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Gene expression profiling of dengue infected human primary cells identifies secreted mediators in vivo

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Abstract

We used gene expression profiling of human primary cells infected in vitro with dengue virus (DENV) as a tool to identify secreted mediators induced in response to the acute infection. Affymetrix Genechip analysis of human primary monocytes, B cells and dendritic cells infected with DENV in vitro revealed a strong induction of monocyte chemotactic protein 2 (MCP-2/CCL8), interferon gamma-induced protein 10 (IP-10/CXCL10) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/TNFSF10). The expression of these genes was confirmed in dendritic cells infected with DENV in vitro at mRNA and protein levels. A prospectively enrolled cohort of DENV-infected Venezuelan patients was used to measure the levels of these proteins in serum during three different periods of the disease. Results showed significant increase of MCP-2, IP-10 and TRAIL levels in DENV-infected patients during the febrile period, when compared to healthy donors and patients with other febrile illnesses. MCP-2 and IP-10 levels were still elevated during the post-febrile period while TRAIL levels dropped close to normal after defervescence. Patients with primary infections had higher TRAIL levels than patients with secondary infections during the febrile period of the disease. Increased levels of IP-10, TRAIL and MCP-2 in acute DENV infections suggest a role for these mediators in the immune response to the infection.

Keywords

Dengue virus; IP-10; MCP-2; MIP-1 β ; TRAIL, Affymetrix, Global gene expression

INTRODUCTION

Dengue virus (DENV) is a single-stranded RNA mosquito-borne virus that belongs to the family Flaviviridae and exists as four different serotypes: DENV1, DENV2, DENV3, and DENV4 [Monath, 1994]. All serotypes can infect humans and produce a broad spectrum of clinical manifestations that ranges from an acute self-limited febrile illness (dengue Fever, DF) to various grades of a severe disease (dengue hemorrhagic fever, DHF and dengue shock syndrome, DSS) [Chaturvedi et al., 2006]. Clinical symptoms include fever, headache, myalgias, skin rash, thrombocytopenia, coagulation alterations, hepatic inflammation and hemorrhagic manifestations. Increased vascular permeability that results in vascular leakage is the characteristic event that defines DHF [Rothman and Ennis, 1999]. Infection with one of the serotypes imparts immunity to the infecting serotype. Multiple infections with different

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(heterologous) serotypes can occur during the lifetime and DHF/DSS is usually associated with secondary infections [Halstead et al., 1970] [Guzman et al., 1990].

It has been suggested that the transient vascular leakage observed in vivo could be associated with the response of the endothelium to inflammatory mediators produced in response to DENV infection [Avirutnan et al., 1998], and several published studies have focused on quantifying the levels of chemokines during the acute phase of the disease. Elevated levels of interleukin 8 (IL-8) [Raghupathy et al., 1998] [Juffrie et al., 2000] and monocyte chemotactic protein 1 (MCP-1) [Lee et al., 2006], and IP-10 [Fink et al., 2007] have been reported in DENV-infected patients, especially in more severe cases. The expression of macrophage inflammatory protein-1 alpha (MIP-1 α) and MIP-1 β was reported in peripheral blood mononuclear cells (PBMC) from DENV-infected patients [Spain-Santana et al., 2001]. In vitro DENV infection of different cell types, including PBMC, monocytes, macrophages, mast cells, umbilical vein endothelial cells (HUVEC) and human primary hepatocytes, induces the expression of various chemokines, including IL-8, MIP-1 α/β and regulated upon activation, normal T cell expressed and secreted (RANTES) [Avirutnan et al., 1998; Chen and Wang, 2002; Huang et al., 2000; King et al., 2002; Spain-Santana et al., 2001; Suksanpaisan et al., 2007]. DENV-induced immunopathology is complex and not well known. Potential mediators of disease have been identified in earlier studies, but none of these molecules alone can explain the events that have been documented in DENV-infected patients. Hence, the identification of new mediators up-regulated in response to DENV infection could contribute to the understanding of the immune response against the virus and the related immune-mediated pathology.

