Impairment of CD4$^+$ T Cell Polarization by Dengue Virus–Infected Dendritic Cells

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Background. The production of type I interferon alpha/beta (IFN-α/β) is crucial to viral clearance during dengue virus (DENV) infection; however, in vitro–infected dendritic cells (DCs) exhibit a decreased capacity to respond to IFN-α/β stimulation, and antigen-presenting cells (APCs) isolated from patients with acute DENV infection exhibit defects in T cell priming.

Methods. In order to ascertain the stimulatory capacity of primary human monocyte-derived DCs infected with wild-type DENV isolates, representing a range of genotypes and disease outcomes, we cocultured infected DCs with allogeneic-naive CD4$^+$ T cells. The gene expression patterns of IFN-α/β sensitive genes were quantitated to determine if the infected DCs displayed a blunted IFN-α/β response.

Results. DENV-infected DCs induced the initial proliferation of naive CD4$^+$ T cells but they remained nonpolarized in effector function. The expression of IFN-α/β–stimulated genes was downregulated, revealing that the inhibition of IFN-α/β signaling is conserved among endemic DENV serotype 2 strains.

Conclusions. The failure of naive CD4$^+$ T cells to differentiate into IFN gamma-producing effector T cells when primed by DENV-infected DCs cannot be explained solely by a block in IFN-α/β signaling, suggesting that the ability of DENV to evade the early host response is multifaceted.

Infection by dengue virus (DENV), a member of the *Flaviviridae* family, may result in benign courses such as dengue fever (DF) or life-threatening presentations defined as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DENV is endemic in most of the world's tropical and subtropical areas and causes an estimated 50–100 million infections annually worldwide [1]. Dengue illness presents during acute infection, when viremia levels are high or declining and prior to the antibody response; therefore, innate immunity plays an important role in fighting infection, and several lines of evidence implicate a key role for type I interferon alpha/beta (IFN-α/β) in protection against DENV infection—specifically: (1) hospitalized children with DF and DHF contain high levels of IFN-α in the serum [2], (2) mice lacking both IFN-α/β and IFN-γ receptors succumb to primary DENV infection, whereas their wild-type counterparts survive [3, 4], and (3) DENV-infected human monocytes and monocyte-derived dendritic cells (DCs) secrete IFN-α/β [5–12], which protects against de novo infection [6, 7].

DENV infects and replicates in human DCs [13–15] and has been shown to block IFN-α/β signaling in infected DCs [16]. The ability of DENV to block IFN-α/β signaling was initially observed when DENV was allowed to replicate prior to IFN-α/β treatment, whereas incubation of cells with IFN-α/β protected them from de novo DENV infection [7]. Several DENV nonstructural proteins have been shown to reduce the expression of major components of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway by blocking their phosphorylation or enhancing their degradation [17–21].

DENV antagonism of IFN-α/β signaling, an important component of the host's innate immune response,

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could hamper the immunostimulatory capacity of infected cells. IFN-α/β acts directly on DCs by inducing DC cytokine production, which is necessary for the generation of a $CD4^+$ T-helper cell (Th) effector response [22, 23]. $CD4^+$ T cells that are exposed briefly to antigen can proliferate several times, but optimal Th effector properties do not develop without the appropriate costimulation [24]. It has been shown that APCs isolated from patients during acute dengue infection exhibit a decreased capacity to stimulate proliferation of T cells [25].

Several factors influence the severity of dengue illness such as genotype of the infecting viral strain, host immunity, and immune status [26]. Recent studies conducted with DENV serotype 2 (DENV-2) reference strains have suggested that DENV-infected DCs exhibit defects in maturation and antigen-presenting cell function [5, 9, 10, 12, 27, 28]. Here, we infected primary monocyte-derived DCs with wild-type, low-passage DENV isolates and exposed them to naive $CD4^+$ T cells in order to learn more about how DENV immune evasion strategies affect the priming of adaptive immunity. The data implicate that the inability of DENV-infected DCs and bystander DCs to polarize $CD4^+$ Th type 1 (Th1) effector properties contributes to the inefficient adaptive immune responses observed in patients.

MATERIALS AND METHODS

Isolation and Infection of Monocyte-Derived DCs

Peripheral blood, collected in heparinized bags, was obtained from a commercial source (Tennessee Blood Services). The donors were healthy adult white volunteers who tested negative for a preexisting DENV infection. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using the Ficoll-Hypaque technique. $CD14^+$ monocytes were isolated via positive selection with human CD14 MicroBeads (Miltenyi) and cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1400 U/mL recombinant human (rh) GM-CSF and 1000 U/mL rh IL-4 (Miltenyi). DCs were harvested on day 6 and the immature phenotype ($CD14^-$, $CD1a^+$, $HLA\text{-}DR^+$, $CD83^-$, and $CD209^+$) was confirmed by flow cytometry before each experiment. For all experiments, immature DCs were infected for 1 h with DENV at a multiplicity of infection (MOI) of 5.0 and then washed 3 times in 1X phosphate-buffered saline (PBS). All infections in this study were performed in DCs derived from at least 3 independent donors with 3 replicates per sample. Where noted, IFN-β (PBL InterferonSource) was added at a concentration of 1000 U/mL.

Nine viral strains representing 5 genotypes (SE Asian, Asian I, Cosmopolitan, American, and Sylvatic) of DENV-2 (Table 1) were grown and propagated in C6/36 mosquito cells, and virus supernatant was collected and stored at −80°C. Virus was UV-inactivated by placing an aliquot 5 cm under a germicidal lamp (2300 $\mu W/cm^2$ UVA irradiation at 254 nm). To calculate the MOI for infection assays, virus titers were determined by flow cytometry using a method similar to that described previously [29]. Briefly, Vero cells were plated in 24-well plates and infected with serial dilutions of DENV stock. At 24 h postinfection (PI), cells were fixed, permeabilized, and stained with Alexa Fluor 488-conjugated antiflavivirus E protein mAb 4G2 (kindly provided by Aravinda M. de Silva, UNC) and analyzed by flow cytometry. The titer of the virus was determined using the following formula: fluorescence-activated cell sorting (FACS) infectious units/mL = [(% of infected cells) × (total number of cells per well) × (dilution factor)]/(volume of inoculum added to cells).

FACS Analysis

Cells were stained with directly conjugated monoclonal antibodies specific for CD80, CD83, CD86, CD209 (DC-SIGN), HLA-DR, TNF-α, and IFN-γ or with appropriate isotype controls (BD Pharmingen). For the detection of intracellular cytokines and DENV E protein, cells were permeabilized and fixed in BD Perm/Wash Buffer (BD Biosciences). Flow cytometric analysis was performed by using a FACSCalibur apparatus with CellQuest software (Becton Dickinson).

