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Targeted Delivery of Small Interfering RNA to Human Dendritic Cells To Suppress Dengue Virus Infection and Associated Proinflammatory Cytokine Production▽

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Dengue is a common arthropod-borne flaviviral infection in the tropics, for which there is no vaccine or specific antiviral drug. The infection is often associated with serious complications such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), in which both viral and host factors have been implicated. RNA interference (RNAi) is a potent antiviral strategy and a potential therapeutic option for dengue if a feasible strategy can be developed for delivery of small interfering RNA (siRNA) to dendritic cells (DCs) and macrophages, the major *in vivo* targets of the virus and also the source of proinflammatory cytokines. Here we show that a dendritic cell-targeting 12-mer peptide (DC3) fused to nona-D-arginine (9dR) residues (DC3-9dR) delivers siRNA and knocks down endogenous gene expression in heterogenous DC subsets, (monocyte-derived DCs [MDDCs], CD34$^+$ hematopoietic stem cell [HSC])-derived Langerhans DCs, and peripheral blood DCs). Moreover, DC3-9dR-mediated delivery of siRNA targeting a highly conserved sequence in the dengue virus envelope gene (siFvE^D) effectively suppressed dengue virus replication in MDDCs and macrophages. In addition, DC-specific delivery of siRNA targeting the acute-phase cytokine tumor necrosis factor alpha (TNF-α), which plays a major role in dengue pathogenesis, either alone or in combination with an antiviral siRNA, significantly reduced virus-induced production of the cytokine in MDDCs. Finally to validate the strategy *in vivo*, we tested the ability of the peptide to target human DCs in the NOD/SCID/IL-2Rγ$^{-/-}$ mouse model engrafted with human CD34$^+$ hematopoietic stem cells (HuHSC mice). Treatment of mice by intravenous (i.v.) injection of DC3-9dR-complexed siRNA targeting TNF-α effectively suppressed poly(I:C)-induced TNF-α production by DCs. Thus, DC3-9dR can deliver siRNA to DCs both *in vitro* and *in vivo*, and this delivery approach holds promise as a therapeutic strategy to simultaneously suppress virus replication and curb virus-induced detrimental host immune responses in dengue infection.

Dengue is a mosquito-borne flavivirus infection that has emerged as a serious public health problem worldwide. Four serotypes of dengue virus (DEN-1 to DEN-4) are capable of causing human disease varying in severity from acute self-limiting febrile illness to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The plasma leakage, hemorrhagic manifestations, and shock that characterize DHF/DSS are considered to have an immunological basis, as they are more common during secondary infection with a heterologous dengue virus strain (15, 28, 33). However, severe clinical manifestations can also occur during primary dengue infection, pointing to a contributory role of viral virulence factors. The WHO estimates that more than 20,000 people worldwide, mainly children, die each year from serious complications of dengue. No specific antiviral therapies are currently available for treating the infection, and efforts to develop a safe prophylactic vaccine have been hindered by the complex role of the immune system in disease pathogenesis (39, 52, 57). Thus, novel treatment strategies that block viral replication and/or to attenuate the exaggerated cytokine response associated with DHF/DSS complications are urgently needed.

Potent and specific gene silencing mediated by RNA interference (RNAi) has generated a great deal of interest in development of RNAi as a therapeutic strategy against viral infections (50, 54). Many studies have demonstrated the effectiveness of the RNAi approach to suppress flavivirus infection, including dengue virus replication in experimental cell lines (3, 23, 26, 42, 60). In addition, the versatility of RNAi could also be exploited to block important host mediators that contribute to dengue pathogenesis. However, the existence of

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four distinct dengue virus serotypes and the ability of viruses to develop resistance to RNAi by mutating their sequences will have to be taken into account before clinical use can be contemplated. A more serious hurdle for RNAi therapeutics is the specific delivery of small interfering RNA (siRNA) to relevant cell types.

Even though dengue virus antigens have been detected in many tissues, including liver, spleen, lymph node, and skin of patients with DHF/DSS, macrophages and dendritic cells (DCs) are considered the predominant infected cell types (9, 36, 59). Following the bite of an infected *Aedes* mosquito, the initial local viral replication is believed to take place in the skin DCs, including myeloid DCs and Langerhans cells (31, 53, 59). Dengue-infected DCs play a key role in the immunopathogenesis of DHF/DSS, as, along with macrophages, they release proinflammatory cytokines and soluble factors that mediate plasma leakage, thrombocytopenia, and hypovolemic shock associated with severe dengue infection (14, 15, 29, 38). Therefore, development of a method to introduce siRNA into DCs would be an important step toward using RNAi therapeutically to suppress viral replication and/or to attenuate the vigorous host cytokine responses in dengue infection (7, 19).

To target DCs, we used a previously characterized 12-amino-acid peptide identified from a phage display peptide library that specifically binds to a ligand expressed on DCs (10). In an earlier study, we demonstrated that fusing nucleic acid-binding nine D-arginine residues to a neuronal cell-targeting peptide enabled siRNA delivery to neuronal cells (27). Here, in a similar approach, we synthesized a chimeric peptide consisting of the DC-targeting peptide fused to nona-D-arginines (9dR) to target siRNA selectively to DCs. We investigated whether the DC3-9dR peptide could deliver siRNA targeting a dengue virus envelope sequence to reduce the viral load in DCs. As tumor necrosis factor alpha (TNF-α) is one of the acute-phase cytokines with a major role in inducing plasma leakage in dengue infection (8, 12, 17, 20), we also explored the possibility of reducing TNF-α expression in DC *in vitro* and *in vivo*. Our findings demonstrate the potential of a targeted RNAi-based approach for simultaneously decreasing viral load and reducing aberrant cytokine responses in DCs.

MATERIALS AND METHODS

Peptide and siRNA. DC3-9dR (FYPSYHSTPQRPGGGGSRRRRRRRRR; arginine residues are D-arginines) was synthesized and purified at the Tufts University Core Facility, Boston, MA. The sense-strand sequences of siRNA designed to target the envelope gene of dengue virus type 2 (DEN-2) are as follows: siEnv1, 5′-ATGAAGAGCAGGACAAAAG-3′; siENV2, 5′-ATTGGA TACAGAAAGAGAC-3′; siENV3, 5′-ACACAACATGGAACAATAG-3′; siENV4, 5′-CATAGAAGCAGAACCTCCA-3′; and siFvE^{D}, 5′-GGATGTGG ATTATTTGGAA-3′. siRNAs targeting human cyclophilin B (siCyPB, 5′-TGT CTTGGTGCTCTCCACC-3′), firefly luciferase (siLuci, 5′-TCGAAGTACTCA GCGTAAG-3′), and human TNF-α (siTNFα-1, 5′-GGCGUGGAGCUGAGA GAUA-3′; siTNFα-2, 5′-CCAGAAUGCUGCAGGACUU-3′) were synthesized by Dharmacon, Inc. (Lafayette, CO). For fluorescein isothiocyanate (FITC) uptake experiments, Luci siRNA with FITC label at the 3′ end of the sense strand was used.

Generation of MDDCs, MDMs, and CD34^{+}-derived Langerhans cells. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy donors (Blood Bank, Children's Hospital, Boston, MA) by centrifugation over a Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) density gradient according to standard protocols. Monocytes were selected from PBMCs by immunomagnetic separation using CD14^{+} microbeads (Miltenyi Biotech, Auburn, CA). For generation of monocyte-derived DCs (MDDCs), CD14^{+} cells were seeded at 4×10^5 cells/well in 24-well plate in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml of penicillin, 50 μg/ml of streptomycin, 2 mM L-glutamine, and human recombinant cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems) (1,000 U/ml) and interleukin 4 (IL-4) (R&D Systems) (500 U/ml) for 6 days at 37°C in a CO_2 incubator as described in reference 11. Monocyte-derived macrophages (MDMs) were generated by standard methods (46). Briefly, human PBMCs (5×10^6 per well) were seeded in 24-well plates at 37°C for 16 h, nonadherent cells removed by repeated gentle washing with warm medium, and the adherent population allowed to differentiate for an additional 5 days in the presence (or absence) of recombinant human M-CSF (R&D Systems) (20 ng/ml) (46). Over 90% purity was obtained with both cell populations as assessed by CD11c and CD14 expression on MDDCs and MDMs, respectively (data not shown).

