J Clin Immunol (2011) 31:1065–1078
DOI 10.1007/s10875-011-9578-7

Immunosuppressive Effects and Mechanisms of Leflunomide in Dengue Virus Infection of Human Dendritic Cells

Wan-Lin Wu · Ling-Jun Ho · Pei-Chih Chen · Yi-Ting Tsai · Seng-Ting Hsu · Deh-Ming Chang · Jenn-Haung Lai

Received: 27 May 2011 / Accepted: 21 July 2011 / Published online: 17 August 2011

Abstract
Background Dengue virus (DENV) infection is a serious public health issue without specific treatment. We examined the potential immunomodulatory effects of leflunomide, a dihydroorotate dehydrogenase inhibitor commonly prescribed for arthritis, in DENV-stimulated monocyte-derived dendritic cells (mo-DCs).
Methods mo-DCs were prepared from purified monocytes. Cytokine and chemokine concentrations were determined by enzyme-linked immunosorbent assay. Expression of cell surface markers or viral E protein was measured by flow cytometry. The activation of transcription factors and kinases was determined by electrophoretic mobility shift assays, Western blotting, or immunoprecipitation kinase assays. Chemotaxis assays were used to determine cell migration.
Results Leflunomide at therapeutic concentrations inhibited cytokine and chemokine production from DENV-infected mo-DCs. Leflunomide suppressed mo-DC maturation by downregulating the expression of both CD80 and CD86. In addition, leflunomide inhibited DENV-induced mo-DC migration and mo-DC response to chemoattractants CCL19 and CCL21. Inhibition of mo-DC migration was likely due to the suppression of CCR7 expression on mo-DCs. These events were associated with the suppression of nuclear factor kappa B and activator protein-1 signaling pathways by leflunomide.
Conclusions Leflunomide preserves immunosuppressive effects, inhibiting activation of DENV-stimulated mo-DCs. Leflunomide may be helpful in the development of therapeutics for DENV infection.

Keywords Dendritic cell · dengue virus · inflammation · leflunomide

Abbreviations

DENV	dengue virus
mo-DCs	dendritic cells
DHF	dengue hemorrhagic fever

W.-L. Wu · L.-J. Ho · S.-T. Hsu
Institute of Cellular and System Medicine,
National Health Research Institutes,
Zhunan,
Miaoli County 350 Taiwan, Republic of China

P.-C. Chen
Rheumatology, Department of Medicine, Chi Mei Medical Center,
Tainan, Taiwan, Republic of China

Y.-T. Tsai
Division of Cardiovascular Surgery, Department of Surgery,
Tri-Service General Hospital, National Defense Medical Center,
Taipei, Taiwan, Republic of China

D.-M. Chang · J.-H. Lai (✉)
Division of Rheumatology/Immunology and Allergy,
Department of Medicine, Tri-Service General Hospital,
National Defense Medical Center,
Neihu,
114, Taipei, Taiwan, Republic of China
e-mail: haungben@tpts5.seed.net.tw

J.-H. Lai
Institute of Preventive Medicine,
National Defense Medical Center,
Taipei, Taiwan

L.-J. Ho
Graduate Institute of Basic Medical Science, PhD Program for Aging,
China Medical University,
Taichung 40402, Taiwan, Republic of China

DSS	dengue shock syndrome
DHODH	dihydroorotate dehydrogenase
IKK	IκB kinase
NF-κB	nuclear factor kappa B
MAPK	mitogen-activated protein kinase
JNK	c-Jun N-terminal kinase
ERK	extracellular signal-regulated kinase
AP-1	activator protein-1
MCP-1	monocyte chemoattractant protein-1
MIP-1α	macrophage inflammatory protein-1α
RANTES	regulated upon activation, normal T cell expressed and secreted

Introduction

Dengue viruses (DENV) are positive-strand RNA viruses belonging to the mosquito-borne *Flaviviridae* family. DENV infection has been a major public health concern worldwide, especially in tropical and subtropical countries. The clinical manifestations after DENV infection include dengue fever and potential fatal phenomenon such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Epidemiological analysis reveals that the estimated annual occurrence worldwide is around 50,000,000–100,000,000 cases for dengue fever and 250,000–500,000 cases for DHF [1]. Efforts devoted to identify host target cells permissive to DENV replication reveal many potential candidates, including B cells, human muscle satellite cells, hepatocytes, mast cells, and dendritic cells (DCs) [2–6]. Among these potential targets for DENV, DCs serve as the best professional antigen-presenting cells (APCs) and play critical roles in both innate and adaptive immune responses. Therefore, they may be the most important and efficient DENV hosts.

After infection by DENV, DCs undergo maturation process and become activated. They then migrate from periphery to lymphoid organs where DCs have the opportunity to interact with a high number of T cells. Along with these inflammatory processes which appear to be dependent in part on cyclooxygenase-2 (COX-2) signaling cascades [7], DCs also begin to secrete cytokines, metalloproteinases, and chemokines [8–10]. In this regard, the levels of many chemokines, including interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and regulated upon activation, normal T cell expressed and secreted (RANTES) have been shown to be elevated in DENV-infected patients [11–14]. According to Dejnirattisai et al. [15], there appears to be a very complicated interaction among viruses, DCs, T cells, and cytokines during the course of DENV infection. The interaction among target immune effectors and cytokines or chemokines may lead to the development of severe clinical manifestations like DHF [16]. Unfortunately, given the high probability of severe consequences in DENV infection, there have been neither effective vaccines nor therapeutics available for the treatment aside from symptomatic treatment [17, 18].

Dihydroorotate dehydrogenase (DHODH) is the rate-limiting enzyme within the pyrimidine synthetic process [19]. Because there is a significant increase of need for pyrimidine in proliferating cells [20], blockade of pyrimidine synthesis by leflunomide (inhibitor of DHODH) has been shown as a powerful immunomodulatory approach in the treatment of rheumatoid arthritis and seronegative spondyloarthropathy [21]. In addition to its uses in autoimmune disorders, leflunomide has also been reported to have antiviral effects. The most commonly reported target for leflunomide is the polyomavirus, a virus closely associated with nephropathy after renal transplantation [22]. Leflunomide under therapeutic concentrations can suppress human immunodeficiency virus replication and reduce herpes simplex virus production [23, 24]. There are therapeutic benefits of leflunomide in cytomegalovirus infection as well as ganciclovir-resistant cytomegalovirus infection [25, 26]. Furthermore, leflunomide is helpful in inhibiting respiratory syncytial virus-mediated reduction of alveolar fluid clearance [27].

Although there are occasional reports on leflunomide in virus infection, the effects and possible mechanisms by which leflunomide work on DENV infection have not been investigated. Given the importance of DCs in DENV infection, we determine in this report whether leflunomide has any effect in DENV infection of mo-DCs. The results suggest that leflunomide did not have any effect on DENV production; instead, leflunomide at therapeutic concentrations suppressed DENV-induced activation of mo-DCs in several different immunological aspects.

Materials and Methods

Culture Medium and Reagents

The cell culture medium consisted of RPMI 1640 (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1,000 U/ml penicillin–streptomycin (Gibco). Recombinant interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from R & D (Minneapolis, MN, USA). Antibodies against c-Jun N-terminal kinase (JNK), phosphorylated p38, and phosphorylated extracellular signal-regulated kinase (ERK) were purchased from Cell Signaling

(Beverly, MA, USA). The antibodies against IkappaBalpha kinase alpha (IKKα), p65, p50, or upstream stimulatory factor-2 were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-CCR7 antibodies and chemoattractants, including CCL19 and CCL21, were purchased from R & D. Fluorescence-conjugated anti-CD80 and anti-CD86 antibodies were purchased from PharMingen (San Diego, CA, USA). Unless specified, the rest of the reagents were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Establishment of mo-DCs

mo-DCs were established from purified $CD14^+$ monocytes as described [28]. In brief, buffy coat (equivalent to 500 ml of whole blood for each) from a blood bank was mixed with Ficoll-Hypaque; after centrifugation, the layer of peripheral blood mononuclear cells (PBMCs) was collected. PBMCs were then incubated with anti-CD14 microbeads at 4–8°C for 15 min. After washing, the $CD14^+$ cells were isolated using a MACS cell isolation column (Miltenyi Biotech). The purified monocytes were cultured in the medium containing 800 U/ml GM-CSF and 500 U/ml IL-4 at a cell density of 1×10^6 cells/ml. The culture medium was changed every other day and the cells after 5–7 days of culture with purity higher than 92% were used for experiments [28].

