when a higher affinity FcR is utilized.** Immune complexes (ICs) generated using WNV RVPs and serial dilutions of a humanized (IgG1) version of E16 (hE16) were used to infect activated Jurkat-TREx-FcRIA or -FcRIIA.LR cells. The resulting infectivity revealed a marked difference in the stoichiometric requirements for ADE (Fig. 2A). In data collected over several independent experiments, both the amount of antibody required to enhance infection (Fig. 2B; n=4, p=0.0045) and the amount of antibody required to neutralize infection (Fig. 2C; n=4, p=0.0017) are significantly lowered on cells expressing FcRIA compared to cells expressing FcRIIA. Of note, the lowest concentrations of hE16 that support ADE of Jurkat-TREx-FcRIA cells correspond to the vast majority of antibody-bound virions having only a single antibody bound, implying that a single antibody molecule may be sufficient to mediate ADE of FcRIA-expressing cells. This is supported by the biology of FcRIA, which is believed to bind to monomeric antibody molecules free in the blood (241).

**Antibody isotypes that bind to FcR with higher affinity mediate ADE and neutralization at lower virus occupancies.** Since FcRIA binds to antibody with higher affinity than FcRIIA, it seemed plausible that the increased binding affinity alone may be responsible for both shifted stoichiometric thresholds. In order to examine this idea further, we chose to study another host factor that can adjust this affinity, antibody
isotype. Previous work has shown that antibody isotypes that bind with higher affinity to FcRs also show augmented neutralizing potency against flaviviruses (242). As with FcR subtype, the contribution of isotype to the stoichiometric threshold for attachment remains unknown. To further characterize the lower threshold, we utilized previously described humanized versions of the anti-DIII-LR mAb E24 (46). ICs were generated using WNV RVPs and serial dilutions of the IgG3 (highest affinity) or IgG4 (lowest affinity) isotypes. The myelogenous leukemia cell line K562 is positive for only a single FcR, the low-affinity, inflammatory receptor FcRIIA (243). The ICs were incubated with K562 cells, revealing that IgG3-mediated infection occurs at very different concentrations of antibody than infection mediated by the IgG4 isotype (Fig. 3A). Performing a similar analysis to that described in Figure 2 over several independent experiments revealed that both the amount of antibody required to enhance infection (Fig. 3B; n=4, p=0.0003) and the amount of antibody required to neutralize ADE (Fig. 3C; n=4, p=0.0003) are significantly lowered when the IgG3 isotype is used compared to the IgG4 isotype. In contrast, no significant difference was observed in the neutralizing potency of the mAbs on FcR-negative Raji-DCSIGNR cells (Fig. 3D, n=3, p=0.0678).

Both antibody isotype and FcR subtype affect the stoichiometric thresholds for attachment and neutralization in a manner that is directly predictable from the affinity of their interactions. Therefore, we decided to examine other potential factors that might affect this more subtly. One such factor that seems of particular relevance is the expression level of FcR, since this is known to change substantially as a function of cell differentiation and immune activation (244), as well as from patient to patient (245). Coinfections resulting in immune activation are associated with higher risk of severe
DENV-induced disease (246, 247), and terminally differentiated mature dendritic cells are the cells that are most susceptible to ADE in primary cell experiments (166).

**Blood-derived monocytes and dendritic cells express substantially different levels of FcRIIA.** To understand what contribution FcR expression level could have to ADE, we measured the amount of FcRIIA present on primary monocytes (CD14+, CD1a-) and blood-derived dendritic cells (CD14+, CD1a+) purified from peripheral blood of several donors by elutriation. The amount of FcRIIA was quantified using flow cytometry and comparing the signal from staining under conditions of saturation to a standard curve generated using beads with defined numbers of antibody binding sites as described previously (Quantum Simply Cellular™, Bangs Laboratories) (235). Monocytes show significantly lower FcRIIA expression than dendritic cells (Fig. 4A; n=6, p=0.0014). We then took advantage of the inducible nature of the Jurkat-TREx-FcRIIA.LR cells, and induced the cells with tetracycline to levels that approximate the FcRIIA expression level of monocytes (henceforth referred to as “Low FcRIIA”) or dendritic cells (“High FcRIIA”) (Fig. 4B). These cells were then used to assess the relative contribution of expression to ADE in the absence of other confounding receptors.

**FcRIIA expression level modulates the enhancing but not the neutralizing potency of antibodies.** When these cells were incubated with ICs of either WNV (Fig. 4C) or DENV (16007 strain) (Fig. 4F) RVPs and an appropriately targeted mouse anti-DIII-LR mAb (mE16 or mE106, respectively), infection profiles were distinctly different as a function of FcRIIA expression. When analyzed over a number of independent experiments, these experiments revealed that FcRIIA expression level altered the amount of antibody required to get ADE (Fig. 4D; n=6, p=0.0012; and Fig. 4G; n=4, p=0.0214).
In contrast, the amount of antibody required to neutralize ADE was unaffected (Fig. 4E, n=6 p=0.1141; and Fig. 4H, n=4 p=0.7245). This effect was independent of the epitope targeted or the neutralizing potency of the mAb, as the poorly neutralizing antibodies mE121 (domain I) and mE53 (domain II-fusion loop) show the same phenotype (Fig. 4I; n=5, p=0.0194 and 0.0081, respectively).

In order to rule out the unlikely possibility that the observed phenotype was a side effect of the Jurkat-based, tetracycline inducible system, we performed a loss of function experiment using blocking antibodies to control the amount of available FcRIIA on the constitutively expressing cell line, K562. The well characterized anti-FcRIIA mAb IV.3 is known to block FcRIIA function (248-250). Consequently, preincubation of IV.3 with K562 cells is capable of completely blocking ADE in an IV.3 concentration-dependent manner (Fig. 5A). ICs of mE16 and WNV RVPs were then added to cells that had been pretreated with either sufficient IV.3 to reduce ADE by half (Fig. 5A, green dot, ~5x10^{-10} M) or control media. Analysis of infection revealed that IV.3 selectively blocks ADE of ICs generated with relatively low antibody:virus ratios (Fig. 5B), an identical phenotype to that described using the Jurkat-TREx-FcRIIA.LR cells. This experiment was also performed using an additional blocking antibody for FcRIIA (6C4, (251), n=2) with similar results (data not shown).

**ADE occurs most efficiently on K562 cells with high FcRIIA expression.**

Given the results described above, we wondered if FcRIIA expression could affect which cells in a heterogeneous population are infected during conditions where the amount of virus is limiting, as would be expected early during secondary DENV infections. If true, this would imply that elevated FcRIIA expression could alter viral tropism early during
infection and may provide insight into the crucial early events in the development of severe disease. In order to test this idea, we infected K562 cells via ADE and compared the FcRIIA expression level of the cells that were infected to that of the total population of cells in the same well, most of which remained uninfected. Briefly, ICs were generated using WNV RVPs and serial dilutions of mE16, generating a conventional ADE curve (Fig. 6A). Analysis was carried out at the peak enhancing titer (PET) of mE16 (~9.75x10^{-12} M) (Fig. 6A, red dot). The FcRIIA expression level of the infected cells (Fig. 6B, red curve) was fairly high compared to the expression level of the total cells in the well (Fig. 6B, grey curve) (p<0.0001, n=3, chi-squared probability binning). In repeated experiments, the number of FcRIIA molecules required to get infection varied, fluctuating around 10,000 molecules (n=3).