The aim of the present study was to identify the expression of novel soluble mediators that may be involved in the inflammatory response to DENV or may be part of the regulatory feedback loop to control inflammation in vivo. An experimental approach based on gene expression analysis of primary human cells infected with DENV in vitro was used. Three cytokine / chemokine genes up-regulated in vitro (MCP-2, IP-10, and TRAIL) were studied in clinical samples. The results showed higher circulating levels of MCP-2, IP-10, and TRAIL in serum from DENV-infected patients during the febrile period of the disease, when compared to both healthy donors and patients with other febrile illnesses. Interestingly, sera from patients with primary infections showed higher levels of TRAIL than sera from patients with secondary infections during the febrile period. While increased levels of MCP-2 and IP-10 could be part of immune mechanisms triggered in response to DENV infection, increased levels of TRAIL could be part of regulatory mechanisms triggered to control the inflammatory response to infection.

MATERIALS AND METHODS

Cell Preparation and Culture

Blood was obtained from healthy U.S. volunteers at the University of Massachusetts Medical School (UMMS), Worcester, MA, USA. Dendritic cells (DC) were prepared from peripheral blood-derived CD14-positive cells. Briefly, PBMC were isolated using a density gradient centrifugation over Ficoll-Paque™ Plus, 1.077g/dl (GE Healthcare). Positive selection of CD14+ cells was performed using the CD14-positive selection magnetic cell sorting kit (Miltenyi Biotec, Inc.). Monocytes were incubated in RPMI 1640 supplemented with 10% FCS, 500 U/ml of IL-4 (Pepro-Tech) and 800 U/ml of granulocyte-macrophage colony stimulation factor (GM-CSF) (Pepro-Tech). The percentage of immature DC (iDC) conversion (CD1a+ / CD14-) was determined after 6 days in culture, using flow cytometry. Monocytes and B cells for GeneChip hybridization experiments were negatively selected from blood using a rosetting antibody precipitation kit (StemCell) and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). The purity (CD14-positive or CD19-positive cells) was determined using flow cytometry.

DENV infection

Cells for gene expression analysis were infected with DENV (DENV2 – New Guinea C, DENV2-NGC) at a multiplicity of infection (M.O.I.) of 1 for monocytes and B cells and M.O.I. of 0.1 for DC. Cells were incubated with virus for 2 hours at 37°C/5% CO₂ and then washed thoroughly, resuspended in RPMI 1640 supplemented with 10% FCS (monocytes and B cells) or RPMI 1640 supplemented with 10% FCS plus 500 U/ml IL-4 and 800 U/ml GM-CSF (DC) and incubated for 48 hours. For the time-course experiment, DC were infected with DENV2-NGC at M.O.I. of 0.1 and incubated for 12, 24 or 48 hours; cells and supernatant were collected at each time point. For TRAIL pre-treatment experiments, DC were treated with 20 µg/ml of recombinant TRAIL (rTRAIL, Biomol International LP) for 24 hours, and then infected with DENV2-NGC at M.O.I of 0.1; cells and supernatants were collected at 48 hours post-infection.

Flow Cytometry

Cells were surface-stained using monoclonal antibodies anti-CD14-Pacific blue, CD-19-APC, anti-CD1a-APC and/or anti-CD83-PE (all from BD). Intracellular detection of DENV antigen was performed in fixed and permeabilized cells, using the Cytofix/Cytoperm kit (BD) and stained with anti-DENV complex antibody conjugated to FITC (Chemicon). Cells were analyzed using the BD FACS Aria™ and Flow-Jo software.

RNA Preparation, Affymetrix GeneChip Hybridization and Quantitative RT-PCR

Total cellular RNA was prepared using the RNeasy kit (Qiagen). Affymetrix genechip hybridization was performed as previously described [Warke et al., 2003] from biotin-labeled total cellular RNA, hybridized to human oligonucleotide microarrays (Affymetrix HG-U133A). Normalized signal values were computed from Affymetrix HG-U133A chips using Robust Multichip Average (RMA) [Irizarry et al., 2003]. Genes were normalized to expression levels in controls for each cell type.

