MAJOR ARTICLE

# Mechanisms of Immune Evasion Induced by a Complex of Dengue Virus and Preexisting Enhancing Antibodies

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**We have found that dengue virus (DENV) not only uses preexisting enhancing antibodies to promote its entry into Fc receptor–bearing cells but also exploits enhancing antibodies for intracellular immune evasion through 2 mechanisms. In the first mechanism, entry of DENV-antibody complexes into human monocytic cells activates negative regulators, dihydroxyacetone kinase and autophagy-related 5–autophagy-related 12, which then disrupt the retinoic acide incucible gene I and melanoma differentiation associated gene 5 signaling cascade and disable type 1 interferon production, leading to suppression of interferon-mediated antiviral responses. In the second mechanism, the immune evasion was found to be mediated by the suppressive cytokine interleukin 10 (IL-10). High levels of IL-10 activated expression of suppressor of cytokine signaling 3 gene, which subsequently inactivated the Janus kinase–signal transducer and activator of transcription pathway. Inhibition of IL-10 production by small interfering RNA down-regulated suppressor of cytokine signaling 3 gene expression, restored inducible nitric oxide synthase gene expression, and suppressed DENV replication. Importantly, we were able to demonstrate that these 2 loops of suppression occurred in patients with severe secondary dengue infection (dengue hemorrhagic fever) but not in patients with mild secondary dengue infection (dengue fever).**

Dengue is caused by a single-stranded RNA virus, the dengue virus (DENV), which exists as 4 closely related serotypes. During the past decades, DENV have evolved into one of the world's major arthropod-borne viruses, a fact that is revealed by the dramatically increasing number of dengue cases not only in areas where the virus is endemic but also among travelers who have visited tropical regions outside of Africa [1–3]. This spike in dengue cases has been driven by factors facilitating the proliferation of the mosquito vector, which suggests that the epidemic of dengue will continue to expand in the absence of a protective dengue vaccine.

A life-threatening form of DENV infection, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), is strongly associated with heterotypic sequential DENV infections and a high level of circulating viruses [4, 5]. This suggests that DHF/DSS may be a result of preexisting immune mediators, which not only fail to neutralize but actually facilitate DENV infection. Examples of such activity are T cell dysfunction due to T cell antigenic sin and premature death of T cells, T-helper cell 2 phenotype biasing, cytokine storm, and the enhancing activity of subneutralizing heterotypic antibodies [6–9]. Interestingly, among these events, antibody-dependent enhancement of DENV infection is the only model that can explain the occurrence of DHF/DSS in the first dengue infection in infants. These infants are predisposed to DHF/DSS when the maternal titer of DENV antibody decreases below a protective level and before their own cellular immune responses have been fully developed. However, after the complete

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degradation of maternal antibodies, infants lose their enhanced susceptibility to DHF/DSS [10]. These observations strongly suggest that preexisting subneutralizing antibodies, in the absence of DENV-specific cellular immunity, are sufficient to induce DHF/DSS.

How the preexisting subneutralizing antibodies increase disease severity has not been fully elucidated. The severity of dengue is strongly associated with a high viral load, and enhancing antibodies are indeed one of the key mediators of increased viral production from Fc$\gamma$ receptor–bearing cells, the main target cell of DENV [11, 12]. This role played by enhancing antibodies is also demonstrated in other virus systems [13–15]. Preexisting antibodies enhance DENV entry into Fc receptor–bearing cells (a process designated "DENV antibody-dependent enhancement" [DENV-ADE]), resulting in increasing DENV-infected cell mass; therefore, more DENV virions are produced. This has been demonstrated in both in vitro and in vivo primate models [16, 17]. In addition to the extrinsic role of the antibodies, investigators have demonstrated that enhancement of virus infection by subneutralizing antibodies activates immunosuppressive mediators such as interleukin 10 (IL-10) and prostaglandin E2, but attenuates T-helper 1 cell mediators such as interleukin 12 and interferon $\gamma$ [18–20]. This immune modification may be the result of an alteration of intracellular signals that is induced by the virus-antibody complex infection. Supporting this notion is the observation that infection via DENV-antibody complexes inhibits signal transducer and activator of transcription 1 and interferon regulatory factor 1 activation, leading to suppression of nitric oxide radical production in THP-1 cells [18]. Taken together, these results suggest that infection by virus-antibody complexes may not only expedite deployment of viruses into target cells but may also alter some intracellular signaling pathways, resulting in their switching from antiviral mode into viral-facilitating mode.

In this study, experiments were built on the foundation of our earlier work: our finding that infection by DENV-subneutralizing antibody complexes suppresses the innate immune responses [18]. Therefore, the objective of the present work was to investigate the mechanisms of innate immune suppression during DENV-antibody complex infection. Experiments were performed using an in vitro model, THP-1 monocytic cells, and an ex vivo system, peripheral blood mononuclear cells (PBMCs) from naturally infected patients with dengue fever (DF) or dengue hemorrhagic fever (DHF). We were able to show that infection facilitated by subneutralizing antibodies preferentially activated the negative regulators of the innate response–stimulating pathways. These alterations divert intracellular machinery in Fc receptor–bearing monocytic cells toward an immunosuppressive mode, which in turn allows DENV to replicate freely.

## PATIENTS AND METHODS

***Clinical samples.*** Blood samples were obtained from DENV-infected patients at the Queen Sirikit National Institute of Child Health, Bangkok, Thailand. The protocol for patient enrollment was approved by the Committee on Human Rights Related to Human Experimentation of Mahidol University. Blood samples were collected twice for each patient: during the acute phase of disease, which was on the first day of hospitalization, and during the convalescent phase, for which samples were acquired on day 30 after the day of defervescence. Plasma and PBMCs were separated immediately and were frozen at −80°C. On the basis of severity, the disease was graded as either DF or DHF, according to World Heath Organization criteria [21].

Secondary infection was defined as a $\geqslant$4-fold increase in hemagglutination inhibition antibody titer against any DENV serotype in paired acute and convalescent serum samples and a dengue-specific ratio of immunoglobulin M to immunoglobulin G of <1.8 as measured using immunoglobulin M and immunoglobulin G capture enzyme-linked immunosorbent assay.

***THP-1 cell culture.*** THP-1 cells were obtained from the American Type Culture Collection (ATCC) Cell Biology Collection and were cultured as described elsewhere [18].

***Enhancing antibody.*** The enhancing antibody was convalescent serum from a patient infected with DENV serotype 3 [18].

