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Structural perspectives of antibody-dependent enhancement of infection of dengue virus

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Dengue virus (DENV) consists of four serotypes. Sequential serotype infections can cause increased disease severity, likely due to antibody-dependent enhancement (ADE) of infection. Here, we review two recent papers showing major advancements in the understanding of the ADE mechanism for both mature and immature DENV. The surface of both mature and immature DENV contains E and another protein — M in mature and prM in immature virus. On mature DENV, the orientation of anti-E antibody with respect to the virus surface determines the antibody enhancement properties. On the immature virus, binding of anti-prM antibody aids the dissociation of pr from the fusion loop of E protein allowing virus-endosomal membrane interaction, thus overcoming the hurdle in the early step of fusion.

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Introduction

Dengue virus, a member of the family *Flaviviridae*, is a major human pathogen infecting 390 million people per year, causing disease that can range in severity from asymptomatic to mild dengue fever and to the severe dengue hemorrhagic fever [1,2]. The development of dengue therapeutics and vaccines are therefore urgently needed. Thus far, although there is a licensed vaccine, its efficacy is poor [3]. The development of DENV vaccine is complicated by the fact that the virus is composed of four different serotypes (DENV1–4), and also within a serotype the virus can display different morphologies and therefore have different antigenic properties [4]. In addition, the development of the severe form of the disease has been observed to occur more frequently in secondary infection with a serotype different from the first infection due to the phenomenon dubbed antibody-dependent enhancement (ADE) of infection [5]. ADE is thought to occur when non-neutralizing antibodies or sub neutralizing concentrations of antibodies form a complex with the virus, and the antibody then enhances infection by binding to Fcγ receptors (FcγR) on cells, such as myeloid cells, facilitating the virus:antibody complex entry [5]. Here we review two recent studies that show major advances in the understanding of the mechanism behind ADE infection of both immature (immDENV) and mature DENV and why some antibodies are inherently unable to cause ADE. Understanding the mechanism of ADE will help optimize the development of antibody therapeutics and vaccines.

The mature and immature DENV structures

DENV are small (50 nm), enveloped, positive-sense single-stranded RNA viruses. The ~11 kb genome encodes three structural proteins — capsid (C), envelope (E), and precursor membrane (prM or in its mature form M), and seven nonstructural proteins [6]. On the surfaces of both immature and mature DENV particles are 180 copies of the icosahedrally-arranged E glycoprotein. The E proteins form a complex with the M protein in the mature virus, and they in turn form dimers with other E–M complexes. Three of these dimers form a raft and 30 such rafts are arranged in a herringbone pattern on the surface of the virus [7,8]. The E and M proteins are anchored to the viral lipid bilayer membrane via their transmembrane regions. In addition, there are interactions between the helical stem regions of both E and M proteins to the viral membrane making a distinct polygonal shape of the membrane [7,9,10]. The central core of the virus particle contains the RNA genome complexed with multiple copies of the capsid proteins.

E protein is the major antigenic protein, and is involved in receptor binding and endosomal membrane fusion. The ectodomain of the E protein is mainly composed of β-strands which are organized into three domains: DI, DII, DIII. DI is positioned between the elongated finger-like DII and the IgC-like DIII. DII possesses the fusion loop on its tip, which in the mature virus remains buried within a hydrophobic pocket formed by the DI/DIII domains of the opposite E protein protomer within the dimer. Two hinge regions, one between DI and DIII and the other between DI and DII, are involved in

conformational changes of the E protein necessary for fusion [11]. The E ectodomain is connected to the E stem and the transmembrane regions [12]. The stem region contains two helices that lie flat on the viral membrane. The transmembrane region contains two anti-parallel helices that transverse to and from the lipid bilayer membrane.

The M protein is a small transmembrane protein that interacts with the E protein on the mature virus. On the immature virus, the M protein has an extension at its N-terminus forming a globular β-barrel domain called the pr domain — the whole molecule is named prM [13]. During the maturation process, the pr domain caps the fusion loop of the E protein, preventing the newly synthesized immature virus from fusing back into the cell when moving through the acidic compartments of the *trans*-Golgi network [13]. At neutral pH, the immature particle has a completely different structure compared to the mature virus. Its surface consists of 60 spikes composed of three E proteins, each in complex with a prM. At 600 Å, the diameter of the immature particle is larger than that of the mature virus [13,14•]. The structural changes of the virus particle in the context of its life cycle from infection to immature assembly and maturation is described below.