Quantitative real time polymerase chain reaction (qRT-PCR) was performed from total cellular RNA using Taqman Reverse Transcription kit, universal PCR Master Mix 2X and specific primers and probes (all from Applied Biosystems). The PCR reaction was performed in the 7300 Taqman PCR System (Applied Biosystems). β -actin was used as an endogenous control and relative quantification (Rq) was done using the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen, 2001].

Subjects and Specimens

Patients were enrolled in a study protocol conducted by UMMS and Banco Municipal de Sangre del Distrito Capital (BMS), Caracas, Venezuela. Written informed consent was obtained from all subjects. Febrile patients with no clinical evidence of other defined infections were enrolled. Patients attended the clinic daily until 2 days after the fever resolved. A convalescent visit was performed at least 2 weeks after the onset of symptoms. Physical exam and routine laboratory tests were performed as described previously [Becerra et al., 2008]. Fever day zero (0) was defined as the day of defervescence. The febrile period included days before defervescence and the post-febrile period included defervescence and days after defervescence. Healthy donors from BMS and UMMS were used as controls.

Dengue cases were defined following the World Health Organization (WHO) guidelines [WHO]. DENV genomic RNA was isolated from febrile serum samples using the QIAmp Viral RNA kit (QIAGEN). DENV serotype-specific reverse transcription and polymerase chain reaction (RT-PCR) was performed using the One-step PCR kit (QIAGEN) and primers previously described [Lanciotti et al., 1992] adapted to a one-step RT-PCR, using reverse primer and serotype specific forward primers. DENV-specific antibodies were measured in

paired serum samples (enrollment and convalescence samples, S1 and S2), using IgM-ELISA and hemagglutination inhibition assay (HI) at the Instituto Nacional de Higiene “Rafael Rangel”, Caracas, Venezuela. Patients were classified as dengue or as other febrile illness (OFI) based on the detection of DENV RNA, presence of IgM antibodies and/or at least a four-fold increase in HI titers in S2 compared to S1. The HI levels were used to further classify dengue patients as a primary infection (HI titer $\leq$ 1:1280) or secondary infection (HI titer $>$ 1:1280) [2003].

Quantification of Proteins in Serum or Culture Supernatants

Levels of MCP-2, IP-10, MIP-1 β , and TRAIL in serum from patients and MCP-2, IP-10 and IFN- α in DC culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (Ray Biotech Inc., R&D Systems) following the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney U test was used for comparisons between two groups for continuous variables not normally distributed. The paired t-test was used for comparisons between percentages of infection. Spearman r test was used to test correlations. We used the software SPSS 15.0 for Windows (Copyright SPSS Inc. 1989-2005) for the statistical analysis.

RESULTS

Gene Expression Analysis in vitro

Gene expression analysis using Affymetrix HG-U133A chips was performed on primary human monocytes, B cells and DC infected with DENV in vitro. To identify the genes that were up regulated by DENV infection, genes induced at least 1.6-fold in all 3 cell types (using means of duplicate measurements for DC and monocytes, and a single measurement for B cells) were selected. Then, from this list, genes that showed statistically significant differences in expression (Welch t-test with Benjamini false discovery rate multi gene correction, $p < 0.05$) in infected versus normal samples were selected. The resulting list included 198 probe-sets representing 157 different genes (Supplementary Table). Greater than 10-fold induction was observed for 24 of the 157 genes and 5 of the 157 genes were in the category of secreted genes with cytokine/chemokine activity. Three cytokine/chemokine genes were in the group of 24 genes with more than 10-fold induction (MCP-2/CCL8, IP-10/CXCL10 and TRAIL/TNFSF10). Two cytokine/chemokine genes, RANTES/CCL5 and MIP-1 β /CCL4, were less than 10-fold induced. The expression of the cytokines/chemokines genes is shown in Table 1. Highly induced MCP-2/CCL8, IP-10/CXCL10 and TRAIL/TNFSF10, as well as the marginally induced MIP-1 β /CCL4, were selected for further study. MCP-2 was strongly induced in monocytes and DC in response to DENV infection. IP-10 was strongly induced in DC and to a lesser extent in monocytes and B cells. MIP-1 β was weakly induced in monocytes, B cells and DC. TRAIL was strongly induced in DC and to a lesser extent in monocytes and B cells.

