Role of Dendritic Cells in antibody dependent enhancement of dengue infection

Kobporn Boonnak, Bonnie M. Slike, Timothy H. Burgess, Randall M. Mason, Shuenn-Jue Wu, Peifang Sun, Kevin Porter, Irani Fianza Rudiman, Djoko Yuwono, Pilaipan Puthavathana, Mary A. Marovich

1Department of Retrovirology, Walter Reed Army Institute of Research and Henry M. Jackson Foundation for the Advancement of Military Medicine, Rockville, MD 20850; 2Viral Disease Department, Naval Medical Research Center, Silver Spring, MD 20889; 3Department of Virus Diseases, Walter Reed Army Institute of Research, Silver Spring, MD 20889; 4Department of Internal Medicine, Hasan Sadikin Hospital, Bandung, Indonesia, 5National Institute of Health Research and Development, Indonesian Ministry of Health, Jakarta, Indonesia, 6Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok-noi, Bangkok 10700, Thailand, 7Uniformed Services University of the Health Sciences, Department of Medicine, Bethesda, MD 20814

*Corresponding author:
Mary A. Marovich, M.D., DTM&H
Associate Professor, Department of Medicine
Uniformed Services University of the Health Sciences
Division of Retrovirology, Department of Vaccine R&D
Walter Reed Army Institute of Research
13 Taft Ct., Suite 200, Rockville, MD 20850
Phone: 301-251-8337
Fax: 301-762-4422
E-mail: mmarovich@hivresearch.org
Abstract

Dengue viruses (DV), composed of 4 distinct serotypes (DV1-4), cause 50-100 million infections annually. Durable homotypic immunity follows infection but may predispose to severe subsequent heterotypic infections, a risk conferred in part by the immune response itself. Antibody-dependent enhancement (ADE), a process best described in vitro, is epidemiologically linked to complicated DV infections, especially in Southeast Asia. Here we report for the first time the ADE phenomenon in primary human dendritic cells (DC), early targets of DV infection, and human cell lines bearing Fc receptors. We show that ADE is inversely correlated with surface expression of Dendritic Cell-Specific Intercellular adhesion molecule-3 Grabbing Nonintegrin (DC-SIGN) and requires FcRγIIa. Mature DC exhibited ADE whereas immature DC, expressing higher levels of DC-SIGN and similar FcRγIIa levels, did not undergo ADE. ADE results in increased intracellular de novo dengue protein synthesis, increased viral RNA production and release, and increased infectivity of the supernatants in mature DC. Interestingly, TNF-α and IL-6, but not IL-10 or IFN-γ were released in the presence of dengue sera but generally only at enhancement titers suggesting a signaling component of ADE. FcRγIIa inhibition with monoclonal antibodies abrogated antibody dependent enhancement and associated downstream consequences. DV versatility in entry routes (FcRγIIa or DC-SIGN) in mature DC broadens target options and suggests additional ways for DC to contribute to the pathogenesis of severe dengue infection. Studying the cellular targets of DV infection and their susceptibility to ADE will aid our understanding of complex disease and contribute to the field of vaccine development.
Introduction

Dengue viruses (DV 1-4) are single stranded positive polarity RNA viruses. Symptomatic infection ranges from a self-limited febrile illness (dengue fever: DF) to a life-threatening syndrome (dengue hemorrhagic fever/dengue shock syndrome: DHF/DSS). The pathogenesis of complicated DV infection is not clearly understood, but viral, host and immune factors likely influence disease severity (12, 18, 48). Antibody dependent enhancement (ADE) of DV infection is often implicated in the pathogenesis of dengue hemorrhagic fever (24, 36). Presumably, sub-neutralizing concentrations of a heterologous antibody leads to ADE which increases the number, intensity and/or types of cells infected, thereby increasing viremia and consequent disease severity (17, 49). DHF is recognized as the best example of in vivo ADE, largely based on key epidemiological studies showing increased risk of DHF in individuals with prior DV infections (6, 46, 49). This hypothesis was strengthened by the increased incidence of DHF associated with the recent introduction of DV2 into Cuba (19) after prior remote infection with Dengue 1. DHF/DHS did not occur in individuals with primary infections, e.g. with DV1 or 2 only. Passive transfer experiments conducted in non-human primates (NHP) showed increased viremia in subsequent dengue infection (14, 20), however DHF has not been recapitulated in the NHP model (14). Further support for the role of ADE in complex disease is provided by the increased incidence of DHF during primary DV infection in the first year of life in infants, born to dengue-immune mothers, who acquire DV antibody across the placenta (23).

One of the main challenges for dengue vaccine development is the requirement for concurrent protective immunity to all 4 serotypes – or vaccination itself could pose
additional risks. There are gaps in our understanding of antibody-mediated entry in susceptible cells, and types of sera, antibodies or other molecules promoting enhancement and downstream functional effects. While new animal models are developed, we build upon the foundation of epidemiologic data and use *in vitro* cellular based studies to move forward. Many different primary cells and cell lines are reportedly infected by dengue viruses, including monocyte/macrophages, B cells, T cells, endothelial cells, hepatocytes and neuronal cells (1). A new murine model confirms that both macrophages and dendritic cells (DC) are cellular targets (25). Human DC are the most susceptible primary cells to direct dengue infection (unaided by antibody) and are considered early cellular targets (15, 33, 39, 51). The recently identified role of the DC-SIGN molecule in facilitating viral entry further supports the involvement of DC in dengue infection (37, 45).

