Dengue Virus Infection Induced NF-κB-dependent Macrophage Migration Inhibitory Factor Production

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Abstract: Dengue virus (DV) infection can cause mild dengue fever or severe dengue hemorrhage fever and dengue shock syndrome. Macrophage migration inhibitory factor (MIF) is a cytokine that plays an important role in the modulation of inflammatory and immune responses and serum levels of MIF are correlated with disease severity in dengue patients. However, the mechanism that induces MIF production during DV infection is unclear. In this study, we showed that DV infection, but not UV-inactivated DV stimulation, dose-and time-dependently induced MIF secretion in human A649 epithelial cells. MIF promoter assays and RT-PCR demonstrated that MIF gene transcription was activated during DV infection. Furthermore, DV infection induced NF-κB activation, and the NF-κB inhibitors dexamethasone and curcumin inhibited DV-induced MIF production. Finally, we found that different cells have different abilities to release MIF after DV infection. Interestingly, DV infection and MIF production in the human monocytic cell line THP-1 and peripheral blood mononuclear cells increased in the presence of antibodies against DV. Taken together, these results suggest that DV infection of human cells induces NF-κB activation and MIF production, which can be increased in the presence of pre-existing antibodies.

Key words: Cytokine; inflammation; infection

INTRODUCTION

Dengue viruses (DV) are mosquito-borne flaviviruses subgrouped into four antigenically related serotypes: DV types 1, 2, 3, and 4[1]. It is estimated that over 50 million DV infections occur globally each year[2]. DV infection generally causes mild symptoms such as fever, headache, and muscle and joint pain, which is called dengue fever (DF). In some cases, especially during secondary infection with a different serotype of DV, the infection may progress to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS)[3]. DHF is a severe febrile disease characterized by abnormalities in homeostasis and increased capillary leakage that can progress to hypovolemic shock (DSS)[4]. Even though the process leading to DHF/DSS is not fully understood, antibody-dependent enhancement (ADE) has been proposed to explain the mechanisms by which heterogeneous serotype DV infection can induce DHF/DSS in secondary

infection[5, 6]. According to the ADE hypothesis, the antibody generated during primary dengue infection can neutralize only DV with the same serotype but not those with different serotypes during secondary infection. In addition, the non-neutralizing antibodies may increase the virus uptake by macrophages through Fc receptor and even worsen the disease. However, antibody against DV pre M protein (anti-pre M Ab) can also augment dengue virus infection in a concentration-dependent but serotype and FcR-independent manner[7].

Macrophage migration inhibitory factor (MIF) is a cytokine important in the modulation of inflammatory and immune responses[8]. MIF is released by different cells in many tissues in response to a variety of stimuli[9]. Once released, MIF augments the secretion of TNF-α and counteracts the anti-inflammatory action of glucocorticoids[10]. Interestingly, low concentrations of glucocorticoids induce rather than inhibit MIF production in macrophages[11]. In our previous study[12],

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we found that serum levels of MIF were correlated with disease severity in dengue patients. The mechanism that induces MIF production during DV infection is unclear, however. In this study, we show that DV infection of the human epithelial cell line A548 directly induced MIF production via NF-κB activation. We also explored the effects of DV infection on the production of MIF in other human cell lines, as well as in primary cells such as human umbilical vein endothelial cells (HUVEC) and peripheral blood mononuclear cells (PBMC), with or without pre-existing antibodies against DV.

MATERIAL AND METHODS

Preparing virus stock and virus titration: Dengue type 2 (DV2) strain PL046 and JEV (strain RP-9) viruses were propagated in C6/36 cells. Briefly, monolayers of C6/36 were inoculated with the virus at a multiplicity of infection (MOI) of 0.1 and incubated at 26°C and 5% CO_2 for 5 days. The culture medium was harvested, and cell debris was removed using centrifugation at $900 \times g$ for 10 min. After further centrifugation at $16,000 \times g$ for 10 min, the virus supernatant was collected and stored at -80°C until use. Virus titer was determined using a plaque assay with the BHK-21 cell line. Briefly, a 10-fold serial dilution of virus was added to BHK-21 monolayer and then incubated at 37°C at 5% CO_2 for 5 days. Plaque numbers were counted after crystal violet staining. UV inactivation of DV (UV-DV) was done in a UV crosslinker (Stratalinker; Stratagen, La Jolla, CA) using short wavelength ultraviolet radiation (UVC, 254 nm) at a distance of 5 cm for 30 min on ice, as previously described[13].