The expression of MCP-2, IP-10, MIP-1 β and TRAIL in response to DENV was then confirmed in the model of DENV-infected DC in vitro. Relative quantification of mRNA in uninfected and DENV-infected DC at 12, 24 and 48 hours post-infection was performed using qRT-PCR (Table 2). A strong induction of IP-10, TRAIL and MCP-2 mRNA at 24 and 48 hours post-infection was found; MIP-1 β mRNA was weakly up-regulated at both 24 and 48 hours. Accumulated levels of MCP-2 and IP-10 proteins in cell culture supernatants at each time point were measured by ELISA (Figure 1). Higher levels of MCP-2 ($p = 0.001$) and IP-10 ($p = 0.022$) were found in supernatants from DENV-infected DC compared to uninfected DC at 48 hours.

Characterization of Patients

The characteristics of the patients enrolled in the study are shown in Table 3. The study included a total of 11 OFI and 31 dengue patients. Among the dengue patients, the predominant infecting serotype was DENV3 (22 patients); 8 patients were infected with DENV1 and 1 with DENV2. Among the 31 dengue patients, 15 had primary infections and 15 had secondary infections (1 dengue patient could not be classified as primary or secondary infection); 24 dengue patients had hemorrhagic manifestations (petechiae, epistaxis, gum bleeding). 21 had platelet counts lower than 100,000/ μ l, two had greater than 20% hemoconcentration, and three had other evidences of vascular leakage (pleural effusion, gall-bladder edema). Two of the dengue patients met the WHO criteria [2003] for DHF.

MCP-2, IP-10, MIP-1 β , and TRAIL levels in Serum from Patients

Levels of MCP-2, IP-10, MIP-1 β and TRAIL in serum from DENV-infected patients and OFI are shown in Figure 2. MCP-2 levels were measured in serum from 22 DENV-infected patients and 8 OFI (Figure 2-A). Increased MCP-2 levels were found during the febrile days of the disease, followed by a progressive decrease at post-febrile days to reach values close to normal by the convalescent visit. Levels of MCP-2 were significantly higher in DENV-infected patients compared to OFI during the febrile ($p=0.015$) and early post-febrile ($p=0.001$) days of the disease. There were no significant differences in MCP-2 levels at any period of the disease between patients with primary ($N=9$) and secondary ($N=11$) infections or between patients with ($N=18$) and without ($N=4$) hemorrhagic manifestations. Serum levels of MCP-2 in healthy donors were 33.7 ± 8.8 ($N=8$). There were no significant differences in levels of MCP-2 in serum between OFI and healthy donors.

IP-10 serum levels (Figure 2-B) were markedly increased in DENV-infected patients during the febrile and early post-febrile days of the disease. Levels of IP-10 decreased by the convalescent visit, but still remained elevated compared to OFI and healthy donors. Levels of IP-10 were significantly higher in DENV-infected patients ($N=19$) compared to OFI ($N=7$) at febrile ($p<0.001$), early post-febrile ($p<0.001$) and convalescent ($p=0.022$) periods of the disease. There were no significant differences in IP-10 levels at any period of the disease between patients with primary ($N=8$) and secondary ($N=11$) infections. Higher IP-10 levels were found in patients with hemorrhagic manifestations ($N=15$) as compared to patients without hemorrhagic manifestations ($N=4$); the difference was significant during the post-febrile period ($p=0.037$). Serum levels of IP-10 in healthy donors were 0.25 ± 0.08 ($N=5$) ng/ml. Higher IP-10 levels were found in serum from OFI during the febrile days of the disease when compared to healthy donors ($p=0.014$).

