Infection of Human Dendritic Cells by Dengue Virus Causes Cell Maturation and Cytokine Production

Ling-Jun Ho, Jaang-Jiun Wang, Men-Fang Shaio, Chuan-Liang Kao, Deh-Ming Chang, Shou-Wha Han and Jenn-Haung Lai

*J Immunol* 2001; 166:1499-1506; doi: 10.4049/jimmunol.166.3.1499

http://www.jimmunol.org/content/166/3/1499

---

**Why The JI?**

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

**References**

This article cites 70 articles, 36 of which you can access for free at:

http://www.jimmunol.org/content/166/3/1499.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Infection of Human Dendritic Cells by Dengue Virus Causes Cell Maturation and Cytokine Production

Ling-Jun Ho,*† Jaang-Jiun Wang,‡ Men-Fang Shaio,§ Chuan-Liang Kao,¶ Deh-Ming Chang,* Shou-Wha Han,§ and Jenn-Haung Lai*‡

Dengue virus (DV) is an arthropod-borne flavivirus that causes serious human diseases all over the world, especially in Asia. There are four serotypes of DV (DV1, -2, -3, and -4), and infection by each of them may result in either a relatively benign febrile course called Dengue fever (DF) or fatal outcomes, such as Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). In 1998 DF was the most important tropical infectious disease after malaria. It is estimated that DV infection results in 100 million cases of DF, 500,000 cases of DHF, and 25,000 deaths annually (1). Nevertheless, although there are many proposed mechanisms, the pathogenesis of DHF and DSS is still far from understood (2, 3).

After DV infection most patients are viremic in the early febrile phase, and the viruses are quickly cleared from the blood after defervescence (4). The activation of both humeral and cellular immune responses is considered to be involved in virus clearance; the former response is shown by detecting the increased serum neutralizing Ab levels, and the latter is correlated with the generation and activation of CD4+ and CD8+ T lymphocytes that recognize serotype-specific, Dengue serotype cross-reactive epitopes (4–7). The activation of T lymphocytes and other immune effector cells than leads to increased levels of TNF-α, soluble TNF receptor (sTNFR/75), IL-8, IFN-γ, and other mediators that may be associated with the pathogenesis of increased vascular permeability, a characteristic picture seen in DHF/DSS patients (8–11). Importantly, not only the activation of CD8+ T lymphocytes but also the level of cytokines and the production of viruses, compared with those in DF patients, are significantly higher in DHF patients (7, 11, 12). Because both virus replication in organs such as brain, skin, and lymph nodes and host factors such as cytokines influence the pathogenesis of DV infection in humans, the identification of the natural hosts for DV as well as the subsequent consequences of the infected cells and their interactions with T lymphocytes appear to be critical for effective therapy of DV infection.

Dendritic cells (DC) are potent APCs that, after infection, migrate from peripheral tissues to the lymph nodes and activate CD4+ and CD8+ T lymphocytes (13–16). By using mutational analysis, this migration step has been shown to be crucial for viral pathogenesis (17). The T lymphocyte stimulatory capacity is mediated through both the increased expression of B7-1 and B7-2 costimulatory molecules and the secretion of a variety of cytokines (16, 18). Reciprocally, DC may be activated or regulated by environmental factors, chemokines as well as cytokines (reviewed in Refs. 19–21). Furthermore, DC can efficiently help maintain the protective antiviral cytotoxic T cell memory (22). Because DC have been shown to bind and allow replications of many viruses (23–26; reviewed in Refs. 27 and 28), we postulated that DC could be infected with DV and possibly play a role in the pathogenesis of DV infection through an increase in virus load and cell activation.

In the present study we show that DV could efficiently infect DC and produce virus particles. After infection, DC were activated to secrete TNF-α and IFN-α and were driven to undergo the maturation process. Given that DC underwent spontaneous apoptosis in...
the absence of feeding cytokines and that the infection of DC by DV resulted in accelerated cell apoptosis in the early phase after infection, both cell maturation and cytokine production could probably produce an anti-apoptotic effect in the late phase of infection.

Materials and Methods

Culture medium and reagents

The cell culture medium consisted of RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS, 2 mM glutamine, and 1000 U/ml penicillin-streptomycin (Life Technologies, Gaithersburg, MD). Recombinant GM-CSF and IL-4 were purchased from R&D Systems (Minneapolis, MN). mAb to viral E protein was obtained from supernatants as detailed in our previous work (34). All determinants were purchased from BD Pharmingen (San Diego, CA); and anti-CD11b from Dako (Carpinteria, CA). The isotype-matched control mAb. The gated areas represent the monocyte-derived DC populations. Each density plot or histogram is generated using at least 10^6 events.

