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## Maternal Anti-Dengue IgG Fucosylation Predicts Susceptibility to Dengue Disease in Infants

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### SUMMARY

Infant mortality from dengue disease is a devastating global health burden that could be minimized with the ability to identify susceptibility for severe disease prior to infection. Although most primary infant dengue infections are asymptomatic, maternally derived anti-dengue immunoglobulin G (IgGs) present during infection can trigger progression to severe disease through antibody-dependent enhancement mechanisms. Importantly, specific characteristics of maternal IgGs that herald progression to severe infant dengue are unknown. Here, we define 10% afucosylation of maternal anti-dengue IgGs as a risk factor for susceptibility of infants to

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#### AUTHOR CONTRIBUTIONS

T.T.W., D.H.L., N.K.T., and R.C.B. designed experiments. T.T.W., N.K.T., R.C.B., R.S., S.Z., S.B., K.G.E., N.S.R., J.K.T., M.M., A.J.G., and P.J. collected and/or analyzed and/or interpreted data. T.T.W. and N.K.T. wrote the manuscript.

#### DECLARATION OF INTERESTS

The authors declare no competing interests.

#### SUPPLEMENTAL INFORMATION

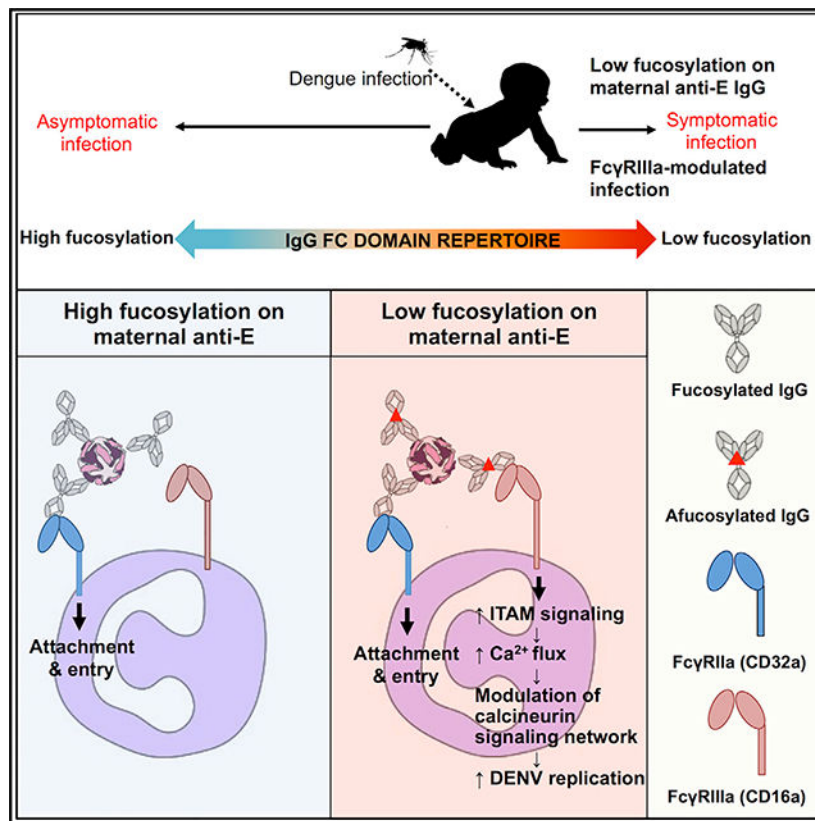
Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.107642>.

symptomatic dengue infections. Mechanistic experiments show that afucosylation of anti-dengue IgGs promotes FcγR1a signaling during infection, in turn enhancing dengue virus replication in FcγR1a+ monocytes. These studies identify a post-translational modification of anti-dengue IgGs that correlates with risk for symptomatic infant dengue infections and define a mechanism by which afucosylated antibodies and FcγR1a enhance dengue infections.

### In Brief

Thulin et al. show that reduced fucosylation (afucosylation) of maternally derived anti-dengue IgGs is associated with symptomatic dengue infections in infants. Afucosylation of dengue immune complexes promotes FcγR1a signaling in monocytes, in turn enhancing infection through a post-entry pathway that is dependent on the calcineurin signaling network.

### Graphical Abstract



### INTRODUCTION

Antibody-mediated inflammatory responses are critical in immunity against infectious organisms. These responses can promote pathogen clearance but can also exacerbate symptoms during infections (Bournazos et al., 2017). Antibody-mediated inflammation is triggered when pathogens or infected cells are bound by immunoglobulin G (IgG) antibodies, forming immune complexes that signal through Fc gamma receptors (FcγRs) on effector cells. The outcome of effector cell responses depends on the balance of activating to

inhibitory (A/I) Fc $\gamma$ R signaling that arises from interactions with Fc domains within immune complexes. Activating Fc $\gamma$ Rs contain immunoreceptor tyrosine-based activation motifs (ITAMs), while the inhibitory Fc $\gamma$ R balances this activity through immunoreceptor tyrosine-based inhibitory motif (ITIM) signaling. One factor that modulates the ratio of A/I Fc $\gamma$ R signaling is the glycosylation state of the IgG Fc domains within immune complexes. For example, sialylation of the Fc promotes anti-inflammatory effector responses, whereas absence of core fucosylation, afucosylation, of the Fc is pro-inflammatory due to increasing affinity of the Fc for the activating Fc $\gamma$ RIIIa, found on natural killer (NK) cells, as well as on subsets of macrophages and monocytes (Anthony et al., 2011; Bournazos et al., 2017; Rafiq et al., 2013). The second major determinant of A/I Fc $\gamma$ R signaling by immune complexes is the distribution of IgG subclasses within the complex, with IgG1 being the dominant subclass promoting pro-inflammatory responses and IgG2 signaling through the inhibitory Fc $\gamma$ R (Pincetic et al., 2014).

Dengue virus infections are unusual in that non-neutralizing anti-dengue virus IgGs can play a central role in triggering progression to the severe forms of disease through antibody-dependent enhancement (ADE) mechanisms (Anderson et al., 2014; Burke et al., 1988; Chau et al., 2009; Guzmán et al., 1990; Halstead et al., 1970; Katzelnick et al., 2017; Libraty et al., 2009; Sangkawibha et al., 1984; Wang et al., 2017). ADE can occur in the presence of reactive, non-neutralizing IgGs, as are found in secondary heterologous dengue infections or in primary infections in infants of dengue-immune mothers due to acquisition of anti-dengue virus IgGs during gestation (Halstead et al., 1970; Kliks et al., 1988; Simmons et al., 2007). These antibodies are thought to promote disease by forming immune complexes with the virus that modulate infection in Fc $\gamma$ R-bearing cells, primarily monocytes and macrophages, leading to higher viral titers and altered cytokine production during infection (Aye et al., 2014; Durbin et al., 2008; Kou et al., 2008; Thein et al., 1997). Still, the vast majority of dengue infections that occur in the presence of non-neutralizing IgGs are asymptomatic, and specific features of antibodies that enhance one's basal susceptibility for dengue disease are unknown. Importantly, mortality rates in severe dengue can exceed 20% when patients are not hospitalized, but can be reduced to <1% with in-patient care (Anderson et al., 2014; Gordon et al., 2013). Therefore, the identification of serologic markers for increased baseline susceptibility to dengue disease could dramatically reduce mortality associated with these viruses by enabling early hospitalization of those at highest risk for disease progression.

Because maternal IgGs are transferred to the fetus during gestation, we hypothesized that the repertoire of maternal anti-dengue virus IgG Fc domains might impact basal susceptibility of infants to disease during dengue virus infections. To address this, we performed a global analysis of maternal anti-dengue IgG Fc domain features from a prospective cohort of mothers and infants in a dengue-endemic region of the Philippines in order to define characteristics that predicted progression to significant infant dengue disease. We found that maternal anti-dengue IgGs enriched for afucosylated Fc glycoforms predicted clinically significant disease in their infants during primary dengue infections. Because afucosylation enhances affinity of the Fc for Fc $\gamma$ RIIIa, this suggested a role for this receptor in the pathogenesis of dengue disease. We therefore performed studies to dissect the mechanism by which Fc $\gamma$ RIIIa and afucosylated IgGs impact dengue virus infections. Our findings identify

a pathway by which dengue replication can be enhanced through a Fc $\gamma$ RIIIa signaling mechanism requiring the calcineurin signaling network. These studies identify a mechanistic basis for differential susceptibility to dengue disease based on the presence of afucosylated anti-dengue IgGs and suggest novel strategies for the treatment of dengue virus infections.

## RESULTS

### Maternal Anti-Dengue Fucosylation Predicts Susceptibility to Clinically Significant Disease during Infant Dengue Infections

Maternal IgG levels in the serum of newborns generally match or exceed those in maternal serum (1,012 mg/mL IgG), and the half-life of maternal IgGs in infants is >40 days (Watanaveeradej et al., 2003). Thus, when a mother is seropositive for dengue viruses, maternal anti-dengue IgGs have the potential to modulate infant dengue infections for over 1 year after birth (Alkan Ozdemiret et al., 2016; Nicoara et al., 1999; Simmons et al., 2007; Thompson et al., 2016).