Allogeneic Mixed Lymphocyte Reactions

Allogeneic-naive $CD4^+$ T cells were isolated by magnetic-negative selection using the human naive $CD4^+$ T cell isolation kit II (Miltenyi), incubated with 1 μM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at room temperature, and washed 4 times. DCs were mock-infected or infected with DENV-2 isolates for 24 h and washed 3 times before coculture. CFSE-labeled naive T cells were cocultured with infected or mock-infected DCs in 96-well round-bottomed plates (Corning) at a T:DC ratio of 5:1. On days 4 and 6, proliferation of naive T cells was measured by CFSE dilution using a FACSCalibur system with CellQuest software. All coculture assays were set up in triplicate, and the results were expressed as a pooled mean of at least 3 independent donors.

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction

1×10^5 infected or mock-infected monocyte-derived DCs were either left untreated or treated with 1000 U/mL of IFN-β (PBL) at 15 h prior to infection (pretreatment) or at 1 or 6 h PI. Supernatants were collected at 9 and 24 h PI and viral RNA output was quantified using a previously published TaqMan real-time reverse-transcription polymerase chain reaction (RT-PCR) protocol [30, 31]. Serial dilutions of in vitro–generated DENV-2 RNA transcripts were used to create a standard curve and calculate a linear regression where we determined that 1 genome copy is equivalent to 1 RNA transcript [31].

To quantify interferon-stimulated gene (ISG) transcription, cells were lysed at 24 h PI, messenger RNA (mRNA) was extracted with the RNeasy Plus Micro Kit (Qiagen) and reverse transcribed using random hexamers and the SuperScript III

Table 1. Dengue Serotype 2 Viruses Used in This Study

Genotype	Virus strain	Passage history[a]	Isolation/clinical diagnosis[b]	Country, year of isolation	Titer (FIU/mL)[c]	Accession no.[d]
Asian I	16681	C4/MK21/C6	Human/DHF	Thailand, 1964	7.1 x 107	U87411
	CO168	C7	Human/DHF	Thailand, 1996	7.2 x 106	GQ868544
SE Asian	15957	C9	Human/DHF	Venezuela, 1996	1.8 x 107	EU726775
	19966	C8	Human/DF	Venezuela, 1996	2.2 x 105	EU687220
American	Ven2	C12	Human/DF	Venezuela, 1987	1.0 x 106	AF100465
	PR159	high passage	Human/DF	Puerto Rico, 1959	3.0 x 107	M19197
	IQT2913	C7	Human/DF	Peru, 1996	2.5 x 107	AF100468
Cosmopolitan	ARA6894	S4/C4	Mosquito	Burkina Faso, 1986	6.2 x 107	GU131843
Sylvatic	Dak Ar 510	S4/C5	Mosquito	Ivory Coast, 1980	1.4 x 106	EF105381

NOTE. [a] C indicates C6/36 mosquito cell line; S, suckling mice; MK2, Rhesus monkey kidney cells.

[b] DHF indicates dengue hemorrhagic fever; DF dengue fever.

[c] FIU indicates fluorescence-activated cell sorting (FACS) infectious units.

[d] GenBank accession number.

First-Strand synthesis system (Invitrogen). For the quantitation of MX1, RSAD2, and OAS1 gene expressions, we used a commercially available kit (TaqMan Assay-on-Demand; Applied Biosystems). To normalize for RNA input, the sample content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified with the TaqMan reagents for GAPDH (Applied Biosystems).

Statistical Analysis

The significance for all comparisons was calculated using 2-tailed Student *t* test, assuming unequal variance. Spearman rank correlation was used to determine the correlation between 2 variables. Statistical significance is defined as a P value of $\leq .05$.

Phylogenetic Analysis

Complete genome nucleotide sequences of representative endemic and sylvatic DENV-2 strains were aligned by ClustalW in MEGA 4 (Koichiro Tamura and Sudhir Kumar). Maximum likelihood analyses and bootstrap values of 1000 replicates were calculated with the PAUP* software package (Sinauer Associates) under the best-fit substitution model estimated by MODELTEST v3.07 (parameters available on request). Homologous nucleotide sequences from DENV serotypes 3 and 4 were used as outgroups to root the DENV-2 tree.

RESULTS

DENV Infectivity of Immature Monocyte-Derived DCs Is Donor Dependent

To study the pathogenicity of DENV-2 wild-type strains, we generated immature monocyte-derived DCs in culture and infected them with DENV at an MOI of 5.0. Nine DENV-2 isolates representing 5 genotypes were used during the course of the study (Table 1 and Figure 6). Prior to infection, the immature DC phenotype was confirmed with a panel of fluorescently-conjugated monoclonal antibodies against common DC markers, HLA-DR, CD80, CD83, CD86, and DC-SIGN. We observed an overall upregulation of markers of maturation on the DENV-infected DC cultures at 48 h PI compared with the mock-infected controls. The expression of DC-specific marker CD83 was significantly upregulated along with the costimulatory molecules CD80 and CD86. The infected, mature DC cultures maintained a high level of major histocompatibility complex (MHC) Class II expression. DC-SIGN (the receptor for DENV [14, 15, 32]) staining was high on uninfected, immature DC cultures with a slight decrease on the infected, mature DC cultures (Figure 1A). Further staining of infected cultures with anti-DENV E mAb revealed a nonsignificant decrease of DC-SIGN on the infected DCs from 3 donors (median fluorescence intensity of 76 $\pm$ 24 vs 115 $\pm$ 31 on uninfected DCs, data not shown). The percentage of infected cells was estimated by FACS analysis using an Alexa Fluor 488-conjugated antiflavivirus E protein mAb 4G2. Although the same MOI was utilized for all DENV infections, the percentages of infected DCs varied markedly within donors and ranged from 4% to 37%, depending on virus strain and donor cells (Figures 1B and C). Interdonor variation among the levels of DENV infection of human DCs has been described previously [33–36]. Immature DCs from 3 different donors were infected with DENV isolates representative of the American (PR159), Asian I (16681), Cosmopolitan (ARA6894), and Sylvatic (DakAr510) genotypes, and expression of intracellular TNF-α was measured by flow cytometry at 48 h PI. The expression of TNF-α, a proinflammatory cytokine implicated in DENV disease severity [37, 38], was found to positively correlate with percent infection ($P = .01$) (Figure 1D). A more robust production of TNF-α has been demonstrated in infected DCs when compared with bystander or mock-infected DCs [8–12, 27, 33].