Establishment of DC from human PBMC

The generation of DC from human PBMC was performed as described by Romani et al. (30). In brief, whole blood (20–50 ml) from healthy donors (>80 participants) was mixed with Ficol-Hypaque, and after centrifugation the layer of mononuclear cells was collected. The adherent mononuclear cells were cultured and maintained in complete medium containing IL-4 and GM-CSF. After culturing for 3, 5, 7, and 10 days, the detached cells were stained with CD1a or isotype-matched control mAb. The first-day cells (collected from scratching the attached cells with a policeman) and the seventh-day cells (detached) were stained with both CD14 and CD1a or isotype-matched control mAb. The gated areas represent the monocyte-derived DC populations. Each density plot or histogram is generated using at least 10^6 events.

Preparation of DV

The preparation of DV has been described previously (31). In brief, four serotypes of DV used in this study were described previously or obtained as follows: DV1, Hawaii strain (American Type Culture Collection); DV2, New Guinea C strain (32); DV3, H87 strain (American Type Culture Collection); and DV4, H241 strain (American Type Culture Collection). Unless otherwise specified, DV2 New Guinea C strain was used as the source of DV to infect DC throughout this paper.

Infection of DC with DV

DC cultured for 7–10 days were infected with mock (C6/36 mosquito cell culture supernatants) or different DV serotypes at various multiplicities of infection (MOIs) for 4 h at 37°C as modified from the report by Hung et al. (31). Cells were then washed and cultured in six-well plates (Costar) with culture medium supplemented with or without cytokines as indicated in the figure legends. Cell density was maintained at 1 x 10^6/ml in 0.5–2 ml of the medium.

Measurement of virus titers

The supernatants from DV-infected DC were collected at various time points as indicated. The production of virus particles in supernatants was determined by titration on adherent BHK cells, permissive for four serotypes of DV. The numbers of plaques were counted and shown as PFU per milliliter. Virus titers <10^3 were taken as 10^2 for convenience.

Confocal microscope and electron microscope examinations

After infection, DC were collected at 24 and 48 h and fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 30 min. The DC cell suspensions were adhered to a polylysine-coated slide, permeabilized by incubating with 1% Triton X-100 for 5 min twice, and stained with anti-NS1 mAb (34). After washing, the cells were stained with goat anti-CD1a polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by biotin-conjugated anti-sheep/goat mAb (Amersham Pharmacia, Piscataway, NJ), which was finally labeled with streptavidin-Cy3 (Amersham Pharmacia). For electron microscopy, DC cell pellets were collected and fixed in 2.5% glutaraldehyde and 0.1 M cacodylate buffer. After dehydrations with ethanol, the cells were embedded in eponate-12 as described previously (33), and examined under a transmission electron microscope (JEOL 1230; JEOL, Peabody, MA).

Determination of cytokine production and cell surface molecule expression

Standard ELISAs were used to measure cytokine concentrations in harvested supernatants as detailed in our previous work (34). All determinants

FIGURE 1. Generation and identification of DC from human peripheral blood. A. Whole blood was obtained from healthy donors. After mixing with Ficol-Hypaque and centrifugation, the layer containing mononuclear cells was collected. The adherent mononuclear cells were cultured and maintained in complete medium containing IL-4 and GM-CSF. After culturing for 3, 5, 7, and 10 days, the detached cells were stained with CD1a or isotype-matched control mAb. B. The first-day cells (collected from scratching the attached cells with a policeman) and the seventh-day cells (detached) were stained with both CD14 and CD1a or isotype-matched control mAb. The gated areas represent the monocyte-derived DC populations. Each density plot or histogram is generated using at least 10^6 events.
were performed in duplicate (IL-6 and IFN-α) or triplicate (TNF-α and IL-12) and expressed as the mean ± SD. The detection limits for these cytokines are: TNF-α, 5 pg/ml (R&D Systems); IFN-α, 10 pg/ml (Endogen, Woburn, MA); IL-6, 3 pg/ml (R&D Systems); and IL-12, 7 pg/ml (R&D Systems). The expression of cell surface molecules was determined by flow cytometric analysis (35). Each histogram or density plot comprised at least 10^4 events.

