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TITLE: Cross-reactive antibodies facilitate innate sensing of dengue and Zika viruses

Authors and affiliations:
Laura K. Aisenberg1*, Kimberly E. Rousseau1*, Katherine Cascino1, Guido Massaccesi1, William H. Aisenberg2, Wensheng Luo3, Kar Muthumani4, David B. Weiner4, Stephen S. Whitehead5, Michael A. Chattergoon1, Anna P. Durbin3, Andrea L. Cox1

1Johns Hopkins University School of Medicine, Department of Medicine, Division of Infectious Disease, Baltimore, MD, USA
2Johns Hopkins University School of Medicine, Department of Medicine, Division of Neurology, Baltimore, MD, USA
3Johns Hopkins University Bloomberg School of Public Health, Center for Immunization Research, Baltimore, MD, USA
4The Wistar Institute, Vaccine & Immunotherapy Center, Philadelphia, PA, USA
5National Institute of Allergy and Infectious Diseases, NIH, Laboratory of Viral Diseases, Bethesda, MD, USA

*Authors contributed equally to the manuscript. Order determined alphabetically.

Corresponding Author:
Andrea L. Cox
855 North Wolfe Street, Room 551
Baltimore, MD, USA, 21205
(410)-502-2715
acox@jhmi.edu
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ABSTRACT:

The *Aedes aegypti* mosquito transmits both dengue (DENV) and Zika (ZIKV) viruses. Individuals in endemic areas are at risk for infection with both viruses as well as repeated DENV infection. In the presence of anti-DENV antibodies, outcomes of secondary DENV infection range from mild to life-threatening. Further, the role of cross-reactive antibodies on the course of ZIKV infection remains unclear.

We assessed the ability of cross-reactive DENV monoclonal antibodies or polyclonal immunoglobulin isolated after DENV vaccination to upregulate type I interferon (IFN) production by plasmacytoid dendritic cells (pDCs) in response to both heterotypic DENV- and ZIKV- infected cells. We found a range in the ability of antibodies to increase pDC IFN production and a positive correlation between IFN production and the ability of an antibody to bind to the infected cell surface. Engagement of Fc receptors on the pDC and Fab binding of an epitope on infected cells was required to mediate increased IFN production by providing specificity to and promoting pDC sensing of DENV or ZIKV. This represents a mechanism independent of neutralization by which pre-existing cross-reactive DENV antibodies could protect a subset of individuals from severe outcomes during secondary heterotypic DENV or ZIKV infection.
INTRODUCTION:

Dengue virus (DENV) and Zika virus (ZIKV) are viral diseases transmitted by the *Aedes aegypti* mosquito (1, 2). DENV is a rapidly emerging, mosquito-borne viral infection, with an estimated 400 million infections occurring annually. ZIKV is linked to an increased risk of neurologic complications in adults and complications in pregnancy that include microcephaly, preterm birth, and miscarriage (3, 4). Both are members of the *Flaviviridae* family, genus *flavivirus*, with highly conserved structural protein identity across the two viruses (5–7). While DENV infections can be caused by four distinct serotypes of virus, ZIKV has been shown to only exist as a single serotype (8). The role of antibodies in DENV outcomes, particularly those following a secondary infection, is complex. Primary DENV infection generates an antibody (Ab) response that generally protects against a subsequent symptomatic homotypic infection but does not always provide protection against heterotypic infection. In fact, serotype cross-reactive antibody can pose a risk for clinically worsened infection (9–13). Why some heterotypic infections result in severe disease while the majority do not remains incompletely understood.

Anti-DENV monoclonal antibodies (mAbs) can be cross-reactive with ZIKV, which has raised the additional question of how prior exposure to DENV might impact ZIKV infection. Prior studies have investigated the potential impact of ZIKV cross-reactive anti-DENV mAbs on ZIKV infection outcome (14–17). Specifically, data from in vitro models and immunodeficient mouse models have shown that anti-DENV antibodies can increase ZIKV replication (14, 18, 19). However, non-human primate (NHP) models and human clinical data are not consistent with these findings (20–27). NHP models demonstrate no worsened clinical outcomes with pre-existing DENV antibodies, with some studies demonstrating shortened clinical ZIKV infection...
after prior DENV exposure (21, 22, 25). Epidemiologic data and analysis of infected people suggests that anti-DENV antibodies may protect against ZIKV infection (24, 26–28).

The type I interferon (IFN) response is critical to viral control. It has been shown to be directly restrictive of DENV (29–31) and ZIKV (32) replication and to induce a cytokine milieu conducive to generating a subsequent adaptive immune response mediating viral control (33). Both viruses have evolved multiple mechanisms to evade the IFN pathway (34–40), demonstrating the importance of the selective pressure of IFN-mediated restriction of viral replication. Specifically, there is extensive literature demonstrating that the DENV NS5 protein inhibits STAT2 and subsequent IFN production (35, 36). Additional work suggests that DENV subgenomic RNA inhibits TRIM25, further limiting the production of type I IFNs (38). ZIKV has also developed several similar mechanisms, including blocking STAT2 activity via NS5 (39, 40). Additional mechanisms by which Zika evades the IFN pathway include inhibition of TBK1 (41), cleavage of cGAS (42), and blockade of the IFN promoter (43). Consistent with an essential role for IFN, individuals with severe cases of dengue fever often have lower IFN levels relative to patients with less severe infections (44–47), and Zika infected fetuses are better protected from severe neurologic sequelae when a robust IFN response is generated (48). Notably, more virulent strains of Zika virus have been demonstrated to induce lower IFN responses than less virulent strains (49).

As in many other viral infections, plasmacytoid dendritic cells (pDCs) are a major producer of type I IFNs in DENV infection (50–54). Prior studies on DENV and ZIKV (55–58) demonstrate that in order for a type I IFN response to be generated, pDCs require cell-to-cell contact with DENV or ZIKV-infected cells (59–61). The cell adhesion molecules ICAM-1 (CD54) and αL-integrin are required for the establishment of this cell-to-cell contact (60). Once
contact between the pDC and a target cell is established, PAMPs are transferred via an ‘interferogenic synapse’ from the target cell to pDC with pDC sensing of viral RNA occurring via TLR7 and subsequent downstream signaling (60).

Despite prior work suggesting that anti-viral antibodies can directly alter innate immune signaling pathways (62) and evidence that demonstrates that recent DENV infection offers increased ZIKV control in the setting of increased pDC frequency (63), it is unknown whether antibodies against DENV modulate pDC sensing of either DENV or ZIKV. We sought to determine the effects of anti-DENV antibodies on the production of type I IFN by pDC upon DENV or ZIKV exposure, including whether antibodies generated in DENV infection can upregulate IFN production in response to heterotypic DENV or to ZIKV, potentially increasing protection.