Cell culture: A human lung cancer epithelial cell line (A549) and a human hepatoma cell line (HepG2) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), and 2 mM L-glutamine. A human T cell line (Jurkat), a myelomonocytic cell line (K562), and a monocytic cell line (THP-1) were grown at 37°C in 5% CO_2 in RPMI 1640 medium with 10% FBS. Human peripheral blood mononuclear cells (PBMC) from healthy blood donors with no antibodies against DV2 were isolated using lymphocyte separation medium (Histopaque-1.077; Sigma-Aldrich Co., St Louis, MO). Human umbilical vascular endothelial cells (HUVEC) were isolated from human umbilical cord veins and cultured as previously described[14].

Viral infection and ADE: Cells were pretreated with or without dexamethasone (Dex, 10^{-6} M) (Sigma-Aldrich) or curcumin (Cur, 60 μM) (Sigma-Aldrich) for 1 h before incubation with DV2 at an MOI of 10 or as indicated. Viruses were allowed to be absorbed by the cells for 2 h at 37°C. After that, unbound viruses were removed by washing with medium. Infected cells and culture supernatants were collected at different time intervals. Human PBMC or THP-1 cells were infected with DV2 at the MOI of 10 in the presence of the diluted dengue type 3 patient serum (1:6000) or anti-pre M monoclonal antibody 70-21 (1 μg)[7].

Immunofluorescent staining and flow cytometric assay: Cells (1×10^6) were infected with DV2 at an MOI of 10 for 24 h. After they had been incubated, the cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.5% Triton X-100 for 10 min. The cells were then washed with PBS and blocked with 0.05% BSA in PBS. Fixed cells were stained with primary antibody at 4°C for 1 h. After being washed, the cells were incubated with secondary antibody and observed under a fluorescent microscope (Olympus, Tokyo, Japan) or for FACSCalibur analysis (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed using WinMDI 2.8 software. Anti-dengue mAb (anti-E, anti-pre M, and anti-NS1, a gift from Dr. Huan-Yao Lei), MIF rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and NF-κB p65 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. FITC-conjugated goat-polyclonal anti-mouse IgG antibody (1:200 dilution; Jackson ImmunoResearch, West Grove, PA), Alexa Fluor 594 conjugate goat-polyclonal anti-Rabbit IgG antibody (1:200 dilution; Molecular Probes, Inc., Eugene, OR) or Alexa Fluor 488 conjugate goat-polyclonal anti-Rabbit IgG antibody (1:200 dilution; Molecular Probes) were used as secondary antibodies.

Reverse-transcription polymerase chain reaction (RT-PCR): RNA was extracted using an isolation reagent (TRIzol; Invitrogen) and quantified at 260 nm. Reverse-transcription (RT) was done using a kit (Invitrogen) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was a control. The primer sequences were:

MIF (sense): 5'-TCCTTCTGCCATGCCGA-3'
MIF (antisense): 5'-TGCGGCTCTTAGGCGAAGGT-3'
GADPH (sense): 5'-CACGGCAAGTTCAATGGCACA-3';
GADPH (antisense): 5'-GAATTGTGAGGGAGAGTGCTC-3'.

A total reaction volume of 20 μl contained 4 μl of RT product, 2.5 units of Taq DNA polymerase, 20 μM of dNTP, 0.1 μM of primer, and 1× Taq DNA polymerase buffer (Promega, Madison, WI). The reaction mixture was incubated in a thermocycler (Perkin-Elmer, Fremont, CA) programmed to pre-denature at 95°C for 5 min, denature at 95°C for 30 s, anneal at 56°C for 45 s, and extend at 72°C for 1 min. for a total of 30 cycles. After the last cycle, the resulting mixture was incubated at 72°C for 7 min and cooled to 4°C.

ELISA for cytokines: Cells (1×10^6) were infected with DV2 or UV-DV2 at an MOI of 10, as described above. Culture supernatants were collected at different intervals post-infection as indicated. The concentrations of MIF in the supernatants as well as in patient sera were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

MIF promoter-luciferase vector: From -1170 to +3-bp of the human MIF promoter was PCR-amplified from genomic DNA (Jurkat cells) and subsequently cloned into pGL2 Basic (Promega) between the Xho I and Hind III sites to generate pMIF-luc. The sequences of the primers used in the PCR were the following:

Primer-1, 5'-CTCGAGCTGCAGGAACCAATACCCAT-3'
and
Primer-2, 5'-AAGCTTGGCATGATGGCAGAAGGACC-3'.

