**DC-SIGN (CD209) Promoter −336 A/G Polymorphism Is Associated with Dengue Hemorrhagic Fever and Correlated to DC-SIGN Expression and Immune Augmentation**

Lin Wang¹,⁵, Rong-Fu Chen²,³, Jien-Wei Liu³, Ing-Kit Lee³, Chiu-Ping Lee², Ho-Chang Kuo¹,⁵, Shau-Ku Huang⁴, Kuender D. Yang¹,²,⁵*

1 Department of Pediatrics, Chang Gung Memorial Hospital-Kaohsiung Medical Center (CGMH-KMC), Kaohsiung, Taiwan, 2 Department of Medical Research, CGMH-KMC, Kaohsiung, Taiwan, 3 Division of Infectious Diseases, Department of Internal Medicine, CGMH-KMC, Kaohsiung, Taiwan, 4 Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland, United States of America, 5 Graduate Institute of Clinical Medical Sciences, Chang Gung University College of Medicine, Kaohsiung, Taiwan

---

**Abstract**

**Background:** The C-type lectin DC-SIGN (CD209) is known to be the major dengue receptor on human dendritic cells, and a single nucleotide polymorphism (SNP) in the promoter region of CD209 (−336 A/G; rs4804803) is susceptible to many infectious diseases. We reason that variations in the DC-SIGN gene might have a broad influence on viral replication and host immune responses.

**Methods and Findings:** We studied whether the rs4804803 SNP was associated with dengue fever (DF) and/or dengue hemorrhagic fever (DHF) through genotyping analysis in a Taiwanese cohort. We generated monocyte-derived dendritic cells (MDDCs) from individuals with AA or AG genotype of rs4804803 to study the viral replication and immune responses for functional validation. A total of 574 DNA samples were genotyped, including 176 DF, 135 DHF, 143 other non-dengue febrile illnesses (OFI) and 120 population controls. A strong association between GG/AG genotypes of rs4804803 and risk of DHF was found when compared among DF, OFI, and controls ($p = 0.004$, $3 \times 10^{-5}$ and 0.001, respectively). The AA genotype was associated with protection against dengue infection compared with OFI and controls ($p = 0.002$ and 0.020, respectively). Moreover, MDDCs from individuals with AG genotype with a higher cell surface DC-SIGN expression had a significantly higher TNFα, IL-12p40, and IP-10 production than those with AA genotype in response to dengue infection. However, the viral replication in MDDCs with AG genotype was significantly lower than those with AA genotype. With both genotypes, MDDCs revealed an increase in viral replication following the addition of anti-IP-10 neutralizing antibody.

**Conclusions/Significance:** The rs4804803 SNP in the CD209 promoter contributed to susceptibility to dengue infection and complication of DHF. This SNP with AG genotype affects the cell surface DC-SIGN expression related to immune augmentation and less viral replication.

---

**Introduction**

Dengue viruses (DEN) are arthropod-borne flaviviruses that cause dengue fever (DF) with significant morbidity and mortality in tropical and subtropical regions of the world. There are four serotypes of dengue viruses (DEN types 1–4). Classic DF is a self-limited illness characterized by fever, headache, myalgia, arthralgia, and abdominal pain. Since the 1950s, a more severe form of the disease, dengue hemorrhagic fever (DHF), has been recognized worldwide [1]. Patients who develop DHF typically have initial symptoms similar to those in DF patients, but develop cytokine-storm-like plasma leakage manifested by hemoconcentration, thrombocytopenia, ascites, and pleural effusion near the deleterious stage [2]. DHF pathogenesis has been attributed to viral virulence versus immune enhancement; however, that has been the subject of debate for many years [2,3].

The innate immune system is the first line of host defense against pathogens and is involved in early recognition and uptake of microbes by the host’s professional phagocytes such as dendritic cells (DCs) and macrophages, through germine-encoded recep-
Author Summary

Dengue fever (DF) is an arthropod-borne disease that is prevalent in tropical and subtropical regions of the world. DC-SIGN [dendritic cell-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin] is a major receptor for dengue infection. DC-SIGN, also called CD209, expresses on dendritic cells (DCs) that bind to ICAM-3, which is expressed on T cells to facilitate the initial interaction between DCs and T cells. Variations in the CD209 promoter (−336 A/G; rs4804803) genotype are involved in the pathogenesis of human infectious diseases. Here we found that patients with dengue hemorrhagic fever (DHF) had a higher frequency of the AG or GG genotype of rs4804803 than DF or controls. Functional studies determined that monocyte-derived DCs (MDDCs) from individuals with AG genotype had significantly higher cell surface DC-SIGN expression, associated with higher TNFα, IL-12p40, and IP-10 production, but lower viral replication than those with AA genotype. An increase in DEN-2 replication in MDDCs was observed following the addition of anti-IP-10 neutralizing antibody. These findings highlight the fact that the rs4804803 SNP in the CD209 promoter is associated with DHF and correlated to DC-SIGN expression and immune augmentation.

Materials and Methods

Ethics statement and subjects studied

This study was approved by the Institution Review Board (IRB) of Chang Gung Memorial Hospital-Kaohsiung Medical Center, Taiwan. The dengue patients were recruited as described previously in the 2002–2003 DEN-2 outbreak in Taiwan [19–23]. A larger retrospective cohort was designed and re-approved by an additional IRB review (Document No.: 97-2111B). To validate cell surface expression and immune functions of rs4804803 SNP, we obtained informed consent to collect blood leukocytes from normal volunteers with AA or AG genotypes of rs4804803.

