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CD40 Ligand Enhances Dengue Viral Infection of Dendritic Cells: A Possible Mechanism for T Cell-Mediated Immunopathology

Peifang Sun, *† Christina M. Celluzzi, † Mary Marovich, †† Hemavathy Subramanian, *† Michael Eller, †* Susana Widjaja, † Dupeh Palmer, † Kevin Porter, †† Wellington Sun, † and Timothy Burgess, ††*†

We have previously shown that dengue virus (DV) productively infects immature human dendritic cells (DCs) through binding to cell surface DC-specific ICAM-3-grabbing nonintegrin molecules. Infected DCs are apoptotic, refractory to TNF-α stimulation, inhibited from undergoing maturation, and unable to stimulate T cells. In this study, we show that maturation of infected DCs could be restored by a strong stimulus, CD40L. Addition of CD40L significantly reduced apoptosis of DCs, promoted IL-12 production, and greatly elevated the IFN-γ response of T cells, but yet did not restore T cell proliferation in MLR. Increased viral infection of DCs was also observed; however, increased infection did not appear to be mediated by DC-specific ICAM-3-grabbing nonintegrin, but rather was regulated by decreased production of IFN-α and decreased apoptotic death of infected DCs. Because CD40L is highly expressed on activated memory (but not naive) T cells, the observation that CD40L signaling results in enhanced DV infection of DC suggests a possible T cell-dependent mechanism for the immune-mediated enhancement of disease severity associated with some secondary dengue infections. The Journal of Immunology, 2006, 177: 6497–6503.

Dengue viruses (DV) are single positive-stranded RNA flaviviruses consisting of four serologically distinct serotypes, DV-1, -2, -3, and -4. DV infections are self-limited and viremia usually resolves within a period of a few days (1). Infection results in clinical manifestations that range from mild dengue fever (DF) to a variety of symptoms of severe pathology characterized by capillary leakage known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Cases of DHF/DSS are commonly associated with heterologous secondary infection, and are thought to be responsible for 200,000–500,000 deaths each year (2).

Several studies demonstrated that dendritic cells (DCs) are more permissive to DV infection compared with monocytes and macrophages (3, 4). DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), a cell surface C-type lectin expressed on DCs, plays a critical role in DV binding and infection (5, 6). Inflammatory cytokines that induce DC maturation, such as TNF-α and IFN-α (7), can down-regulate DC-SIGN expression and thus render DCs less permissive to infection. Our previous study (8) showed that although DV exposure induces TNF-α production and maturation of bystander (uninfected) cells, it renders the infected DCs refractory to TNF-α stimulation, thereby blocking their maturation. Such effects on DC maturation are commonly observed among a number of DC-targeting viruses including HSV, Ebola and Lassa viruses, CMV, and HIV (9–14). Maturation is essential for DC to become potent APCs. Therefore, interference with this process may be detrimental to immune responses and lead to viral immune invasion. We found that DV-exposed DCs diminish T cell proliferation in a MLR. Clinical studies have shown that DV infection causes APC dysfunction, which may account for the loss of mitogenic T cell proliferation and reduced blood lymphocyte counts observed in acutely infected patients (15, 16).

CD40/CD40L, members of the TNF and TNFR superfamily, play a critical role in modulating immune responses. CD40L is expressed mainly on activated T cells, while its receptor CD40 is expressed on a variety of accessory cells, including B cells, DCs, monocytes, macrophages, mast cells, fibroblasts, follicular DCs, and endothelial cells. In vivo, CD40/CD40L plays an important role in T cell priming, memory formation, and induction of a Th1 immune response (17). Using blocking Abs and murine gene knockout models, CD40/CD40L interaction was shown to be required for protective immunity in several parasitic, bacterial, and viral disease systems (17). In vitro ligation of CD40 on the surface of DCs by soluble CD40L or CD40L-transfected L cells (CD40L-1 cells) stimulates DCs to increase expression of co-stimulatory and MHC molecules, produce IL-12 and -15, and prevent apoptosis (18–20). Because of these properties, CD40L has been used to generate mature DCs as APCs to expand CD8+ CTL specific for viral and bacterial Ags (18, 21–23).

