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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

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Abstract

In vitro studies have attempted to identify dengue virus (DEN) target cells in peripheral blood; however, extensive phenotyping of peripheral blood mononuclear cells (PBMCs) from dengue patients has not been reported. PBMCs collected from hospitalized children suspected of acute dengue were analyzed for DEN prM, CD32, CD86, CD14, CD11c, CD16, CD209, CCR7, CD4, and CD8 by flow cytometry to detect DEN antigen in PBMCs and to phenotype DEN-positive cells. DEN prM was detected primarily in activated monocytes ($CD14^+$, $CD32^+$, $CD86^+$, $CD11c^+$). A subset of samples analyzed for DEN nonstructural protein 3 (NS3) confirmed that approximately half of DEN antigen-positive cells contained replicating virus. A higher percentage of PBMCs from DHF patients expressed prM, CD86, CD32, and CD11c than did those from DF patients. Increased activation of monocytes and greater numbers of DEN-infected cells were associated with more severe dengue, implicating a role for monocyte activation in dengue immunopathogenesis.

Keywords

dengue; immunopathogenesis; activated monocytes; PBMCs; infection; replication; Nicaragua

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Introduction

The four serotypes of dengue virus (DEN1–4), members of the *Flavivirus* genus of the *Flaviviridae* family, are positive-sense RNA viruses (Lindenback and Rice, 2001). All serotypes can cause the full disease spectrum of dengue, which ranges from undifferentiated febrile illness to classic dengue fever (DF) to potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Burke and Monath, 2001). Severity of illness is classified according to the World Health Organization (WHO) scheme, which incorporates several key clinical manifestations including thrombocytopenia, bleeding, and signs of vascular leak with and without associated hypotension (WHO, 1997). DEN causes tens of millions of DF cases and hundreds of thousands of DHF/DSS cases annually worldwide (Gibbons and Vaughn, 2002; WHO, 1997). Although dengue affects the entire population, children bear the majority of the burden of severe disease, as more than 90% of DHF/DSS cases occur in children less than 15 years of age (WHO, 1997). Dengue activity continues to increase, with over 60 countries reporting cases of DHF/DSS and more than 100 countries endemic for dengue, including most of South and Southeast Asia, Central and South America, the Caribbean, and South Pacific regions (Halstead, 2002).

Secondary DEN infection with a different serotype from that which caused primary infection has been identified as a major epidemiologic risk factor for DHF/DSS (Burke et al., 1988; Halstead and Yamarat, 1965). Halstead proposed that the presence of heterotypic anti-DEN antibody, induced by either previous heterotypic DEN infection or the transfer of maternal antibody in infants, is not only unable to neutralize DEN, but may actually increase viral load by opsonization and replication of the virus in monocytes and macrophages, a phenomenon designated as antibody-dependent enhancement (ADE) (Halstead et al., 1973; Halstead and O'Rourke, 1977). ADE of DEN infection has yet to be definitively demonstrated in vivo in humans, although higher levels of viremia in dengue patients have been correlated with increased disease severity (Murgue et al., 2000; Vaughn et al., 2000). It is unclear whether or not monocytes/macrophages are the primary target cell of DEN, as it is difficult to infect these cells in vitro in the absence of antibody (Kou et al., 2008), and other cell types such as dendritic cells (DCs) are more susceptible to DEN (Jessie et al., 2004; Marovich et al., 2001; Wu et al., 2000). Early reports described DEN infection of peripheral blood leukocytes in DHF patients but did not rigorously identify the target cells (Scott et al., 1980; Waterman et al., 1985). In a limited analysis, King et al. (1999) identified replicating DEN primarily in B cells as well as monocytes, NK cells, and T cells from DHF patients. Dengue antigen was detected by immunohistochemical (IHC) staining in the Kupffer cells of the liver, splenic lymphoid cells, alveolar macrophages, and renal tubular cells in biopsies obtained pre- and post-mortem from DHF/DSS patients (Jessie et al., 2004). Blood clots from 8 of 20 dengue patients revealed DEN antigen primarily in peripheral monocytes, but also in some lymphocytes. Only splenic lymphoid cells and peripheral blood monocytes (and some lymphocytes) were positive for DEN antigen by in situ hybridization in 7 of 8 of these patients (Jessie et al., 2004). However, studies to date have detected DEN structural proteins or positive-strand viral RNA, which may indicate phagocytosis of the virus and not necessarily active replication.