DENV life cycle

After receptor binding, DENV enters the cell through receptor-mediated endocytosis [15] (Figure 1). Exposure to low pH in the endosome induces a large conformational change at the surface of flaviviruses; this involves the dissociation of the E protein dimer and then exposure of their DII fusion loops, allowing interaction with the endosomal membrane [15,16]. The E proteins then trimerize to facilitate the fusion of the viral and endosomal membrane [16]. After fusion, the viral genome is released into the cytoplasm [17].

The immature particles are first assembled by budding of the RNA-capsid complex into ER membrane coated with prM and E proteins [11] (Figure 1). At this point, the prM-E complexes on the newly synthesized particles are arranged as 60 trimeric spikes (Figure 2). During transport through the *trans*-Golgi network, the low pH environment triggers a rearrangement of the trimeric spikes into E protein dimers similar to that observed on the mature virus (Figure 1). This exposes the furin cleavage site on the prM, allowing furin to cleave pr from M, and both of which remain attached to the viral surface due to the low pH environment [18]. When virus particles are secreted into the extracellular environment, the neutral pH allows dissociation of pr from the virus, thus uncapping the E protein fusion loop, leading to a fully mature virus particle (Figures 1 and 2).

DENV structural heterogeneity

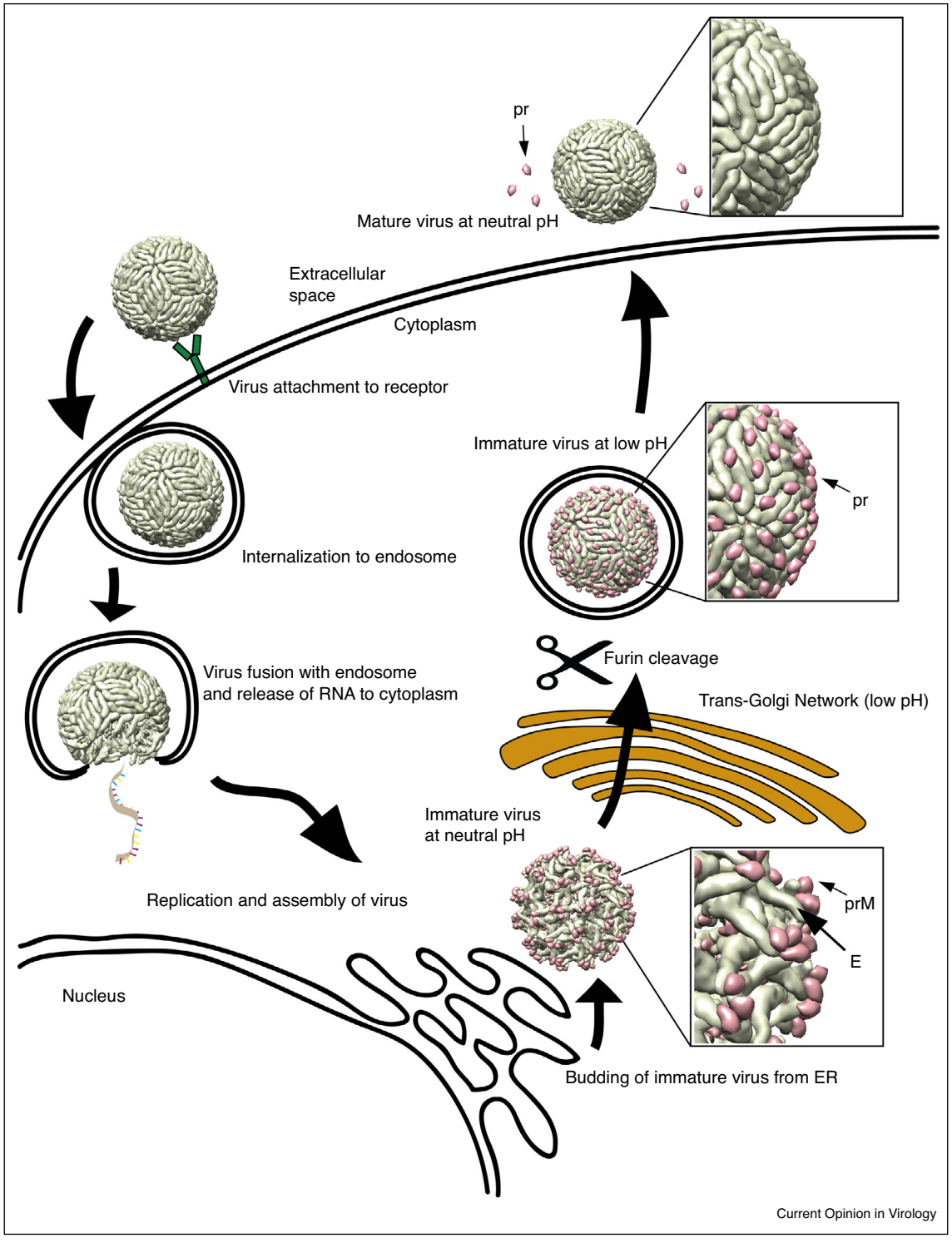
The overall picture of a mature icosahedral flavivirus of 50 nm is incomplete, as this is only an average from a selection of particles — in reality, there appears to be a continuum of mature virus morphologies [19], as well as some virus particles with incomplete cleavage of prM [20]. Some strains of DENV2 have been shown to undergo dramatic structural rearrangements from the compact smooth to bumpy surfaced particles when the incubation temperature is increased from 28°C to 37°C [21•,22]. CryoEM reconstruction of the 37°C DENV2 sample [21•] showed four structural classes (Class I–IV) with different radii representing various stages of expansion. Class I had the same structure as the smooth compact mature structure. Class IV particles possessed a smaller diameter than the other classes, and the E protein layer was not present in the 2D averages, suggesting the E proteins may have flipped out resulting in the disorderness of the E protein layer [23]. For the class II and III particles, most of the E protein dimers remain intact, except for those at the icosahedral twofold vertices. All inter-dimer interactions were completely broken leading to a looser E protein shell (Figure 2). These expanded particles therefore have different antigenic characteristics compared to the previously compact virus structure as their E proteins are now more solvent accessible.

