# Human peripheral blood mononuclear cells as an *in vitro* model for dengue virus infection

Félix Giovanni Delgado Tiria[1], Adriana Marisol Pérez Acosta[1], Jaime E. Castellanos[1]

## SUMMARY

To date, there are no appropriate animal models for the study of the pathophysiology and clinical manifestations of the disease caused by dengue virus infection; therefore, experimental models are required for that purpose. The objective of the present work was to establish a model of *in vitro* infection with DENV-2. To this end, human peripheral blood mononuclear cells (PBMC) were obtained using a Ficoll gradient, and infected with DENV-2 using a low multiplicity of infection. The cell populations infected and responsible for the production of cytokines were identified using a multiparametric analysis by flow cytometry. As a result, PBMC were permissive to infection that was detected 24 hours after virus inoculation. Additionally, at this same time, CD14+ cells, but not CD3+ or CD19+ cells, were preferentially infected and responsible for the production of TNF-α and IL-6. In conclusion, we established a model of *in vitro* infection using unfractionated PBMC, in which CD14+ cells were identified as the primary target cells for infection with DENV-2, and the production of proinflammatory cytokines.

## KEY WORDS

*Dengue Virus; Flow Cytometry; IL-6; Mononuclear Cells; TNF-α*

## RESUMEN

**Descripción de un modelo de infección *in vitro* con virus dengue empleando células mononucleares humanas de sangre periférica**

No existen modelos animales apropiados para el estudio de la fisiopatología y las manifestaciones clínicas de la enfermedad causada por la infección con virus dengue, por lo que para ello se requiere desarrollar modelos experimentales. El propósito del presente trabajo fue establecer un modelo de infección *in vitro* con virus dengue serotipo-2 (DENV-2). Para esto se obtuvieron células mononucleares de sangre periférica (CMSP) usando un gradiente de Ficoll, y se las cultivó e infectó con DENV-2 a una baja multiplicidad de infección. La subpoblación celular que se infectó y produjo citocinas se identificó empleando un análisis multiparamétrico por

[1] Grupo de Virología, Universidad del Bosque, Bogotá, Colombia.
Corresponding author: Félix Giovanni Delgado Tiria; delgadofelix@unbosque.edu.co

Received: August 9, 2013
Accepted: October 11, 2013

citometría de flujo. Como resultado, las CMSP fueron permisivas a la infección, que se detectó a las 24 horas de inoculado el virus. Además, en este mismo tiempo, los monocitos CD14+, pero no los linfocitos CD3+ o CD19+, fueron la subpoblación celular preferencialmente infectada y responsable de la producción de TNF-α e IL-6. En conclusión, se estableció un modelo de infección *in vitro* usando CMSP no fraccionadas, en el que se identificó a los monocitos CD14+ como la principal célula blanco de la infección con DENV-2 y productora de citocinas proinflamatorias.

## PALABRAS CLAVE

*Células Mononucleares; Citometría de Flujo; IL-6; TNF-α; Virus Dengue*

## INTRODUCTION

Dengue virus (DENV) is a member of the *Flavivirus* genus and the *Flaviviridae* family, and it has four different serotypes, named DENV 1 to 4. This virus is transmitted by bites from the female *Aedes aegypti* mosquito in a man-mosquito-man cycle (1). The virus genome is a positive single stranded RNA of approximately 11 kb in length. The genome codes for one precursor polyprotein, which is cleaved into ten proteins: three structural proteins, C (capsid), M (membrane, synthesized as the precursor prM) and the glycoprotein E (envelope); and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5), which have a role in virus replication (2).

Several details about DENV infection remain unclear, including the extent of the susceptibility of different cells to DENV infection *in vivo* and which tissues are the primary sites of viral replication. It has been suggested that viral replication starts in the mosquito bite site, possibly in the skin Langerhans cells (3,4), and then spreads out to reach the regional lymphatic nodes, spleen and liver using both the lymphatic and the blood pathways (5,6). Data obtained from experimentally infected Rhesus monkeys and histopathological studies in fatal human cases noted that *in vivo* DENV replication occurs in phagocytic-monocytic lineage cells (7-10). Some studies have tried to detect the viral antigen or RNA in peripheral blood mononuclear cells (PBMCs) as an easy way to confirm the infection or evaluate the cell response.

In patients with acute infection, DENV is detected more frequently in the PBMCs (11,12), and there is a direct relationship between the severity and number of antigen-positive cells (11). Additionally, both negative- and positive-sense RNA have been detected in PBMCs of patients, revealing active viral replication in these cells (13,14). Furthermore, *ex vivo* DENV infection of PBMCs has been detected by plaque assay, with a virus replication peak at 48 h post-infection (15).