Definition of cases and controls

DEN infection was confirmed by clinical dengue symptoms and signs along with detection of DEN-2 RNA by quantitative RT-PCR in blood, detection of IgM to DEN or at least a 4-fold increase in dengue-specific hemagglutination inhibition titers in convalescent serum compared with that in acute-phase serum [20,21]. In those with DEN-2 infection, blood was drawn at least once a day subsequent to admission into the hospital to measure the platelet counts and hematocrit levels. A Chest X-ray and abdominal ultrasonography were performed routinely in individuals without evidence of hemoconcentration or hypoalbuminemia to refine the differential diagnosis of DHF vs. DF based on pleural effusion or ascites. A clinical diagnosis of DHF was assigned according to the DHF criteria of the World Health Organization (WHO); including a reduced platelet count (<100,000/mm3), petechiae, hemorrhagic manifestation, and plasma leakage showing hypoalbuminemia (peak hematocrit ≥20% above the mean for the population, or an increase in hematocrit of 20% or more), pleural effusion, ascites, or hypoalbuminemia [24]. Patients with DF were defined by detectable DEN-2 RNA by RT-PCR or DEN-specific IgM, but without evidence of DHF. Primary or secondary DEN infections were identified using previously established serologic criteria for IgM/IgG ELISAs [19].

Patients with other non-dengue febrile illnesses (OFI) were defined by febrile illness with no detectable DEN-specific IgM, no detectable DEN RNA, and no obvious or reported bacterial etiology for their illness during the same study period. Population controls were healthy, unrelated volunteers from the same community, with neither signs nor previous history of dengue infection, with a DEN IgG sero-positive rate of 1.37% (1/73).

Genotyping of CD209 rs4804803 SNP

Genomic DNA was isolated from heparin-antiocoagulated blood samples using a standard phenol-chloroform extraction followed by 70% alcohol precipitation. Genotyping for the CD209 variant (−336 A/G; rs4804803) was carried out using Custom TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The primer sequences were 5’-GGACAGTGCTTCCAG-GAACT-3’ (forward) and 5’-CTGGTTTACCACCCCTCCAC-TAG-3’ (reverse). The TaqMan minor groove binder probe sequences were 5’-TACCTGGCTACCCCTG-3’ (forward) and 5’-CTGGCCACGGCTTG-3’. The probes were labeled with the TaqMan fluorescent dyes VIC and FAM, respectively. The PCR was conducted in total volume of 15 μL using the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 20 s, followed by annealing and extension at 60°C for one minute. After the PCR, the genotype of each sample was determined by measuring the allele-specific fluorescence in the ABI Prism 7500 Sequence Detection System, using SDS 1.1 software for allele discrimination.
**Table 1.** Demographic data of dengue patients with DF and DHF.

|              | DF (n = 176) | DHF (n = 135) | p value |
|--------------|--------------|---------------|---------|
| Age (years)  | 41.7 ± 1.6   | 45.7 ± 1.3    | <0.001  |
| Sex (male/female) | 86/90    | 60/75         | 0.439   |
| Hematocrit (%)| 37.0 ± 0.1   | 39.7 ± 3.0    | 0.021   |
| Hemoconcentration<sup>1</sup> (%) | 6.0 ± 0.6 | 20.7 ± 1.3   | <0.001  |
| Platelet (<×10<sup>9</sup>/mm<sup>3</sup>) | 10.9 ± 0.7 | 26.0 ± 0.3   | <0.001  |
| WBC (×10<sup>9</sup>/mm<sup>3</sup>) | 4.13 ± 0.19 | 4.37 ± 23.8  | 0.425   |
| GOT (U/mL)   | 70.1 ± 8.1   | 313.8 ± 74.6  | 0.002   |
| GPT (U/mL)   | 67.1 ± 11.3  | 142.7 ± 21.9  | 0.003   |
| Serum albumin level (g/dL) | 3.7 ± 0.2 | 3.1 ± 0.1    | 0.001   |
| Pleural effusion (%) | 0/59 (0) | 57/75 (76) | <0.001  |
| Ascites (%)   | 1/68 (1)     | 47/85 (55)    | <0.001  |
| Secondary infection (%) | 63/173 (36) | 78/120 (65) | <0.001  |

<sup>1</sup>Values are the mean ± SEM or number (%). P values were determined by application of Student’s t-test for continuous variables and by Χ<sup>2</sup> test or Fisher exact test for categorical variables.

**Table 2.** Distribution of CD209 –336 A/G (rs4804803) genotype in patients with dengue infection (DEN) and other non-dengue febrile illnesses (OFI) or population controls.