***Antibody dependence of DENV infection in THP-1 cells.*** An antibody dependence test of DENV serotype 2 16681 was performed as described elsewhere [18]. Briefly, THP-1 cultures were infected with complexes of DENV serotype 2 16681 and a 1:10,000 dilution of enhancing antibody, or infected with DENV serotype 2 16681 with a multiplicity of infection of 0.01. The supernatants and infected cells were harvested every 24 h (or at the indicated time points) for 3 consecutive days and were subjected to detection of viral genome synthesis, IL-10 production, and interferon $\beta$ (IFN-$\beta$) production. Harvested cells were used for detection of melanoma differentiation associated gene 5 (MDA-5), retinoic acide incucible gene I (RIG-I), and other signaling molecules, using specific antibodies.

***Viral RNA copy-number titration by fluorogenic real-time reverse-transcription polymerase chain reaction.*** RNA was extracted from culture supernatants by means of a NucleoSpin RNA virus kit (Macherey-Nagel). The purified RNA was then subjected to reverse-transcription polymerase chain reaction by means of a QuantiTect probe kit (Qiagen), as described by Houng et al [22].

***Detection of cytokine production by enzyme-linked immunosorbent assay.*** Levels of IL-10 and IFN-$\beta$ production were quantitated using a Quantikine enzyme-linked immunosorbent assay kit (R&D Systems) according to the protocol recommended by the manufacturer.

***Detection of protein production by immunoblot assay.*** DENV-infected and DENV-ADE–infected THP-1 cells and PBMCs obtained from patients with secondary DF and from patients with secondary DHF were lysed, subjected to electrophoresis, and stained with specific monoclonal antibodies as described elsewhere [18].

***Inhibition of IL-10 gene expression by small interfering RNA.*** IL-10 gene silencing was performed using IL-10 SMARTpool (Dharmacon) according to the protocol recommended by the manufacturer and as described elsewhere [23]. Briefly, $3 \times 10^5$ THP-1 cells were cultivated for 18 h before incubation with 1 mL of transfection solution containing 100 nmol/L small interfering RNA and lipid conjugates at 37°C for 4 h. After 4 h of transfection, cultures were infected with the immune complex of DENV-enhancing antibody or were infected with DENV alone. DENV replication, IL-10 and suppressor of cytokine signaling 3 (SOCS-3) production, and inducible nitric oxide synthase (iNOS) gene expression were monitored at the genomic and proteomic levels. THP-1 cells with either DENV infection or DENV-ADE infection were included in this experiment.

***Complementary DNA array analysis of the Janus kinase–signal transducer and activator of transcription (JAK-STAT)–specific pathway.*** Labeled single-strand complementary DNA synthesized from RNA samples that were isolated from DENV-infected THP-1 cells, from DENV-antibody complex–infected THP-1 cells, and from mock-infected THP-1 cells were hybridized with the JAK-STAT pathway oligoneucleotide array (SABiosciences) according to the manufacturer's instructions. The hybridized membranes were autoradiographed. Array images were acquired and quantified in TIFF format by means of Image Master TotalLab software (version 2.00; Amersham Pharmacia). Densities of hybridized spots were analyzed using AtlasImage software (version 2.7; ClonTech). The densities of the spots were normalized to the expression of the housekeeping gene $\beta$2-microglobulin before being subjected to statistical analysis. The statistically significant changes in transcriptional level were determined on the basis of 2-fold cutoff criteria and results of the Student *t* test. The differences in gene expression levels were further validated by means of semiquantitative reverse-transcription polymerase chain reaction.

***Statistical analysis.*** Values are expressed as means ± standard deviations of the 3 independent observations. The differences were tested using the Student *t* test, with results for which $P < .05$ considered to be statistically significant.

## RESULTS

***Infection by DENV-subneutralizing antibody complexes suppressing IFN-$\beta$ production while up-regulating IL-10 synthesis.*** As demonstrated earlier by our group, infection by DENV-immune complexes suppresses production of secondary antiviral responses [18]. We then questioned whether this suppression was mediated by the immunosuppressive cytokine IL-10 and/or by suppression of other first-line defenses such as type 1 interferon (IFN). To answer this question, DENV replication by subneutralizing antibodies was enhanced in THP-1 cells. As shown in Figure 1*A*, infection with virus and antibody complexes facilitated DENV genome synthesis ($P <$ .05). This process was partially blocked by anti-CD32 antibody but not by anti-CD64 antibody, which suggests that this enhancing phenomenon occurred partly through the Fc$\gamma$ receptor IIA entry site.

To determine whether increasing DENV replication was due to the suppression of antiviral mediators, levels of IFN-$\beta$ and IL-10 production were monitored using enzyme-linked immunosorbent assay. We found that the production level of IFN-$\beta$ was statistically significantly suppressed within 6 h after DENV-ADE infection (Figure 1*B*). In contrast, the level of IL-10 expression gradually increased within 6 h and statistically significantly up-regulated within 18 h, and the production level of this cytokine was dramatically increased by 24 h (Figure 1*C* and 1*D*). Treatment with a 1:10,000 dilution of antibody had no effect on IFN-$\beta$ and IL-10 production levels in cells (data not shown). These data suggest that infection via DENV-immune complexes diminished the first-line intracellular antiviral defense while promoting early production of immunosuppressive mediators.

***Signaling responses required for IFN-$\beta$ transcription disrupted by DENV-ADE infection.*** Viral replication requires successful negotiation and/or negative regulation of innate responses in order to bypass cellular regulatory mechanisms [24]. Although several viruses block the signaling pathway that directs IFN transcription, some viruses choose to disrupt specific signaling molecules required for IFN transcription [25]. During DENV infection, expression of the IFN-$\beta$ gene is activated through the interferon $\beta$ promoter stimulator protein 1 (IPS-1)–regulated pathway [26, 27]. Given that production of IFN-$\beta$ was strongly suppressed during DENV-ADE infection, we then asked whether decreasing IFN-$\beta$ production was due to suppression of the RIG-I or MDA-5 pathways or due to specific blocking of some of the signaling molecules along these pathways. To answer this question, the expression levels of RIG-I, MDA-5, and the downstream signaling molecules (ie, IPS-1, tumor necrosis factor receptor associated factor 3, IKK-related kinase $\epsilon$, tank binding kinase 1, and interferon regulatory factor 3) in THP-1 cells infected with DENV-antibody complexes were compared with the expression levels of these molecules in cells infected with DENV alone. As shown in Figure 2*A* and 2*B*, expression levels of MDA-5 and RIG-I were statistically significantly increased in cells infected with DENV alone, whereas very low expression levels were found in cells with DENV-ADE infection. Similar results were demonstrated for the expression