The PCR conditions were 94°C for 5 min and then 35 cycles at 94°C, 55°C and 72°C, each for 1 min. The PCR product was gel-purified and then digested by the restriction enzymes XhoI and HindIII ligated into the XhoI/HindIII sites of the pGL2 vector (Novagen, Madison, WI) to produce pGL2-MIF. The nucleotide sequence was confirmed using DNA sequence analysis.

Transient cell-transfection and assay for luciferase activity: The cells were transfected with reporter plasmid and the internal control vector thymine kinase. After transient transfection, the cells were treated with DV2 for two days and then cultured in an atmosphere of 5% CO_2 and 95% air at 37°C for 72 h. The cells were then harvested, and luciferase activities in the cell extracts were determined using a Lucilight (Perkin-Elmer Life Sciences, Norwalk, CT) and read using a microplate scintillation and luminescence counter (TopCount NXT; Perkin-Elmer). All experiments were performed on triplicate samples and were independently repeated four times.

The promoter regions were repeated and turned into a luciferase reporter system. For the reporter assay, plasmid DNA was transfected into A549 cells using a cationic lipid reagent (Lipofectamine 2000; Invitrogen). In brief, cells were plated 24 h before transfection at a density of 1×10^5 cells/well (12-well plate). Five hours after transfection, the cells were replenished with culture medium and then incubated for various time periods. NF-κB-Luc luciferase reporter plasmid was from Clontech (Palo Alto, CA). The pRL-TK reporter construct (Promega), encoding *Renilla* luciferase driven by the HSV thymidine kinase promoter, was used as an internal control. Five hours post-transfection, cells were infected with DV2 or JEV (MOI = 10). At various times after infection, cell lysates were collected for dual-luciferase assays (Promega). Relative firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis: Data are expressed as means ± SD. Student's *t* test was used to analyze the significance of the difference between the test and the control groups. Statistical significance was set at $P < 0.05$.

RESULTS

DV2 infection induced MIF production in A549 cells: To assess whether DV2 infection induces MIF production, we incubated A549 cells with DV2 for 48 h at an MOI of 10. Antibody against DV2 E antigen as well as antibody against MIF were used to double-stain both DV2 and MIF in cells. DV2 infection of A549 cells was confirmed by the presence of E antigen in the cytoplasmic region of the infected cells. Furthermore, DV infection induced MIF expression in both the nuclei and cytoplasm of these cells (Figure 1A). On the other hand, MIF expression in mock-infected cells was found only in some cells and mainly in nuclei (Figure 1B).
MIF was time- and dose-dependently released from DV-infected A549 cells: We next assessed whether DV2-infection-induced MIF production is time- and dose-dependent. No significant increase of MIF release was found in DV2-infected A549 cells than in mock-infected cells after 24 h of infection (Figure 2A); however, after 48 h of infection, the MIF concentration were significantly elevated in the supernatants of the DV2-infected cells but not in the mock-infected cells. DV2-infected A549 cells continued to release MIF for at least 72 h after DV infection (Figure 2A). MIF was also dose-dependently secreted after DV infection (Figure 2B), but A549 cells

(A) DV2 infected A549 cells

| Anti-E | MIF | DAPI |

(B) Mock-infected A549 cells

| Anti-E | MIF | DAPI |

Fig. 1: DV2 infection induced MIF production in A549 cells. A549 cells were mock- or DV2-infected for 48 h (MOI = 10). The cells were then immunostained with anti-MIF (Alexa 594 conjugated secondary antibodies), anti-DV-2 E (FITC-conjugated secondary antibodies) antibodies, and DAPI to stain nuclei, as described in Materials and Methods. Cells were observed using fluorescent microscopy at ×400 magnification.

treated with UV-inactivated DV did not release a significant amount of MIF (Figure 2B).

DV infection increased the MIF mRNA expression of A549 cells: To further understand whether MIF production was caused by a *de novo* synthesis of MIF RNA or the release of pre-formed cytokines stored inside cells, we used RT-PCR to analyze the MIF mRNA levels in DV-infected A549 cells. Although background expression of MIF mRNA levels was detected in the mock-treated cells (Figure 3A). the expression of MIF RNA was dramatically increased in DV2-infected cells. Transfecting A549 cells with a construct containing the MIF promoter and luciferase reporter gene provided further evidence that DV

infection activates MIF-gene transcription. When A549 cells were transiently transfected with an MIF-Luc minimal promoter/reporter and analyzed for luciferase expression 48 h after infection, the luciferase activity of DV2-infected cells increased 1.75 times compared with mock-infected cells (Figure 3B) which confirmed that DV2 infection induced MIF gene activation. JEV-infected A549 cells, on the other hand, did not induce MIF gene activation.