Antibody occupancy of the virion is inversely correlated with the number of FcRIIA required to mediate ADE. FcRIIA-mediated ADE seems to require a number of antibody-FcR contacts for stable attachment (156). We wondered if the number of FcRIIA molecules/cell required to mediate ADE might be a function of the number of antibodies engaged per virion, as this could imply altered cellular tropism as a function of antibody concentration. To test this, we compared FcRIIA expression of K562 cells infected by ICs generated from WNV RVPs and varying concentrations of mE16 (Fig. 7A and 7B). As expected, when ICs were generated with lower levels of antibody (shown in blue and indicated with the number 1), the cells required more FcRIIA to be infected than when the ICs were generated with higher occupancy (shown in red and indicated with the number 2). In order to see if this was a generally applicable phenomenon, we used the murine antibody E60 (mE60), which targets a cross-reactive epitope in the
Domain II-fusion loop (DII-FL) of many flaviviruses (46). This epitope is believed to be poorly accessible on WNV, and the antibody shows poor neutralizing potency (202). When serial dilutions of E60 are used to generate ICs that are then added to K562 cells, the resulting infection shows similar enhancing properties to those observed with mE16, but even at saturating concentrations neutralizing potency is not achieved (Fig. 7C).

Analysis of the FcRIIA expression of infected cells reveals that increasing antibody occupancy decreases the number of FcRIIA required to get ADE, as with mE16 (Fig. 7D). When the concentration of mE60 becomes saturating, additional antibody does not affect the amount of infection (Fig. 7C, navy and orange points). At these concentrations, no differences are observed in the number of FcRIIA molecules required to get infection (Fig. 7D, navy and orange lines), confirming that the effect is not affected by the addition of free antibody to the well, but only by changes in the antibody occupancy of the virus particle.

When examining the three factors that affect functional affinity of ICs for FcR-bearing cells, two separate outcomes are observed. FcR subtype changes and antibody isotype changes affect both stoichiometric thresholds of ADE, as both the amount of antibody required to mediate ADE and to neutralize infection are changed (Figs. 2-3). In contrast, changes to the FcRIIA expression level only seem to affect the amount of antibody required to mediate ADE, but not the antibody requirements for neutralization (Figs. 4-5). Since all three of these factors are expected to change the avidity of ICs for cells, it was interesting that the two factors (FcR subtype and antibody isotype) which controlled the strength of each molecular bridge had a different neutralization phenotype than the factor that simply controlled the number of receptors available to make bridges.
This led us to wonder if it was possible that increased FcR expression did not necessarily guarantee that more antibody-FcR bridges could be formed. Since individual ICs (<75nm diameter) are much smaller than Jurkats or monocytes (~15000nm diameter), the amount of antibody-FcR bridges which can be constructed is more defined by the local density of the FcR at the site of IC binding than by the total FcR expression level of the cell. In order to identify what large-scale changes in FcRIIA expression mean at the local level that would affect a single IC, we examined the FcRIIA expression of activated Jurkat-TREx-FcRIIA.LR cells by confocal microscopy.

**FcRIIA expression is localized to distinct, punctate microdomains within the membrane.** When an image is taken of a single plane through the cell, FcRIIA is distributed in a manner similar to what has been previously described (167) (Fig. 8A). However, when a three dimensional reconstruction was performed by compressing a z-stack of the same cell, images revealed that the majority of cell surface FcRIIA is not uniformly distributed but rather localized within distinct clusters on the cell membrane (Fig. 8B). This phenomenon is true even if staining is carried out using only F_{AB} fragments or if cells are prefixed in PFA (data not shown). Additionally, monocytes show a similar distribution (Fig. 8C). When a spot detection algorithm is applied to analyze the clusters of FcR, both monocytes (n=20) and Jurkat-TREx-FcRIIA.LR cells induced to low FcRIIA expression as shown in Figure 4 (n=34) have similar numbers of clusters per cell (Fig. 8D, p=0.0843). Furthermore, the resulting clusters have similar cluster sizes (Fig. 8E, p=0.1173). In order to get an idea of the number of FcRIIA molecules within a cluster, the beads used to quantify receptor expression by flow cytometry (Bangs Laboratories) were also mounted and imaged by confocal. Using the beads to construct a
standard curve, the approximate number of FcRIIA molecules in a cluster can be calculated from the fluorescence intensity. It should be noted that the antibody used for this experiment (6C4) cannot differentiate between FcRIIA and FcRIIB, which are both present on monocytes (though the inducible cells only express FcRIIA). This reveals that clusters have an average of 132.7+/-79.4 FcRIIA molecules in them on Jurkat-TREx-FcRIIA.LR cells, assuming that each antibody binds a single FcRIIA molecule. On monocytes, the clusters have an average of 195.0+/-102.9 FcRIIA and FcRIIB molecules, a difference that is significant (Fig. 8F, p=0.0186). This is an intriguing number, since the average diameter of a cluster is ~0.3 µm. If a single-virion IC is approximated to be 75 nm in diameter, the footprint of a spherical immune complex is ~1/15 the size of a cluster. For a cluster with 200 molecules, that suggests that an IC would have a maximum of ~13 FcRIIA molecules available for binding. This number is close to previous estimates that 15 antibodies are required per virion for ADE (156), and is appealing in that only half of the 15 antibodies would be expected to face the cell during an IC-cellular collision, allowing some buffer for binding.

Armed with the knowledge that cells with higher FcRIIA expression outcompete lower-expressing cells for virus attachment (Figs. 6-7) and that FcR clusters seemed to have about the right number of molecules, it seemed reasonable that the clusters of FcR are the primary sites for engagement of ICs. Therefore, we next examined the effect of changing FcRIIA expression on the observed clusters directly.

Elevating FcRIIA expression of cells increases the number of FcRIIA clusters on the surface but does not change the density within each cluster. We induced Jurkat-TREx-FcRIIA.LR cells to the low (Fig. 9A) or high (Fig. 9B) FcRIIA expression
levels described in Figure 4 and imaged the cells with confocal microscopy. In three independent experiments analyzing at least 30 cells in each group, the number of clusters per cell (Fig. 9C), average amount of FcRIIA per cluster (cluster intensity) (Fig. 9D), average cluster volume (Fig. 9E), and average density of the receptor within the cluster (Intensity/Volume) (Fig. 9F) were analyzed. The number of clusters (n), cluster intensity, and cluster volume were all significantly higher in high FcRIIA-expressing cells than in low FcRIIA-expressing cells (p<0.0001 for all three variables). In contrast, the density of the receptor within each cluster was not significantly changed (Figs. 9F, p=0.4597). This was consistent over all three experiments (Fig. 9G), suggesting that FcRIIA density is not affected by increases in FcRIIA expression level. Since an IC containing a single virus particle would be predicted to be much smaller than a cluster, the number of antibody-FcR bridges that can be formed is likely to primarily depend upon the molecular density within the cluster, which is unchanged. Therefore, increasing the FcRIIA expression level of cells (as would be predicted to occur in immune activation and differentiation) would change the number of places an IC could bind to a cell, but might not affect the number of physical bonds that occurred. This would be consistent with the observation that increased neutralizing potency observed with higher affinity interactions are dependent on tighter binding of ICs to cells (as with FcR subtype and antibody isotype), while the increased enhancing potency is only dependent on higher probability of stable attachment. Accordingly, FcR expression only affects the enhancing potency of antibodies but not the neutralizing potency.
3.5 Conclusions

