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MCP-1 and MIP-1α expression in a model resembling early immune response to dengue

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ABSTRACT

Dengue virus has become endemic in most tropical urban areas throughout the world, and DHF has appeared concomitantly with this expansion. The intensity of dengue virus replication during the early stages of infection could determine clinical outcomes; therefore, it is important to understand the impact of dengue virus infection on the earliest immune defense against microbial infection, which also strongly regulates the adaptive immune responses. This study was aimed at evaluating the expression of the CC-chemokines MIP-1α/CCL3 and MCP-1/CCL2 in peripheral blood leukocytes using an *ex vivo* model resembling dengue infection *in vivo*, in subjects with a well characterized dengue immune background, due to the exceptional Cuban epidemiological situation in dengue. The expression of IFNγ, TNFα and IL10 was also evaluated, giving insight about the role of MCP-1 and MIP-1α in the interplay between innate and adaptive immunity. From individuals with different dengue immune background after dengue virus challenge, increased and different expression of the chemokines and cytokines studied was verified in peripheral blood mononuclear cells, thus demonstrating that the previous immunity to a dengue virus serotype has a strong influence on the early immune response after dengue re-infection.

1. Introduction

Patients infected with dengue virus (DV)[1] show multiple clinical manifestations; these can be total absence of symptoms, mild dengue fever or life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [1,2]. Besides the participation of the viral mechanism, like virus variation and viral load [3–6], the involvement of the immune response in dengue pathogenesis has also been hypothesized [7–14] through the activation of different cells of the immune system and the production of diverse cytokines and chemokines [15–32].

Chemokines are a superfamily of small proteins with a crucial role in immune and inflammatory reactions. Induction of leukocyte migration is the eponymous function of the chemokines, which, at the same time, affect angiogenesis, collagen production and proliferation of haematopoietic precursors.

Monocytes/macrophages [33], mast cells [34], dendritic cells [35], endothelial cells [36] and Kupffer cells [37] have shown to be permissive cells able to produce chemokines in response to dengue infection. Likewise, several studies have reported that DV infection induces the overexpression of many chemokines and cytokines in monocytes [38,39]. There are few studies about the possible role of monocyte chemoattractant protein-1 (MCP-1/CCL2) and macrophage inflammatory protein-1α (MIP-1α/CCL3) in dengue infection. We consider that the role of these chemokines in dengue infection is probably underestimated. They act over the migration and infiltration of monocytes, memory T lymphocytes and natural killer cells; all of which are deeply involved in dengue disease pathogenesis. They also act on the endothelial cells, causing permeability change, contributing to plasma leakage which is a critical event in DHF clinical picture [40–42].

Considering all the above-mentioned facts, we analyzed here the expression of the chemokines MIP-1α, also known as CCL3, and MCP-1, also named CCL2, in the course of a 24 h-*ex vivo* infection of peripheral blood mononuclear cell (PBMC) from individuals with different dengue immune backgrounds.

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[1] *Abbreviations:* DF, dengue fever; DHF, dengue hemorrhagic fever; MIP-1α, macrophage inflammatory protein-1 alpha; MCP-1, monocyte chemoattractant protein-1; IFNγ, interferon gamma; TNFα, tumor necrosis factor alpha; IL-10, interleukin 10.

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Taking into consideration that there is compelling evidence that MIP-1α and MCP-1 are involved in TH1/TH2 polarized immune responses [43,44], and have also importance in the equilibrium between the pro-inflammatory and regulatory branches of the immune response, we evaluated the expression of IFNγ, TNFα and IL10, IL4 and IL5 to give insights about the role of MCP-1 and MIP-1α in the early interplay between innate and adaptive immunity.

2. Materials and methods

2.1. Study subjects

Forty healthy Cuban individuals from Havana City (16 female and 24 male, ages 20–60, mean equal to 35.8 years) were studied. Ten individuals were non-immune to dengue. Of the remaining 30 individuals, 10 showed a history of DV1 infection, 10 history of DV2 infection, and 10 history of DV3 infection during the epidemics occurred in Havana, Cuba in 1977, 1981 and 2001, respectively. All of them had dengue virus antibody titers of 1/40 or higher obtained by ELISA [45] and were primary dengue infection cases as shown by means of the plaque reduction neutralization assay [46]. These 30 subjects were clinically classified, according to PAHO/WHO Guidelines for Dengue Prevention and Control, as dengue fever without hemorrhagic manifestations during the outbreaks. In all the cases, the infection was detected by IgM determination in serum samples collected in the early convalescent phase of the disease and confirmed by viral isolation in the C636 mosquito cell line.

This study was conducted according to the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects and was approved by the Institutional Ethical Review Committees of the Institute of Tropical Medicine "Pedro Kourí" and the Cuban National Academy of Sciences. Written informed consent was obtained from each individual upon enrolment in the study.