CD34-derived Langerhans cells were obtained by culturing cord blood-enriched CD34^{+} cells in a two-step culture as described in reference 49. In the first expansion step, 2×10^5 enriched CD34^{+} cells were seeded in a 25-cm^2 flask and cultured in RPMI medium (with 10% FBS) supplemented with 50 ng/ml stem cell factor (SCF), 10 ng/ml thrombopoietin (TPO), and 50 ng/ml Flt-3L (R&D Systems) for 10 days. The medium was refreshed every other day by partial depletion throughout the culture period. In the second differentiation step, expanded CD34^{+} cells (2×10^6) were seeded in a 25-cm^2 flask in 5 ml RPMI (with 10% FBS) supplemented with 50 ng/ml GM-CSF, 10 ng/ml TNF-α, and 100 ng/ml Flt-3L. On day 3, cultures were fed by adding 1.5 ml fresh medium with cytokines. On day 5, cultures were split into two flasks with fresh medium and cytokines, and on day 10, cells were harvested for experiments. About 40 to 50% cells were positive for expression of Langerhans cell markers (CD1a^{high}, CD11c^{high}, and CD14^{low}) (data not shown).

siRNA binding and silencing experiments using DC3-9dR peptide. To test siRNA binding, 100 pmol of siRNA was complexed with different amounts of DC3-9dR peptide for 15 min and analyzed by 2% agarose gel electrophoresis with uncomplexed siRNA as a control. To test delivery, uptake of FITC-labeled siLuc was measured. FITC-siRNA (200 pmol) was incubated with various amounts of DC3-9dR in serum-free RPMI medium for 20 min at room temperature. The complexes were then added to MDDCs (4×10^5 cells/well in 24-well plates) and incubated at 37°C for 4 h, after which the medium was replaced with fresh medium supplemented with 10% FBS. After incubation for an additional 16 h at 37°C, cells were subjected to flow cytometric analysis. Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as a positive control. Cellular uptake of FITC-labeled siRNA complexed with DC3-9R peptide was also tested in phytohemagglutinin (PHA)-stimulated T lymphocytes and cell lines, including Jurkat, HeLa, and K562, as described above. For gene-silencing experiments, DC3-9dR peptide complexed with 200 pmol of siCyPB at a molar ratio of 10:1 was added to 2×10^5 MDDCs, CD34-derived DCs, or freshly isolated blood DCs, and cyclophilin B mRNA levels were assessed after 24 h of treatment by quantitative real-time reverse transcription-PCR (RT-PCR).

Transfection of siRNAs targeting the envelope gene of DEN-2. To identify an optimal siRNA for DEN-2 inhibition, we designed five different siRNAs targeting different regions of the envelope gene of DEN-2 and tested them in the BHK-21 cell line. BHK-21 cells (ATCC, Manassas, VA) were seeded at 1×10^5 cells/well in six-well plates at 16 h before transfection. Lipid-siRNA complexes were prepared by incubating 300 pmol of the indicated siRNA with Lipofectamine 2000 in the volume recommended by the manufacturer. Lipid-siRNA complexes were added to the wells in a final volume of 0.5 ml of serum-free Dulbecco modified Eagle medium (DMEM) (Gibco BRL). After incubation for 6 h, cells were washed and reincubated in DMEM containing 10% FBS for additional 18 h and then infected with DEN-2 (multiplicities of infection [MOIs] of 0.5 and 1.5). Another round of siRNA transfection was repeated 24 h after viral infection. The effect of siRNA postinfection was assessed by infecting BHK-21 cells with DEN-2, followed by one round of siRNA transfection either 24 h or 48 h postinfection. Viral infection was measured by flow cytometry at 72 h after infection. siRNA transfections in MDCCs and MDMs (4×10^5 cells/well in 24-well plates) were performed as described above.

DEN-2 infection. Viral stocks generated from the New Guinea C (NGC) strain of DEN-2, obtained from ATCC, were plaque titrated using BHK-21 cell lines. For infection studies, BHK-21 cells (1×10^5 cells/well in six-well plates) or MDDCs (4×10^5 cells/well in 24-well plates) were infected with DEN-2 (NGC) at the indicated MOIs or mock infected. For DEN-2 infection of MDMs, DEN-2 was premixed with the enhancing anti-prM antibody (clone 70-21 used at 0.1 ng/ml) (21) (a gift from Huan-Yao Lei, National Cheng Kung University, Taiwan) at 37°C for 2 h and then added to MDMs (4×10^5 cells/well in 24-well plates). After 2 h (for BHK-21 cells) or 4 h (for MDDCs and MDMs), cells were

washed twice with fresh medium and further incubated at 37°C for the indicated times before harvesting for RNA isolation or flow cytometric analysis.

For infectivity studies, culture supernatants from infected MDDCs were serially diluted, added to fresh BHK-21 monolayers (5×10^4 cells/well in 12-well plates), left for 2 h, washed, incubated in fresh medium with 2.5% FBS for 96 h, and then subjected to flow cytometric analysis for dengue infection.

Qualitative and quantitative RT-PCR. Total RNA was isolated from MDDCs and MDMs using an RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse transcribed with the SuperScript III first-strand synthesis kit (Invitrogen, Carlsbad, CA) using random hexamers in accordance with the manufacturer's protocol. RT-PCR was performed on 2 μl of cDNA with the QuantiTect SYBR green PCR kit (Qiagen) according to the manufacturer's instructions. Amplification conditions were as follows: 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s using the iCycler iQ real-time PCR detection system (Bio-Rad, Philadelphia, PA). Primers used were for cyclophilin B (5′-CATGGACAAGATGCCAGGAC-3′, forward; 5′-GTACCTGTTCTACG GTCCTG-3′, reverse) and β-actin (5′-TGAGTCTGACGTGGACATC-3′, forward; 5′-ACTCGTCATACTCCTGCTTG-3′, reverse). Relative mRNA expression was calculated using the ΔC_T method.

For quantitation of dengue virus RNA, serial dilutions of the PCR product generated by reverse transcribing viral RNA extracted from DEN-2-infected BHK21 cells were used to plot a standard curve. Samples and standards were amplified with primers (forward, CAATATGCTGAAACGCGAGAGAAA; reverse, CCCCATCTATTCAGAATCCCTGCT) (41) using the amplification conditions mentioned above.

For qualitative detection of negative-strand DEN-2 RNA, a "tag" sequence was incorporated during first-strand cDNA synthesis and PCR performed as described by Kyle et al. (32).

Flow cytometry. Dengue infection in MDDCs and BHK-21 cells was assessed by intracellular staining and flow cytometry. Briefly, cells were fixed and permeabilized using Perm/Wash buffer (BD Pharmingen) at room temperature for 20 min and then incubated with dengue virus type 2 monoclonal antibody (D2800-10; U.S. Biologicals) for 1 h, followed by staining with phycoerythrin-conjugated goat anti-mouse polyclonal antibody (R0480; DakoCytomation, Glostrup, Denmark) for 20 min.

Viral plaque assay. BHK-21 cells were seeded in 24-well plates (2×10^4 cells/well) in DMEM with 10% FBS overnight. Serial dilutions of MDDC or MDM supernatant were added to the cells in a final volume of 200 μl/well. After 2 h of incubation at 37°C, α-minimal essential medium (α-MEM) containing 2% FBS and 1% low-melting-point agarose was added and plates incubated at 37°C for 4 days. Plaques were visualized after removal of the agarose plug and staining with 0.1% crystal violet. Viral titers were determined as PFU per milliliter.