In summary, we examined the contribution of three host factors to the enhancing and neutralizing potency of antibodies in the context of ADE. We show for the first time that higher affinity interactions are associated with exacerbated infection at very low levels of antibody. This is true for all three means of adjusting the affinity and is of particular note, since several of these factors (FcR subtype and antibody isotype) have been suggested to play a protective role in studies which examined conditions with higher levels of antibody (53, 167, 169, 242, 252). We also confirm the results of a number of previous studies, showing that the neutralizing potency of antibodies is enhanced at high concentrations of antibody by higher affinity binding interactions. Additionally, we show that FcR expression is not uniform over the surface of primary cells or cell lines. Since these factors can have a large effect on whether or not cells are protected by antibody, this implies that the neutralizing and enhancing potency of antibodies may vary depending on the distribution of receptor present at the site of initial contact with an IC. As technologies for resolving single virus infection events are developed further, it will be interesting to see how the cellular landscape can affect the outcome of virus infections.

It is not known which levels of antibody are most relevant as susceptibility factors during secondary infection, so it is possible that the affinity of antibody-FcR interactions can be both a protective factor and a risk factor for severe disease, depending on the conditions. This is especially important to consider in the infants of DENV-immune mothers, who have elevated risk of severe disease between six to eight months of age as the maternally-transferred antibodies are degraded (101, 113). In this case, the risk of severe disease is likely to be decreased by higher affinity interactions at around six
months, as the enhanced neutralizing potency would lengthen the time the infant is protected. In contrast, higher affinity binding would be predicted to increase the risk of severe disease at around eight months, when antibody levels have diminished and higher affinity binding decreases the amount of antibody required to mediate stable attachment and ADE. Furthermore, there are a number of variants within the human population that can affect the affinity of interactions with FcRs (reviewed by (139)). Some of these have been examined in the context of risk of severe DENV-induced disease, and severe disease correlates strongly with the higher affinity binding genotype (125). This suggests that susceptibility to severe disease in secondary infection may be more likely to be governed by the lower stoichiometric threshold for attachment than by the neutralization threshold, since higher affinity binding is protective at high concentrations of antibody where the neutralization threshold is dominant. If this is true, generation of antibody responses with higher affinity interactions with FcRs may actually exacerbate the risk of severe disease, rather than induce protection as has been proposed.

This study underscores the complex nature of ADE, and suggests that further work is needed identifying the components of protective and enhancing antibody responses in vivo. Such work will inform our understanding of protection in polyclonal responses and will inform future vaccine design.

3.6 Materials and Methods

Growth and maintenance of cell lines. Raji-DCSIGNR and K562 cells were maintained in RPMI-1640 medium (Life Technologies) supplemented with 7% heat-inactivated fetal bovine serum (HyClone) and 50 U/ml penicillin-streptomycin (Life Technologies) (complete RPMI). Jurkat-TREx cells were maintained in complete RPMI
supplemented with 10 μg/ml Blasticidin (Life Technologies), and Jurkat-TREx cells engineered to inducibly express FcRIIA or FcRIA were maintained in complete RPMI supplemented with 10 μg/ml Blasticidin and 1000 μg/ml Geneticin (Life Technologies). HEK-293T cells were passaged in Dulbecco’s modified Eagle medium (DMEM) (Life Technologies) supplemented with 7% heat-inactivated fetal bovine serum (HyClone) and 50 U/ml penicillin-streptomycin (Life Technologies).

**Generation of Jurkat cell lines that inducibly express FcRIIA or FcRIA.** Jurkat T cell lines that express either human FcRIIA or FcRIA under the control of a tetracycline-inducible CMV promoter were constructed using the T-Rex system (Life Technologies). The coding sequence of the low responder form of human FcRIIA (NM_001136219.1) or FcRIA (NM_000566) was PCR amplified from the plasmid pCMV6-Neo-FcRIIA or pCMV5-XL5 (Origene Technologies) and cloned into the pT-Rex-DEST30 vector using Gateway technology (Life Technologies) to generate the expression clones pCD32A.LR-EXP30 and pCD64A-EXP30. Expression clones were electroporated into Jurkat-T-Rex cells using the Amaxa Nucleofector® system (Lonza) according to the manufacturer’s specifications. Stable integrations were selected by repeatedly passaging the cells for nine weeks in complete RPMI supplemented with 5 μg/ml Blasticidin and 1000 μg/ml Geneticin, followed by sorting to enrich for high expressing cells using a FACS-Aria (BD Biosciences). Enriched cells were then cloned by limiting dilution. Individual clones were screened for FcRIIA or FcRIA expression and susceptibility to ADE, of which a single clone of each line was selected to proceed.

**Production of WNV and DENV reporter virus particles.** Pseudo-infectious RVPs were produced by cotransfection of plasmids encoding a WNV sub-genomic
replicon and the structural genes of the desired virus as has been described previously (156, 219). Briefly, a 1:3 mixture (by mass) of two plasmids which encode a GFP-expressing WNV replicon (pWNIIrepGZ) and either the WNV (NY99) or DENV1 (Western Pacific-74) structural genes were transfected into HEK-293T cells using Lipofectamine LTX (Life Technologies) as recommended by the manufacturer. After 12 hours, the transfection media was replaced with a low-glucose formulation of DMEM supplemented with 3% FBS and 50 U/ml penicillin-streptomycin. RVPs were collected after either 48 hours at 37°C (WNV) or 72 hours at 30°C (DENV1), aliquoted, and stored at -80°C until further use.

**Neutralization and enhancement of reporter virus particles.** Neutralization and enhancement assays were performed as previously described (37, 40, 41, 156, 235). Briefly, RVPs were incubated with serial dilutions of antibody for one hour at room temperature and then added to cells. Cells were then incubated for an additional 48 hours with immune complexes at 37°C. After incubation, cells were either stained as described below or fixed by resuspension in 2% paraformaldehyde (PFA) in Dulbecco’s phosphate-buffered saline (PBS) (Life Technologies). Infection was monitored as a function of GFP expression by flow cytometry using a FACSCaliber (BD Biosciences). Data was analyzed using FlowJo software (Treestar, Inc.). Statistics of enhancing and neutralizing potency were estimated by a least-squares minimization non-linear regression using Prism software (GraphPad).

**Staining and quantification of cellular receptors by flow cytometry.** Cellular expression level of FcRs was quantified as previously described for DC-SIGNR (235). Briefly, cells were stained on ice for one hour in 1% BSA with excess antibody and
10µg/ml nonspecific mouse antibody, washed once in cold PBS, and fixed in 2% PFA. The number of FcR molecules on each cell was estimated by comparison with a standard curve generated using Quantum Simply Cellular beads, which have a defined number of antibody binding sites (Bangs Laboratories). Beads were stained under the same conditions and on the same day as cells. Use of this curve assumes each FcR molecule is engaged by a single antibody. Data was collected using a FACSCaliber (BD Biosciences).

**Elutriation, purification, and analysis of primary monocytes from peripheral blood.** Monocytes were purified from the peripheral blood of donors by elutriation in the NIH Department of Transfusion Medicine. Cells were then subjected to an additional positive selection step using beads labeled with anti-CD14 and an AutoMACS system (Miltenyi Biotec) according to the manufacturer’s specifications. Cells were kept on ice, and staining occurred within six hours of collection as described above.