DENV Preparation

The preparation of DENV has been described previously [28]. Although the mouse-adapted, neurovirulent prototype New Guinea C (NGC) strain of DENV2 has undergone a large number of passages in vitro and, therefore, has acquired tissue culture adaptations, it remains to be a commonly chosen viral strain in many DENV studies [29, 30]. In brief, DENV2 NGC strain (American Type Culture Collection, Rockville, MD, USA) was propagated in C6/36 mosquito cells in RPMI containing 5% heat-inactivated fetal calf serum (FCS) and maintained at 28°C in a 5% CO_2 atm for 7 days. The supernatants were collected, virus titers determined, and then stored at −70°C until use. To determine virus titers, the culture supernatants were harvested for plaque-forming assays. Various virus dilutions were added to 80% confluent baby hamster kidney (BHK-21) cells and incubated at 37°C for 1 h. After adsorption, cells were washed and overlaid with RPMI 1640 containing 1% agarose (SeaPlaque; FMC BioProducts) and 1% FCS. After incubation for 7 days, cells were fixed with 10% formaldehyde and stained with 0.5% crystal violet. The numbers of plaques were counted and the results were shown as plaque forming units per milliliter. To prepare mock-infected supernatants, all of the procedures were identical except that buffered saline was substituted for the virus inoculation.

DENV Infection

mo-DCs cultured for 5–7 days were infected with mock or DENV at multiplicity of infection (MOI) 5 for 4 h at 37°C [28]. After viral absorption, cells were then washed and cultured in six-well plates (Costar, Cambridge, MA, USA) with culture medium in the presence of exogenously added cytokines for various periods of time as indicated in the figure legends. For treatment, the cell density was maintained at 1×10^6/ml in culture medium.

Cell Toxicity Measurement

Aside from trypan blue exclusion assay, the release of lactate dehydrogenase (LDH) after damage of plasma membrane indicating cell death was also measured according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). The percent cytotoxicity was calculated as (sample value−medium control)÷(high control−medium control)×100, where the sample value is the average of absorbance values of the triplicates of drug-treated cell supernatants after subtraction from each of the absorbance values obtained in the background control. Similarly, the average of absorbance values of untreated cell supernatants, used as medium control, was calculated [31]. Equal amounts of cells treated with 1% Triton X-100 were used as the high control 100%.

Determination of Chemokines by ELISA

Standard enzyme-linked immunosorbent assay (ELISA) methods were used to measure concentrations of cytokine TNF-α and chemokines, including RANTES, MCP-2, and MIP-1α (R & D). All determinants were performed in triplicates and expressed as means±standard deviations (SD).

Flow Cytometry Analysis

The determination of the expression of cell surface markers, including CD80, CD86, and CCR7, has been described [28]. For the determination of intracellular viral E protein expression, cells were permeabilized with 0.25% saponin (Sigma). After incubation for another 20 min, the anti-DENV E protein antibodies were added. After washing, the goat antimouse antibodies conjugated with fluorescein isothiocyanate were added and incubated for another 30 min. Finally, the samples were analyzed by flow cytometry [28].

Nuclear Extract Preparation

Nuclear extracts were prepared as described [28]. Briefly, the treated cells were left at 4°C in 50 μl of buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiotheritol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 3.3 μg/ml aprotinin] for 15 min with occasional gentle vortexing. Swollen cells were centrifuged at 15,000 rpm for 3 min. After the removal of the supernatants (cytoplasmic extracts), the pelleted nuclei was washed with 200 μl buffer A and, subsequently, cell pellets were resuspended in 30 μl buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol, 1 mM DTT, 0.5 mM PMSF, and 3.3 μg/ml aprotinin) and incubated at 4°C for 30 min with occasional vigorous vortexing. The mixtures were centrifuged at 15,000 rpm for 20 min, and the supernatants were used as nuclear extracts.

Electrophoretic Mobility Shift Assays

The electrophoretic mobility shift assay (EMSA) was performed as described [28]. The oligonucleotides containing NF-κB and AP-1 binding sites were purchased and used as DNA probes (Promega). The probes were radiolabeled with [γ-^{32}p]ATP using T4 kinase (Promega). For the binding reaction, the radiolabeled NF-κB or AP-1 probe was incubated with 5 μg of nuclear extracts. The binding buffer contained 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1 mM $MgCl_2$, 4% glycerol, and 2 μg poly(dI-dC). Binding reaction proceeded for 20 min at room temperature. If unradiolabeled competitive oligonucleotides were added, they were preincubated with nuclear extracts for 30 min before adding radiolabeled probes.

Western Blotting

ECL Western blotting (Amersham) was performed as described [28]. Briefly, equal amounts of proteins were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with nonfat milk buffer for 1 h and then blotted with antisera against individual proteins overnight. After washing with milk buffer, the filter was incubated with secondary antibodies conjugated to horseradish peroxidase for 1 h. The filter was then incubated with the substrate and exposed to an X-ray film.

Immunoprecipitation Kinase Assay

The immunoprecipitation kinase assay was performed as described [32]. The GST–IκBα and GST–c-Jun fusion proteins were used as substrates for IKKα and JNK, respectively. The antibodies for kinase assays were purchased from Cell Signaling (anti-JNK) or Santa Cruz (anti-IKKα). To perform immunoprecipitation kinase assays, 50–100 μg of the cellular extract was mixed with 2 μl of specific antibodies in incubation buffer containing 25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μM leupeptin, and 400 μM PMSF overnight. After precipitation with protein A beads and subsequent wash, the beads were resuspended in 40 μl kinase buffer containing cold ATP (30 μM), substrates (GST–c-Jun and GST–IκBα for JNK and IKKα, respectively), and 10 μCi of [γ-^{32}P] ATP. The mixture was incubated at 30°C (room temperature) with occasional gentle mixing for 30 min. The reaction was then terminated by resuspending in 1% SDS solubilizing buffer and boiled for 5 min and analyzed in SDS-PAGE.

Chemotaxis Assay

The chemotaxis assays were performed according to a previous report [33]. In brief, mo-DCs treated under different conditions as indicated migrated through a polycarbonate filter of 5 μm pore size in 24-well transwell chambers (Corning Costar). The lower chamber of a transwell cassette contained serum-free RPMI 600 μl with 100 ng/ml CCL19 or CCL21 (R & D). mo-DCs 1×10^5 in 100 μl serum-free medium were loaded in the upper chamber and incubated for 2 h at 37°C. Alternatively, to determine the chemoattractive activity of the culture supernatant, THP-1 cells were loaded into the upper chambers and the lower chambers were loaded with the culture supernatants and chemotaxis assays were performed accordingly. Then, cells migrating from the upper chamber to the lower chamber were counted by flow cytometry. The acquired events for a fixed time period of 60 s in a FACScan were determined using CellQuest software.

Statistics

When necessary, the results were expressed as the mean ±SD. The statistical analysis was performed using one-way analysis of variance with Bonferroni post hoc tests for multiple comparisons and Student's t test for comparison between two groups. $P<0.05$ was considered significant.

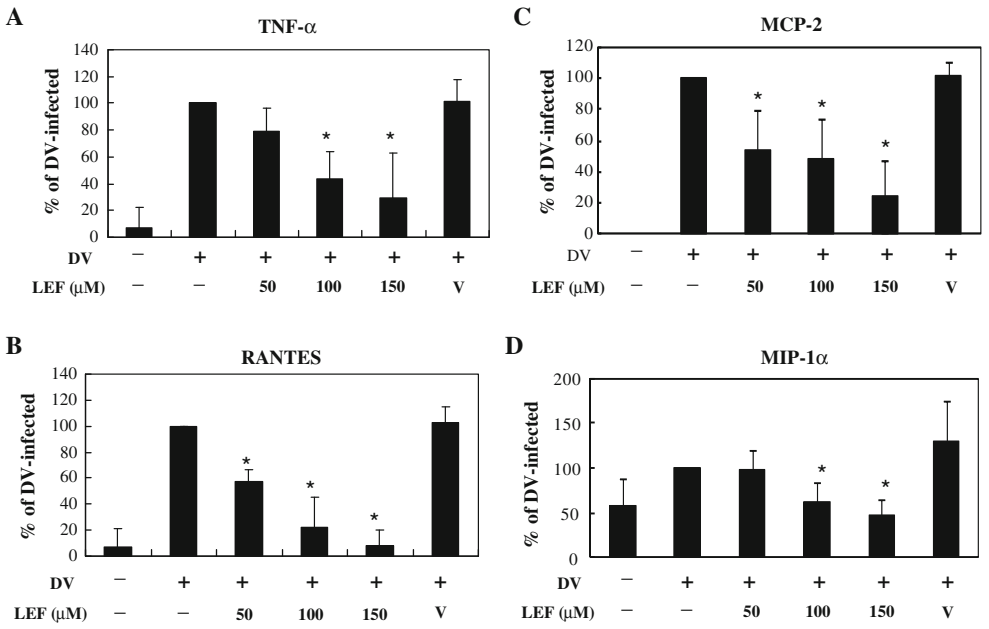

Fig. 1 Leflunomide suppressed cytokine and chemokine production from DENV-infected mo-DCs. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The supernatants were collected and the concentrations of cytokine or chemokines, including TNF-α (**a**), RANTES (**b**), MCP-2 (**c**), and MIP-1α (**d**) were determined by ELISA. The data shown are from at least three independent experiments using different donor mo-DCs. The level of cytokine or chemokines from DENV-infected mo-DCs in the absence of leflunomide treatment was taken as 100%. *$P<0.05$ compared to the DENV-stimulated mo-DCs. *V* vehicle, *LEF* leflunomide