In this pilot study, we evaluated peripheral blood mononuclear cells (PBMCs) recovered from pediatric DF and DHF cases for the presence of DEN antigen and compared the phenotypic markers of DEN-infected cells from these two patient populations. Using monoclonal antibodies to both structural and nonstructural DEN proteins, we found that activated monocytes ($CD86^+$, $CD32^+$, $CD14^+$, $CD11c^+$) were the main target of DEN infection regardless of immune status. We also observed significantly increased monocyte activation ($CD86^+$) and a higher prevalence of $CD32^+$, $CD14^+$, $CD11c^+$, and DEN prM^+ cells in DHF patients compared with DF patients, which has implications for ADE and the immunopathogenesis of DHF.

Results

This study attempts to characterize differences in the PBMC populations of DF and DHF patients and to determine which cell types contain significant amounts of DEN antigen. Sufficient viable PBMCs were recovered and analyzed from 14 children hospitalized with acute DF and from 4 children hospitalized with DHF. All DHF patients had serologic evidence of a secondary DEN infection, compared to 10 of the 14 DF patients. Eighty-seven percent of samples had a viability of greater than 50% by live-dead marker. There was no correlation between viability and expression of prM or disease severity (data not shown). Virus serotype was identified by virus isolation and/or RT-PCR from the blood of all but four patients (three DF patients and one DHF patient), yielding 11 DEN2 and 3 DEN1 infections.

Cell types expressing DEN antigen

Two cocktails of monoclonal antibodies (mAbs) were utilized to detect DEN antigen in the PBMCs of hospitalized dengue patients and to phenotype those cells positive for DEN antigen. DEN prM antigen was detected in approximately 11% of all live PBMCs (Table 1). Because of the different scatter and size characteristics of monocytes and lymphocytes in flow cytometry, gating was initially performed based on morphology; it was then observed that the great majority of DEN antigen-positive live PBMCs were $CD86^+$ (82%) in cocktail 1 and $CD32^+$ (78%) in cocktail 2 (Table 1). Since these cell surface markers distinguish predominately monocyte/macrophage populations more reliably than morphological characteristics alone, subsequent analyses were performed on $CD32^+$ and $CD32^-$ cells (cocktail 2) and $CD86^+$ and $CD86^-$ cells (cocktail 1) separately. DEN antigen was detected primarily in cells that were phenotypically monocytes ($CD14^+$, $CD32^+$) and not lymphocytes ($CD4^+$, $CD8^+$) (Table 1, Figure 1). Cells positive for DEN antigen in cocktail 2 were also substantially positive (92%) for CD11c (iC3b receptor) (Table 1, Figure 1A). The proportion of PBMCs expressing CD32 was significantly associated with the proportion of DEN prM-positive live cells (p=0.0005). Approximately 2% of all live cells were $CD32^-/prM^+$ (Table 1, Figure 1B); these cells were additionally positive only for CD11c.

The majority (82%) of live cells stained with cocktail 1 containing DEN prM expressed CD86 (B7-2) (Table 1, Figure 2A); these cells were not positive for the mature DC marker CD83. Although CD209 expression was detected at a low rate in cells positive for DEN antigen (Table 1, Figure 2A), the $CD86^+$ cells expressing CD209 were significantly associated with those cells with detectable DEN antigen (p = 0.0015). CD209 expression was not detected in prM-negative samples. CD16 (Fcγ receptor III)-expressing cells that contained DEN antigen were distributed between CD86-positive (2.4%) and CD86-negative live cells (3.9%). Only a small percentage of $CD86^+/prM^+$ cells expressed CCR7 (DC homing/trafficking chemokine, Table 1). Approximately 2% of live cells stained with cocktail 1 were $CD86^-/prM^+$ (Table 1, Figure 2B); these cells were negative for all other markers in cocktail 1 except for CD16. Because of the limited number of markers that could be studied, no specific B cell or NK cell marker was included in either cocktail.

