Dengue: a trilogy of people, mosquitoes and the virus. Current epidemiology and pathogenesis in (non-)endemic settings
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HIGH INCIDENCE OF PERIPHERAL BLOOD PLASMACYTOSIS IN PATIENTS WITH DENGUE VIRUS INFECTION: A PROSPECTIVE STUDY

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**Background:** Polyclonal peripheral blood plasmacytosis has occasionally been described in dengue virus (DENV) infected patients. We initiated this prospective observational study to quantify and describe the kinetics and phenotype of peripheral blood plasma cells (PCs) in these patients.

**Methods:** Morphological examination and flow cytometric (FC) analysis for the characterization and immunophenotyping of lymphocyte subsets and PCs were performed in 35 and 31 patients suspected of DENV infection, respectively.

**Results:** Our results show that blood plasmacytosis is a very common haematological finding. Depending on the days of illness at presentation, blood plasmacytosis was observed in 64% to 73% of patients. Blood plasmacytosis was the most pronounced before 7 days of illness and declined rapidly thereafter, to completely disappear after 14 days of illness. Blood plasmacytosis was higher in secondary DENV infection. The majority of CD138⁺ PCs (89%) had a shared immunophenotype (CD45⁻/CD19⁻/CD56⁻) and in all cases the PCs were polyclonal.

**Conclusions:** Blood plasmacytosis, characterized by a transient presence of polyclonal PCs in the circulation, is a common event in DENV infection. Blood PCs may play a role in the humoral immune response to and pathogenesis of dengue.

### 1. INTRODUCTION

Dengue is the infectious disease which is caused by dengue virus (DENV). DENV transmission primarily takes place through bites by the mosquito vectors *Aedes aegypti* and *Aedes albopictus*.\(^{112,244}\) DENV is a single-stranded, positive-sense RNA virus. There are four DENV serotypes which, based on antigenic and genetic differences could be regarded as four distinct virus species (DENV1-4). All serotype are capable of infecting and replicating in numerous human cells, including dendritic cells, monocytes/macrophages, B cells, T cells, endothelial cells, hepatocytes and neuronal cells in vivo. Although a consensus that the mononuclear phagocyte lineage cells (monocytes and macrophages) constitute the primary targets for replication based on clinical and autopsy studies, there is still controversy about the primary target cell type(s) targeted in humans.\(^{137}\)

Infection by one of the four DENV serotypes cannot be distinguished on clinical grounds; all serotypes can cause a spectrum of clinical manifestations that varies from mild undifferentiated febrile illness to severe dengue, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) of which the latter is the most life threatening.\(^{309}\) The pathogenesis of severe DENV infection is poorly understood. One of the characteristic features of dengue is the occurrence of leucopenia and thrombocytopenia, probably resulting from virus induced bone marrow suppression.\(^{166}\) This is non-specific and not exclusively seen in dengue. Thrombocytopenia may worsen as a result of coagulopathy and vasculopathy in patients with severe disease. Despite the general bone marrow suppression, blood plasmacytosis has been reported in a few patients with DENV infection.\(^{21,75}\) However, the frequency of blood plasmacytosis in patients with dengue infection, the origin of these plasma cells (PCs) and the mechanisms by which they appear in the blood are not known.

Blood plasmacytosis is an unusual haematological finding that is most commonly seen in plasma cell leukaemia or advanced stage multiple myeloma, in which case the plasma cells are part of the malignant clone and thus are monoclonal. Non-malignant reactive peripheral plasmacytosis is occasionally found in a variety of diseases, such as tumours, autoimmune disorders, and infectious diseases including sepsis, primary infection and reactivation of Epstein-Barr virus, acute respiratory infections, parvovirus B19 infection, rubella and hepatitis virus A infection.\(^{136,148,150,214,223,231}\)

In this study, we prospectively quantified and described blood plasmacytosis in returned travellers with DENV infection. We also characterized the immunological phenotype of these PCs by flow cytometry and explored associations with viral load, the appearance of anti-DENV IgG antibodies, and the presence of primary or secondary DENV infection.

### 2. MATERIALS AND METHODS

#### 2.1 STUDY SITE AND STUDY POPULATION

Returned travellers presenting with a history of less than 14 days of fever, clinically suspected of dengue, were included at the Department of Tropical Diseases, Academic Medical Center, Amsterdam, the Netherlands. Blood samples were collected from each patient for routine diagnostic procedures. All samples were tested by enzyme-linked immunosorbent assay (ELISA) for anti-DENV IgM and IgG antibodies and DENV real time reverse transcriptase polymerase chain reaction (RT-PCR). Morphological examination and flow cytometric (FC) analysis of blood were performed within 24 hours after the blood was drawn. All patients at this department are routinely informed that their data can be used for observational studies. All patients consented to participate in this study.