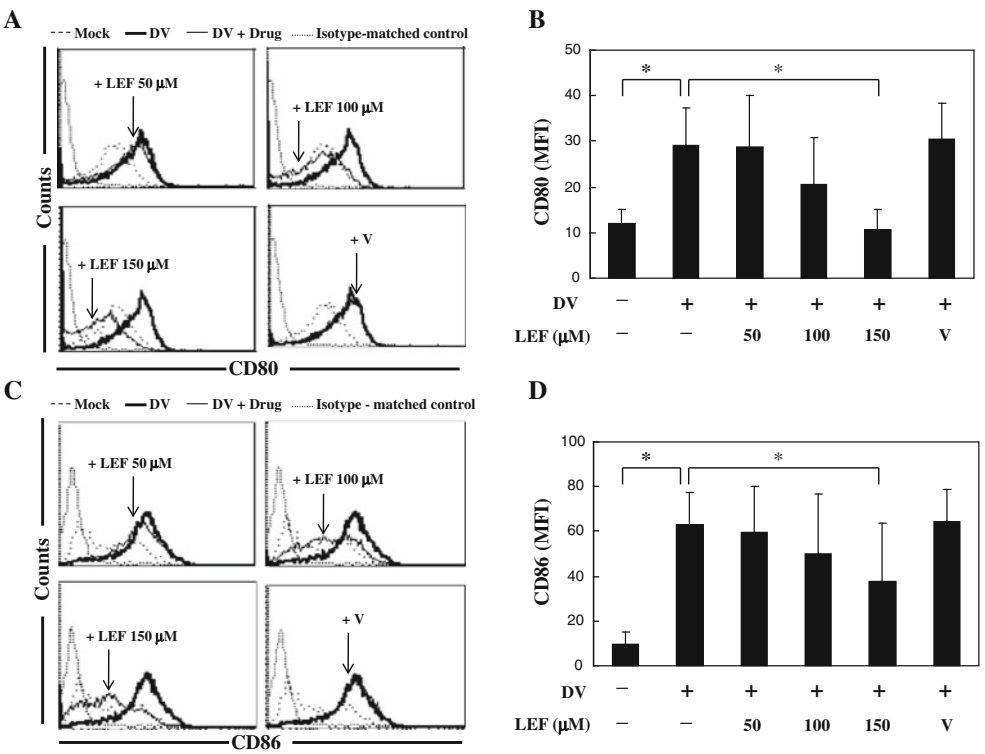

Fig. 2 Leflunomide inhibited DENV-induced mo-DCs maturation. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for another 46 h. Cells were collected and the expression of cell surface markers CD80 (**a**, **b**) and CD86 (**c**, **d**) were determined by flow cytometry as described in the "Materials and Methods" section. The representative results and the analysis pooled from at least three independent experiments using different donor mo-DCs were also shown. *$P<0.05$ compared to the DENV-stimulated mo-DCs. *V* vehicle, *LEF* leflunomide

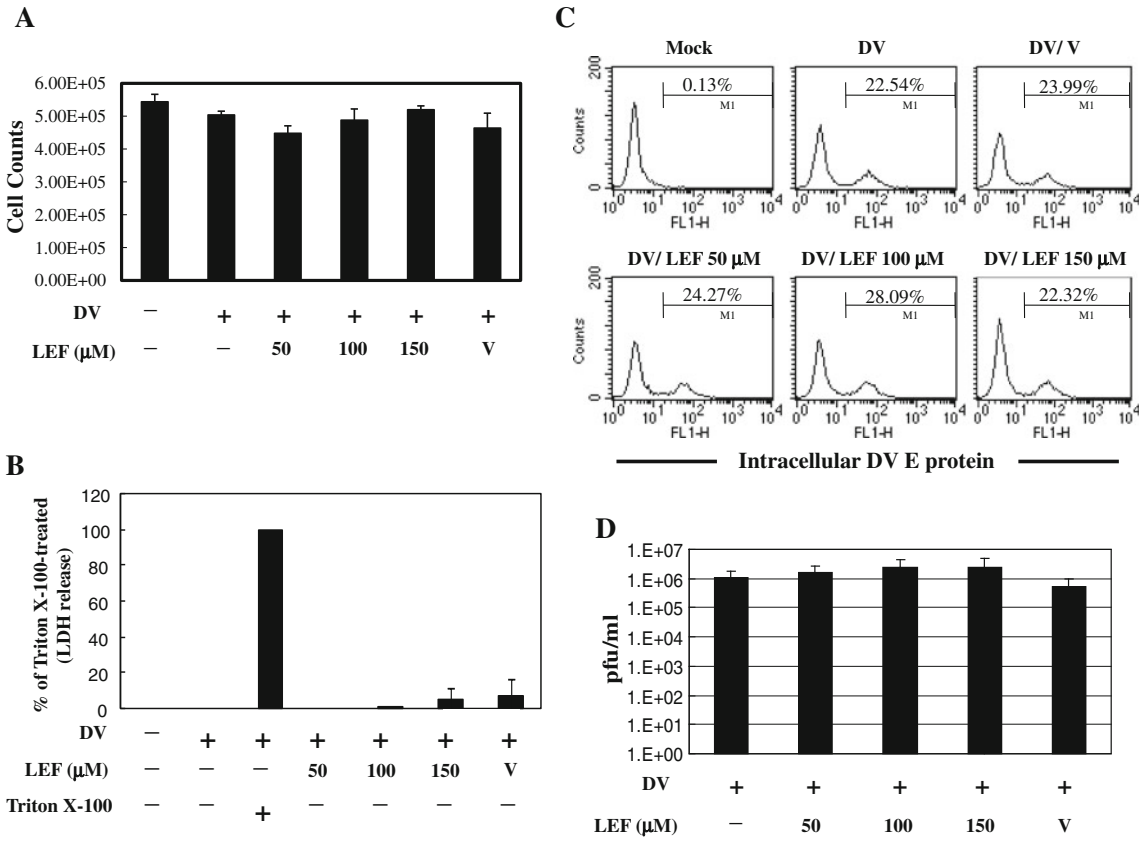

Fig. 3 Leflunomide did not affect DENV production. To determine the possible cytotoxic effects of leflunomide, mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for another 24 h. The cells were collected for trypan blue exclusion assay (**a**). The supernatants were collected for measurement of the release of LDH (**b**) as another indicator of cell death. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The expression of intracellular viral E protein was determined by flow cytometry (**c**). The culture supernatants were collected and the levels of viruses were determined by plaque assays (**d**). The representative results (**c**) and the polled analysis from at least three independent experiments (**a**, **b**, **d**) were shown. *V* vehicle, *LEF* leflunomide

Results

Leflunomide Inhibited DENV-Induced Cytokine and Chemokine Production and Maturation of mo-DCs

One potential fatal outcome in patients infected by DENV is the development of cytokine storm in which oversecreted cytokines and chemokines trigger overwhelming inflammatory responses. Indeed, after DENV infection, the abortively infected DCs or bystander DCs are stimulated and produce high levels of inflammatory mediators. This causes a vicious cycle in immune system activation and inflammatory responses [10, 34–37]. We determined whether leflunomide has an effect on cytokine and chemokine secretion from DENV-infected mo-DCs. mo-DCs were pretreated with various doses of leflunomide for 2 h and then infected by DENV at MOI 5. Figure 1 shows that treatment with leflunomide decreased the production of the cytokine TNF-α and chemokines, including MCP-2, RANTES, and MIP-1α, from mo-DCs infected by DENV. After DENV infection, DCs undergo maturation and express several markers on their cell surface, as reported in our previous study [8]. The possible effects of leflunomide in DENV-induced mo-DCs maturation were examined. By flow cytometry analysis, we demonstrate that the maturation process of mo-DCs induced by DENV infection was suppressed by leflunomide because the DENV-induced expression of CD80 (Fig. 2a, b) and CD86 (Fig. 2c, d) on infected mo-DCs significantly and dose-dependently decreased with leflunomide treatment.