NF-κB was activated after DV infection of A549 cells: Because NF-κB activation is implicated in activating many pro-inflammatory cytokines, we next examined whether NF-κB was activated after A549

(A)

MOCK   DV2
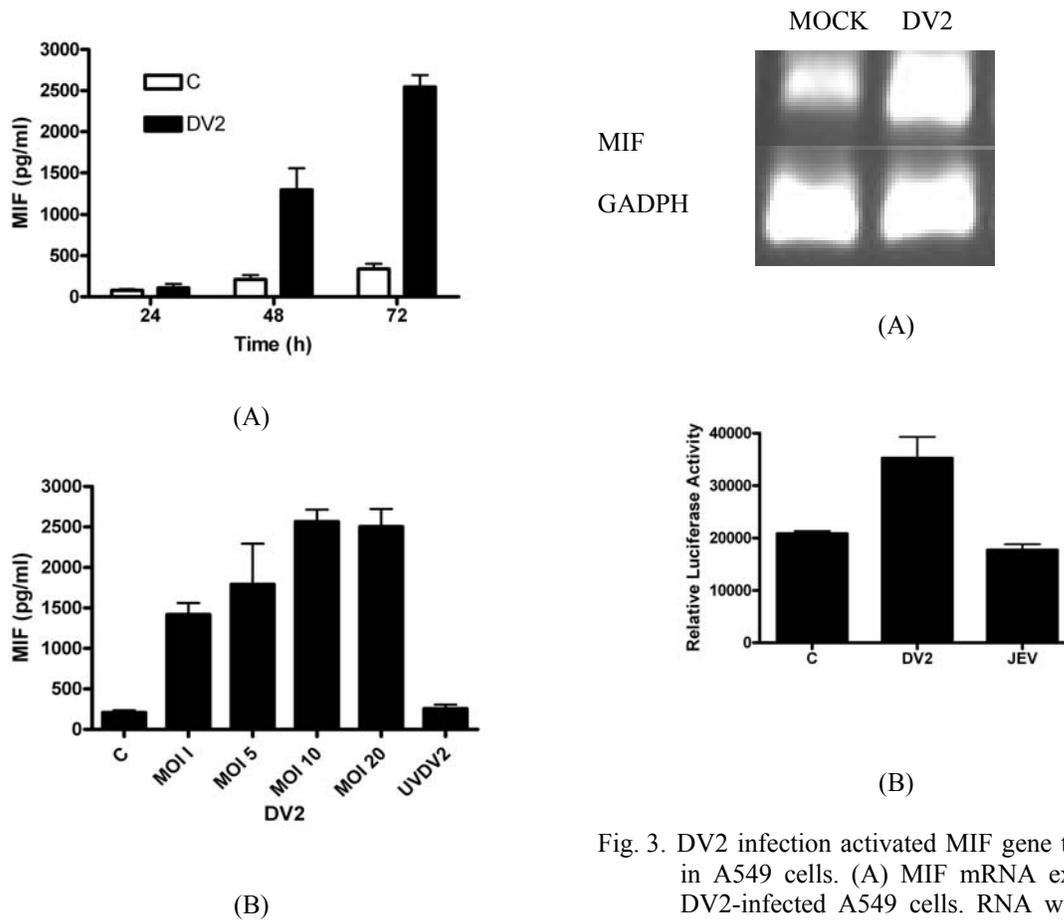
MIF

GADPH

(A)

(B)

(B)

Fig. 2. DV2 time and dose-dependently induced MIF production in A549 cells. (A) A549 were infected with DV2 (MOI = 10) or mock-infected. Supernatants were harvested at 24, 48, and 72 h, and the amount of MIF released into the supernatants was assessed using ELISA. (B) A549 cells were infected with DV2 at MOIs of 1, 5, 10, and 20 or stimulated with UV-inactivated-DV2 at an MOI of 10. Supernatants were collected after 72 h and the amount of MIF was assessed using ELISA.