| Genotype distribution | Case vs. OFI | Cases vs. Control | DHF vs. DF |
|------------------------|--------------|-------------------|------------|
|                        | OR (95% CI)  | p                 | OR (95% CI) | p       | OR (95% CI) | p       |
| OFI (143)              |              |                   |            |         |            |         |
| GG (0)                 | 0 (0)        | 1                 | 3.68 (1.67–8.09) | 0.001  | 2.46 (1.32–4.59) | 0.004  |
| AG (5.6)               | 135 (94.4)   | 1                 |            |         |            |         |
| AA (0)                 |              |                   |            |         |            |         |
| Control (120)          |              |                   |            |         |            |         |
| GG (0)                 | 0 (0)        | 1                 | 3.68 (1.67–8.09) | 0.001  | 2.46 (1.32–4.59) | 0.004  |
| AG (7.5)               | 111 (92.5)   | 1                 |            |         |            |         |
| AA (0)                 |              |                   |            |         |            |         |
| DEN (311)              |              |                   |            |         |            |         |
| GG (0.6)               | 2 (0.6)      | 1                 | 2.36 (1.12–4.97) | 0.020  |            |         |
| AG (15.4)              | 48 (83.9)    | 3.23 (1.49–7.02)  | 0.002      |         |            |         |
| AA (0)                 |              |                   |            |         |            |         |
| DF (176)               |              |                   |            |         |            |         |
| GG (0)                 | 0 (0)        | 1                 | 1.49 (0.65–3.42) | 0.342  | 1          |         |
| AG (10.8)              | 19 (89.2)    | 2.04 (0.87–4.81)  | 0.097      |         | 1          |         |
| AA (0)                 |              |                   |            |         |            |         |
| DHF (135)              |              |                   |            |         |            |         |
| GG (1.5)               | 2 (1.5)      | 3.03 (2.22–11.40) | 0.001      | 2.46 (1.32–4.59) | 0.004  |
| AG (21.5)              | 29 (21.5)    | 3.10 (21.5)       | 0.001      |         | 2.46 (1.32–4.59) | 0.004  |
| AA (0)                 |              |                   |            |         |            |         |

Values are number (%) studied. P value was determined by Χ<sup>2</sup> test or Fisher exact test based on GG or GA vs. AA comparison OR, odds ratio. CI, confidence interval.

doi:10.1371/journal.pntd.0000934.t002

Expression of markers was measured by flow cytometer using specific antibodies and their corresponding isotype controls.

**DCs infection with DEN-2 and endogenous IP-10 neutralizing experiments**

Unless otherwise stated, monocyte-derived dendritic cells (MDDCs) were infected with DEN-2 at a multiplicity of infection (MOI) of 5 for 2 h at 37°C and 5% CO2. Cells were washed twice to remove cell-free virus, and cultured in complete RPMI medium (without cytokines) at a density of 2×10<sup>5</sup> cells/mL in 48-well plates. Cells and supernatants were removed and analyzed at 24, 48, and 72 h post-infection. For the neutralization experiments, cells were incubated in the medium alone or in the medium with the addition of anti-human CXCL10/IP-10 antibody (R&D Systems, Minneapolis, MN, USA) at 10 μg/mL for 30 min. Cells and supernatants were harvested and analyzed 24 h post-infection.

**Detection of CD209 mRNA by real time RT-PCR and cell surface expression on MDDCs by flow cytometry**

Total RNA extracted from MDDCs was subjected to quantitative RT-PCR to assess levels of mRNA corresponding to CD209 and β2-microglobulin (B2MG) using the ABI PRISM 7500 instrument (Applied Biosystems). The forward primer, reverse primer sequence for detecting CD209 and B2MG were 5’-AACGAGCTGAGGCCCTTGGA-3’, 5’-GGGACCATGCGCCAAGACA-3’, and 5’-AATTGAAAAAGTGGAGCATCAG-3’, 5’-GGCTGTGACAAAGTCATGCTGT-3’, respectively. The PCR cycling parameters were 40 cycles of PCR reactions at 94°C for 20 s, and 60°C for one minute. The results were detected in real-time and recorded on a plot showing fluorescence vs. time. RT-PCR products were also visualized on ethidium bromide-stained 1.5% agarose (Pierce Co., Rockford, IL, USA) gel with a 100-bp ladder (Pharmacia Biotech, Piscataway, NJ, USA) as a reference.

To measure the CD209 cell surface expression, MDDCs were stained with FITC-conjugated mAbs specific for DC-SIGN (R&D Systems, Minneapolis, MN, USA). An isotype-matched FITC-labeled control, mouse IgG2b (clone MOPC195, Immunotech, Beckman Coulter, Fullerton, CA, USA) was included in each experiment.

**Quantitation of viral replication in MDDCs by real time RT-PCR**

Total RNA extracted from MDDCs was subjected to assess DEN-2 RNA viral copies. Fluorescent RT-PCR was performed in an ABI 7500 quantitative PCR machine (Applied Biosystems) for 40 cycles using TaqMan technology as previously described [21].

(both Applied Biosystems). To validate the genotyping by real-time PCR analysis, 100 PCR products were subject to restriction fragment length polymorphism (RFLP) analysis with MscI restriction enzyme (New England Biolabs, Beverly, MA, USA) and showed 100% identical result between these two genotyping systems.

**Generation of DCs from individuals with AA or AG phenotype of rs4804803**

Peripheral blood mononuclear cells were collected from peripheral blood of 20 healthy, DEN-specific IgM or IgG seronegative volunteers with AA or AG genotype. CD14<sup>+</sup> monocytes were isolated by positive selection according to the manufacturer’s specifications using CD14 microbeads and a magnetic cell separator (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched CD14<sup>+</sup> cells (purity>95%) were cultured for 6 days in six-well plates in complete RPMI medium in the presence of 10 ng/mL rhGM-CSF and 5 ng/mL rhIL-4 at 37°C, and 5% CO2. On day 3, half of the medium was replaced with fresh medium supplemented with rhGM-CSF and rhIL-4.