Leflunomide Did Not Affect Viral Production in DENV-Infected mo-DCs

To exclude the possibility that the observed effects were due to potential cytotoxic effects, both trypan blue exclusion assays and LDH release assays were conducted. We show that leflunomide, at concentrations similar to therapeutic concentrations in humans, did not reduce cell survival by both trypan blue exclusion

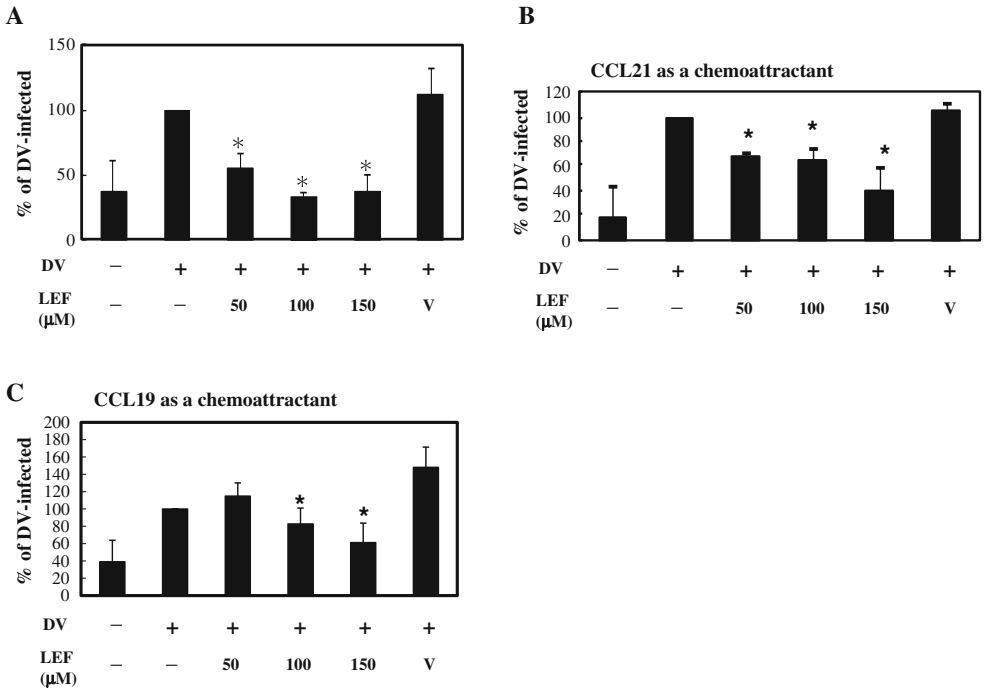

Fig. 4 Leflunomide inhibited DENV-induced mo-DCs migration. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The supernatants were collected and loaded onto the lower chamber of the transwell cassette and THP-1 cells were loaded onto the upper chamber. The cells migrating from the upper chamber to the lower chamber were collected and counted with flow cytometry as described in the "Materials and Methods" section (**a**). Similarly, mo-DCs were infected by mock or DENV in the presence or absence of various concentrations of leflunomide for 48 h. The cells were collected and loaded onto the upper chamber and either CCL21 (**b**) or CCL19 (**c**) was added into the lower chamber. The cells migrating from the upper chamber to the lower chamber were collected and counted with flow cytometry. The pooled data out of at least three independent experiments were shown. *$P<0.05$ compared to the DENV-stimulated mo-DCs. *V* vehicle, *LEF* leflunomide

assays (Fig. 3a) and LDH release assays (Fig. 3b). Incubation of DENV-infected mo-DCs with leflunomide for 48 h did not significantly increase cell toxicity compared to control (data not shown). Because leflunomide preserves antiviral effects, we evaluated whether such an antiviral activity could also be detectable in DENV infection of mo-DCs. By flow cytometry analysis, the results indicate that leflunomide treatment did not affect the level of viral E protein (Fig. 3c) or the level of viral nonstructural protein 4B (data not shown) intracellularly in DENV-infected mo-DCs. We also observed that leflunomide did not affect the production of viral particles in plaque assays (Fig. 3d).

Inhibition of DENV-Induced Migration of mo-DCs by Leflunomide

Functionality of DCs depends on their interaction with other immune effector cells after processing viral antigens. The first step for DCs to accomplish this process is to migrate from peripheral tissues to lymphoid organs, a phenomenon demonstrated in many virus infection models. Several complicated processes, such as a dynamic modulation of secretion of adhesion molecules and expressions of their respective receptors, as well as changes in cytoskeletal proteins, are involved. As demonstrated previously by our group, DENV infection can drive migration of DCs and this process is dependent on COX-2 signaling cascades [7]. Because leflunomide was capable of inhibiting chemokine production, we determined whether leflunomide treatment had an effect on the migration of THP-1 cells towards culture mediums collected from DENV-infected mo-DCs. By chemotaxis assays, the results suggest that the culture medium collected from leflunomide-treated cells attracted less THP-1 cells (Fig. 4a). When CCL21 or CCL19 was used as a chemoattractant and placed in the lower chamber of the transwell cassette, DENV-infected mo-DCs treated by leflunomide migrated relatively slower towards the chemoattractant than the untreated mo-DCs (Fig. 4b, c).

Leflunomide Inhibited DENV-Induced Expression of CCR7 on mo-DCs

We previously demonstrated that DENV-infected DCs express increased levels of CCR7, a critical lymphoid homing receptor, on their cell surfaces [7]. This step also plays a critical role in the immunopathogenesis of DENV

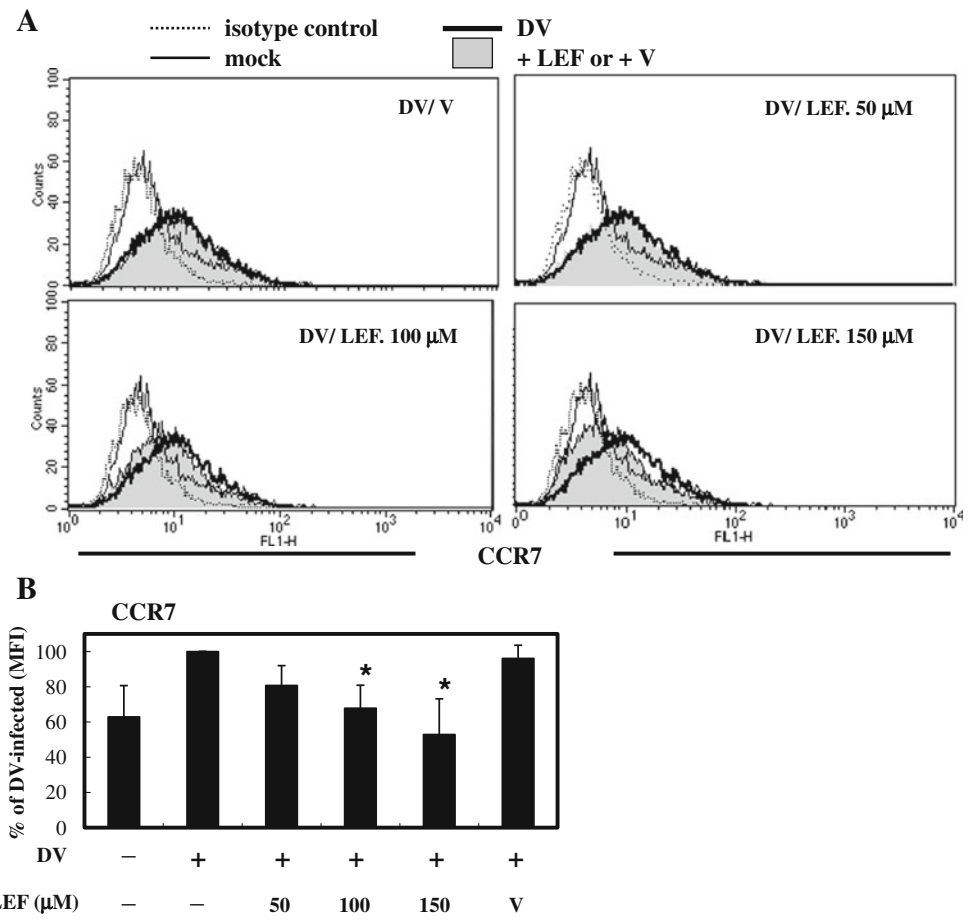

Fig. 5 Leflunomide inhibited DENV-induced CCR7 expression on mo-DCs. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 48 h. The expression of CCR7 was determined by flow cytometry (**a**). The pooled data out of at least three independent experiments were shown (**b**). *$P<0.05$ compared to the DENV-stimulated mo-DCs. *V* vehicle, *LEF* leflunomide, *MFI* median fluorescence intensity

infection. The effects of leflunomide on DENV-induced CCR7 expression on mo-DCs were examined. The results suggest that leflunomide reduced the expression of CCR7 on the surface of mo-DCs infected by DENV (Fig. 5a, b).