DENV Isolates Replicate in the Presence of IFN-β

In vitro studies with DENV-2 reference strains, New Guinea C and 16681, have indicated that DENV is capable of replicating in

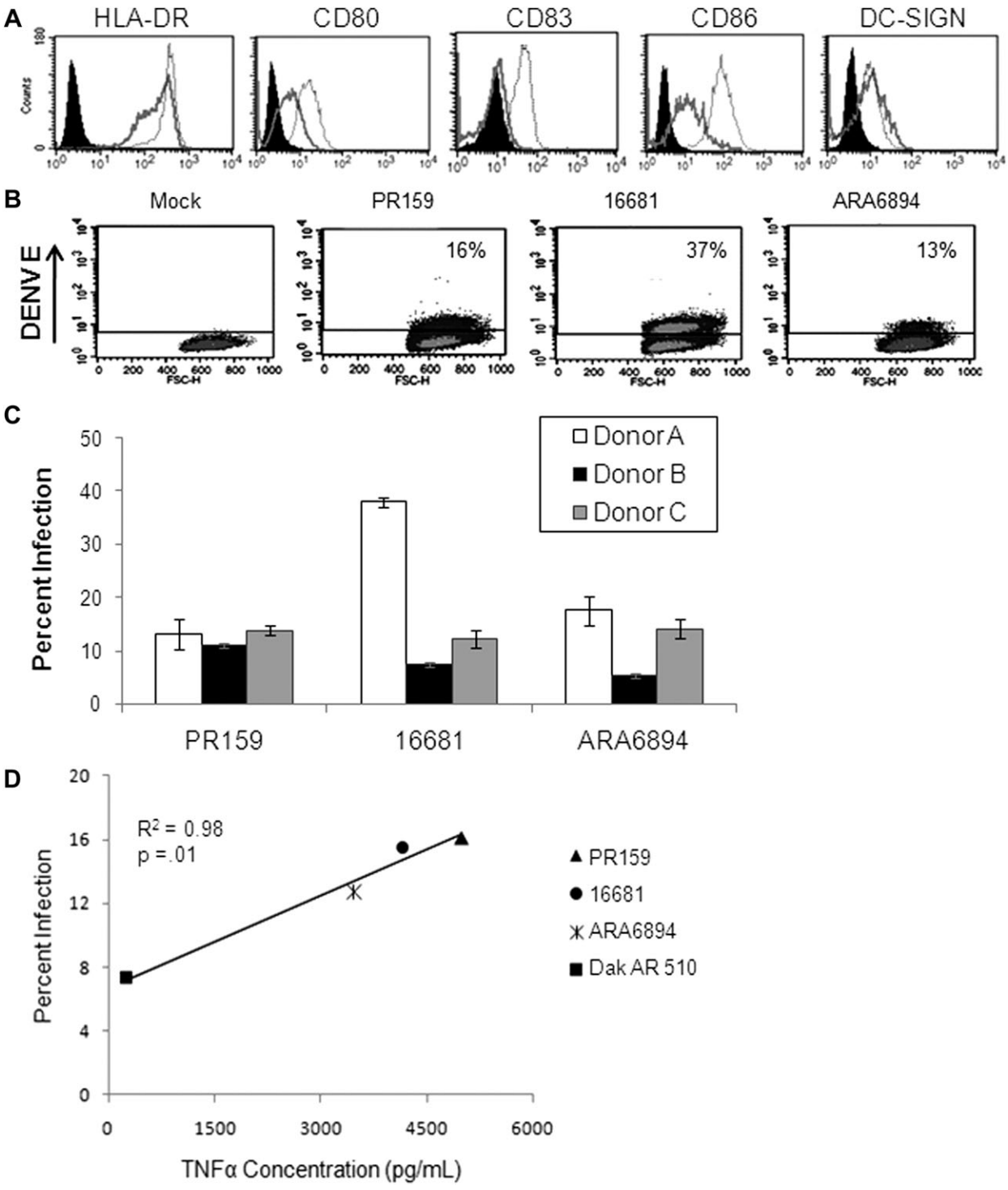

Figure 1. Interdonor variation in susceptibility of immature monocyte-derived DCs to DENV infection. *A,* Cell surface staining of DCs at day 2 PI with DENV-2 strain PR159. Black-filled peaks, isotype control; gray peaks, mock-infected DCs; dotted-line peaks, infected DCs. *B,* DCs from a representative donor were mock-infected or infected with DENV-2 strains PR159 (American), 16681 (Asian I), and ARA6894 (Cosmopolitan) and stained for intracellular DENV with an anti-E protein mAb. Results are representative of 1 of 3 experiments from an individual donor. *C,* DCs from 3 different donors were infected with DENV-2 strains (PR159, 16681, or ARA6894) at an MOI of 5.0. Cells were harvested at 24 h PI and infected cells were detected by intracellular staining with an anti-E protein mAb followed by flow cytometry. Values represent the pooled means ± SEM of samples performed in triplicate from at least 3 experiments. *D,* Correlation between intracellular TNF-α expression and DENV infection in DCs. At 48 h PI, cells were stained for intracellular TNF-α and DENV-2 and analyzed by flow cytometry. The *P* value indicates the level of significance. Values represent the means of samples performed in triplicate and are representative of 1 of 3 experiments. Note: DCs indicates dendritic cells; DENV, dengue virus; PI, post infection; DENV-2, DENV serotype 2; MOI, multiplicity of infection; SEM, standard error of the mean.

the presence of type I IFNs, IFN-α, and IFN-β [7, 16]. Several DENV nonstructural proteins have been shown to inhibit IFN-α/β signaling by blocking the activities of transcription factors STAT1 and STAT2 [17–21]. To determine whether DENV-2 isolates from other genotypic groups are able to overcome the effects of IFN-α/β on the host cell, we infected immature DCs with DENV isolates representative of the American (PR159 and IQT2913), Southeast Asian (19966), and Sylvatic (DakAr510) genotypes. DENV-infected DCs were either left untreated (no tx) or IFN-β was added into the culture medium of DCs at 15 h prior to infection (pre tx), 1 h PI (early tx), or at 6 h PI (late tx), and supernatants were collected for viral titer determination by quantitative real-time RT-PCR at 9 and 24 h PI [30]. As shown in Figure 2A, treatment with IFN-β either prior to PR159 infection or immediately following infection (1 h PI) significantly reduced viral output at 24 h PI when compared with untreated or late-treated (6 h PI) cultures ($P \leq .01$). IFN-α/β establishes an antiviral state within cells and when these cytokines are added several hours before DENV infection, they render the cells resistant to viral replication [7, 16]. The addition of IFN-β at 6 h PI allowed time for sufficient production of DENV nonstructural proteins, whereby the virus was able to overcome the antiviral effects of IFN-β and replicate to significantly higher titers, similar to titers seen without treatment (Figure 2A). Next, we tested low-passage DENV-2 isolates from the American (IQT2913), Southeast Asian (19966), and Sylvatic (DakAr510) genotypes and found that, similar to other DENV isolates, they are capable of replicating in the presence of IFN-β when added at 6 h PI (Figure 2B).