#### 2.2 DENGUE DIAGNOSTICS

Serum samples were routinely tested for dengue antibodies with direct IgG enzyme-linked immunosorbent assay (ELISA) and IgM-Capture ELISA (Pambio Tech Co, Brisbane, Australia).

For real time RT-PCR, RNA was isolated from blood plasma as described elsewhere.\(^{29}\) RNA was reverse transcribed and amplified using an internally controlled and quantitative serotype-specific, TaqMan-based assay as described elsewhere.\(^{150}\) Quantitative results were expressed as cDNA equivalents per ml. The RT-PCR and ELISA results were used for diagnosis and classification of dengue infection.
2.3 CLASSIFICATION OF PRIMARY AND SECONDARY DENGUE

Acute primary DENV infections were confirmed by RNA detection and/or detection of dengue specific IgM antibodies in acute samples, in the absence of dengue serum specific IgG antibodies. A negative first acute sample for dengue serum specific IgM antibodies with seroconversion for IgM antibodies within 7 to 14 days was also considered as primary dengue. Acute secondary DENV infections were confirmed on acute samples (less than 14 days) with positive IgG results and detection of viral RNA, either with or without detectable dengue serum specific IgM antibodies.

For patients who presented with both positive IgM and IgG results in the first acute sample, but without detectable RNA by RT-PCR, the ratio between the IgM and IgG concentrations was used to distinguish primary and secondary infections. A ratio of anti-dengue IgM to IgG of greater than or equal to 1.8 was the criterion for primary dengue infection; values lower than 1.8 were considered to indicate secondary infections. The patients for whom the diagnosis DENV infection was not confirmed were grouped as other febrile illness (OFI) and included in this study as controls.

2.4 MORPHOLOGICAL EXAMINATION

A complete blood count with differential was performed on an automated cell counter (Sysmex XE-5000, Sysmex Corporation, Kobe, Japan). Blood smears were fixed and stained first with May-Grünwald eosine-methylene blue and then after rinsing with Giemsa’s azur eosine methylene blue solution (Merck). 100 white blood cells per slide were scored by one experienced technician blinded for disease outcome with a binocular microscope at x100 magnification. Lymphocytes were scored as normal lymphocytes, atypical lymphocytes (with abundant cytoplasm), plasmacytoid lymphocytes or PCs.

2.5 FLOW CYTOMETRY ANALYSIS OF PERIPHERAL BLOOD

Erythrocytes were lysed using ammoniumchloride and washed twice with phosphate-buffered saline. Thereafter, the cell suspension (100 μl) was mixed in tubes with 5 μl of undiluted monoclonal antibodies (mAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), R-phycocerythrin coupled to the cyanine dye Cy5™ (PE Cy5), R-phycocerythrin coupled to the cyanine dye Cy7™ (PE Cy7), allophycocyanine (APC), allophycocyanine coupled to the cyanine dye H7 (APC-H7) and Horizon™ or Anemone Majano cyanine (AmCyan) for the following cell surface markers: CD3, CD5, CD8, CD10, CD14, CD19, CD45, kappa and lambda Lights chains F(ab’)2 (DAKO A/S, Denmark), CD4 (Sanquin, the Netherlands) CD138, CD16/56 (Immuno Quality Products, the Netherlands) in a four-tube assay. After adding the mAbs, the cells were incubated in the dark for 15 minutes at room temperature. Fluorescence intensity was determined with an eight-color FACSCanto™ (BD Biosciences, USA) flow cytometer. Mononuclear cells were gated on the basis of forward and side light-scattering properties and subtyped on the basis of cluster of differentiation (CD) molecule expression. Isotype-matched control mAbs were used to define background fluorescence. On average, 1 x 10^4 events were acquired and analyzed using CellQuest software (BD Biosciences, USA). For demonstration of PCs a lower limit of detection of 0.2 % of cells expressing the surface marker CD138 was applied.