To define specific maternal anti-dengue IgG features that herald risk for infant dengue disease, we performed a global analysis of anti-dengue envelope (E) IgGs in mothers of infants with symptomatic (febrile) or asymptomatic primary dengue infections. To do this, we studied samples from a prospective cohort in San Pablo, Laguna, Philippines, a dengue-endemic region. After delivery, infants were followed rigorously for primary dengue infections, including documentation of subclinical infections evidenced by seroconversion (Capeding et al., 2010; Libraty et al., 2009).

Given the role of Fc $\gamma$ Rs in ADE of dengue virus infections, we first asked whether any maternal anti-E Fc glycosylation patterns could predict susceptibility to infant dengue disease. Characterization of anti-E IgG Fc glycoforms showed that mothers of infants who had clinically significant (symptomatic) primary dengue infections had significantly increased levels of afucosylated anti-E Fc glycoforms compared with mothers of infants who had asymptomatic primary infections (Figure 1A). Indeed, 10% afucosylated Fc glycans was predictive of elevated risk for symptomatic infant dengue infections; this threshold predicted symptomatic infections in infants with a positive predictive value of 88%. Specificity of this marker was 93%, indicating that it nearly always predicted symptomatic dengue infections in infants. Sensitivity was lower, 46%, as many, but not all, symptomatic infections could be predicted using this serologic marker. Even omitting the sample with the highest level of afucosylation in the symptomatic group, the 10% threshold remained predictive with unchanged specificity (93%) and slightly reduced sensitivity (43%). Interestingly, this specific threshold of afucosylation was also identified in a prior study of hospitalized dengue patients; this prior study found that anti-E IgG with 10% afucosylated Fc glycans was predictive of the most severe disease outcomes during acute dengue infections that occurred in the presence of reactive, non-neutralizing IgGs (Wang et al., 2017). Aside from Fc fucosylation, other Fc glycoforms did not vary between mothers of infants with symptomatic and asymptomatic primary dengue infections (Figure 1B).

Besides Fc glycosylation, the other features that can impact the A/I ratio of Fc-Fc $\gamma$ R interactions were also measured. Extensive characterization of the maternal anti-E IgG

response showed that symptomatic infant dengue infections could not be predicted by the IgG1/IgG2 ratio of anti-E subclasses, a measurement that accounts for the most abundant IgG subclasses, with IgG1 having increased affinity for activating Fc $\gamma$ R3 over IgG2 (Figure 1C). The distribution of all maternal anti-E IgG sub-classes, the titer of anti-E IgGs, and the binding avidity of anti-E IgGs (Figures 1D-1F) were also not different between groups. In addition, there was no correlation between either symptomatic infant dengue infections or the levels of maternal anti-E afucosylation with infant sex, age at infection, days of fever during symptomatic infections, or serotype of the infecting dengue virus strain (Table S1). Because anti-NS1 IgGs can modulate the severity of dengue infections, we further assessed whether the maternal titer of anti-NS1 IgGs predicted symptomatic dengue infections in their infants and found no correlation (Figure S1A) (Hertz et al., 2017; Jayathilaka et al., 2018). In addition, there was no significant difference in the abundance of anti-NS1 ortho- tal IgG Fc fucosylation from mothers of infants with symptomatic or asymptomatic primary dengue infections (Figure S1B). The presence of 10% afucosylated glycans on anti-E IgG was the only factor identified in our characterizations that predicted risk for dengue disease in infants. Because Fc afucosylation increases affinity for Fc $\gamma$ R3, this finding pointed toward a role for Fc $\gamma$ R3 in antibody-enhanced dengue infections.

### **Fc $\gamma$ R1a Signaling Is Driven by the Fucosylation Level in Dengue Immune Complexes and Enhances Dengue Virus Replication in Human U937 Monocytes**

To study how Fc $\gamma$ R3 signaling by dengue immune complexes might be impacted by variable levels of anti-E fucosylation, we used the maternal IgGs to generate dengue immune complexes and defined their signaling capacity in a Fc $\gamma$ R3 reporter assay. This showed a linear correlation between endogenously produced levels of anti-E afucosylation and Fc $\gamma$ R3-mediated signaling (Figure 2A). Next, we generated dengue virus immune complexes using pooled maternal IgGs with 10% or <10% afucosylation of anti-E IgG; immune complexes with 10% afucosylation drove significantly higher Fc $\gamma$ R3 signaling (Figure 2B). To assess whether anti-E domain specificity might modulate Fc $\gamma$ R3 signaling activity, we expressed monoclonal antibodies (mAbs) that bound to distinct domains of the E protein with fucosylated or afucosylated Fc glycans. All mAbs triggered significantly enhanced Fc $\gamma$ R3 signaling when expressed with afucosylated Fc glycoforms regardless of their anti-E binding specificity (Figure 2C; Figure S2; Table S2).

Although Fc $\gamma$ R3 is present on macrophage and pro-inflammatory monocyte subsets that are associated with dengue disease, Fc $\gamma$ R3 is not expressed on human monocytic or other granulocytic lineage cell lines that are often used to study ADE of dengue virus infections, such as THP-1, K562, or U937 cells (Fleit et al., 1982; Tridandapani et al., 2002; van de Winkel and Anderson, 1991). These cell lines express the high-affinity activating Fc $\gamma$ R, Fc $\gamma$ R1 (except for K562 cells), along with the low-affinity Fc $\gamma$ Rs, activating Fc $\gamma$ R2 and inhibitory Fc $\gamma$ R1b. Further, primary cells and *in vivo* mouse models are not robust systems for studying the role of Fc $\gamma$ R3 in dengue infections because Fc $\gamma$ R3 is unstable on primary monocytes/macrophages that are cultured *ex vivo* (Ong et al., 2019; Wang lab, unpublished data), and mouse models that are humanized for Fc $\gamma$ R expression, and thus express Fc $\gamma$ R3, are immunocompetent, rendering them incompatible with current dengue infection models (Smith et al., 2012; Zellweger and Shresta, 2014). Thus, to dissect the role

of Fc $\gamma$ RIIIa in ADE of dengue infections, we generated human U937 monocytes expressing different combinations of the activating low-affinity Fc $\gamma$ Rs, Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa (Figures S3A–S3F). Fc $\gamma$ RIIa was previously shown to have a role in ADE of dengue infections (Boonnak et al., 2013), but a role for Fc $\gamma$ RIIIa has not been defined.

First, U937 monocytes expressing different combinations of Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa were infected with immune complexes made from dengue virus and five different maternal IgGs from the San Pablo cohort. Single-cycle infection was enhanced by up to 300% in monocytes expressing Fc $\gamma$ RIIIa (Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>-</sup>) compared with those expressing only Fc $\gamma$ RIIa (Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>-</sup>) (Figure 3A). Next, we determined whether Fc $\gamma$ RIIIa ITAM signaling was required for Fc $\gamma$ RIIIa-mediated ADE, using cells expressing signaling-competent (Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>-</sup>) or signaling-null [Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>(ITAM<sup>-</sup>)</sup>] versions of Fc $\gamma$ RIIIa (Figure S3A) (Blázquez-Moreno et al., 2017). Interestingly, the Fc $\gamma$ RIIIa-mediated enhancement of infection required ITAM signaling (Figure 3A). To assess whether anti-E domain specificity might contribute to the involvement of Fc $\gamma$ RIIIa in ADE, we tested the panel of afucosylated anti-E mAbs for their ability to modulate dengue infections. In all cases, anti-E mAbs mediated significantly increased infection in Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> cells over U937 monocytes expressing only Fc $\gamma$ RIIa. As observed with the maternal IgG, Fc $\gamma$ RIIIa-mediated ADE required Fc $\gamma$ RIIIa signaling in all cases (Figure 3B). Overall, these studies showed that increasing the cellular A/I Fc $\gamma$ R signaling ratio via Fc $\gamma$ RIIIa ITAM signaling promoted ADE of dengue virus infections.

We next asked whether modulating cellular A/I Fc $\gamma$ R signaling in parallel with dengue infection might impact ADE. Crosslinking individual Fc $\gamma$ Rs in Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells showed that increased signaling by the activating, ITAM-containing Fc $\gamma$ RIIa or Fc $\gamma$ RIIIa enhanced cell infectibility, whereas signaling through the inhibitory, ITIM-containing Fc $\gamma$ RIIb reduced infection (Figure 3C). These results are consistent with increased A/I signaling ratios promoting ADE of dengue infections, whereas reducing the A/I ratio via Fc $\gamma$ RIIb signaling diminished ADE. Additive activating/ITAM signaling through crosslinking Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa together resulted in the greatest enhancement of infection (Figure 3D). These data supported a mechanism whereby infection was enhanced by cellular calcium flux, which is triggered by ITAM signaling (Bolland and Ravetch, 1999; Hippen et al., 1997). To verify this, we treated cells with the calcium ionophore, ionomycin, prior to infection. This treatment increased ADE of infection in a dose-dependent manner (Figure 3E).