To investigate this hypothesis, we adapted previously defined in vitro models of DENV and ZIKV sensing by pDC (59, 60). With anti-DENV monoclonal antibodies (mAbs) and unique plasma specimens from DENV-naïve human subjects challenged with DENV after administration of live attenuated DENV vaccines, we demonstrate that some anti-DENV antibodies facilitate pDC sensing of cross-serotype DENV or of ZIKV and promote type I IFN production. We show that a subset of anti-DENV antibodies function in place of or in concert with cell adhesion molecules to tether DENV- or ZIKV- infected cells to pDCs. This link occurs via Fab-epitope binding on infected cells and Fc binding to pDC Fc receptor (FcR), offering a specificity to cell-to-cell contact and generating a more robust anti-viral IFN response.
RESULTS:

pDCs sense DENV in a cell-to-cell contact-dependent manner facilitated by cell adhesion molecules ICAM-1 and $\alpha_\text{L}$-integrin

Primary human pDCs produce type I IFN when co-cultured with DENV-2-New Guinea C (NGC) infected Huh 7.5.1 hepatoma cells. Work by others has demonstrated that pDC sensing of DENV requires direct cell-to-cell contact with infected cells (59, 60). We confirmed that pDC production of IFN is much more robust in the setting of cell-to-cell contact by incubating pDCs for 24 hours with uninfected Huh 7.5.1 cells, with DENV infected Huh 7.5.1 cells, with DENV alone, with supernatant collected from DENV infected Huh 7.5.1 cells (Figure 1A), or with the TLR7/8 agonist resiquimod (REQ) as a positive control. Supernatant from DENV infected Huh 7.5.1 cells was titered to confirm the presence of infectious viral particles (Supplemental Figure 1). We then measured IFN-$\alpha$2a production by ELISA. There is significant donor-to-donor variability between experiments, and it is not feasible to use the same pDC donor for repeated experiments given the anonymous nature of the leukapheresis donation. Therefore, the amount of IFN generated is reported as a percentage of the pDC response to DENV alone and as the average of at least three replicates (Figure 1A). All conditions were tested in the same experiment using the same pDC donor. To demonstrate that pDCs are required for type I IFN production, we also assessed IFN-$\alpha$2a from DENV infected Huh 7.5.1 cells without pDCs added. We observed that only the pDCs co-cultured with infected Huh 7.5.1 cells produced significant amounts of type I IFN, confirming the previous finding that pDCs are required for type I IFN production and that pDCs produce IFN much more robustly when in contact with DENV infected cells (Figure 1A). Given these data, all subsequent experiments used this co-culture model (Figure 1B).
The cell-to-cell contact required for pDC sensing of DENV depends on the cell adhesion molecules ICAM-1 and αL-integrin, and prior data suggest that these cell adhesion molecules establish transient contact between pDCs and infected cells (60). If pathogen-associated molecular pattern (PAMP) transfer occurs at these transient contact sites, long-term contacts are established, and TLR7-mediated IFN signaling occurs (60). To validate this, we treated infected Huh 7.5.1 with Ab blockade of ICAM-1 (Figure 1C) or αL-integrin (Figure 1D). We demonstrated that blocking either of these cell adhesion molecules results in the complete loss of IFN-α2a production, confirming a requirement for these adhesion molecules in pDC sensing (Figure 1C-D).

Monoclonal anti-DENV antibodies against a subset of DENV epitopes enhance in vitro IFN production by pDCs

Given our previous study demonstrating that envelope (E)-specific antibodies can alter innate immune signaling of HIV by pDCs (62), we hypothesized that pre-existing antibodies in a DENV-exposed individual might alter the innate immune response to a subsequent heterotypic DENV or to subsequent ZIKV infection. To test this hypothesis, we assessed the effect of anti-DENV mAbs on pDC sensing of DENV in our in vitro model using a panel of previously characterized cross-reactive antibodies. We first used mAb DV87.1 (DVSF-3), previously defined as a DENV-1 antibody specific for E domain III and cross-reactive for multiple DENV serotypes (64). To assess the effect of mAb DV87.1, DENV-2-infected Huh 7.5.1 cells were incubated with anti-DENV DV87.1 for 1 hour and then co-cultured with pDCs, with levels of IFN-α2a assessed after 24 hours. The addition of antibody after infection was designed to allow replication to occur in the absence of antibody to assess the effects of DENV-specific antibodies on sensing, rather than on infection. The results demonstrate that IFN production by pDCs is
upregulated in the presence of anti-DENV mAb DV87.1 in a dose-dependent manner (Figure 2A). DV87.1 antibody did not trigger IFN-α2a production in a pDC/Huh 7.5.1 co-culture system in the absence of virus, nor did it trigger IFNα2a production when incubated with virus and then subsequently incubated with primary pDCs (Supplemental Figures 2 and 3).

Having established that DV87.1 increases type I IFN production, we assessed a panel of broadly cross-reactive DENV-specific mAbs to assess epitope specificity of the effect. We used mAbs C8 and C10, human mAbs specific for DENV envelope (E) epitope EDE1 and previously characterized as highly DENV serotype cross-reactive and neutralizing (65). We also used the murine mAbs 2H2 and 4G2, specific for pre membrane (prM) and E, respectively, and also cross-reactive for multiple DENV serotypes (66, 67). We assessed the ability of each mAb to promote pDC sensing of DENV as described in Figure 2A. C8 significantly increased IFN-α2a production, though not as robustly as DV87.1, while C10 showed a trend toward increased IFN-α2a production (p=0.0867) (Figure 2B). In contrast, murine anti-E mAb 4G2 and anti-prM mAb 2H2 did not demonstrate a significant effect on IFN-α2a production (Figure 2B).

To determine whether the lack of effect of 2H2 on pDC sensing was due to this antibody’s specificity for prM and not E, we assessed an additional panel of human anti-prM antibodies (4G21, 1H10, 2H21, 1E23, and 1B22) previously characterized as being prM specific without cross-reactivity for envelope (characterized in Supplemental Table 1, adapted from Smith et al.) (68). The experiment was performed as described in Figure 2A, with a range of effects observed. prM specific mAbs 4G21 and 1H10 did not significantly increase pDC IFN production relative to virus alone. However, 2H21, 1E23, and 1B22 significantly upregulated IFN generated by pDC sensing of DENV, although less robustly than DV87.1 (Figure 2C). These data demonstrate that mAbs targeting diverse DENV E and prM epitopes can enhance
pDC sensing of DENV, but that this is not a universal characteristic of all antibodies that target these proteins. Thus, it is clear that antigen specificity is not the only determinant of upregulated pDC sensing.

Modulation of IFN production by anti-DENV antibodies is dependent on the amount of Fab/epitope binding on the surface of infected cells

Although DENV virions are not thought to bud from infected cells (69), it has been shown that excess viral proteins stud the surface of DENV-infected cells (70–72). This suggests that DENV epitopes might be available at the surface of target cells and provide a target for anti-DENV antibodies. Given the range of enhancing effects of our mAbs targeting diverse E and prM epitopes, we hypothesized that the amount of mAb binding to the infected cell surface determines the capacity of the mAb to enhance pDC sensing.