CD40L-induced signaling can be interrupted by viruses. Measles virus (MV) suppresses CD40L-induced DC maturation and production of proinflammatory cytokines, IL-12, and IL-1αβ (9, 24). MV replication is significantly higher in DCs treated with

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CD40L compared with untreated ones, resulting in increased DC apoptosis. Impaired T cell responses and T cell death in DC-T cell cocultures correlate with enhanced DC infection. HIV also shows more rigorous replication in DCs in the presence of CD40L signaling (25). Extensive deletion of T cells during infection is proposed to be a potential mechanism for systemic immunosuppression caused by MV and HIV infection. Therefore, CD40L, as it enhances viral replication and causes more T cell apoptosis, may play a role in viral immunopathogenesis.

In dengue, DHF and DSS are almost exclusively found in secondary infection (26, 27), when memory/effector T cells, elicited by primary infection, are already present (28). T cell responses, particularly inflammatory cytokine responses, have been suggested by Ennis and colleagues (27) to be “double-edged,” by reducing viral replication in most circumstances but also mediating disease severity in others. In this study, we examined the effect of CD40L on DV infection of DC, maturation of DC, and activation of T cells. We found that CD40L enhanced DV infection of DC. CD40L restored DC maturation and IL-12 production. We also found that CD40L significantly elevated IFN-γ response in T cells cocultured with DV-exposed DC; however, T cell proliferation did not correlate with IFN-γ response. Together these results suggest a potential mechanism of T cell-mediated immunopathogenesis through CD40-CD40L costimulation.

Materials and Methods

Virus preparation

DV-2 (strain 16803, originally isolated by S. Halstead et al., and maintained at the Walter Reed Army Institute of Research) was grown and propagated in mycoplasma-free vero cell lines. The viral titer was determined by limiting dilution plaque assays on vero cells. The presence of contaminating LPS in the virus stock and culture supernatants was evaluated by the Limulus Amebocyte Lysate test (BioWhittaker). Mycoplasma contamination of the virus stocks and culture supernatants was evaluated by the Mycoplasma Rapid Detection System (Gen-Probe). All virus stocks and culture supernatants used in the present study were free from LPS and mycoplasma.

Generation of DCs

PBMCs were isolated from whole blood units obtained from normal, healthy, seronegative consenting deidentified donors at the American Red Cross. All cell cultures were conducted in complete medium consisting of RPMI 1640, 1% t-glutamine, 1% penicillin/streptomycin, 1% essential amino acids, and 10% heat-inactivated FBS (all from Invitrogen Life Technologies). DCs were generated according to a protocol described elsewhere (4). Briefly, monocytes were obtained by adhering PBMCs in Primaria tissue culture dishes (100 × 20 mm, Falcon; BD Labware) at 5 × 10^6 cells/dish for 2 h, followed by vigorous washing to remove nonadherent cells. The nonadherent cells were cryopreserved for T cell isolation, whereas the adherent cells were further cultured in the presence of recombinant cytokines. 100 ng/ml recombinant human (rh) GM-CSF (Leukine: Immunex) and 50 ng/ml rhIL-4 (R&D Systems). Cultures were maintained in a 37°C, 5% CO₂ humidified incubator. On day 3, half of the medium was replaced with fresh medium supplemented with cytokines. At day 5, cells were harvested by gentle pipetting and placed into fresh medium and cytokines, and seeded into 6-well plates (Corning). At day 7, cells were identified to be typical immature DC as indicated by the presence of characteristic cell surface markers (CD11c<sup>low</sup>, CD11b<sup>hi</sup>, CD14<sup>pos</sup>, CD83 negative, CD80, and CD86<sup>int</sup>). DCs generated using this procedure had ≥95% purity.