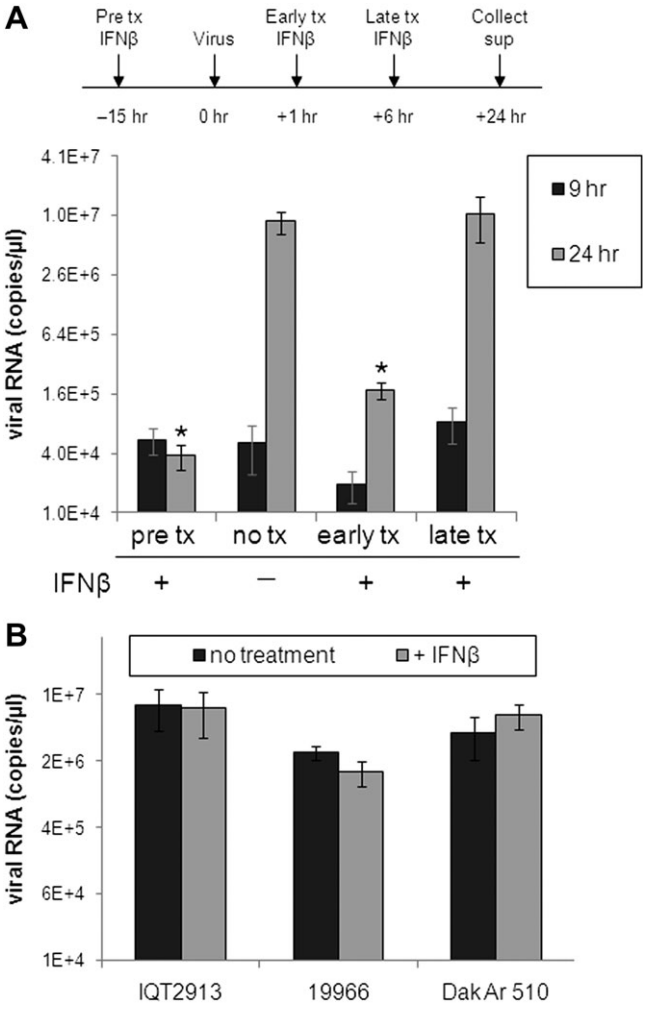

Figure 2. DENV-2 isolates replicate in the presence of IFN-α/β. Mean viral RNA copy number from the supernatant of DENV-2–infected DCs was measured via real-time qRT-PCR. *A,* DCs were either infected with DENV-2 strain PR159 and left untreated (no tx) or treated with 1000 U of IFN-β at 15 h prior to infection (pre tx), 1 h PI (early tx) or 6 h PI (late tx), and supernatant was collected at 9 and 24 h PI for viral RNA output measurements. Viral RNA copy number was significantly lower in pre tx and early tx groups in comparison to both untreated and late tx groups (*, $P \leq .01$). *B,* DCs were infected with different DENV-2 isolates and left untreated or treated with 1000 U of IFN-β at 6 h PI. Values represent the means ± SEM of samples performed in triplicate. The data shown are representative of 3 independent experiments using different donor DCs with similar results. Note: DENV-2 indicates dengue virus serotype 2; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; DCs, dendritic cells; PI, post infection; SEM, standard error of the mean.

DENV-Infected DCs Induce T Cell Proliferation

DENV-infected, monocyte-derived DCs have been shown to stimulate proliferative responses of allogeneic-naive T cells [9, 28]. One of the primary effector functions of $CD4^+$ T cells during a viral infection is the secretion of the Th1 cytokine, interferon gamma (IFN-γ) [24]. To determine whether our panel of DENV-2 isolates are capable of inducing a functional Th1 response with IFN-γ secretion, we cocultured DENV-infected monocyte-derived DCs with allogeneic-naive $CD4^+$ T cells. The T cells were labeled with CFSE in order to differentiate them from the infected, mature DCs, which have been shown to express small amounts of IFN-γ [39]. In comparison to mock-infected DCs, infection with DENV-2 isolates significantly increased the capacity of DCs to induce $CD4^+$ T cell proliferation (Figures 3A and B). No proliferation was observed in T cells alone (Figure 3A).

DENV-Infected DCs Fail to Induce a Th1 Effector Response

Interestingly, the $CD4^+$ T cells proliferated in response to DENV-infected DC stimulation but did not express cytokines indicative of a Th1 response. IFN-γ and TNF-α protein expression by $CD4^+$ T cells, detected by intracellular staining, were measured daily up to day 6 following coculture with mock-infected DCs or DCs infected with DENV-2 isolates. Only 1 strain, ARA6894, consistently induced a significant Th1 response in infected DC-T cell cocultures (Figure 4A). Treatment of the DCs with UV-inactivated ARA6894 before exposure to $CD4^+$-naive T cells did not result in production of Th1