Given the likelihood that DC are DV targets, and DC-SIGN facilitates viral entry, we studied the relationship between DC-SIGN and ADE. We previously reported that immature DC did not undergo ADE despite expressing similar levels of Fc receptors (FcγRs) when compared to positive control K562 cells (33). We next studied the role of the C-type lectins in entry (45) and questioned whether the abundance of DC-SIGN on immature DC overrides the effect of enhancing immune sera, either by preventing ADE altogether or obscuring its effects. Since DC-SIGN levels are lower on mature DC, we evaluated their susceptibility to ADE. Fc receptors are identified as key molecules mediating ADE in DV infections (5, 22, 27, 43). We and others have previously shown that human DC mainly express FcγRII (3, 4, 28, 33). In this study, we tested dengue immune sera for a variety of ADE effects in a high throughput and reproducible assay
using relevant primary cell targets for DV infection, including primary DC and other Fc and non-Fc receptor bearing cells.

Here we show that mature DC display an enhanced infection pattern in the presence of dengue immune serum. We report for the first time that viral output on a per cell basis is increased dramatically under conditions of ADE. This ADE pattern is detected after down regulation of DC-SIGN upon DC maturation, and requires cell surface expression of FcγRIIa. The data suggest that DV uses at least two routes of entry into the same cell type, depending on the milieu, with different outcomes. We show that the route of viral entry in mature DC influences the intensity of cellular infection, viral output, transmissibility and downstream cytokine secretion.
Materials and Methods

Viral stocks and cell lines. The Burma Den-2 isolate, S16803 (S. Halstead, personal communication), was used for all experiments. Cell lines bearing FcR included the human erythroleukemic cell line K562, the human monocytic cell line U937, and the human Raji B cell line (ATCC, Manassas, VA). The K562, U937 and NIH3T3 transfectants were graciously provided by Dr. Vineet Kewalramani (NCI, Ft. Detrick, MD). These were maintained in RPMI supplemented with 10% heat inactivated FCS (Gemini Bio-Products, Sacramento, CA) with 2mM L-glutamine, 100 U/ml penicillin and 100 ug/ml streptomycin, referred to as supplements (Quality Biological, Gaithersburg, MD). The non FcR bearing murine fibroblast cell line NIH3T3 (ATCC) was maintained in DMEM (Quality Biological) with 10% heat inactivated FCS and supplements.

Monoclonal antibodies (mAb) and dengue immune serum. Intracellular dengue infection was measured using the 2H2 mAb (kindly provided by Dr. Robert Putnak, WRAIR, Silver Spring, MD), an IgG2a mouse specific anti prM that is conserved on serotypes 1-4, indicating de novo protein production as described previously (45). We accessed small aliquots of a well-characterized Den-1 immune sera collection from an IRB approved Indonesian cohort obtained to study ADE. The Den-1 sera were tested in a PRNT-70 assay and found to neutralize only Den-1 virus (personal communication, S-J Wu). Purified 4G2 (IgG2a) monoclonal antibody and 3H5 (IgG1) ascites monoclonal antibodies were obtained through the Naval Medical Research Center (Silver Spring, MD). Normal Human AB serum was purchased from Gemini Bio-Product, West Sacramento, CA.
**Monocyte isolation.** Primary human monocytes were prepared using the Dynal® Monocyte Negative Isolation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Briefly, $10^7$ PBMC were incubated with blocking reagent and antibody mix for 10 minutes at 2-8°C. Depletion Dynabeads (100 ul) were added and incubated for 15 minutes at 2-8°C. The labeled cells were removed with a magnet (Dynal MPC®) leaving untouched, highly purified monocytes (90-95% CD14⁺ as determined by flow cytometry).

**Monocyte-derived DCs.** Peripheral blood mononuclear cells (PBMC) were cultured as described previously (34, 47) with some modification. PBMC isolated from leukapheresis products from healthy donors (BRT Laboratories, Baltimore, MD) were cryopreserved allowing repeat experiments. PBMC were adhered to tissue culture dishes for 60 min and after several RPMI washes, adherent cells were cultured in 10 ml complete media (CM) with $2 \times 10^4$ U/ml rhuGM-CSF (Fisher Clinical Services, Allentown, PA) and $2 \times 10^4$ U/ml IL-4 (R&D Systems, Minneapolis, MN) for 7 days at 37°C, 5% CO₂. On Day 6, 50 ul of MCM mimic (15 μg/ml IL-6 (Peprotech Rocky Hill,NJ), 500 ng/ml IL-1β, 500 ng/ml TNF-α (Sigma St.Louis, MO) and 100 μg/ml PGE₂ (CaymanChemical, AnnArbor, MI) was added to mature the cells. The phenotypes of all DC were confirmed by flow cytometry before use. Specifically, DCs lack CD3, CD19 or 20 and CD14, but express high levels of HLA-DR and DC-SIGN. Mature cells additionally express CD25, CD83 and CD86 but much lower levels of DC-SIGN (34,45).

**Antibody dependent enhancement assay.** Dengue immune serum, 4G2 or 3H5 mAb, were serially diluted from 1/10 to 1/163840 in a volume of 50 ul. Virus, at a
multiplicity of infection (MOI) of 1, unless otherwise noted, was placed into the antibody
dilution tubes and incubated for 60 min at 37ºC, 5% CO₂ to allow immune complex
formation. The content of each tube was then added to 0.5x10⁶ cells and incubated for 2
hrs. The exposed cells were washed with CM to remove excess dengue immune
complexes. The cells were resuspended in CM, and incubated for an additional 48 hrs.