Despite efforts to establish an appropriate animal model, there are many difficulties in studying the *in vivo* pathogenesis, clinical manifestations and testing of antiviral drugs for DENV infection. For instance, the virus can replicate in the *Cercopithecus* family of monkeys, but at very low titers, and the monkeys do not exhibit clinical symptoms of dengue infection (16). The aim of this work was to establish an *in vitro* dengue infection model to evaluate drugs intended to either control viral replication or regulate the immune response induced by the infection.

## METHODOLOGY

**Cell lines and virus.** The epithelial *Rhesus* monkey kidney cell (LLC-MK2) and the human pro-monocytic cell line U-937, obtained from a diffuse histiocytic lymphoma, were used. Both cell lines were maintained with pyrogen-free Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in a 5% $CO_2$ atmosphere. The mosquito *Aedes albopictus* cell line C6/36 was maintained under the same conditions, but at 33 °C. The dengue virus serotype 2 was kindly donated by Jairo A. Mendez from the Colombian Instituto Nacional de Salud and harvested from C6/36 cells 6 days post-infection. After freezing and thawing of the monolayer, the culture medium was centrifuged, aliquoted and stored at -80 °C until use. Additionally, the mock inoculum was prepared from uninfected and lysed C6/36 cells. The viral titer was obtained by inoculating serial dilutions of viral stock on LLC-MK2 monolayers, which were covered with carboxymethylcellulose to reveal the infected cells for focusing and counting, following the protocol described elsewhere (17).

**Peripheral blood mononuclear cell (PBMC) purification.** Blood from healthy donors who previously

authorized the use of the cells was the source of PBMCs. The study was approved by the Universidad El Bosque Ethics Committee. All the sera were previously tested and shown to be negative for anti-dengue IgM and IgG antibodies using Dengue Duo Cassette (Panbio Diagnostics). PBMCs were isolated by using a Ficoll density gradient centrifugation (Histopaque-1077, Sigma) and cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS and antibiotics. To avoid adhesion, $1x10^6$ cells/mL were maintained in 15 mL blue-capped polypropylene tubes (Becton Dickinson) in a 5% $CO_2$ incubator at 37 °C using pyrogen-free certified reagents (endotoxin level below 0.3 EU/mL).

**PBMC immunocompetence.** PBMC immunocompetence was evaluated by adding 5 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma) and 500 ng/mL ionomycin (calcium salt ionomycin, Sigma) as a stimulus to induce lymphocyte activation and 50 ng/mL *E. coli* lipopolysaccharide to activate the monocytes. The stimulus was added simultaneously with 3 mg/mL Brefeldin (eBioscience). Following 12 hours of stimulation, $5x10^5$ cells were recovered and stained to detect intracellular IL-6 and TNF-α cytokines.

**PBMC infection with DENV.** A low multiplicity of infection (MOI=0.1) was used to simulate natural infection. Negative control cells were treated with uninfected C6/36 cell lysate (mock). Time points of 12 and 24 hours post-infection (h.p.i.) were used for different experiments. For detecting intracellular cytokines, 3 mg/mL Brefeldin was added 12 hours before cell collection. The cells were stained to evaluate the phenotype (CD14, CD3 and CD19), viral antigen or intracellular cytokines (IL-6 and TNF-α). The 24 h.p.i. PBMC supernatants were used to infect LLC-MK2 cells. Replication of virus in the LLC-MK2 cells was detected by peroxidase immunocytochemistry. For dengue virus detection by flow cytometry, both infected C6/36 and U-937 cells were used as positive controls.

**Dengue RNA detection by polymerase chain reaction.** Whole RNA was isolated using Trizol (Invitrogen) following the manufacturer's instructions. RNA was reverse transcribed using 0.5-1 mg RNA and the MMLV enzyme (Promega) with random hexanucleotides. The cDNA was amplified with GoTaq polymerase (Promega) and the primers mD1 5´-TCA ATA TGC TGA AAC GCG AGA GAA ACC G-3´ and D2 5´-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3 to obtain a 511 bp product corresponding with a fragment of the C-prM viral genes, as described by Chien and colleagues (18).

**PBMC flow cytometry.** Infected cells were stained with an indirect staining protocol using 10 mg/mL of a flavivirus monoclonal antibody (Chemicon, MAB8744, IgG2a mouse isotype) and a FITC-coupled secondary anti-IgG2a antibody (8 mg/mL, Becton Dickinson). The following antibodies are IgG1 isotype: anti-CD3 Pacific blue (8 mg/mL, Becton Dickinson), anti-CD19 APC-Cy7 (5 mg/mL, Becton Dickinson) and anti-CD14 PerCP-Cy5.5 (8 mg/mL, eBioscience). Anti-TNF-α APC (5 mg/mL, Becton Dickinson) and IL-6 PE (5 mg/mL, eBioscience) were used to detect intracellular cytokines.