MIP-1 β levels (Figure 2-C) were slightly increased in serum from DENV-infected patients ($N=23$) as compared to OFI ($N=11$) during the febrile days of the disease ($p=0.024$). Levels of MIP-1 β in DENV-infected patients at the post-febrile and convalescent days were no different than those from OFI and healthy donors. Slightly increased levels of MIP-1 β in primary infections ($N=8$) as compared to secondary infections ($N=15$) were found during the post-febrile period of the disease and the difference was statistically significant ($p=0.016$). There were no significant differences in MIP-1 β levels between patients with ($N=6$) and without ($N=17$) hemorrhagic manifestations, at any period of the disease. Serum levels of MIP-1 β in healthy donors were 105.2 ± 10.4 ($N=11$) pg/ml. There were no significant differences between MIP-1 β levels in serum from OFI and healthy donors.

TRAIL levels in serum from DENV-infected patients ($N=19$) were increased during the febrile days of the disease as compared to TRAIL levels in serum from OFI ($N=4$); the difference was statistically significant ($p=0.009$) (Figure 2-D). Levels of TRAIL in DENV-infected patients dropped close to normal shortly after defervescence. Higher TRAIL levels were found in

patients with primary infections (N=10) as compared to patients with secondary infections (N=7) during the febrile period of the disease; the difference was significant ($p=0.050$). There were no significant differences in TRAIL levels between patients with (N=4) and without (N=15) hemorrhagic manifestations, at any period of the disease. Serum levels of TRAIL in healthy donors were 33.7 ± 8.8 (N=8) pg/ml. There were no differences in TRAIL levels in serum from OFI and healthy donors.

TRAIL Modulation of DC Response to DENV Infection

TRAIL-mediated inhibition of DENV infection in vitro has been previously reported [Warke et al., 2007]. Pre-treatment of DC with rTRAIL before infection with DENV resulted in reduced mRNA expression of several cytokines and chemokines (data not shown) as well as decreased levels of cytokines and chemokines in cell culture supernatants at 48 hours post-infection and a lower percentage of DENV-infected DC (Figure 3). Among the cytokines and chemokines suppressed by rTRAIL pre-treatment, significant differences for MCP-2 ($p=0.050$), IP-10 ($p=0.021$) and IFN- α ($p=0.025$) were observed. Additionally, levels of IFN- α in culture supernatants were positively correlated with the percentage of DENV-infected DC ($r=0.73$; $p<0.003$).

DISCUSSION

Several cytokines and chemokines have been reported to be induced in response to DENV infection in vitro and in vivo. It has been suggested that the short-lived vascular leakage observed in DENV infections in vivo is most likely mediated by transiently released/produced soluble mediator(s) [Avirutnan et al., 1998]. The effect of chemokines is not restricted to the regulation of local trafficking of leukocytes, but also would have an effect on systemic targets [Farber, 1997]. A combination of different chemokines signaling through different receptors in different cells or tissues, rather than an individual molecule, determines the final outcome. Hence, it is believed that the over-expression of different chemokines and cytokines would probably contribute to the DENV-induced immune-mediated pathology, including endothelial cell dysfunction.

In this study, gene expression analysis was used to identify additional cytokines and chemokines that could be involved in the cellular response to DENV infection in vitro and in vivo. Gene expression analysis showed three genes with cytokine/chemokine activity (MCP-2/CCL8, IP-10/CXCL10 and TRAIL/TNFSF10) up-regulated more than 10-fold in human primary immune cells infected with DENV in vitro; one gene (RANTES) was up-regulated 5-fold and one gene (MIP-1 β /CCL4) was marginally up-regulated. MCP-2/CCL8 has not been previously described in DENV infections in vitro or in patients. IP-10/CXCL10 has been shown to be induced in vitro and recently was reported to be increased in serum of DENV-infected patients [Fink et al., 2007]. TRAIL/TNFSF10 expression has been reported in human primary cells in response to DENV infection in vitro [Warke et al., 2007]. MIP-1 β mRNA expression has been found in PBMC from DENV-infected patients [Spain-Santana et al., 2001] and serum levels were recently reported [Bozza et al., 2008].