Cell viability was checked using Trypan Blue exclusion at 24 hr and by flow cytometry
using propidium iodide at 48 hrs. Tissue culture grade IgG₁ (DAKO, Glostrup,
Denmark), IgG₂a and IgG₂b (R&D Systems, Minneapolis, MN) and Normal Human Sera
(Gemini Bio-Product, West Sacramento, CA) were included as additional negative
controls for dengue immune sera.

**Flow Cytometry.** A FACS® Calibur (BD Biosciences, San Jose, CA) was used
to monitor cell surface staining with a panel of PE-conjugated mAbs to HLA-DR, CD80,
CD86, CD3, CD14, CD20, CD25, CD1a (BD Biosciences, San Jose, CA), CD83
(Beckman Coulter, Fullerton, CA), and matched isotype controls. To detect intracellular
*de novo* dengue protein production, cells were permeabilized with Cytofix/Cytoperm (BD
Biosciences, San Jose, CA) and stained with 2H2 (anti DV-prM mAb) conjugated to
AlexaFluor-488 (Invitrogen, Carlsbad, CA) 48 hours post-viral exposure.

**Viral RNA Quantification.** A viral RNA standard was prepared by amplifying a
170bps fragment of the Dengue-2 16803 with primers 5’-AATATGCTGAAACGCGAGAGAAACCGCG-3’
(corresponding to genome position 136 to 163) and 5’-CACCAACACAGCGGATATTG-3’
(corresponding to genome position 278 to 305). The resulting PCR product was ligated into a TA cloning vector
using the pGEM-T Easy System (Promega, Madison, WI) and the sequence was
confirmed using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Plasmid DNA was linearized with EcoRI and RNA transcripts were generated using Megascript Kit (Ambion, Austin, TX) according to the manufacturer’s specifications. The concentration of transcribed RNA was estimated by UV spectrophotometry. Primers (Den-IF: 5’-GCTGAAACGCAGAGAAACC-3’ and Den_IR: 5’-CAGTTTTAATGGTCCTCGTCCCT-3’) and probe (Den_PR: 5’FAM-CATTCCAAAGTAGAATCTCTTTTGTCAACTGTTGT BHQ1-3’) were designed to target the region of the Den-2 capsid gene which is highly conserved among the four dengue viruses, but not in other flaviviruses (50). Amplification was performed using an ABI Prism 7700 or 7500 detection instrument (Applied Biosystems, Foster City, CA).

The RT-PCR thermal cycles were performed as follows: 50 ºC for 30 min, 95 ºC for 15 min, followed by 40 cycles of 95 ºC for 15s and 60 ºC for 1 min. RNA copy number was calculated from a standard curve generated by an in vitro transcribed RNA standard.

**Vero cell plaque assay**

The Vero cell plaque assay was performed as described previously (11). Six 10-fold serial dilutions (10^{-1}-10^{-5}) were made from each supernatant sample and inoculated into quadruplicate wells of six-well tissue culture plates containing confluent Vero cell monolayers. After virus adsorption for 1 hr, the Vero monolayer was overlaid with complete MEM media (Cellgro, Manassas, VA) containing low melting agarose (Invitrogen, Carlsbad, CA) to restrict dissemination of progeny virions. The cells were incubated for 5 days at 37°C for 5 days and overlaid with the vital stain, neutral red (Sigma St. Louis, MO). Plaques were counted by visual inspection at 24 hrs after neutral
red overlay to determine the number of plaque forming units of DV per milliliter of supernatant.

**Measurement of Cytokine Levels.** Cytokines were measured in cell-free supernatants using the Cytometric Bead Array Flex Set (BD Biosciences, San Jose, CA) per manufacturer’s instructions. Briefly, Multiscreen 1.2 µm hydrophilic filter plates (Millipore, Bedford, MA) were pre-wet with wash buffer and aspirated. Capture beads for each of the four cytokines (IL-6, IL-10, IFN-γ, TNF-α) were combined with 50 uL of supernatant obtained from the ADE experiments. The plate was incubated for 1 hr at room temperature. PE detection reagent for each cytokine was pooled and added to the wells. The plate was incubated for 2 hrs at room temperature. Following incubation, the plate was washed and the beads were resuspended in wash buffer. Samples were read on an LSRII flow cytometer and analyzed using FCAP Array Software (BD Biosciences, San Jose, CA).

**Blocking of FcγRs on mature dendritic cell.** Mature DC were pretreated with 10ug/ml anti-human FcγIIa blocking antibody (clone IV.3, ATCC, Manassas, VA), anti-human FcγIIb blocking antibody (clone 2B6; kindly provided by Macrogenics, Rockville, MD) or IgG1, IgG2a, IgG2b controls for 1 hour at 37°C. Treated cells were washed twice with complete media before use in ADE assays.
UV irradiated DV preparation. The DV viral stock was placed in a Petri dish and exposed to short wavelength UV light (540 nm) for 20 min. The lack of infectivity of UV exposed DV was confirmed in highly susceptible Raji-DCSIGN cells before using in the ADE assay.