DC3-9dR/siRNA delivery in humanized mouse. $CD34^+$-engrafted NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (HuHSC) (22, 25) mice were injected intraperitoneally with recombinant human GM-CSF and IL-4 (10 μg/mouse each) for five consecutive days to mobilize dendritic cell precursors into the circulation (4, 48). On day 5 mice were intravenously (i.v.) injected with DC3-9dR/siRNA complexes at a 10:1 molar ratio at a dose of 50 μg siRNA per injection in 5% glucose in a volume of 200 μl three times at 6-h intervals. Sixteen hours after the third injection, animals were injected with poly(I:C) (Invivogen, San Diego, CA) intraperitoneally (300 μg in 1× phosphate-buffered saline [PBS]). Serum obtained from mouse blood drawn at the indicated time points after poly(I:C) treatment was used for measuring TNF-α levels, using a human TNF-α enzyme-linked immunosorbent assay (ELISA) kit (BioLegend, San Diego, CA).

Statistical analysis. All statistical analyses comparing groups of mice were performed by one-way analysis of variance and then Bonferroni's post hoc test. The Student *t* and Mann-Whitney tests were used for other experiments. A *P* value of <0.01 was considered significant. Error bars indicate standard deviations.

RESULTS

DC3-9R peptide delivers siRNA to various dendritic cell subsets. A short, 12-mer peptide identified through a phage display library has been reported to bind specifically to human MDDCs but not T or B lymphocytes (8). We have previously demonstrated the ability of nona-arginines (9dR) fused to a cell-targeting peptide or antibody to deliver siRNA to specific cell types (25, 27). Here we used a similar approach for targeted delivery of siRNA to human MDDCs. The chimeric DC3-9dR peptide consisting of a DC3 peptide (10), a spacer, and a 9dR was synthesized and tested for siRNA binding using different peptide/siRNA ratios in a gel shift assay. DC3-9dR was able to bind siRNA as determined by a gel shift and decrease of fluorescence intensity due to quenching by peptide binding, and at peptide/siRNA ratios above 1:10, siRNA was no longer visible (Fig. 1A). To test siRNA transduction, FITC-labeled siRNA was mixed with the peptide and incubated with MDDCs. DC3-9dR peptide was able to transduce siRNA to human MDDCs in a dose-dependent manner (Fig. 1B). A 1:10 molar ratio of siRNA to DC3-9dR was found to be optimal for siRNA uptake, with efficiency similar to that for Lipofectamine transfection (Fig. 1C), and this ratio was used for further studies. Unlike Lipofectamine, DC3-9dR could not transduce FITC-labeled siRNA into primary T cells (Fig. 1D) or non-DC human cell lines such as Jurkat, HeLa, and K562 (Fig. 1E), showing its specificity for MDDCs.

Next we tested the ability of DC3-9dR-delivered siRNA to mediate specific gene knockdown. MDDCs were transduced with cyclophilin B (siCyPB) or an irrelevant siRNA complexed to DC3-9dR, and CyPB mRNA levels were quantitated 24 h later by real-time PCR using gene-specific primers. Specific gene knockdown comparable to that achieved with Lipofectamine was detected in MDDCs treated with CyPB siRNA but not with a control luciferase (Luci) siRNA (Fig. 2A, left panel). Further, DC3 9dR-mediated delivery of siRNA did not silence CyPB expression in PHA-treated T cells (Fig. 2A, right panel). These results indicate that DC3-9dR is able to deliver siRNA and silence gene expression in a DC-specific manner.

Cultured monocyte-derived DCs (MDDCs) may not represent all *in vivo* DC subsets. Thus, we also evaluated whether DC3-9dR could target a broader subset of DCs, since this would be an important criterion for clinical applications. Peripheral blood DCs, which consist of a mixture of myeloid and plasmacytoid DCs, were isolated ex vivo from human PBMCs by immunomagnetic selection using a blood dendritic cell isolation kit (Miltenyi Biotech). Enriched blood dendritic cells were treated with CyPB or control siRNA complexed to DC3-9dR as described above and tested for gene knockdown in cells by quantitative PCR at 24 h posttransfection. While mock and irrelevant siRNA controls did not show any gene silencing, gene expression was knocked down ~55% with CyPB siRNA (Fig. 2B), suggesting that DC3-9dR can deliver siRNA to freshly isolated circulating blood DCs. The modest level of gene silencing is consistent with the enrichment efficiency of the isolated blood DCs in this experiment, which was approximately 50 to 60% as assessed by flow cytometry for expression of dendritic cell markers (HLA-DR^+, $CD11c^+$, and $CD123^+$) (data not shown).

$CD34^+$ hematopoietic stem cells (HSC) have been shown to give rise to Langerhans cells after culture in an appropriate cytokine milieu (49). To determine whether these cells can be targeted with DC3-9dR, $CD34^+$ cells immunomagnetically purified from cord blood mononuclear cells were expanded and differentiated in a two-step manner as described in Materials and Methods. After 10 days of culture, when CD34-derived DCs were phenotypically characterized by staining for expression of Langerhans cell markers ($CD1a^{high}$, $CD11c^{high}$, and $CD14^{low}$), 40 to 50% of cells expressed these markers (data not shown). The $CD34^+$ HSC-derived DCs were treated with

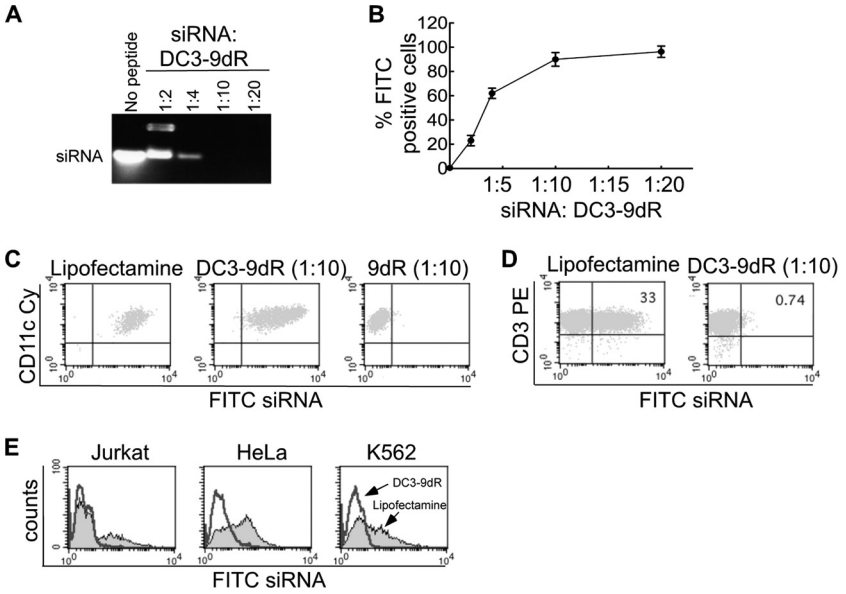

FIG. 1. DC3-9dR peptide delivers FITC siRNA specifically to MDDCs. (A) siRNA was incubated with DC3-9dR peptide at the indicated molar ratios for 15 min and electrophoretic mobility tested by agarose gel electrophoresis. (B) MDDCs were incubated with FITC-siRNA (200 pmol) complexed with the indicated molar ratios of DC3-9dR, and siRNA uptake was assayed 16 h later by flow cytometry. (C) Representative results for MDDCs treated with FITC-siRNA complexed with Lipofectamine, DC3-9dR, or 9dR at a 1:10 molar ratio. (D and E) PHA-stimulated T lymphocytes (D) or indicated cell lines (E) were transfected or transduced with FITC-labeled siRNA using Lipofectamine or DC3-9dR peptide, respectively, and analyzed 16 h later for siRNA uptake. The shaded histogram in the overlay in panel E represents Lipofectamine transfection, and the open histogram represents DC3-9dR transduction.

DC3-9dR/CyPB siRNA complexes. CyPB siRNA, but not Luci siRNA, was able to silence CyPB (~40%) in CD34-derived DCs (Fig. 2C). Taken together, these results indicate that DC3-9dR can deliver siRNA to silence gene expression in different dendritic cell subsets such as monocyte-derived DCs, blood DCs, and CD34-derived Langerhans cells and thus might have potential for *in vivo* application.