Having found that Fc $\gamma$ RIIIa ITAM signaling could increase dengue infections, we next sought to determine whether Fc $\gamma$ RIIIa might have a separate role in mediating attachment of dengue immune complexes. To investigate this, we used well-characterized blocking mAbs against Fc $\gamma$ RI, Fc $\gamma$ RIIa, or Fc $\gamma$ RIIIa to prevent immune complex attachment to cells prior to infection (Boruchov et al., 2005; Fleit et al., 1982; Mandelboim et al., 1999; Tamm and Schmidt, 1996; Zeller and Sullivan, 1993). In Fc $\gamma$ RIIa<sup>+</sup> Fc $\gamma$ RIIIa<sup>+</sup> U937 cells, blocking either Fc $\gamma$ RI or Fc $\gamma$ RIIa during immune complex attachment prevented ~50% infection, whereas blocking Fc $\gamma$ RIIIa alone with the blocking mAb, 3G8, did not inhibit infection (Figure 4A) (Boruchov et al., 2005; Fleit et al., 1982; Mandelboim et al., 1999; Tamm and Schmidt, 1996; Zeller and Sullivan, 1993). Combining blocking mAbs for Fc $\gamma$ RI and

FcγRIIa inhibited infection by ~80%, and inclusion of the FcγRIIIa blocking mAb did not further diminish infection. In freshly isolated primary monocytes, blocking attachment of immune complexes to FcγRIIa prior to entry prevented nearly all infection, whereas blocking FcγRI, FcγRIIb, or FcγRIIIa did not reduce infection (Figure 4B). These results are consistent with studies showing that FcγRIIa on monocytes mediates dengue immune complex attachment and entry (Boonnak et al., 2013; Kou et al., 2008; Littaua et al., 1990) and suggested that the FcγRIIIa-mediated enhancement is due to modulation of a post-attachment step of infection.

### **Inhibitors of the Calcineurin Signaling Network Block Dengue Virus Replication**

Engagement and crosslinking of the activating FcγRs trigger phosphorylation of ITAM domains by Src family kinases and intracellular calcium flux (Bournazos and Ravetch, 2015; Hippen et al., 1997). Calcium flux, in turn, activates calcineurin, a serine/threonine phosphatase that recognizes a host of substrates, scaffolds, and regulator proteins through specific docking motifs (Roy and Cyert, 2009; Roy et al., 2007). To dissect how enhanced A/I ratios contribute to ADE of dengue infections, we treated cells with inhibitors of the ITAM signaling pathway prior to infection. First, FcγRIIa<sup>+</sup>FcγRIIIa<sup>+</sup> monocytes were treated with PRT, an inhibitor of Syk tyrosine kinase activity, required for initiation of ITAM signaling (Lowell, 2011; Reilly et al., 2011). This inhibited dengue infection (Figure 5A), supporting the requirement for ITAM signaling in ADE of dengue virus infection.

Next, we tested the hypothesis that ADE of dengue infection is enhanced by calcium flux via the calcineurin signaling network. The small-molecule inhibitor INCA-6 (Bretz et al., 2013) and the peptide inhibitor 11R-VIVIT (VIVIT) (Aramburu et al., 1999) act selectively to prevent the association of calcineurin with substrates that bind via the calcineurin PxIxIT docking motif (Roy et al., 2007). Both inhibitors prevented dengue virus infection in U937 monocytes (Figures 5B and 5C). Inhibitors were used at concentrations that did not impact cell viability (Figure S4). To assess the specificity of this observation for dengue infections, we studied the impact of the VIVIT inhibitor on infection of U937 cells by influenza A viruses. Although dengue serotypes 2 and 3 were robustly inhibited by VIVIT, influenza A viruses (H1N1 A/Netherlands/602/2019 and H3N2 A/HK/1/1968 strains) were not affected by this inhibitor of the calcineurin signaling network (Figure 5D). We next treated cells with the VIVIT inhibitor during or at different time points after dengue infection to characterize the kinetics of the required calcineurin network activity. Treatment of cells up to 6 h after entry significantly reduced infection, consistent with disruption of a post-entry stage of infection (Figure 5E). Interestingly, FK506 and cyclosporin A, two clinically used drugs that block calcineurin through mechanisms distinct from INCA-6 and VIVIT (Ho et al., 1996), did not prevent dengue infections (Figure S5). These studies show that calcineurin-PxIxIT site interactions are required for maximal dengue virus infections, suggesting new clinical strategies for treatment of dengue infections.

### **IgG Fucosylation Is Stable over Time, Suggesting Utility as a Predictive Marker for Dengue Disease Risk**

To be a useful marker of dengue disease susceptibility, the abundance of Fc afucosylation would have to be stable over time and withstand immune perturbation. To assess stability of

this IgG modification, we performed a longitudinal study of IgGs from healthy adults in a North American cohort who were challenged with influenza A virus (Memoli et al., 2015). We characterized IgG afucosylation prior to and over a period of 60 days after influenza virus challenge. The abundance of afucosylated glycans was remarkably durable over this period and, despite virus challenge (Figure S6), supporting utility of this serologic marker to guide clinical care of dengue patients.

## DISCUSSION

Here, we show that elevated afucosylated Fc glycans ( ~ 10%) on maternal anti-E IgGs is one risk factor for susceptibility of infants to disease during primary dengue virus infections. Mechanistic studies performed to identify how afucosylated anti-E IgGs can impact dengue infections support a model whereby the presence of afucosylated dengue immune complexes triggers a Fc $\gamma$ RIIIa-modulated infection characterized by increased ITAM signaling. Enhanced ITAM signaling, in turn, promoted dengue infections in U937 monocytes through an intrinsic ADE mechanism (Figure 5F) (Halstead et al., 2010). On a cellular level, the data show a role for Fc $\gamma$ RIIIa in enhancing ADE of dengue virus infections through cooperativity with Fc $\gamma$ RIIa. Although Fc $\gamma$ RIIa acts in virus immune-complex attachment and entry (extrinsic ADE) (Halstead et al., 2010), we show that the increased cellular A/I signaling ratio triggered by afucosylated immune complex-Fc $\gamma$ RIIIa interactions supports dengue virus infection through calcineurin signaling network interactions requiring the PxxIT docking motif (Figure 5G). This mechanism suggests novel treatment strategies for dengue infections that target this pathway.

An important topic for future studies on the pathogenesis of dengue disease is the cellular activities associated with afucosylated IgGs during dengue infection that were not studied here. We have used percent infection as the readout of activity conferred by anti-dengue Fc afucosylation and of Fc $\gamma$ RIIIa signaling in ADE of dengue virus infections, yet Fc $\gamma$ RIIIa signaling activity likely impacts disease pathogenesis through multiple mechanisms, including modulation of cytokine production by Fc $\gamma$ RIIIa-expressing cells.

Because mortality rates in severe dengue cases can be nearly eliminated with intensive clinical monitoring (Anderson et al., 2014; Gordon et al., 2013), identifying markers for susceptibility to dengue disease could dramatically improve the clinical management of dengue patients. Here we show that maternal IgG Fc fucosylation could be used in the cohort studied to identify a significant subset of susceptible infants. Prospective cohort studies are now needed to examine testing of antibody fucosylation to guide the treatment of primary infant dengue infections and acute dengue infections in non-infant populations.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:



## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
FITC conjugated anti-human Fc $\gamma$ RIIa, clone IV.3	STEMCELL Technologies	Cat#60012FI; RRID: AB_2722545
Brilliant Violet 711 conjugated anti-human Fc $\gamma$ RIIIa, clone 3G8	Biolegend	Cat#302044; RRID: AB_2563802
Anti-human Fc $\gamma$ RIIb, clone 2B6	J. Ravetch, Rockefeller University	N/A
APC anti-human Fc $\gamma$ RI, clone 10.1	Biolegend	Cat#305014; RRID: AB_1595428
Influenza A NP Monoclonal Antibody (D67J), FITC	Invitrogen	Cat#MA17322; RRID: AB_1017747
anti-E antibody, clone D1-4G2-4-15	ATCC	Cat#VR-1852; RRID: AB_827205
Fucosylated and afucosylated human monoclonal antibody 23-5G2D2747(4)	(Sasaki et al., 2013)	N/A
Fucosylated and afucosylated human monoclonal antibody B7	(Rouvinski et al., 2015)	N/A
Fucosylated and afucosylated human monoclonal antibody 2D22	(de Alwis et al., 2012)	N/A
Fucosylated and afucosylated human monoclonal antibody 753(3) C10	(Rouvinski et al., 2015)	N/A
Fucosylated and afucosylated human monoclonal antibody 5J7	(de Alwis et al., 2012)	N/A
anti- Fc $\gamma$ RIIIa, clone 3G8	Biolegend	Cat#302057; RRID: AB_2572005
anti-Fc $\gamma$ RIIa clone IV.3	Stem Cell Technology	Cat#60012; RRID: AB_2722545
<b>Bacterial and Virus Strains</b>		
DENV2 New Guinea C	BEI Resources	NR-84
A/Hong Kong/1/1968, H3N2	BEI Resources	NR-28620
A/Netherlands/602/2009, H1N1	Wang Lab Stock	N/A
DENV3 C0360/94	BEI Resources	NR-48800
<b>Biological Samples</b>		
Sera collected at birth from 29 dengue seropositive mothers of infants who had primary dengue infections	(Libraty et al., 2009)	<a href="https://clinicaltrials.gov/ct2/show/study/NCT00377754">ClinicalTrials.gov: NCT00377754</a>
Sera from 8 individuals challenged with Ca/04/2009/H1N1 Vero Grown Virus. Sera were collected prior to challenge as well as on day 30 and 60 post-challenge.	(Memoli et al., 2015)	<a href="https://clinicaltrials.gov/ct2/show/study/NCT01646138">ClinicalTrials.gov: NCT01646138</a>
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Dengue Envelope Protein Serotype 1	Prospec	DEN-021
Dengue Envelope Protein Serotype 2	Prospec	DEN-022
Dengue Envelope Protein Serotype 3	Prospec	DEN-023
Dengue Envelope Protein Serotype 4	Prospec	DEN-024
11R-VEET	Thermo Fisher	Cat#CM102281.2
11R-VIVIT	Thermo Fisher	Cat#CM102281.1