Leflunomide Inhibited Activation of NF-κB in DENV-Infected mo-DCs

Aside from viral production, several important cellular events in DENV-infected mo-DCs were suppressed by leflunomide. It suggests the possibility that certain common and crucial signaling pathways are potentially targeted by leflunomide. It has been demonstrated by us and other researchers that activation of the NF-κB signaling pathway is critical in DENV-infected mo-DCs and other tissue cells [7, 38]. We determine whether leflunomide has any effect on these signaling pathways. mo-DCs were pretreated with various doses of leflunomide for 2 h and the cells were infected by DENV in the presence or absence of various doses of leflunomide. The nuclear extracts were prepared and the NF-κB DNA-binding activity was determined by EMSA. In Fig. 6a, we show that leflunomide reduced DENV-induced NF-κB DNA-binding activity in mo-DCs. Analysis of nuclear extracts revealed the reduced nuclear translocation of both p65 and p50 from the cytosol in the presence of leflunomide treatment (Fig. 6b). Because we were not able to detect degradation of IκBα in DENV-infected mo-DCs probably because of its rapid kinetics [7], we determine whether leflunomide may affect the activity of IKK, an enzyme regulating IκBα degradation and NF-κB nuclear translocation. The results in Fig. 6c show a significant reduction of DENV-induced IKKα kinase activity in the presence of leflunomide treatment.

Leflunomide Inhibited Activation of AP-1 in DENV-Infected mo-DCs

In addition to the NF-κB signaling pathway, we also observed the activation of the MAPK–AP-1 signaling pathway after DENV infection of mo-DCs [7]. The potential effects of leflunomide on DENV-induced activation of AP-1 in mo-DCs were determined. The results in Fig. 7a show that the DNA-binding activity of AP-1

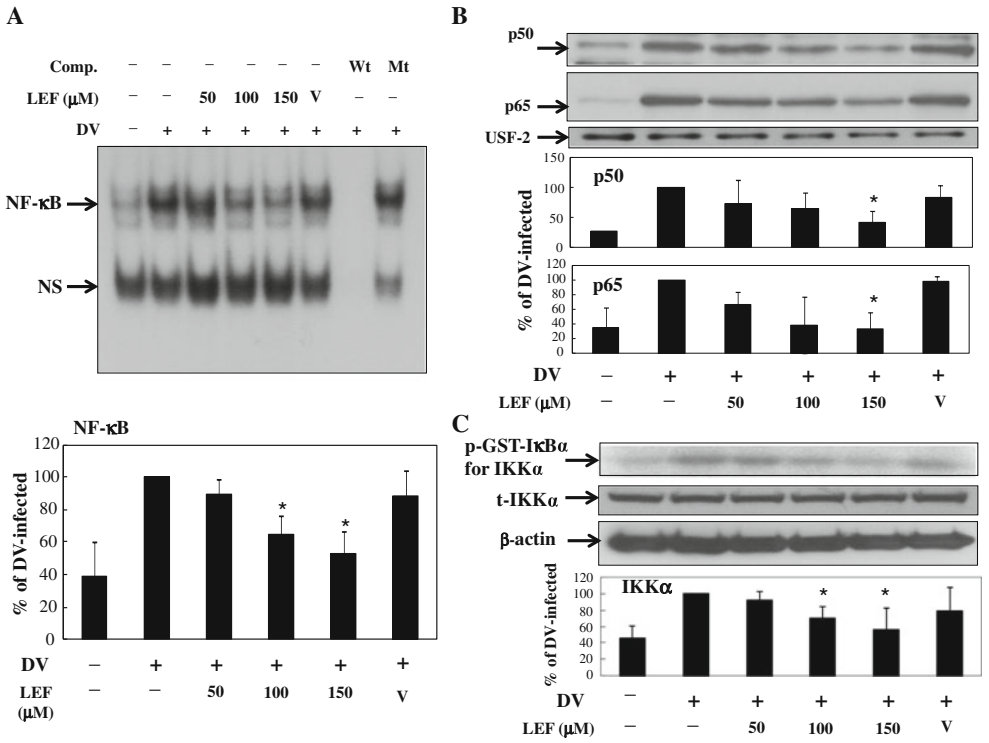

Fig. 6 Leflunomide inhibited DENV-induced IKKα–NF-κB activation in mo-DCs. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The cells were collected and the DNA-binding activity of NF-κB in nuclear extracts was determined by EMSA (**a**). The statistics of densitometric measurements of band intensities from at least three independent experiments were shown in the *lower panel*. The expressions of p65 and p50 in the nuclear extracts were determined by Western blots (**b**). The statistics of densitometric measurements of band intensities from three independent experiments were shown. Meanwhile, the total cell lysates were prepared and immunoprecipitated with antibodies against IKKα and the kinase assays were performed using GST–IκBα as a substrate (**c**). The densitometric measurements of band intensities from at least three independent experiments were shown. *Comp.* competitor, *Wt* wild type, *Mt* mutant, *V* vehicle, *LEF* leflunomide

induced by DENV infection was significantly suppressed by leflunomide. Meanwhile, the DENV-induced activation of MAPK, including ERK, p38, and JNK, was also significantly suppressed by leflunomide treatment (Fig. 7b).

The Immunomodulatory Effects of Leflunomide Could Not Be Reversed by Adding 50 μM Uridine

Uridine is a well-known component used in the salvage route to generate pyrimidines. We tested if exogenously added uridine could reverse leflunomide-mediated immunomodulatory effects. Uridine at 50 μM was added concomitantly with leflunomide in the culture medium of DENV-infected mo-DCs. The results indicate that the reconstitution of the pyrimidine synthase pathway could reverse neither the leflunomide-mediated reduction of cytokine TNF-α and chemokine RANTES production (Fig. 8a, b) nor the activation of NF-κB and AP-1 in DENV-infected mo-DCs (Fig. 8c). These results suggest that leflunomide suppressed DENV-induced chemokine and cytokine production and activation of both NF-κB and AP-1 through a uridine-independent pathway.

Discussion

Oral intake of leflunomide results in its rapid uptake in the gastrointestinal tract and subsequent conversion to A771726, an active metabolite of leflunomide. For therapeutic purposes in autoimmune disorders like rheumatoid arthritis, the suggested dose includes a loading dose of 100 mg/day for 3 days consecutively, followed by 20 mg/day. Under this therapeutic plan, the concentration of converted metabolite, A771726, approximately 63 μg/ml (corresponding to 233 μM) can be achieved [39]. In therapy for patients with renal allografts and biopsy-proven polyomavirus BK (BKV) nephropathy, much higher doses of leflunomide with plasma concentrations of A771726 reaching up to 50 to 100 μg/ml (corresponding to 150 to 300 μM) are needed [40]. Under such high