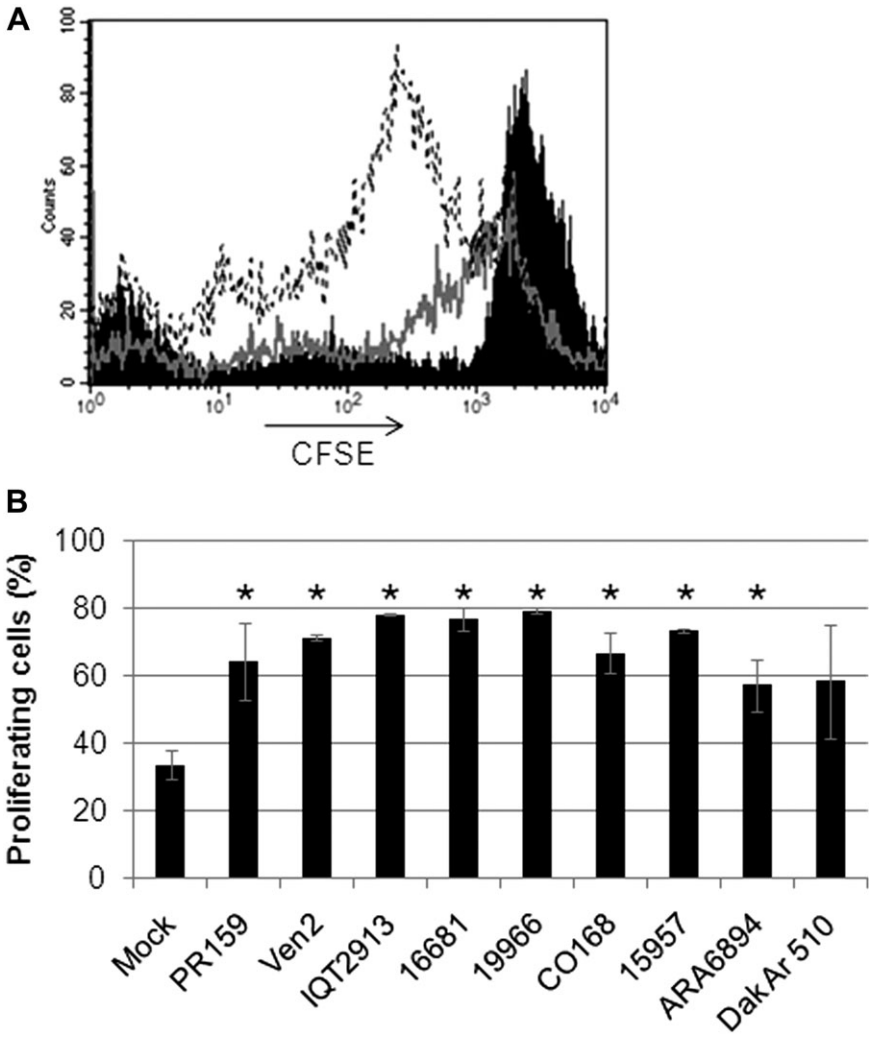

Figure 3. DENV-2–infected DCs induce robust proliferation of allogeneic-naive CD4+ T cells. DCs were mock-infected (Mock) or infected with DENV-2 isolates at an MOI of 5.0. Naive CD4+ T cells were labeled with CFSE and added to DCs at a T:DC ratio of 5:1, and proliferation of T cells was measured by CFSE dilution on day 4 post coculture. *A,* Naive CD4+ T cells were either cultured alone (black fill) or cocultured with mock-infected DCs (gray line) or DENV (strain 15957)–infected DCs (dotted line). T cells were identified by gating on CD4-positive cells. *B,* Summary of the percent of proliferating CD4+ T cells relative to naive T cells alone following coculture with mock-infected DCs or DCs infected with DENV-2 isolates. Significantly higher levels of proliferation by T cells exposed to DCs infected with DENV-2 isolates versus mock-infected DCs are shown with an asterisk (*, $P \leq .01$). Data are the pooled means ± SEM from experiments performed in 3 different donor DCs. Note: DENV-2 indicates dengue virus serotype 2; DCs, dendritic cells; MOI, multiplicity of infection; SEM, standard error of the mean; CFSE, carboxyfluorescein succinimidyl ester.

cytokines, indicating that uptake of infectious virus is required for induction of the Th1 response (Figure 4B). Additionally, DCs were treated with IFN-β both before and after DENV infection and then exposed to naive $CD4^+$ T cells; however, neither IFN-β pretreatment or posttreatment reversed the inhibition of Th1 polarization (data not shown). These results suggest that the DENV-2–infected DC cultures prime $CD4^+$ T cells to proliferate but the T cells are not receiving the appropriate costimulation and do not develop effector properties. Expression of costimulatory molecules by mature DCs is required for efficient MHC-antigen complex presentation to naive $CD4^+$ T cells, and IFN-α/β induces DCs to secrete soluble mediators of T cell activation [22, 23, 40].

DENV Inhibition of Interferon-Stimulated Gene Transcription

Other groups have shown that DENV isolates differ in their ability to inhibit IFN-α/β signaling [41, 42]. Therefore, we tested our panel of DENV-2 wild-type strains for their ability to block IFN-α/β signaling in DCs in order to determine whether the failure of ARA6894 to evade adaptive immunity is due to its inability to block the effects of IFN-α/β. In order to measure IFN inhibition, we quantified the transcript levels of 3 interferon-stimulated genes (ISGs), MX1, OAS1, and RSAD2 (viperin), in infected and mock-infected DCs. MX1, OAS1, and RSAD2 transcription, quantified via a quantitative real-time RT-PCR assay, were upregulated when 1000 U of IFN-β was added to mock-infected DCs. Contrary to the MX1 upregulation