Fig. 3. DV2 infection activated MIF gene transcription in A549 cells. (A) MIF mRNA expression in DV2-infected A549 cells. RNA was extracted and MIF expression was analyzed using semi-quantitative RT-PCR with specific primers for MIF (see Materials and Methods) using 30 amplification cycles. The gene expression of GADPH was used as the internal control. Lane 1: Mock (uninfected). (B)A549 cells were transfected with MIF-Luc (0.5 μg) and pRL-TK (0.05 μg) for 24 h, then infected with DV2 (MOI = 10) for 48 h. The cell lysates were then harvested for dual-luciferase assays. Luciferase activity is shown as the relative counts per second of firefly luciferase normalized to *Renilla* luciferase.

cells had been infected with DV2. We first assessed whether DV2 infection had activated an NF-κB dependent reporter gene. Luciferase expression in DV2-infected cells increased 1.87 times compared with mock-infected cells (Figure 4A) but not in cells transfected with UV-inactivated DV2.

We next sought further evidence of NF-κB activation by assessing NF-κB nuclear translocation using immunostaining to detect p65/NF-κB.

Immunofluorescent microscopy analysis of DV2-infected and mock-infected cells. DV2-infected A549 cells showed clear evidence of NF-κB nuclear translocation, but mock-infected cells did not (Figure 4B). To further confirm NF-κB translocation occurred in DV2-infected cells, A549 cells were double stained with DV E protein and NF-κB. Co-localization of DV E protein in the cytoplasm and NF-κB in the
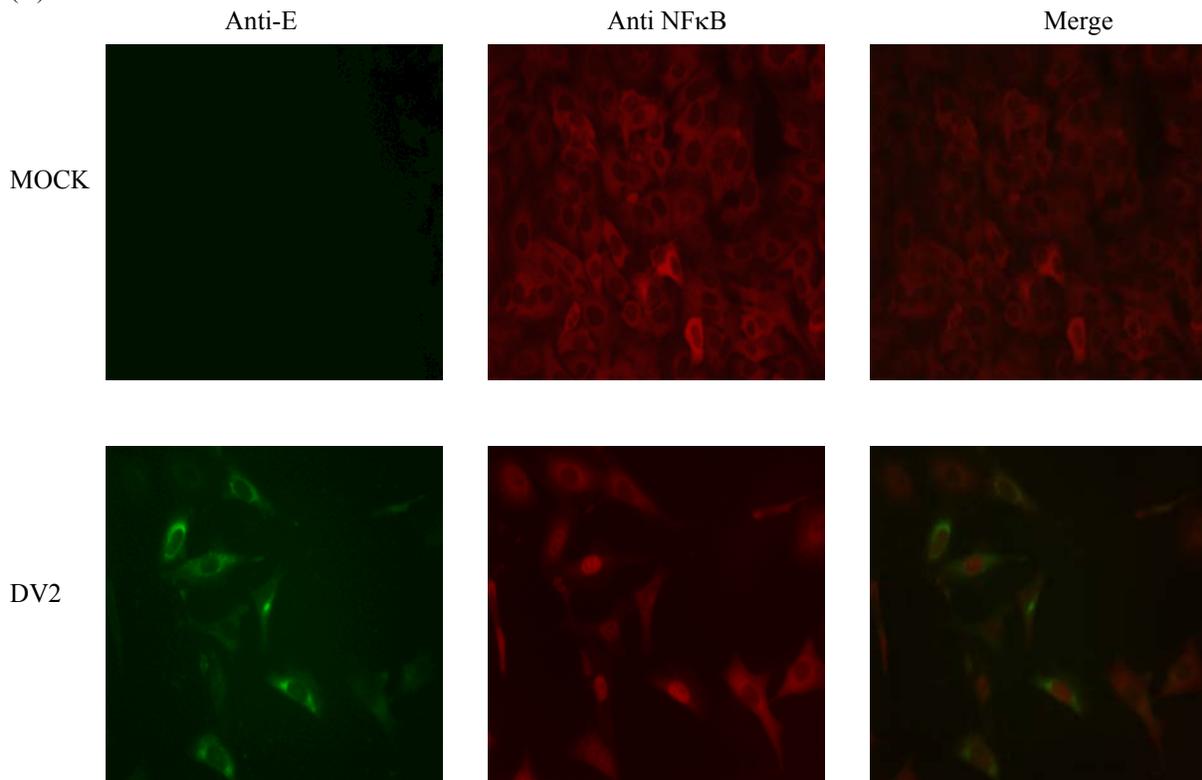
(A)