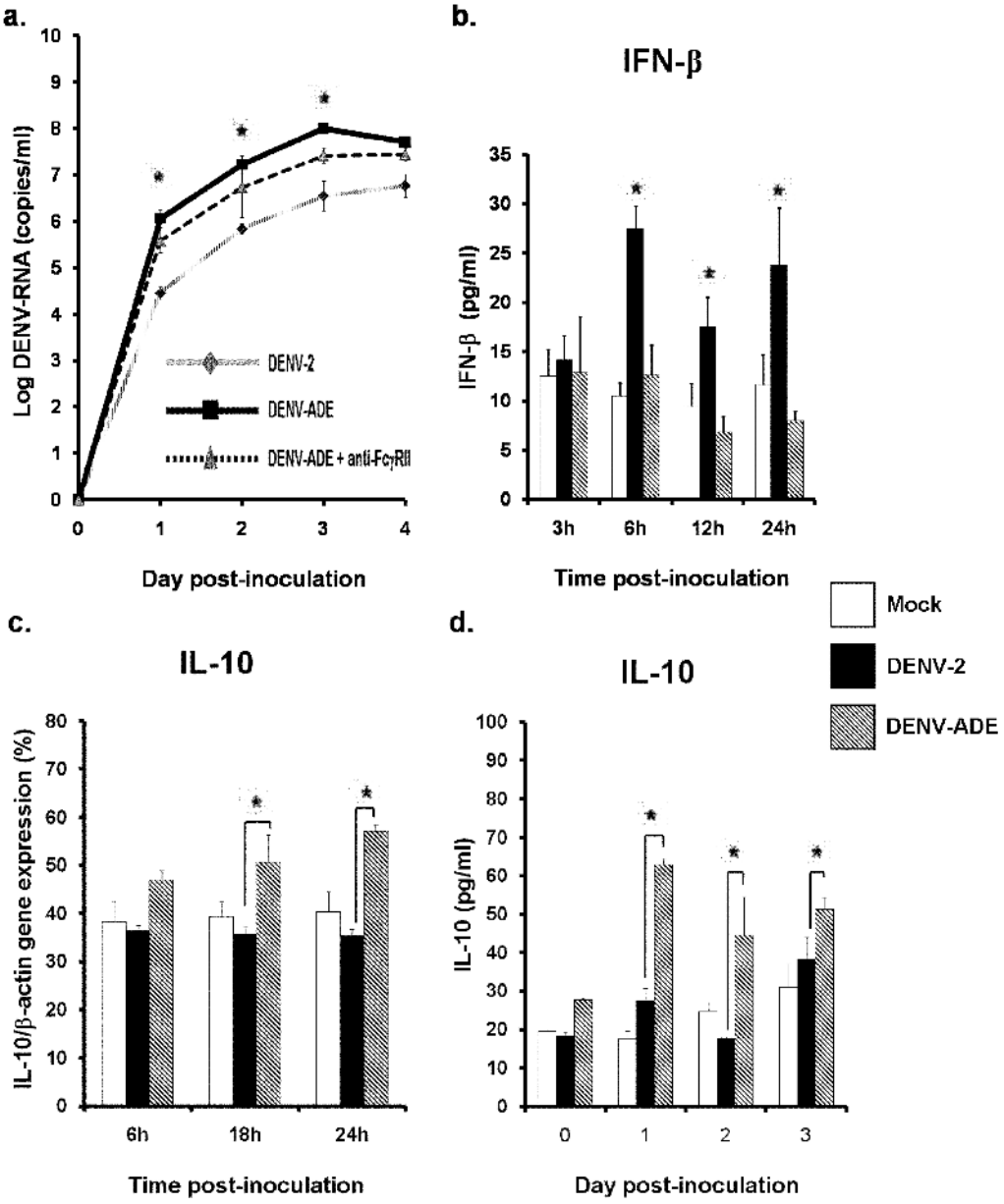


**Figure 1.** Antibody-dependent enhancement of dengue virus (DENV) infection mediated via Fc$\gamma$ receptor II (Fc$\gamma$RII) on THP-1 cells. *A*, Viral load of THP-1 cells infected with DENV or DENV-antibody complexes in the presence or absence of 100 $\mu$g of anti-CD32 antibody. Numbers of viruses in supernatants were quantified on the basis of RNA copies by use of quantitative reverse-transcription polymerase chain reaction (RT-PCR). DENV antibody-dependent enhancement (DENV-ADE) infection suppressed innate immune responses. *B*, *C*, Levels of interferon $\beta$ (IFN-$\beta$) and interleukin 10 (IL-10), respectively, in supernatants of DENV-ADE–infected THP-1 cultures, of THP-1 cells infected with DENV alone, or of mock-infected THP-1 cells, quantified by enzyme-linked immunosorbent assay. *D*, Levels of IL-10 gene expression at various time points, quantified by RT-PCR. DENV-2, dengue virus serotype 2.

levels of IPS-1 and downstream signaling molecules, in which infection with DENV-ADE complexes did not stimulate expression of these molecules but infection with DENV alone did (Figure 3). These data suggest that DENV-ADE infection attenuated the helicase sensors, resulting in inhibition of type 1 IFN production.

***Stimulation of negative regulators of helicase sensors during DENV-ADE infection.*** Expression of RIG-I, MDA-5, and the downstream signaling molecules are regulated by several negative regulators [28]. To further dissect the suppressive mechanism of IFN-$\beta$ production, expression levels of type 1 IFN negative regulators, including laboratory of genetic and phys-