**Preparation, imaging, and analysis of cellular expression by confocal microscopy.** Cells were stained and prepared as described above for flow cytometry. After fixation, cells were mounted by placing 2 µl of cells into 8 µl of ProLong Gold with DAPI mounting medium (Life Technologies) directly on a glass coverslip. This was then placed on a glass slide and dried overnight at room temperature. Imaging was performed using an SP5 confocal microscope (Leica) outfitted with custom hybrid detectors (HyD, Leica). Cells were selected for imaging by eye, using only the DAPI channel to avoid potential bias based on FcR expression. Full z-stacks were taken using a 0.2 µm step size, and images were taken at 12 bit resolution for quantification. Number of receptor molecules (when calculated) was estimated by comparison to a standard curve of beads.
as described for flow cytometry above. Images were reconstructed into three dimensions, and clusters were analyzed using a spot detection algorithm in Imaris (Bitplane).

**Phagocytosis Assays.** The phagocytic capacity of cells was evaluated by use of a commercially available kit (Life Technologies). Briefly, the cells were incubated with fluorescently inert DQ-BSA or immune complexes of DQ-BSA and anti-BSA antibody. DQ-BSA becomes fluorescent only if cleaved by proteases in the late endosome, and phagocytic capacity is calculated as the fold increase in fluorescence of the cells incubated with ICs over those incubated with the BSA-only control.