1. Demographic data of dengue patients with DF and DHF.
2. Distribution of CD209 –336 A/G (rs4804803) genotype in patients with dengue infection (DEN) and other non-dengue febrile illnesses (OFI) or population controls.
**Table 3. Distribution of CD209 –336 A/G (rs4804803) allele in patients with dengue infection (DEN) and other non-dengue febrile illnesses (OFI) or population controls.**

| Allele distribution | Cases vs. OFI | Cases vs. Control | DHF vs. DF |
|---------------------|---------------|-------------------|------------|
|                     | G | A  | OR (95% CI) | p   | G | A  | OR (95% CI) | p   | G | A  | OR (95% CI) | p   |
| OFI                 |   |    |             |     |   |    |             |     |   |    |             |     |
|                    | 8 (2.8) | 278 (97.2) | 1 |     | 8 (2.8) | 278 (97.2) | 1 |     | 56 (10.8) | 464 (89.2) | 3.34 (1.63–6.84) | 0.002 |
| Control             | 9 (3.8) | 231 (96.2) | 1.35 (0.51–3.57) | 0.538 | 9 (3.8) | 231 (96.2) | 1 |     | 62 (11.0) | 482 (89.0) | 1.94 (1.07–3.47) | 0.027 |
| DEN                 | 52 (8.4) | 570 (91.6) | 3.17 (1.49–6.77) | 0.002 | 52 (8.4) | 570 (91.6) | 3.17 (1.49–6.77) | 0.002 | 29 (10.8) | 234 (89.2) | 2.46 (1.36–4.45) | 0.004 |
| DF                  | 19 (5.4) | 333 (94.6) | 1.98 (0.86–4.60) | 0.105 | 19 (5.4) | 333 (94.6) | 1.98 (0.86–4.60) | 0.105 | 10 (2.4) | 384 (97.6) | 1.80 (0.65–5.26) | 0.351 |
| DHF                 | 33 (12.2) | 237 (87.8) | 4.84 (2.19–10.68) | 2×10⁻³ | 33 (12.2) | 237 (87.8) | 4.84 (2.19–10.68) | 2×10⁻³ | 12 (5.2) | 218 (94.8) | 5.03 (2.03–12.47) | 0.001 |

Values are number (%) studied. P value was determined by χ² test or Fisher exact test OR, odds ratio. CI, confidence interval.

**Detection of cytokine/chemokine production by ELISA**
Cytokine/chemokine production and viral replication were determined at 24, 48, and 72 h post-infection. Cell-free culture supernatants TNFα and macrophage chemoattractant protein 1 (MCP-1) concentrations were measured using ELISA kits from eBioscience Inc. (San Diego, CA, USA); IL-12p40 and IFN-inducible protein 10 (IP-10) were measured using ELISA kits from R&D Systems as per manufacturer’s instructions.

**Statistical Analyses**
Data are presented as mean ± SEM values. Alleles and genotypes distribution of rs4804803 are presented as numbers (percentages). Conformance of the allele frequencies to Hardy-Weinberg equilibrium proportions was tested to compare the observed and expected frequencies of heterozygotes and homozygotes. Differences among patients with DEN, DF, DHF, OFI, and population controls were determined using two-sided Chi-square test or Fisher exact test. Odds ratio (OR) values were calculated with 95% confidence intervals (CI). The Student’s t-test or Mann-Whitney U test was used for statistical comparison between continuous variables. The Wilcoxon signed-rank test was used for statistical comparison of the neutralization experiments. All analyses were performed using SPSS 15.0 (SPSS Inc. Chicago, IL, USA).

**Results**

**Demographic characteristics of patients with DF and DHF**
During a large DEN-2 outbreak in southern Taiwan between June 2002 and January 2003, a hospital-based case-control study was used to identify the risk immune parameters [19–23]. Employing the decoded DNA samples from that same cohort of the population that study has been extended to investigate the association of rs4804803 SNP with DF, DHF, viral replication, and immune response. Based on the previous case-control study design, we have included DNA samples from 135 DHF, 176 DF, and 143 OFI patients in this expanded study. The main characteristics of the study population are summarized in Table 1. There were no significant differences in sex or total leukocyte counts between patients with DF and those with DHF. However, age (41.7 ± 1.6 years vs. 45.7 ± 1.3 years, p = 0.001), serum GOT levels (70.1 ± 8.1 U/mL vs. 313.8 ± 74.6 U/mL, p = 0.002) and GPT levels (67.1 ± 11.3 U/mL vs. 142.7 ± 21.9 U/mL, p = 0.003) were significantly higher in the DHF group (Table 1). A patient manifested with abdominal pain had ascites as evidenced by abdominal ultrasonography was classified as DF because the patient revealed no thrombocytopenia (<100,000/ mm³), petechia or hemorrhagic manifestation during the admission period.

**Table 4. Distribution of CD209 –336 A/G genotype in patients with primary and secondary dengue infection.**

|            | DF   |          | OR (95% CI) | p   |            |          | OR (95% CI) | p   |
|------------|------|----------|-------------|-----|------------|----------|-------------|-----|
|            | GG   | AG       | AA          |     |            |          |             |     |
| Primary    | 0 (0) | 13 (11.8) | 97 (88.2) | 1    | 0 (0)      | 6 (14.3) | 36 (85.7)   | 1    |
| Secondary  | 0 (0) | 5 (7.9)  | 58 (92.1)  | 0.64 (0.22–1.90) | 0.409 | 2 (2.6) | 16 (20.5) | 60 (76.9) | 1.80 (0.65–4.95) | 0.251 |

Values are number (%) studied. P value was determined by χ² test or Fisher exact test based on GG or GA vs. AA comparison. OR, odds ratio. CI, confidence interval.

Primary and secondary dengue infections are defined by detectable DEN-specific IgM and IgG, respectively, within one week of the illness.
OR = 2.34, \( p = 0.018 \); Table 3). Moreover, the frequency of G allele of rs4804803 was significantly higher in DHF patients (12.2\%) than OFI patients or population controls (OR = 4.84 and 3.57, \( p = 2 \times 10^{-5} \) and 0.001), and higher than DF patients (5.4\%, OR = 2.44, \( p = 0.002 \), Table 3). Few DHF patients (n = 6) had dengue shock syndrome in this cohort; one of them carrying AG genotype.