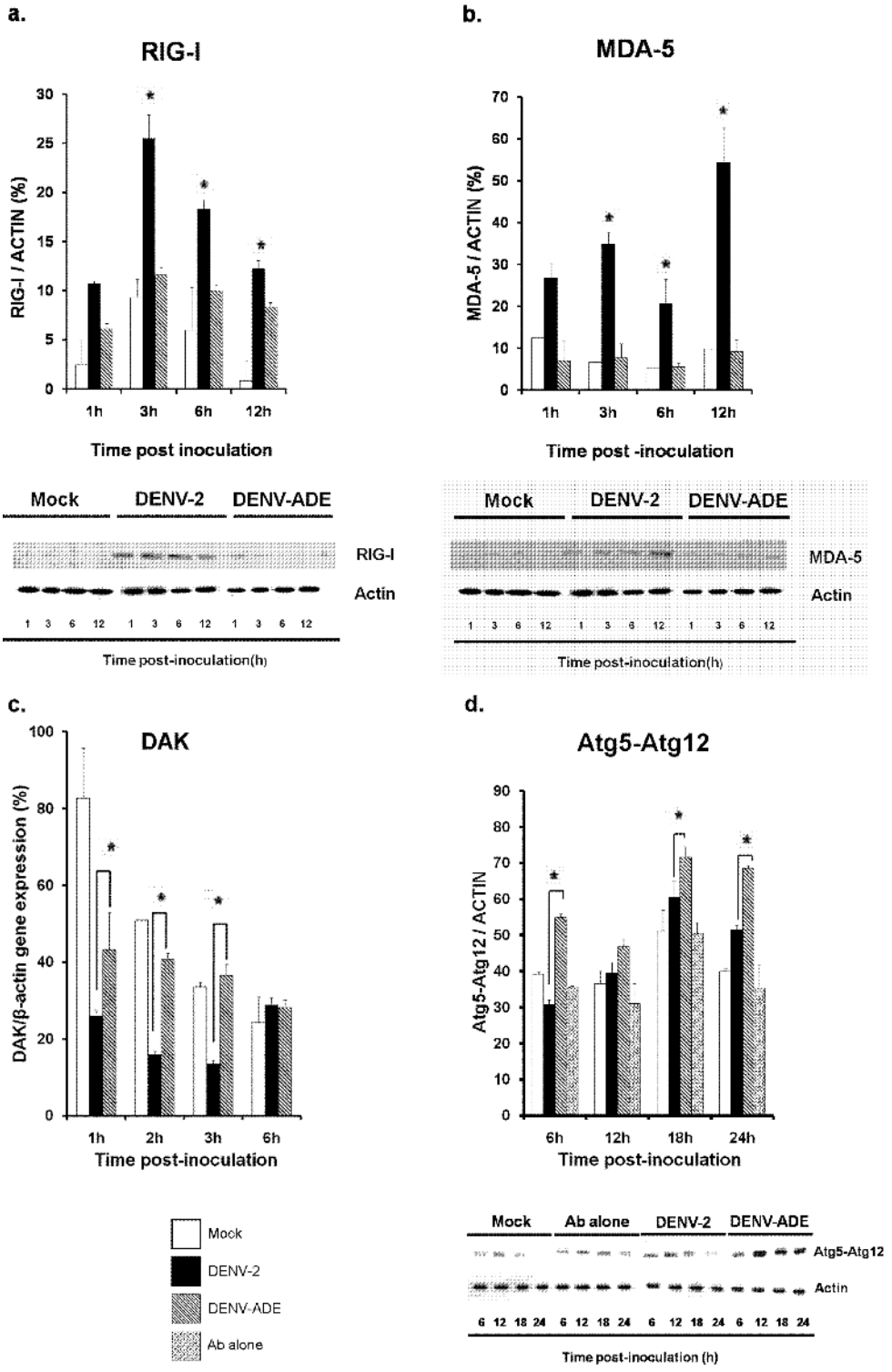


**Figure 2.** Suppression of retinoic acide incucible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) signaling pathways during dengue virus (DENV) antibody-dependent enhancement (DENV-ADE) infection but not during DENV infection. THP-1 cells were infected with DENV alone, infected with DENV-antibody complexes, or mock infected. Lysates of infected cells were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and stained with specific antibodies. Densities of protein bands were determined using a densitometer. Levels of RIG-I and MDA-5 expression are expressed as percentages relative to $\beta$-actin. *A*, *B*, Expression levels of RIG-I and MDA-5 negative regulators during DENV-antibody complex infection. Expression levels of RIG-I and MDA-5 were determined using specific antibodies. THP-1 cells were infected with DENV alone, infected with DENV-enhancing antibody complexes, or mock infected. Expression levels of the dihydroxyacetone kinase (DAK) gene (*C*) and production levels of autophagy-related 5–autophagy-related 12 (Atg5-Atg12) protein (*D*) were determined at indicated time points by use of semiquantitative reverse-transcription polymerase chain reaction and specific antibodies, respectively. Levels of expression are expressed as percentages relative to $\beta$-actin. Ab, antibody; DENV-2, dengue virus serotype 2.

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

**Figure 3.** Suppression of interferon $\beta$ promoter stimulator protein 1 (IPS-1), tumor necrosis factor receptor–associated factor 3 (TRAF-3), IKK-related kinase $\epsilon$ (IKK-i), tank binding kinase 1 (TBK-1), and phosphorylated interferon regulatory factor 3 (IRF-3) during dengue virus (DENV) antibody-dependent enhancement (DENV-ADE) infection but not during DENV infection.

iology 2, dihydroxyacetone kinase (DAK), ring finger protein 125, and autophagy-related 5–autophagy-related 12 (Atg5-Atg12), were monitored and compared between mock-infected THP-1 cells, DENV-ADE–infected cells, and cells infected with DENV alone. We found that DENV infection and DENV-ADE infection had no effect on the expression levels of laboratory of genetic and physiology 2 and ring finger protein 125 (data not shown). In contrast, the production level of Atg5-Atg12 was statistically significantly up-regulated during DENV-ADE infection. DAK gene expression was suppressed in DENV infection but not in DENV-ADE infection (Figure 2*C* and 2*D*). These data indicate that infection with DENV-antibody complexes specifically up-regulates the negative regulators, whereas infection with DENV alone tends to suppress these regulators.

***Suppression of RIG-I and MDA-5 signaling pathways in patients with secondary DHF.*** Because a monocytic cell line such as THP-1 may not be an ideal physiological model of the native responses, PBMCs obtained from DENV-infected patients were used to confirm the phenomenon observed in THP-1 cells. Expression levels of RIG-I, MDA-5, and IPS-1 were determined in PBMCs from patients with secondary DF and in PBMCs from patients with secondary DHF. As demonstrated in Figure 4*A*–4*C*, expression levels of RIG-I, MDA-5, and IPS-1 were statistically significantly suppressed in PBMCs from patients with secondary DHF, whereas these molecules were induced in PBMCs from patients with DF. This finding was supported by the plasma level of IFN-$\beta$, demonstrated in Figure 4*D*, in which patients with DF synthesized higher amounts of IFN-$\beta$ ($P < .05$) than did patients with DHF. These data suggest that suppression of interferon production in patients with DHF may partly occur via inhibition of the RIG-I and MDA-5 signaling pathways, which corroborates the phenomenon demonstrated in the THP-1 system.

***IL-10 produced from DENV-ADE infection as a suppressor of the JAK-STAT signaling pathway.*** During DENV-ADE infection, IL-10 production was rapidly induced (Figure 1*C*). We hypothesized that a high level of IL-10 may suppress the JAK-STAT signaling pathway through SOCS-3 activities, resulting in down-regulation of secondary antiviral responses, such as nitric oxide radical production [29]. To determine whether IL-10 could block the JAK-STAT signaling pathway during DENV-ADE infection, we first investigated whether up-regulation of IL-10 was accompanied by increased production of SOCS-3 protein. As illustrated in Figure 5*A* and 5*B*, the level of suppressor of cytokine signaling 1 (SOCS-1) gene expression and the level of SOCS-3 production increased 3 and 6 h after infection, respectively, in THP-1 cells infected with DENV-antibody complexes but not in cells infected with DENV alone. These data imply that IL-10, at least in part, activated the suppressor of cytokine signaling (SOCS) system, especially SOCS-3. Moreover, PBMCs obtained from patients with secondary DHF expressed SOCS-3 more strongly than did PBMCs from patients with DF (Figure 5*C*).