**Induction and activation of Jurkat-TREx-FcRIA or -FcRIIA.LR cells.** Cells were induced with tetracycline by incubating the cells in complete RPMI supplemented with either 250 ng/ml (high expression) or 2.5 ng/ml (low expression) of tetracycline for 44 hours. At this point, the media was additionally supplemented with 100nM phorbol 12-myristate 13-acetate (PMA) and 100 nM Ionomycin to activate the cells. At 48 hours, the cells were washed once and then resuspended in warm, complete RPMI before use in assays.
Figure 3.1. Expression of FcRIA or FcRIIA causes activated Jurkat T cells to become phagocytic and susceptible to ADE. In order to measure ADE quantitatively, a cell line with little to no background infection was required. Jurkat T cells are not permissive to infection with WNV or DENV due to a defect in attachment, so inducible lines expressing either FcRIA or FcRIIA were generated. (A) Analysis by staining and flow cytometry confirmed that the Jurkat-TREx-FcRIIA.LR line does not express FcRIIA unless stimulated with tetracycline. Cells were incubated for 48 hours with tetracycline or control media. The expression level was quantified by comparison with a standard curve of beads with defined numbers of antibody binding sites (Bangs Laboratories), and expression is shown. (B) Expression of FcRIIA produces a functional receptor, as measured by induction of phagocytosis. Jurkat-TREx-FcRIIA.LR cells or the parental line were stimulated with tetracycline or control media for 48 hours and then activated. (C) FcRIIA expression causes Jurkat cells to become highly susceptible to ADE. ICs were generated by incubating a mouse antibody against the domain III of WNV E protein (mE16) with GFP-expressing WNV RVPs at room temperature for an hour. ICs were generated with serial dilutions of mE16, then added to Jurkat-TREx-FcRIIA.LR cells pre-incubated with tetracycline or control media. Infection was detected as a function of GFP expression by flow cytometry. (D) As in (A), staining and flow cytometry confirm that
Jurkat-TREx-FcRIA cells express FcRIA only as a function of tetracycline induction. (E) Expression of FcRIA in activated Jurkat cells also produces a functional receptor as measured by phagocytic capacity. Jurkat-TREx-FcRIA or the parental line were prepared as in (B) and the phagocytic capacity was determined using DQ-BSA. The cells were not phagocytic if the cells were not activated (data not shown). (F) ICs of mE16 and WNV RVPs are infectious via ADE on Jurkat-TREx-FcRIA cells only if the cells are induced to express FcRIA. ICs were generated as in (C) and added to cells prepared as described above. Infection was monitored by flow cytometry and results are shown. In all panels, tetracycline-stimulation was performed at 250 ng/ml for 48 hours, and activation was achieved with 100 nM PMA and 100 nM Ionomycin. Error bars show the standard error of the mean.
Figure 3.2. FcRIA and FcRIIA mediate enhancement and neutralization at different concentrations of the same mAb. To simultaneously investigate the effect of FcR subtype on both the number of antibodies required for enhancement and the number of antibodies required for neutralization, immune complexes were generated with serial dilutions of hE16 (IgG1) and WNV RVPs. These were added to Jurkat cells induced to express either FcRIA or FcRIIA at similar levels. After 48 hours, infection was analyzed as a function of reporter gene expression. (A) Results are shown normalized to the maximum infection observed, and error bars represent the standard error of the mean of triplicate wells. One representative experiment of four independent experiments is shown. (B) The minimum amount of antibody required to enhance infection to 50% of the peak enhancement is shown for four independent experiments. Error bars represent the standard error of the mean. (C) The amount of antibody required to neutralize ADE to 50% of the peak enhancement is shown for four independent experiments, and error bars represent the standard error of the mean. ** = p < 0.01.
Figure 3.3. Antibody isotypes with higher binding affinity for FcR both enhance and neutralize infection more potently than a lower affinity isotype. ICs generated using two isotypes of the same humanized antibody (hE24) and WNV RVPs were added to Jurkat cells induced to express FcRIIA. 48 hours later, infection was monitored as a function of GFP expression. (A) Results are shown normalized to the peak enhancement observed, and error bars display the standard error of the mean of triplicate wells and are present unless too small to see. Shown is one representative experiment of four independent experiments. (B) The minimum amount of antibody required to enhance infection to 50% of the peak enhancement is shown. (C) The amount of antibody required to neutralize ADE to 50% of the peak enhancement is shown. (D) The amount of antibody required to neutralize 50% of infection on Raji-DCSIGNR cells over 3 experiments is shown. The two isotypes show no significant difference in neutralizing potency. B and C represent a summary of the four independent experiments described in A, and error bars represent the standard error of the mean. *** = p < 0.001, NS = not significant.
Figure 3.4. FcRIIA expression affects the enhancing potency of antibodies at levels observed on cells in human blood. (A) Monocytes (CD1a-) and dendritic cells (CD1a+)
were isolated from human blood by elutriation and CD14 positive selection. Cells were stained and FcR expression was quantified by comparison to a standard curve generated using beads with known numbers of antibody binding sites. The antibody used cannot distinguish between FcRIIA and FcRIIB. (B) Jurkat-TREx-FcRIIA.LR cells were induced with tetracycline to levels that approximate the expression seen in monocytes (Low) or dendritic cells (High). (C) ICs of mE16 and WNV RVPs were added to Jurkat-TREx-FcRIIA.LR cells induced as shown in (B), revealing a marked difference in the amount of FcRIIA required to mediate ADE. (D-E) When analyzed over several experiments, the minimum amount of mE16 required to enhance infection to 10% of the peak ADE was significantly reduced (D), while the minimum amount of mE16 required to neutralize ADE to half of the peak was unaffected (E). (F) ICs of mE106 (anti-DENV1 DIII-LR) and DENV RVPs (strain 16007) were added to Jurkat-TREx-FcRIIA.LR cells induced as shown in (B). There is a marked difference in the amount of antibody required to mediate ADE. (G-H) The minimum amount of mE106 required to enhance infection to 10% of the peak enhancement is significantly lower on High FcRIIA cells than on Low FcRIIA expressing cells (G), but the neutralizing potency is unaffected (H). (I) Antibodies to distinct epitopes on WNV show a similar phenotype. mE53 (anti-WNV DII-LR) and mE121 (anti-WNV DI) were used to generate ICs with WNV RVPs. The ICs were added to cells induced to express FcRIIA at the levels shown in (B), and the minimum amount of antibody required to enhance infection to 10% of the maximum infection observed was recorded. Cells expressing High FcRIIA are susceptible to enhancement at lower concentrations of antibody than cells expressing Low FcRIIA. * = p < 0.05, ** = p < 0.01, NS = not significant.
Figure 3.5. Blocking FcRIIA accessibility increases the amount of mAb required for ADE. As a loss-of-function experiment, the amount of FcRIIA available on K562 cells was modulated using the blocking antibody IV.3. (A) To determine appropriate FcRIIA blocking conditions, WNV RVP ICs were generated using mE16 at the peak enhancing titer (PET) (~1.5 x 10^{-11} M mE16). ICs were used to infect K562 cells pre-incubated with serial dilutions of the antibody IV.3. The percent of RVP-infected K562 cells is shown, demonstrating that ADE can be completely abrogated by anti-FcRIIA antibodies on K562 cells. The concentration of IV.3 that reduces ~50% of infection is indicated with a green dot (~5 x 10^{-10} M). (B) The effect of blocking FcRIIA on K562 cells was determined by pre-treating K562 cells with 5 x 10^{-10} M IV.3 or control media prior to infection with WNV ICs generated at a variety of mE16 concentrations. ADE profiles of IV.3-treated (green curves) or untreated (orange dots) are shown. All panels show one representative experiment of at least two independent repeats.
Figure 3.6. Not all FcRIIA-expressing cells are susceptible to ADE. We investigated the relationship between FcRIIA expression level and the propensity of cells to be infected via ADE. (A) ICs were generated by incubating serial dilutions of the DIII-specific antibody mE16 with RVPs for one hour at room temperature. ICs were then added to K562 cells and incubated for an additional 48 hours. Infection was enumerated as a function of GFP expression using flow cytometry. The percent of K562 cells positive for GFP is shown as a function of the concentration of E16 used to generate the ICs. The approximate PET of E16 in this assay is shown with a red dot (156). (B) We next compared the amount of FcRIIA on RVP-infected and uninfected cells by flow cytometry. After the 48-hour incubation, cells were stained for FcRIIA, and the number of FcRIIA molecules/cell was approximated by comparison with a standard curve generated using beads with pre-defined numbers of antibody binding sites (Quantum Simply Cellular Kit, Bangs Laboratories, Inc.). The expression of the RVP-infected cell population (GFP+) is shown in red. The FcRIIA expression level of the entire population of cells in the well is shown in grey. Shown is one of three independent experiments.
Figure 3.7. An inverse relationship between antibody occupancy and the number of FcRIIA required for ADE. (A) To understand how the number of antibodies bound to the virion affects the number of FcRIIA required for ADE, ICs generated from serial dilutions of mE16 and WNV RVPs were used to infect K562 cells. The percentage of infected cells is shown. (B) RVP-infected cells were stained for FcRIIA expression as described in Figure 1. FcRIIA expression of cells infected by RVP ICs produced with E16 at a concentration corresponding to point #1 (blue dot) or point #2 (red dot) from panel A are shown. Best fit curves generated using a gaussian approximation were fit to the ADE profiles using GraphPad Prism. (C) ICs of WNV RVPs and the DII-FL reactive antibody mE60 were then used to infect K562 cells. The percent of RVP-infected K562 cells is shown, demonstrating that E60 shows little neutralizing potency against WNV RVPs, even at saturating concentrations (202). (D) RVP-infected cells were stained for FcRIIA as described above. FcRIIA expression of cells infected by RVP ICs produced with E60 and the concentrations that correspond to the colored dots in panel C are shown.
There is an inverse relationship between the amount of antibody and the number of FcRIIA required for infection. Panels A and B are one representative experiment of the three experiments described in Figure 1, and panels C and D are one representative of three independent experiments.
Figure 3.8. FcRIIA is localization is confined to punctate microdomains within the cell membrane. To understand how FcRIIA expression may affect ADE at the level of a single virion, we examined the cellular distribution of FcRIIA by confocal microscopy. Jurkat-TREx-FcRIIA.LR cells were induced, activated, and stained as described in previous figures. The cells were then mounted and imaged in three dimensions using confocal microscopy with an optimized z-step. (A) An image taken through a single focal plane reveals FcRIIA localized at the cell surface with varying density. (B) When the same cell is viewed in a three dimensional reconstruction, the punctate nature of much of the FcRIIA expression is more clearly observed. (C) Clustering occurs in primary monocytes as well. Monocytes were isolated from human blood, stained, and mounted for confocal under identical conditions. Imaging was performed using the same settings as Jurkat-TREx-FcRIIA.LR cells imaged on the same day. (D-F) In order to verify that the inducible cells clustered in a similar way to the monocytes, a spot detection algorithm
in Imaris (Bitplane) was used to identify the clusters on monocytes (orange dots, n=20) or Jurkat-TREx-FcRIIA.LR cells induced to monocyte-like expression (Low) as described in Figure 4 (blue dots, n=34). Each cell imaged is represented as a single dot in each panel, and error bars show the standard error of the mean. The cells show similar numbers of clusters per cell (D) and cluster sizes (E). To compare the amount of FcR per cluster, the fluorescence intensity of each cluster was compared to a standard curve generated from beads with a defined number of antibody binding sites (Bangs Laboratories) that were stained, mounted, and imaged simultaneously. (F) The average number of antibodies bound per cluster is shown for each cell, and it is only slightly elevated on monocytes. Note that the antibody used binds both FcRIIA and FcRIIB, which are both present on monocytes, which may account for the elevated number of FcRs per cluster. * = p < 0.05, NS = not significant.
Figure 3.9. Elevating FcRIIA expression results in more FcRIIA clusters, but does not increase molecular density of FcRIIA. Jurkat-TREx-FcRIIA.LR cells were induced to monocyte-like (Low) and dendritic cell-like (High) expression levels as described in Figure 4 and then stained and imaged by confocal microscopy as described in Figure 8. Staining and imaging were performed simultaneously for both Low and High cells, and the same imaging conditions, settings, and spot detection algorithm were used. (A-B) One representative cell of at least 100 collected over three independent experiments is shown for both Low FcRIIA (A) and High FcRIIA (B). Images in (A) and (B) have been compressed by the same factor to allow comparison of variant expression levels. (C-F) In order to compare directly the clusters of High (red dots) and Low (blue dots), the spot detection algorithm was used to identify the clusters on every cell. For each cluster, the total amount of FcRIIA (as measured by fluorescence intensity), volume of the cluster, and density of the cluster (=intensity/volume) were recorded. For a representative experiment of three independent repeats (>30 cells per condition per repeat), the total number of clusters per cell and the average of those three parameters are shown. Each dot represents a single cell, and error bars show the standard error of the mean. High FcRIIA
expression resulted in more clusters per cell (C), more FcRIIA per cluster (D), and larger clusters (E). (F) The density of the receptor was not significantly different. (G) The fold increase in the mean of each variable between the Low and High cells is shown for three independent experiments. Statistical significance is determined by performing a one-sample t test to determine if the mean is significantly different from one. *** = p < 0.0001, * = p < 0.05, NS = not significant.
CHAPTER 4

DISCUSSION, IMPLICATIONS, AND FURTHER WORK

4.1 Introduction

The strange protective-pathogenic duality of antibodies in flavivirus infections has been of broad interest for many years, now; but the mechanisms by which antibodies contribute to severe disease remain elusive. There is a need for a protective human vaccine against a number of flaviviruses (19, 228), and since antibodies are also the strongest correlate of protection, it is crucial to understand the variables that control the balance between pathogenesis and protection by antibodies. Previous work has shown that neutralization and ADE are related by the amount of antibody present (reviewed by (191)), but the division between the two and the mechanisms that drive each are still unclear.