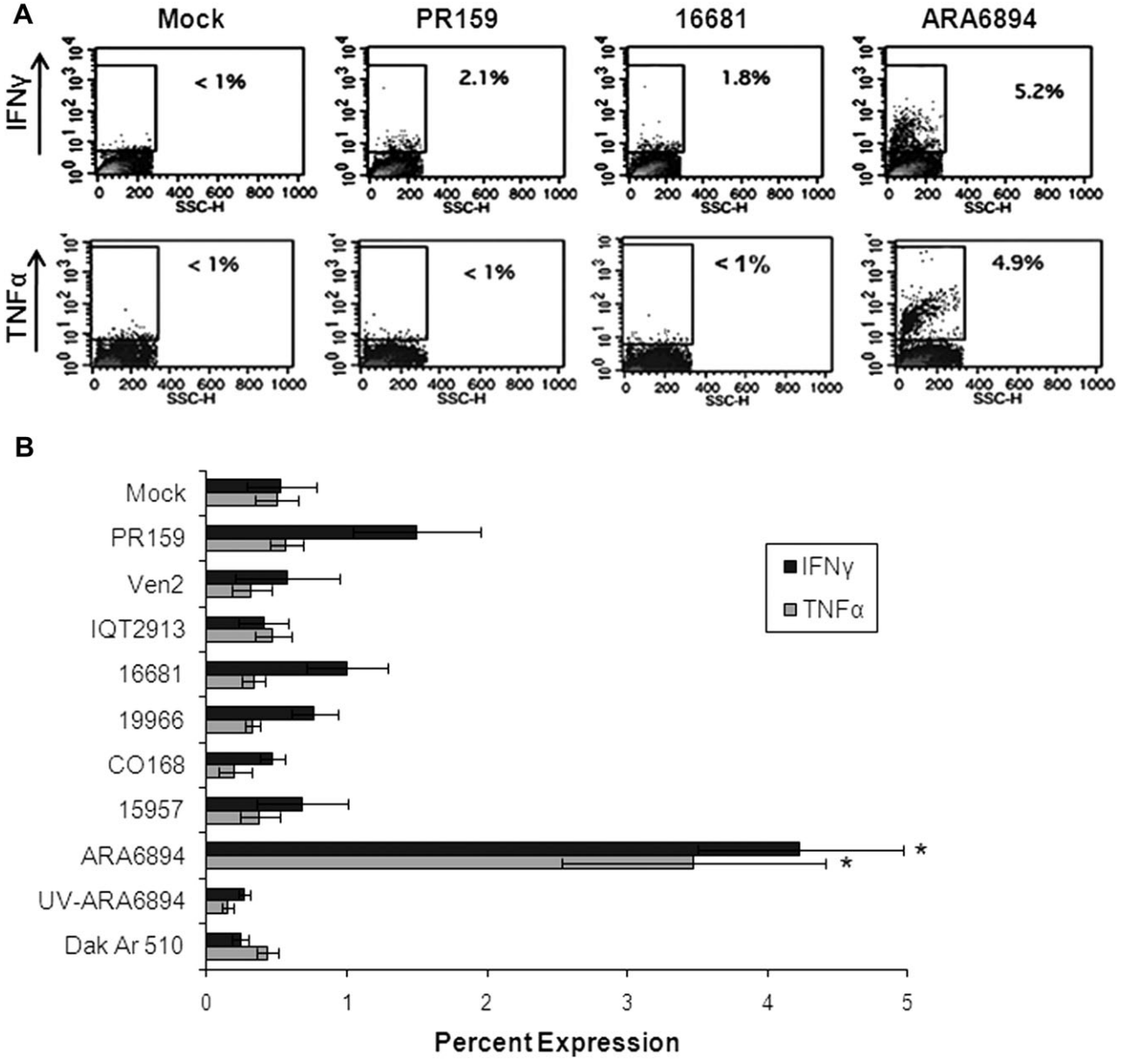

Figure 4. DENV-infected DCs fail to prime T cell effector responses. DCs were mock-infected (Mock) or infected with DENV-2 isolates at an MOI of 5.0. Naive CD4+ T cells were labeled with CFSE and added to DCs at a T:DC ratio of 5:1, and intracellular IFN-γ and TNF-α were detected in CD4+ T cells on day 4 post coculture. *A,* Density plots gated on CD4+ cells are shown for T cells that were exposed to mock-infected DCs or DCs infected with DENV-2 isolates representative of the American (PR159), Asian I (16681), and Cosmopolitan (ARA6894) genotypes. *B,* Summary of intracellular IFN-γ and TNF-α protein expression by T cells following coculture with mock-infected DCs or DCs infected with DENV-2 isolates or UV-inactivated ARA6894. Significantly higher levels of intracellular IFN-γ and TNF-α were detected in T cells exposed to DCs infected with the ARA6894 isolate versus mock-infected DCs or DCs infected with other isolates of DENV-2 (*, $P \leq .05$). Data are the pooled means ± SEM from experiments performed in 3 different donor DCs. Note: DENV indicates dengue virus; DCs, dendritic cells; DENV-2, DENV serotype 2; MOI, multiplicity of infection; CFSE, carboxyfluorescein succinimidyl ester; SEM, standard error of the mean.

observed in mock-infected DCs, we identified significantly lower transcript levels of MX1 in DCs that were infected with several DENV-2 isolates prior to IFN-β treatment, suggesting that they are able to block IFN-α/β signaling (Figure 5A). Pretreatment with IFN-β 15 hr prior to infection abrogated the virus-mediated block in IFN-α/β signaling, and comparable levels of ISG transcription were seen in mock and infected samples (data not shown). The difference in ISG inhibition in mock versus DENV-2–infected DCs was expressed as a percent inhibition, calculated as follows: (ISG transcription in mock-infected DCs – ISG transcription in DENV-infected DCs)/ISG transcription in mock-infected DCs. Inhibition of MX1 transcription was tested

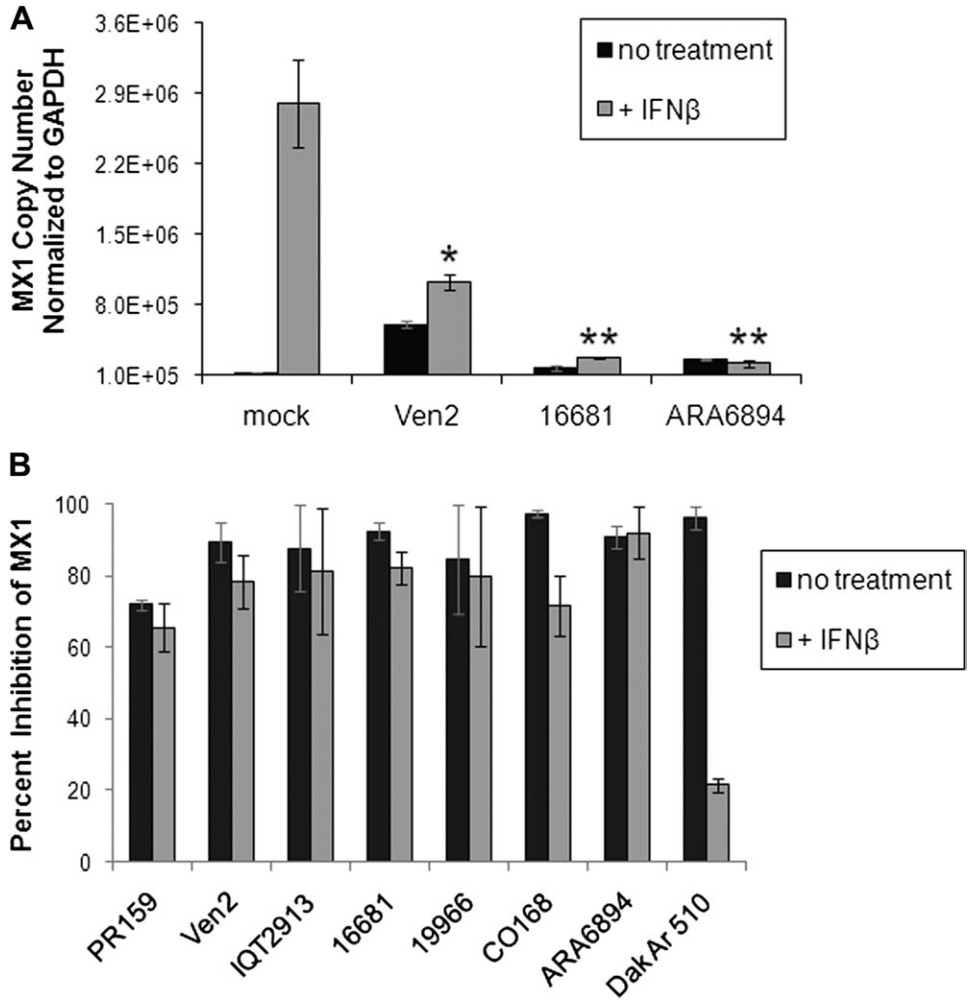

Figure 5. DENV-2 isolates inhibit IFN-α/β signaling. DCs were mock-infected or infected at an MOI of 5.0 and left untreated or treated with 1000 U of IFN-β at 6 h PI. Cells were harvested at 24 h PI and lysed for real-time qRT-PCR analysis of ISG transcription. *A,* Mean MX1 copy numbers in mock-infected and DENV-2–infected DCs. Significantly lower levels of MX1 transcription were detected in IFN-β-treated, DENV-2-infected DCs compared with levels in IFN-β-treated, mock-infected DCs (*, $P \leq .05$; **, $P \leq .01$). *B,* Percent inhibition of MX1 transcription by DENV-2 isolates. *C,* Percent inhibition of MX1, OAS1, and RSAD2 by DENV-2 isolates. The percentage of inhibition of ISG transcription was calculated as follows: (ISG transcription in mock-infected DCs − ISG transcription in DENV-2-infected DCs)/ISG transcription in mock-infected DCs. The percentage of inhibition in the ``no treatment'' group was determined by comparing ISG transcription in DENV-2-infected DCs to ISG transcription in mock-infected, IFN-β-treated DCs from the same donor. *D,* Infected DCs were examined by FACS analysis at 48 h PI with antiflavivirus E mAb 4G2 to determine the percentage of infected cells. Data are the pooled means ± SEM from experiments performed in 3 different donor DCs with each sample being performed in triplicate. Note: DENV-2 indicates dengue virus serotype 2; DCs, dendritic cells; MOI, multiplicity of infection; PI, post infection; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; ISG, interferon-stimulated gene; FACS, fluorescence-activated cell sorting; SEM, standard error of the mean; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

for all DENV-2 isolates in DCs from at least 3 different donors (Figure 5B). ARA6894 along with other Cosmopolitan and SE Asian genotypes demonstrated strong inhibition of IFN-α/β signaling, whereas viruses from the American genotype were significant yet less potent inhibitors of IFN-α/β. DakAr510, a sylvatic isolate, did not inhibit IFN-α/β signaling when IFN-β was added. The percent infection for DakAr510 was consistently less than 10% in DCs, which means that expression of MX1 by uninfected DCs may have overshadowed any inhibition by DakAr510 (Figure 5D). In addition to MX1, the inhibition of 2 other ISGs, OAS1 and RSAD2, was observed in DENV-2-infected DCs (Figure 5C).