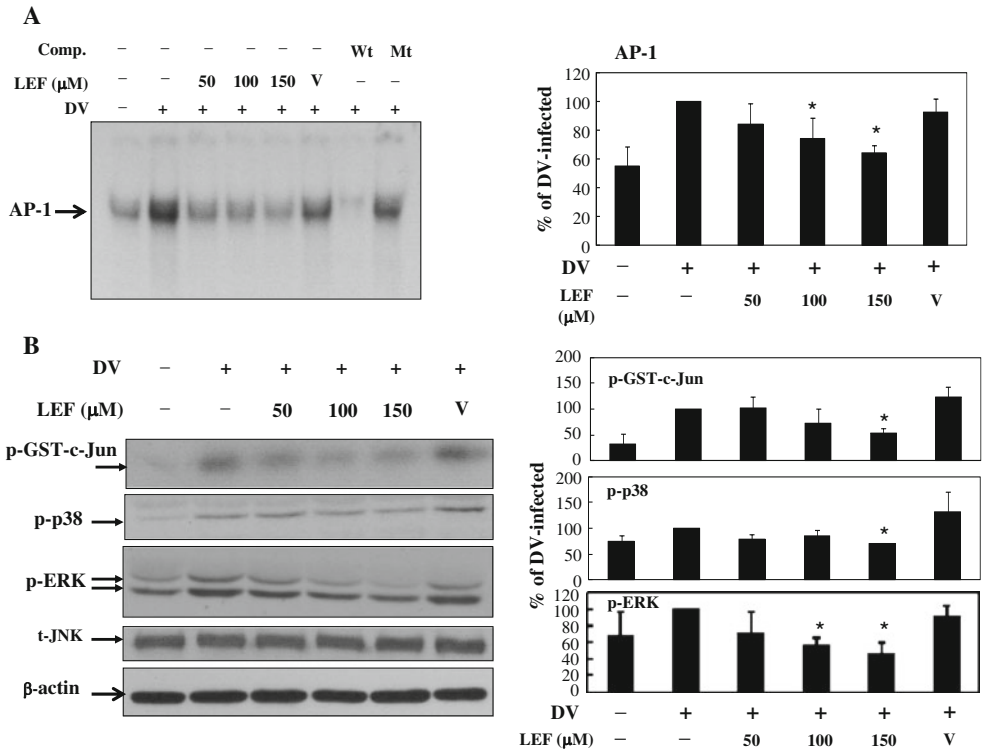

Fig. 7 Leflunomide inhibited DENV infection-activated MAPK–AP-1 signaling pathway. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The cells were collected and the DNA-binding activity of AP-1 in nuclear extracts was determined by EMSA (**a**). The statistics of densitometric measurements of band intensities from at least three independent experiments were shown in the *right panel*. Total cell lysates were collected and immunoprecipitation kinase assay using GST–c-Jun as a substrate (for JNK) or Western blots determining phosphorylated kinases (for both ERK and p38) was performed to evaluate the kinase activities of these molecules as described in the "Materials and Methods" section (**b**). The representative results and the statistics of densitometric measurements of band intensities from at least three independent experiments (*right panel*) were shown. *Comp.* competitor, *Wt* wild type, *Mt* mutant, *V* vehicle, *t-JNK* total JNK, *LEF* leflunomide

dosages with approximately 40 mg/day, leflunomide appears to be well tolerated [40]. The concentrations of leflunomide used in this study were, therefore, within the therapeutic ranges.

The antiviral effects of leflunomide can be demonstrated in several examples [22–27]. Nevertheless, both cellular events and molecular mechanisms underlying the therapeutic effects of leflunomide have rarely been investigated. According to Waldman et al. [25], leflunomide has no effect on the transcription and expression of immediate early and late cytomegalovirus genes. Leflunomide does not inhibit the accumulation of cytomegalovirus DNA in infected cells; however, it prevents tegument acquisition by viral nucleocapsids during late stages of virion assembly [25]. In another example, leflunomide reduces BKV production in human renal tubular epithelial cells [41]. Investigation into the mechanisms reveals leflunomide affects extensive processes during viral replication and production that include viral genome replication, late protein expression, virion assembly, and release and maintenance of the nuclear replication architecture [41]. In this report, we could not detect any suppressive effects of leflunomide on DENV replication or production through measuring both expression of intracellular viral E protein by flow cytometry and production of viral particles by plaque assays. In contrast, many cellular and molecular consequences in DENV-infected DCs were indeed targeted by leflunomide: leflunomide treatment suppressed DENV-induced activation of mo-DCs by inhibiting secretion of cytokines and chemokines, cell migration, and cell maturation. These results demonstrate that leflunomide had variable effects on different viral infections in different cell types. Interestingly, by a high-throughput screen assay on more than 60,000 small-molecular-weight compounds, Hoffmann et al. [42] identified a novel compound A3 preserving broad-spectrum antiviral activity against RNA viruses, DNA viruses, and retroviruses that also targets de novo pyrimidine biosynthesis. Different from leflunomide, A3 actively inhibits viral RNA synthesis and this process can be reversed specifically by the addition of uracil [42].

The clinical manifestations of severe DENV infection consequences such as DHF are highly correlated with high levels of cytokines and chemokines in blood [43–45]. As the most important host of DENV, the production of these

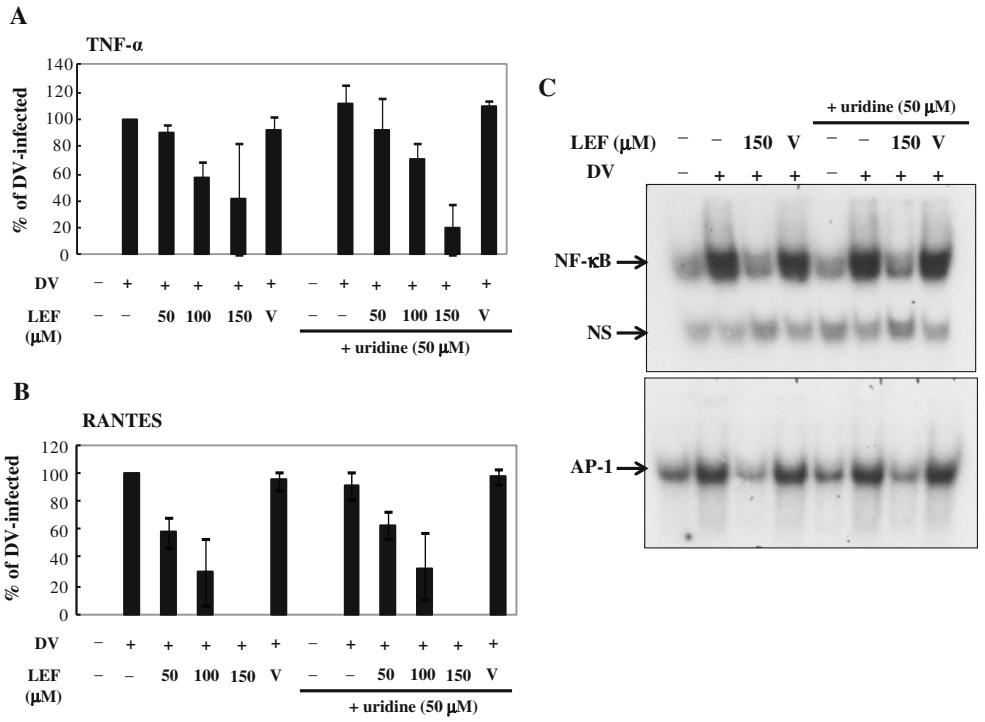

Fig. 8 DENV infection of mo-DCs in the absence or presence of leflunomide with or without 50 μM uridine. mo-DCs at 1×10^6 cells/ml were pretreated with various doses of leflunomide in the presence or absence of exogenously added 50 μM uridine for 2 h, then infected by mock or DENV at MOI 5 for 24 h. The supernatants were collected and the concentrations of cytokine TNF-α (**a**) or chemokine RANTES (**b**) were determined by ELISA. The data shown are from at least three independent experiments using different donor mo-DCs. The level of cytokine or chemokine from DENV-infected mo-DCs in the absence of leflunomide treatment was taken as 100%. In parallel, the nuclear extracts were prepared and the DNA-binding activity of NF-κB and AP-1 was determined by EMSA (**c**). *V* vehicle, *LEF* leflunomide