No association of CD209 −336 A/G polymorphism (rs4804803) with primary and secondary dengue infection

To investigate whether the rs4804803 SNP in the promoter region of CD209 associated with the primary and secondary DEN infection, we used serological methods to detect DEN antibodies for differentiation into primary and secondary dengue infection. Of the 293 DEN patients, 141 (48\%) had secondary DEN infections, based on detectable DEN-2 virus RNA and DEN IgG. As shown in Table 1, secondary DEN-2 infection was more frequently found in patients with DHF than in those with DF (65\% vs. 36\%, \( p < 0.001 \)). We found the rs4804803 GG/AG genotypes were found in 12.5\% of patients with primary DEN infection and 16.3\% of patients with secondary DEN infection, which did not reach significantly different (OR = 1.36; \( p = 0.352 \)). As shown in Table 4, there was no association between rs4804803 SNP and primary or secondary dengue infection in DF patients (OR = 0.64; \( p = 0.409 \)), or in DHF patients (OR = 1.80; \( p = 0.251 \)). In addition, there was no association between allele distribution and primary or secondary dengue infection in DF and DHF patients (data not shown).

Increase of DC-SIGN expression on MDDCs from individuals with AG genotype of rs4804803

Due to the low frequency of GG genotype in our population (2 cases, 0.6\%), we could not recall the patients because the data file

Figure 1. CD209 mRNA and cell surface expression on MDDCs with AA or AG genotype of rs4804803. (A) Quantitative RT-PCR analysis of CD209 mRNA in MDDCs was run parallel to β2-microglobulin (B2MG) mRNA expression (control) on the real-time RT-PCR tracing and the gel view of RT-PCR products. (B) Subjects with rs4804803 AG genotype had higher CD209 mRNA expression in MDDCs than those with AA genotype (\( p = 0.032 \)). (C) Flow cytometric analysis of CD209 surface expression on MDDCs from individuals with the rs4804803 AG genotype (blue line; MFI = 34.1) and with the AA genotype (black line; MFI = 30.4). (D) MDDCs from individuals with AG genotype had significantly higher CD209 cell surface expression than those with AA genotype (\( p = 0.029 \)). The results are shown as mean ± s.e.m. from subjects with AA (n = 10) or AG (n = 10) genotype in three independent experiments. Statistical values were determined by Mann-Whitney U test. Asterisk (*) indicates a significant difference (\( p < 0.05 \)).

doi:10.1371/journal.pntd.0000934.g001
CD209 -336A/G Correlated to DHF & Altered Immunity

Figure 2. Kinetic surface CD209 expression and viral replication from DEN-infected MDDCs with AA or AG genotype. (A) Kinetic CD209 surface expression on MDDCs from individuals with AA or AG genotype of rs4804803 by flow cytometry. The surface CD209 expression declined along with infection in subjects with both genotypes. (B) Changes in the yield of DEN-2 viral replication (copies/10^5 cells) from individuals with AA or AG genotype of rs4804803 by flow cytometry. The surface CD209 expression declined rapidly with DEN-2 infection on MDDCs from both genotypes' subjects, which showed no difference at 24, 48, and 72 h post-infection (Fig. 2A).

Lower viral replication in MDDCs from individuals with AG genotype of rs4804803

To investigate whether the rs4804803 SNP was correlated to viral replication, we measured DEN-2 RNA copies in MDDCs with AA or AG genotype of rs4804803 at 24, 48, and 72 h post-infection. DEN-2 replication was significantly higher in MDDCs from individuals with AA genotype than those with AG genotype at 48 h post-infection (1.07±0.43×10^6 copies/10^5 cells vs. 3.90±0.67×10^6 copies/10^5 cells, p=0.006) and 72 h post-infection (4.83±0.70×10^6 copies/10^5 cells vs. 2.32±0.68×10^6 copies/10^5 cells, p=0.003; Fig. 2B). Viral replication, as measured at 72 h post-infection, increased more remarkably in MDDCs at MOIs of 5 and 10 (p<0.001 and 0.002, respectively; Fig. 2C).

Elevation of cytokines TNFα, IL-12p40, and chemokine IP-10 production in DEN-infected MDDCs with AG genotype of rs4804803

To investigate whether higher cell surface DC-SIGN expression was correlated with immune response, we investigated kinetic cytokine/chemokine production by MDDCs from individuals with AA or AG genotype of rs4804803. Results showed that MDDCs with AG genotype had significantly higher TNFα production than those with AA genotype at 24 and 48 hr post-infection (303.51±66.75 pg/mL vs. 143.97±68.80 pg/mL and 202.35±19.33 pg/mL vs. 73.00±9.55 pg/mL; p=0.021 and 0.002, respectively; Fig. 3A). IL-12p40 production significantly increased by MDDCs with AG genotype than those with AA genotype at 24, 48, and 72 h post-infection (322.32±9.55 pg/mL vs. 243.02±17.56 pg/mL; p=0.001, 0.0007 and 0.001, respectively; Fig. 3B). We also measured the concentration of two chemokines, MCP-1 and IP-10, which had been implicated in the recruitment and stimulation of monocytes, macrophages, dendritic cells, NK cells, and T lymphocytes [25]. It was found that IP-10, but not MCP-1, production was significantly higher by MDDCs with AG genotype than those with AA genotype at 24, 48, and 72 h post-infection (68.80 pg/mL and 202.35 pg/mL vs. 19.35 pg/mL; p=0.034, 0.009 and 0.010, respectively; Fig. 3C). The MCP-1 levels peaked at 48 hr in subjects with both genotypes, but there was no significant difference between AA genotype and AG genotype (550.72±60.73 pg/mL vs. 463.92±66.80 pg/mL, p=0.157; Fig. 3D).
IP-10 production by MDDCs involved in viral replication of DEN infection