As noted above, MCP-2 has not been previously associated with DENV infections. Previous studies have shown higher levels of MCP-1 in patients with DHF and the authors hypothesized that the mechanism of vascular leakage in their in vitro model using DENV2-infected HUVECs was partially dependent on MCP-1 [Lee et al., 2006]. MCP-1 and MCP-2 are co-expressed, although MCP-1 is produced in higher quantities and lower concentrations of MCP-1 are required to induce chemotaxis of monocytes and activated T lymphocytes [Proost et al., 1996]. However, MCP-1 binds only to CCR2 while MCP-2 is able to bind to CCR1, CCR2 and CCR5. Therefore, MCP-1 and MCP-2 could have independent effects during DENV infection. Interestingly, a recent report showed that the MCP-2 – CCR2 interaction counteracts

the activation signaling normally generated by other chemokines [O'Boyle et al., 2007]. MCP-2 might contribute to the immune response to DENV infection by engaging with different receptors in various cell types and/or suppressing the effect of other chemokines.

IP-10 was among the highest up-regulated genes in DC and was also up-regulated in other primary cells in response to DENV infection in vitro. Increased levels of IP-10 were also found in DENV-infected patients. Interestingly, a tendency of higher levels of IP-10 in DENV-infected patients with hemorrhagic manifestations was found, but no differences in IP-10 levels in serum from patients with primary and secondary DENV infections. Recently, higher serum levels of IP-10 in DENV-infected patients were also reported by others [Fink et al., 2007]. The data presented here confirms the data published by Fink et al in a different cohort of patients (Venezuela vs. Singapore). IP-10 is induced by IFN- γ and IFN- α/β and is chemoattractant to activated T lymphocytes and NK cells [Farber, 1997]. IP-10 has been found to be induced in various viral infections in vivo and in vitro [Chen et al., 2006; Diago et al., 2006; Roe et al., 2007; Warke et al., 2007]. It has been suggested that IP-10 has a role in the protective immune response against DENV, competing with the virus for the binding to heparan sulfate, and blocking entry and replication [Chen et al., 2006] [Hsieh et al., 2006]. On the other hand, high serum levels of IP-10 have been found in patients with chronic inflammatory conditions [Laine et al., 2007]. Thus, IP-10 could have a protective role against DENV, but could also be associated with the potentially damaging inflammatory response if produced in high amounts and in an uncontrolled manner.

Gene expression analysis showed a slight increase of MIP-1 β expression in various primary human cells infected with DENV. MIP-1 β levels were slightly increased in serum from DENV-infected patients during the febrile period of the disease. Interestingly, MIP-1 β levels were higher in patients with primary infections during the post-febrile period of the disease. Higher levels of MIP-1 β in primary infections may suggest a protective role of MIP-1 β in dengue infections. Recently, Bozza et al [Bozza et al., 2008] reported elevated levels of MIP-1 β associated with mild dengue disease. MIP-1 β is induced in response to LPS, TNF- α , IFN- γ , and viral infections [Maurer and von Stebut, 2004] and induces chemotaxis of monocytes, T lymphocytes, NK cells and immature DC. MIP-1 β expression was induced in K562 cells in response to DENV infection in vitro and also was found in PBMC isolated from DENV-infected patients [Spain-Santana et al., 2001]. The induction of MIP-1 β in response to DENV might be limited to certain cell types or restricted to specialized areas, and hence systemic levels or cell-specific levels might underestimate the role of MIP-1 β in vivo.