We used two methods to test the capacity of our antibodies to bind cell surface antigen. The first method was surface immunofluorescence staining to assess whether the antibodies that enhanced IFN production also bind to the surface of DENV-infected Huh 7.5.1 cells. Surface labeled cells were counterstained with a murine anti-E mAb (4G2) or a human anti-E mAb (DV87.1) to label intracellular DENV E protein and to identify successfully infected cells. Imaging of these stained cells revealed DENV E present on the surface of infected Huh 7.5.1 cells, labeled robustly by DV87.1 (Figure 2D). Staining was compared to the well-characterized surface stain wheat germ agglutinin to validate that the staining pattern observed was consistent with cell surface binding (Supplemental Figure 4). DV87.1, which is highly effective at upregulating IFN production, bound specifically to the surface of infected, but not uninfected, cells. Two additional mAb capable of enhancing pDC sensing of DENV, C8 and C10, bound the surface of infected cells robustly (Supplemental Figure 5). In contrast, the mAb 2H2, which did
not enhance pDC sensing, was unable to bind the surface of infected cells despite being able to
bind intracellular viral protein (Figure 2D). Collectively, these data support the hypothesis that
enhanced pDC sensing of virally infected cells requires binding of an epitope present on the
infected cell’s surface.

We used flow cytometric analysis of infected Huh 7.5.1 cells to detect surface DENV E
and prM epitopes as a second method to verify these findings. DENV-infected cells were stained
and analyzed for expression of prM and E on the cell surface and compared to uninfected
controls; data is quantified as percent of DENV positive cells for all tested mAbs (gating strategy
as demonstrated Supplemental Figure 6). Surface staining with DV87.1, C8, and C10 confirmed
that E epitopes were accessible on the surface of infected cells (Figure 2E). The degree to which
these antibodies bind the surface of infected cells correlates with their ability to boost type I IFN
production – namely, C8 and C10 bound much less robustly and also generated a less robust
increase in IFN. Surface staining with 2H21, 1E23, and 1B22 confirmed that some prM epitopes
were also accessible on the surface of infected cells. However, the prM-specific mAbs that did
not significantly upregulate pDC sensing, 4G21 and 1H10, did not bind infected cells (Figure
2E), either because the epitopes were not expressed on the cell surface or the mAb avidity was
too low to bind these epitopes on the cell surface. The dramatic differences in binding among
anti-E and anti-prM antibodies suggest that epitope accessibility or antibody affinity for surface-
expressed epitopes differ. Importantly, the degree to which a given antibody binds to infected
cells as quantified by flow cytometry correlates with their IFN-promoting activity (Figure 2C)
for the vast majority of antibodies in our panel, suggesting that upregulation of pDC-mediated
IFN activity is at least in part defined by the ability of the antibody to bind to infected cells.

These data support our hypothesis that antibody binding to viral epitopes accessible on the
surface of infected cells is necessary to increase IFN production, with a direct correlation
between the degree of antibody binding to the infected cell surface and the level of increased
pDC IFN production.

We then established a hypothesis that antibodies can upregulate pDC IFN production in
response to DENV infection by enhancing cell-to-cell contact between the sensing pDC and the
DENV-infected cell, tethering the pDC specifically to an infected cell as opposed to the non-
specific cell-to-cell interaction afforded by ICAM-1 engagement of αL-integrin.

**Anti-DENV mAb facilitates the interaction of pDCs with infected cells**

Our data thus far suggest that the Fab/epitope interaction on the surface of an infected
cell is required for increased production of type I IFN by pDCs. To test our hypothesis that
DENV antibodies that bind the surface of infected cells would lessen the dependence of pDC on
adhesion molecules to sense infection, we reassessed the requirement for ICAM-1 and αL-
integrin in the presence of these mAb (Figure 3A). Specifically, we treated DENV-infected Huh
7.5.1 cells with anti-αL-integrin antibody, followed by DV87.1 mAb. As in Figure 1B, treatment
with anti-αL-integrin antibody in the absence of mAb led to a complete loss of IFN-α2a
production by pDCs co-cultured with infected Huh 7.5.1. However, the addition of the DV87.1
mAb rescued IFN-α2a production in a dose-dependent manner (Figure 3B). The same effect was
observed when Huh 7.5.1 were cultured with anti-ICAM-1 and then treated with DV87.1 (Figure
3C). These data suggest that DENV-specific mAbs enhance pDC sensing of DENV by altering
interactions between pDCs and infected cells, thereby reducing dependence on non-specific
adhesion molecules, potentially through higher affinity or prolonged cell-to-cell interactions.

**Modulation of IFN production by anti-DENV antibodies requires Fc/FcR interactions**
Consistent with our proposed model, we hypothesized that Fc engagement of FcR on the sensing pDC is required for upregulated IFN production. Our previous research demonstrated that HIV mAbs enhance pDC sensing and IFN production in an Fab- and FcγR2a-dependent manner (62). We used three different methods to determine whether binding of the Fc portion of antibody to Fc-gamma receptor (FcγR) is necessary for increased pDC sensing of DENV. First, we used a variant of the mAb DV87.1 with two leucine to alanine mutations that abrogate binding to FcγRs (LALA DV87.1) (64). In contrast to the wild type DV87.1, the LALA DV87.1 mAb did not enhance IFN production when incubated with infected Huh 7.5.1 cells prior to coculture with pDCs (Figure 4A). Next, we usedFcBlock to compete with the Fc portion of DV87.1 mAb for binding to all FcγRs on the pDC surface. When pDCs were pre-incubated with FcBlock, DV87.1 mAb failed to enhance IFN production (Figure 4B). Finally, we used a specific anti-FcγR2a (CD32a) blocking antibody to determine whether this effect was specifically dependent on this FcγR (62). When pDCs were pre-incubated with anti-FcγR2a, DV87.1 mAb no longer enhanced IFN-α2a production (Figure 4B). Taken together, these data support that Fc binding to FcγR, specifically FcγR2a, is required for antibody-mediated upregulation of type I IFN production by pDCs.

To determine if bypassing cell adhesion molecules by antibody requires the engagement of FcγR2a on the pDC as well as Fab binding to the infected cell, we assessed the role of FcγR in rescuing IFN production as in Figures 4A and 4B. In contrast to DV87.1 mAb, the addition of LALA DV87.1 mAb failed to rescue IFN production in the presence of ICAM or αL-integrin blockade, suggesting that rescue is dependent on Fc/FcγR binding (Figure 4C-D). To further test this requirement for FcγR engagement, we blocked pDC FcRs with anti-FcγR2a while treating infected Huh 7.5.1 with anti-ICAM antibody and DV87.1. In the presence of anti-FcγR2a,
DV87.1 was incapable of rescuing IFN production, further validating that FcγR2a binding is critical to rescuing IFN production in the absence of adhesion molecule engagement (Figure 4E). Finally, we performed a competition experiment in which we treated infected Huh 7.5.1 with anti-ICAM antibody, followed by treatment with DV87.1 and increasing concentrations of LALA DV87.1. LALA DV87.1 mAb competes for E binding on the surface of infected hepatoma cells in a dose-dependent fashion but is incapable of binding pDC FcRs, including FcγR2a. The DV87.1 mAb binds FcR, but is outcompeted for Fab binding in the presence of high amounts of LALA DV87.1. With increasing amounts of LALA DV87.1 added, the capacity for WT DV87.1 to rescue IFN production in the presence of ICAM blockade was abrogated (Figure 4F). The results of this competition experiment suggest that bridging the pDC to the infected cell via the same antibody molecule is necessary, not simply that Fab binding to infected cells and Fc binding to pDCs in isolation triggers increased IFN production. The Fab portions of these antibodies bind E protein epitopes accessible on the surface of infected cells with the Fc portion tethering the pDCs to these target cells and providing pDCs specificity for infected target cells. This contrasts with a naïve host environment in which random, non-specific cell interactions must result in sufficient contact to trigger sensing. Anti-ICAM antibodies used extensively in our experiments do not mediate an increased IFN response to infected cells as they are murine in origin and thus are not expected to bind human pDC FcR. Furthermore, the binding of anti-ICAM is not specific for infected cells.