In addition to these different mature virus morphologies, in many mature virus preparations, there were also contaminations with fully or partially immature virus particles suggesting that the maturation process is inefficient [20,24,25] (Figure 2). *In vitro* tests showed that anti-fusion loop and anti-prM antibodies play major roles in ADE. The anti-prM antibody will bind to partially or fully immature virus particles. The anti-fusion loop antibody binds well to immature virus [26]. The E proteins in the expanded mature particles compared to the compact mature particles are more solvent accessible and, therefore, will likely allow fusion loop antibodies to bind [21•,22,23,27].

Structural insights into ADE of mature DENV and why some antibodies are inherently unable to cause ADE

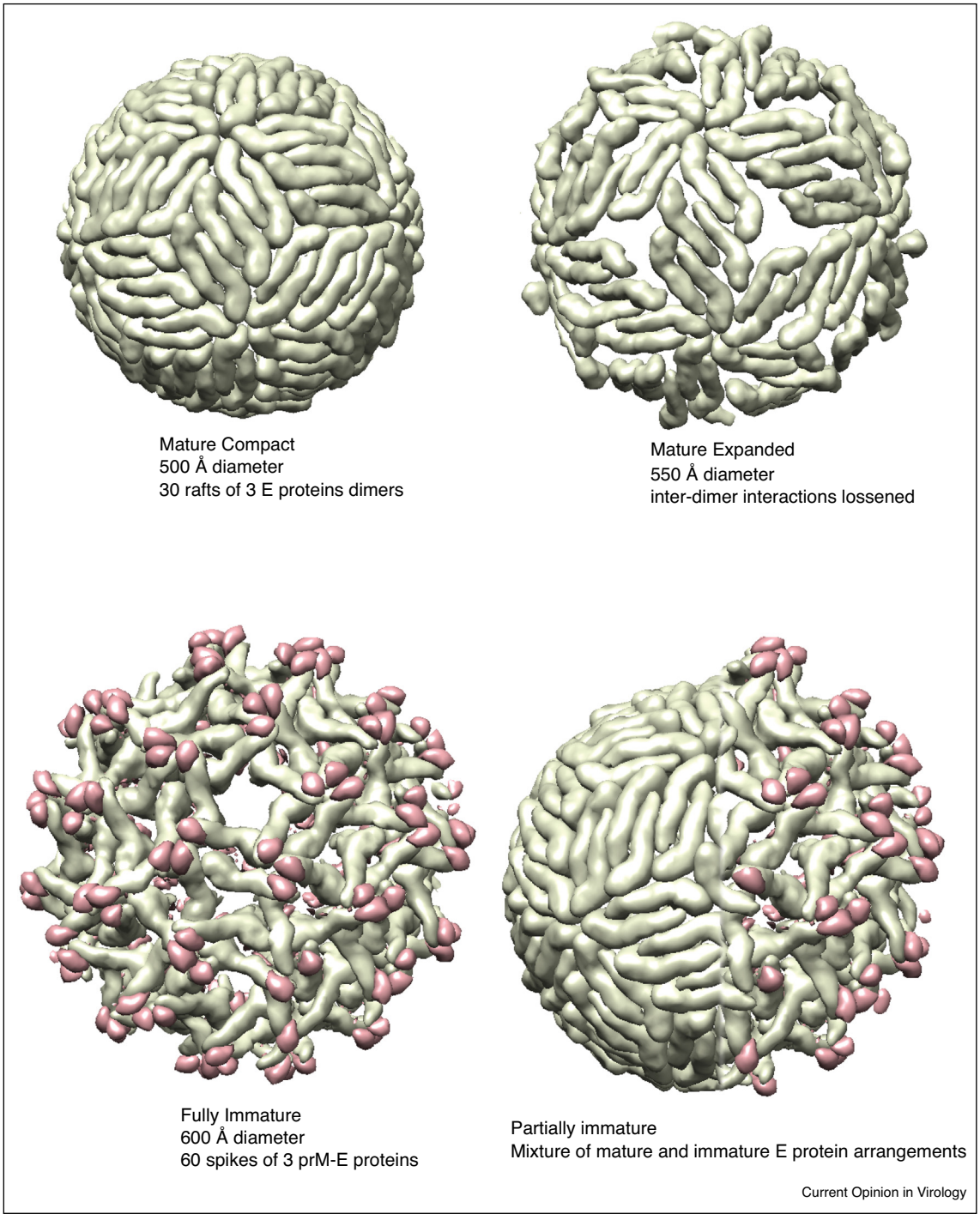
Renner *et al.* characterize two potently neutralizing antibodies to DENV2, but with very different ADE profiles [28••]. While 2C8:DENV complex showed the antibody supporting ADE at wide antibody concentrations, the 3H5:DENV complex had no ADE activity at any concentrations. This was true even when the Fc region of 3H5 (an IgG1 subtype) was switched to that of 2C8 (IgG2a isotype), indicating the differences are not due to different IgG subtypes. Binding assays with immobilized FcγR showed strong binding for 2C8:DENV, but the 3H5:DENV was unable to bind to the receptor. Crystal structures of the two Fab fragments in complex with DIII showed the epitopes largely overlap; however, the orientation of the Fab to DIII is different. Superposition of the Fab:DIII crystal structures into the previously determined cryoEM uncomplexed compact mature DENV2 structure showed 2C8 extends perpendicularly relative to the surface of the virus, while 3H5 lies flat