IP-10, produced by non-infected bystander DCs in response to DEN infection, is a potent chemoattractant for activated T and NK cells [26], and the modulation of adaptive immune response [27]. IP-10 has also been known to inhibit the binding ability of DEN in immortalized cells [28]. In our MDDC model, cells from individuals with AG genotype exhibited an augmented innate immune reaction, showing higher IP-10 production, post-infection (Figure 3C). Based on these results, we hypothesized that DEN-infected MDDCs with AG genotype produced higher levels of IP-10, which might block viral entry or viral replication in MDDCs. We used an anti-IP-10 neutralizing mAb to block endogenous IP-10 production by MDDCs. With both genotypes, the viral replication 24 h post DEN infection increased significantly more in the presence of neutralizing antibody than in the absence of neutralizing antibody ($p = 0.034$ and $0.040$, respectively; Fig. 4A). IP-10 production by MDDCs from individuals with AG genotype significantly decreased ($795.3 \pm 368.1$ pg/mL vs. $273.8 \pm 87.8$ pg/mL; $p = 0.037$), but it did not decrease in MDDCs from individuals with AA genotype ($329.8 \pm 114.2$ pg/mL vs. $201.8 \pm 37.0$ pg/mL; $p = 0.091$; Fig. 4B). These results suggest that IP-10 produced by MDDCs is involved in the viral replication of DEN infection.

Discussion

DC-SIGN has been shown to be an important receptor for DEN and a number of viruses, including HIV, Helicobacter pylori, and Mycobacterium tuberculosis and hepatitis C virus (HCV) [29]. Some studies have demonstrated that genetic variations of CD209 (rs4804803) were associated with the susceptibility to HIV [14], Mycobacterium tuberculosis [17], HCV [30], and dengue [16]. Few studies have demonstrated how the rs4804803 SNP is involved in viral replication or immune response. We are the first in the field to demonstrate the relationship among functional cell surface expression, viral replication, and immune responses in DEN-infected MDDCs from subjects with rs4804803 SNP. Here we found that rs4804803 SNP was strongly associated with the risk of DHF vs. DF and controls. Functional studies have determined that
MDDCs from individuals with AG genotype have a significantly higher cell surface DC-SIGN expression than from those with AA genotype. MDDCs with AG genotype produced higher TNF-α, IL-12p40, and IP-10 levels but lower viral replication in response to dengue infection.

Because the physiopathology of various manifestations of DHF is not fully understood, several studies have supported the supposition that secondary dengue infection [31], age [32], a number of preexisting chronic diseases such as diabetes and bronchial asthma [33], and host genetic factor [16,22] increase the risk of progression to DHF. This indicates that multiple factors are involved in the development of DHF. Our findings regarding rs4804806 SNP associated with DHF vs. OFI control ($p = 3 \times 10^{-5}$; Table 2) in a case-control association study suggests that rs4804806 SNP contributes in part to the development of DHF.

Our study shows that the GG/AG genotypes of rs4804803 were associated with susceptibility to DHF, compared with DF, which is consistent with the observation of Sakuntabhai et al. [16]. In our study, the AA genotype was associated with protection against DHF, compared with OFI and population controls, while G allele was associated with protection against DF in Sakuntabhai’s observation. The inconsistency between these studies regarding the protection for DHF or DF may result from two possibilities. First, the frequency of G allele in Chinese population is 3.8%; while in Thailand, it is 9.5–10.4% [16,34]. Second, definition of DF and DHF might be also different. We defined DF and DHF according to WHO criteria, while in the study by Sakuntabhai et al., DF was defined by criteria of severe dengue fever syndrome with hemorrhage but no plasma leakage, excluding patients with flu-like symptoms or those having only fever. Moreover, the rs4804803 SNP was demonstrated to be in linkage disequilibrium with three other intronic polymorphisms in a Thai population, and these might also have contributed to the susceptibility of DHF [16]. Our results suggest that humans carrying the rs4804803 AG genotype have a higher DC-SIGN expression and lower DEN-2

![Figure 4. IP-10 production by MDDCs involved in the viral replication of DEN infection.](image)

MDDCs were mock-infected or DEN infected (MOI = 5) after preincubation, with and without anti-IP-10 neutralizing antibody (10 μg/mL) for 30 min. Total RNA was extracted from MDDCs and the supernatants were collected 24 h post-infection. The percentage of viral replication (A) and the production of IP-10 levels (B) following DEN-2 infection were significantly higher by MDDCs from individuals with AG genotype than from individuals with AA genotype. Following DEN-2 infection, the production of IP-10 by MDDCs from individuals with AA genotype was not significantly different between those with and without anti-IP-10 neutralizing antibody. The results are shown as mean ± SEM from three independent experiments (n = 7). Statistical values were determined by Wilcoxon signed-rank test. Asterisk (*) indicates a significant difference ($p < 0.05$). doi:10.1371/journal.pntd.0000934.g004
replication in MDDCs. These results differ from a previous study by Loach et al. who demonstrated that the DC-SIGN expression levels on Raji cells after transfection of various DC-SIGN cDNA constructs were significantly correlated to the infection rate of DEN-1 [33]. DC-SIGN is an endocytic receptor shown to induce endocytosis of several pathogens, including dengue [36–38]. The difference between these two studies might be due to different cell types and ex vivo culture systems. In our study, it was found that MDDCs from subjects with rs4804803 AG genotype had higher surface DC-SIGN expression with higher production of chemokines such as IP-10, which could limit DEN-2 replication (Fig. 4A); however, the higher surface DC-SIGN expression in subjects with AG genotype decreased remarkably 24 h post-infection (Fig. 2A). In the study by Loach et al., ectopic expression levels of DC-SIGN on Raji cells enhanced DEN-1 replication, which might be related to a higher quantity of receptors or lower production of IP-10 favoring DEN replication. Results from these studies suggest that the correlation of viral replication to higher or lower DC-SIGN expression depends on genetic factors in the host, cell type, and dynamic changes in the receptor following DEN infection.