Overall, DEN antigen was primarily detected in cells that were phenotypically monocytes: $CD86^+$, $CD32^+$, $CD14^+$, $CD11c^+$, $CD83^-$, $CD4^-$, $CD8^-$, $CD209^-$. However, a small percentage of prM-positive cells did express CD209. In addition, a population of cells containing DEN antigen did not express CD86, lymphocyte markers CD4 or CD8, or monocyte markers CD14 or CD32. They did, however, express CD16 (cocktail 1) or CD11c (cocktail 2). These cell populations may represent NK cells ($CD86^-/CD16^+$) and dendritic cells ($CD32^-/CD14^-/CD11c^+$).

To restrict the analysis to replicating virus only, analysis with anti-NS3 mAbs was performed on samples from a subset of 11 patients (7 DF, 4 DHF) from whom sufficient cells were

available. NS3 was detected in samples from all patients. Overall, 3.4% ± 0.8% (standard error) of total PBMCs were NS3-positive (see Figure 3A for a representative sample), compared with prM detection in 4.5% ± 0.9 % of PBMCs in this limited analysis. Although only a restricted analysis of the cell type in which NS3 was detected could be performed, NS3 was detected primarily (85%) in $CD14^+$ cells (Figure 3B). In a cumulative analysis of all samples, 79.6% ± 2.8% of PBMCs with detectable NS3 antigen expressed CD14.

Comparison of the phenotype of cells from DF and DHF patients

The expression of CD86, CD83, CD209, CCR7, CD16, and DEN prM was analyzed in 20 serial samples recovered from 14 DF patients and in 9 serial samples recovered from 4 DHF patients. Ten samples from 4 DHF patients and 15 samples from 12 DF patients were analyzed for the expression of CD4, CD8, CD32, CD14, CD11c and DEN prM. Samples from two febrile patients who were subsequently confirmed as negative for DEN infection were included for comparison. Overall, no significant difference was found in the expression of CD4, CD8, CCR7, CD83, or CD16 in PBMCs recovered from patients with DHF compared to DF. However, a significant increase was observed in the expression of CD86, CD32, CD14, CD11c, and DEN prM in PBMCs from DHF patients compared with DF patients (Figure 4). For instance, a significantly higher percentage of DEN-antigen containing PBMCs from DHF patients expressed CD32 compared with those from DF patients (43.7% ± 3.3 vs. 31% ± 2.7, $\alpha = 0.01$); likewise for CD86 (35.0 ± 3.8 vs 19.8 ± 2.4, $\alpha = 0.01$). Samples from two dengue-negative patients were included in these experiments as negative controls. Significance testing was not performed on the negative-control samples because of the limited number of patients; however, the expression of surface markers on PBMCs from these patients is included in Figure 4 for comparison. No significant differences were detected with any marker in relation to immune status (primary versus secondary DEN infection) or when stratified by infecting virus serotype. When primary DEN infections were removed from the analysis, the difference in the expression of CD86, CD32, CD14, CD11c, and DEN prM remained significant between DHF and DF patients.

Discussion

Monocytes and macrophages have long been considered the primary target cells of DEN infection, although the recovery of DEN or DEN antigen from monocytes of dengue patients has met with variable success (Halstead et al., 1977; Jessie et al., 2004; King et al., 1999; Morens et al., 1991; Scott et al., 1980; Waterman et al., 1985). To date, DEN antigen has been recovered from mixtures of peripheral blood cells, but the target cells in infected humans have not been defined by detailed phenotypic characterization. Other purported target cells of DEN include immature DCs in skin and lymph nodes (Kyle et al., 2007; Marovich et al., 2001; Wu et al., 2000). In this initial study, we characterized the phenotype of PBMCs from acute pediatric dengue cases using mAbs to both structural and nonstructural DEN proteins together with two mAb cocktails staining for numerous cellular markers, including DC-SIGN (purported co-receptor for DEN (Tassaneetrithep et al., 2003)). We found the vast majority of cells containing DEN antigen expressed the phenotype typical of activated peripheral blood monocytes, even in primary DEN infections. In a limited analysis, we demonstrated that replicating DEN virus, as evidenced by the presence of NS3 protein, was found in predominantly in $CD14^+$ cells. Finally, we found that expression of CD86, CD32, CD14, CD11c, and DEN prM was significantly increased in PBMCs from DHF patients as compared with DF patients.