For phenotype evaluation, $5x10^5$ cells were recovered by centrifugation and resuspended in FACSFlow (Becton Dickinson) and stained with a viability stain (LIVE/DEAD, Fixable Aqua Dead Cell stain, Invitrogen) and phenotype antibodies simultaneously for 30 min at 4 °C before treatment with the Cytofix/Cytoperm reagent (Becton Dickinson) for 20 min and then washed with PermWash (Becton Dickinson). For the detection of dengue virus, cells were incubated with anti-flavivirus antibody for 1 hour at 37 °C and the secondary FITC antibody for 30 min at 4 °C. In an independent set of cells, the antibodies against cytokines were incubated for 30 min at 4 °C, then washed with PermWash three times and collected for analysis on a FACSCanto II flow cytometer (Becton Dickinson) using FACSDiva software.

## RESULTS

### CD14+ monocytes are the only cells within the PBMCs permissive to dengue infection

In this model, we used non-fractionated PBMCs and a low MOI (0.1) to simulate natural infection. PBMC infection was confirmed by RT-PCR (figure 1A) and the presence of infectious virions in the supernatants, which were detected in LLC-MK2 cells by immunoperoxidase staining (figure 1C). Neither viral RNA nor antigens were detected in mock-infected cultures (figures 1A (line 1) and B).

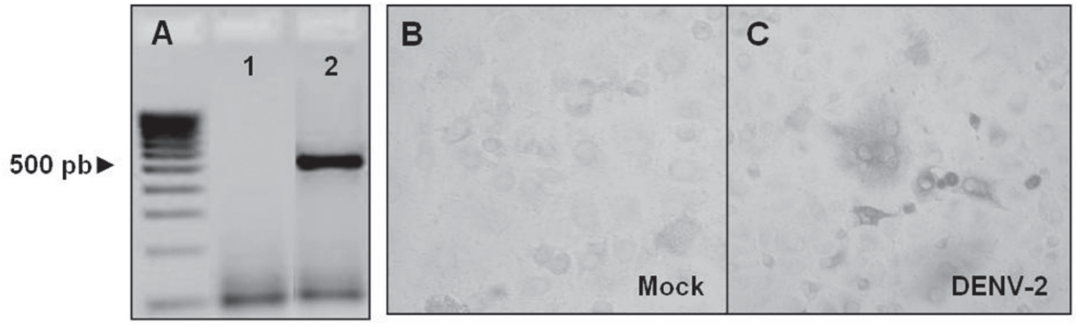


**Figure 1. Peripheral blood mononuclear cells (PBMCs) were susceptible to in vitro infection by dengue virus serotype 2 (DENV-2).** A. Results of a RT-PCR from RNA obtained from PBMCs inoculated with mock (lane 1) or DENV-2 (lane 2). It was detected the characteristic 511 bp amplicon from the C-prM segment in infected cells. B and C. Peroxidase immunostaining of LLM-CK2 cells inoculated with supernatants of DENV-2 infected PBMCs or mock infected

Prior to analysis of the PBMC subpopulation infected by DENV, we standardized the use of an indirect fluorescence assay for DENV detection by flow cytometry using mosquito C6/36 and human U-937 monocytic cell lines. A high percentage (74.8%) of mosquito cells were infected, while 7.3% of monocytic cells were infected (figure 2). In mock-infected cells, only 0.2% of cells had non-specific staining. After setting the parameters for virus detection by flow cytometry, we applied the following gating strategy in the PBMCs (figure 3): i) we first selected the "singlets," the individual cells passing in front of the laser beam, which have a uniform forward scatter area (FCS-A) and width (FCS-W), discarding the cell clusters; ii) in these singlets, we identified the lymphocyte and monocyte subpopulations according to size (forward scatter) and complexity (side scatter); iii) in these two subpopulations, we identified non-viable cells using cytoplasmic staining that reacts with the amine group in cells with disrupted membranes, and we selected the cells with low fluorescence for this staining; and iv) on the viable lymphocytes, we detected the T-lymphocytes using CD3 expression, and on the CD3-negative group, we identified the B-lymphocyte population using CD19 expression. Monocytes were identified as CD14+. This strategy allowed us to identify all of the target subpopulations (CD3+, CD19+, CD14+) for the evaluation of both DENV infection and cytokine expression.

After the analysis, we found that the CD14+ monocyte subpopulation had a significantly higher percentage of DENV+ cells (2.2% vs. 0.5% in mock-infected cells). In contrast, the T-lymphocytes (CD3+) or B-lymphocytes (CD19+) did not show evidence of DENV infection (figure 4).