**Measurement of cellular apoptosis by flow cytometric analysis**

The mock-treated or DV-infected DC, after washing with cold PBS, were pelleted and resuspended in binding buffer containing HEPES-buffered PBS supplemented with 2.5 mM CaCl2. Then 10 μl of annexin V-FITC at a concentration of 10 μg/ml and 10 μl of propidium iodide (PI) at a concentration of 50 μg/ml were added to each sample and incubated for 15 min at room temperature. After washing, the cells were analyzed by a flow cytometer (Becton Dickinson). The annexin V^+ and PI^+ population represents early apoptosis, and the late apoptotic or necrotic population is shown to be annexin V^− and PI^−.

**Nested RT-PCR**

To determine the specificity of DV2, a nested RT/PCR method described by Lanciotti et al. (36) was exactly adapted. In brief, the viral RNA was isolated from the supernatants of DV-infected DC. After RT of RNA to cDNA, samples were subjected to PCR. The consensus primers 5'−TCAATATGCTGAAACGCGCGAGAAACCG-3' (D1 primer, genomic positions 134−161) and 5'−TTGCAACACAGTAAATCTTCCAAGGTTC-3' (D2 primer, genomic positions 616−644) were used to amplify 511-bp DNA products (our unpublished observations). After the first-round amplification reaction with consensus primers, a second-round amplification reaction was conducted, except that the D2 primer was replaced with the DV2-specific primer 5'−CGCCACAAGGGCCATGAC-3' (genomic positions 232−252). The RT-PCR working conditions have been detailed previously (36). The final products were analyzed on a 4% agarose gel, stained with ethidium bromide, and visualized under UV light. After the second-round PCR, a 119-bp product specific for DV2 was detected.

**Statistics**

When necessary, the results were expressed as the mean ± SD. A paired (for comparison of cytokine production) or unpaired (for comparison of virus titers) Student's t test was used to determine the difference, which was considered significant at p < 0.05.

**Results**

**Establishment of DC from human PBMC**

DC were propagated from culturing adherent human PBMC in the presence of GM-CSF and IL-4. The detached cells were then removed at different time points, stained with anti-CD1a or isotype-matched control mAb, and analyzed in a flow cytometer. As shown in Fig. 1A, after culture for >7 days, >95% of the gated monocyte-derived DC population expressed CD1a. In contrast, the expression of CD14 was barely detectable after 7 days of culture (Fig. 1B). All of the following experiments were performed using DC cultured for 7−10 days. At this time point, DC routinely represented 65−95% of the total cell population. Such an estimation is a conservative, low measure of the DC population, because a significant number of unstained “cells” are acellular debris, as characterized by their size and light scatter properties in flow cytometric analysis.

**Infection of DC by DV**

Because DC were established in the culture medium containing IL-4 and GM-CSF, we first investigated the effects of these cytokines on virus production. After infection with DV2 New Guinea C strain at an MOI of 1, DC were maintained in the culture medium with or without cytokines. As shown in Fig. 2A, DC could provide efficient replications of DV and yielded infectious virus particles in both conditions. In the presence of cytokines, higher titers of virus particles were produced. To minimize the effects of cytokines, the rest of the experiments were conducted without supplementing the culture medium with cytokines after DV infection.
The specificity of the productive infection of DV2 was subsequently confirmed with nested RT-PCR that detects a 119-bp product specific for DV2 in the culture supernatants (Fig. 2B). These results exclude the possibility that the productive infection of DC by DV2 is because of the contamination by other DV serotypes or other flaviviruses. The infection of DC by DV was further investigated by staining both the intracellular viral NS1 protein and the cell surface CD1a molecule and then was analyzed under a confocal microscope. As illustrated in Fig. 2C, only DV-infected, but not mock-treated, DC coexpressed CD1a and NS1 proteins. As predicted, CD1a was localized on the cell surface, whereas NS1 was located in the cytosol (Fig. 2C, lower panel, showing better contrast). In our preliminary studies we also found that neither purified B lymphocytes nor T lymphocytes, two major populations contaminating DC purity, could be infected by DV (our unpublished observations). Therefore, these results strongly suggest that DV could infect cultured human DC.