An siRNA targeting a highly conserved sequence in the dengue virus envelope region has potent antiviral activity pre- or postinfection. We identified five sequences in the envelope region of DEN-2 virus that are well conserved among different strains within the serotype. One of these (FvE^D siRNA) corresponds to a region in the envelope gene of an siRNA that we used in an earlier study to suppress West Nile virus (26), but with incorporation of nucleotide changes homologous to the DEN-2 NGC strain. In initial studies, we compared the abilities of the five siRNAs to suppress virus replication in BHK-21 cells. The cells were transfected with siRNA using Lipofectamine at 1 day before and 1 day after DEN-2 challenge. The extent of infection was assessed by flow cytometry after staining with DEN-2 envelope-specific antibody at 72 h and 96 h after viral infection. Compared to mock and irrelevant Luci siRNA-transfected BHK-21 cells, which showed high levels of infection, dengue virus envelope-directed siRNAs reduced viral loads, although to different extents (Fig. 3A). The highest protection was seen with FvE^D siRNA, with near complete abrogation of virus replication at 72 h (Fig. 3B, upper panel) that persisted even at 96 h after infection (Fig. 3B, lower panel).

We also tested whether the FvE^D siRNA afforded protection when transfected postinfection. BHK-21 cells were first infected with DEN-2 (MOI of 1.5), and 24 h or 48 later, cells were transfected with FvE^D or Luci siRNA; protection levels were assessed at 72 h postinfection. Indeed, the FvE^D siRNA treatment reduced infection levels, with maximal protection when siRNA was transduced at 24 h postinfection and protection persisting even when treatment was at 48 h postinfection (Fig. 3C). These results demonstrate that FvE^D is a potent antiviral siRNA and can protect against DEN-2 both pre- and postinfection.

DC3-9R-mediated delivery of FvE^D suppresses DEN-2 infection in MDDCs. Our next objective was to test whether DC3-9dR-mediated FvE^D siRNA delivery inhibits dengue virus replication in MDDCs. First we tested the susceptibility of MDDCs to dengue infection by infecting with DEN-2 (at various MOIs ranging from 0.1 to 10) and assessing infection 72 h later by intracellular staining with DEN-2 antibody and flow cytometric analysis. An infection rate of 12 to 16% was reached with an MOI of 2 and did not significantly increase with higher MOIs (Fig. 4A). To test antiviral effects, the MDDCs were either mock treated or treated with FvE^D siRNA or Luci siRNA complexed to DC3-9dR twice on two consecutive days and then infected with DEN-2 virus. When viral replication was assessed 72 h later by flow cytometry and quantitative PCR, cells pretreated with $siFvE^D$, delivered either by DC3-9dR or by the positive control Lipofectamine, showed >80% inhibition, while no inhibition was seen with Luci siRNA (Fig. 4B). Viral positive-strand RNA copy numbers were reduced in FvE^D siRNA-treated MDDCs compared to controls (Fig. 4C), as was the negative-strand DEN-2 RNA (Fig. 4D), indicating inhibition of actively replicating virus. Viral plaque assay performed on BHK-21 monolayer cells exposed to serial dilutions

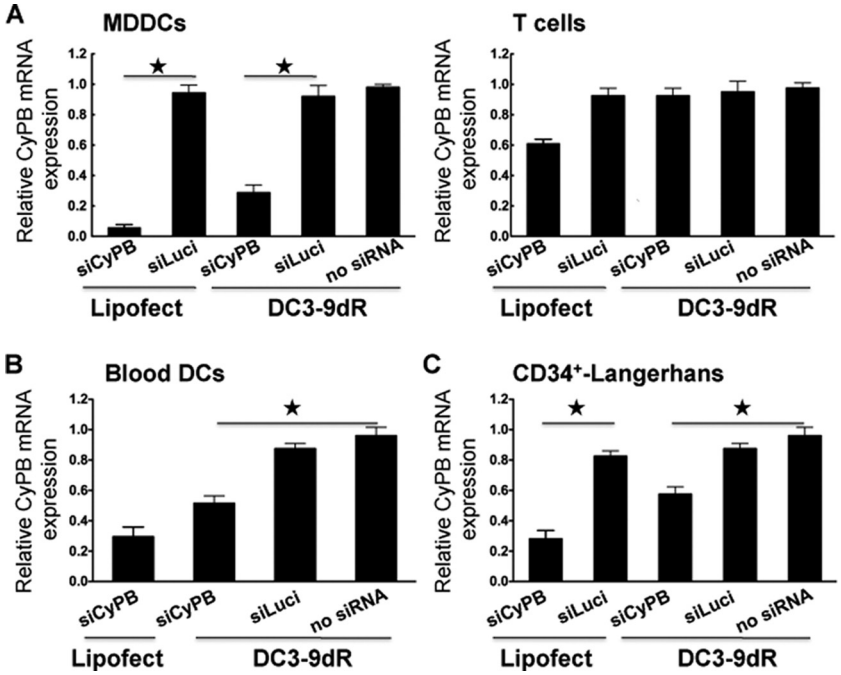

FIG. 2. DC3-9dR-delivered siRNA silences gene expression specifically in dendritic cell subsets. Human MDDCs or PHA-stimulated T cells (A) ($n = 4$), blood dendritic cells (B) ($n = 2$), and CD34-derived dendritic cells (C) ($n = 3$) were transfected with CyPB siRNA using Lipofectamine or transduced with DC3-9dR-complexed CyPB siRNA, and specific gene knockdown was assayed by quantitating mRNA levels by real time-PCR 24 h later. Error bars indicate standard deviations. *, $P < 0.5$.

of the supernatant from siRNA-treated or control MDDCs indicated that FvE^D siRNA-treated infected MDDCs released fewer virus particles in the supernatant ($<10^2$ PFU/ml) than did controls ($\sim 10^3$ PFU/ml) (Fig. 4E). In parallel, flow cytometric analysis of the infected BHK-21 cells revealed greater than 10-fold-lower infection with FvE^D siRNA-treated infected MDDC supernatants at a 1:100 dilution compared to controls (Fig. 4F). These results indicate that DC3-9dR-delivered antiviral siRNA inhibits DEN-2 infection in human DCs.

The DC3-9dR/siFvE^D complex inhibits DEN-2 replication in MDMs. Macrophages are also prime targets of dengue infection and are considered to be a major source of cytokines and chemokines that affect disease pathogenesis (9, 12). In preliminary experiments we found that unlike Lipofectamine, DC3-9dR peptide was unable to deliver siRNA to the adherent macrophages isolated ex vivo from human PBMCs (Fig. 5A, left panel), with only background levels of FITC-labeled siRNA uptake. However, it has recently been shown that dengue infection results in a strong upregulation of the M-CSF receptor in monocyte-derived macrophages (40). To mimic dengue infection, we treated macrophages with M-CSF and then tested for DC3-9dR-mediated siRNA delivery. Uptake of FITC-labeled siRNA in M-CSF-treated MDMs using Lipofectamine, DC3-9dR, and 9dR demonstrated that DC3-9dR was indeed able to deliver more FITC-labeled siRNA to M-CSF-treated MDMs (35%) than to untreated MDMs (9%) (Fig. 5A, right panel). Further gene-silencing experiments with CyPB siRNA showed that specific gene knockdown was about 45 to 50% with DC3-9dR in M-CSF-treated MDMs versus <5% in untreated MDMs (Fig. 5B).