## PBMC infection with DENV induced TNF-α and IL-6 in CD14+ monocytes

The immunocompetence of each volunteer's PBMCs in culture was initially confirmed by stimulating the cells with PMA/ionomycin (for T and B lymphocytes) or LPS (for monocytes) and evaluating the intracellular cytokine expression (figure 5). Approximately 1.4% and 1.3% of PMA/ionomycin-stimulated lymphocytes produced TNF-α and IL-6, respectively (figure 5B and G). LPS stimulation induced TNF-α and IL-6 in 45% and 30.9% of the CD14+ monocytes, respectively (5D and I), confirming the ability of PBMCs to be activated.

Cytokine production was then evaluated in the PBMCs inoculated with DENV-2 or mock supernatant for 12 or 24 hours using flow cytometry. The CD14+ monocytes were the only cell subset that produced TNF-α and IL-6; for example, at 24 h p.i., 7.5% and 35% of CD14+ cells produced TNF-α and IL-6 respectively (figure 6), while these molecules were not detected in T or B lymphocytes (data no shown).

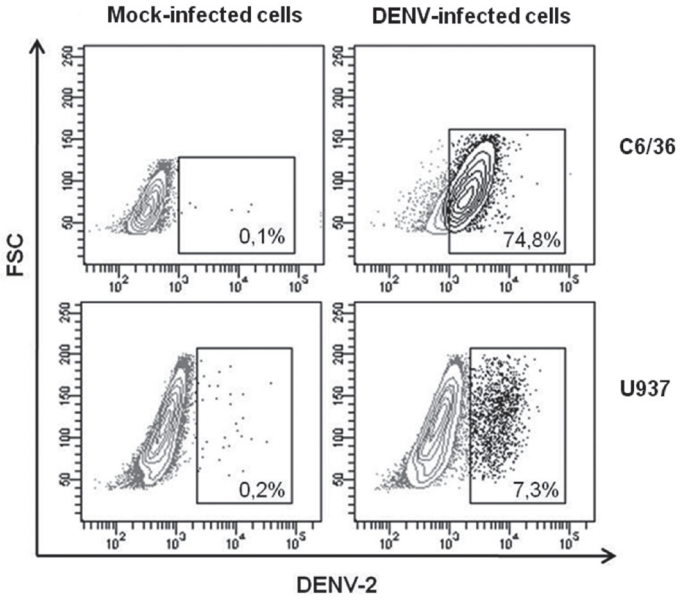


**Figure 2. DENV-2 viral antigen was detected by indirect flow cytometry in the infection positive control cells.** C6/36 mosquito cells and U937 human macrophages were infected by 5 and 2 days, respectively. Mosquito cells are more permissive to DENV-2 infection (74.8%) than U937 cells (7.3%). Less than 0.2% of positive cells were detected in mock infected cells. This graph is representative of three independent experiments

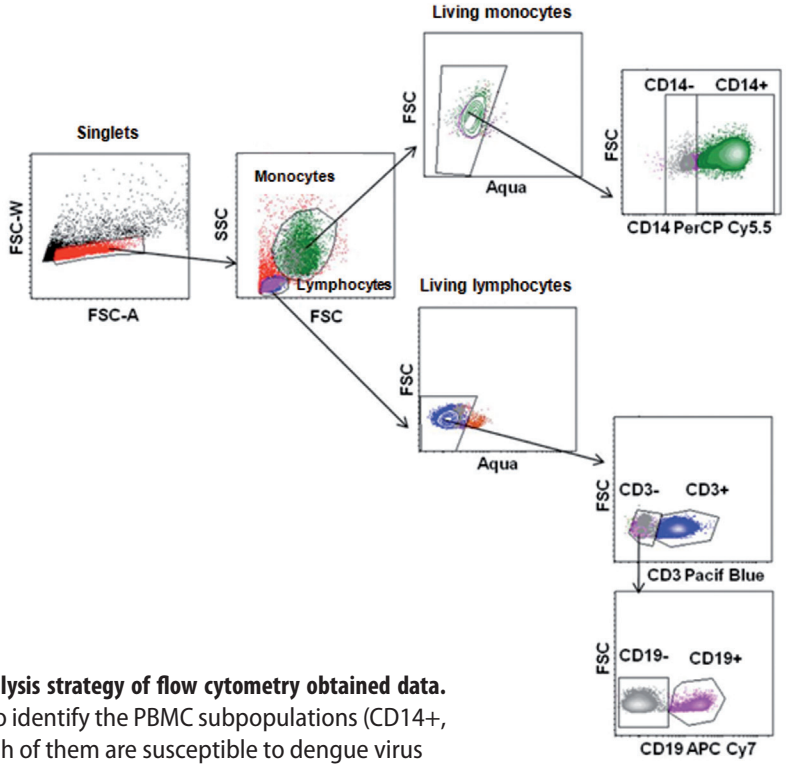


**Figure 3. Chart of the analysis strategy of flow cytometry obtained data.** This strategy allows us to identify the PBMC subpopulations (CD14+, CD3+, CD19+) and which of them are susceptible to dengue virus