Figure 1



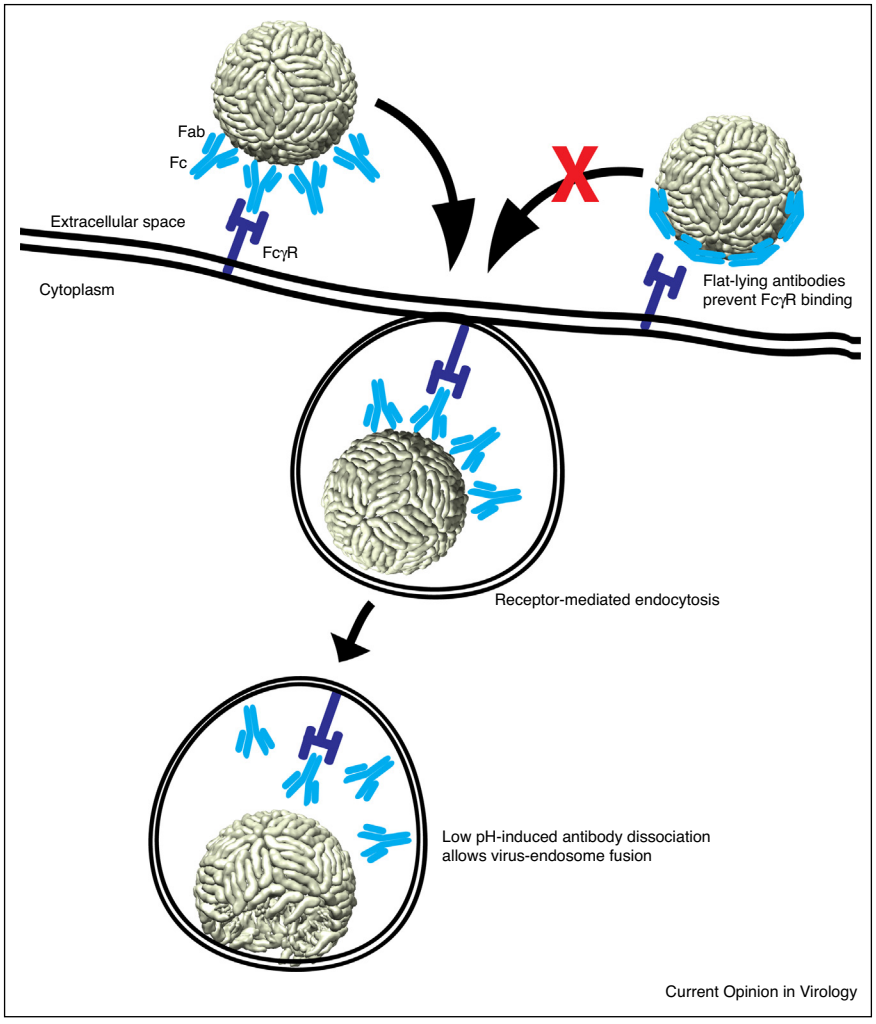
DENV life cycle. DENV infection begins with viral-receptor binding and receptor-mediated endocytosis; the low-pH environment of the endosome triggers fusion of virus with endosome, releasing the RNA genome into the cytoplasm. After replication and assembly of the immature virus by budding from the ER, the immature virus transits through the *trans*-Golgi network. The low pH environment of the *trans*-Golgi network induces dramatic rearrangement of the viral surface proteins (E and prM proteins) leading to the exposure of the furin cleavage site on the prM allowing furin protease to cleave the pr from the M protein. However, the pr remains associated with the E protein. Secretion into the extracellular environment provides the final step of maturation, where the neutral pH causes dissociation of pr from E and the formation of a fully mature viral particle. PDBID of mature, neutral-pH immature, and low-pH immature virus: 3J27 [7], 4B03 [13], and 3C6R [18].

Figure 2



Possible DENV particle morphologies. DENV can exist as a mixed population of virus particles: the compact smooth mature particle, with a diameter of 500 Å and a surface arrangement of 30 rafts of 3 E protein dimers; the bumpy expanded mature particle (in some DENV2 strains), with a diameter of 550 Å and with a surface organization in which the inter-dimer interactions between E dimers are loosened; the fully immature particle with a diameter of 600 Å and a surface consisting of 60 spikes of three prM-E protein dimers; and partially immature particles in which cleavage of prM is incomplete, resulting in a surface with both mature and immature characteristics. PDBID of compact mature, mature expanded, and fully immature DENV: 3J27 [7], 3ZKO [21•], and 4B03 [13].

Figure 3



ADE mechanism of antibody:mature DENV complex and why some antibodies are inherently unable to cause ADE. Antibodies that bind perpendicularly to the virus surface (such as 2C8) have their Fc region of the antibody fully exposed thus allowing FcγR to bind, facilitating entry into the cell. In contrast, antibodies such as 3H5, which lay flat on the virus surface, could sterically prevent interaction with FcγR on cells. Thus this antibody is unable to cause ADE. In the case of 2C8, when the antibody-virus complex is successfully endocytosed, the exposure to the low-pH environment causes a reduction in affinity of the antibody to the virus, thereby releasing the antibodies from the virus, allowing virus to fuse to the endosomal membrane.