(B)
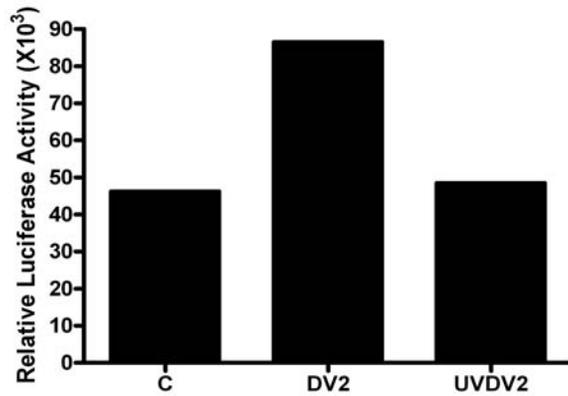
Fig. 4. NF-κB activation in DV2-infected A549 cells. (A) A549 cells were transfected with NF-κB-Luc (0.5 μg) and pRL-TK (0.05 μg) for 24 h, and then infected with DV2 (MOI = 10) for 48 h. The cell lysates were then harvested for dual-luciferase assays. Luciferase activity is shown as the relative counts per second of firefly luciferase normalized to *Renilla* luciferase. (B) A549 cells were mock-infected or DV2-infected for 48 h (MOI = 10). The cells were then immunostained with anti-NF-κB p65 and anti-DV-2 E antibody, as described in Materials and Methods.

(A)

(B)
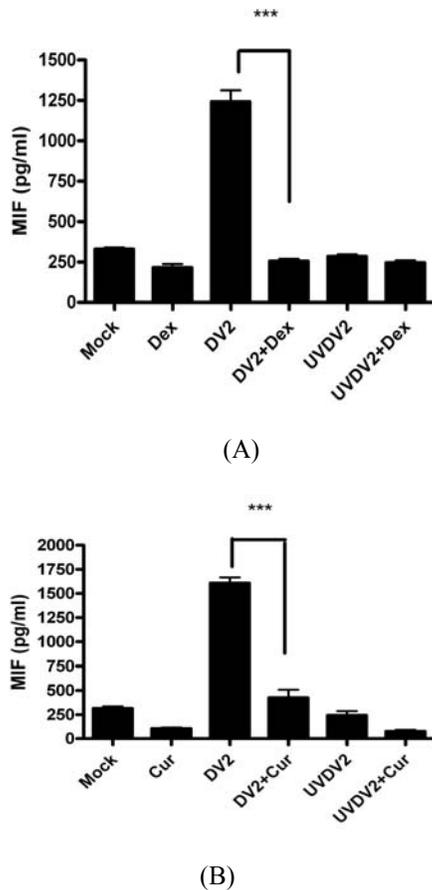
Fig. 5.   Effects of dexamethasone and curcumin on the production of MIF induced by DV2 infection of A549 cells. A549 cells were incubated with or without DV2 or UV-V2, as described in Materials and Methods, with or without (A) dexamethasone (Dex, 10^{-6} M) or (B) curcumin (Cur, 60 μM). The levels of MIF in the supernatants were assayed using ELISA. ***P < 0.001.

nuclei of the same cells was clearly evident under fluorescent microscopy (Figure 4B).

Inhibiting NF-κB blocked DV2-infection-induced MIF production in A549 cells: To assess whether NF-κB activation was involved in MIF production, we used two NF-κB activation inhibitors, dexamethasone[15] and curcumin[16]. We found that DV2-infected A549 cells treated with 10^{-6} M of dexamethasone or 50 μg/ml of curcumin significantly (P < 0.001) inhibited MIF production (Figure 5). We tested the effect of dexamethasone and curcumin at these
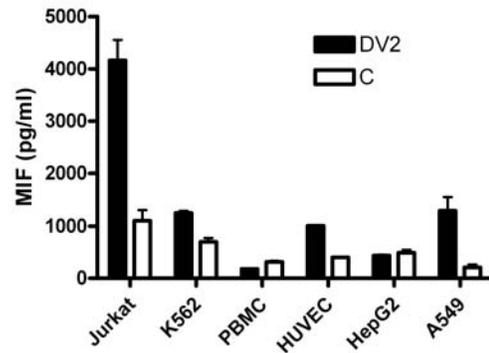

Fig. 6. DV2 infection in different types of cells induced different capacities to produce MIF. (A) Human Jurkat, K562 HepG2, and A549 cell lines, as well as primary PBMCs and HUVECs, were infected with DV2 at an MOI of 10. The levels of MIF in the supernatants were assayed at 48 h post-infection using ELISA

concentrations because they had previously proved to have the maximal effect without any cell toxicity in A549 cells[15, 16].