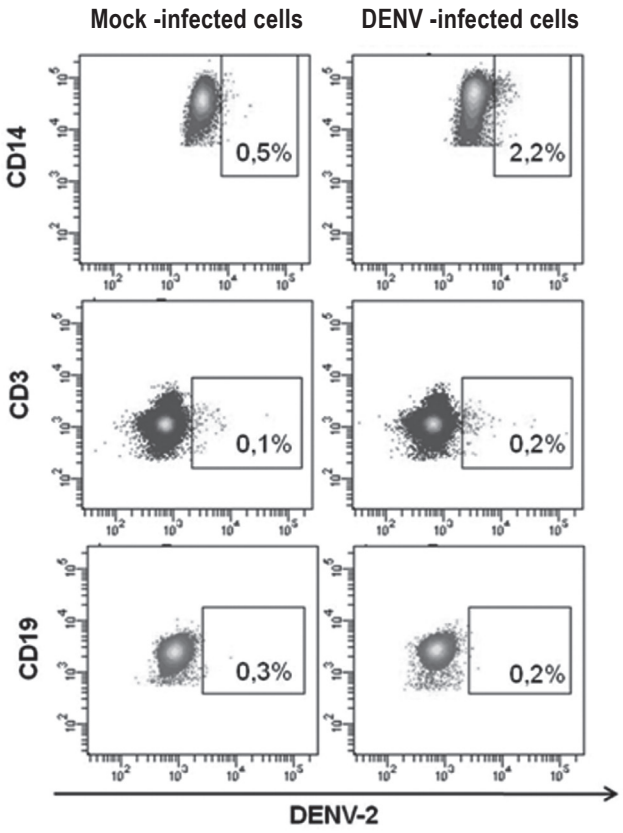


**Figure 4. Monocyte cells were susceptible to DENV-2 infection.** Only the CD14+ cells were susceptible to DENV-2 infection (2.2% positive cells vs. 0.5% in mock infected cells). A low DENV-2 inoculum was used (MOI=0.1) for 24 hours. There was no viral antigen staining in T or B lymphocytes. This graph is representative of three independent experiments

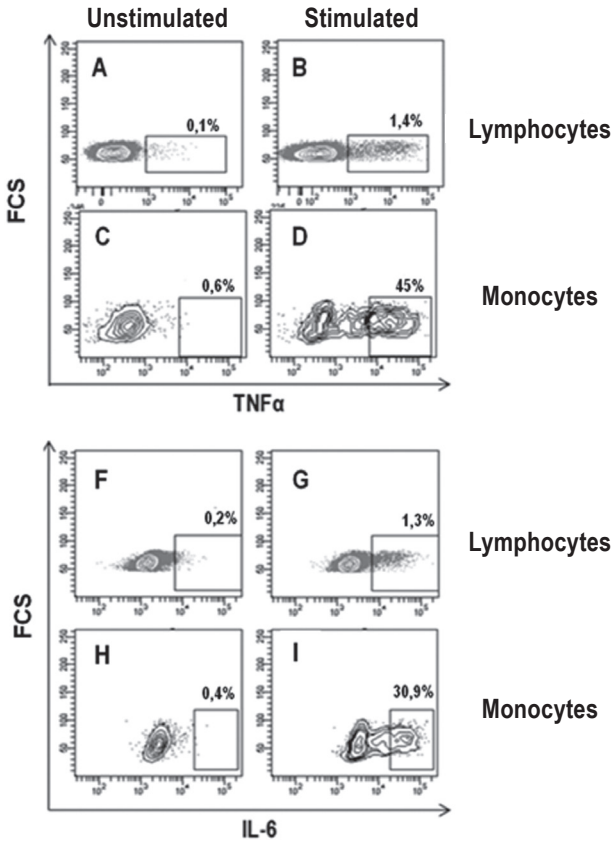


**Figure 5. Immunocompetence of PBMCs.** The PBMCs were stimulated with PMA/Ionomycin (to lymphocytes) and LPS (to monocytes) and treated with brefeldin, then stained to intracellular cytokines: TNF-α (A-D panels) and IL-6 (F-I panels). Data are representative of three independent experiments

## DISCUSSION

In this study we established an *in vitro* DENV infection model to identify the main PBMC subset that is susceptible to infection as well as the cell type responsible for cytokine production. We used non-fractionated PBMCs and a low MOI, and we cultured the cells in polypropylene tubes to maintain them in suspension, resulting in better recovery for cytometric analysis (19). This methodology has previously been used to study monocyte biology (19-21). To avoid the neutralizing or enhancing effect in pre-immune cells (22, 23), the blood donors were negative for dengue antibodies. PBMCs were infected for 24 hours because it is known that this time is needed for the first round of replication (24), and in longer infections, such as 48 h, monocytes express apoptotic markers, such as phosphatidylserine and Fas receptor (25). Most of the cells were viable after 24 h p.i., as only 4.1% and 3.4% of them were positive with the Aqua stain in the mock and infected cell groups, respectively.