**Anti-DENV antibodies cross-reactive for ZIKV facilitate pDC sensing of ZIKV**

The impact of anti-DENV antibodies in ZIKV immunity remains an open question. To address this, we assessed whether DENV-specific mAb altered type I IFN production in response to ZIKV infected cells. Many mAbs binding to the EI or EII domains of DENV E or the E dimer
epitope (EDE) regions of DENV are cross-reactive with ZIKV, providing the possibility for these antibodies to increase sensing of ZIKV. However, some mAbs directed to the less homologous EIII domain are not cross-reactive with ZIKV (14, 17). We assessed whether a subset of our panel of anti-DENV mAbs that boost the pDC response to DENV also augment pDC sensing of ZIKV infected cells. C8 and C10 mAbs, both specific for the EDE1 region of DENV E and previously characterized as cross-reactive with ZIKV (14), increased IFN-α2a production when added to a co-culture of pDCs and ZIKV Nicaragua- or ZIKV Sao Jose do Rio Preto (SJRP)-infected Huh 7.5.1 cells (Figure 5A-B). However, when the non-cross reactive mAb DV87.1 (an EIII specific mAb) or 2H2 (an anti-prM DENV mAb) were added into the co-culture system, neither upregulated IFN-α2a production in response to ZIKV (Figure 5A-B). We then assessed the panel of mAbs for binding to the surface of ZIKV-infected Huh 7.5.1 cells, as tested previously with DENV-infected cells. Consistent with what we had observed for DENV, all antibodies that enhanced type I IFN production in the pDC co-culture system (with C10 shown as a representative example here) bound the surface of ZIKV-infected cells, while those that did not increase IFN production (DV87.1, for example) did not bind the surface of ZIKV infected cells (Figure 5C, supplemental Figure 7). While DV87.1 bound the surface of DENV-infected cells and was highly upregulating of pDC mediated DENV sensing (Figure 2A), it did not bind the surface of ZIKV-infected cells and did not boost IFN production in our ZIKV co-culture system (Figure 5C), consistent with its binding to the EIII epitope, an epitope less conserved between DENV and ZIKV. Unlike DENV, ZIKV-infected Huh cells were not highly stimulating of pDC type I IFN in our assays. Thus, there is no baseline type I IFN production to rescue after cell adhesion molecule blockade. However, type I IFN upregulation in the presence of ZIKV Nicaragua or SJRP can be blocked in a dose-dependent fashion by anti-ICAM antibody
when also cultured in the presence of C8 or C10 monoclonal antibodies (Figure 5D-E, Supplemental Figure 8). These data are consistent with our model that antibody binding of epitopes expressed on the infected cell surface tether infected cells to pDC and increase IFN production from baseline. These data suggest that this phenomenon is not restricted to innate sensing of DENV and that antibodies generated in response to DENV may modulate the innate immune response to ZIKV. Notably, significant differences were not observed in the pDC response generated in the presence of antibodies between ZIKV strains.

**Polyclonal IgG isolated from individuals with immunity to DENV can alter pDC sensing of DENV and ZIKV**

Having demonstrated that anti-DENV mAbs can alter the pDC response to both DENV and ZIKV, we next hypothesized that individuals exposed to DENV of one serotype might generate cross-reactive antibodies that can alter the type I IFN response to a second serotype of DENV or to ZIKV. To test this hypothesis across multiple DENV serotypes, we isolated polyclonal IgG (pIgG) from human subjects enrolled in a randomized placebo-controlled double-blinded DENV challenge study (NCT# 02433652) and assessed the effect of this pIgG on pDC sensing of DENV. Twenty-four individuals who were naïve to DENV were randomized to receive either placebo (6 individuals) or vaccination (18 individuals) with a live attenuated admixture containing DENV serotype 1, 3, and 4 viruses. Twenty-one individuals (6 placebo recipients; 15 vaccine recipients) were then challenged with DENV-2. Vaccinees were expected to generate a broad humoral response to DENV-1, -3, and -4, and a subset would develop cross-reactive pIgG that binds DENV2. Given that plaque reduction neutralization titer 50% (PRNT50) antibody titers against DENV 1-4 were highest on average at day 28 post-vaccination, we isolated pIgG from the serum of vaccine and placebo recipients on day 28 and assessed their
effect on our in vitro pDC/DENV-infected Huh 7.5.1 model. Total pIgG samples were isolated
from two placebo recipients (subjects P1 and P2), six vaccinees who were well-protected post-
challenge (no detectable viremia by culture – subjects VP1-6), and three vaccinees who were not
protected (detectable viremia, rash post-challenge – VV1-3) and were assessed. Of these 11
individuals, pIgG from subject VP3, a well-protected vaccine recipient, strongly boosted type I
IFN production in our in vitro assay while pIgG from placebo recipients (subjects P1 and P2) and
from subjects who were not well protected against challenge (VV1-3) did not (Figure 6A).
However, pIgG from a subset of the subjects protected from challenge also failed to enhance IFN
production, demonstrating that antibodies induced by vaccination and challenge are not
uniformly able to increase IFN production (Figure 6A). When these pIgG were assessed for their
ability to restore IFN production in the presence of cell adhesion molecule blockade, IgG from
subject VP3 was the only pIgG capable of rescuing pDC sensing of DENV-infected cells (Figure
6B). This is consistent with the pattern established with mAbs, in which antibodies that
upregulated IFN activity were capable of rescuing IFN production when ICAM/integrin
interactions were blocked (Figure 6B).

We next assessed the pIgG of these same individuals in our model of ZIKV sensing to
assess whether the effects of monoclonal anti-DENV antibodies on ZIKV sensing might be
replicated by polyclonal antibodies generated in individuals exposed to DENV. When we co-
cultured pIgG from the same individuals described above, we observed the same patterns of
altered IFN-α2a production. pIgG from subject VP3 strongly upregulated IFN-α2a production in
our assay in response to ZIKV, while IgG from placebo recipients and other vaccine recipients
did not significantly alter IFN-α2a production (Figure 6C).
To validate whether the modulation of type I IFN by pIgG is consistent with the mechanism demonstrated with monoclonal antibodies, we assessed the isolated pIgG for binding to the surface of infected cells via immunofluorescence as performed with mAb against DENV. As predicted, pIgG from subject VP3 robustly bound the surface of DENV-infected cells, while pIgG isolated from subjects that did not increase IFN production did not bind the surface of these cells (Figure 6D). These data show that, as with mAb, there is a heterogeneity in the ability of pIgG generated in DENV vaccination to increase IFN in response to subsequent DENV or ZIKV infection.
DISCUSSION:

The type I IFN pathway is critical in establishing the early anti-viral immune response to DENV and ZIKV by directly restricting viral replication and shaping the downstream adaptive immune response. Our data suggest that anti-DENV antibodies that bind specifically to epitopes accessible on the surface of DENV- or ZIKV-infected cells increase the type I IFN response by pDCs independent of neutralization potential. Antibody-mediated upregulation of type I IFN production by pDCs requires Fab binding to an epitope present on infected cells and Fc – FcγR2α engagement on the pDC. Our work suggests that virus-specific antibody can function instead of or in concert with the naturally occurring ICAM-1/αL-integrin interactions to bridge the pDC to the infected cell, leading to more specific cell-to-cell contacts and greater IFN production (Figure 7). We also show that the presence of cross-reactive anti-DENV antibodies increased type I IFN production when pDCs interact with ZIKV infected cells, offering novel information about cross-reactive immunity between DENV and ZIKV. Finally, as demonstrated with monoclonal antibodies, we show that anti-DENV pIgG from an individual given a live attenuated DENV1/3/4 vaccine increased the production of type I IFN by pDCs in response to serotype 2 DENV and ZIKV infection. In summary, these data suggest a role for non-neutralizing antibodies to offer protection via modulation of the innate immune response to DENV and ZIKV.