Infection of DCs with DV

Immature DCs were pulsed with DV-2 at various multiplicities of infection (MOIs) for 2 h at 37°C in a 5% CO₂ atmosphere. Cells were washed twice to remove cell-free virus and cultured further in complete medium at a density of 0.5 × 10^5 cells/ml in 12-well plates without cytokine. In some experiments, irradiated L cells transfected to express CD40L (CD40L<sup>L</sup>-L cells) were added to DCs at ratios of 1:5 or 1:10 of DC:CD40L<sup>L</sup>-L. DCs and culture supernatants were harvested at indicated culture periods (24 or 48 h) for assay analyses.

CD40L<sup>L</sup>-L cells and CD40L blocking Ab

The CD40L-transfected L cell line (CD40L<sup>L</sup>-L) cell was provided by Dr. A. Granelli-Piperno (Rockefeller University, New York, NY) and anti-CD40L mAb (M90) was provided by Amgen. CD40L<sup>L</sup>-L were gently treated with 2 mM EDTA and irradiated at 10,000 rad using a cobalt irradiator. For blocking CD40L-CD40L interaction, mAb M90 was incubated with irradiated CD40L L cells for 1 h at 4°C before coculture with DCs. Mouse IgG1 mAb (clone MOPC) (BD Pharmingen) was included as an irrelevant isotype control Ab.

DC surface marker phenotyping

For measurement of cell surface expression of costimulatory and HLA molecules, DCs were stained with PE-conjugated mAbs specific for: CD40 and CD83 (Coulter Immunotech), CD80 (clone L307.4), CD86 (clone IT2.2), HLA-A,B,C (clone G46-2.6), and HLA-DR (clone G46-6) (BD Pharmingen). Isotype-matched PE-labeled controls, mouse IgG1 (clone MOPC-21C), and mouse IgG2b (clone 27-35) (BD Pharmingen) were included in each experiment.

Detection of virus intracellularly and in cell supernatants

Surface marker-labeled DCs were fixed and permeabilized with Cytofix and CytoPerm (BD Pharmingen) with CellQuest software. During sample acquisition, infected cells were analyzed separately from uninfected ones by drawing distinctive gates on 2H2<sup>pos</sup> and 2H2<sup>neg</sup> subsets. Virus titers produced by infected cells in culture supernatants were determined by plaque assay on Vero cells.

Analyses of cell apoptosis

The viability of DV-exposed DCs in the presence or absence of CD40L was compared at 24 and 48 h. Cells were stained with propidium iodide (PI) and FITC-conjugated annexin V using the Annexin v<sup>FITC</sup> Apoptosis Detection kit 1 (BD Pharmingen). Nuclear matrix protein release was measured by an ELISA kit according to the manufacturer’s instruction (BD Pharmingen).

Cytokine production in culture supernatants

IFN-α was measured by ELISA according to the manufacturer’s recommendations (PBL Biomedical Laboratories). Samples were read on a SpectraMax 340 plate reader (Molecular Devices). All other cytokines were quantified by cytometric bead array (CBA) with inflammatory or Th1/Th2 CBA kits on a FACSscan using CBA software (BD Pharmingen).

T lymphocyte stimulation

DCs were exposed for 24 h to live DV-2 at indicated MOI or with 10 μg/ml Staphylococcus aureus Cowan strain (Calbiochem). T cells were negatively selected from PBMCs by magnetic microbeads using a Pan-T cell isolation kit (Miltenyi Biotec), and cocultured with differently treated DCs (DCs unexposed or exposed to DV in the presence or absence of CD40L) in triplicate, in complete RPMI 1640 medium containing 5% of low endotoxin FBS (HyClone) for 5 days at DC:T ratios of 1:3. Proliferation was measured by adding 0.5 μCi [³H]thymidine/well for the last 18 h of culture. Radioisotope incorporation was assessed using a 1450 Microbeta Liquid Scintillation and Luminescence Counter (PerkinElmer Life Sciences). Supernatants of T cell and DC cocultures were collected at different time points and IFN-γ was measured.

Statistical analysis

The paired t test was used to determine the significance of differences between experimental and control groups. A p value <0.05 was considered significant.