To confirm the immunosuppressive role of IL-10, IL-10 production during DENV-ADE infection was inhibited using small interfering RNA specific to the IL-10 gene. Then the levels of IL-10 and SOCS-3 production and of iNOS gene expression were monitored. As shown in Figure 6*A*–6*D*, THP-1 cells infected with DENV-antibody complexes and treated with small interfering RNA showed a statistically significant decrease in IL-10 gene expression level, IL-10 production level, and SOCS-3 protein synthesis level, whereas iNOS gene expression level increased. These data indicate that IL-10 produced during DENV-antibody complex infection exerted an immunosuppressive effect through the SOCS system, which may then have inhibited the JAK-STAT pathway.

To further confirm the immunosuppressive role of IL-10 during DENV-ADE infection, the replication efficiency of DENV in THP-1 cells infected with DENV-antibody complexes in the presence or absence of small interfering RNA specific for IL-10 was monitored. As shown in Figure 6*E*, DENV-ADE infection statistically significantly facilitated replication of DENV from day 1 of infection onward. However, inhibition of IL-10 production by small interfering RNA treatment statistically significantly reduced DENV-RNA synthesis within 24 h. This suppression could be detected up to 48 h after infection. THP-1 cells infected with DENV alone and treated with small interfering RNA specific to IL-10 showed no effect on DENV replication (data not shown). These data indicate that IL-10 facilitated DENV replication, possibly through suppression of intracellular antiviral response.

***Up-regulation of several JAK-STAT inhibitors during DENV-ADE infection revealed by complementary DNA array screening of the JAK-STAT pathway.*** To further confirm the down-regulation of the JAK-STAT pathway during DENV-ADE infection, complementary DNA array expression of the JAK-STAT signaling pathway was performed. Expression levels of 18 of 128 genes present on the array were statistically significantly altered. Among the altered genes, protein inhibitor of activated signal transducer and activator of transcription 1,

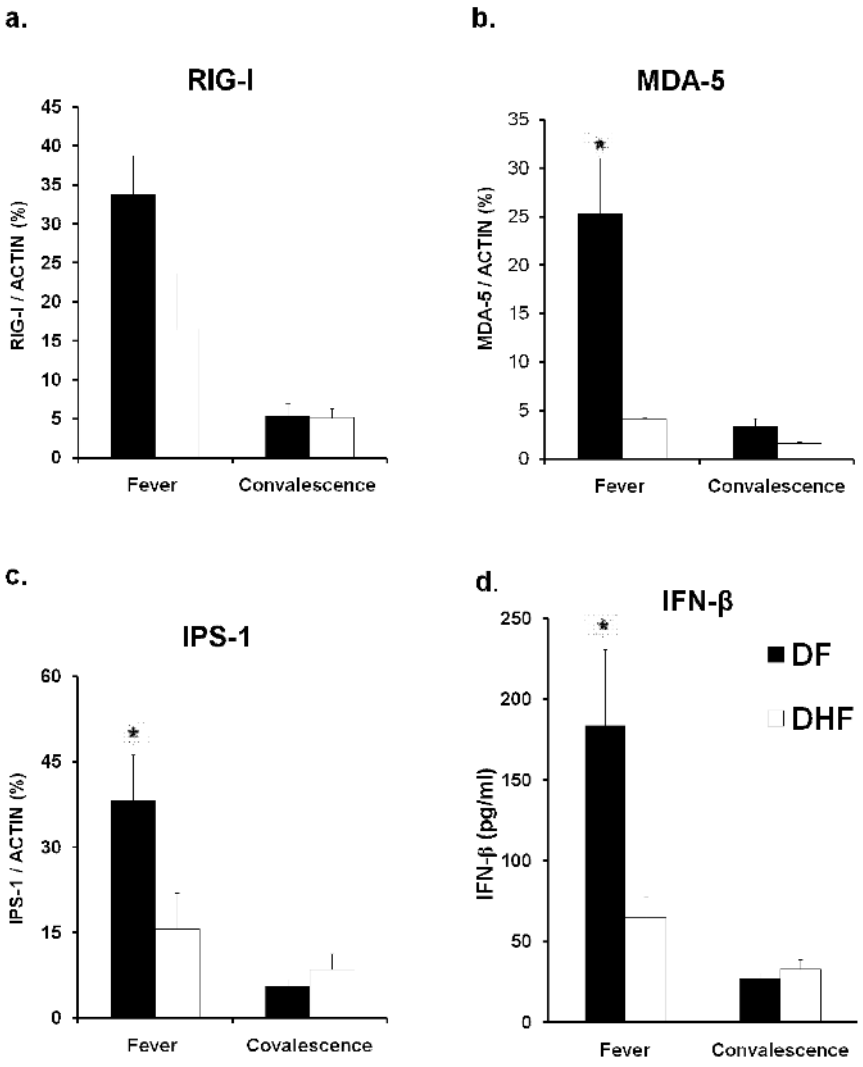


**Figure 4.** *A–C*, Retinoic acide incucible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA-5), and interferon $\beta$ promoter stimulator protein 1 (IPS-1) expression, respectively, suppressed in peripheral blood mononuclear cells (PBMCs) obtained from patients with acute or convalescent secondary dengue fever (DF) or dengue hemorrhagic fever (DHF). The day −4 acute phase PBMCs obtained from patients with secondary DF or from patients with secondary DHF ($n = 4$ for each group) were lysed and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Levels of RIG-I, MDA-5, and IPS-1 were determined using specific antibodies. *D*, Levels of plasma interferon $\beta$ (IFN-$\beta$) measured on serum samples from patients with acute or convalescent secondary DF or DHF ($n = 15$ for each group).

specificity protein 1 transcription factor, C-reactive protein, colony-stimulating factor 2 receptor $\beta$, IL-10 receptor, SOCS-1, SOCS-3, and oncostatin M are known to either directly or indirectly inhibit the JAK-STAT pathway (Table 1). Up-regulation of these genes was validated by means of reverse-transcription polymerase chain reaction and found to confirm the complementary DNA array data (Figure 7). One of the altered genes, interferon regulatory factor 1, was down-regulated during DENV-ADE infection. Inactivation of interferon regulatory factor 1 gene expression supports the notion that the JAK-STAT pathway was down-regulated. These data suggest that DENV-antibody complex infection attenuated the JAK-STAT signaling pathway, which is in agreement with the role of IL-10 mentioned above.