When stained simultaneously by mAbs directed to structural and nonstructural DEN proteins, approximately half of those cells that contained DEN prM protein were also positive for NS3. The lower level of NS3 detection is not unexpected, for several reasons. First, prM will be

detected intracellularly in both infecting virions as well as replicating genomes, whereas NS3 derives only from replicating genomes. Thus, it is likely that NS3 is more difficult to detect by intracellular staining and may sometimes be below the limit of detection. Second, a greater number of monocytes would be expected to contain residual phagocytosed DEN antigen than to be supporting active viral replication. Nonetheless, the detection of NS3 primarily in $CD14^+$ cells demonstrates that monocytes are targets of DEN infection and active replication in vivo. Approximately 20% of cells with detectable NS3 were $CD14^-$, indicating that viral replication is occurring to a much lesser extent in cells other than monocytes. These cells may represent B cells (King et al., 1999), dendritic cells, or possibly NK cells.

Two small subpopulations staining with DEN antigen were not characteristic of monocytes. The first ($CD86^-/CD16^+$, cocktail 1; $CD32^-/CD11c^+/CD4^-/CD8^-$, cocktail 2) may represent NK cells; however, because of the limited number of cells available for staining, NK cell marker CD56 was not included in the analysis. The second subpopulation was comprised of DC-SIGN-positive cells. $CD14^+$ DC precursors (preDC), which also express DC-SIGN, have been derived from CD34 progenitor cells and are thought to be closely related to dendrocytes (Kwan et al., 2005). In addition to CD14 and DC-SIGN, these cells express CD86, CD11c, and CD32 and support DEN infection and replication, albeit less effectively than DCs (Kwan et al., 2005). Although DEN infects interstitial $CD14^+$ cells less efficiently than Langerhans cells, these $CD14^+$ cells are more numerous in the skin and may provide a greater opportunity for DEN cell entry and replication via binding to DC-SIGN (Kwan et al., 2005). Interestingly, $CD14^+/CD1a^-$ monocyte-derived DC precursors are refractory to DEN infection (F.B. dos Santos, C.M. Mueller, and E. Harris, unpublished). Whether or not the small numbers of DC-$SIGN^+$ cells that were found to contain DEN antigen represent preDCs or monocyte-derived dendritic cells could not be determined in this study, as there were not sufficient cells to stain for DEN antigen, CD14, and DC-SIGN together in a separate cocktail.

Importantly, significantly higher percentages of DEN antigen-positive cells from DHF patients expressed CD32, the FcγII receptor on monocytes, as compared to DF patients. In vitro studies have demonstrated that although peripheral blood monocytes are infected poorly by DEN, non-neutralizing antibody greatly enhances infection (Halstead et al., 1973; Halstead and O'Rourke, 1977) by forming immune complexes with the virus and enabling its entry via the FcγII receptor (ADE) (Littaua et al., 1990). ADE is thought to contribute to the development of more severe disease in secondary DEN infections (Kliks et al., 1989), and in vitro studies as well as passive antibody studies in non-human primate models have substantiated the potential role of ADE in enhanced viremia (Goncalvez et al., 2007; Halstead, 1979). In addition, interferon (IFN)-γ, the levels of which are significantly increased in the serum of DEN-infected patients in the acute phase of illness (Azeredo et al., 2001; Green et al., 1999b; Kurane et al., 1995; Kurane et al., 1991), may contribute to increased entry of DEN into monocytes through higher numbers of FcγII receptors (Kontny et al., 1988; Rothman and Ennis, 1999). Although absolute viral load was not measured in patients in this study, DEN antigen was detected in a higher proportion of PBMCs from DHF patients, and there was a significant correlation between the proportion of CD32-positive cells and cells positive for DEN antigen in these samples ($p=0.0005$).