Results

DV infection in the presence of CD40L

To determine whether CD40L affected infection, DCs were exposed to DV at different MOIs in the presence or absence of CD40L<sup>L</sup>-L cells at ratios of 1:5 and 1:50 DC:L cells. FITC-2H2 staining revealed an elevated percentage (>2-fold) of DV-infected...
FIGURE 1. CD40L, enhancement of DV infection: immature DCs were pulsed with DV at MOI of 0.3 and 1 for 2 h. Cells pulsed with noninfected Vero cell supernatants were used as mock controls. DCs were washed to remove cell-free viruses and cultured in the presence or absence of CD40L-L cells. Two concentrations of CD40L-L cells were used: 1 L cell/50 DC (low) and 1 L cell/5 DC (high). At 48 h, DCs were collected to measure infection with 2H2-FITC by flow cytometry. Culture supernatants were harvested to measure infection with 2H2-FITC by flow cytometry. Data are presented as mean ± SEM (n = 3). The unpaired Student t test was performed to determine the significance of the difference between presence/absence of CD40L treatment: A, Percentage of 2H2+ cells (p = 0.029 and 0.016 at MOI of 0.3 and 1, respectively); B, MFI of 2H2+ cells (p = 0.0001 and 0.0019 at MOI of 0.3 and 1, respectively); C, PPV of culture supernatants (p = 0.035 and 0.043 at MOI of 0.3 and 1, respectively); D, Percentage of 2H2+ cells following addition of blocking Ab to CD40L.

cells at 48 h in CD40L-L cell-treated DCs compared with untreated cells (Fig. 1A). CD40L-treated cultures expressed significantly higher 2H2+ mean fluorescence intensity (MFI) compared with untreated cells (Fig. 1B), indicating the amount of intracellular premembrane expression among the infected cells was elevated by CD40L treatment. Infectious viruses secreted in the culture supernatants were quantified using a Vero cell plaque assay. An increase of >1 log PFUs in the supernatant of CD40L-treated cells was observed (Fig. 1C). These results indicate that CD40L significantly increases DV infection of DCs. Enhancement was dose dependent: the more CD40L provided, the greater the observed infection. Enhancement of infection could be blocked by addition of M90, a mAb specific to CD40L (Fig. 1D), but not by the isotype control Ab (data not shown).

Effect of CD40L on DC-SIGN expression

DC-SIGN is thought to mediate, in part, the infection of immature DC by DV (5, 6). We previously showed that levels of DC-SIGN expression positively correlated with infection of immature DCs (5). In this study, we examined whether there was a correlation between CD-SIGN expression and infection when cells were exposed to CD40L. In our experiments (data not shown), DV-infected and mock control DCs cocultured with or without CD40L-L cells were stained with PE-conjugated anti-DC-SIGN mAb. DCs kept in GM-CSF and IL-4 were used as the control, because DC-SIGN expression is dependent on IL-4 (7). At 24 h, DC-SIGN expression levels were lower on both mock-control and DV-exposed DCs compared with DCs cultured in IL-4 and GM-CSF; however, the difference was not significant (p > 0.05). Addition of DV and CD40L caused a further decrease in DC-SIGN levels, but the difference was not significant (p > 0.5). There appeared to be no direct correlation between DC-SIGN expression and levels of enhanced dengue infection.

Influence of CD40L on DC cytokine production

We previously reported that DV-exposed DC produce inflammatory cytokines, TNF-α and IFN-α, but fail to produce IL-12p70 (8). These cytokines can affect DC maturation and polarization of T cell immune responses. We sought to determine whether CD40L stimulation modulates the production of inflammatory cytokines by DC in the context of DV infection, thereby affecting DC infectivity and function. As CD40L treatment alone promoted cytokine production, the t test was performed to determine whether the changes in cytokine response in the presence of both CD40L and virus were significantly different from cytokine responses with CD40L alone. We show that DCs produced TNF-α in response to DV infection (Fig. 2A) as reported previously (8), and that CD40L further augmented this response. CD40L alone was able to promote TNF-α production in the absence of viral Ag, but this response was significantly higher in the presence of DV (p < 0.05). By contrast, CD40L treatment negatively affected the IFN-α response (Fig. 2B). IFN-α was not induced by CD40L alone. It was observed in DV-infected DCs at 48 h, but when CD40L was added to infected cells, the response was markedly reduced (p < 0.05).