As the M-CSF receptor is upregulated in MDMs at 6 h after dengue infection (40) and MDMs treated with M-CSF are amenable to DC3-9dR-mediated siRNA delivery (Fig. 5A and B), we hypothesized that DC3-9dR would be able to deliver antiviral siRNA to dengue-infected MDMs. To test this, first dengue infection was established in MDMs by treating them with DEN-2 complexed with the enhancing anti-prM antibody (0.1 ng/ml) (21). The infection levels were negligible in the absence of antibody but rose to ~20% in the presence of the enhancing antibody (Fig. 5C). To test the effect of siRNA on viral replication, at 24 h postinfection MDMs were treated with DC3-9dR/siFvE^D or siLuci complexes. The viral load was quantitated by quantitative RT-PCR at 48 h posttransduction. Viral RNA in MDMs transduced with FvE^D siRNA complexed to DC3-9dR was reduced to levels comparable to those after Lipofectamine transfection, whereas no change in viral load was seen with the mock or irrelevant control siRNA (Fig. 5D). Further, in comparison to control MDMs, reduced amounts of negative-strand DEN-2 RNA could be detected in FvE^D siRNA-treated MDMs by qualitative RT-PCR (Fig. 5E). Infection was also assayed by exposing BHK21 cells to supernatants from siRNA- and mock-treated MDMs. About a 10-fold reduction in plaque numbers was observed with supernatants from FvE^D siRNA-treated MDMs compared to the mock control (Fig. 5F). Flow cytometric analysis of the infected BHK-21 cells confirmed the results of the plaque assay; there was about a 4-fold-lower infection with FvE^D siRNA-treated MDM supernatants than with Luc siRNA and mock controls (Fig. 5G). These results demonstrate that DC3-9dR peptide-mediated siRNA delivery inhibits viral replication in dengue-infected MDMs. The results underscore the potential usefulness of the delivery approach for treatment of dengue infection, as mac-

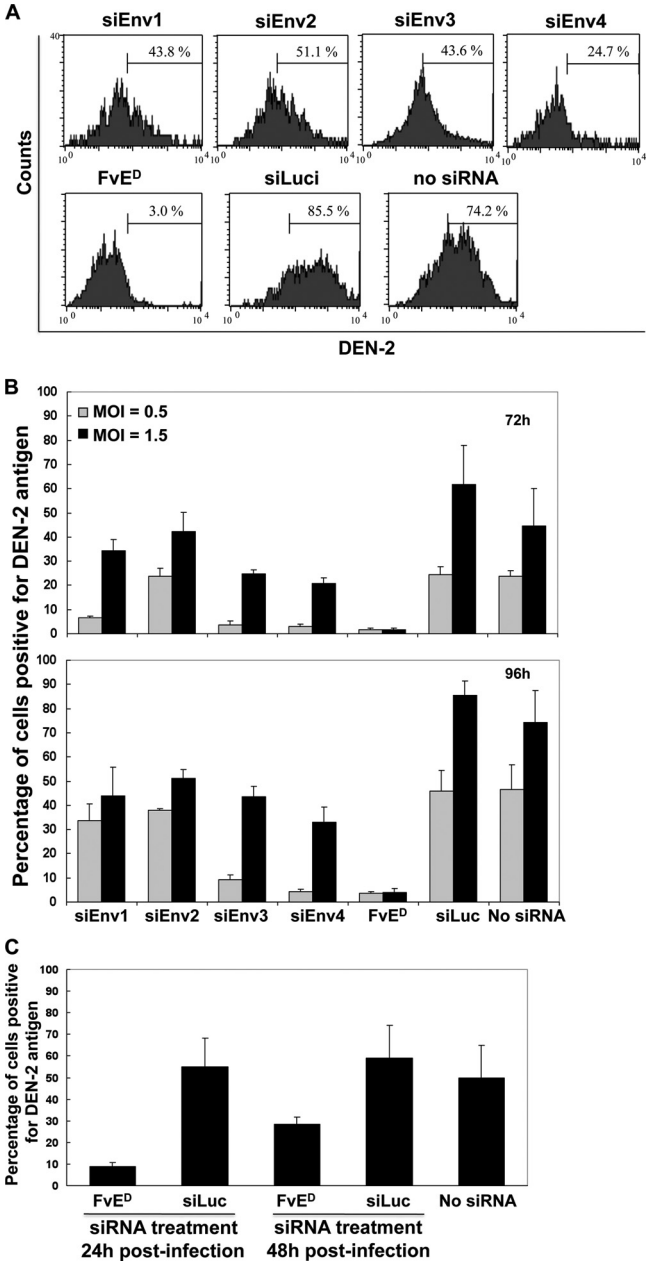

FIG. 3. FvE^D siRNA potently suppresses dengue virus replication in BHK-21 cells. (A and B) BHK-21 cells were transfected with the indicated siRNAs using Lipofectamine, and after 24 h cells were challenged with DEN-2 (MOI of 0.5 or 1.5), followed by another round of siRNA transfection 24 h postinfection. Viral replication was monitored at 72 h (B, top panel) or 96 h (B, bottom panel) postinfection by flow cytometry. Representative results after 72 h of infection (MOI of 1.5) are shown in panel A, and cumulative data from three independent experiments are shown in panel B. (C) BHK21 cells were first infected with DEN-2 (MOI of 1.5) and 24 h or 48 h later were transfected with control siLuci or FvE^D siRNA. Infection levels were monitored at 72 h postinfection. Error bars indicate standard deviations.

rophages are important targets of the virus and a major source of proinflammatory cytokines (12).

DC3-9dR-mediated siRNA delivery to inhibit DEN-2-induced TNF-α expression. TNF-α produced by immune cells is thought to play a major role in the pathogenesis of DHS/DSS by its well-known ability to induce plasma leakage (12). Also, elevated levels of this cytokine have been detected in the blood of dengue virus-infected individuals (18, 20). We therefore used the DC3-9dR peptide to test the feasibility of silencing dengue virus-induced TNF-α production in MDDCs. In a preliminary experiment, the kinetics of TNF-α induction by dengue virus was examined. TNF-α mRNA and protein levels in immature MDDCs were measured at various times after dengue infection (MOI of 2) with concurrent quantitation of viral RNA by real-time PCR. TNF-α mRNA as well as protein levels peaked at around 12 h postinfection and dropped at later time points (Fig. 6A), but viral RNA levels increased with time (Fig. 6B). These data indicate that dengue virus induces TNF-α production by myeloid DCs.

Next we tested two siRNA sequences targeting different regions of the human TNF-α gene. To confirm the silencing activity of siRNAs, MDDCs were transfected with graded doses of TNF-α siRNAs using Lipofectamine, and 24 h later treated cells were stimulated with lipopolysaccharide (LPS) (100 ng/ml) for 10 h and TNF-α mRNA levels measured. TNF-α mRNA levels were reduced >80% with one of the siRNAs (Fig. 6C, siTNFα-1), which was used for all further experiments. To test whether DC3-9dR-mediated siRNA delivery suppressed dengue virus-induced TNF-α production in MDDCs, the cells were first transduced with TNF-α siRNA/DC3-9dR complex on two consecutive days and then 16 h later infected with DEN-2 (MOI of 2). After 10 h, cells were harvested for RNA extraction to detect TNF-α mRNA levels. Treatment with TNF-α, but not Luci siRNA, reduced both TNF-α mRNA and protein induction after dengue infection (Fig. 6D).

In addition to its powerful proinflammatory role, TNF-α has also been suggested to have an antiviral role against several viruses (13). Since dengue-infected MDDCs produce TNF-α, we investigated whether silencing this cytokine had an effect in enhancing viral replication. MDDCs were transfected with DC3-9dR/TNF-α siRNA and infected with DEN-2 (MOI of 2) as described above, and viral RNA levels were quantitated 48 h later. No significant difference in viral replication was detected between control and TNF-α-silenced cultures (Fig. 6E). These data indicate that our strategy of targeted suppression of endogenous TNF-α in dengue-infected MDDCs would likely serve to reduce TNF-α-associated pathogenesis in DHF/DSS without in itself increasing the viral load.

We also used the DC3-9dR peptide delivery strategy to simultaneously target both host and viral genes, as this might also serve as a useful therapeutic approach for ameliorating DSS/DHF. MDDCs were transduced with a combination of TNF-α and FvE^D siRNAs twice and infected with DEN-2. RNA was extracted from cells at 10 h and 48 h postinfection as described above. Treatment with dual siRNAs was effective at simultaneously decreasing TNF-α levels (Fig. 6F) and reducing the viral load (Fig. 6G), suggesting that DC-specific delivery could be useful for blocking many aspects of dengue pathogenesis.