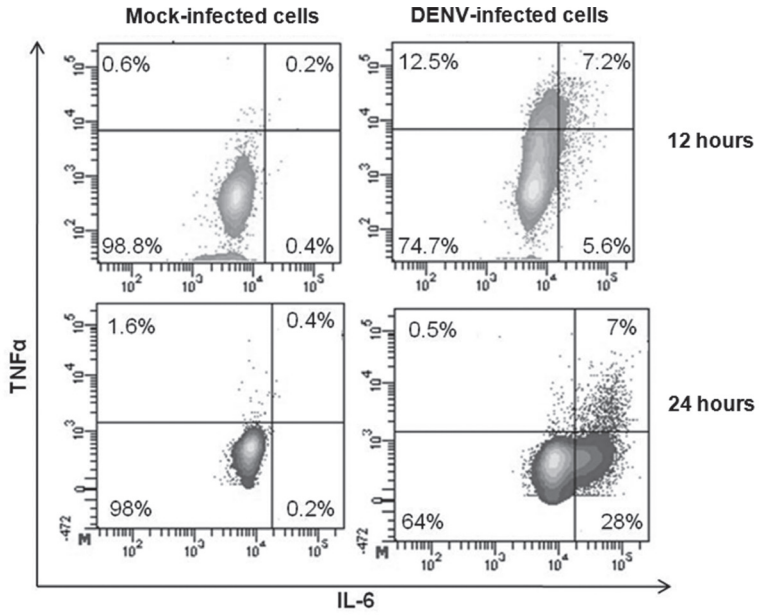


**Figure 6. Monocytes CD14+ were responsible for cytokine production during the *in vitro* DENV-2 infection.** PBMCs were infected by 12 or 24 hours and then stained to detect intracellular TNF-α and IL-6 in the different cell subsets (CD14+, CD3+ and CD19+). Data representative of three independent experiments

Keeping in mind that there are no anti-dengue antibodies coupled with fluorochromes, we used indirect fluorescence to detect dengue infection. We used two cell lines with different permissiveness, the highly susceptible mosquito cell line C6/36 (24) and the low-susceptibility human pro-monocyte cell line U-937 (26), which were infected at high (74.8%) and low (7.3%) proportions, respectively, while the mock-infected control cells were only 0.1% positive. Our multiparametric analysis strategy allowed us to simultaneously detect individual viable cells, improving the sensitivity and accuracy of the data (27). In light of the above, we observed that among the identified monocytes, B and T lymphocytes, only the CD14+ cells were infected, as reported in previous studies (6, 28). The percentage of infected monocytes, 2.2%, was similar to the 1 to 5% reported by Wu and colleagues (3) using an MOI of 2 and the 3.5% reported by Kou et al. using an MOI of 5 (6).

Primary human cells are infected at a lower level than cell lines, and the kinetics of infection depend on the viral strain or the MOI used (29). In naturally infected patients, the CD14+ monocytes were the only identified cell subset positive for DENV using flow cytometry (30,31). In contrast, some studies suggest that DENV could replicate in both T and B lymphocytes and that these white cells could play an important role in dengue pathogenesis (32-34), although we did not find CD3+ or CD19+ cells that were positive for DENV antigen.

The most important characteristic of severe dengue infection is the damage to the endothelial function mediated by an aberrant immune response (35). The sera of severely infected patients have significantly elevated TNF-α and IL-6 levels (36-39). These proinflammatory cytokines are associated with the acute phase response, which results in the release of chemotactic peptides, fever and endothelial cell activation, leading to an increase in vascular permeability, the dominant sign of severe dengue (40). It has been shown that DENV-2 infected B lymphocytes and monocytes are the main source of TNF-α and IL-6 cytokines; even

T CD4+ lymphocytes exposed to DENV-2 antigens *in vitro* also produce these immune mediators (41). The present study demonstrates that only the monocytes were infected by DENV-2 and that they were the only cell subset that produced TNF-α and IL-6. These results are consistent with those from natural infections, where a high percentage of severely dengue-infected patient monocytes are activated (31).

In conclusion, in this study we established an *in vitro* model using non-fractioned PBMCs and a very low MOI to simulate natural infection. Despite the low quantity of challenging virus, we detected the infection in monocytes, which were activated to produce proinflammatory cytokines that may be related with the characteristic immunopathogenesis of the dengue virus infection.

## CONFLICT OF INTERESTS

The authors declare that no conflicting financial interests exist.