along the viral surface. This suggests the 3H5 epitope is partially hidden and the binding of 3H5 antibody may lead to the distortion of viral surface. Indeed, cryoEM 2D class-averages of the DENV2 particles complexed to these two Fabs supported these observations. They suggest this mode of binding, with the Fab 3H5 lying parallel to the viral surface, interferes with FcγR binding, hence preventing ADE [28••] (Figure 3). Separately, Lok [29••] superimposed the same crystal structures into the expanded mature DENV2 structure and showed both 3H5 and 2C8 epitopes are fully accessible on some of the E proteins and the 3H5 Fab did not clash with neighboring E proteins when bound to the E proteins near the icosahedral twofold vertices. The orientation of the Fab, however, remains similar, 3H5 laterally and 2C8 perpendicularly to the virus surface. Superposition of the whole IgG:Fc receptor complex onto the Fab 3H5:mature expanded particles model showed that, due to the lateral orientation of the Fab 3H5, the organization of the whole immunoglobulins will be too crowded to allow FcγR to bind. The upright conformation of 2C8, on the other hand, would allow the Fc regions of the whole immunoglobin to be fully exposed, thus allowing FcγR to bind (Figure 3).

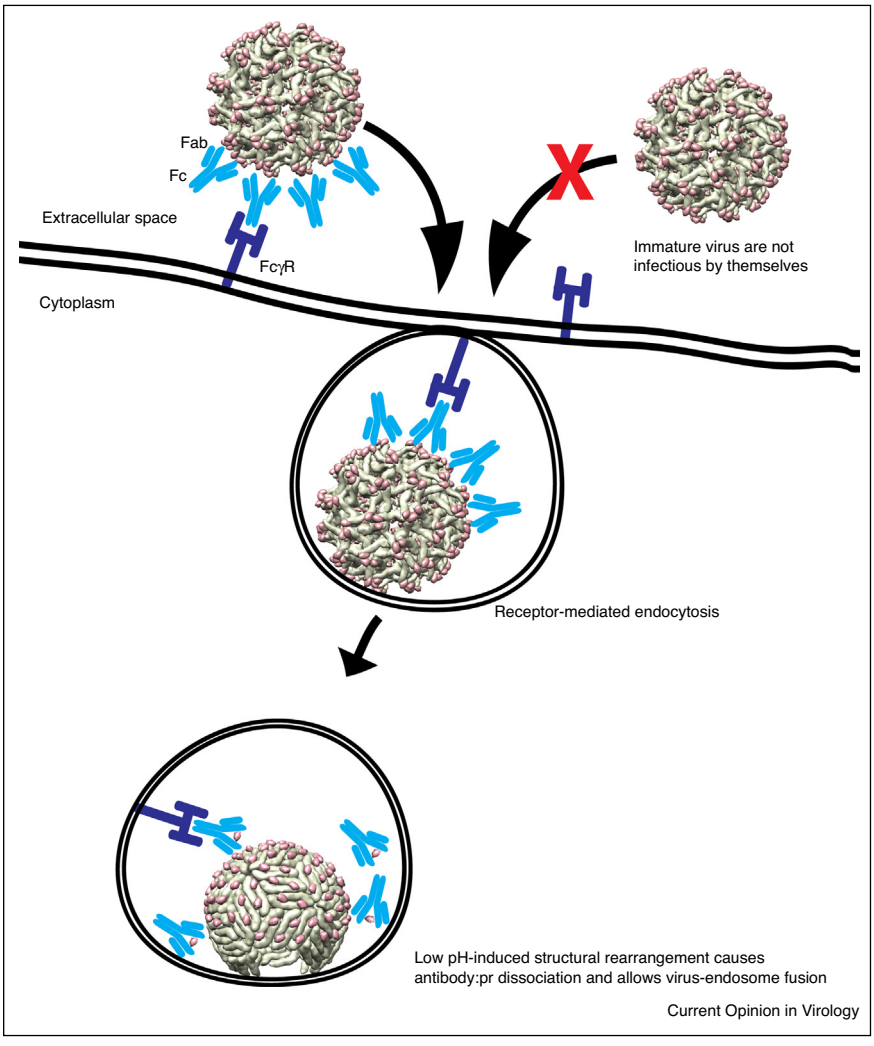
Another interesting finding by Renner *et al.* is the differential binding of 2C8 and 3H5 at different pH [28••]. While 3H5 shows strong binding at both neutral pH and

low pH, 2C8 shows drastically reduced binding at low pH. As the late endosome has a low pH environment, suggesting that 2C8 may dissociate from the viral surface, allowing infection and hence an explanation for the mechanism of ADE in mature dengue particles [30] (Figure 3). This paper thus shows that the nature of the antibody binding and positioning on the viral surface could influence ADE.