Intravenous treatment of siRNA complexed to DC3-9dR prevents poly(I:C)-induced TNF-α production in HuHSC mice. The ability of DC3-9dR to deliver siRNA to dendritic cells *in vivo* was tested in CD34$^+$ engrafted HuHSC mice,

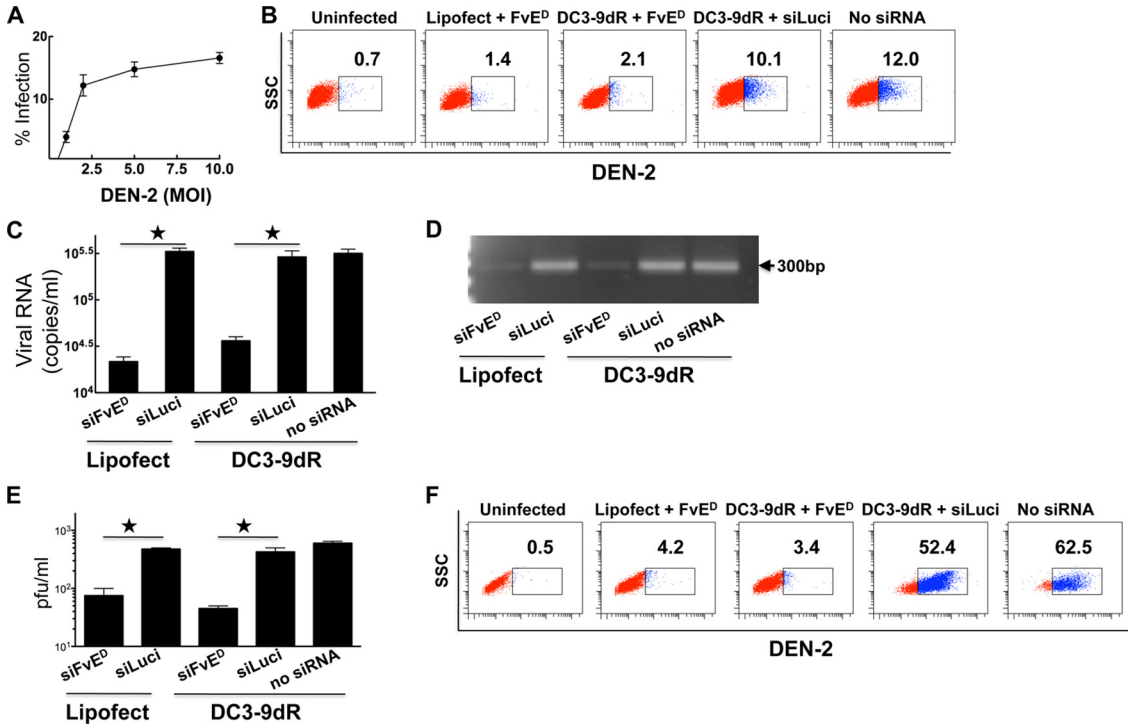

FIG. 4. DC3-9dR-mediated delivery of FvED suppresses DEN-2 infection in MDDCs. (A) MDDCs were infected with DEN-2 at the indicated MOIs and viral replication monitored after 72 h by flow cytometry. (B, C, and D) MDDCs were transduced with DC3-9dR-complexed siFvE^D twice on two consecutive days before challenge with DEN-2 at an MOI of 2. Viral replication was monitored at 72 h postinfection either by flow cytometry after intracellular staining (B) or by quantitating viral positive-strand RNA load by real-time RT-PCR (C) or detection of DEN-2 negative strand RNA by the tagged RT-PCR method using primers targeting the NS3 region of the genome (D). (E and F) BHK-21 cells were infected with serially diluted supernatants used for panel B, and infection was monitored after 96 h by plaque assay (E) or flow cytometry (F). The results shown in panel F are for the 1:100 dilution. Error bars indicate standard deviation. ⋆, $P < 0.5$.

which reconstitute all components of the human adaptive immune system, including dendritic cells. In an initial experiment, we sought to determine whether dendritic cells or macrophages are the primary source of TNF-α production upon TLR3 ligation by incubating human MDDCs and MDMs with poly(I:C) (50 μg/ml) *in vitro* and assaying supernatants for TNF-α protein levels at various time points. The results clearly indicate that the TNF-α signal was mostly confined to dendritic cells and peaked at around 2 h postinduction (Fig. 7A).

We then tested whether DC-3-9dR was able to deliver siRNA to DC *in vivo*. The mice were first treated with recombinant human GM-CSF (rhGM-CSF) and rhIL-4 (10 μg/mouse each) for 5 days to increase the dendritic cell precursor number in circulation. The animals were then given three intravenous (i.v.) injections of DC3-9dR/siLuci or siTNFα complex (at 6-h intervals). Sixteen hours after the last siRNA injection, the mice were given poly(I:C) (300 μg/ml) intraperitoneally and bled at the indicated time points to assay serum TNF-α levels. In control mice, poly(I:C)-induced TNF-α production was detected beginning at 0.5 h, peaked at 1 h, and was still detectable at 6 h. DC3-9dR-mediated delivery of siTNFα, but not siLuci, resulted in a significant reduction of poly(I:C)-induced-TNF-α production in these mice (Fig. 7B). The data suggest that DC3-9dR was able to deliver siRNA to DCs *in vivo*. However, further refinements of the humanized mouse model will be required to directly assess the DC-targeting capability of the peptide and its usefulness as a potential tool for RNAi therapy in dengue infection.

DISCUSSION

Here we describe a novel tool for DC-specific delivery of siRNA that uses a chimeric peptide, consisting of a DC-targeting 12-mer peptide and nonamer polyarginine residues, that binds nucleic acids by charge interaction. Since DCs play a key role in initiating dengue virus infection *in vivo*, we tested the feasibility of this approach for suppressing viral replication in these cells. This strategy could be used to suppress dengue virus replication as well as virus-induced TNF-α production in MDDCs. Finally, as proof of concept, we demonstrated that DC3-9dR is able to deliver siRNA to human dendritic cells *in vivo* in humanized NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ mice.

RNA interference (RNAi) is a posttranscriptional gene-silencing mechanism that has emerged as a powerful laboratory tool for abrogating gene expression in a sequence-specific manner (16). RNAi is a natural antiviral defense mechanism in plants, nematodes, and insects (2, 54). Although its natural antiviral role in mammals is still debated, several studies have investigated viral RNA and replication intermediates as potential RNAi targets that could be harnessed for combating viral infections. RNAi-mediated inhibition has also been success-