## ACKNOWLEDGMENTS

The authors sincerely thank the Fundación Hematológica Colombia and all individuals who participated in this study for their generosity. This work was supported by Universidad El Bosque and Instituto Colombiano para el Fomento de la Ciencia y la Tecnología (Colciencias, Grant 1308-493-26111).

## REFERENCES

1. Velandia ML, Castellanos JE. Virus del dengue: estructura y ciclo viral. Infectio. 2011; 15:33-43.
2. Henchal E, Putnak J. The Dengue viruses. Clin Microbiol Rev. 1990; 3: 376-96.
3. Wu S, Grouard-Vogel G, Sun W, Mascola J, Brachtel E, Putvatana R, et al. Human skin Langerhans cells are targets of dengue virus infection. Nature Med. 2000; 6: 816-20.
4. Tassaneetrithep B, Burgess T, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W, et al. DC-SIGN (CD209) Mediates Dengue Virus Infection of Human Dendritic Cells. J Exp Med. 2003; 197: 823–9.
5. Jessie K, Fong M, Devi S, Lam S, Wong K. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis. 2004; 189: 1411–18.
6. Kou Z, Quinn M, Chen H, Rodrigo W, Rose R, Schlesinger J, et al. Monocytes, But Not T or B Cells, Are the Principal Target Cells for Dengue Virus (DV) Infection Among Human Peripheral Blood Mononuclear Cells. J Med Virol. 2008; 80:134–46.
7. Halstead SB, O'Rourke EJ. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by non-neutralizing antibody. J Exp Med. 1977; 146: 201–17.
8. Bhamarapravati N, Tuchinda P, Boonyapaknavik V. Pathology of Thailand hemorrhagic fever: A study of 100 autopsy cases. Ann Trop Med Parasitol. 1967; 61: 500-10.
9. Marchette N, Halstead SB, Falkler WA, Nash D. Studies on the pathogenesis of dengue infection in monkeys. III. Sequential distribution of virus in primary and heterologous infections. J Infect Dis. 1973; 128: 23–30.
10. Morens D, Marchette N, Chu M, Halstead SB. Growth of dengue type 2 virus isolates in human peripheral blood leukocytes correlates with severe and mild dengue disease. Am J Trop Med Hyg. 1991; 45: 644–51.
11. Kittigul L, Meethien N, Sujirarat D, Kittigul C, Vasanavat S. Comparison of dengue virus antigens in sera and peripheral blood mononuclear cells from dengue infected patients. Asian Pac J Allergy Immunol. 1997; 15: 187–91.
12. Fung C, Lee Y, Kuo B, Yang S, ChanY , Liu C, et al,. Using buffy coat for reverse transcriptase-polymerase chain reaction in the diagnosis of dengue virus infection: preliminary study. J Microbiol Immunol Infect. 2000; 33: 217-22.
13. Wang W, Lee C, Kao C, Lin Y, and King C. Quantitative competitive reverse transcription-PCR for quantification of dengue virus RNA. J Clin Microbiol. 2000; 38: 3306-10.
14. Wang W, Sung T, Tsai Y, Kao C, Chang S, King C. Detection of Dengue Virus Replication in Peripheral Blood Mononuclear Cells from Dengue Virus Type 2-Infected Patients by a Reverse Transcription–Real-Time PCR Assay. J Clin Microb. 2002; 40: 4472–78.
15. Rabablert J, Wasi C, Kinney R, Kasisith J, Pitidhammabhorn D, Ubol S. Attenuating characteristics of DENV-2 PDK53 in flavivirus-naïve peripheral blood mononuclear cells. Vaccine. 2007; 25: 3896-905.

16. Bente DA, Rico-Hesse R. Models of dengue virus infection. Drug Discov Today Dis Models. 2006;3:97-103.

17. Velandia-Romero ML, Acosta-Losada O, Castellanos JE. In vivo infection by a neuroinvasive neurovirulent dengue virus. J Neurovirol. 2012;18:374-87.

18. Chien LJ, Liao TL, Shu PY, Huang JH, Gubler DJ, Chang GJ. Development of real-time reverse transcriptase PCR assays to detect and serotype dengue viruses. J Clin Microbiol. 2006;44:1295-304.

19. Baran J, Kowalczyk D, Ozog M, Zembala M. Three-Color Flow Cytometry Detection of Intracellular Cytokines in Peripheral Blood Mononuclear Cells: Comparative Analysis of Phorbol Myristate Acetate-Ionomycin and Phytohemagglutinin Stimulation. Clin Diagn Lab Immunol. 2001; 8:303-13.

20. Van Der Bruggen T, Nijenhuis S, Van Raaij E, Verhoef J, Van Asbeck B. Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Production by Human Monocytes Involves the Raf-1/MEK1-MEK2/ERK1-ERK2 Pathway. Infect Immun. 1999;67: 3824–29.