In this dissertation, we provide the first systematic, quantitative analysis of the contribution of host factors to these phenomena at the level of the single cell. Specifically, we examine the role of cellular avidity in mediating both protection and enhanced infection by flaviviruses. We show that affinity of binding can both exacerbate and protect against infection, depending on the conditions. Furthermore, our data suggests that cellular avidity for immune complexes can work at two distinct levels. Factors that affect the probability of binding have one effect, while factors that control the strength of binding have another. This work provides new insights into the mechanisms of antibody-mediated neutralization, and suggests a new set of potential risk factors for severe antibody-induced disease.
4.2 The effect of cellular factors on the neutralizing potency of antibodies

The prevailing models for antibody mediated neutralization of animal viruses involve neutralization as a stoichiometric property of the immune complex (reviewed by (130)). Once a critical antibody density is reached, the infectivity of an individual virus particle is inhibited. In general, this model views the infectious nature of an individual immune complex as defined strictly by the immune complex itself. Historically, this has been challenging to reconcile with the large body of evidence for cell type-dependent neutralization of viruses (see Chapter 1 for full discussion). Recently, this has been suggested to be the result of highly heterogeneous populations of viruses. For flaviviruses, it has been shown that maturation can affect cellular tropism independently of its effects on antibody neutralization—the result being an apparent cell type-dependent neutralization phenotype (253).

While this may explain many of the phenotypes in the literature, it still does not explain the observations with influenza A. Selection of influenza in the presence of neutralizing antibody responses results in the acquisition of mutations in the hemagglutinin binding pocket that confer increased binding affinity to cells (151, 152). These mutations are structurally distant from the binding sites of the antibody and are deleterious to virus growth; removal of the antibody results in reversion mutants (151, 254). This suggests that there may be a role for general cellular avidity in defining the effectiveness of an antibody response. In this work, we have examined this using both a direct attachment factor for WNV and DENV (Chapter 2) and the indirect attachment mechanism of ADE (Chapter 3). The results and implications are discussed below.
4.2.1 The role of general (direct) attachment factor avidity

In Chapter 2, we examined the role of the avidity of direct virus receptors using the expression level of DC-SIGN and DC-SIGNR. In a number of various experiments, we saw no difference in the ability of antibodies to neutralize WNV or DENV as a function of attachment factor expression level. This provided a crucial control for subsequent experiments—we knew that the potency of antibodies would not be affected by changing the number of places a virus could bind to a cell. However, it is a challenging experiment to use for drawing large-scale conclusions about avidity, for reasons detailed below.

The avidity of an interaction is a description of the stability (both spatially and temporally) of the interaction. It measures the probability that a binding event will occur (and not fail) within a certain unit of time. In the context of virus-receptor interactions, this is primarily defined by how well the interaction prevents dissociation of virus from the cell (255). By increasing the expression level of an attachment factor, the probability of stable attachment can be manipulated two ways. First, a larger fraction of the cell surface now has an attachment factor present, so the likelihood of a virus collision with the cell to result in attachment is increased. Additionally, the density of the factor may be increased (or not, see Chapter 3), which would increase the strength of binding and decrease the likelihood of virion dissociation.

Chapter 2 clearly demonstrates that the first of these two variables is occurring in the context of DC-SIGNR. When DC-SIGNR expression level is elevated, a larger fraction of cells is infected, and post-infection analysis reveals that infection is biased towards the highest-expressing cells. This increase in the probability of stable binding
clearly does not affect the ability of antibodies to neutralize, but one could ask why it would be expected to. In these studies, the antibodies have been prebound to the virus before addition to cells. (In practice, this is true in every neutralization experiment, since antibody binds so much more quickly to virus than virus binds to cells. See (37)). Therefore, virus particles have either reached the critical antibody density (hence infection is blocked) or not—whether they bind to the cell is probably irrelevant. Incidentally, several of the antibodies used in Chapter 2 are primarily attachment-blocking in their mechanism, and even these antibodies show no altered potency as a function of DC-SIGNR expression.

In contrast, we did not examine the density of DC-SIGNR on the cell surface in these studies. It is therefore possible that increasing DC-SIGNR expression has an effect similar to that of FcRIIA expression (see Chapter 3), where more of the cell surface becomes available for virion binding, but the strength of binding is unaffected. If this is the case, it is still formally possible that increasing the strength of each DC-SIGNR-virus bond could confer resistance to antibodies. This seems unlikely, as it would imply that antibodies neutralize virus by different mechanisms as function of the strength of virion binding, since high affinity-binding virus would require a denser coat of pre-bound antibody to interfere in the infection process (see section 5.4). However, given the data collected with influenza, such a possibility cannot be ruled out.

4.2.2 The role of indirect attachment factor avidity

In Chapter 3, we studied the affect of variables that modified the avidity of an indirect attachment factor on neutralization. Interestingly, we found that both factors tested that directly modified the affinity of immune complex interactions with cells
showed substantial effects on the neutralizing potency of antibodies. This corroborated a number of previous studies in systems where quantification was much more challenging. In contrast, increasing the FcRIIA expression level had no effect on neutralizing potency, as would be predicted by a model where neutralizing potency of antibodies can be affected by the strength of binding but not by the probability of binding.

The fact that high affinity cellular interactions confers increased neutralizing potency is interesting, and has some precedent with FcRIA in other virus systems (e.g. (256)). This has often been ascribed to the induction of phagocytosis (257-260), but data with DENV and WNV from our unpublished work and the work of others (53) suggests that this is independent of the signaling capacity of FcRIA. Additionally, we show in Chapter 3 that FcRIIA can mediate a similar effect when IgG3 antibodies are used with WNV, in agreement with published work on DENV (242). The lack of signaling dependence and the association with other isotypes implies that the effect is simply the result of the increased affinity. Additionally, some limited studies with fluorescently labeled DENV suggest that binding is still mediated efficiently to THP-1 cells under these conditions (167).

One explanation proposed in the HIV system is that FcR1A concentrates antibody at the cell surface, creating an artificially high antibody concentration (256). This would be consistent with the results seen using altered antibody isotypes, since the high affinity binding of IgG3 could presumably concentrate free antibody molecules at the cell surface. Another potential explanation is that virus at the cell surface may require some mobility for infection to occur. This could be required either to locate some currently unidentified coreceptor, or to locate a preformed clathrin pit for entry (65).
high occupancy binding, the tighter associations could limit this mobility, blocking the chance the virus has to mediate entry. This is consistent with results described for some antibodies to HIV (261).