We propose that upregulation of type I IFN production is not the sole, but one of several, mechanisms of protection provided by cross-reactive antibodies. DENV1/3/4 vaccinated individuals were protected from severe infection with heterotypic DENV2. One of the individuals who was well protected without detectable viremia by culture following vaccination generated pIgG that mediated increased type I IFN production and bound the surface of infected
cells as measured by immunofluorescence, suggesting that the described phenomenon may have contributed to viral control. However, pIgG isolated from other protected individuals failed to alter IFN production and did not bind the surface of infected cells, indicating that this is not the only, but likely one of many, mechanisms of protection which may include neutralizing antibodies and T cell responses resulting from vaccination. Our data highlight that the polyclonal response to DENV infection is composed of antibodies with diverse features and that the resulting population of antibodies may shape the individual’s clinical outcome through neutralizing and non-neutralizing means.

Our model demonstrates dependence on the availability of viral epitopes on the surface of both DENV- and ZIKV- infected cells for IFN enhancement. The accessibility of DENV epitopes at the surface of infected cells could be the result of viral protein accumulation at the plasma membrane or of binding of intact virions to the cell membrane, either on entry into a target cell or on exocytosis from an infected cell. Our data do not distinguish between these possibilities; however, the existing literature and our current data demonstrate that viral epitopes are present on the infected cell surface (70–72). Further, our data that pDCs do not produce IFN when cultured with virus pre-incubated with antibody demonstrate that IFN enhancement by anti-DENV antibodies is not due to enhanced pDC internalization of free virus. This supports the proposed model and the dependence on interactions between pDCs and infected cells expressing viral epitopes on the cell surface.

Our data extend the existing literature on the non-neutralizing effects of antibody responses to DENV and ZIKV. Monocytic cells, a secondary producer of type I IFN in DENV infection, have been the focus of previous studies and show reduced type I IFN production in the presence of pre-existing cross-reactive anti-DENV antibodies, thus worsening measured viral
infection (73–75). However, the primary type I IFN producing cell in many viral infections is the pDC (51–53), making the pDC critical in our understanding of the type I IFN response in secondary DENV or ZIKV infection. Our data are consistent with the limited literature that exists on pDC response to DENV as well as the data on the in vivo IFN response post-exposure (57–61). Specifically, studies demonstrating that people with severe DENV have decreased peripheral IFN and a suppressed pDC response supports a mechanism of protection by which pre-existing antibodies upregulate IFN production (57).

Our data demonstrate for the first time the effect of cross-reactive antibodies on the pDC viral sensing pathway following DENV or ZIKV exposure and adds to existing literature establishing the concept of the ‘interferogenic synapse’ in pDC sensing of DENV and ZIKV (59, 60). This antibody effector mechanism may be relevant in protection against a variety of viruses, as pDCs generate large quantities of type I IFNs when they interact with liver cells infected with DENV, ZIKV, and other important pathogens (59, 61). The released IFNs directly function to restrict viral replication (29, 30, 61). We established that a specific subset of cross-reactive antibodies increased IFN production, leading to more robust innate immune protection relative to primary infection in a mechanism that relies on contact between innate sensing and infected cells. However, our data do not distinguish whether the antibody-mediated increase in IFN production results from increasing the number or the duration of pDC/infected cell contacts.

Our work defines a novel mechanism independent of neutralization by which cross-reactive antibodies present at the time of secondary DENV or ZIKV infection might be protective. Our in vitro model specifically utilizes a system in which infection and replication occur for 48 hours prior to the addition of antibodies, bypassing the infection neutralization effect of these antibodies. Importantly, the ability of an antibody to neutralize DENV or ZIKV
infection did not segregate completely with IFN upregulation, and notably, not all neutralizing antibodies upregulate IFN production. It is well known that non-neutralizing antibodies can enhance disease severity, but the vast majority of secondary DENV infections are not severe. Antibodies that increase IFN production may mitigate the risk of worsened clinical outcomes in a subset of individuals with antibodies to DENV. These data further highlight the complexity of and potential for diverse effects of antibodies in clinical outcomes.

We have demonstrated that the IFN innate immune signaling cascade can be upregulated in DENV and ZIKV infections in vitro by the presence of pre-existing cross-reactive anti-DENV antibodies, suggesting a possible mechanism for enhanced protection against these viruses following primary infection. This work advances our understanding of the role of non-neutralizing antibodies in viral infection, flavivirus and otherwise, and particularly in understanding the interplay between antibody and innate immune responses and highlights the multifaceted role of antibodies in clinical outcomes.
METHODS:

Expansion of DENV and ZIKV cell culture strains

DENV 2 – New Guinea C (DENV2-NGC), Zika (ZIKV) Nicaragua/2015, and ZIKV Sao Jose do Rio Preto (SJRP)/2015 were obtained from Dr. Stephen Whitehead (NIAID, Bethesda, MD). The viruses were amplified as has been previously described (76). Vero cells were plated and infected with 0.01 MOI of DENV2-NGC or 0.001 MOI of ZIKV SJRP/2015 or ZIKV Nicaragua/2015. Infected cells were cultured for 5-7 days. Vero culture supernatant was collected and aliquoted. PFU/mL was determined by modified a plaque assay and immunostaining. Briefly, Vero cells were plated in 24-well plates and cultured to 90% confluency. Virus was plated in duplicate in serum-free OPTIMEM at 6 serial 10-fold dilutions and incubated for 1 hour. An overlay of 1% methylcellulose in OPTIMEM was applied to cultures. Plates were incubated for 4-5 days. Titration plates were fixed with methanol and stained with anti-DENV antibodies (2H2 & 4G2, Dr. Stephen Whitehead, supplied by NIAID, Bethesda, MD), followed by a secondary goat-anti-mouse HRP-conjugated antibody (VWR 074-1806). TrueBlue KPL substrate (VWR 50-78-02) was used to develop plaques.

DENV monoclonal antibodies

The following antibodies were obtained from Dr. Stephen Whitehead (NIAID, Bethesda, MD), originally produced by LakePharma: 4G2, C8, and C10 (65, 66, 77, 78). Dr. Whitehead also provided the 2H2 antibody, originally purchased from the ATCC as hybridoma D3-2H2-9-21 (ATCC HB-114). The following antibodies were obtained from Drs. Kar Muthumani and David Weiner (Wistar Institute, Philadelphia, PA): pDV87.1, pDV87.1 LALA (64). pDV87.1 LALA is identical to pDV87.1 except for two leucine-to-alanine mutations (L234A, L235A) in the Fc portion of the antibody, which abrogate its binding to Fc receptors(79). The following antibodies
were obtained from Dr. Aravinda de Silva (UNC, Chapel Hill, NC): 4G21, 1H10, 2H21, 1E23, and 1B22 (68).