Statistics

The student’s paired T test, Spearman’s Rank test, Mann-Whitney U test and use of non linear fit one phase exponential decay for curve fitting were applied to the data with Prism software (GraphPad Software Inc., SanDiego, CA).
Results

Flow cytometry assay shows anti-DV1 immune sera enhancement of DV2 infection

We refined a flow cytometry assay that permits high throughput, quantitative and reproducible assessment of the *in vitro* ADE phenomenon (33). As in earlier studies (16, 24, 36), we focused on two assay parameters: Peak Enhancement Titer (PENT) and Power of enhancement (Fig.1A). We tested ADE in K562 cells using the following antibodies: 4G2, a broadly reactive flavivirus mAb; 3H5, a DV2 envelope specific mAb; and a well-characterized DV1 immune serum collection. We used a 2H2-AlexaFlour ® conjugate to detect *de novo* intracellular dengue prM antigen production as described previously (45). The dengue immune serum generally mediated at least a two-fold higher infection rate at PENT compared to 4G2 while ADE was not observed with 3H5 (Fig. 1B). The concentration of 4G2 at PENT ranged from 6.25-0.4 ug/ml. Similar results were observed using U937 cells (data not shown) (16). Thus, we routinely used 4G2 to screen for ADE, conserving precious immune sera and accruing experience with a prediction model. Next, we looked for ADE in a variety of cell lines (U937, 3T3 and K562) with or without DC-SIGN transfection to test the role of DC-SIGN. The U937 and K562 wild type (WT) cell lines were previously shown to undergo ADE (16, 24, 30, 33). All cells were phenotypically characterized prior to virus exposure using flow cytometry (Fig. 1C, insets). In the absence of enhancing antibodies, the flow cytometric assay showed very low baseline levels of infection in U937-WT (1%) (dotted line, Fig. 1C upper left panel). With sub-neutralizing concentrations of immune serum, U937-WT cells did undergo ADE (solid line, peak infection 8.6% as shown in Fig. 1C, upper left panel). Interestingly, we did not detect dengue infection under any experimental
conditions in 3T3-WT cells, a cell line without Fc receptors (Fig.1C, upper right panel). Under conditions of high DC-SIGN expression, high baseline infection rates were observed in both U937-DS and 3T3-DS cell lines, 38% and 47% 2H2 expression, respectively (Fig. 1C, dotted line lower panels). However, ADE was not observed in cells with high levels of DC-SIGN at any immune sera dilution suggesting that high levels of this molecule obscured the ADE phenomenon.

DC-SIGN levels influence ADE

We next asked whether ADE occurred in the presence of lower levels of DC-SIGN in the Fc-bearing K562 cells. We compared the infection rates in K562 cells expressing high (Hi), low (Lo) and no (WT) DC-SIGN. K562-Hi cells were 98% positive and showed a Mean Fluorescence Intensity (MFI) =331 and K562-Lo were 36% positive with an MFI=88 for surface DC-SIGN expression (Fig.2A). As expected, the K562-WT cells undergo ADE with peak infection at 17.2% (Power = 30) at PENT 1:640 (Fig. 2B, left column). Interestingly, K562-Lo cells showed enhancement, albeit at lower power, with peak infection at 11.6% (Power = 8), and PENT shift from 1:640 to 1:160 (Fig.2B, middle column). Although K562-Hi cells showed the highest baseline infection (24.9%) without immune serum, we did not appreciate ADE at any tested sera dilution (Fig.2B, right column). There is a strong negative correlation between surface levels of DC-SIGN and enhancement power (r = -0.98, p<0.0001) as determined by Spearman correlation, see inset for non-linear fit curve (Fig. 2C).
ADE was observed in monocytes and mature DC but not in immature DC

We extended the study of ADE to more physiologically relevant Fc receptor bearing primary cells. Previous work showed that monocytes, but not autologous immature DC, demonstrated ADE (33). Here we concurrently assessed ADE in immature DC (immDC), mature DC (matDC) and monocytes from multiple autologous donors. Figure 3A shows the relative levels of DC-SIGN expression on the three cell types from a representative donor. Mature DC express approximately 50% less DC-SIGN than immDC, and as expected, monocytes do not express this molecule (Fig. 3A-B). We detected ADE in both monocytes (power ~10) and matDC (power ~2.5) (Fig.3C). Despite the apparent lower power of enhancement in mature DC, the overall enhancement effect was dramatic because the baseline infection is at least a log-fold higher in mature DC compared to K562 cells or monocytes (Fig. 3C, left and middle panels). No enhancement was observed in immDC under any conditions tested (Fig.3C, right panel). Next, we extended the study of ADE using additional samples of DV1 immune sera (n=5) complexed with DV2 virus in immature and mature DC prepared from 5 different blood donors. A 5x5x2 matrix (sera x donors x cell types) was planned although only 46/50 data points (92%) were collected due to limited cell availability from one donor. Mature DC from all donors demonstrated ADE with different degrees of enhancement (power range = 2-10; mean = 4.4), see Table 1. Peak enhancement occurred at serum dilution ranges from 1:640 - 1:2560 (Table 1). Representative data from a single donor is shown in figure 3C. Although neutralization was seen at dilution 1:10 and a higher percent infection (dotted line) was shown in immDC in the absence of immune sera, ADE was not observed at any titer of immune sera (Fig. 3C, right panel).
ADE in mature DC increases viral output and pro-inflammatory cytokines