CD86 was also detected in a higher proportion of PBMCs from DHF patients, as compared with DF. CD86 (B7-2) is a costimulatory molecule important for the activation of T cells and is upregulated on antigen-presenting cells such as monocytes, macrophages, DCs, and B cells following stimulation and interaction with CD28 (Bhatia et al., 2006; Reiser and Stadecker, 1996). IFN-γ, presumably from activated T cells (Kurane et al., 1991), also increases the expression of CD86 on monocytes (Creery et al., 1996). The significantly higher levels of CD86 expression in DHF patients may therefore be a result of increased antigen load and activation, a consequence of up-regulation by IFN-γ, or a combination of the two. Elevated levels of cytokines have been detected in DHF/DSS patients and may play a causal role in the

vascular leak and coagulopathy syndromes characteristic of severe dengue (Bethell et al., 1998; Green et al., 1999a; Hober et al., 1996; Hung et al., 2004; Kurane and Ennis, 1994; Pinto et al., 1999). Specifically, elevated levels of TNF-α, which is produced by activated monocytes and DCs (Chaturvedi et al., 1999), are associated with more severe dengue in patients (Azeredo et al., 2001; Hober et al., 1996; Hung et al., 2004) and increase vascular permeability in in vitro (Dewi et al., 2004) and in vivo models (Atrasheuskaya et al., 2003; Shresta et al., 2006) of DEN infection. Although we did not specifically measure TNF-α levels in these patients, the upregulation of CD32, CD11c, and CD86 is indicative of monocyte activation, and patients with DHF demonstrated evidence of greater monocyte activation than did those with DF. Our data is thus consistent with a model in which a feedback loop initiated by elevated levels of IFN-γ produced by cross-reactive T cells induces the expression of CD32 on peripheral blood monocytes (Kurane and Ennis, 1992). Antibody-virus complexes, via the FcγII receptor, gain entry into monocytes, where viral replication and antigen load result in activation and increased expression of CD32 and CD86, allowing for even greater viral entry. The release of immunomodulatory cytokines, including TNF-α, from activated monocytes then contributes to increased permeability across endothelial cells and the hallmark vascular leak syndrome. Eventually, the virus is cleared and the feedback loop is abrogated.

Although our results suggest a role for monocyte activation in DHF, the study had several limitations. Because of the amount of blood that may be safely drawn from acutely ill pediatric patients is small, only a limited number of PBMCs were available for analysis. For this reason, we were unable to determine if DEN antigen was present in NK cells or B cells or if the DEN antigen detected in other cell populations represented replicating virus. B cell infection and activation by DEN cannot be excluded in this study because B cells can express both CD86 and CD32. DEN virus has been isolated from B cells obtained from DHF patients and may be an important target cell during infection (King et al., 1999). Because the study was limited to phenotyping of cells expressing DEN antigen, intracellular staining of IFN-γ or other cytokines was not performed. However, as described above, other studies have implicated IFN-γ in the upregulation of CD32. Finally, we were not able to compare NS3 and prM expression directly in each of the samples analyzed.

Nonetheless, this initial in vivo phenotyping study was able to substantiate monocytes as the predominant target cell in the peripheral blood of dengue patients. Importantly, this demonstrates that DEN targets the same cell type in PBMCs in both primary and secondary infections. In addition, our data supports a role for activated monocytes in the immunopathogenesis of DEN. These studies are being extended to further evaluate the activation phenotype of peripheral blood monocytes following DEN infection via intracellular cytokine staining, to further characterize the identity of DC-SIGN-positive cells, to explore the possible role of peripheral blood NK cells and B cells in DEN infection, and to further characterize the phenotype of cells in the peripheral blood that are supportive of active DEN replication.