**Localization of DV particles**

To investigate the organelle of DC where DV resided, the infected DC were examined under an electron microscope. As shown in Fig. 3a, after infection for 24 h DV particles were obvious in cystic vesicles, vacuoles, and the endoplasmic reticulum. Fig. 3B clearly shows the structures of the rough endoplasmic reticulum with virus particles inside. This is consistent with the characters of flavivirus infection (37). In addition, virus-induced hypertrophy and proliferation of the rough endoplasmic reticulum as well as swollen mitochondria were observed (Fig. 3, a and b). We could not find similar pictures in mock-treated DC (our unpublished observations). These findings may suggest a cell undergoing apoptosis, although in this particular picture of EM, the nuclear condensation has not developed yet (38) (Fig. 3a). However, we did observe many DV-infected cells showing pictures of nuclear condensation (our unpublished observation). Because mock-treated DC also showed patterns of nuclear condensation, whether these findings in DV-infected DC were due to DV infection or as were the result of a spontaneous consequence in the absence of feeding cytokines was not clear. When the cells were examined 48 h after infection, bulky virus particles inside the vesicle were detected, and virus release out of the cell membrane became evident (Fig. 3c).

**Infection of DC by different DV serotypes**

There are four serotypes of DV (DV1–4), and their infectivities in DC were examined. As shown in Fig. 4, although the production

**FIGURE 3.** Localization of DV in DC. After infection with DV at an MOI of 10 for 24 h (a and b) or an MOI of 20 for 48 h (c), DC were collected and visualized under an electron microscope as described in Materials and Methods. Bars: a, 0.5 μm; b, 0.5 μm; c, 0.3 μm. N, Nucleus; m, mitochondria; rer, rough endoplasmic reticulum; arrowheads, virus particles.
of virus titers was different, four serotypes of DV at an MOI of 1 could infect DC.

Maturation of DC after DV infection

After confirming the infection of DC by DV, the possible outcomes of DC after infection were investigated. There are two stages in DC development. In the immature stage, DC primarily phagocytose and process Ag, whereas in the mature stage, DC function as the best Ag presenters to T lymphocytes by up-regulating the expression of costimulatory and HLA-DR molecules (16). We determined whether DV infection of DC could enhance the expression of these cell surface molecules. After mock infection or infection with DV at an MOI of 1 for 48 h, cells were collected, and the gated DC population was analyzed for cell surface molecule expression. As shown in Fig. 5, compared with mock-treated DC, DV infection induced significant expression of B7-1, B7-2, CD11b, HLA-DR, and CD83 on DC. Thus, DV infection of DC induced cellular maturation.

Differential regulation of cytokine production from DV-infected DC

Aside from the expression of costimulatory molecules, DC also initiate a potent immune response through the secreted cytokines. The supernatants from mock-treated or DV-infected DC were collected at 48 h after infection and assayed for several cytokine concentrations. As shown in Fig. 6A, the cultured DC spontaneously produced a variety of cytokines, consistent with other reports (39, 40). In the presence of DV infection, DC produced higher levels of IFN-α and TNF-α, but not IL-12 and IL-6. The production of IFN-γ was not detectable (our unpublished observations). The induction of TNF-α production could also be observed in four serotypes of DV infection (Fig. 6B).
Anti-apoptotic effect of cell maturation and cytokine production in DV-infected DC

In the absence of feeding cytokines, DC died through an apoptotic pathway, similar to that observed in spontaneous apoptosis of monocytes (41) (our unpublished observations). We found that compared with mock treatment, DV infection resulted in a higher percentage of cell death in the early phase of infection (see below). Such an accelerated apoptotic mechanism appeared not to involve Fas/Fas ligand interaction, because these two molecules were not induced on DV-infected DC (our unpublished observations). It has been shown that mature DC are more resistant than immature DC to vaccinia virus-induced cell apoptosis; we determined whether this could also be observed in DV infection (26, 42). Both mock-treated and DV-infected DC cultured for longer periods of time in the absence of exogenous cytokines were stained with annexin V-FITC and PI and examined with a flow cytometer. As shown in Fig. 7, compared with mock-treated DC, DV infection induced more DC showing annexin V

Discussion

It remains elusive which tissue cells are the natural targets in which DV reside and which produce virus particles. Both immune effector cells such as monocytes and T lymphocytes and nonimmune cells such as hepatocytes, endothelial cells, and brain cells were reported to be potential hosts (9, 43, 44). However, none of these cells could efficiently present virus Ags to T lymphocytes. Although human Kupffer cells, effective APCs, can be infected by DV, no viral progeny synthesis is detected (45).