TRAIL levels were increased in serum from DENV-infected patients during the febrile period of the disease. Interestingly, higher levels of TRAIL were found in patients with primary infections. Recently, a potent antiviral effect of TRAIL in DENV infection in vitro was reported [Warke et al., 2007]. TRAIL pre-treatment of DENV-infected DC in vitro also suppressed the production of pro-inflammatory chemokines and cytokines. These results could suggest a protective role of TRAIL in DENV infections in vivo. TRAIL is a member of the TNF family of proteins, originally identified as a promoter of apoptosis in tumor cell lines and some primary tumors [Ashkenazi et al., 1999]. Recent studies have shown that TRAIL can induce proliferation of T lymphocytes and negatively regulate inflammation and the innate immune response through an apoptosis-independent mechanism [Diehl et al., 2004; Secchiero et al., 2005; Song et al., 2000; Vassina et al., 2005]. The mechanism of action of TRAIL to reduce the production of cytokines and chemokines is not known. The effect of TRAIL as an antiviral could be responsible for the suppression of DENV-induced chemokines and cytokines, as lower levels of virus might account for lower induction of these soluble mediators, but we cannot rule out a direct anti-inflammatory role of TRAIL. Further investigation should be conducted to elucidate the mechanisms involved in TRAIL-mediated control of the virus and the inflammatory response.

The clinical events that characterize DHF occur around the time of defervescence [Kalayanaroj et al., 1997]. Higher levels of MCP-2 and IP-10, as well as other mediators, around the time of defervescence could have a role promoting and increasing the potentially damaging inflammatory response and thereby contribute to the events that lead to endothelial cell dysfunction, vascular leakage and altered coagulation. The specific up-regulation of TRAIL in DENV-infected patients and the previous observation that TRAIL has an antiviral effect in DENV-infections in vitro, suggests a role of TRAIL in the control of virus infection. The tendency of higher serum TRAIL levels in primary infections could suggest a protective role, limiting virus replication and consequently the transition to a severe disease and a potential therapeutic use for TRAIL in limiting the damaging inflammatory response triggered by DENV in severe cases. Further investigation, using a larger cohort of patients and patients with different grades of severity should be conducted to confirm these observations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

MCP-2 and IP-10 protein levels in DC culture supernatants. Levels of MCP-2 and IP-10 in supernatants from uninfected (white bars) and DENV-infected (black bars) DC (N=3), at 12, 24 and 48 hours post-infection. Mean values $\pm$ standard error of mean are shown. Mann–Whitney statistical analysis between uninfected and DENV-infected: MCP-2 (48 hours, $p=0.001$); IP-10 (48 hours, $p=0.022$).

Figure 2.

Serum levels of MCP-2 (A), IP-10 (B), MIP-1 β (C) and TRAIL (D) in DENV-infected patients (solid black) and OFI (solid white); in patients classified as primary (horizontal lines) and secondary infections (solid light gray); and in patients classified according to the absence or presence of hemorrhagic manifestations, as not bleeding (solid dark gray) and bleeding (downward diagonal lines). Results are expressed as a mean value $\pm$ standard error of mean, for each patient group and each disease day. Mann-Whitney statistical analysis between OFI and dengue at each disease day: MCP-2 (febrile $p=0.015$; post-febrile $p=0.001$); IP-10 (febrile $p<0.001$; post-febrile $p<0.001$); MIP-1 β (febrile $p=0.024$); TRAIL (febrile $p=0.009$); Mann-Whitney statistical analysis between primary and secondary infections: MIP-1 β (post-febrile

p=0.016); TRAIL (febrile p=0.050); Mann–Whitney statistical analysis between absence and presence of hemorrhagic manifestations: IP-10 (post-febrile p=0.037). Conv.: convalescence.

Figure 3.

Effect of rTRAIL pre-treatment of DENV-infected DC. Untreated or r-TRAIL pre-treated DC were infected with DENV for 48 hours (see Methods). The percentage of infected DC was assessed by flow cytometry (A); levels of IFN- α (B), MCP-2 (C) and IP-10 (D) were measured by ELISA in cell culture supernatants. Results are expressed as a mean value $\pm$ standard error of mean (N=3-7). Mann-Whitney statistical analysis between untreated and r-TRAIL pre-treated: IFN- α (p=0.025); MCP-2 (p=0.050); and IP-10 (p=0.021).