REAGENT or RESOURCE	SOURCE	IDENTIFIER
INCA-6	Abcam	Cat#ab145864
PRT062607	Fisher Scientific	Cat#NC0664362
Ionomycin	Invivogen	Cat#inh-ion
2F-Peracetyl-Fucose	EMD Millipore	Cat#344827
Critical Commercial Assays		
Protein G Sepharose 4 Fast Flow resin	GE Healthcare	Cat#17061802
NHS agarose resin	ThermoFisher	Cat#26196
Experimental Models: Cell Lines		
FcγRIIa <sup>+</sup> FcγRIIIa <sup>+</sup> U937 Cells	This paper	N/A
FcγRIIa <sup>-</sup> FcγRIIIa <sup>-</sup> U937 Cells	This paper	N/A
FcγRIIa <sup>+</sup> FcγRIIIa <sup>(ITAM<sup>-/-</sup>)</sup> U937 Cells	This paper	N/A
U937 Cells	ATCC	CRL-1593.2
Vero cells	ATCC	CCCL-81
Expi293 cells	ThermoFisher	Cat#A14527
Recombinant DNA		
pLJM1-EGFP plasmid	Addgene	Plasmid #19319
Human codon optimized cas9 DNA	Addgene	Plasmid #41815
Software and Algorithms		
GraphPad PRISM 9	GraphPad Software, La Jolla, California, USA	<a href="https://www.graphpad.com">https://www.graphpad.com</a>
Flowjo	FlowJo, LLC	<a href="https://www.flowjo.com/">https://www.flowjo.com/</a>
Analyst 1.6.1 software	SCIEX	<a href="https://sciex.com">https://sciex.com</a>
MultiQuant 2.1.1	SCIEX	<a href="https://sciex.com">https://sciex.com</a>

## RESOURCE AVAILABILITY

**Lead Contact**—The lead contact for this study is Taia T. Wang (taiawang@stanford.edu).

**Materials Availability**—This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact.

**Data and Code Availability**—This study did not generate any unique datasets or code. An unpaired Student's t test was utilized to calculate p value: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001.

**Key Resources Table**—See attached file

## Experimental Model And Subject Details

Clinical cohorts and samples: Samples from mothers of infants who had primary dengue infections were from a study that was approved by the institutional review boards of the Research Institute for Tropical Medicine, Philippines, and the University of Massachusetts

Medical School. Mothers and their healthy infants were recruited and enrolled after providing written informed consent ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00377754) NCT00377754) (Libraty et al., 2009). Symptomatic dengue was defined as febrile illness with a positive result by dengue serotype-specific reverse-transcription PCR in acute phase sera (Lanciotti et al., 1992). Clinically inapparent dengue infection (asymptomatic infection) was determined using a hemagglutination inhibition assay (HAI assay); a four-fold increase in HAI against one or more dengue serotypes in the absence of febrile illness confirmed asymptomatic infection (Libraty et al., 2009). The outcome assessed was clinical phenotype during dengue virus infection (symptomatic infection versus asymptomatic infection) (Table S1). Samples from the controlled influenza A virus challenge study have been previously described (Memoli et al., 2015). The study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01646138) NCT01646138) was approved by the National Institute of Allergy and Infectious Diseases institutional review board and underwent an ethics review by the NIH Department of Bioethics prior to being conducted. The study was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

## METHOD DETAILS

IgG Fc glycan and IgG subclass analysis: Methods for relative quantification of Fc Glycans and IgG subclasses have been previously described (Wang et al., 2015; Wang et al., 2017). Briefly, IgGs were isolated from serum by protein G purification. Antigen-specific IgGs were isolated on NHS agarose resin (ThermoFisher; 26196) coupled to the protein of interest. Mixed envelope proteins from the four DENV serotypes were used (ProSpec; DEN-021, DEN-022, DEN-023, DEN-024). Following tryptic digestion of purified IgG bound to antigen-coated beads, nanoLC-MS/MS analysis for characterization of glycosylation sites was performed on an Ulti-Mate3000 nanoLC (Dionex) coupled with a hybrid triple quadrupole linear ion trap mass spectrometer, the 4000 Q Trap (SCIEX). MS data acquisition was performed using Analyst 1.6.1 software (SCIEX) for precursor ion scan triggered information dependent acquisition (IDA) analysis for initial discovery-based identification.

For quantitative analysis of the glycoforms at the N297 site across the three IgG subclasses (IgG1, IgG2 and IgG3/G4), multiple-reaction monitoring (MRM) analysis for selected target glycopeptides, was applied using the nanoLC-4000 Q Trap platform to the samples which had been digested with trypsin. The m/z of 4-charged ions for all different glycoforms as Q1 and the fragment ion at m/z 366.1 as Q3 for each of transition pairs were used for MRM assays. A native IgGs tryptic peptide (131-GTLVTVSSASTK-142) with transition pair of, 575.9<sup>+2</sup>/780.4 was used as a reference peptide for normalization. IgG subclass distribution was quantitatively determined by nanoLC-MRM analysis of tryptic peptides following removal of glycans from purified IgGs with PNGase F. Here the m/z value of fragment ions for monitoring transition pairs was always larger than that of their precursor ions to enhance the selectivity for unmodified targeted peptides and the reference peptide. All raw MRM data were processed using MultiQuant 2.1.1 (SCIEX). All MRM peak areas were automatically integrated and inspected manually. In the case where the automatic peak integration by MultiQuant failed, manual integration was performed using the MultiQuant software.

U937 cell lines: Wild-type U937 cells are Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>-</sup>; Fc $\gamma$ RIIa was deleted from the wild-type line using CRISPR/Cas9 methods to generate Fc $\gamma$ RIIa<sup>-</sup>Fc $\gamma$ RIIIa<sup>-</sup> monocytes. Wild-type cells were stably transfected for expression of Fc $\gamma$ RIIIa generating the Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup>. A final cell line, Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>(ITAM<sup>-/-</sup>)</sup>, expresses Fc $\gamma$ RIIa and a variant of Fc $\gamma$ RIIIa that does not associate with the Fc receptor gamma chain, and thus lacks ITAM signaling (Blázquez-Moreno et al., 2017).

The gRNAs to target Fc $\gamma$ RIIa were designed utilizing DESKGEN software, and inserted into sequence containing U6 promoter and gRNA scaffold (Yang et al., 2014). The gRNA sequence to target human Fc $\gamma$ RIIa was 5' GATGTATGTCCCAGAAACCTG3'. The final fragment (Integrated DNA Technologies) was cloned into the pCR-BluntII-Topo vector. Human codon optimized cas9 DNA was obtained from (Addgene, Plasmid #41815). To generate the knockout cell line, U937 cells were transfected with 1.5 $\mu$ g hCas9 and 0.5 $\mu$ g of gRNA via electroporation. The U937 cells were electroporated using Lonza Nucleofector 2b (program W-001) and the Nucleofection Kit C (Lonza, VPA-1004). After electroporation, U937 cells were incubated at 37°C for 48 hours. Following incubation, cells were synchronized at the early S phase using a double thymidine block. The cells were stained with IV.3-FITC, and Fc $\gamma$ RIIa negative cells were bulk sorted on SH800 sorter. After one bulk sort, the cells were single cell sorted based on negative Fc $\gamma$ RIIa expression. To generate Fc $\gamma$ RIIIa<sup>+</sup> cells, the wild-type cells were nucleofected with pLJM1-EGFP plasmid (Addgene, Plasmid #19319) that has EGFP replaced with Fc $\gamma$ RIIIa or Fc $\gamma$ RIIIa<sup>(ITAM<sup>-/-</sup>)</sup>, while the Fc $\gamma$ RIIa<sup>-</sup> cells were nucleofected with pLJM1-Fc $\gamma$ RIIIa only. Stably transfected clones were selected using 1 $\mu$ g/mL puromycin.

Fc $\gamma$ RIIIa signaling assay: U937 cells were infected with DENV2 New Guinea C (BEI Resources; NR-84) at MOI2 and 0.2 $\mu$ g/ml 235WT. 48h post infection, cells were washed once with PBS. Infected U937 cells were added to anti-dengue mAbs or purified maternal IgGs. Infected U937 cell immune complexes were washed and added to 100 $\mu$ l of Jurkat-Lucia NFAT CD16 (Invivogen; jktl-nfat-cd16) cell suspension at a concentration 1 $\times$ 10<sup>6</sup>cells/ml in IMID media and incubated at 37°C for 24h. 20 $\mu$ l of assay supernatant was then transferred to a 96-well white polystyrene clear flat bottom plate. 50 $\mu$ l Lucia detection medium QUANTI-Luc (Invivogen; rep-qlc1) was added and luciferase activity was measured using Tecan Infinite M100 with a 0.1 s reading time.