The lack of IL-12p70 production by DCs after DV exposure that we and others previously reported was restored by CD40L stimulation. Fig. 2C shows that IL-12p70 was nearly undetectable (<6.3 pg/ml) in infected cells without CD40L stimulation, but
when CD40L was added, the levels increased to >30 pg/ml by 24 h and >60 pg/ml by 48 h (Fig. 2C). CD40L alone also induced IL-12p70; however, the levels were significantly higher in response to DV infection \( (p < 0.05) \).

**Decreased DC apoptosis in the presence of CD40L**

CD40-CD40L costimulation is known to enhance DC survival through activation of antiapoptosis molecular pathways. We showed previously that DV-infected DCs are prone to apoptosis (8). Because apoptotic elimination of infected cells can serve as a mechanism to control infection (29), we examined the level of DV-induced apoptosis in the presence and absence of CD40L stimulation to determine whether the increased infection seen with CD40L treatment was due to CD40L-mediated “rescue” of DV-infected cells. FACS analysis showed annexin V/PI staining was lower in CD40L-treated DV-infected and uninfected DCs compared with untreated DCs (Fig. 3A and the table). Nuclear matrix proteins released in the supernatant were also lower in CD40L-treated cultures compared with untreated cultures (Fig. 3B). These data indicate that CD40L signaling is able to decrease cell apoptosis in DCs exposed to DV.

**Up-regulation of costimulation markers on DCs by CD40L**

Exposure of DCs to DV induces maturation of bystander cells, but not infected cells. Unlike bystander cells, infected DCs are unable to undergo maturation in response to exogenous TNF-α treatment (8). In the current study, we show that following DV exposure, CD40L stimulation enhanced expression of costimulatory molecules on DCs. Fig. 4 shows that expression of CD80, CD83, and CD86 on the mock-control DCs as well as the DV-exposed DCs (including both the 2H2+ infected and the 2H2− bystander cells). The paired Student t tests indicated that without the CD40L stimulation, only the 2H2− cells had significant up-regulation of the three markers \((p\) values not shown), confirming the reported observation (8). Significant up-regulation of all three markers were shown following CD40L treatment on the mock-control cells and the infected cells (tabled \( p \) values showing the differences between the CD40L untreated and treated). The up-regulation of the markers was not obvious on bystander cells after CD40L treatment, because bystander cells were already showing phenotypic maturation in the absence of CD40L treatment. Levels of expression of these molecules in CD40L treated cells were similar among 2H2+, 2H2−, and mock-infected controls, indicating that infected cells proceed to maturation with a similar magnitude as the bystander cells in the presence of DV infection.

**T cell proliferation**

As reported previously, DV-exposed DCs have a reduced capacity to induce T cell proliferation in a MLR, likely due to the abrogated DC maturation induced by viral infection (8). Fig. 4 shows that...
CD40L induced Th1 type cytokine production

We showed in Fig. 3 that CD40L costimulation allows IL-12p70 production in response to DV infection. To evaluate the T cell cytokine response induced by CD40L-treated DCs, IFN-γ production in MLR was measured. Surprisingly, although there was suppression of T cell proliferation, DV-infected DCs up-regulated IFN-γ production (from 33.8 to 92.7 pg/ml at 72 h), indicating induction of a Th1 type of T cell response to DV infection (Fig. 6A). This response was greatly enhanced by CD40L treatment of DCs (Fig. 6B).

Discussion

DHF and DSS represent severe clinical manifestations of DV infection. Although the mechanism controlling development of DHF and DSS is complicated, one consistent observation is that the increased risk of DHF is associated with a secondary heterologous DV infection (27). Ab-dependent enhancement (ADE), increased viral infection caused by subneutralizing Abs, has been used to explain how pre-existing suboptimal DV-specific Abs increase DV infection. ADE has been demonstrated in vivo in infants born to DV seropositive mothers (30) and in vitro in peripheral leukocytes (26) as well as the monocytic cell line K562 (4). ADE, however, cannot be demonstrated with immature DCs (4). DCs are permissible to DV, possibly serving as primary targets for early infection. Enhancing infection of DCs via mechanisms similar to ADE could impact the course of disease in dengue infection.