Table 1

Gene expression analysis of human primary immune cells infected with DENV: Genes with cytokine/chemokine activity up-regulated more than 1.6-fold after DENV infection

Gene	Affy ID	GenBank	Description	Fold Change ^{a,b}			Mean ^c
				DC	Mo	B	
TRAIL	202688_at; 202687_s_at; 214329_x_at	NM_003810	tumor necrosis factor (ligand) superfamily, member 10	42.1	9.7	11.6	21.1
IP-10	204533_at	NM_001565	chemokine (C-X-C motif) ligand 10	128.0	3.7	6.3	46.0
MCP2	214038_at	A1984980	chemokine (C-C motif) ligand 8	26.2	16.4	6.6	16.4
RANTES	204655_at	NM_002985	chemokine (C-C motif) ligand 5	13.9	3.3	1.8	6.3
MIP1B	204103_at	NM_002984	chemokine (C-C motif) ligand 4	1.6	2.2	2.7	2.2

^aValues from single probe sets (MIP-1β: 204103_at; IP-10: 204533_at; MCP-2: 214038_at; RANTES: 204655_at) or the mean from three probe sets (TRAIL: 202687_s_at, 202688_at, and 214329_x_at) on HG-U133A chips are presented.

^bGene expression fold-change in DENV-infected cells compared to mock-infected cells. N=2 for DC and Mo and N=1 for B cells.

^cMean values listed are geometric means.

Table 2Gene Expression Changes in Primary Human Dendritic Cells Induced by DENV determined using qRT-PCR^a

Alias	Fold change ^b		
	12 hours	24 hours	48 hours
MIP-1β	1.0	1.6	1.5
MCP-2	1.0	12.0	60
IP-10	1.0	337	18084
TRAIL	1.0	105	168

^aRelative quantification (Rq) of mRNA using qRT-PCR and the $2^{-\Delta\Delta C_t}$ method; β -actin was used endogenous control and uninfected DC samples as calibrator.

^bFold-change in RNA expression in DENV-infected DC as compare to uninfected DC (expression in uninfected DC set at 1.0). Values are average of three independent experiments.

Table 3

Clinical Profile of Patients Enrolled in the Study Protocol

Classification ^a	OFI	Dengue			
		All	Primary ^b	Secondary ^b	No hemorrhage Hemorrhage ^c
N	11	31	15	15	7 24
Sex distribution (female:male)	6:5	12:19	8:7	3:12	3:4 9:15
Age ^d	25 (9-56)	20 (7-55)	20 (7-37)	25 (11-55)	20 (9-25) 22 (7-55)
Laboratory Parameters ^e					
Minimum WBC (x10 ³ /ul)	4.8±0.6	2.6±0.2	2.3±0.2	3.0±0.3	3.3±0.4 2.4±0.2
Minimum Platelet Count (x10 ³ /ul)	188±13	88±8	108±11	68±11	122±18 78±8
Maximum ΔTT (sec)	2.6±0.5	11.1±2.2	6.4±0.8	15.8±4.2	5.6±1.2 12.8±2.8
Maximum AST (U/ml)	36.5±4.9	206.0±35.3	176.1±57.6	229.2±45.2	88.3±23.1 240.2±42.7
Maximum ALT (U/ml)	35.5±8.9	174.5±29.7	170.5±54.4	180.0±30.8	51.9±8.9 210.3±35.1

^a Patients were classified as OFI or Dengue according to DENV RNA and IgM detection. Dengue patients were positive for both DENV RNA and IgM; OFI patients were negative for both parameters.

^b Dengue patients classified according to HI titer in primary (HI ≤ 1:1280) or secondary (HI > 1:1280).

^c Hemorrhagic manifestations: petechia, epistaxis, gum bleeding.

^d Age in years (median, range).

^e Average values ± standard error.