## DISCUSSION

In this study, we found that DENV complexed with preexisting antibodies and then penetrated monocytic THP-1 cells via CD32 ligation. This ligation not only is a critical part of the entry process but also plays a vital role in antiviral evasion. Antibodies are indicators of a properly functioning adaptive immune response, meaning that infectious agents that are recognized by the antibodies will be cleared and an immune mem-

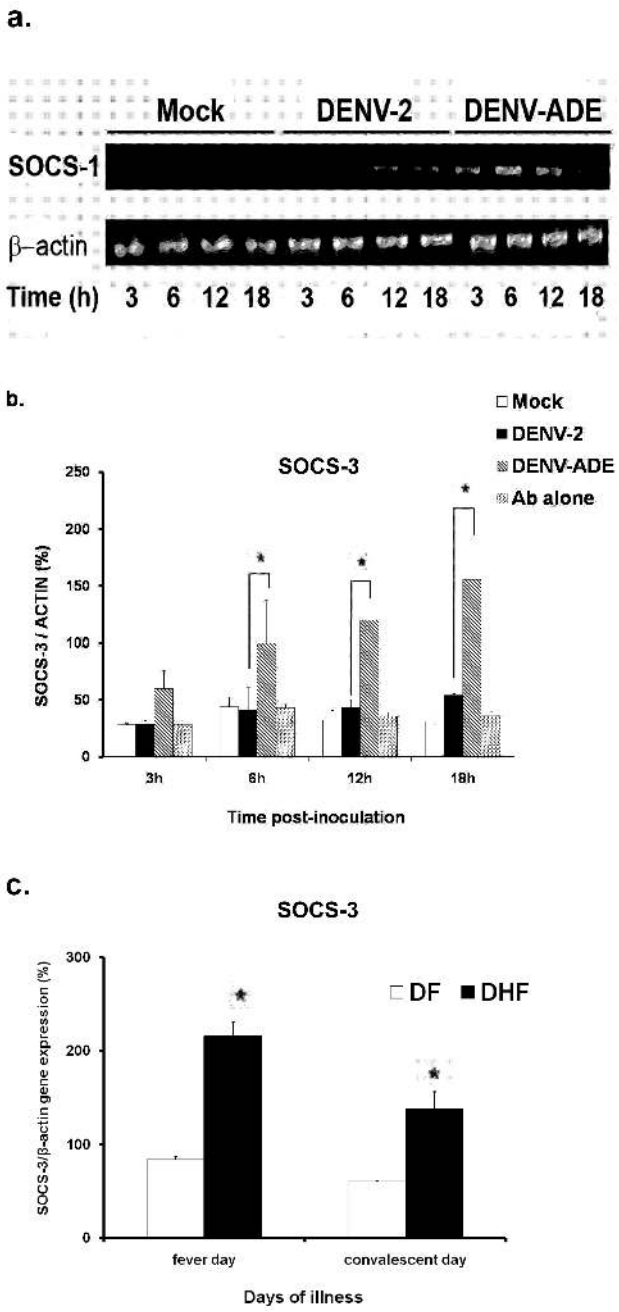


**Figure 5.** Up-regulation of the suppressor of cytokine signaling system during infection with dengue virus (DENV)–enhancing antibody complexes. THP-1 cells were either infected with DENV-antibody complexes, infected with DENV alone, or mock infected. The expression of suppressor of cytokine signaling 1 (SOCS-1) was monitored using reverse-transcription polymerase chain reaction (RT-PCR) (*A*), whereas suppressor of cytokine signaling 3 (SOCS-3) production was shown by use of specific antibody (*B*). Peripheral blood mononuclear cells (PBMCs) obtained from patients with secondary dengue fever (DF) or from patients with secondary dengue hemorrhagic fever (DHF) strongly produced SOCS-3 protein (*C*). PBMCs were obtained from patients on fever day and day 30 after defervescence ($n = 4$ for each group). Levels of SOCS-3 gene expression were determined by semiquantitative RT-PCR. Ab, antibody; DENV-2, dengue virus serotype 2; DENV-ADE, dengue virus antibody-dependent enhancement.

ory will be established. However, this traditional view of antibodies has been reconsidered because several infectious agents exploit antibodies for more efficient infection [30–32]. The discovery of the enhancing activity of antibodies has given rise to a worldwide concern regarding vaccine development and antibody-based treatment [33]. Therefore, it is important to understand the intracellular alterations that occur in virus-antibody complex infections and to be able to link these alterations to the exacerbation of disease.

Human cells are equipped with 2 distinct innate immune mechanisms that detect viral infection and trigger inductions of type 1 interferon and proinflammatory cytokines [34]. Indeed, the effectiveness of these responses has led many viruses to develop specific mechanisms that antagonize the production and/or the action of this first line of defense in order to replicate effectively. For example, Nipah virus and herpes simplex virus inhibit IFN signaling through specific interaction with signal transducer and activator of transcription and interferon regulatory factor 3, respectively, whereas Japanese encephalitis virus and tickborne encephalitis virus are able to block signal transducer and activator of transcription 1 phosphorylation [35–38]. DENV is able to antagonize IFN signaling, via either preventing signal transducer and activator of transcription 1 phosphorylation or inducing signal transducer and activator of transcription 2 degradation [39, 40]. In this study, we were able to show that DENV used preexisting antibodies to subvert IFN production, which in turn inhibited IFN-stimulated antiviral responses. This subversion of type 1 IFN production was traced back and found to be due to a high level of DAK and Atg5-Atg12 upon entry of DENV-antibody complexes into target cells. These regulators negatively regulated the RIG-I and MDA-5 signaling pathways. To our knowledge, this is the first study to show the mechanism by which DENV uses preexisting antibodies to evade the innate immune response. Importantly, this study also showed that this mechanism of innate immune evasion also occurred in natural DENV infection, because levels of IFN-$\beta$, RIG-I, MDA-5, and IPS-1 were statistically significantly suppressed in PBMCs obtained from patients with secondary DHF but not in PBMCs from patients with secondary DF.