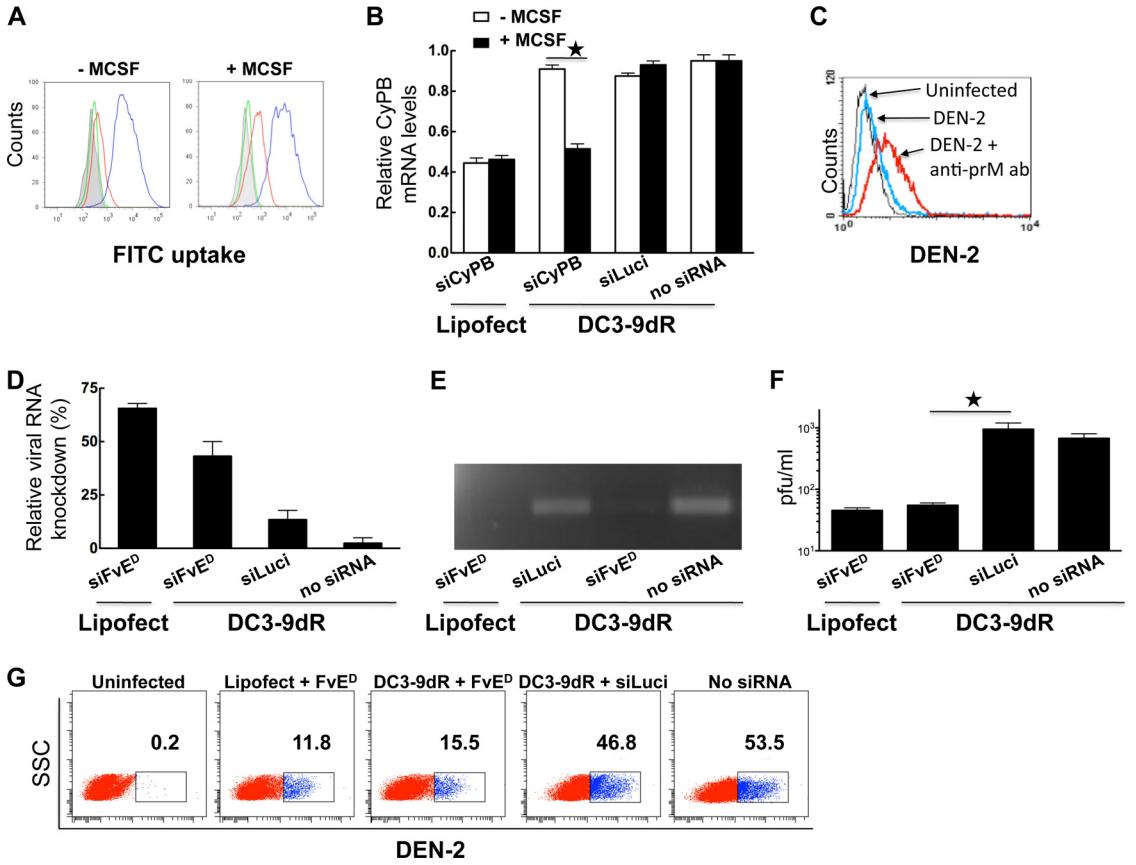

FIG. 5. The DC3-9dR peptide can deliver siRNAs to M-CSF-treated and DEN-2-infected MDMs. (A) Monocytes were cultured in the absence (left panel) or presence (right panel) of M-CSF (20 ng/ml). Five days later MDMs were transfected/transduced with Lipofectamine (blue histogram)-, DC3-9dR (red histogram)-, or 9dR (green histogram)-complexed FITC-labeled siRNA, and uptake was quantitated 16 h later. (B) M-CSF-treated or untreated MDMs were transduced with siCyPB/siLuci using Lipofectamine or DC39dR and assayed for gene knockdown by quantitative PCR 24 h later. Expression relative to mock and irrelevant siRNA-treated cells is shown. (C) MDMs were infected with DEN-2 (MOI of 2) previously complexed with anti-prM antibody (0.1 ng/ml) and tested for infection by flow cytometric analysis at 72 h postinfection. (D and E) Dengue-infected-MDMs were transduced with the DC3-9dR/FvE^D siRNA complex at 24 h postinfection and viral replication monitored 48 h later by quantitation of positive-strand DEN-2 RNA by real-time RT-PCR (D) or negative-strand DEN-2 by a tag amplification method using primers targeting the NS3 region (E). (F and G) BHK-21 cells were infected with serial dilutions of the viral supernatants from panel D and infection monitored after 96 h by plaque assay (F) or by flow cytometry (G). The results shown in panel G are for the 1:100 dilution. Error bars indicate standard deviations. *, $P < 0.5$.

fully demonstrated for many arthropod-borne flaviviruses, including dengue virus (3, 26, 60).

We identified a potent siRNA for DEN-2 targeting a sequence in the cd loop-coding sequence within domain II of the viral envelope protein. The sequence may be constrained for mutation because of its important role in membrane fusion (1). We were aided by the availability of a related lead sequence determined in an earlier study that conferred robust protection against experimental murine encephalitis caused by the closely related Japanese encephalitis and West Nile viruses (26). The siRNA was synthesized to be homologous to the corresponding target sequence in DEN-2, which differed at positions 10 and 12.

DCs are of special relevance to dengue infection, as they are the initial cells in the skin to become infected during transmission of the virus by infected mosquito bite (12), and the proinflammatory cytokines that they produce play a significant role in dengue immunopathogenesis (13, 14, 38). Viral vectors have been used successfully for intracellular expression of siRNA in DCs. In fact, one study used adeno-associated virus-encoded short hairpin RNA (shRNA) to reduce dengue virus replication in cultured DCs (60). However, a significant reduction could be achieved only at a high vector PFU of 10^9/ml. In addition, long-term transgene siRNA expression may not be necessary for an acute viral infection such as dengue, particularly given the attendant pathogenic risks such as oncogenicity, immune response, and toxicity related to the viral vectors themselves (reviewed in reference 35). The use of siRNA as a small-molecule drug would overcome many of the limitations associated with vector-enforced endogenous siRNA expression.

Delivery of synthetic siRNAs to DCs by electroporation and chemical transfection *in vitro* supports the feasibility of using RNAi to modulate gene expression in these cells (37, 44, 45). However, for application of RNAi as a dengue therapeutic, an effective and cell-specific *in vivo* delivery system is required. High-pressure hydrodynamic injection of siRNA or even more practical approaches (such as conjugating siRNA to a cholesterol moiety or packaging it in liposomes) are nonselective