This study is the first to show the enhancement of DV replication in DCs in the presence of CD40-CD40L signaling. DC-SIGN is a possible binding receptor for DV, and infectivity correlates with DC-SIGN expression levels on the cell surface (5). IL-4 is critical to the expression of DC-SIGN on immature DCs derived from monocytes (7). IFN-αβ and TNF-α can interfere with the IL-4-dependent Stat6 and JAK2/JAK3 gene transcription pathways (31), and thus can negatively regulate DC-SIGN expression (7, 32) during differentiation of monocytes. Our monocyte-derived DCs were generated in GM-CSF and IL-4 for ~6 days. At this time, DC-SIGN expression varied within individuals, from 100 to 900 MFI (data not shown). Consistent with other studies (32), we report production of IFN-α and TNF-α from DV-exposed DCs. CD40L treatment further increased TNF-α production, but decreased IFN-α response. The inflammatory cytokine milieu may down-regulate DC-SIGN expression among cells exposed to DV and CD40L. In fact, we did observe less DC-SIGN expression after DCs were exposed to DV. Not surprisingly, DC-SIGN levels were somewhat lower upon exposing DCs to CD40L (although the differences were not significant). In light of our previous work showing that infectivity of DCs with DV correlates directly with DC-SIGN expression (5), these results demonstrate that the increased infectivity observed with CD40L signaling is not related to changes in DC-SIGN expression induced by CD40L. We found that CD40L treatment decreases IFN-α secretion by DCs, and that effect correlates with increased DV infection, suggesting a potential mechanism whereby decreased IFN-α production in DV-exposed DCs receiving CD40L treatment may be responsible for reducing antiviral activity against dengue replication and survival. Diamond et al. (33) and others have shown that IFN-α inhibits DV infection and replication, while Garcia-Sarstre and colleagues (34, 35) and Jones et al. (36) have reported that DV inhibits IFN-α signaling. Interestingly, Ho et al. (37) found that DC are refractory to the viral inhibitory action of IFN-α but not IFN-γ following DV infection. We are continuing to explore the role of the IFN response in the context of DV infection, in particular with respect to different strains of DV, and a future manuscript is forthcoming. In addition to the effects of type 1 IFN, apoptosis can serve as a mechanism to eliminate dengue-infected cells, thereby controlling virus spread (29). The decreased DC apoptosis in the presence of CD40L signaling demonstrated in this study may serve as another explanation for the observed enhancement of DV infection.

Before our study, rapid (within 2 h) increase in expression of HIV p24 and gag transgenes in DCs exposed to vCP205 (a canarypox vector inserted with g24 and gag genes) via CD40-CD40L signaling was observed (Dr. M. Marovich, unpublished observations). Unlike DV, MV and HIV, Canarypox is replication incompetent (38). Others have demonstrated an increase in transgenic protein expression in DCs transfected with naked DNA by engaging CD40 on cell surface (39), and suggested that the increased levels of NF-κB, AP-1, and cAMP-responsive element, are

FIGURE 5. CD40L improves T cell stimulation and enhances IFN-γ production in DV-infected DCs. DCs pulsed with or without (mock) DV were cultured in the presence or absence of CD40L−L−L−L− cells. Cells were harvested at 24 h and washed. T cells were added to the DCs at a DC:T ratio of 1:3. Cell proliferation was measured by [3H]thymidine incorporation at day 6. Stimulation index = cpm of T cells + DC/cpm of T cells (n = 3).