Another explanation that is appealing is that high affinity binding of cellular factors reduces the stoichiometric threshold for neutralization, similar to what has been described for the complement protein C1q or anti-Antibody Fab₂ (159). This explanation is dependent on a model of antibody neutralization where antibody neutralizes virus by blocking the necessary E protein motion in the endosome. This is not unreasonable, since at the concentrations of antibody where the increased neutralization occurs, attachment is not blocked (167). Furthermore, it is known that at least some anti-flavivirus antibodies neutralize infection at a post-attachment step (83). In this model, the stoichiometric threshold for neutralization would be reduced because a lower density of antibody is required to block infection. Presumably this would be the result of crosslinked antibodies restricting the mobility of the E protein in the endosome more efficiently. Interestingly, we find that FcRIA-mediated ADE is unaffected by C1q independent of the antibody isotype used, data that is consistent with the stoichiometric threshold already being reduced in that situation (unpublished data). However, C1q is quite efficient at suppressing IgG3-mediated ADE, so this situation may be more complex than it first appears (unpublished data).

4.2.3 The role of the distribution of factors which modulate antibody potency

The distribution pattern of FcRs discussed in Chapter 3 has some interesting implications. Since FcR affinity can clearly modulate the neutralizing potency of antibodies, it seems likely that the neutralizing potency of antibodies is partially
modulated by the location on the cell that the virus initially binds to. This further suggests that a potential mechanism of antibody neutralization could involve restricting the virus to sections of the cellular landscape that are less efficient in supporting the virus entry process. This particularly interesting in light of the role of anti-glycan antibodies in HIV infection, which slow the kinetic process of entry (261); a similar kinetic inhibition has been noted for WNV (262).

Future work on both the biology of virus entry and the mechanisms of antibody neutralization will be required to fully appreciate the contribution of receptor distribution on cells. Historically, this has been challenging due to the lack of technologies which can resolve such phenomena quantitatively or in real time. However, recent advances in optical imaging technologies make this a possibility in the coming years (263-266), and the work presented in Chapter 4 will form a basis for numerical quantification of this data.

**4.2.4 The role of FcRIIB in neutralization**

It has been suggested by Chan et al. that the mechanism of neutralization in the context of ADE is distinct from the mechanism of neutralization of FcR-independent infection. This conclusion was based upon the observation that larger immune complexes had reduced infectivity on primary monocytes and THP-1 cells (168). As discussed in the Chapter 1, this is hard to explain in the context of humans who do not express FcRIIB in myeloid cells. In this dissertation, we have shown that neutralization of ADE is independent of FcRIIB. In two different cell lines which do not express FcRIIB (Jurkat and K562), we see that antibody neutralization is easily observed. Additionally, neutralization of ADE corresponds to virion occupancies that are neutralizing on FcR-
negative cells (e.g. Raji-DCSIGNR). This strongly suggests that FcRIIB does not affect the neutralizing potency of antibodies in the context of ADE, a concept that is supported by the observation that neutralization of ADE in monocytes corresponds with a decrease in the efficiency of attachment to the cells (159, 232). Additionally, the results of Chan et al. could also be explained by a decrease in the infectivity of total immune complexes due to cross-linking reducing the number of potential infectious units.

4.3 The role of cellular factors in defining the enhancing potency of antibodies

The role of cellular factors in defining the enhancing potency of antibodies is more obvious than the contribution to neutralizing potency. Factors that increase the probability of stable attachment will increase the enhancing potency of antibodies. Therefore, for every protective factor described above, there is a balancing contribution to the enhancing potency of the antibodies. This presents a paradox for understanding protective antibody responses. When the responses are robust and there is a high concentration of anti-flavivirus antibodies, tight binding to FcRs is desirable as it increases the neutralizing potency of the response. Unfortunately, these protective characteristics will enhance infection under low antibody conditions, suggesting that the most crucial requirement for DENV vaccine development will be the induction of antibody responses that don’t wane over time.

4.3.1 The role of receptor avidity

Changes that affect the affinity of the FcR-Fc interaction increase the enhancing potency of antibodies (see Chapter 3). Additionally, increasing the FcR expression level seems to do the same thing. This supports the idea that ADE is defined by the efficiency of antibody-mediated attachment to cells.
4.3.2 Contributions of cell biology

Despite the steps to understand ADE quantitatively in this dissertation, the cell biology of the process remains poorly understood. It is possible that ADE may also alter the cell biology of virus infection. Early studies on flavivirus identified that virus could be observed in several distinct endosomal compartments, in the context of ADE at least some of these were not clathrin-mediated (67, 161). It has been reported that ADE may alter the cellular immune response (reviewed by (267)), but this remains contentious (233). The changes observed in many of these studies could be fully explained simply by the changing efficiency of virus infection. If multiple infectious genomes were provided to each cell, the ability of the virus to rapidly depress the immune response would be expected to be stronger. Therefore, given the variation in FcR expression seen even between donors, it would be challenging to distinguish the role of multiple FcRs in controlling the number of genomes each cell is exposed to without a very carefully controlled low MOI infection.

Another potential role for ADE in enhancing pathogenesis has been proposed, in which fully immature virus particles (not infectious in traditional infection assays) can be rendered infectious by the addition of non-neutralizing concentrations of antibody. This has been proposed to be the result of furin cleavage of prM molecules during the entry process (reviewed by (268)). While this idea is appealing, in practice it is difficult to show that virus is truly 100% immature. At least with WNV, no role has been found for furin in mediating cleavage during entry (40). The results previously described could be explained by a simple antibody-dependent increase in the efficiency of virus binding. On cells bearing TIM or TAM receptors as the primary means of attachment, the efficiency
of binding of high-prM particles is quite low (40), so the addition of FcR-mediated attachment could potentially cause a substantial increase in infection just by increasing the efficiency of infection of a large subset of the virus population.

The work in this dissertation suggests that future work on the dynamic exchange of receptors for flaviviruses may be important. Studies using simple colocalization experiments suggest that virus may preferentially bind to FcRIIA when immune complexes are generated at high antibody:virus ratios (86). It would be fascinating to examine this in the context of a cell that expresses both FcRs and the direct attachment factor DC-SIGN, such as immature dendritic cells. It has long been observed that immature dendritic cells do not seem to support efficient ADE, despite an impressive array of FcRs(214, 217). However, the result has been difficult to interpret, as it is not clear if this represents a DC-SIGN-dependent suppression of FcR activity (269) or simply the efficient nature of DC-SIGN-mediated infection masking the enhancing effect. If the virus can be passed from one receptor to another, then it is also possible that this represents an ability of DC-SIGN clusters to “steal” infectious immune complexes from FcRs.