Different cells had different abilities to release MIF after DV2 infection: Because human mononuclear, endothelial, and hepatic cells are all permissive to DV2 infection in vitro, we tested whether DV2 infection of these cells induces MIF production. A significant increase in MIF release was found in human endothelial cells, HUVECs, K562 cells (a human erythroleukemia cell line, and Jurkat cells 48 h after DV2 infection. However, no significant increase of MIF release was found in HepG2 cells or PBMCs (Figure 6).

Antibodies against DV increased DV2 infection and MIF production in human immune cells: Because monocytes and macrophages are probably the primary targets of DV infection in vivo, and because antibodies against DV have been reported to increase DV infection, we tried to assess whether antibodies against DV in these cells increases MIF production. We first used the human monocytic cell line THP-1 and a pre M monoclonal antibody, 70-21, which has been reported to increase DV infection. After we double-stained THP-1 cells with antibodies against DV E antigen and MIF, we used flow cytometry to analyze the effects. We found a significant increase of DV and MIF double-positive cells in the presence of anti-pre M mAb but not DV infection alone (50.04% vs. 0.98%) (Figure 7A). We found a similar antibody-dependent increase in MIF production in PBMCs infected with
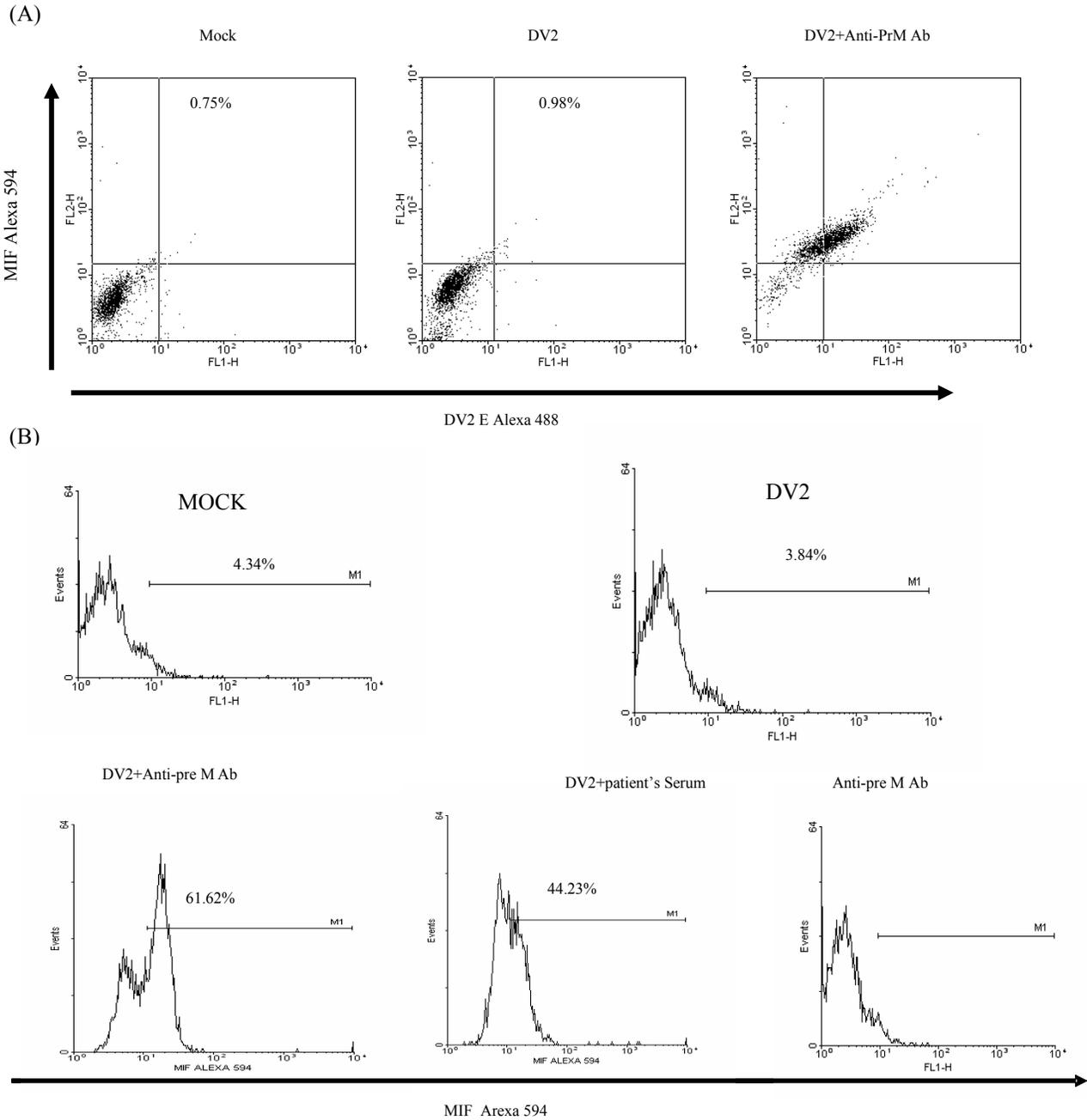
(A)