Results from our ADE assay demonstrated enhanced infection of DV2 under the influence of DV1 immune serum in matDC. We next looked for changes in viral production using absolute quantitative real time PCR to measure the viral RNA copy number in the culture supernatants from the ADE assays. We observed a 100-fold increase in viral RNA production comparing baseline infection (no immune serum) with infection at PENT ($2.1 \times 10^6$ vs. $5.6 \times 10^8$), see Fig. 4A. This result indicated that viral production was also enhanced by dengue immune serum. Parallel studies were performed to further investigate if the released virions in these supernatants retained infectivity. DC-SIGN transfected Raji cells (Raji-DS) are highly susceptible to dengue infection and were used previously in a standardized dengue neutralization assay (35). To assess infectivity, Raji-DS cells were exposed to the supernatants collected from each condition in the ADE assays in mature DC (Fig. 4B). The pattern of Raji-DS infection after exposure to the supernatants correlates closely with the enhancement effects observed in mature DC. A similar infectivity pattern was observed by standard vero cell plaque assay (Fig.4C). We next asked whether ADE in mature DC was accompanied by cytokine release as cytokine storms are implicated in the pathogenesis of DHF/DSS (40). We measured the cytokine levels in culture supernatants from mature DC exposed to sera alone, DV alone, and to different concentrations of dengue immune serum complexes. Increased levels of TNF-$\alpha$ and IL-6 coincided with PENT (Fig. 4D). IL-10 and IFN-$\gamma$ were undetectable under any conditions tested (data not shown).
FcγRIIa mediates ADE in matDC

FcγRs reportedly mediate ADE in monocytes/macrophages and cell lines (43). FcγRs are expressed constitutively on monocyte-derived DC, and FcγRII expression predominates over FcγRI or FcγRIII in both immDC and matDC. (2, 4, 33). Interestingly, DC express both FcγRIIa, with an intracytoplasmic activatory internalization motif (ITAM), and FcγRIIb, with an inhibitory motif (ITIM). Figure 5A shows the percent expression of FcγRIIa and IIb in paired DC donors. FcγRIIa levels were maintained throughout DC maturation, but FcγRIIb was significantly down regulated, p<0.001 (Fig. 5A, histogram shows representative donor), consistent with previous reports (4, 28). Because K562 cells only express FcγRIIa, we suspected, and confirmed with blocking studies, that it was the sole mediator of ADE in K562 cells (data not shown). We next studied the roles of FcγRIIa and FcγRIIb on ADE in matDC. We pre-treated matDC with either monoclonal antibodies (mAb) against FcγRIIa or FcγRIIb, before subjecting them to the ADE assay. Mature DC treated with the FcγRIIb specific mAb or the IgG1, IgG2a and IgG2b control antibody exhibit a similar ADE pattern (Fig.5B). However, the ADE effect was abrogated when matDC were pre-treated with the FcγRIIa specific mAb. Finally, we tested the supernatants collected from these blocking studies for cytokine production (Fig. 5C). As expected, TNF-α and IL-6 were detected in the control Ab and the FcγRIIb studies at PENT, but no cytokines were detected if ADE was inhibited with FcγRIIa blocking mAb.
Cytokine production required active virus

We questioned whether the cytokine production detected at PENT required live, replicating virus or was simply a result of FcR cross-linking by dengue immune complexes. DV2 was inactivated with UV irradiation before use in the ADE assay. Figure 6A shows loss of infectivity (and no ADE) when using UV irradiated DV2, while the active virus shows enhancement in matDC. The supernatants from matDC infected with active virus contained TNF-α and IL-6 but not IL-10 or IFN-γ at enhancement titer, while UV irradiated DV2 did not elicit production of these cytokines (Fig. 6B). These results suggest that viral replication post antibody-enhanced entry, not solely FcR cross-linking, is required for enhanced cytokine production. In the absence of dengue immune sera, DC-SIGN facilitates viral entry into DC (Fig 6C, left panel). TNF-α and IL-6 levels were detectable, but there was no consistent increase in cytokines with higher % infection under these conditions (Fig. 6C, right panel). Next, we conducted a head-to-head comparison of the infection rates, cytokine production and infectivity of progeny virions from supernatants of matDCs undergoing ADE or a matched direct infection (no sera) using a higher MOI. Similar infectivity rates were observed when matDCs were infected with high inoculums (MOI=5) or MOI=1 + 1/640 immune sera at PENT titer (Fig.7A). Mean fluorescence intensity of intracellular 2H2 levels, de novo dengue antigen production in high MOI (5) v MOI (1) + 1/640 immune sera (209.1 ± 21.7 v 196.3 ± 16.1, respectively), were comparable as well (data not shown). However, the same pattern of elevated IL-6 and TNF-α were observed under in ADE conditions (Fig.7B) indicating that the cytokine signaling component required both live DV and dengue immune sera at sub-neutralizing concentrations. Finally, in a Vero-cell plaque assay comparison of
supernatants, we found that virions released from matDC under ADE conditions (MOI=1+ 1/640 sera) or inoculated at MOI=5 showed similar levels of infection (plaques) in Vero cells (Fig.7C), 5.9x10^7 v 8.0x10^7 pfu/ml. However, the infections with matched MOI (1) with or without dengue immune sera showed a substantial 2.3 log increase in ADE viral output, 2.6x10^5 v 5.9x10^7 pfu/ml. These data indicate that while the % of DC infected increased by 3-fold (Fig 7A, 9 to 25% 2H2+) under ADE conditions, there was a much greater output of virus in a per cell basis, (1.5-2.0 log-fold increase in plaque formation and viral copy number).
Discussion