FACS staining of U937 cell lines: U937 cell lines were stained with a fixable live/dead stain (ThermoFisher; L10119). To assess Fc $\gamma$ R expression cells were stained at 1:100 with a FITC conjugated anti-human Fc $\gamma$ RIIa antibody, clone IV.3 (STEMCELL Technologies; 60012FI); Brilliant Violet 711 conjugated anti-human Fc $\gamma$ RIII, clone 3G8 (Biolegend; 302044); an Fc $\gamma$ RIIb antibody clone 2B6 (gifted by the Ravetch lab, Rockefeller University) conjugated to Alexa Fluor 647 (ThermoFisher; A20186) and an APC conjugated anti-human Fc $\gamma$ RI, clone 10.1 (Biolegend, 305014) for 30 minutes at 4°C.

Cross-linking of Fc $\gamma$ Rs for receptor signaling detection: U937 cell lines were treated with mouse anti-Fc $\gamma$ RIIIa/b, clone 3G8 (Biolegend; 302057) at 10 $\mu$ g/ml in Optimem on ice for 30 min. Anti-mouse Fc was then added at 1.25 $\mu$ g per 1 million cells and incubated at 37°C for 3 min to crosslink mAb 3G8. Cells were then lysed in a one to one ratio of cell lysate

buffer containing 100mM TRIS-HCL/300mM NaCL/2% Triton X-100 and a protease/phosphatase inhibitor cocktail (ThermoFisher; 88668). Protein concentrations were quantified using BCA Protein Assay Kit (ThermoFisher; 23227) and lysates were treated by boiling for 5 min with Laemmli SDS sample buffer containing 5%  $\beta$ -mercaptoethanol. Cell lysates were run on Mini-PROTEAN® TGX Precast Protein Gels (Bio Rad; 456903). Blots were blocked 5% BSA in TBS buffer 20mM Tris-HCL/150mM NaCl pH 7.6 for 2 hours and were subsequently incubated with anti-phosphotyrosine secondary antibody (Biolegend; 309302) at 1:1,000 in 5% BSA in TBS, 0.2% Tween-20 (TBST) overnight at 4 °C. Blots were washed and incubated with anti-mouse IgG Fc-HRP (Southern Biotech; 1030-05) at 1:10,000 for 1 hour at room temperature. Blots were developed with Western Lightning ECL Pro (Perkin Elmer; NEL120001EA) according to manufacturer's instructions. The membrane was imaged using the Amersham Imager 600 (GE Life Sciences) with auto exposure under chemiluminescent detection settings. Post-imaging, the membrane was washed extensively with TBST before treating with Restore™ Western Blot Stripping Buffer (ThermoFisher; 21059) for 15 min at room temperature. To analyze protein loading, the membrane was re-blocked with 5% BSA in TBST for 2 hour at room temperature before probing with a 1:200 dilution anti-vinculin 7F9 (Santa Cruz Biotechnology; sc-73614) in 5% BSA/TBST for 1 hour at room temperature followed by anti-mouse IgG HRP secondary, as above. The membrane was then treated with chemiluminescent substrate and imaged as described above.

Cross-linking of Fc $\gamma$ R in parallel with infection: Non-TC treated round bottom plates were coated with Fc $\gamma$ R specific antibodies in PBS for 1h at 37°C. Coating antibodies: anti-Fc $\gamma$ RIIa clone IV.3 (Stem Cell Technology; 60012) (at 5ug/ml individually or 2.5ug/ml in conjunction with other antibodies), anti-Fc $\gamma$ RIIb clone 2B6 (generously provided by J. Ravetch, Rockefeller University) (at 5ug/ml individually or 2.5ug/ml in conjunction with other antibodies); and anti-Fc $\gamma$ RIIIa, clone 3G8 (Biolegend; 302057) (at 10ug/ml individually or 5ug/ml in conjunction with other antibodies). Plates were coated at 10ug/ml in PBS of human IgG2/kappa (Sigma Aldrich; AG504) to control for protein coat. Plates were washed twice and 50,000 Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells/well diluted in OPTI-MEM were added to plate and incubated at 4°C for 30min. Immune complexes composed of DENV2 New Guinea C (BEI Resources; NR-84) and 0.2ug/ml mAb 235 were then added to plate and incubated at 37°C for 2h. Cells were washed twice in PBS and re-suspended in OPTI-MEM followed by an incubation for 22 h at 37°C. Percent infection was detected by FACs.

Monoclonal antibody production: Antibodies: 23-5G2D2747(4)(Sasaki et al., 2013), B7 (B7)(Dejnirattisai et al., 2015; Rouvinski et al., 2015; Swanstrom et al., 2016), 2D22(de Alwis et al., 2012; Fibriansah et al., 2015a; Swanstrom et al., 2016), and 753(3) C10 (C10) (Rouvinski et al., 2015; Swanstrom et al., 2016), 752-2 C8(de Alwis et al., 2012; Rouvinski et al., 2015; Swanstrom et al., 2016), and 5J7(de Alwis et al., 2012; Fibriansah et al., 2015b; Swanstrom et al., 2016) were expressed in Expi293 cells as follows. For afucosylated variants, Expi293 cells (ThermoFisher; A14527) were split to  $2.0 \times 10^6$  cells/mL in a 10 mL volume 24h prior to transfection and treated with 100  $\mu$ M of afucosyl transferase inhibitor, 2F-Peracetyl-Fucose (Rillahan et al., 2012) (EMD Millipore, 344827). On the day of transfection, 5  $\mu$ g filter-sterilized DNA (2.5  $\mu$ g each of heavy and light chain-encoding

plasmids) was added to 1 mL of Expi293 medium (ThermoFisher Scientific; A1435101) in a fresh tube, and mixed. To this, 13  $\mu$ L (1.3  $\mu$ L/mL of total transfection volume) of FectPro transfection Reagent (Polyplus Transfection; 116-001) was added. The DNA-transfection reagent mixture was incubated at room temperature for 10 minutes before adding dropwise to 10 mL of the Expi293 cells. The cells were then additionally treated with a final concentration of 4g/L glucose and 3 mM valproic acid. The cells were then grown under shaking conditions (125 rpm) at 37°C for 3 days before receiving an additional 4g/L final concentration of glucose. The cells were harvested on day 6 post-transfection, the cell pellet discarded, and the supernatant media filtered before purification. MAbs were purified over Protein G Sepharose 4 Fast Flow resin (GE Healthcare; 17061802). The antibodies were buffer exchanged into PBS pH 7.4 prior to use in any cell-based assays.

Cell-based ELISA: Wells of a 96 well plate were seeded with  $3 \times 10^4$  Vero cells (ATCC; CCL-81). The next day cells were infected with DENV2 New Guinea C (BEI Resources; NR-84) or DENV3 C0360/94 (BEI Resources; NR-48800) MOI1 for 1 hour, washed, and incubated for 48 h in MEM, 1% methylcellulose, 2% inactivated FBS, antibiotics, and L-glutamine. Cells were fixed with 4% formaldehyde for 30 minutes and permeabilized in 2% inactivated FBS/0.1% saponin/PBS. For analysis of clinical samples, serially diluted maternal sera were incubated for 2 hours at room temperature on cells infected with the virus serotype of the paired infant's dengue virus infection. For mAb binding assays, mAbs were titrated and incubated for 2 hours at room temperature on DENV2 infected cells. Cells were washed with PBS-0.1% Tween 20 and incubated with mouse anti-human IgG Fc-HRP (Southern Biotech; 9040-05) for 1 hour. Following incubation, the wells were washed and TMB Liquid Substrate (Sigma; T0440) was added and stopped with 1M HCL after approximately 5 minutes. Absorbance at 450 nm was measured; ELISA performed in triplicate.

Antibody-dependent infections: Anti-E mAbs were incubated with dengue viruses for 30 minutes at 37°C to form immune complexes. Afucosylated anti-E mAbs were used where indicated in the text.  $5 \times 10^4$  U937 cells were added to the immune complexes (MOI: 4) in RPMI containing 2% heat-inactivated FBS, antibiotics, MEM nonessential amino acid solution, 2-mercaptoethanol, and HEPES. Cells were infected for 2 hours at 37°C, washed, then resuspended in infection media and incubated for an additional 4–24 h at 37°C. Infected cells were stained with a fixable violet live/dead stain (ThermoFisher; L34955) for 30 min, fixed with 4% form-aldehyde and permeabilized in 2% inactivated FBS, 5mM EDTA, 0.1% saponin in PBS. Cells were stained using an anti-E antibody, clone 4G2 (ATCC; VR-1852) conjugated to Alexa Fluor 647 (ThermoFisher; A20186) at 1:400 for 30min. The frequency of infected cells was determined using flow cytometry, defined as the percentage of live, single cells that were positive for mAb 4G2 staining. For infections involving inhibitors or activators of ITAM signaling, U937s were treated with titrated inhibitors/activators: Ionomycin (Invivogen; inh-ion), PRT062607 (Fisher Scientific; NC0664362), INCA-6 (Abcam; ab145864), 11R-VIVIT (Thermo Fisher; CM102281.1), or 11R-VEET (Thermo Fisher; CM102281.2) in complete RPMI media for 1h-6h at 37C prior to, during or following infection as indicated. Immune complexes were formed at 37°C for 30min between hIgG1 mAb 235 and DV2 (MOI4) at 37°C. U937 were washed 2 times with PBS and then added to the immune complexes for a 2h infection at 37°C. Cells were then washed

2x in PBS and re-suspended in infection media followed by incubation for an additional 22 h at 37°C.