Polyclonal IgG from DENV challenge cohort subjects

Serum samples were generously shared from the NIAID Trivalent Vaccination Cohort Study and were obtained from the two sites involved in the cohort: Dr. Anna Durbin’s group at Johns Hopkins University (Baltimore, MD) and Dr. Beth Kirkpatrick’s group at the University of Vermont (Burlington, VT) (Clinicaltrials.gov NCT02433652). The study is a challenge study designed to assess the efficacy of a trivalent live attenuated DENV vaccine formulation containing DENV-1, -3, and -4 vaccine components and was sponsored by NIAID contract No. HHSN272200900010C. The study was approved by Western Institutional Review Board (WIRB) and the University of Vermont IRB and conducted under an FDA investigational new drug application. Subjects were vaccinated or received placebo on day 0 of the study. On day 180, subjects were all challenged with the recombinant DENV-2 challenge virus rDEN2Δ30. Of the 25 subjects enrolled in the cohort, 20 completed the course of the challenge study. We obtained day 28 post-vaccination/placebo serum for 16 individuals (14 vaccinees and 2 placebo recipients). Polyclonal antibody (pAb) was isolated using the Pierce Protein A IgG isolation columns (Thermo 44667) per the manufacturer’s instructions. Each 1mL elution fraction was neutralized using 100uL of binding buffer. Fractions containing IgG as determined by nanodrop and SDS-PAGE were concentrated on a 50kDa Amicon Ultra 5mL filter (Millipore UFC805024) and quantified using Nanodrop Protein A280 measurement.

Cell lines and primary plasmacytoid dendritic cell isolation

Huh 7.5.1 hepatoma cells were originally obtained from Dr. Charles Rice (Rockefeller Institute, New York, NY) and were cultured in Dulbecco’s Modified Eagles Medium (DMEM) with 10%
fetal bovine serum (FBS) and 1% non-essential amino acids. For primary human cell culture and pDC isolation, freshly collected de-identified human blood Leuko Paks (LPS) were obtained from the Anne Arundel Medical Blood Donor Center (Anne Arundel, MD). PBMCs were isolated using Ficoll-Hypaque gradient centrifugation. PBMCs were then subjected to magnetic separation, and pDCs were isolated by negative selection per the manufacturer’s protocol (Miltenyi Biotec 130-097-415). pDCs were collected and cultured in RPMI 1640 media supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 1% HEPES buffer.

**Co-culture plasmacytoid dendritic cell (pDC) experiments**

On day 0, Huh 7.5.1 were plated at approximately 6e5 cells/well in a 6-well cell culture plate. Huh 7.5.1 cells carry a point mutation in the gene encoding RIG-I, which reduces the host innate immune response to viral RNA and renders cells more permissive to viral infection (80, 81). On day 1, Huh 7.5.1 were confluent at approximately 1.5 x 10^6 cells/well. Huh 7.5.1 were infected on day 1 with 0.1-1 MOI of DENV-2-NGC or ZIKV or left in media alone and cultured for 48 hours. On day 3, Huh 7.5.1 cells were lifted and re-plated at 100,000 cells/well in a 96-well U-bottom cell culture plate. After allowing cells to re-adhere, infected or uninfected Huh 7.5.1 were treated with cell culture compounds or antibody for 1 hour. Primary human pDCs were isolated as described above. pDCs were plated at 20,000 cells/well in a 96-well U-bottom cell culture plate. As needed, pDCs were treated with any cell culture compounds or antibodies for 1 hour. Finally, pDCs were transferred to co-culture with Huh 7.5.1 for 24 hours. After 24 hours, cell culture supernatants were collected for further analysis.

**IFNα measurements**
Human IFNα2a was quantified using the Human IFNα2a Tissue Culture Kit from Meso Scale Discovery (MSD K151ACB-4). Samples were assessed per the manufacturer’s protocol, using 25 ul of supernatant, diluted 1:10 in pDC tissue culture media (described above). Data were acquired on a MESO QuickPlex SQ 120 imager. Data were analyzed using Meso Discovery Workbench software.

**Modulation of IFN by antibody**

As described above, Huh 7.5.1 cells were re-plated on day 3 of the experiment. After adherence in 96-well cell culture plates, 100,000 mock-infected or infected Huh 7.5.1/well were incubated with monoclonal antibodies at 0.1ug/mL-10ug/mL or polyclonal antibodies at 0.001ug/mL-1000ug/mL for 1 hour. Concentrations of DENV anti-env monoclonal antibodies were selected based on their enhancement activity in the K562 assay. Following antibody incubation with mock-infected Huh 7.5.1 cells or DENV2-NGC or ZIKV infected-Huh 7.5.1 cells, pDCs were then co-cultured as described above for 24 hours and IFNα2a was measured in supernatant to assess the effect of antibody on IFN production.

**Modulation of IFN by cell adhesion molecules**

Mock-infected or DENV-2-NGC infected Huh 7.5.1 cells were re-plated on day 3. After adherence, cell adhesion molecules were blocked for 1 hour using anti-ICAM-1 (CD54) antibody (Fisher Scientific 559047) or anti-integrin (ITGAL) antibody (LifeSpan Biotechnologies LS-C134275-100) as described previously(60). After 1 hour, pDCs were co-cultured for 24 hours and IFNα2a was measured in the supernatant following co-culture.

**Assessing FcR requirement**

Freshly isolated pDCs were plated at 20,000 cells/well in a 96-well U-bottom cell culture plate. Prior to co-culture, they were pre-incubated with one of the following reagents to assess Fc
receptor (FcR) usage: FcBlock (BD Biosciences #564219) or anti-FcγR2a (CD32a) antibody (R&D Systems AF1875). Following the blockade of FcRs, pDCs were co-cultured with Huh 7.5.1, which had simultaneously been incubated with a monoclonal antibody. In addition to FcR blockade, LALA variant DV87.1 antibody with intact Fab but Fc regions unable to bind FcR was used to further assess Fc/FcR binding requirements.

**Immunofluorescent imaging of DENV infected cells**

Huh 7.5.1 hepatoma cells were infected with 0.1 MOI of DENV-2-NGC. After 48 hours of infection, cells were replated onto coverslips and then blocked with PBS supplemented with Ca^{2+} and Mg^{2+} with 1% BSA. Then, cells were cultured with the given mAb or pAb (human mAbs DV87.1, C8, C10, murine mAbs 2H2 and 4G2, or polyclonal IgG) in blocking buffer for 1 hour at 4 degrees Celsius to prevent endocytosis and allow for surface staining. Cells were fixed with 4% PFA and permeabilized using TBS with 1% BSA, 0.2% milk, and 0.15% saponin. Intracellular DENV was then stained using a second, different anti-DENV mAb diluted in permeabilization buffer. If surface staining was completed using a human mAb, intracellular staining was done with a murine mAb and vice versa. Stained cells were washed and stained with goat anti-human Dylight 650 (ThermoFisher MA1-016-D650), goat anti-human IgG AF488 (Invitrogen A-11013), donkey anti-mouse AF488 (Life Technologies A21202), or goat anti-mouse IgG AF594 (Abcam ab150116). When relevant, cells were incubated for 10 minutes at 37 degrees Celsius and 5% CO_{2} with 5 ug/mL wheat germ agglutinin-AF594 (Invitrogen W11262) prior to primary antibody incubation or fixation/permeabilization. Cells were counterstained with DV87.1 and a goat anti-human IgG AF488 secondary antibody (Invitrogen A-11013). Images were acquired using a LSM800 confocal (Zeiss) with GaAsP detectors.