FIGURE 6. DV-exposed DCs induce IFN-γ response in T cells, which is enhanced further by CD40L stimulation: DCs pulsed with or without DV were cultured in the presence (B) or absence of CD40L−L−L−L− cells (A). Cells were harvested at 24 h and washed. T cells were added to the DCs at DC:T ratio of 1:3. IFN-γ was measured from culture supernatants and data presented as mean ± SEM (n = 3).
CD40L ENHANCES DV INFECTION OF DCs

Infected DC, unlike bystanders (DV-exposed cells that remained uninfected following exposure), are incapable of undergoing maturation in response to DV (8). In this study, a significant phenotypic maturation of infected DCs following CD40L stimulation was observed. Expression of each individual maturation marker, CD80, CD83, and CD86, on mock-control, DV-infected, and bystander DCs reached nearly the same level. We previously reported that addition of TNF-α to DV-exposed DCs only increased maturation of bystander cells, but not infected cells (8). Therefore, maturation of infected cells may not due to the increased production of TNF-α elicited by CD40L. The NF-κB complex is required for DC maturation (40). Recently, O’Sullivan et al. (40) reported striking differences between TNF-α and CD40L for induction of gene transcription of NF-κB and NF-κB complex: TNF-α induces a rapid transcription quickly followed by attenuation, whereas CD40L induces a slower, higher, and sustained transcription. This suggests that CD40L, compared with TNF-α, delivers a more potent DC maturation signal under conditions tested in this study. This also may explain why CD40L and TNF-α have unequal effects on DV-infected DCs.

IL-12 is not produced by DV-exposed DCs (8). CD40L is a potent signal, which restored DC IL-12 response to DV. IL-12p70 was induced by CD40L-treated DCs and was further increased by DV exposure. TLR3, a pattern recognition molecule specific for dsDNA and a synthetic analog of dsRNA, poly(I:C), is located intracellularly in monocyte-derived immature DCs (41). Activating DCs through TLR3 gives rise to IFN-αβ and IL-12 (42). Because DV replication causes accumulation of dsRNA, it is logical that DV should induce both cytokines. However, without extra stimuli, DV infection of DCs induces only IFN-α but not IL-12 (8), suggesting the possible pathway of TLR3-mediated IL-12 production is hindered. DC-SIGN has been proposed to play a role in DC maturation (40). Currently, we are studying issues related to activation-induced cell death using this in vitro DC model.

We showed a marked enhancement of IFN-γ from T cells in response to DV-exposed DCs by CD40-CD40L signaling. Like other inflammatory cytokines, IFN-γ is suggested to contribute to protective immunity due to its antiviral effects (49), and it is generally thought to correlate with long-term immunological memory. Primary infection with dengue leads to long-term immunity against the homologous serotype. Severe disease emerges during secondary infections with heterologous serotypes. During a secondary infection, memory T cells generated by a primary infection (28) are likely to be activated by the infecting second strain of virus through cross-reacting epitopes. These cells may be activated even more vigorously upon virus re-exposure than naive T cells because they bear memory phenotypes. However, as suggested by others (27, 50), variant peptides from a heterologous virus can act as partial agonists to alter or weaken the function of these cross-reactive T cells. It has been demonstrated that the HLA-A11-restricted NS3 peptide-specific CD8+ T cells appear to bind weakly to peptides of the current serotype, but more strongly to the peptide variants presumably from the previously encountered virus. A high frequency of these T cells do not give IFN-γ ELISPOT responses and display an apoptotic phenotype (51) during the acute phase of secondary infection. These cells are also found more abundantly than those of current strain-specific cells. In a separate study, Rothman and colleagues (52) showed that a variant T cell epitope of NS3 from DV-2 was able to weaken IFN-γ responses of effector CD8+ T cells generated from a previous DV-3 infection. These studies suggest that secondary infections may activate cross-reactive T cells to express CD40L without proper antiviral function. We hypothesize that these T cells will not inhibit viral replication, but enhance infection through CD40L. In fact, higher viral titers during acute infection are associated with high risk of DHF (53), suggesting enhancement of infection is critical to altering the disease course.

In conclusion, we have demonstrated that CD40L enhances DV infection in DCs possibly through regulation of IFN-α secretion and apoptosis. CD40L stimulation restored IL-12 production from DV-exposed DCs and markedly enhanced IFN-γ response from T cells stimulated with DV-infected DC. However, it was not sufficient to restore T cell proliferation from the suppression caused by DV-infected cells.

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Disclosures
The authors have no financial conflict of interest.

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