(B)

Fig. 7. Antibody-enhanced DV2 induced MIF production in THP1 cells and PBMCs. (A) THP-1 cells were infected with DV2 at an MOI of 10 in the presence of anti-pre M (70-21) (10 μg) antibody. The cells were then immunostained with anti-MIF (Alexa 594 conjugated secondary antibodies) and anti-DV-2 E (Alexa Fluor 488-conjugate secondary antibodies). (B) Human PBMCs were infected with DV2 at an MOI of 10 in the presence of diluted dengue type 3 immune serum (1:6000) or anti-pre M (70-21) (10 μg) antibody. The cells were then immunostained with anti-MIF (Alexa Fluor 594-conjugate secondary antibodies). Monocytes in PBMCs were gated and analyzed for antibody binding using flow cytometry as described in Materials and Methods.

DV2 in the presence of anti-pre M antibodies or serum from dengue patients (Figure 7B). Anti-pre M antibody alone, however, had no effect on MIF expression in PBMCs.

DISCUSSION

An increase in the serum level of MIF is correlated with the disease severity in a dengue patient. However, the cellular source and molecular mechanism involved in the induction of MIF during DV2 infection are unclear. In the present study, we investigated the ability and molecular mechanism of DV2 infection to induce MIF production in human epithelial A549 cells. We found that DV2 infection, but not UV-inactivated-DV2 stimulation, dose- and time-dependently induced MIF production, which indicated that active DV2 replication is required to induce MIF release. We also found that not all virus infections of A549 cells can induce MIF production: Japanese encephalitis virus (JEV) did not activate MIF gene promoter activity in A549 cells.

Activating MIF gene transcription requires the participation of several transcription factor complexes such as NF-κB[9]. The activation of NF-κB occurs via the phosphorylation and degradation of an inhibitory protein, IκB, which releases NF-κB from the cytoplasm and allows its translocation into the nucleus. The nuclear translocation of NF-κB p65 was evident in DV2-infected A549 cells, in marked contrast to the mock-infected cells, in which NF-κB p65 was predominantly in the cytoplasmic region. DV2-infection-induced MIF production is NF-κB-activation-dependent was further supported by the inhibition of MIF release in the presence of the NF-κB inhibitors dexamethasone and curcumin. Although JEV infection of A549 cells also activated NF-κB[13], we found no MIF promoter activation in JEV-infected A549 cells. Therefore, DV2-infection-induced NF-κB activation is required but not sufficient to turn on MIF gene expression.

We also found that DV2 infection, but not UV-inactivated-DV2 stimulation, dose-dependently induced MIF production in human endothelial cells, as it did in A549 cells. However, in cells from the human hepatoma HepG2 cell line, which is also permissive to DV2 infection[17], DV2 infection did not induce the release of MIF. Therefore, not all cells permissive to DV2 infection will release MIF after DV2 infection. Human blood mononuclear cells, which are thought to be the main target of DV2 in vivo, show a different susceptibility to DV2 infection and MIF production in

vitro. Both the Jurkat T cell line and the myelomonocytic K562 cell line are highly susceptible to DV2 infection and show high percentage of infection[18, 19]. However, the Jurkat cells release approximately four times more MIF than do K562 cells. This suggests that DV2 infection induces different amount MIF in different cells. Human monocytic THP-1 cell line and peripheral blood mononuclear cells, on the other hand, showed a smaller percentage of infection and no detectable MIF release in the absence of antibodies against DV2.

Antibody-increased DV2 infection in human macrophages in vitro has long been reported [5]. In the present study, we further showed that, in the presence of such antibodies, either a monoclonal pre M antibody or patient serum, MIF production was also upregulated in the DV2-infected cells. These results may explain why secondary infection with DV2 causes more severe DHF/DSS and why higher levels of MIF are found in these patients.

In summary, the present study shows that DV2 infection of cells induced MIF production in a NF-κB dependent manner. Further study to understand the mechanism and the role of MIF production in the immunopathogenesis of DHF/DSS may lead to the development of better therapeutic drugs against DHF/DSS.

ACKNOWLEDGEMENTS

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