Structural insights into ADE of immature DENV

Fully immDENV on its own is not infectious; however, when complexed with anti-prM, immDENV can be rendered infectious to FcγR-positive cells. Furin cleavage was shown to be necessary for the infectivity of the anti-prM antibody:immDENV complex [26], suggesting that the virus needs to mature in the endosomes. The low pH environment and the presence of membrane bound furin in the endosomes could allow a part of the maturation process to occur; however, it is unknown how the pr could dissociate from the virus since the pH of the endosome remains acidic. To investigate this, Wirawan *et al.* used an anti-prM antibody (1H10), observed to induced ADE of immDENV, to form a complex with immDENV at neutral and low pH for cryoEM structural studies [31••]. The 11 Å resolution cryoEM structure of the Fab fragment of 1H10 complexed with immDENV3 at pH 8.0 showed the Fabs bind to prM proteins at full occupancies. At pH 5.0, mimicking the conditions in the

Figure 4



ADE mechanism of prM antibody:immature DENV complex. ADE with immature DENV occurs when anti-prM antibodies bind to the immature virus and are recognized by FcγR, allowing internalization of the virus-antibody complex. The low pH of the endosome induces dramatic rearrangements of the surface proteins of the virus. The combined effect of the bulkiness of the antibody, and its high affinity toward pr, further increases the clashes between the surface proteins thus enhancing the dissociation of pr molecule from the E protein fusion loop. This allows virus-endosomal membrane interaction, thus overcoming the first step of fusion.

late endosomes, two structural classes (class I and II) were observed at about 25 Å resolution, both having reduced number of Fab molecules bound on the viral surface compared to the pH 8.0 structure. In addition, their E proteins in both class I and II structures have undergone rearrangement, likely representing the early and late stages of the maturation process, respectively. These provide glimpses of some structural maturation intermediates.

Using molecular dynamics simulations (MDS) to simulate the movements of only the prM-E proteins from the cryoEM pH 8.0 Fab-immDENV structure to that of the Class I low pH Fab 1H10:immDENV structure showed a prM molecule was 'knocked-off' from one of the E protein molecules within an asymmetric unit even when the Fab molecules were not used in simulations. This is consistent with the liposome co-sedimentation assay showing that in the absence of Fabs, some immature DENV particles could interact with the liposome at low pH, and in the presence of Fabs, the virus-liposome interactions are enhanced. When the Fab molecule was added to the MDS transitional structures, the surface proteins and the Fabs encountered more clashes at an earlier time point, suggesting that more Fab or Fab:pr complexes have dissociated from the E protein fusion loop compared to the simulation without Fabs. To determine whether the Fabs dissociate alone or as a Fab:pr complex at low pH, Wirawan *et al.* [31••] used various methods – ELISA, biolayer interferometry, and hydrogen deuterium exchange mass spectrometry – to show that at low pH, the Fab 1H10 to prM interaction remains strong. Wirawan *et al.* [31••] propose the low pH environment of the endosomes stimulates dramatic structural rearrangement of the surface proteins of immDENV, so that bound prM antibodies cause increased clashes between the surface proteins, allowing the antibody:pr complex to dissociate from the E protein fusion loop and thereby facilitating virus:endosome membrane interaction, overcoming the first step of fusion [31••] (Figure 4).

This study, besides the above mechanism of ADE for immature DENV, provides intermediate structures along the maturation pathway that could be used as a target for drug and antibody therapeutics design.

Conclusions and future directions

One of the great obstacles to effective vaccine development is the phenomenon of ADE. While the possibility of ADE is especially important for DENV due to the presence of multiple DENV serotypes, it may also be important across flaviviruses as there are conserved epitopes across viruses such as DENV and ZIKV [32–34]. However, more research has to be done to determine if the flavivirus cross-reactive antibodies will enhance infection of different flaviviruses in a natural setting.

The two structural and functional studies described in this review will provide much insight into the mechanism of ADE, demonstrating the complex nature of antibody-virus binding and how these structures play a large role in the infectivity of mature and immature DENV [28••,29••,31••]. The ADE mature virus study by Renner *et al.* suggests partially hidden epitopes will more likely stimulate flat-lying antibodies that is inherently unable to cause ADE [28••]; however, there will be a fine balance between stimulating these antibodies and achieving high enough occupancy on the virus for neutralization. Antibodies with such properties — binding laterally to the viral surface and achieving a stoichiometry that allows neutralization could be safer therapeutics candidates for DENV and possibly for other flaviviruses.

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