4.4 The role of human genetic susceptibility

The role of host susceptibility to severe disease remains poorly understood. Since severe antibody-exacerbated disease is reasonably rare (87), host susceptibility factors are likely to be an important factor. Identification of these factors is likely to be the best way to directly affect patient care in the context of DENV, and as such should be a primary focus of ongoing DENV research. The work in this dissertation suggests some areas that may be of interest for future studies, which are discussed below.
4.4.1 Genetic variations that alter affinity

Given that changes in antibody-FcR affinity adjust both the enhancing and neutralizing potency of immune complexes, genetic polymorphisms that adjust this affinity are potential risk factors for severe disease. There is a well characterized single nucleotide polymorphism in FcRIIA that affects the affinity of this interaction for human isotypes of antibody, and has been recently confirmed to adjust the efficiency of immune complex binding to cells (138). Since changes of this nature should modulate both the enhancing and neutralizing potency of antibodies, this provides an interesting opportunity to examine what functions of antibody in ADE are most important for severe disease. If the low affinity form of the SNP correlates with severe disease manifestations, then the neutralizing potency of the antibody in ADE is most important, since the high affinity form of the SNP should be protective at high antibody concentrations. Conversely, if the high affinity form of the SNP is associated with severe disease, then it is more likely that severe disease is defined by the infectivity of immune complexes with very low occupancies, since the low affinity form of the SNP would be protective due to poor efficiency for binding.

This SNP has been examined in a small cohort in Cuba, and symptomatic disease strongly correlates with the high affinity form of the SNP (125). Interestingly, the correlation was less clear between DHF/DSS and traditional DF symptoms, however. The high affinity allele also correlated with a number of long term immunological sequelae in DENV patients, suggesting that the mechanism of susceptibility may be more complex than originally thought (126).
4.4.2 SNPs that alter expression

Given the effects that FcR expression had on when ADE was possible, it seems likely that this could provide a source of risk for disease manifestations. We have observed substantial variation in the FcR expression level of monocytes and blood-derived dendritic cells of a small number of patients (Chapter 3). It is therefore possible that preexisting variation of FcR expression across the human population may be one source of the variation seen in disease symptoms during secondary dengue infection. Further studies on the FcR expression level in the resting state of patients will elucidate this farther.

4.4.3 SNPs that alter distribution

Since the distribution of FcR on the cell surface seems to affect the infectious nature of immune complexes, SNPs that affect this could contribute to whether or not ADE will occur. Currently, a SNP has been described in the transmembrane domain of FcRIIB that has this effect (reviewed in (145)). One allele results in a version of the receptor that fails to associate with lipid rafts, which could affect the ability of the receptor to mediate ADE. We did not examine the role of this receptor in mediating ADE in this dissertation, but it should be considered in future work.

4.5 Closing thoughts

In conclusion, this work suggests that the enhancing and neutralizing properties of antibodies are governed by dynamic stoichiometric thresholds, and the avidity of immune complexes for cells is one factor that can modulate them. Further work is needed to identify what causes these shifts, but a few potential contributors are discussed below.
This is by no means an exhaustive list, but it is inspired by some work with other animal virus and what is known about their neutralization.

4.5.1 Evaluation of the stoichiometric nature of neutralization

The idea of a moving stoichiometric threshold for flavivirus neutralization has been discussed in detail previously (159). One possible explanation for this is that the stoichiometric threshold for neutralization is a structure- and mechanism-dependent number. For example, 30 molecules of E16 can block endosomal fusion of WNV, but blocking attachment may require a larger number (and in fact may not even be possible for E16 on all virions). There is some data that suggests this may be the case, as another antibody that targets essentially the same epitope as E16 but is predicted to bind at a different angle shows a distinct neutralization phenotype (270). Since bound antibodies will have their Fc-regions arrayed outwards in a manner that is presumably epitope dependent, it is not inconceivable that the critical density needed to block infection may be affected.

This would be all the more exaggerated in the context of ADE and FcR-dependent neutralization, where engagement of those arrayed Fc regions is required. If this model is accurate, then the reason the neutralization threshold is decreased when the antibody isotype or FcR subtype is changed (or when C1q is added) could simply be that the mechanism of neutralization is different. The fact that the neutralization potency is increased by a similar amount may be coincidence—flaviviruses are quite small and it 30 molecules of antibody is not a large number. It may be hard to distinguish between a mechanism of neutralization that requires 12 antibodies and one that requires 18. This is consistent with the only other virus where this has been studied quantitatively, HIV. With
flaviviruses, antibodies neutralize ADE about a log better with FcRIIA present than with FcRIIA present. With HIV, this number was found to be antibody epitope-dependent, with a maximum of 5000-fold, consistent with a higher stoichiometric threshold for neutralization due to the larger virus particle. Therefore, the fact that C1q augments neutralization of IgG3 on FcRIIA but not on FcRIA would not be surprising, as one of the entry pathways could allow a place for C1q to interfere while the other did not.

4.5.2 ADE and human disease

Severe disease manifestations in humans are complicated, and it is clear many variables can play a role. If ADE is truly the causative factor, a fair question is why we don’t see ADE in other flavivirus infections. There are a number of theories for why ADE of DENV is associated with an altered disease presentation that has not been observed with other flaviviruses. The most prevalent theory is that this an effect of the evolutionary distance between the flaviviruses. DENV serotypes show a relatively close evolutionary relationship when analyzed over the entire polyprotein (4), but when analyzed over only the structural proteins that are the target of antibody responses, they show surprisingly low amount of conservation (203). Other members of the genus are more closely clustered, such as JEV and WNV; or more distantly related such as YFV or TBEV. Accordingly, JEV immune responses are generally protective against WNV (271, 272), and there is generally little lasting cross-reactivity across the more distant relations (273, 274). The only pairing within the genus that falls a similar distance is between St. Louis Encephalitis virus (SLEV) and the JEV/WNV clade (275). As these are endemic in birds with only periodic emergence into the human population, it seems less likely that individuals would be exposed to subsequent infections by these viruses in this order.
Another potential variable is the cellular tropism of the viruses. In studies with TBEV (176) and WNV (162), animals were specifically primed for ADE but no disease was observed. Since the cell biology of ADE, while poorly understood, seems to be indistinguishable between the flaviviruses (181); it stands to reason that the altered cellular tropism of the viruses may play a role. DENV may show enhanced pathogenesis under conditions of ADE because enhanced infection of monocytes occurs, resulting in systemic inflammation. For WNV or TBEV, it is possible that either a.) the increase in infection of monocytes in the presence of antibody is less drastic or b.) the viruses do not cause the same widespread inflammation or increase in viremia when replicating in those cells. This is supported by data with the unrelated Coronavirus of cats, feline infectious peritonitis virus (FIPV). Cats with preexisting, poorly neutralizing antibodies succumb to a severe hemorrhagic disease that is known to be related to ADE and show similar effects with antibody isotype as is shown in this dissertation with DENV (187, 188, 276-278). In this situation, ADE is associated with an increased level of infection in monocytes which then infiltrate into the peritoneal cavity, mediate release of inflammatory markers, and induce widespread vascular leakage (276). Therefore, ADE may be occurring in the rare individual who is infected with JEV followed by SLEV, but even if a person had the right genetic susceptibility factors these viruses may not be capable of mediating a hemorrhagic form of disease.

4.5.3 Conclusion

This dissertation has examined the contribution of cellular affinity to antibody function in both neutralizing and enhancing conditions. We have shown that a number of factors that modulate this variable have surprising and potent effect on antibody function,
but have only scratched the surface of the complex ways cells may affect the potency of antiviral antibodies. Further studies are needed to examine these factors more fully and in connection with other studies on antibody function and virus entry. As the technology to resolve single virus particles and evaluate their status as infectious or neutralized becomes available, our understanding of antibody mediated neutralization and enhancement will undoubtedly change, bringing light to many potential paths for the development of antiviral therapeutics and vaccine design.
REFERENCES