To ensure a global intracellular innate-immune evasion, infection with virus-antibody complexes also amplified its suppressive effect on another mode of innate response, using IL-10 as a mediator. IL-10 is known to induce T-helper cell 2 cytokine response, diminish IFN-$\gamma$ production, and attenuate iNOS activity [41, 42]. These activities of IL-10 explain the strong association between high levels of IL-10 and increasing disease severity during infection by various types of pathogens [43, 44]. One of them is infection with *Leishmania* amastigote via Fc receptor on macrophages, which induces a high level of IL-10 production, leading to decreases in IFN-$\gamma$ and nitric oxide

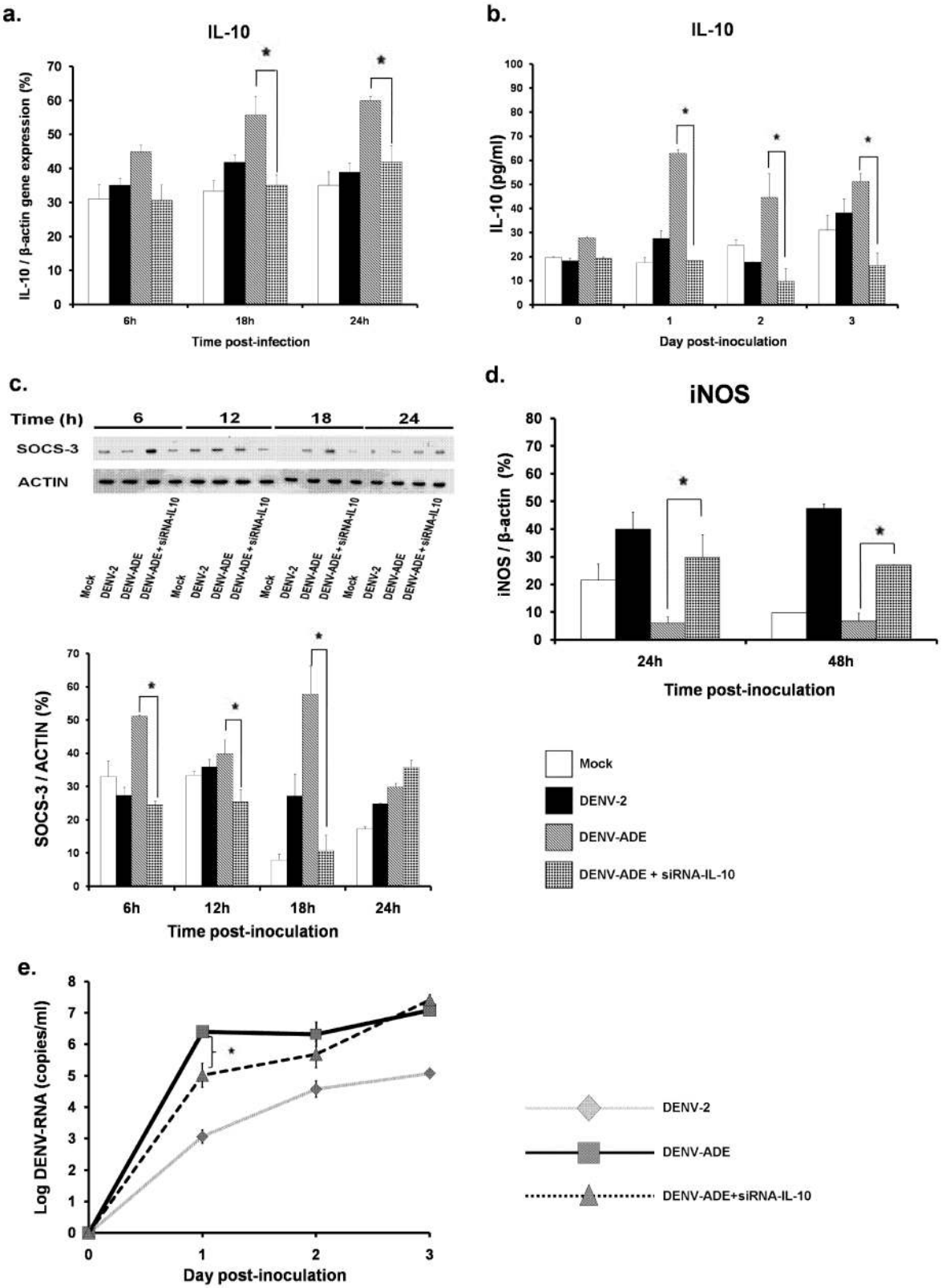


**Figure 6.** Inhibition of interleukin 10 (IL-10) production by small interfering RNA (siRNA). IL-10 inhibition decreased production of suppressor of cytokine signaling 3 (SOCS-3), and levels of nitric oxide radicals were increased. THP-1 cells were transfected with siRNA specific to IL-10 before being infected with dengue virus (DENV)–antibody complexes, infected with DENV alone, or mock infected. Level of IL-10 gene expression (*A*), production of IL-10 (*B*), production of SOCS-3 (*C*), levels of nitric oxide gene expression (*D*), and kinetics of DENV genome synthesis (*E*) were monitored. DENV-2, dengue virus serotype 2; DENV-ADE, dengue virus antibody-dependent enhancement; iNOS, inducible nitric oxide synthase.

**Table 1. Genes with Altered Expression Levels during Dengue Virus Antibody-Dependent Enhancement (DENV-ADE) Infection**

| Expression change, gene | Fold change ± SD | | | Function | Reference(s) |
|---|---|---|---|---|---|
| | DENV vs control | DENV-ADE vs control | DENV-ADE vs DENV | | |
| Up-regulation | | | | | |
| CCAAT/enhancer binding protein (C/EBP) β (*CEPBP*) | 0.82 ± 0.20 | 2.06 ± 1.62 | 2.50 ± 1.82 | Transcription factor required for CRP regulation in response to IL-6, transcription factor required for IL-10 production | 51, 52 |
| C-reactive protein (*CRP*) | 0.72 ± 0.5 | 3.77 ± 3.23 | 4.65 ± 1.16 | Induction of anti-inflammatory responses in human monocyte | 53, 54 |
| Colony-stimulating factor 2 receptor β (*CSF2RB*) | 1.02 ± 0.88 | 4.40 ± 1.38 | 4.40 ± 1.91 | Regulator of Th2 immunity | 55 |
| Interleukin 10 receptor α (*IL10RA*) | 0.96 ± 0.50 | 4.42 ± 1.01 | 4.53 ± 2.96 | Receptor for IL-10, regulation of inflammation | 56, 57 |
| Oncostatin M (*OSM*) | 0.36 ± 0.06 | 3.95 ± 1.03 | 10.72 ± 1.22 | Activation of SOCS-3 expression | 58 |
| Protein inhibitor of activated *STAT1* (*PIAS1*) | 1.18 ± 0.24 | 15.00 ± 7.66 | 12.35 ± 3.96 | Negative regulator of STAT-1, repression of NF-κB p65 | 59, 60 |
| Suppressor of cytokine signaling 1 (*SOCS1*) | 0.75 ± 0.04 | 1.25 ± 0.19 | 1.91 ± 1.30 | Inhibition of type 1 and type 2 IFN signaling | 61–63 |
| Suppressor of cytokine signaling 3 (*SOCS3*) | 0.56 ± 0.10 | 0.92 ± 0.02 | 1.58 ± 0.19 | Inhibition of type 1 and type 2 IFN signaling | 64, 65 |
| Sp1 transcription factor (*SP1*) | 0.73 ± 0.09 | 1.80 ± 0.43 | 2.60 ± 0.14 | Transcription factor required for IL-10 production | 66, 67 |
| Down-regulation | | | | | |
| Interferon regulatory factor 1 (*IRF1*) | 2.27 ± 1.59 | 1.06 ± 0.76 | 2.27 ± 1.08 | Interaction with STAT-1 leads to activation of antiviral response | 68 |