Human PBMC cultured with recombinant IL-4 and GM-CSF were shown to generate DC with the most efficient Ag-presenting function (46). In the presence of DC maturation factor CD40 ligand, these myeloid lineage DC become potent inducers of Th1 differentiation (47). Using different techniques, we show that DC, established from this well-appreciated condition, could provide DV to enter and produce virus particles. In addition, this conclusion was consistently observed in four different serotypes of DV infection (Fig. 4). In our hands, both purified human T and B lymphocytes were poorly infected by DV (unpublished observations). According to Rosen et al. (48), 7 of 16 mesenteric lymph node specimens and 13 of 18 spleen specimens from children dying of DHF contain DV RNA. It is highly possible that DC may be one of the primary targets of DV in these infected tissues. In support of this idea as well as our observations, while this paper was under review Wu et al. (49) provided convincing evidence that DV could infect DC both in vitro and in vivo.

By using different approaches, many known or sequence-identifiable molecules were reported to be potential DV receptors (50–52). Among previous reports, Chen et al. (53), using Vero cells as target cells, presented convincing evidence showing that the highly sulfated glycosaminoglycan (GAG) is the putative cellular receptor for DV. Interestingly, we found that among the GAGs examined, heparin, heparan sulfate, and dermatan sulfate, but not chondroitin sulfate A, could inhibit DV-DC binding (unpublished observations). Examination of the molecular mass (kilodaltons) and the number of sulfate groups per disaccharide unit (SO3/COO ratio) of these compounds shows the order of molecular mass as chondroitin sulfate A (30 kDa) > dermatan sulfate (28 kDa) > heparan sulfate (18 kDa) > heparin (13.6 kDa), and the order of SO3/COO ratio as heparin (2.14) > dermatan sulfate (1.22) > chondroitin sulfate A (1) > heparan sulfate (0.93) (54). Thus, although the sulfation of GAGs is important for virus-DC binding in several examples (53–57), our preliminary observations suggest that, in contrast to Vero cells, the binding of DV to DC may have no strong correlation with the levels of sulfation of GAGs.

DC undergo the maturation process and migrate from peripheral tissues to lymph nodes after being directly infected or phagocytosing apoptotic infected cells (58, 59). The DC maturation process can be regulated by a variety of stimuli such as cytokines, viral products, and CD40 ligand. In the context of infection, microorganisms use the mechanism of modulating the DC maturation process to influence the immune response. For example, the infection of DC by vaccinia virus inhibits DC maturation and therefore down-regulates its stimulatory capacity to T lymphocytes (42). A similar mechanism was demonstrated in herpes simplex virus infection (60). The infection of erythrocytes by Plasmodium falciparum induces the expression of PfEMP-1 Ag, which, after binding to DC, inhibits maturation of immature DC (61). The infection of DC by Trypanosoma cruzi also results in inhibition of the maturation process (62); through the suppression of DC maturation, it
may help to evade the immune regulation and to minimize the immune response. Although some microorganism infections may impair the maturation process of DC, other pathogens could drive DC maturation after infection. As reported by Schuur et al. (63), measles virus infection of DC induces DC maturation that, in turn, causes immunosuppression through as yet unidentified mechanisms. The induction of DC maturation may also result in protection against a viral cytopathic effect and help stabilize the production of viral progeny (26). Similar effects were indeed observed in the example of DV infection of human DC shown in this report (Fig. 7).

In reaction to the entrance of virus, DC may release various cytokines to initiate an immune response and probably leading to the pathological changes seen in virus infection (59). The production of cytokines from DV-infected DC seem to be differentially regulated; only TNF-α and IFN-α, not IL-6 and IL-12, were significantly induced (Fig. 6A). The induction of TNF-α in DV-infected DC was correlated very well with clinical observations expressed in terms of increased TNF-α associated with the induction of MxA protein that exerts antiviral effects. This suggests that type 1 IFN has been shown to mediate the antiviral response (65–67). In the example of DV infection of human DC shown in this report, TNF-α and IFN-α levels in plasma samples from DV-infected patients (11, 64). Importantly, the increase in TNF-α and IFN-α may also contribute to the lesser cell death in DV-infected DC in the late phase of virus infection (Fig. 7).

Whether DV infection results in DC apoptosis is still an open question. In our system because DC were infected with DV in the absence of feeding cytokines, it has been very difficult to discern the apoptotic mechanisms of IFN-α and IFN-α, which are involved in the induction of viral progeny (26). Similar effects were indeed observed in the example of DV infection of human DC shown in this report (Fig. 7).

Acknowledgments
We thank Chien-Ting Lin, Yu-Lin Hsu, Moon-Chung Wu, and Chia-Ho Kuo for excellent technical assistance.

References