Fc $\gamma$ R blocking assay: Afucosylated anti-E monoclonal antibody 235 expressed as a human IgG1 (hIgG1) was incubated with dengue virus for 30 minutes at 37°C to form immune complexes and then cooled on ice for 15 min. Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells or CD14<sup>+</sup> PBMCs were incubated on ice for 30 minutes with different combinations of Fc $\gamma$ R-specific blocking antibodies, described above, each at 4 $\mu$ g/ml (Boruchov et al., 2005; Fleit et al., 1982; Zeller and Sullivan, 1993). Total antibody concentration between determinants was controlled by addition of human IgG2/kappa where needed (Sigma Aldrich; AG504). Cells were washed with ice-cold PBS and added to dengue immune complexes (MOI 5). Infection proceeded for 1 hour on ice. Cells were then washed and resuspended in infection media followed by incubation for an additional 22 h at 37°C. Percent infection was determined by FACs.

Influenza infections of U937 cells: Influenza viruses A/Hong Kong/1/1968, H3N2 (BEI; NR-28620) and A/Netherlands/602/2009, H1N1 in Opti-MEM were added to Fc $\gamma$ RIIa<sup>+</sup> Fc $\gamma$ RIIIa<sup>+</sup> U937 cells for 2h at 37°C. Cells were then washed twice with PBS, resuspended in OPTI-MEM and incubated at 37°C for an additional 22h. Percent infection was determined by FACs. Influenza-infected cells were detected using Alexa488 influenza A NP monoclonal antibody clone D67J (Invitrogen; MA1-7322) at a 1:100 dilution. For infections involving inhibitors of ITAM signaling, U937s were treated with titrated inhibitors: 11R-VIVIT (Thermo Fisher; CM102281.1) or 11R-VEET (Thermo Fisher; CM102281.2) in complete RPMI media for 2h at 37°C prior to infection. Treated cells were washed 2x with PBS before being added to virus.

Avidity ELISA: The avidity ELISA has been described before (Wang et al., 2015). Briefly, Wells of microtiter plates were coated with DENV2 (ProSpec; DEN-022) or DENV3 (ProSpec; DEN-023) E proteins (2.5 $\mu$ g ml<sup>-1</sup> in PBS) for 2 hours at 37°C. Plates were blocked for 30 min with 1% BSA/1% PBS and then incubated with titrated Gentle Ag/Ab Elution Buffer, pH 6.6 (ThermoFisher; 21027) and maternal IgG, which was normalized for optical density of binding to dengue-infected cells and matched for virus serotype of the paired infant's dengue virus infection. Plates were washed with PBS/0.1% Tween 20 and wells were incubated with alkaline phosphatase-conjugated anti-human Fc (Southern Biotech; P7998) followed by 2 hour incubation. Following incubation, the wells were washed and p-nitrophenyl phosphate substrate solution (Sigma; P7998) was solution was added. Absorbance at 405 nm was measured after approximately 45 min; ELISA performed in triplicate.

Western blot of mAbs: Antibodies were run under reducing and denaturing conditions by SDS-PAGE on a gradient gel with Tris-Glycine buffer. The gel was transferred to a 0.45  $\mu$ m nitrocellulose membrane (Bio-Rad) using semi-dry transfer. The membrane was blocked in 5% BSA in TBST for 2 hours at room temperature, before probing with a 1:10,000 dilution of biotinylated Aleuria Aurantia Lectin or AAL (Vector Labs; B-1395) in 5% BSA/TBST for 1 hour at room temperature. After 4–6 washes with TBST, the membrane was incubated with a 1: 2500 dilution of streptavidin-HRP (Southern Biotech; 7100-05) in 5% BSA/TBST

for 1 hour at room temperature. After 4–6 washes with TBST the membrane was treated with chemiluminescent substrate Western Lightning ECL Pro (Perkin Elmer; NEL120001EA) according to manufacturer's instructions. The membrane was then imaged using the Amersham Imager 600 (GE Life Sciences) with auto exposure under chemiluminescent detection settings. Post-imaging, the membrane was washed extensively with TBST before treating with Restore™ Western Blot Stripping Buffer (ThermoFisher; 21059) for 15 min at room temperature. To analyze protein loading, the membrane was re-blocked with 5% BSA in TBST for 2 hours at room temperature before probing with 1:5000 dilution of anti-human IgG-HRP (Southern Biotech) in 5% BSA/TBST for 1 hour at room temperature. The membrane was then treated with chemiluminescent substrate, and imaged as described above.

## QUANTIFICATION AND STATISTICAL ANALYSIS

All *in vitro* experiments were performed using three different biological replicates at least three independent times. Normality of Fc glycan distribution was confirmed by Kolmogorov-Smirnov test. Statistical analysis was performed with an unpaired t test. All the analyses were performed using the GraphPad Prism 9 software (Graphpad Software, San Diego, California, USA).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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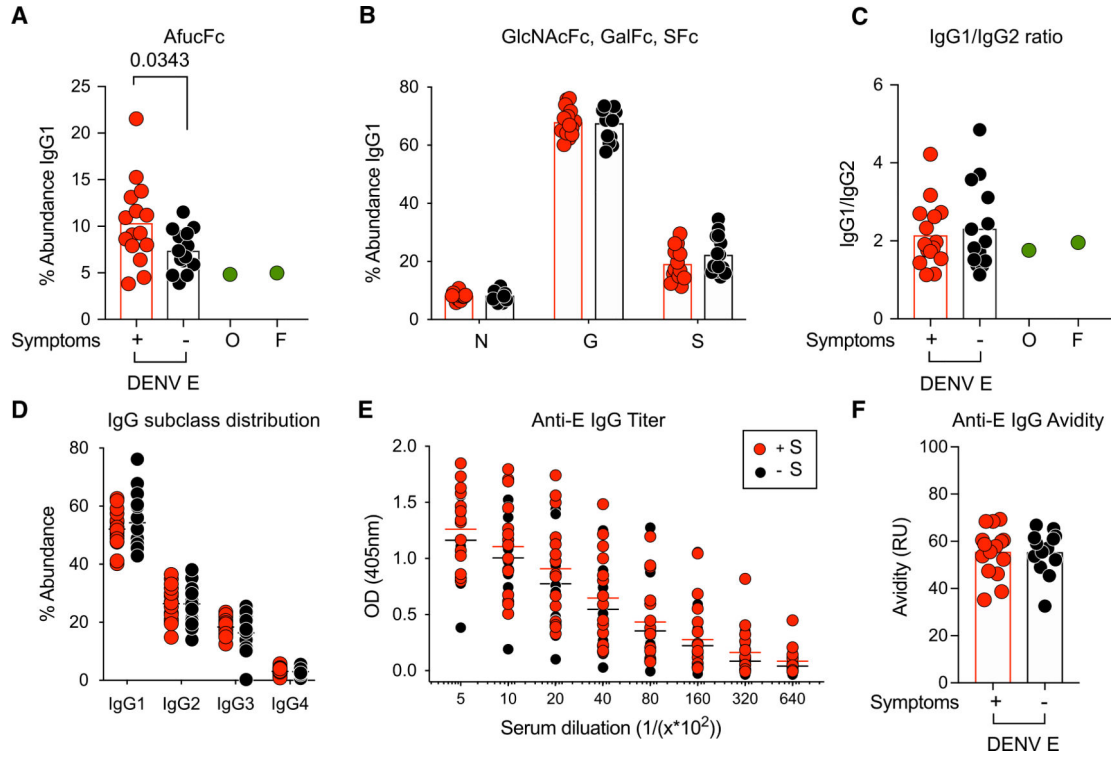
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### Highlights

- Maternal anti-dengue afucosylation predicts symptomatic infections in infants
- Afucosylated IgGs enhance dengue infections by promoting Fc $\gamma$ R1Ma signaling
- Dengue virus replication required calcineurin signaling network interactions