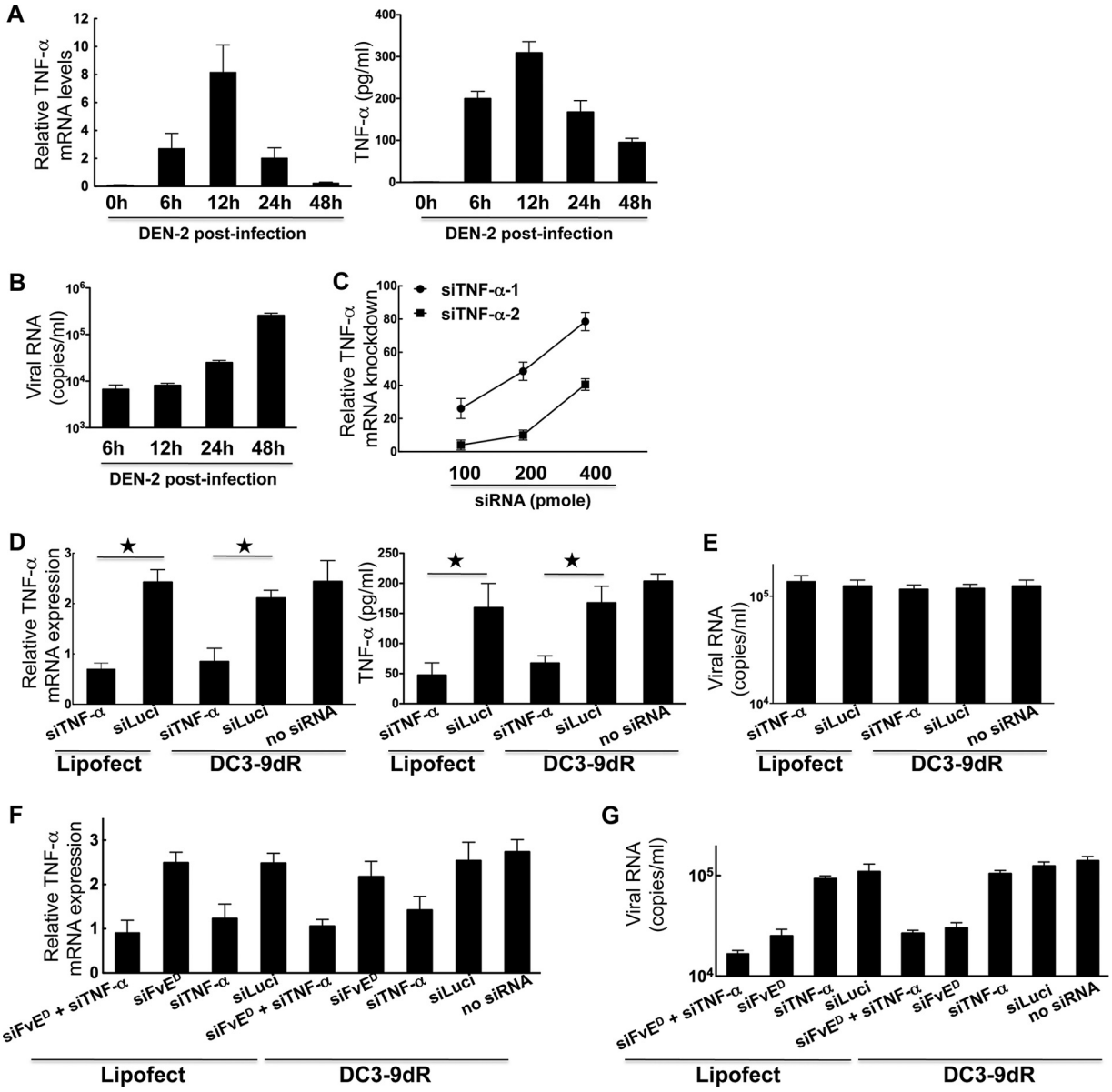

FIG. 6. DC3-9dR-mediated siRNA delivery inhibits DEN-2-induced TNF-α expression without increasing viral replication. (A and B) MDDCs were infected with DEN-2 (MOI of 2) and TNF-α mRNA (A, left panel), and protein levels (A, right panel) as well as viral RNA loads (B) were assayed at the indicated time points. Results from three experiments (means ± standard deviations) are shown. (C) MDDCs were transfected with siRNAs targeting the human TNF-α gene and after 24 h were treated with LPS (100 ng/ml). After overnight culture, TNF-α mRNA levels were measured by quantitative PCR. The percent reduction in TNF-α mRNA relative to those in irrelevant siRNA-treated cells is shown. (D and E) MDDCs were transduced with siLuci or siTNFα using Lipofectamine or DC3-9dR twice on two consecutive days before infection with DEN-2 (MOI of 2). Ten hours later TNF-α mRNA (D, left panel) and TNF-α protein (D, right panel) levels were assayed. At 48 hours postinfection, viral copy numbers (E) were measured by quantitative PCR. (F and G) MDDCs were treated with TNF-α and antiviral FvE^D siRNA singly or in combination and infected with DEN-2 as for panel D. TNF-α mRNA levels (F) and viral loads (G) were quantitated as described above. Error bars indicate standard deviations. *, $P < 0.5$.

methods that have been used successfully for *in vivo* delivery of siRNA to liver and other tissues (26, 43, 51, 58); however, they may not work well for primary hematopoietic cells such as DCs. We and others have used siRNAs coupled to antibodies or peptides that recognize cell surface receptors for targeted delivery to specific cell types, including neurons and T lymphocytes (25, 27, 43). Our novel peptide reagent DC3-9dR can deliver siRNA to a variety of DC populations *in vitro*, including MDDCs, CD34-derived Langerhans cells, and freshly isolated blood DCs, as well as to the DCs *in vivo*, further supporting the practical utility of the approach.

Dengue pathogenesis is characterized by unbridled production of proinflammatory cytokines. Notable among them is TNF-α, which has been implicated in the vascular leakage that characterizes DHF/DSS (13, 15) and whose plasma levels are elevated during acute dengue infection (7, 18, 19). We have

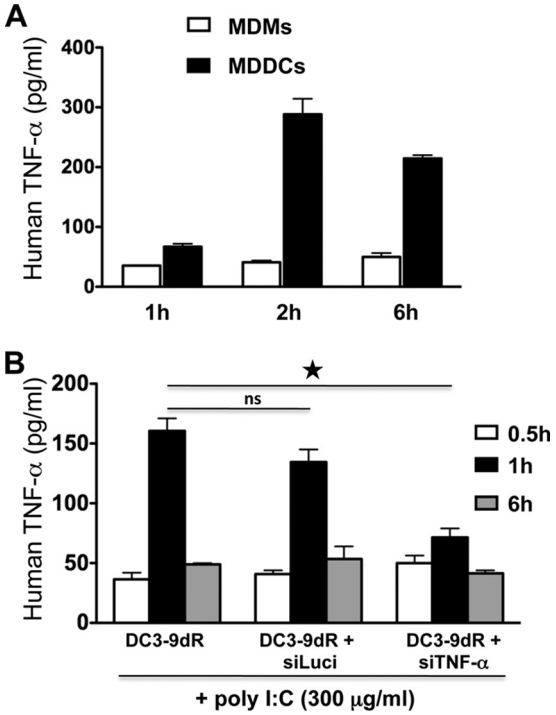

FIG. 7. Intravenous treatment of siRNA complexed to DC3-9dR prevents poly(I:C)-induced TNF-α production in HuHSC mice. (A) Human monocytes were differentiated into MDDCs or MDMs as described in Materials and Methods. At day 5, poly(I:C) (50 μg/ml) was added to the cell cultures, and TNF-α levels in the supernatants collected at the indicated times were assayed by ELISA. (B) HuHSC mice were injected with recombinant human GM-CSF and IL-4 (10 μg/mouse each) daily for 5 days for dendritic cell mobilization. On day 5, mice were intravenously injected thrice (at 6-h intervals) with DC3-9DR/siLuci or siTNFα complex. At 16 hours after the third injection, mice were injected intraperitoneally with poly(I:C) (300 μg/ml), and after the indicated times serum levels of human TNF-α were measured by ELISA ($n = 3$). ns, not significant. ⋆, $P < 0.5$.

demonstrated that our delivery approach effectively curbs virus-induced TNF-α production in DCs. However, an important limitation to be considered for targeting a host molecule such as TNF-α is whether blockade also interferes with a possible antiviral effect that might outweigh its pathogenic potential. Our data show that TNF-α ablation does not lead to augmented virus replication in DCs, suggesting that the cytokine provides a potentially safe therapeutic RNAi target for ameliorating severe immune manifestations of dengue. This is in agreement with a previous demonstration that TNF-α did not alter the course of viral replication in dengue-infected macrophages (56). Apart from TNF-α, other host molecules have been identified to have immunopathogenic roles in dengue virus infection. For example, dengue infection of DC has been reported to induce the expression of the proapoptotic TRAIL molecule in a type I interferon (IFN)-dependent manner (5, 55). Additionally, many other host proteins necessary for dengue infection were recently identified through a genome-wide siRNA screen (24). Thus, if detrimental consequences to the host can be ruled out, many known and newly identified host genes involved in dengue pathogenesis could be therapeutically targeted alongside viral genes.

Animal models for dengue have suffered due to the lack of a system that precisely reflects human dengue infection. Humanized mouse models have been tested for dengue infection with various degrees of success in recapitulating the features of human infection, including fever, viremia, erythema, and thrombocytopenia (6, 30, 41). NOD/SCID mice engrafted with $CD34^+$ human HSC have been shown to be susceptible to subcutaneous infection with DEN-2, manifesting clinical signs of erythema and thrombocytopenia along with detectable viral RNA in tissues of some mice (6). On the other hand, *RAG2*$^{-/-}$ *IL2rγ*$^{-/-}$ mice challenged with a pool of four lab-adapted DEN-2 viruses of different serotypes did not show clinical features of erythema or thrombocytopenia but developed viremia and dengue virus-specific human IgM and IgG responses (30). A more recent analysis uncovered strain-specific differences in virulence and transmission of DEN-2 infection in humanized NOD-*scid IL2rγ*$^{-/-}$ Hu/HSC mice, which may explain the conflicting results. Our attempts to reproduce dengue infection with the lab-adapted DEN-2 NGC strain in NOD-*scid IL2rγ*$^{-/-}$ HuHSC mice were not successful. As an alternative means to validate siRNA delivery by DC3-9dR *in vivo*, we tested Toll-like receptor (TLR)-mediated TNF-α production in these mice. Our studies show that DC3-9dR/siRNA is able to curb poly(I:C)-mediated TNF-α production in HuHSC mice. Unlike LPS, which stimulates both macrophages and DC to secrete the cytokine, poly(I:C) is known to preferentially act on DCs (34, 47) (Fig. 7A), validating the DC3-9dR peptide as a promising tool not only for facilitating RNAi therapeutics for dengue but also in broader clinical applications requiring selective gene ablation in dendritic cells.

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The first two authors contributed equally to this study.

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