This is the first report of antibody dependent enhancement in human primary dendritic cells (DC). Prior studies emphasized the high susceptibility of DC to DV infection, largely owing to their very high expression levels of DC-SIGN (29, 37, 45). Here we show an inverse correlation between DC-SIGN expression levels and ADE effects when tested in an array of cell types, both cell lines and primary cells including monocytes, immature and mature DC. ADE was not observed in the presence of high DC-SIGN levels, as in K562-Hi and U937-DS cells (Figs. 1, 2) or immature DC (Fig. 3). The negative effect of DC-SIGN on ADE was best demonstrated using K562 cells expressing various levels of DC-SIGN, as shown in Figure 2B. K562-WT naturally undergo ADE (33) with maximal ADE infection observed here of about 17%. Low levels of DC-SIGN (K562-Lo) reduced peak infection by about half. High levels of DC-SIGN (K562-Hi) effectively obscured ADE (maximal infection of 20%). These results suggest the following: 1) that the virus preferentially uses DC-SIGN when sufficient levels are present and 2) that Fc receptor mediated entry is not operational under conditions of high levels of DC-SIGN. This effect of high DC-SIGN levels obscuring ADE was recently reported in another flavivirus infection model (14, 41).

The present data reveal in vitro ADE susceptibility in an important target cell of dengue virus infection, primary mature DC. The significance of this observation lies in the fact that DC are increasingly recognized as early targets in infection (25, 37, 51). The DC shows uniquely high susceptibility to DV infection in the absence of sera (51) generally 20-50% “baseline infection” rates (Fig. 3C). Demonstration of enhancement in mature DC suggests an even greater role for viral infection within this cell compartment.
While we did not appreciate ADE in immature DC here, consistent with prior work (33), we know these cells express high levels of DC-SIGN. Thus, the effect of DC-SIGN observed in cell lines was confirmed in relevant primary human cells.

Increased infections under the influence of immune sera were consistently observed in mature DC, with some donor-to-donor variability in power (2-10 fold increases in infection, Fig. 3C, Table 1). These variations could be influenced by genetic polymorphisms between donors. For example, polymorphisms of CD209 (DC-SIGN) were shown to be associated with severity of dengue disease (44). While the 2-10 fold increases in usual ADE effects may seem small, the baseline infection rates of DC must be taken into consideration. It is helpful to consider the area under the curve and the large increase in cellular infection in the presence of sub-neutralizing concentration of antibody. The disease status of serum donors also may have contributed to the different levels of enhancement. Further work using different sources of immune sera and different virus serotypes will directly address this question.

We showed associated ADE effects including a more productive infection of mature DC, as judged by the accumulation of disproportionately higher copy numbers of viral RNA (2-log increase for 3 fold power) and the release of infectious virus into cell supernatants (Fig. 4). The ability of dengue immune sera to modulate infection rates in mature DC expands the potential role of DC in dengue pathogenesis. Immature DC, bearing high levels of DC-SIGN, can act as early direct targets for DVs independent of antibody effects. Under the influence of dengue immune serum, modeling secondary infection, mature DC could become a viral factory, especially in the presence of waning or fluctuating concentrations of heterologous antibody.
In addition to high levels of viremia, cytokine cascades are thought to contribute to severe dengue disease (8, 13, 42). Induction of cytokine signaling was recently reported in post entry events of dengue infection under ADE conditions in monocytic cell lines (7, 33). Interestingly in our study, TNF-α and IL-6 were detected only at peak enhancement titers in mature DC (Figs. 4D, 5C, 6B and 7B). Despite similar levels of infection in the absence of immune sera (baseline infection) using higher MOI, minimal levels of cytokines were detected, suggesting additional signaling in post entry ADE in primary DC. This trend of cytokine production correlates closely with other ADE effects measured by viral antigen production, accumulation of viral RNA in supernatants, the transmissibility of infection to Raji-DCSIGN cells via supernatants and in classic Vero cell based plaque assays. Of note, mature DC typically produce much lower levels or no cytokines when compared to immature DC infected with DV (21). The ability of dengue immune sera to increase infection rates, viral and pro-inflammatory cytokine production in mature DC suggests additional viral entry mechanisms (e.g. FcR mediated) with different signaling components and downstream functional effects.

Fc receptors are often implicated in ADE infection (5, 15, 30, 31). DC constitutively express these receptors, predominantly FcγRII (4, 27, 28, 33). Our study examined the role of two different forms of FcγRII expressed on DC in ADE: the activating (FcγRIIa) and the inhibitory (FcγRIIb) isoforms (Fig5A, B). We and others (4, 28, 41) reported that the ratio of FcγRIIa/FcγRIIb is increased in matDC and propose these changes regulate DC function and control cellular responses (9, 10, 38). Blocking experiments (Fig. 5B) using specific anti-FcγRIIa or FcγRIIb mAbs illustrate the critical role of FcγRIIa on ADE in matDC. We observed a similar role for FcγRIIa in ADE of
K562 cells, as these cells only express FcγRIIa (data not shown). This raises the possibility that in addition to DC-SIGN down regulation, increasing the FcγRIIa/FcγRIIb ratio facilitates ADE in dengue infection in matDC. DC maturation may have independent effects not studied here that play a role in facilitating ADE. Additionally, FcγRIIa blocking also clearly inhibited TNF-α and IL-6 production at enhancement titers (Fig. 5C), suggesting that FcγRIIa mediated entry of DV immune complexes leads to signaling for cytokine production. Cytokine storms are linked to disease severity in severe dengue infections, e.g. high serum levels of TNF-α in patients suffering from DHF/DSS (52). Identifying a new cellular compartment susceptible to ADE with capacity for increased viral replication and pro-inflammatory cytokine release at peak enhancement titers links key pathogenesis concepts.