In functional studies of rs4804803 SNP, we determined that MDDCs with AG genotype had a higher DC-SIGN expression correlated to augmented immune responses with higher TNF-α, IL-12p40, and IP-10, than those with AA genotype, but not MCP-1 production. DEN replication was significantly lower in individuals with AG genotype. The addition of anti-IP-10 neutralizing antibody blocked the production of endogenous IP-10 and significantly enhanced the replication of DEN-2 (Fig. 4A). This suggests that rs4804803 SNP was involved in the DC-SIGN expression associated with augmented immune response, such as the increase in the production of IP-10 that repressed the replication of DEN. This is supported by the fact that altered immune response, but not viral load, was observed in DHF patients [21,39]. CLEC5A-mediated DEN infection in animals that was susceptible to DEN hemorrhagic infection also revealed augmented immune response [40].

In contrast, it is interesting to note that the viral replication in MDDCs from individuals with rs4804803 AA genotype was significantly higher than in individuals with AG genotype following DEN-2 infection. The mechanism by which rs4804803 SNP influences DEN replication in MDDCs is currently unknown. Chan et al. showed that certain polymorphisms of L-SIGN, a DC-SIGN homologue, mediated more efficient viral degradation of SARS-CoV [41]. The clinical implications of screening genotypes to prevent DEN infection might be supported if different viral loads could be demonstrated among humans with various genotypes of rs4804803 in future outbreaks of DEN.

The outcome of DEN infection is determined by a myriad of interactions among viral, immunological, and human genetic factors, as well as kinetic interactions between innate and adaptive immunity. This study provides new evidence that CD209 rs4804803 SNP, correlated to cell surface expression on dendritic cells, mediates augmented immune responses against DEN-2 infection and is implicated in the susceptibility of DHF. Further studies are warranted, particularly with regard to the genetic variants of CD209 on the DC polarization of adaptive immunity, and how they may promote or protect the development of DHF.

Acknowledgments

The authors have benefited from the statistical advice by Dr. Eng-Yen Huang. For technical assistance, we would like to thank Mrs Ya-Ting Lo and Mrs Yu-Ni Su.

Author Contributions

Conceived and designed the experiments: LW KDY. Performed the experiments: LW RFC CPL. Analyzed the data: LW RFC. Contributed reagents/materials/analysis tools: JWL IRL HCX SKH. Wrote the paper: LW RFC KDY.