**Figure 1. Maternal Anti-E IgG Characterization**

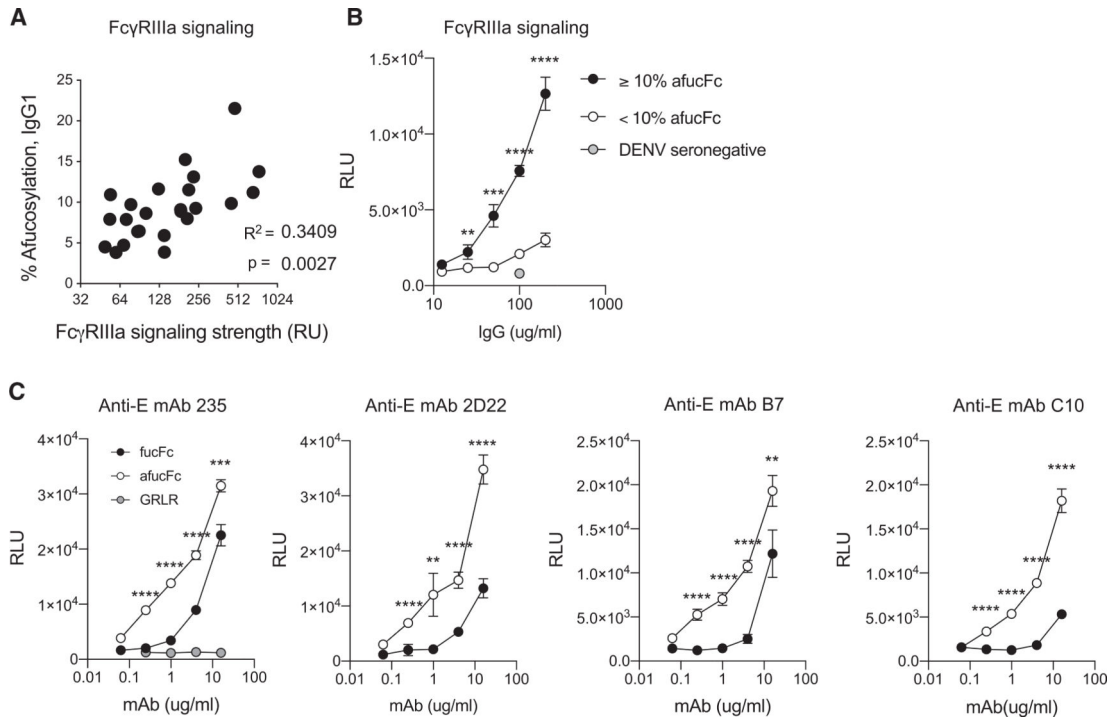
Anti-E Fc glycoforms were characterized.

(A) Fucosylation of Fc glycoforms was significantly different in abundance between mothers of infants with symptomatic (red) and asymptomatic (black) dengue infections, with afucosylated Fc glycans significantly increased in mothers of infants with symptomatic dengue infections. 10% afucosylated Fc glycans was predictive of elevated risk for symptomatic infant dengue infections with a positive predictive value of 88%.

(B) No significant differences in other Fc glycoforms, bisected (N), galactosylated (G), or sialylated (S), were observed.

(C–F) There were also no differences in the maternal anti-E (C) IgG1/IgG2 subclass ratio, (D) IgG subclass distribution, (E) IgG titer on dengue-infected cells, or (F) IgG avidity between mothers of infants who developed symptomatic or asymptomatic primary dengue infections.

Each symbol (A–F) represents an individual donor. Data are representative of one experiment per donor (A–D) or three experiments (E and F); (A–C and F) horizontal bars represent mean of clinical samples. Significance was assessed by unpaired Student’s t test, where  $p < 0.05$  was considered significant; \* $p < 0.05$ .



### Figure 2. Anti-E Fucosylation in Dengue Virus Immune Complexes Determines Fc $\gamma$ RIIIa Signaling by Effector Cells

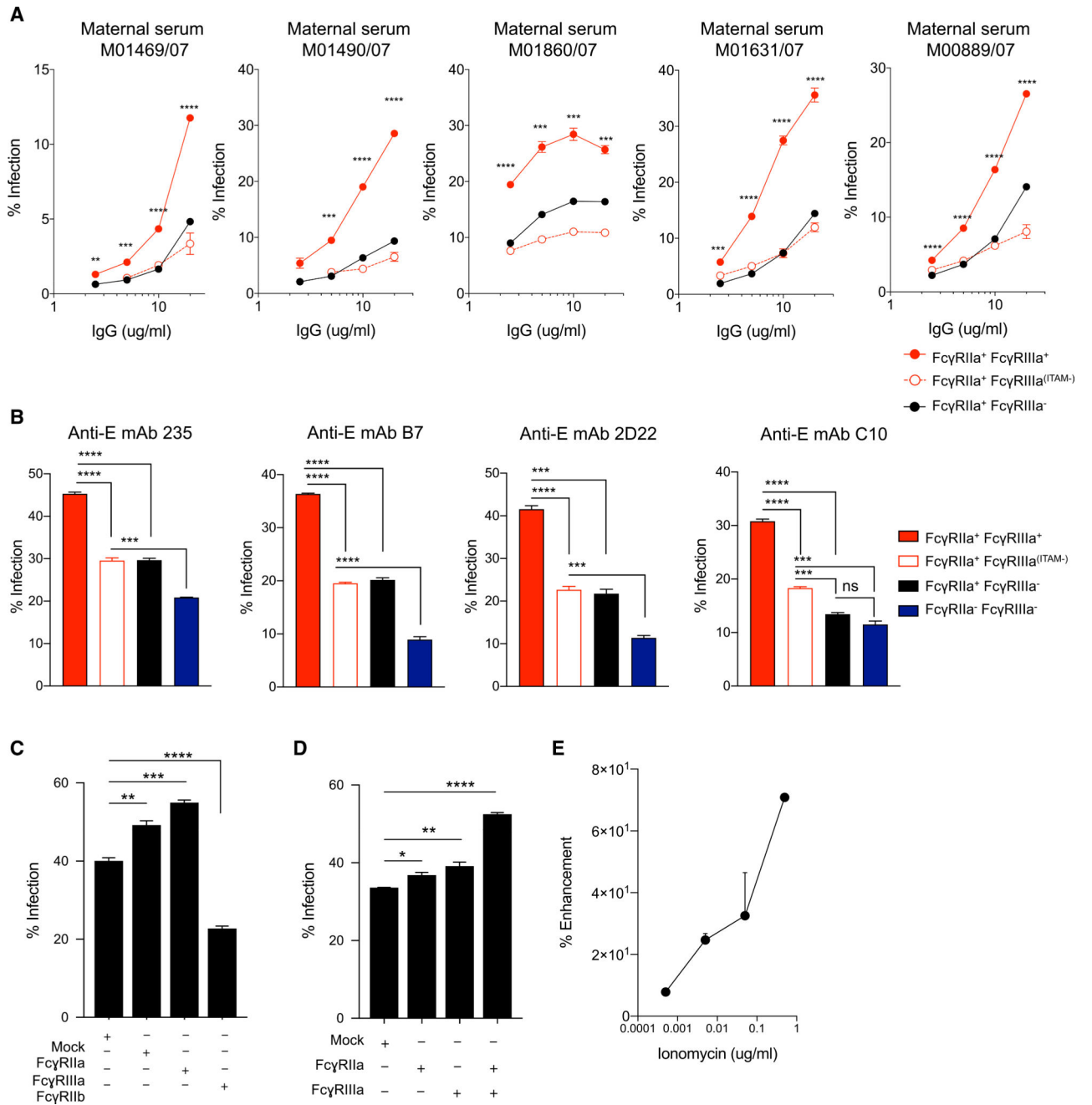
Dengue immune complexes were generated from maternal serum IgGs.

(A) Fc $\gamma$ RIIIa signaling was linearly correlated with the abundance of afucosylated Fc glycoforms of anti-DENV E IgGs within immune complexes,  $n = 29$ .

(B) Dengue immune complexes generated from pooled maternal IgGs with  $\geq 10\%$  anti-E afucosylation triggered significantly more Fc $\gamma$ RIIIa signaling when compared with immune complexes generated from pooled maternal IgGs with  $< 10\%$  anti-E afucosylation. Both pools were generated from sera of mothers of infants who experienced symptomatic primary dengue infections.

(C) Fucosylation of distinct anti-E mAbs modulates Fc $\gamma$ RIIIa signaling. Fucosylated (black circles) or afucosylated (white circles) anti-DENV E mAbs with distinct Fab specificities were tested for the ability to trigger Fc $\gamma$ RIIIa signaling. Enhanced Fc $\gamma$ RIIIa signaling by afucosylated immune complexes was observed in all cases. GRLR is a non-Fc $\gamma$ R-binding Fc variant.

Each point in (A) represents an individual donor. Data shown in (B) and (C) are representative of three experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (unpaired Student's *t* test). Data in (A)–(C) are representative of a minimum of three experiments. Error bars (B and C) represent the standard error of three technical replicates. Replicates were performed at all points; where error bars are not present, the standard error was smaller than the width of the symbol marking the data point.