Phylogenetic Analysis of DENV-2 Isolates

DC infection by ARA6894 was unique in that it was the only DENV-2 isolate that did not counter DC polarization of bystander $CD4^+$ T cells. We therefore compared the complete coding sequence of ARA6894 to other representative endemic and sylvatic DENV-2 strains. We identified 8 nonsynonymous mutations unique to ARA6894: 3 conservative and 5 nonconservative amino

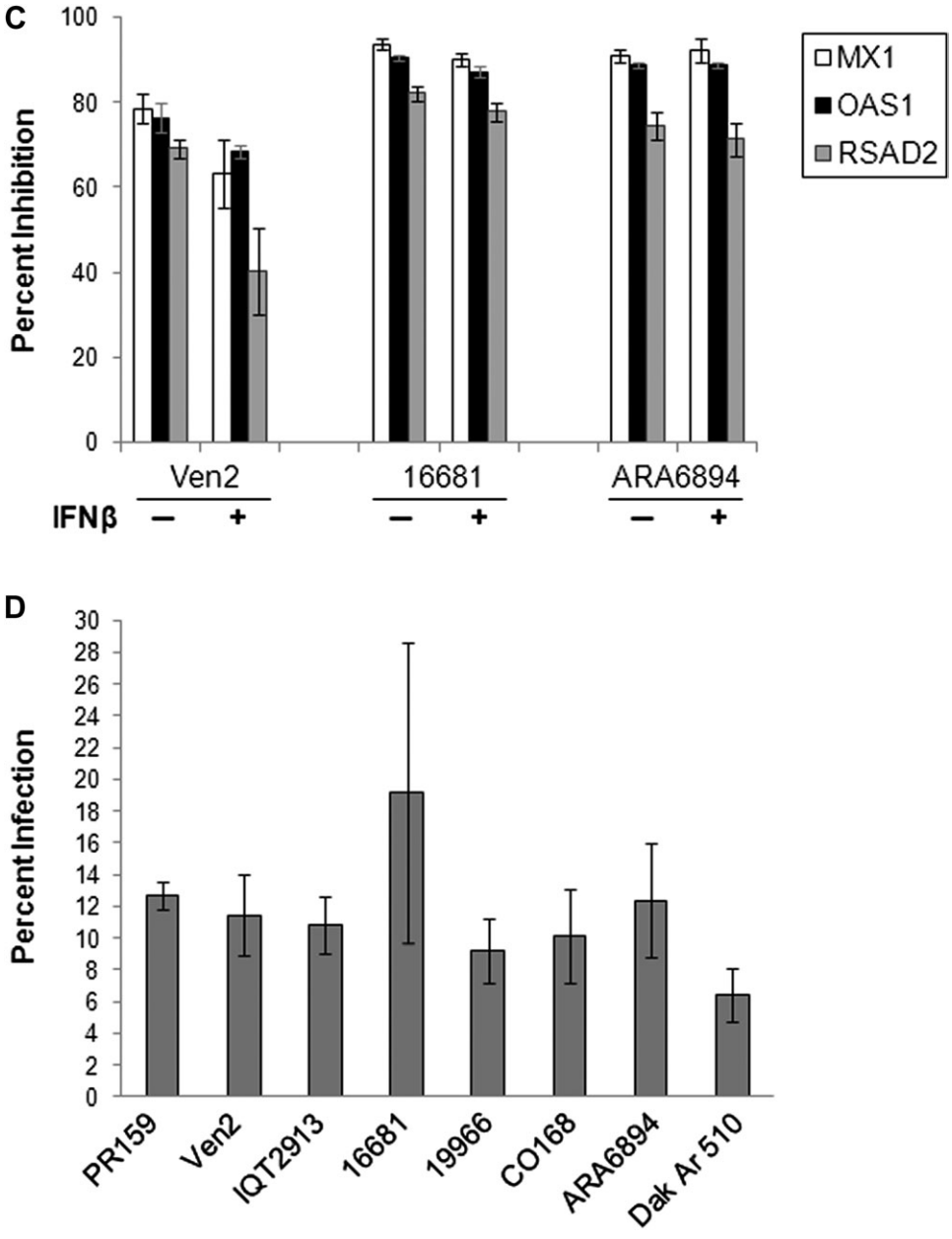

Figure 5. Continued

acid substitutions (Figure 6). We did not expand our analysis to include comparisons between ARA6894 sequence and consensus sequences from each individual genotypic group of DENV-2, but solely identified amino acid substitutions that were unique to ARA6894 and not present in any other endemic or sylvatic DENV-2 strain. The DENV capsid protein (C) contained 2 amino acid changes (I59V and A114T) at sites that were invariant among other endemic or sylvatic sequences. The conservative residue change at position 59 occurred within a conserved internal hydrophobic region (residues 46–66) of DENV capsid proposed to function as an endoplasmic reticulum (ER) membrane anchor domain [43, 44]. A nonconservative amino acid change in a hydrophobic signal sequence was found at position 65 (I65T) of the mature membrane (M) protein. The E protein of ARA6894 contained 1 nonconservative amino acid substitution (T180I) present in domain I. Four nonsynonymous amino acid substitutions were found in 2 nonstuctural proteins of DENV, NS1, and NS2A. The conservative amino acid change at position 220 in NS1 does not seem to affect fitness, as the same substitution (V220I) arose during the continuous evolution of DENV-2 in Puerto Rico in 2000 [45], although it may play a yet unknown role in pathogenesis. NS2A contained 3 amino acid substitutions (H/Q2Y, A37T, and I/T171V). Future work will determine whether