**Flow Cytometry**
Uninfected or DENV New Guinea C infected Huh 7.5.1 cells were gently scraped and washed 1x in cold PBS. Cells were stained for viability with Live/Dead Fixable Aqua dye 1:200 (ThermoFisher Scientific) + FcBlock 1:20 (BD Biosciences) for 20min at 4C in the dark. Cells were washed 1x with cold PBS. Cells were then surface stained with human anti-DENV envelope antibodies DV87.1 (1µg/mL), C8 (0.49µg/mL), C10 (0.29µg/mL), or human anti-prM antibodies 4G21, 1H10, 2H21, 1E23, or 1B22 (1µg/mL) in 100µL staining buffer (cold PBS + 0.5% BSA) for 30min at 4C in the dark. Cells were washed 2x with cold staining buffer. Primary antibody stained cells were then stained with secondary anti-human AF647 1:1000 (Southern Biotech 2048-31) or secondary anti-human Dy488 1:1000 (Abcam ab 97003) in 100uL cold staining buffer for 20min at 4C in the dark. Finally, cells were fixed in 1% paraformaldehyde and run on a 5 laser BD Biosciences Fortessa flow cytometer or a 4 laser Cytek Aurora. Analysis was performed using Flowjo v10 software.

Statistics

One-way ANOVA tests were performed with GraphPad Prism 7.0 software to assess statistical significance. Sidak’s or Tukey’s test was used to correct for multiple comparisons. Differences between groups were considered significant when p<0.05. Data represent mean plus or minus SEM. Data for many experiments were normalized as a percent of the average of replicates of a baseline condition for the experiment (most often virus alone) due to the marked donor-to-donor variability in pDC IFN production – normalization is noted in all figures and figure legends when applicable.

Study Approval

The use of human subjects occurred via the NIAID Trivalent Vaccination Cohort Study (Clinicaltrials.gov NCT02433652). The study was sponsored by NIAID under contract No.
HHSN272200900010C, was approved by Western Institutional Review Board (WIRB) and the University of Vermont IRB and conducted under an FDA investigational new drug application.

**Author contributions:**

LKA designed experiments, conducted experiments, acquired data, analyzed data, and wrote the manuscript. KER conducted experiments, acquired data, contributed to writing the manuscript, and analyzed data. KC conducted experiments, acquired data, and analyzed data. GM conducted experiments and acquired data. WA assisted in conducting experiments, specifically immunofluorescent imaging studies and helped to design these experiments. WL provided many reagents and helped in designing experiments. KM and DW provided critical reagents. SW assisted in designing experiments and shared critical reagents. MC designed experiments and analyzed data. AD designed research studies and experiments, analyzed data, provided critical reagents, and contributed to the manuscript. AC designed research studies and experiments, analyzed data, and contributed to writing the manuscript. The listing order of co-first authors was determined by alphabetical order.
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Figure 1: Contact between DENV infected cells and pDCs is required for type I IFN response

(A) Primary human pDCs were co-cultured with uninfected Huh 7.5.1 cells (first bar), with infected Huh 7.5.1 cells (second and third bar), or with DENV alone (fourth bar). Supernatants were collected and IFN-α2a was measured. To assess the requirement for pDCs in IFN production, infected Huh 7.5.1 cells were cultured alone (seventh bar). To assess the requirement for cell-to-cell contact, pDCs were cultured with supernatant collected from infected Huh 7.5.1 cells (fifth bar). To demonstrate that TLR signaling is intact in the pDCs in the absence of hepatoma cells, pDC were cultured with 1 ug/mL TLR7 agonist resiquimod (REQ) (sixth bar).

This figure represents four independent experiments with distinct pDC donors, with n ≥ 3 per condition. (B) Schematic representation of experimental design to follow: Huh 7.5.1 cells are infected with DENV at 0.1-1 MOI for 48 hours. At 48 hours, antibody treatment is added. pDCs are isolated and co-cultured with treated Huh 7.5.1 one hour after antibody addition. Supernatants are collected after 24 hours and analyzed for IFN-α2a. (C-D) Primary human pDCs were co-cultured with infected Huh 7.5.1 following 1 hour of treatment with (C) anti-ICAM-1 antibody at 2-10 µg/mL or (D) anti-α4 integrin antibody at 0.1-1 µg/mL. Supernatants were collected and IFN-α2a measured. (C) represents seven independent experiments with distinct pDC donors (3-7 donors per condition) with n ≥ 3 replicates per independent experiment. (D) represents seven distinct pDC donor experiments (1-7 donors per condition) with n ≥ 3 replicates per condition per experiment. Y-axis represents IFN relative to baseline level generated after pDC + Huh co-culture with 0.1 MOI DENV. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 2: Antibody-mediated IFN upregulation requires binding to a viral epitope on the surface of infected cells

Huh 7.5.1 cells were infected with DENV at 0.1 MOI for 48 h. (A-C) After 48 h, (A) 0.1-1 µg/mL DV87.1 or (B) 1 µg/mL DV87.1, 0.49 µg/mL C8, 0.29 µg/mL C10, 0.42 µg/mL 4G2, 20.4 µg/mL 2H2, 1 µg/mL isotype control IgG or (C) 1 µg/mL 4G21, 1H10, 1B22, 2H21, 1E23 or DV87.1 were added to infected Huh 7.5.1 cells and incubated for 1 hour. Primary human pDCs were added and co-cultured for 24 hours. Supernatants were collected for IFN-α2a measurement. (D) Infected Huh 7.5.1 cells were treated with DV87.1 or 2H2 for surface binding of DENV epitopes, fixed and permeabilized after 24 hours, and stained intracellularly with 4G2, 2H2, or DV87.1. Representative images are displayed. Scale bars represent 10 µM. (E) Uninfected or DENV infected Huh 7.5.1 cells were surface stained with antibodies against DENV E or prM protein and stained with secondary anti-human AF647 or DY488. The left panels are representative flow plots for uninfected or infected Huh 7.5.1 cells. Right panels quantify flow data on left as percent DENV positive cells stained with each antibody. Significance was determined by comparing the binding of each antibody to infected cells over uninfected cells. Panels represent (A) 17 (10-17 per condition) (B) 4 (1-4 per condition) independent experiments with unique pDC donors with n ≥ 3 per condition per experiment. Panel (C) represents one independent experiment (conducted 4 times) with n ≥ 3 per condition. Panel (D) is representative of 5 total experiments. Panel (E) represents two independent experiments with unique pDC donors for both the top and bottom panels. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 3: Anti-DENV antibodies bypass the requirement for cell adhesion molecules in pDC sensing of virus infected cells