The cytokine signaling cascade could be triggered simply as a result of cross-linking of FcγRs. However, we identified a requirement for active virus to elicit cytokine production that is biologically plausible and supported in the literature (26, 31, 32). UV irradiated DV did not cause ADE nor induce cytokine production (Fig.6). Furthermore, comparable infection levels of matDC unaided by antibody using higher input virus, a DC-SIGN mediated process, did not elicit such cytokine responses (Fig. 6C). Therefore, we propose that the ADE phenomenon described here as measured by increased de novo DV antigen production and cytokine release requires viral replication post antibody facilitated entry, not solely FcγR cross linking. Importantly, these results show the versatility of DV to exploit multiple routes to gain access to the same cellular target.

Limiting access to cellular targets during DV infection is a reasonable goal for a dengue vaccine. Therefore, identifying conditions favoring ADE would contribute to the
field of vaccine development. Data presented here substantiate a major role for FcRγIIa in ADE in mature DC. Though not formally studied here, our results raise the possibility that different isotypes and relative affinities of these isotypes for FcγRII could be further investigated for differential enhancement potential (e.g. 4G2-positive control is an IgG2a isotype versus 3H5 and control sera, both negative controls are IgG1 isotypes). Our results with the dengue 2 specific 3H5 ascites (no ADE) differ from an early report (27) showing 3H5 enhancement of Dengue 2 infection in K562 cells (see Figure 1B). There may be differences in the actual cell line used in this early study given the very high baseline infection in their K562 cells (10-20%) compared to the typical baseline infection rates reported in K562s, <0.5% (16,33). In addition, we used a different viral strain of dengue 2 virus in our study and the type of virus may play an important role. Well-controlled blocking studies shown in Figure 5B indicate that dengue specificity is required for antibody mediated enhancement.

We propose that the K562 DC-SIGN model can be readily used to study serum, antibody or other immunologic effects (e.g. complement) for dengue vaccine advancement. We now routinely monitor ADE using an automated plate-based assay run in replicates with excellent reproducibility. We are currently evaluating ADE in primary DC using all 4 DV serotypes with additional sources of immune sera, as this pilot study focused only on the effects of anti-DV1 immune sera against DV2 infection. While this infection sequence (DV1 followed by DV2) is a recognized risk for predisposition to DHF (18, 20, 48), much more information will be gained by studying different virus sequences, serotypes and serum sources to understand and mitigate risks for immune enhancement of disease.
Figure legends

**Fig.1 Antibody dependent enhancement of dengue infection.** (A). Schematic diagram of the flow cytometry based ADE assay. Control is the percent DV infection in the absence of dengue immune serum (baseline infection). Peak enhancement titer (PENT) is the dilution at which maximum percent infection occurs for the tested serum. Power is the ratio of the percent infection at PENT divided by the percent infection at control. (B) Comparison of ADE effects of DV2 in K562 cells using anti-DV1 dengue immune serum (open circles) and two commercially available anti-dengue antibodies, 4G2 (filled circles) and 3H5 (open triangles) and normal human IgG (filled squares). Three independent experiments were performed in triplicate, and data shown are the means ± SD for all three experiments. (C) Infection of U937-WT / 3T3-WT (upper panels) and U937-DS /3T3-DS (lower panels) by DV2 16803 (MOI=1). The solid line represents the infectivity with dengue immune serum and the dashed line represents infectivity without dengue immune serum in one representative of three independent experiments. Surface expression of DC-SIGN for each cell line as measured by flow cytometry is shown as insets in the upper panels.
Fig. 2 Antibody dependent enhancement in cell lines as a function of DC-SIGN expression. (A) DC-SIGN surface expression on K562-WT (shaded histogram), K562-Lo (dashed line) and K562-Hi (solid line) cells using flow cytometry. (B) ADE patterns obtained from infection of K562-WT (left column), K562-Lo (middle column) and K562-Hi (right column) with or without serial dilutions of dengue immune sera. (C) Percent surface expression of DC-SIGN (bars) versus power of enhancement (solid line) for K562-WT, K562-Lo and K562-Hi cells. The insert graphs the power versus the DC-SIGN MFI of each of the 3 cells types in 3 independent experiments and the correlation ($r^2 = 0.96$) was determined using a non-linear curve fitting algorithm. Data shown are the means ± SD of three independent experiments for each cell line.