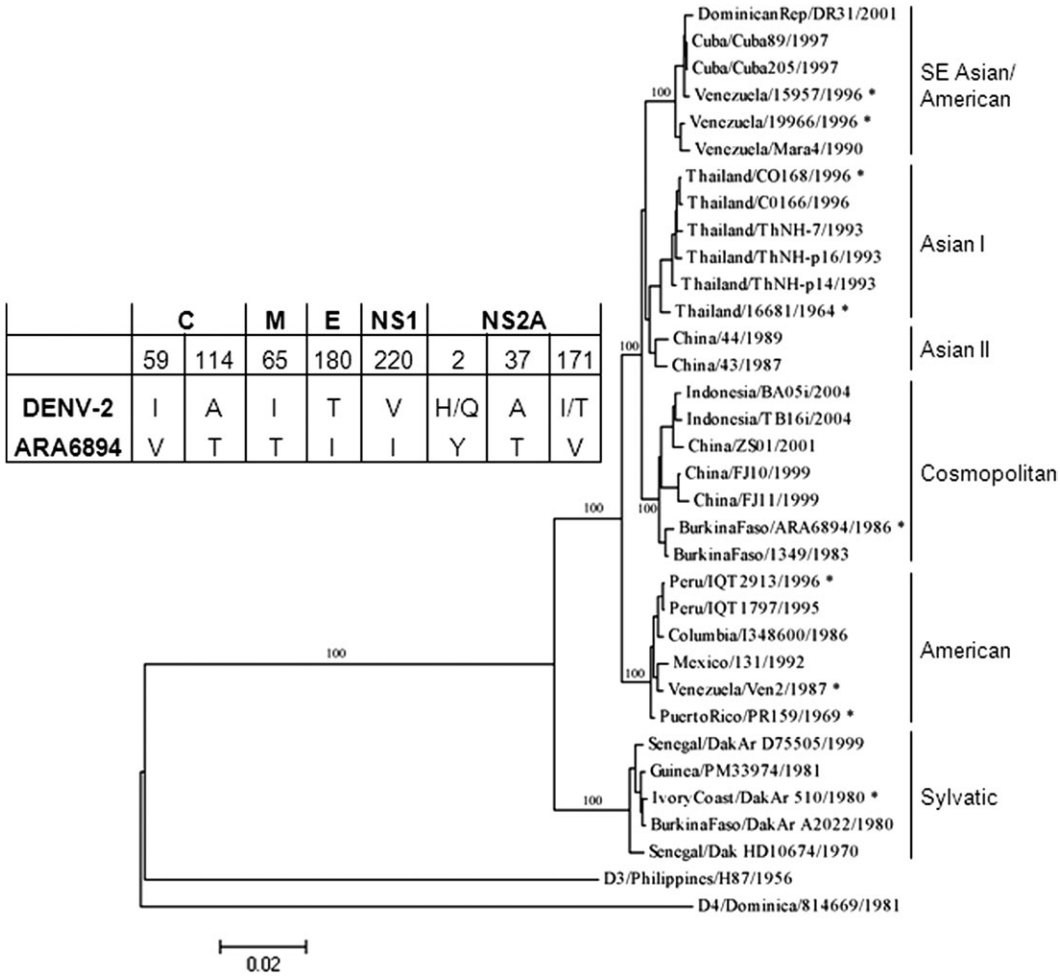

Figure 6. Phylogenetic tree derived from the complete genome nucleotide sequences of representative endemic and sylvatic DENV-2 isolates. Strains are identified by country of isolation, strain name, and year of isolation. Genotypic groups are marked by vertical lines, with names given to the right. Genomic sequences from endemic DENV serotypes 3 (strain H87) and 4 (strain 814699) were used as outgroups to root the DENV-2 tree. Bootstrap values of statistical support for major branches are given as percentage equivalents. The horizontal distance between strains represents the extent of genetic divergence (see scale). Asterisks indicate DENV-2 strains used in experiments. Eight nonsynonymous amino acid substitutions unique to ARA6894 and not present in any other endemic or sylvatic DENV-2 strain are shown in the table to the left of the tree. Numbers below each DENV protein refer to the amino acid position within the respective protein. Note: DENV-2 indicates dengue virus serotype 2.

the aforementioned amino acid substitutions in ARA6894 play a role in the ability of ARA6894-infected DCs to prime effector T cells. Isolates that stimulate adaptive immunity could play an important role in vaccine development.

DISCUSSION

Previous reports using in vitro DENV-infected DCs are varied and have suggested that although DC cultures are activated upon infection, the infected DCs display insufficient maturation and effector properties [5, 9, 10, 12, 27, 28]. We tested numerous DENV-2 isolates and a reference strain (16681) for their ability to trigger adaptive immunity by coculturing infected DCs with allogeneic-naive $CD4^+$ T cells. We found that although naive $CD4^+$ T cells proliferated upon exposure to DENV-infected DCs, they lacked the ability to produce cytokines indicative of a Th1 effector response, suggesting that the necessary costimulation for polarization is lacking.

Uniquely, 1 DENV isolate, ARA6894, lacked the ability to evade stimulation of adaptive immunity, and ARA6894-infected DCs induced IFN-γ secretion by the T cells. It has been suggested that the inability of certain DENV strains to suppress the IFN response may impact their pathogenesis [41, 42]. Thus, we tested the ability of the virus panel to inhibit IFN-α/β signaling in monocyte-derived DCs. Endemic DENV strains blocked IFN-α/β signaling in infected DC populations as evidenced by significant decreases in ISG expression, suggesting that IFN-α/β inhibition by DENV is a conserved function. Type I IFN signaling not only induces the expression of a number of genes that can interfere with viral replication or confer an antiviral state on

uninfected proximal cells but also aids in DC maturation into enhanced stimulators of naive T cells [22]. Interestingly, ARA6894 was a potent inhibitor of IFN signaling, yet resulted in the stimulation of T cell differentiation, suggesting that DENV inhibition of IFN signaling alone cannot explain the inability of infected DCs to prime T cells.

Impaired T cell activation has been shown in PBMCs isolated from patients with acute dengue infection, and the defect has been attributed to the failure of the APC population to provide the necessary stimuli [25]. Yet other studies indicate an important role for mosquito salivary gland extracts in the downregulation of Th1 cytokines [46]. In a recent report, DENV infection of DCs induced IFN-α production but not IL-12; however, the addition of CD40L restored DC maturation and production of IL-12, and greatly elevated the IFN-γ response of T cells [12]. Another study, however, showed that DENV inhibited IFN-α production in infected DCs, thereby impairing the ability of the DCs to prime a Th1 response, and the addition of exogenous IFN-β was sufficient to restore the production of IFN-γ by the T cells [28]. In our system, the addition of IFN-β did not restore T cell effector function. It seems that the inability of DENV-infected DCs to prime T cells is multifactorial, and could be complicated by the percent of infected cells and the contribution of bystander DCs in the coculture system.

ARA6894 did not impede T cell polarization when the T cells were exposed to infected DCs, suggesting that ARA6894 may lack a mechanism that other DENV strains use to evade the priming of an adaptive immune response. Isolates that exhibit immune stimulatory abilities in vitro or in vivo should prove to be useful in deciding what viral signatures should go into vaccine design in order to improve efficacy by stimulating a robust adaptive immune response to the vaccine virus. In addition, identifying the predominant molecular mechanisms that DENV utilizes to delay and disable DC priming capacity will help us to develop effective therapeutics and treatments that aid in augmenting an early host response.

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