2.2. Preparation of purified viruses

DV were prepared as previously described [47]. C636 cell lines from *Aedes albopictus* were grown to confluence, infected at a multiplicity of infection of 0.1 particle forming unit (pfu) per cell with the DV strains DV1-113 Peru 1990, DV-2 A15 Cuba 1981, and DV-3 116 Cuba 2000 and were cultured in Minimum Essential Medium (MEM) supplemented with 2% fetal calf serum onto 25 cm^2 flasks. When a cytopathic effect above 50% was observed, some of the flasks were inactivated by exposure to ultraviolet (UV) light, at a distance of 15 cm, for 15 min [48]. The UV light source used was a Phillips TUV 30W G30T8 UV-C light bulb. The absence of residual infectious virus was confirmed by plaque assay and IF assay [49,50]. Culture supernatant from infected UV-inactivated and non UV-inactivated C636 cells as well as the non-infected cells was clarified by centrifugation at 10,000 rpm for 30 min at 4 °C and titrated using BHK21, clone 15 cell line as previously described [46]. The non-infected C636 cells were used as negative control antigen. The presence in the viral preparations of contaminating lipopolysaccharide was evaluated by the Limulus Amebocyte Lysate test (BioWhittaker Inc., Walkersville, MD). All viral preparations used in the present study were lipopolysaccharide-free.

2.3. Blood sampling and isolation of mononuclear cells

Whole blood (10 ml) from all the individuals included in the study was sampled from cubital vein into sodium citrate-containing tubes. Peripheral blood mononuclear cells (PBMC) were obtained by Histopaque-1077 (Sigma–Aldrich, UK) density gradient centrifugation. The tube was centrifuged at 400*g* for 30 at 22 °C. The layer of the mononuclear cells was collected, washed twice with Hanks solution, and finally counted using trypan blue dye. In addition, cell viability was determined (>97%).

2.4. Ex vivo PBMC stimulation

Mononuclear cells were adjusted to 2×10^6 in 1000 μl of RPMI 1640 supplemented with autologous sera or fetal calf serum and were cultured for 24 h at 37 °C in the presence of infective DV1, DV2 and DV3, respectively, at a multiplicity of infection of 0.01 particle forming unit (pfu) per cell. In order to asses whether induction of MIP-1α and MCP-1 was a direct effect of dengue infection, PBMC cells from dengue non-immune donors were also cultured in the presence of UV-inactivated DV1, DV2 and DV3, as well as in the presence of RPMI 1640 culture medium only. Phytohaemagglutinin (PHA), 5 μg/ml, was used as a standard control mitogen [51]. After 24 h of culture, cells were separated from supernatant and frozen at −80 °C.

2.5. Viral load quantification

Viral load, in terms of particle forming unit (pfu), was determined in supernatants of PBMCs challenged with infective DV1, DV2 and DV3, respectively, using TaqMan real time RT-PCR (LightCycler 1.5, Roche Applied Sciences). Standard curves for DV1, DV2 and DV3 were used, following the protocol of Laue et al. [52], with some modifications by Castellanos-Orta et al. [53], (personal communication, Arbovirus Lab., Virology Department, Pedro Kourí Institute).

2.6. Gene expression analysis

DNase-treated total RNA was isolated from stimulated PBMC by means of RNeasy Mini kit (Qiagen, Hilden, Germany) and evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The cDNA was synthesized from mRNA with poly(dT) primers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) and quantified by real-time PCR analysis using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocols. The expression of MIP-1α, MCP-1, IFNγ, TNFα, IL10, IL4, IL5 and the housekeeping gene hypoxanthine phosphoribosyltransferase-1 (HPRT-1) was analyzed in triplicates. Data was expressed in picograms of cytokine mRNA per nanogram of HPRT-1 mRNA using a standard curve for each cytokine.

2.7. Quantification of cytokine proteins by cytometry beads assay (CBA)

The concentrations of the cytokines and chemokines in the supernatants from PBMC cells were measured using multiplex bead arrays, (BD PharMingen, San Diego, CA, USA). In brief, 10 μL of each cytokine was added to prepare the Mixed Human Cytokine Capture Beads. Fifty microliter of the mixed capture beads were mixed with 50 μL of PE Detection Reagent and 50 μL of culture supernatants and incubated for 3 h at room temperature. The beads were then washed with wash buffer and analyzed by FACScalibur (Becton Dickinson, San Jose, CA, USA). The fluorescence intensity measured is proportional to the concentration of the cytokine in the sample, which is quantified from a calibration curve, generated according to the recombinant standards (calibrators). Each assay had a sensitivity range of 0–2500 pg/ml.