Fig. 3 Analysis of ADE pattern in monocytes, immature and mature dendritic cells. (A) DC-SIGN surface expression on immature (solid line) and mature DC (dashed line) and monocytes (shaded histogram) from a single representative donor. (B) Mean fluorescence intensity (MFI) of surface DC-SIGN expression from paired immature and mature DC (n=5). (C) Infection and ADE patterns obtained in monocytes, immature and mature DC prepared from a single representative donor in the absence (dashed line) and presence (solid line) of dengue immune serum. All 3 cell types (monocyte, immDC and matDC) were tested in 3 independent experiments with 3 different donors.
Fig. 4 Increased viral production and pro-inflammatory cytokines with ADE in mature DC. (A) Detection of viral output in supernatant using quantitative real time PCR (filled circles) compared with intracellular viral antigen detection using 2H2 monoclonal antibody (open circles) in mature DC undergoing ADE. (B) Culture supernatants collected from mature DC undergoing ADE (from Fig. 4A (open circles)) were tested for productive infection by culturing with Raji-DC-SIGN cells (filled circles). (C) Supernatants from mature DC in 4A were tested in parallel in Vero cell plaque assays to confirm Raji-DC-SIGN infectivity data. (D) Enhanced pro-inflammatory cytokines (TNF-α and IL-6) were detected in culture supernatants from mature DC undergoing ADE only at enhancement titers. The dashed line indicates the lower limit of detection for the assay (20 pg/ml). A representative of four independent experiments performed in triplicate is shown. All data points shown are means ± SD. Similar cytokine production patterns were obtained in 5 donors tested across 5 different ADE sera (Table 1).

Fig. 5 Influence of FcγRIIa on ADE in mature DC. (A) Scatter plots show the percentages of FcγRIIa positive cells (circles) and FcγRIIb positive cells (triangles) in immDC (closed circles and closed triangles) and matDC (open circles and open triangles) from 8 paired donors. The black bars represent means. FcγRIIb expression decreases significantly between immDC and matDC (paired t test, p<0.001). The histogram shows a single representative donors’ changes in expression levels of FcγRIIa and FcγRIIb with maturation: immDC (dashed line) and matDC (solid line). (B) Infection and ADE pattern in matDC treated with control IgG1 (open circle), control IgG2a (closed diamond), with control IgG2b (opened diamond), specific anti-FcγRIIa mAb (closed square) and
anti-FcγRIIb mAb (open square). The dashed line represents the baseline infection rate without dengue immune serum. (C) TNF-α (left panel) and IL-6 (right panel) in matDC (white bar) undergoing ADE, under the influence of control IgG1 (black bar), IgG2a (white bar), IgG2b (gray bar), specific anti-FcγRIIa mAb (striped bar) and specific anti-FcγRIIb mAb (hatched bar). The dashed line indicates the lower limit of detection for the assay (20 pg/ml). One representative of three independent experiments using 3 donors with 2 different dengue immune sera is shown. The experiments were performed in triplicate and the bars represent means ± SD.

**Fig.6 ADE requires active virus.** (A) Percent infection in ADE assay in matDC using active DV2 (open circles) versus UV irradiated DV2 (closed circles). (B) Cytokine production in ADE assay supernatants with active DV2 (open bars) versus UV irradiated DV2 (black bars); TNF-α (left panel) and IL-6 (right panel). (C) DV infection without dengue immune serum (left panel) in matDC (white bar) and matDC treated with 10μg/ml anti-DCSIGN mAb (black bar). TNF-α and IL-6 production from mDC and anti-DCSIGN treated matDC (right panel). Experiments were performed in triplicate, expressed as means ± SEM from three different donors. The dashed line indicates the lower limit of detection for the assay (20 pg/ml).
Fig. 7 Comparison of infection infection pattern under the presence and absence of dengue immune serum. (A) %DV infection under the influence of dengue immune serum at PENT and without immune sera (MOI=1, 5). (B) TNF-α (white bar) and IL-6 (gray bar) production from matDCs under ADE conditions and without dengue immune serum. (C) Vero cell plaque assay from supernatants of matDCs undergoing ADE (MOI=1, 1/640 sera) or inoculated with DV2 at MOI=1 or 5. Experiments were performed in quadruplicate, expressed as means ± SEM from three different donors.

Acknowledgements

This work was supported by the Pediatric Dengue Vaccine Initiative (PDVI) and in part by the cooperative agreement DAMD17-98-2-8007, between the U.S. Army Medical Research and Materiel Command, the Henry M. Jackson Foundation for the Advancement of Military Medicine, and the Military Infectious Disease Research Program. The views and opinions expressed herein are those of the authors and do not purport to reflect the official policy or position of the Department of Defense. KB and PP were financial supported from Thailand Research Fund through Thai Royal Golden Jubilee Ph.D Program. We appreciate the critical review of the manuscript and provision of helpful comments by Dr. Mark DeSouza, AFRIMS, Bangkok, Thailand.
References


Neutralization and Enhancement of West Nile Virus Infection. Cell Host and Microbe \textbf{1}:135-145.


Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Table 1. Enhancement of DV2 infection by anti-DV1 immune serum in multiple donors.

<table>
<thead>
<tr>
<th>Serum ID</th>
<th>BC266</th>
<th>BC284</th>
<th>BC287</th>
<th>BC291</th>
<th>BC295</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICDC</td>
<td>mDC</td>
<td>ICDC</td>
<td>mDC</td>
<td>ICDC</td>
</tr>
<tr>
<td>94501293</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>94501396</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>94501310</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>94501326</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*ADE response indicated as ++ (power -5), + (power 2-3), + (power -2) or – (no ADE observed).

*Represents the mean baseline infection (no sera) ± SD were calculated from at least 3 independent infections from each donor.

*Represents the baseline infections (no sera) from 2 independent infections.

We planned a 5x5x2 matrix to test 5 donors, with 5 sera across a dilution range (1:10-1:163840) comparing two different cell types (ICDC and mDC) for a total of 50 data points. Due to limited cell availability, only 46/50 data points are shown (NT, not tested). Experiments were repeated if cells and sera permitted (Sera 94501281-repeated all 5 donors, sera 94501333-repeated 4 donors).