21. Soga F, Katoh N, Inoue T, Kishimoto S. Serotonin Activates Human Monocytes and Prevents Apoptosis. J Invest Dermatol. 2007; 127: 1947–55.

22. Van der Schaar H, Wilschut J, Smit J. Role of antibodies in controlling dengue virus infection. Immunobiology. 2009; 214: 613-29.

23. Chareonsirisuthigul T, Kalayanarooj S, Ubol S. Dengue virus antibody dependent enhancement of infection upregulates the production of anti-inflamatory cytokines, but suppresses anti-dengue virus free radical and pro-inflammatory cytokines production, in THP-1 cells. J Gen Virol. 2007; 88: 365-75.

24. Lambeth C, White L, Johnston R, Silva A. Flow Cytometry-Based Assay for Titrating Dengue Virus. J Clin Microbiol. 2005; 43: 3267–72.

25. Torrentes-Carvalho A, Azeredo E, Reis S, Miranda A, Gandini M, Kubelka C. Dengue-2 infection and the induction of apoptosis in human primary monocytes. Mem Inst Oswaldo Cruz. 2009; 104: 1091-99.

26. Diamond M, Edgil D, Roberts G, Lu B, Harris E. Infection of Human Cells by Dengue Virus Is Modulated by Different Cell Types and Viral Strains. J Virol. 2000; 74: 7814-23.

27. Mahnke Y, Roederer M. Optimizing a Multi-colour Immunophenotyping Assay. Clin Lab Med. 2007; sep. 27: 469-85–v.

28. Sydow F, Santiago M, Neves-Souza P, Cerqueira D, Gouvea A, Lavatori M, et al. Comparison of Dengue Infection in Human Mononuclear Leukocytes with Mosquito C6/36 and Mammalian Vero Cells Using Flow Cytometry to Detect Virus Antigen. Mem Ins Oswaldo Cruz. 2000; 95: 483-89.

29. Cologna R, Rico-Hesse R. American genotype structures decrease dengue virus output from human monocytes and dendritic cells. J Virol. 2003; 77: 3929-38.

30. Neves-Souza P, Azeredo E, Zagne S, Valls-de-Souza R, Reis S, Cerqueira D, et al. Inducible nitric oxide synthase (iNOS) expression in monocytes during acute Dengue Fever in patients and during in vitro infection. BMC Infect Dis. 2005; 5: 64.

31. Durbin A, Vargas M, Wanionek K, Hammond S, Gordon A, Rocha C, et al. Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. Virology. 2008; 376:429-35.

32. King A, Nisalak A, Kalayanrooj S, Myint K, Pattanapanyasat K, Nimmannitya S, et al. B cells are the principal circulating mononuclear cells infected by dengue virus. Southeast Asian J Trop Med Public Health. 1999; 30: 718-28.

33. Lin Y, Wang K, Lei H, Lin Y, Yeh T, Liu H, et al. Virus replication and cytokine production in dengue virus-infected human B lymphocytes. J Virol. 2002; 76: 12242-49.

34. Mentor NA, Kurane I. Dengue virus infection of human T lymphocytes. Acta Virol. 1997; 41: 175-6.

35. Rodenhuis-Zybert I, Wilschut J, Smit J. Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci. 2010; 67: 2773-86.

36. Chaturvedi U, Raghupathy R, Pacsa A, Elbishbishi E, Agarwal R, Nagar R, et al. Shift from a Th1-type response to Th2-Type in dengue haemorrhagic fever. Curr Sci. 1999; 76: 63-9.

37. Pinto L, Oliveira S, Braga E, Nogueira R, Kubelka C. Increased pro-inflammatory cytokines (TNFα and IL-6) and anti-inflammatory compounds (sTNFRp75 and sTNFRp55) in Brazilian patients during exanthematic dengue fever. Mem Inst Oswaldo Cruz. 1999; 94: 1-8.

38. Bethell DB, Flobbe K, Cao XT, Day NP, Pham TP, Buurman WA, et al. Pathophysiologic and prognostic role

of cytokines in dengue hemorrhagic fever. J Infect Dis. 1998; 177: 778-82.

39. Houghton-Triviño N, Martín K, Giaya K, Rodríguez JA, Bosch I, Castellanos JE. Comparison of the transcriptional profiles of patients with dengue fever and dengue hemorrhagic fever reveals differences in the immune response and clues in immunopathogenesis. Biomedica. 2010; 30:587-97.

40. Anderson R, Wang S, Osiowy C, Issekutz A. Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. J Virol. 1997; 71: 4226–32.

41. Gagnon S, Ennis F, Rothman A. Bystander target cell lysis and cytokine production by dengue virus-specific human CD4+ cytotoxic T lymphocyte clones. J Virol. 1999; 73: 3623-29.