**Figure 3. FcγRIIIa Signaling Enhances Antibody-Dependent Infection of U937 Monocytes**  
 (A) Infection by dengue immune complexes generated with maternal IgGs was significantly enhanced in FcγRIIIa<sup>+</sup> monocytes, and FcγRIIIa signaling was required for enhancement in all cases. Red line: FcγRIIa<sup>+</sup>FcγRIIIa<sup>+</sup> cells; black line: FcγRIIa<sup>+</sup>FcγRIIIa<sup>-</sup> cells; broken red line, unfilled circles: FcγRIIa<sup>+</sup>FcγRIIIa<sup>(ITAM-)</sup> cells.  
 (B) The role of FcγRIIIa signaling in ADE was evaluated by comparison of infection in FcγRIIa<sup>+</sup>FcγRIIIa<sup>+</sup> cells (red), FcγRIIIa-signaling null FcγRIIa<sup>+</sup>FcγRIIIa<sup>(ITAM-)</sup> cells (white), and FcγRIIa<sup>+</sup>FcγRIIIa<sup>-</sup> (black) or FcγRIIa<sup>-</sup>FcγRIIIa<sup>-</sup> (blue) cells. Immune complexes were generated from dengue virus and afucosylated anti-envelope mAbs 235, B7,



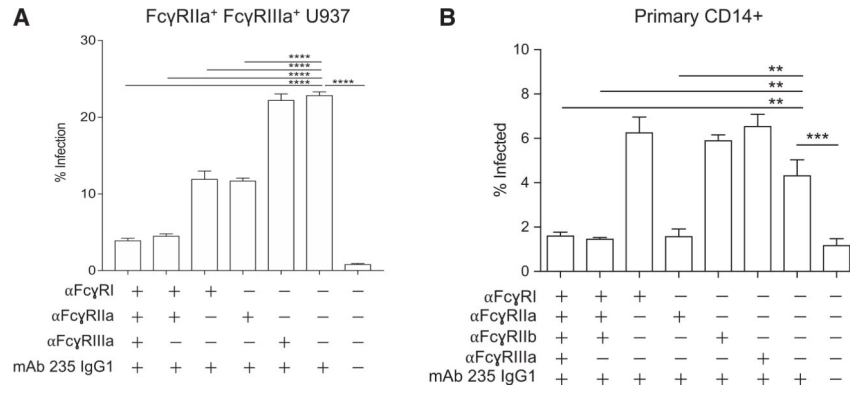
2D22, or C10, which have distinct anti-E binding specificities. In all cases, Fc $\gamma$ RIIIa signaling was required for maximal infection.

(C) Signaling induced by crosslinking of Fc $\gamma$ RIIa or Fc $\gamma$ RIIIa promoted infection of Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells, whereas crosslinking the inhibitory Fc $\gamma$ R, Fc $\gamma$ RIIb, reduced infectibility of cells.

(D) Combined signaling by activating Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa during infection resulted in maximal infectibility of Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells.

(E) Increasing cellular calcium flux promoted infection of Fc $\gamma$ RIIa<sup>+</sup>Fc $\gamma$ RIIIa<sup>+</sup> U937 cells.

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 (unpaired Student's t test). Data are representative of a minimum of three experiments. Error bars represent the standard error of three technical replicates. Replicates were performed at all points; where error bars are not present, the standard error was smaller than the width of the symbol marking the data point.

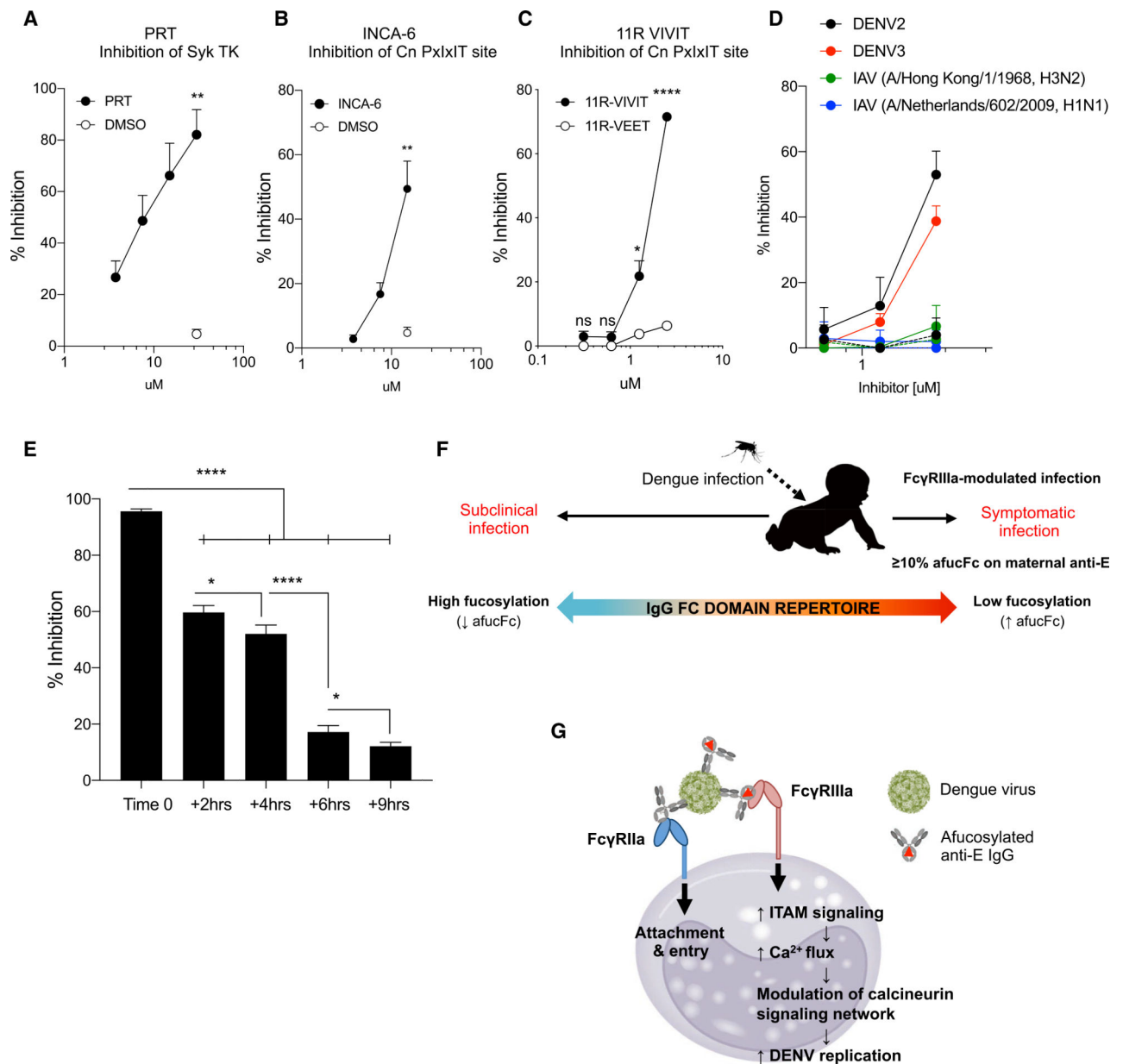


**Figure 4. FcγRIIIa Does Not Mediate Virus Immune Complex Attachment during ADE in Monocytes**

(A) Individual or combined FcγRs were blocked during the viral attachment step of infection in FcγRIIIa<sup>+</sup> FcγRIIIa<sup>+</sup> U937 monocytes. Blocking FcγRIIIa and/or FcγRI during immune complex attachment significantly reduced infection, whereas blocking FcγRIIIa did not impact infection.

(B) Blocking FcγRIIIa alone during attachment in primary monocytes prevented their infection, whereas blocking FcγRI, FcγRIIb, or FcγRIIIa did not impact infection.

\*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 (unpaired Student's t test). Data are representative of a minimum of three experiments. Error bars represent the standard error of three technical replicates.



**Figure 5. Inhibition of Dengue Infection through Targeted Inhibition of ITAM Signaling Pathway Elements**

(A) A small molecule that targets Syk tyrosine kinase (Syk TK), PRT, inhibited dengue infection in FcγRNA<sup>+</sup>FcγRNA<sup>+</sup> U937 monocytes.

(B and C) A small-molecule inhibitor, INCA-6 (B), and a peptide inhibitor, 11R-VIVIT (VIVIT) (C), which act by blocking calcineurin-substrate interactions at the calcineurin PxlIT site, inhibited dengue infections. A scrambled peptide containing the amino acids found in VIVIT (11R VEET) did not inhibit infection (open circles).

(D) Inhibition of dengue viruses (DENV2, DENV3), but not H1N1 or H3N2 influenza viruses (IAVs), was observed using the VIVIT inhibitor (solid lines); scrambled VEET peptide in dashed lines.

(E) Treatment of cells with VIVIT up to 6 h after infection by dengue immune complexes significantly inhibited infection.

(F) Model for susceptibility to clinically significant dengue infections in infants. Infants of mothers who are dengue-immune and have 10% afucosylated anti-E IgGs are at elevated risk for symptomatic primary dengue infections due to modulation of Fc $\gamma$ RIIIa-expressing cells during infection.

(G) Model for the roles of Fc $\gamma$ RIIa, Fc $\gamma$ RIIIa, and afucosylated dengue immune complexes in ADE. While Fc $\gamma$ RIIa supports a majority of viral attachment and entry, Fc $\gamma$ RIIIa signaling increases cellular ITAM signaling. ITAM signaling, in turn, triggers calcium flux and modulates the calcineurin signaling network, which supports dengue virus replication. \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001 (unpaired Student's t test). Data are representative of a minimum of three experiments. Error bars represent the standard error of three technical replicates. Replicates were performed at all points; where error bars are not present, the standard error was smaller than the width of the symbol marking the data point.