(A) Schematic representation of experimental design: As established in Figure 1, blockade of ICAM-1 or αL-integrin abrogates IFN production by sensing pDCs. The experiments that follow in panels (B) and (C) evaluated the role of ICAM-1 or αL-integrin in the presence of anti-DENV antibody (represented in purple). (B-C) Infected Huh 7.5.1 cells were incubated for 1 hour with (B) anti-αL integrin at 0.1 µg/mL or (C) anti-ICAM-1 antibody at 5 µg/mL or 2 µg/mL with or without DV87.1 at 0.1 µg/mL or 1 µg/mL. pDCs were added and co-cultured for 24 hours. Supernatants were collected and assessed for IFN-α2a. Panel (B) represents a single pDC donor experiment with n ≥ 3 per condition. Panel (C) represents 11 pDC donor experiments (1-11 per condition) with n ≥ 3 per condition. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 4: Antibody-mediated upregulation of pDC sensing and IFN production requires Fc engagement of FcγR2a on pDCs

Huh 7.5.1 cells were infected with DENV at 0.1 MOI for 48 hours. (A) Infected Huh 7.5.1 were treated with DV87.1 or LALA DV87.1 antibody for 1 hour, followed by pDC co-culture for 24 hours. (B) Infected hepatoma cells were cultured with DV87.1 for 1 hour while pDCs were treated with FcBlock or anti-FcγR2a antibody for 1 hour. pDCs were then co-cultured with Huh 7.5.1 for 24 hours. (C-D) Huh 7.5.1 were incubated with (C) anti-ICAM-1 antibody at 2 µg/mL or (D) anti-αL integrin antibody at 0.1 µg/mL with or without DV87.1 (0.1 µg/mL or 1 µg/mL) or LALA DV87.1 (0.1 µg/mL or 1 µg/mL), following which they were co-cultured with primary human pDCs for 24 h. (E) Huh 7.5.1 cells were treated with anti-ICAM-1 blocking antibody at 2 µg/mL with 0.1 µg/mL or 1 µg/mL of DV87.1 while pDCs were treated with anti-FcγR2a blocking antibody at 10µg/mL. (F) Infected Huh 7.5.1 cells were incubated with anti-ICAM-1 antibody at 2 µg/mL with 1 µg/mL of DV87.1 and increasing amounts of LALA DV87.1 (0.1 µg/mL or 10 µg/mL) for 1 hour, and then co-cultured with pDCs. All supernatants were collected and assessed for IFN-α2a by MSD analysis. Each panel represents (A) 15 & (B) 3 (2-3 per condition) independent experiments with unique pDC donors with n ≥3 per condition per experiment. Panels (C), (E), and (F) each represent at least two independent experiments with unique pDC donors and n ≥3 per condition per experiment. Panel (D) represents a single pDC donor experiment with n ≥3 per condition. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 5: Cross-reactive anti-DENV mAbs increase IFN production in pDC exposed to ZIKV

Huh 7.5.1 cells were plated and infected with 0.1-1 MOI of (A) ZIKV Nicaragua/2015 or (B) ZIKV SJRP/2015 for 48 hours. Infected Huh 7.5.1 cells were replated and pre-incubated with anti-DENV monoclonal antibodies C8, C10, 2H2, and DV87.1 for 1 hour. Primary human pDCs were added and co-cultured with infected and antibody-treated Huh 7.5.1 cells for 24 hours, after which supernatants were assessed for IFN-α2a by MSD analysis. (C) Huh 7.5.1 cells were infected with ZIKV and then replated for 24 h, at which point they were stained with DV87.1 and C10 mAbs for 1 h at 4°C. Following surface Ab staining, the cells were fixed and permeabilized and stained for intracellular DENV E with mouse mAb 4G2. Representative images for 4 conducted experiments are displayed (ZIKV SJRP used in displayed images). Scale bars represent 10 µM. (D-E) Huh 7.5.1 cells were plated and infected with 0.1 MOI of (D) ZIKV Nicaragua/2015 or (E) ZIKV SJRP/2015 for 48 h. Infected cells were replated and pre-incubated for 1 hour with the anti-ICAM antibody at 0.1-5 µg/mL and (D) C8 or (E) C10 monoclonal antibodies. After pre-incubation, primary human pDCs were isolated and co-cultured with infected and antibody-treated Huh 7.5.1 cells for 24 hours, after which supernatants were assessed for IFN-α2a by MSD analysis. Each figure panel represents at least two independent experiments with unique pDC donors with n ≥ 3 per condition per experiment. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 6: Polyclonal IgG from DENV-immune individuals can enhance IFN activity of pDC exposed to DENV or ZIKV.

Huh 7.5.1 cells were infected with (A) 0.1 MOI of DEN2-NGC for 48 hours. Polyclonal IgG isolated from DENV-vaccinated (subjects VV1-3, VP1-6) or DENV-naïve (subjects P1, P2) subjects 28 days after vaccination with live attenuated DENV 1/3/4 admixture or placebo was added to culture after 48 hours. VV represents vaccinated and viremic subjects, VP represents vaccinated and protected subjects, and P represents placebo-treated subjects. After 1 hour of pre-incubation with polyclonal IgG, primary human pDCs were co-cultured for 24 hours. IFN-α2a was assessed in the supernatant. The figure represents two independent pDC donor experiments with n ≥ 2 per condition.

(B) Infected Huh 7.5.1 cells were pre-incubated with the anti-ICAM antibody at 2 µg/mL for 1 hour. Polyclonal IgG isolated from DENV-vaccinated (subjects VV1, VP1-6) were added for 1 hour. Primary human pDCs were isolated and co-cultured for 24 h. IFN-α2a was assessed in supernatants. (C) Huh 7.5.1 cells were infected with ZIKV SJRP or ZIKV Nicaragua for 48 h. They were then cultured with polyclonal IgG isolated from DENV-immune subjects (subjects VV1, VP1-4) for 1 hour and then co-cultured with pDCs for 24 h. IFN-α2a was measured in the supernatant. Panels (B) and (C) each represent a single pDC donor experiment with n ≥ 3 per condition.

(D) Infected Huh 7.5.1 cells were treated with polyclonal IgG from VP3, P1, and VP4 to assess binding of polyclonal IgG to the surface of infected cells. Cells were fixed, permeabilized, and stained intracellularly with 4G2 murine anti-DENV E antibody. Representative images for 4 total experiments are displayed. Scale bars represent 10uM. Statistical significance determined by one-way ANOVA. * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, ns = not significant.
Figure 7: A proposed model for antibody-mediated specificity in pDC sensing of DENV and subsequently enhanced IFN production.

In the absence of anti-DENV antibodies, random interactions (represented on the left-hand side of the schematic) result in pDCs interacting with infected and uninfected cells randomly, forming transient contacts via ICAM-1/integrin interactions. When a pDC randomly encounters an infected cell, PAMP transfer occurs, and TLR7 signaling and IFN production ensue. When the anti-DENV antibody is present (represented on the right-hand side of the schematic), antibody-directed contact with DENV- or ZIKV-infected cells occurs. This mechanism works in concert with ICAM/integrin interactions to bind pDCs specifically to the infected cell, possibly increasing the number of pDC interactions with infected cells, the duration, or the avidity of pDC interactions with infected cells.