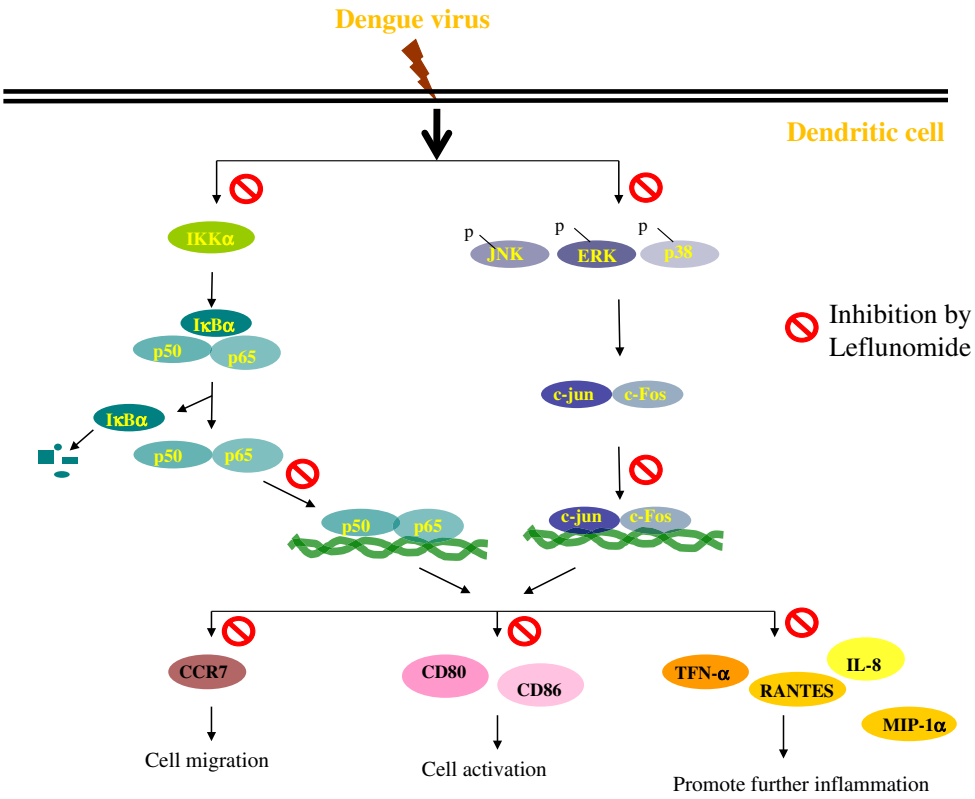

Fig. 9 Effects of leflunomide in DENV-induced activation of mo-DCs. After DENV infection, several signaling cascades like IKKα–IκBα–NF-κB and MAPKs–c-Jun/c-Fos were activated in mo-DCs. The inhibition of these signaling pathways by leflunomide resulted in the reduction of cell activation (through reducing CD80 and CD86 expression), cell migration (through reducing CCR7 expression), and likely further exaggerated immune responses (through reducing cytokine and chemokine production)

mediators from virus-infected DCs is critical because many downstream events are initiated after this step. The suppressive effects of leflunomide on DENV-induced production of chemokines and cytokines from mo-DCs, therefore, helped reduce subsequent dangerous outcomes. The therapeutic benefits of leflunomide were strengthened by the inhibition of DENV-induced mo-DCs maturation. The interaction between APCs and T cells requires full expression of costimulatory molecules on APCs that help prevent apoptosis or anergy of T cells. The reduced expressions of both CD80 and CD86 on DENV-infected mo-DCs were likely to attenuate the activation of interacting T cells and consequently decrease inflammatory responses. Such assumptions stand because T cells play crucial roles in the immunopathogenesis and development of cytokine storms and DHF [46, 47]. Moreover, our results extend the usefulness of leflunomide in the therapy of DENV infection by demonstrating the suppressed migration of DENV-infected mo-DCs. Meanwhile, DENV-induced expression of CCR7, a receptor essential for DCs migration to the T cell area of draining lymph nodes [48, 49], on mo-DCs was also inhibited by leflunomide. Suppression of these cellular events along the process of DENV infection by leflunomide may support its potential in therapeutics for DENV infection, especially for those with autoimmune disorders that are already on the drug prior to infection.

To address how leflunomide suppressed DENV-induced inflammatory responses in mo-DCs, we investigated both NF-κB and AP-1 signaling pathways. These are crucially involved in DENV-induced activation of mo-DCs and many important processes in inflammatory responses after DENV infection [7, 50]. As expected, leflunomide was a potent suppressor of DENV-stimulated IKKα–NF-κB and MAPK–AP-1 signaling pathways. This helps to explain why so many cellular events were inhibited by leflunomide. By suppressing the NF-κB signaling pathway, leflunomide treatment could inhibit maturation and T cell priming activity of mo-DCs, a mechanism that was also observed by other researchers [51]. NF-κB blockade using decoy oligodeoxynucleotides in vivo has been shown to reduce Langerhans cell migration and impair T cell responses [52]. Because there are several functional NF-κB binding sites in both human and mouse CCL19 promoters in DCs [53], the inhibition of NF-κB is expected to be associated with the reduction of CCL19 [53, 54]. The suppression of NF-κB also likely explains the decrease of CCR7 expression as observed in cytomegalovirus-infected DCs [55]. Interestingly, the observed reduction of chemokine and cytokine in DENV-infected mo-DCs by leflunomide could not be reversed by the exogenously added uridine. The findings are supportive for many reported effects of leflunomide that are also independent of pyrimidine depletion [56–58].

There are certain limitations in this study. In natural DENV infection, when a mosquito seeks a blood meal and lands at a prospective host, approximately 40% of the time, the proboscis does not cannulate directly at a blood vessel. During this period, it probes for the blood vessel, and as a consequence, saliva is released into the epidermis where DCs are residing. However, the majority of the time, the proboscis directly cannulates and saliva and virus are released into the bloodstream. This issue was not addressed in this report. In addition, we do not know the effect of leflunomide in the presence or absence of non-neutralizing levels of heterologous antibodies. Nevertheless, this report explains the effects and mechanisms of leflunomide and identifies its potential therapeutic benefits in DENV infection (Fig. 9). Given that patients with autoimmune disorders like systemic lupus erythematosus, rheumatoid arthritis, and vasculitis have a high tendency to develop viral infections [59] that cause disease flare-up, disease-modifying drugs like leflunomide preserving antiviral activity should be beneficial, in addition to their control of autoimmune disorders.

Acknowledgements This report was supported by a grant from the Institutional Collaboration Projects raised by both Chi Mei Medical Center and National Defense Medical Center (CMNDMC99 and CMNDMC10008) as well as from Institute of Preventive Medicine, National Defense Medical Center. The authors' work was supported in part by grants from the National Science Council (NSC 96-2314-B-016-015-MY3, NSC 96-2314-B-400-003-MY2, and NSC 98-2314-B-400-002-MY3).

Competing Interests The authors declare that they have no competing interests.

References

1. Guzman MG, Kouri G. Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. J Clin Virol. 2003;27:1–13.
2. Lin YW, Wang KJ, Lei HY, et al. Virus replication and cytokine production in dengue virus-infected human B lymphocytes. J Virol. 2002;76:12242–9.
3. King CA, Anderson R, Marshall JS. Dengue virus selectively induces human mast cell chemokine production. J Virol. 2002;76:8408–19.
4. Jessie K, Fong MY, Devi S, et al. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis. 2004;189:1411–8.
5. de Macedo FC, Nicol AF, Cooper LD, et al. Histologic, viral, and molecular correlates of dengue fever infection of the liver using highly sensitive immunohistochemistry. Diagn Mol Pathol. 2006;15:223–8.
6. Warke RV, Becerra A, Zawadzka A, et al. Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells. J Gen Virol. 2008;89:1605–15.
7. Wu WL, Ho LJ, Chang DM, et al. Triggering of DC migration by dengue virus stimulation of COX-2-dependent signaling cascades