**NOTE.** CCAAT, cytidine-cytidine-adenosine-adenosine-thymidine box motif; CRP, C-reactive protein; DENV, dengue virus; IFN, interferon; IL-6, interleukin 6; IL-10, interleukin 10; NF-κB, nuclear factor κB; SD, standard deviation; SOCS-3, suppressor of cytokine signaling 3; Sp1, specificity protein 1; STAT-1, signal transducer and activator of transcription 1; Th2, T-helper 2 cells.

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases*.

**Figure 7.** Validation of Janus kinase–signal transducer and activator of transcription pathway–specific screening.

production, which subsequently results in inability to resolve infection in both human and mouse models [45, 46]. A similar phenomenon may occur in patients with secondary DHF/DSS, because these patients have statistically significantly higher IL-10 levels than those in patients with DF [47, 48]. In the present study, we found that DENV-ADE infection induced early IL-10 production in accordance with SOCS activation. SOCS can be activated by IL-10 and others, such as oncostatin M, to inhibit the JAK-STAT pathway. This role of IL-10 was confirmed by means of treatment with small interfering RNA, in which inhibition of IL-10 production restored the synthesis of nitric oxide radicals through attenuation of SOCS-3. Restoration of nitric oxide radical production then suppressed DENV gene synthesis. This loop of immune suppression during DENV-ADE infection was further confirmed by means of JAK-STAT-specific pathway array, in which we found that DENV-ADE infection up-regulated transcription factors of IL-10 and IL-10 receptor genes, increased expression of negative regulators of JAK-STAT (protein inhibitor of activated signal transducer and activator of transcription 1, SOCS-3, and SOCS-1), and increased expression of the activator of the SOCS system. However, this role of IL-10 may depend on the cell system, because DENV-ADE infection of dendritic cells did not up-regulate IL-10 production [49].

In conclusion, our present study demonstrates that DENV-ADE infections circumvent the intracellular antiviral state of the host cell via 2 related immunosuppressive loops. The first loop is mediated through negative regulators of the Toll-like receptor–independent pathway, which results in IFN-$\beta$ suppression (Figure 8). This immune suppressive state is further amplified by the IL-10 autocrine, which leads to suppression of secondary antiviral responses, such as synthesis of nitric oxide radicals. The synergism between intracellular antiviral suppression and the extrinsic role of enhancing antibodies on viral uptake is responsible for an observed increase in viral load in infected cells in vitro and possibly also in vivo in patients with DHF/DSS. Moreover, the phenomenon found in the present study also implies that DENV-ADE infection has the potential to stimulate expression of other host genes that assist in the survival of DENV.

How do these enhancing antibody-mediated activities help in understanding the pathophysiological changes that occur during DHF/DSS infection? Certainly, a high level of viremia, a low level of type 1 IFN production, an increase in production

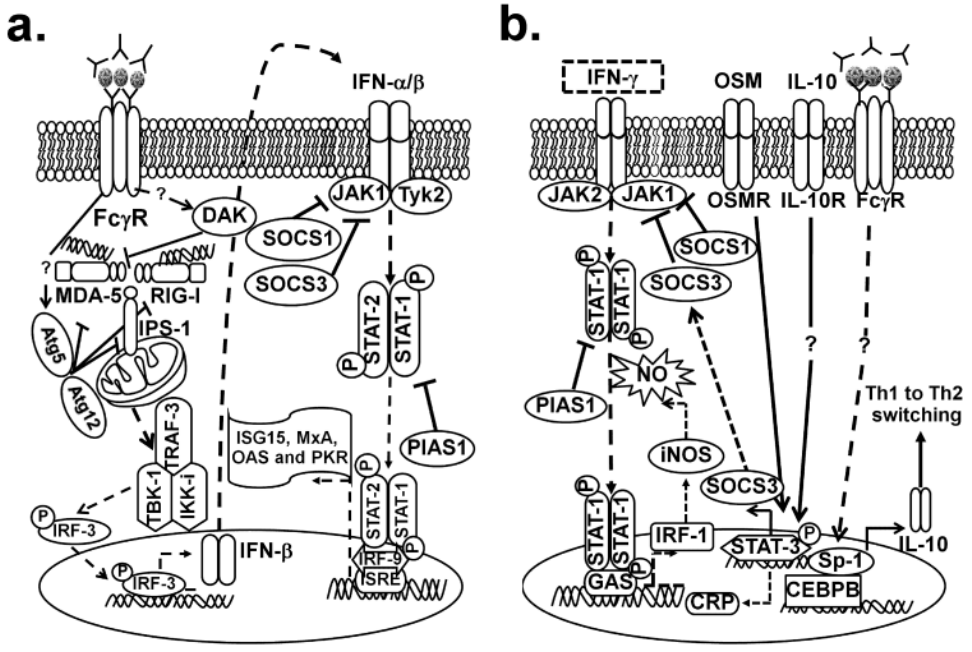


**Figure 8.** Model illustrating 2 antiviral evasion loops stimulated by infection with complexes of dengue virus–enhancing antibody. *A*, Evasion of type 1 interferon production and suppression of interferon-stimulated antiviral mediators. *B*, Down-regulation of anti–dengue virus responses mediated by interleukin 10 (IL-10), a suppressive cytokine. Atg5-Atg12, autophagy-related 5–autophagy-related 12; DAK, dihydroxy acetone kinase; IKK-i, IKK-related kinase $\epsilon$; MDA-5, melanoma differentiation–associated gene 5; RIG-I, retinoic acide incucible gene I; STAT, signal transducer and activator of transduction; TRAF3, tumor necrosis factor receptor–associated factor 3.

of IL-10, and a T-helper cell 2 response biasing are prevalent in patients with secondary DHF, in contrast to patients with secondary DF [5, 7, 50]. Indeed, all of these phenomena are dominant in antibody-dependent enhancement of DENV infection, as shown in the present study.

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