2.6 STATISTICAL ANALYSIS

Statistical calculations were performed using SPSS (version 17.0, SPSS Inc. Illinois). Results were summarized in terms of medians and ranges for continuous data and non-parametric tests were used to compare within groups. For dichotomous variables, Fisher’s exact test was performed. The Mann-Whitney test was used for non-normally distributed variables. A two-tailed p-value of 0.05 was considered to be statistically significant.

3. RESULTS

3.1 STUDY POPULATION

Morphological examination of the blood smear was performed in 35 returned travellers with suspected DENV infection presenting at our hospital between September 2008 and June 2009, of whom FC analysis for immunophenotyping of PCs was done in 31 patients. Demographic information and travel history of the 35 returned travellers is shown in table 1. DENV infection was confirmed in 28 of 35 patients, of whom 15 had primary infection and 13 had secondary infection and seven patients were classified as OFI.
From the 35 patients, 47 blood samples, collected on 0 to 32 days after the onset of symptoms, were morphologically examined. White blood count (WBC) subsets, stratified by days after onset of symptoms are presented in figure 1A. Overall, blood plasmacytosis was demonstrated in 16/28 (57%) returned travellers with confirmed DENV infection. The frequency of plasmacytosis was 73% (11/15) among patients from whom blood was collected during the first 7 days of illness. Among patients from whom a blood sample was collected during the first 14 days of illness (DOI), plasmacytosis occurred in 64% (16/25). Plasmacytosis was not observed in any of the 7 patients with OFI.

### 3.2 KINETICS OF PLASMACYTOSIS

During the first 7 days of illness, the median plasma cell count in DENV patients was 2.5% (25-75% interquartile range (IR): 0 - 8 %) of the total white blood cell count (figure 1A); the median absolute plasma cell concentration was 8.4 x10^7/L (25-75 IR: 0 - 3.5 x 10^7/L). The PC distribution by days of illness, including eight patients with DENV infection for whom sequential samples were available, is shown in figure 1B and D. Figures 1C show a typical example of a PC and plasmacytoid lymphocyte in a patient with secondary DENV infection.

### 3.3 IMMUNOPHENOTYPES OF CD138^-PCS IN DENV INFECTION

The antigen profiles of lymphocytes and plasma cells (CD19, CD16/CD56, CD3, CD4, CD8, CD138) are presented in figure 2 for 25 patients with DENV infection and 6 patients with OFI. The lymphocyte subsets did not differ significantly between patients with DENV infection and patients with OFI, with the exception of PCs, characterized by being CD138^-$. In patients with DENV infection, PCs were detected during 15 days after onset of illness. The PCs were polyclonal in all cases with a mean kappa/lambda ratio of 1.3 (49.3:37.9). Subsequent immunophenotyping of PCs in the 19 patients with plasmacytosis revealed a shared phenotype: in 17/19 cases the majority of the PCs was CD138^-/CD45+/CD19^-/CD56^-$. A linear correlation was observed between the percentage of PCs as assessed by morphology and by flow cytometry (R2 = 0.85).
3.4 ASSOCIATION BETWEEN PLASMACYTOSIS WITH VIRAL LOAD, ANTI-DENV IgG ANTIBODIES, PRIMARY AND SECONDARY DENV INFECTION

Viral RNA was detectable in 18 of 28 DENV patients, of whom 12 patients had primary and 6 had secondary DENV infection. The viral RNA levels ranged from $6.2 \times 10^2$ to $4.7 \times 10^9$/mL in DENV-1 infection and $8.9 \times 10^4$ to $1.6 \times 10^9$/mL in DENV-2 infection. There was no correlation between DENV load and the percentage of blood PCs or with blood PCs concentration (data not shown).

Morphological examination and anti-DENV IgG serology was performed on the same day in 12 samples from 10 patients with secondary DENV infection. Figure 3A shows percentages of PCs by anti-DENV IgG optical density ratios (ODR) stratified by days of illness. The anti-DENV IgG ODR increases beyond five days of illness, which is indicative of an increase of anti-DENV IgG concentration in serum. A negative correlation was observed between IgG ODR and the percentage of PCs, but this did not reach significance ($P = 0.053$).

The median percentage of PCs was significantly higher in secondary than in primary dengue (4.5% versus 1.0%, respectively, $P = 0.05$, figure 3B) when day of illness was not taken into account. The difference was even more striking during the first 7 days of illness (15% versus 0.0%, respectively, $P = 0.005$).