in vitro highlights the significance of these cascades beyond inflammation. Eur J Immunol. 2009;39:3413–22.
8. Ho LJ, Wang JJ, Shaio MF, et al. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J Immunol. 2001;166:1499–506.
9. Tassaneetrithep B, Burgess TH, Granelli-Piperno A, et al. DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med. 2003;197:823–9.
10. Luplertlop N, Misse D, Bray D, et al. Dengue-virus-infected dendritic cells trigger vascular leakage through metalloproteinase overproduction. EMBO Rep. 2006;7:1176–81.
11. Green S, Vaughn DW, Kalayanarooj S, et al. Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis. 1999;179:755–62.
12. Juffrie M, van Der Meer GM, Hack CE, et al. Inflammatory mediators in dengue virus infection in children: interleukin-8 and its relationship to neutrophil degranulation. Infect Immun. 2000;68:702–7.
13. Chau TN, Quyen NT, Thuy TT, et al. Dengue in Vietnamese infants—results of infection-enhancement assays correlate with age-related disease epidemiology, and cellular immune responses correlate with disease severity. J Infect Dis. 2008;198:516–24.
14. Sierra B, Perez AB, Vogt K, et al. MCP-1 and MIP-1alpha expression in a model resembling early immune response to dengue. Cytokine. 2010;52:175–83.
15. Dejnirattisai W, Duangchinda T, Lin CL, et al. A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections. J Immunol. 2008;181:5865–74.
16. Martina BE, Koraka P, Osterhaus AD. Dengue virus pathogenesis: an integrated view. Clin Microbiol Rev. 2009;22:564–81.
17. Noble CG, Chen YL, Dong H, et al. Strategies for development of dengue virus inhibitors. Antiviral Res. 2010;85:450–62.
18. Murrell S, Wu SC, Butler M. Review of dengue virus and the development of a vaccine. Biotechnol Adv. 2011;29:239–47.
19. Cherwinski HM, Cohn RG, Cheung P, et al. The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. J Pharmacol Exp Ther. 1995;275:1043–9.
20. Fairbanks LD, Bofill M, Ruckemann K, et al. Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of de novo synthesis inhibitors. J Biol Chem. 1995;270:29682–9.
21. Nandi P, Kingsley GH, Scott DL. Disease-modifying antirheumatic drugs other than methotrexate in rheumatoid arthritis and seronegative arthritis. Curr Opin Rheumatol. 2008;20:251–6.
22. Wu JK, Harris MT. Use of leflunomide in the treatment of polyomavirus BK-associated nephropathy. Ann Pharmacother. 2008;42:1679–85.
23. Schlapfer E, Fischer M, Ott P, et al. Anti-HIV-1 activity of leflunomide: a comparison with mycophenolic acid and hydroxyurea. AIDS. 2003;17:1613–20.
24. Knight DA, Hejmanowski AQ, Dierksheide JE, et al. Inhibition of herpes simplex virus type 1 by the experimental immunosuppressive agent leflunomide. Transplantation. 2001;71:170–4.
25. Waldman WJ, Knight DA, Lurain NS, et al. Novel mechanism of inhibition of cytomegalovirus by the experimental immunosuppressive agent leflunomide. Transplantation. 1999;68:814–25.
26. Avery RK. Update in management of ganciclovir-resistant cytomegalovirus infection. Curr Opin Infect Dis. 2008;21:433–7.
27. Davis IC, Lazarowski ER, Chen FP, et al. Post-infection A77-1726 blocks pathophysiologic sequelae of respiratory syncytial virus infection. Am J Respir Cell Mol Biol. 2007;37:379–86.
28. Ho LJ, Hung LF, Weng CY, et al. Dengue virus type 2 antagonizes IFN-alpha but not IFN-gamma antiviral effect via down-regulating Tyk2-STAT signaling in the human dendritic cell. J Immunol. 2005;174:8163–72.
29. Stein DA, Huang CY, Silengo S, et al. Treatment of AG129 mice with antisense morpholino oligomers increases survival time following challenge with dengue 2 virus. J Antimicrob Chemother. 2008;62:555–65.
30. Rothwell C, Lebreton A, Young Ng C, et al. Cholesterol biosynthesis modulation regulates dengue viral replication. Virology. 2009;389:8–19.
31. Cheng SM, Yang SP, Ho LJ, et al. Down-regulation of c-jun N-terminal kinase-activator protein-1 signaling pathway by *Ginkgo biloba* extract in human peripheral blood T cells. Biochem Pharmacol. 2003;66:679–89.
32. Ho LJ, Juan TY, Chao P, et al. Plant alkaloid tetrandrine downregulates IkappaBalpha kinases-IkappaBalpha-NF-kappaB signaling pathway in human peripheral blood T cell. Br J Pharmacol. 2004;143:919–27.
33. Scandella E, Men Y, Legler DF, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. Blood. 2004;103:1595–601.
34. Ho LJ, Shaio MF, Chang DM, et al. Infection of human dendritic cells by dengue virus activates and primes T cells towards Th0-like phenotype producing both Th1 and Th2 cytokines. Immunol Invest. 2004;33:423–37.
35. Bosch I, Xhaja K, Estevez L, et al. Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. J Virol. 2002;76:5588–97.
36. Chen YC, Wang SY. Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. J Virol. 2002;76:9877–87.
37. Libraty DH, Pichyangkul S, Ajariyakhajorn C, et al. Human dendritic cells are activated by dengue virus infection: enhancement by gamma interferon and implications for disease pathogenesis. J Virol. 2001;75:3501–8.
38. Lin CF, Chiu SC, Hsiao YL, et al. Expression of cytokine, chemokine, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J Immunol. 2005;174:395–403.
39. Rozman B. Clinical pharmacokinetics of leflunomide. Clin Pharmacokinet. 2002;41:421–30.
40. Josephson MA, Gillen D, Javaid B, et al. Treatment of renal allograft polyoma BK virus infection with leflunomide. Transplantation. 2006;81:704–10.
41. Bernhoff E, Tylden GD, Kjerpeseth LJ, et al. Leflunomide inhibition of BK virus replication in renal tubular epithelial cells. J Virol. 2010;84:2150–6.
42. Hoffmann HH, Kunz A, Simon VA, et al. Broad-spectrum antiviral that interferes with de novo pyrimidine biosynthesis. Proc Natl Acad Sci USA. 2011;108:5777–82.
43. Spain-Santana TA, Marglin S, Ennis FA, et al. MIP-1 alpha and MIP-1 beta induction by dengue virus. J Med Virol. 2001;65:324–30.
44. Chen JP, Lu HL, Lai SL, et al. Dengue virus induces expression of CXC chemokine ligand 10/IFN-gamma-inducible protein 10, which competitively inhibits viral binding to cell surface heparan sulfate. J Immunol. 2006;177:3185–92.
45. Mathew A, Rothman AL. Understanding the contribution of cellular immunity to dengue disease pathogenesis. Immunol Rev. 2008;225:300–13.
46. Screaton G, Mongkolsapaya J. T cell responses and dengue haemorrhagic fever. Novartis Found Symp. 2006;277:164–71. discussion 71–6, 251–3.
47. Rothman AL. Cellular immunology of sequential dengue virus infection and its role in disease pathogenesis. Curr Top Microbiol Immunol. 2010;338:83–98.

48. MartIn-Fontecha A, Sebastiani S, Hopken UE, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med. 2003;198:615–21.
49. Ohl L, Mohaupt M, Czeloth N, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity. 2004;21:279–88.
50. Ceballos-Olvera I, Chavez-Salinas S, Medina F, et al. JNK phosphorylation, induced during dengue virus infection, is important for viral infection and requires the presence of cholesterol. Virology. 2010;396:30–6.
51. Moore F, Buonocore S, Aksoy E, et al. An alternative pathway of NF-kappaB activation results in maturation and T cell priming activity of dendritic cells overexpressing a mutated IkappaBalpha. J Immunol. 2007;178:1301–11.
52. Isomura I, Tsujimura K, Morita A. Antigen-specific peripheral tolerance induced by topical application of NF-kappaB decoy oligodeoxynucleotide. J Invest Dermatol. 2006;126:97–104.
53. Pietila TE, Veckman V, Lehtonen A, et al. Multiple NF-kappaB and IFN regulatory factor family transcription factors regulate CCL19 gene expression in human monocyte-derived dendritic cells. J Immunol. 2007;178:253–61.
54. Jimenez F, Quinones MP, Martinez HG, et al. CCR2 plays a critical role in dendritic cell maturation: possible role of CCL2 and NF-kappa B. J Immunol. 2010;184:5571–81.
55. Moutaftsi M, Brennan P, Spector SA, et al. Impaired lymphoid chemokine-mediated migration due to a block on the chemokine receptor switch in human cytomegalovirus-infected dendritic cells. J Virol. 2004;78:3046–54.
56. Korn T, Magnus T, Toyka K, et al. Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide—mechanisms independent of pyrimidine depletion. J Leukoc Biol. 2004;76:950–60.
57. Stuhlmeier KM. Effects of leflunomide on hyaluronan synthases (HAS): NF-kappa B-independent suppression of IL-1-induced HAS1 transcription by leflunomide. J Immunol. 2005;174:7376–82.
58. Kirsch BM, Zeyda M, Stuhlmeier K, et al. The active metabolite of leflunomide, A77 1726, interferes with dendritic cell function. Arthritis Res Ther. 2005;7:R694–703.
59. Witt MN, Braun GS, Ihrler S, et al. Occurrence of HSV-1-induced pneumonitis in patients under standard immunosuppressive therapy for rheumatic, vasculitic, and connective tissue disease. BMC Pulm Med. 2009;9:22.