References

1. Wilder-Smith A, Schwartz E (2005) Dengue in travelers. N Engl J Med 353: 924–932.
2. Pang T, Cardosa MJ, Guzman MG (2007) Of cascades and perfect storms: the immunopathogenesis of dengue hemorrhagic fever-dengue shock syndrome (DHF/DSS). Immunol Cell Biol 85: 41–45.
3. Clyde K, Kyle JL, Harris E (2006) Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 80: 11410–11431.
4. Kimbell DA, Beutler B (2001) The evolution and genetics of innate immunity. Nat Rev Genet 2: 256–267.
5. Lee MS, Kim YJ (2007) Signaling pathways downstream of pattern-recognition receptors and their cross talk. Ann Rev Biochem 76: 447–480.
6. Rappocciolo G, Jenkin EJ, Hensler HR, Piazza P, Jain M, et al. (2006) DC-SIGN is a receptor for human herpesvirus 6 on dendritic cells and macrophages. J Immunol 176: 1741–1749.
7. Camili A, Giжен K, de Vries JM, Roemmers R, Joosten B, et al. (2003) The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for Candida albicans on dendritic cells. Eur J Immunol 33: 532–538.
8. Tassaneinphiphat B, Burgess TH, Granelli-Piperno A, Trumpfheller C, Fauci A, et al. (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 197: 823–829.
9. Gijzen K, Tacken PJ, Zimmerman A, Joosten B, de Vries J, et al. (2007) Relevance of DC-SIGN in DC-induced T-cell proliferation. J Leukoc Biol 81: 729–740.
10. Weinborn K, Helmus Y, Lahn K, Jones C, Lasokova A, et al. (2006) Migration of immature mouse DC across resting endothelium is mediated by ICAM-2 but independent of beta2-integrins and murine DC-SIGN homologues. Eur J Immunol 36: 2781–2794.
11. Geijtenbeek TB, Krooshoop DJ, Bleijs DA, van Vliet SJ, van Duijnhoven GC, et al. (2006) DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking. Nat Immunol 1: 333–337.
12. Geijtenbeek TB, van Vliet SJ, Engering A, H Hart BA, van Kooyk Y (2004) Self- and nonself-recognition by C-type lectins on dendritic cells. Annu Rev Immunol 22: 33–54.
13. Zhou T, Chen Y, Hao L, Zhang Y (2006) DC-SIGN and immunoregulation. Cell Mol Immunol 3: 279–283.
14. Martin MP, Lederman MM, Hutton CR, Goddert JJ, Nelson GW, et al. (2004) Association of DC-SIGN promoter polymorphism with increased risk for parenchymal, but not mucosal, acquisition of human immunodeficiency virus type 1 infection. J Virol 78: 14053–14056.
15. Liu H, Hwangbo Y, Holte S, Lee J, Wang C, et al. (2004) Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. J Infect Dis 190: 1055–1058.
16. Sakuntabhai A, Turbicalpoon C, Casademont I, Chuanumrit A, Lowhout T, et al. (2005) A variant in the CD209 promoter is associated with severity of dengue disease. Nat Genet 37: 507–513.
17. Barreiro LB, Neiroles O, Babli CB, Tailleux L, Quach H, et al. (2006) Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. PLoS Med 3: e29.
18. Vannberg FO, Chapman SJ, Khor CC, Tosh K, Floyd S, et al. (2008) CD209 genetic polymorphism and tuberculosis disease. PLoS One 3: e1388.
19. Yeh WT, Chen RF, Wang I, Liu JW, Shiao MF, et al. (2006) Implications of previous subclinical dengue infection but not virus load in dengue hemorrhagic fever. J Infect Dis 194: 755–1058.
20. Chen RF, Yang KD, Wang I, Liu JW, Chiu CC, et al. (2007) Different chronic and laboratory manifestations between dengue hemorrhagic fever and dengue fever with bleeding tendency. Trans R Soc Trop Med Hyg 101: 1106–1113.
21. Chen RF, Liu JW, Yeh WT, Wang I, Chang JC, et al. (2005) Altered T helper 1 reaction but not increase of virus load in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 48: 84–90.
22. Chen RF, Wang I, Yeh WT, Chang JC, et al. (2009) Combination of CTLA-4 and TGFbeta1 gene polymorphisms associated with dengue hemorrhagic fever and virus load in a dengue-2 outbreak. Clin Immunol 131: 494–499.
23. Wang I, Chen RF, Liu JW, Hu HR, Kuo HC, et al. (2007) Implications of dynamic changes among tumor necrosis factor-alpha (TNF-alpha), membrane...
TNF receptor, and soluble TNF receptor levels in regard to the severity of dengue infection. Am J Trop Med Hyg 77: 297–302.

24. Bandyopadhyay S, Lum LC, Kroeger A (2006) Classifying dengue: a review of the difficulties in using the WHO case classification for dengue haemorrhagic fever. Trop Med Int Health 11: 1238–1253.

25. Baggiolini M, Devauld B, Moser B (1997) Human chemokines: an update. Annu Rev Immunol 15: 675–705.

26. Nightingale ZD, Patkar C, Rothman AL (2008) Viral replication and paracrine effects result in distinct, functional responses of dendritic cells following infection with dengue 2 virus. J Leukoc Biol 84: 1028–1038.

27. Palmer DR, Sun P, Celluzzi C, Bisbing J, Pang S, et al. (2005) Differential effects of dengue virus on infected and bystander dendritic cells. J Virol 79: 2432–2439.

28. Chen JP, Lu HL, Lai SL, Campanella GS, Sung JM, et al. (2006) 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 177: 3185–3192.

29. van Vliet SJ, Garcia-Vallejo JJ, van Kooyk Y (2008) Dendritic cells and C-type lectin receptors: coupling innate to adaptive immune responses. Immunol Cell Biol 86: 380–387.

30. Ryan EJ, Dring M, Ryan CM, McNulty C, Stevenson NJ, et al. (2010) Variant in CD209 promoter is associated with severity of liver disease in chronic hepatitis C virus infection. Hum Immunol 71: 829–832.

31. Halstead SB (2007) Dengue. Lancet 369: 1644–1652.

32. Lee IK, Liu JW, Yang KD (2008) Clinical and laboratory characteristics and risk factors for fatality in elderly patients with dengue hemorrhagic fever. Am J Trop Med Hyg 79: 149–153.

33. Figueiredo MA, Rodrigues LC, Barreto ML, Lima JW, Costa MC, et al. (2010) Allergies and diabetes as risk factors for dengue hemorrhagic fever: results of a case control study. PLoS Negl Trop Dis 4: e699.

34. Wichuckhind A, Kitamura Y, Rojanasirivat A, Nakayama EE, Song H, et al. (2007) The polymorphisms in DC-SIGNR affect susceptibility to HIV type 1 infection. AIDS Res Hum Retroviruses 23: 686–692.

35. Lozach PY, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, et al. (2005) Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J Biol Chem 280: 23698–23708.

36. Ludwig IS, Lekkerkerker AN, Depla E, Bosman F, Musters RJ, et al. (2004) Hepatitis C virus targets DC-SIGN and L-SIGN to escape lysosomal degradation. J Virol 78: 8322–8332.

37. van Kooyk Y, Geijtenbeek TB (2003) DC-SIGN: escape mechanism for pathogens. Nat Rev Immunol 3: 697–709.

38. Mukhopadhyay S, Kuhn RJ, Rosmann MG (2005) A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3: 13–22.

39. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangkawornnaik N, et al. (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9: 921–927.

40. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, et al. (2008) CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453: 672–676.

41. Chan VS, Chan KY, Chen Y, Poon LL, Cheng AN, et al. (2006) Homozygous L-SIGN (CLEC4M) plays a protective role in SARS coronavirus infection. Nat Genet 38: 38–46.