4. DISCUSSION

This is the first prospective study demonstrating that blood plasmacytosis, characterized by a transient increased amount of polyclonal PCs in the circulation, is a common hematological event in DENV infection. The plasmacytosis was most pronounced during the first week of disease and disappeared completely within two weeks. The PCs were all polyclonal by origin; the predominant immunophenotype was Cd138+/Cd45+/Cd19-/Cd56-.

Bone marrow PCs are long-lived non-proliferating cells generated as part of the humoral immune response to infections. Memory B cells that are activated by antigen in the secondary lymphoid organs differentiate into plasmablasts that migrate to the bone marrow where they develop into PCs. In contrast to bone marrow PCs, plasmablasts are short-lived proliferating cells and plasmablasts normally represent less than 0.1% of peripheral blood mononuclear cells (PBMCs). Also PCs are usually not observed in peripheral blood and non-malignant blood plasmacytosis in DENV infection thus far has only been described in two reports.

Circulating (Cd138+) PCs can be regarded as PC progenitors or early or mature PCs. The differentiation of B cells into PCs is regulated by transcription factors such as BCL6, PAX5, Blimp-1, IRF-4 and XBP-1 and is characterized by a number of changes in B-cell surface molecules. CD19, CD20, CD21, CD22, and CD45 expression are down regulated, whereas...
CD38 and CD138 expression are upregulated. In fact, CD138 is the only antigen that discriminates plasmablasts (CD138) from early and mature PCs (CD138+). The surface phenotypes of PCs in the majority of DENV infected patients (89%) were CD138+/CD45+/CD19+/CD56-. This differs from bone marrow and blood PCs from patients with reactive plasmacytosis, which are usually CD19+. Cd138 is the only antigen that discriminates plasmablasts (CD138-) from early and mature pCs (CD138+). The surface phenotypes of PCs in the majority of DENV infected patients (89%) were CD138+/CD45+/CD19+/CD56-. This differs from bone marrow and blood PCs from patients with reactive plasmacytosis, which are usually CD19+. Cd19 is a protein that is invariable present on B cell lineage cells but is lost during maturation to plasma cells. This could point at redistribution as the cause of increased number of plasma cells in peripheral blood. However, increased production may also play a role as Cd45 are expressed. During DENV infection, virus and cytokines are detectable in blood and PBMCs, similar to what is seen in many other virus infections. Both B cells and monocytes can secrete IL-6. IL6 has pro-and anti-inflammatory activities but is also growth, proliferation and survival factor for nonmalignant and malignant PCs. Polyclonal blood PCs have been observed in patients with IL-6 producing malignancies such cardiac myxoma and gastric carcinoma. IL-6 has the ability to prevent plasma cells from going into apoptosis and thus increases the survival of PCs. Other cytokines than IL-6 can also be involved in the development of plasmacytosis. The IL-6 receptor subunit glycoprotein 130 is shared with receptors of IL-11, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor. IL-11 has been detected as a growth factor of IL-6-dependent plasmacytoma, and IL-10 has been reported to stimulate myeloma cells via oncostatin M. DENV infected monocytes produce IL-1, which is a known inducer of IL-6 and tumor necrosis factor-α and can induce an inflammatory response and the maturation of B cells into PCs. Moreover, increased levels of IL-6 have been associated with dengue disease severity, particularly in patients with DHF and DSS. Production of IL6 is also a common response in a variety of diseases that are not characterized by blood plasmacytosis. This implies that either IL6 production is not the sole explanation for blood plasmacytosis or that plasmacytosis does occur much more often than has been hitherto recognized.

B cells and monocytes in PBMCs support virus replication. B cells contribute to dengue pathogenesis by producing high titers of antiplatelet auto-antibodies and anti-endothelial cell antibodies, which could induce increased vascular permeability. The parallel time course of DENV and peripheral PCs with normalization within two weeks after onset of symptoms, at the time when anti-DENV IgG antibodies started to rise, taken together with the higher percentage of peripheral blood PCs in secondary DENV infection suggests that excess stimulation of B cells and excess proliferation or release of PCs play an important role in the pathogenesis of severe dengue infection.

Although our study provides insight into the biology and kinetics of blood plasmacytosis in DENV infection, the mechanism and possible role in dengue pathogenesis remain unclear. Important unanswered questions in this respect are whether active DENV replication takes place in peripheral PCs and whether PCs are redistributed and released from the bone marrow into the circulation or whether they represent an increased production and if this is virus or cytokine driven? Research into these questions can further address the contribution of peripheral PCs to dengue pathogenesis and expand our knowledge of DENV infection.

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