Materials and Methods

Study Design and Population

PBMCs were obtained from an ongoing study at the Infectious Disease Unit of the Hospital Infantil Manuel de Jesús Rivera (HIMJR), the Nicaraguan National Pediatric Reference Hospital in Managua. Suspected dengue cases that presented to the HIMJR within four days since onset of fever between August, 2005 and February, 2006 and who completed the required informed consent and assent procedures were enrolled. Study participants included 15 females and 13 males 7 to 14 years old. Twenty-six patients were laboratory-confirmed as positive for DEN infection and 2 as dengue-negative; of the confirmed dengue cases, 6 (23%) were classified as DHF and 20 (77%) were diagnosed as DF. Seven cases (27%) were defined as

primary and 19 cases (73%) as secondary DEN infections.. The serotype was determined in 20 cases (77% of positive cases), with 4 DEN1 and 16 DEN2 infections.

Serological and virological assays to detect DEN infection were performed at the National Virology Laboratory of the Ministry of Health in Managua. A positive case was defined by IgM seroconversion, a 4-fold or greater increase in total anti-DEN antibodies as measured by Inhibition ELISA (Balmaseda et al., 2006; Fernandez and Vazquez, 1990), detection of viral RNA by RT-PCR, and/or virus isolation (Hammond et al., 2005; Harris et al., 2000). In accordance with the WHO classification scheme, DHF was defined as fever with hemorrhagic manifestations, thrombocytopenia, and hemoconcentration or elevated hematocrit for age and sex or other signs of plasma leakage (e.g., pleural effusion, ascites); DSS was defined as DHF plus either hypotension for age (systolic pressure <80 mmHg for those <5 years of age and <90 mmHg for those ≥5 years of age) or narrow pulse pressure (<20mmHg) in the presence of clinical signs of shock (e.g., slow capillary filling, cold clammy skin) (WHO, 1997). Primary and secondary DEN infections were defined by an antibody titer by Inhibition ELISA of <2560 or ≥2560 in convalescent samples, respectively (Harris et al., 2000). This study was approved by the Institutional Review Boards at the University of California Berkeley, the Nicaraguan Ministry of Health, and the HIMJR.

PBMC preparation

Daily blood specimens were obtained from patients (average 2.2 samples, range 1–4), along with a convalescent/discharge sample. Analyzed samples were obtained 4, 5, or 6 days following the onset of symptoms (mean of 4.9 ± 0.2 days). Six mL of blood were collected in CPT tubes (Becton-Dickenson, Franklin Lakes, NJ); the transport temperature (~28°C), time of sample collection, transport, reception, and processing (total = ~2.5 hours) were strictly controlled using personal data assistants (PDAs) with barcode scanners. Upon receipt in the National Virology Laboratory, the tubes were centrifuged for 30 minutes at 765 × *g* at room temperature. The cells and plasma were mixed by inversion, centrifuged for 15 minutes at 340 × *g*, the plasma was removed, and the cell pellet was washed with RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) containing 2% FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin and centrifuged for 15 minutes at 340 × *g*. The supernatant was discarded, the pellet was resuspended in 10mL of RPMI 1640 with 2% FBS and 1% penicillin/streptomycin, cells were counted, and 5mL was removed for use in another study. The remaining 5mL were centrifuged for 15 minutes at 340 × *g* and resuspended in the volume of 90% FBS/10% dimethyl sulfoxide calculated to result in a final concentration of 5×10^6 cells/mL. Average yield was 7.2×10^6 cells (range $1.4 – 29.5 \times 10^6$). Cryovials containing the cell suspension were placed in isopropanol containers (Mr. Frosty, Nalgene, Rochester, NY) at −80°C overnight and then transferred to liquid nitrogen.

Monoclonal Antibodies

Monoclonal antibody (mAb) hybridoma clone D3-2H2-9-21, which recognizes the prM protein of all four DEN serotypes, was purchased from the American Type Culture Collection (Manassas, VA). MAb was produced from the D3-2H2-9-21 clone at Biovest International, (Minneapolis, MN) and conjugated to Alexa-488 at Molecular Probes (Eugene, OR). 2H2-Alexa-488 was used at a final dilution of 1:600. A premixed cocktail of conjugated mAbs CD86-PE, CD83-APC, and CD209-PerCP Cy5.5, individual conjugated mAbs CCR7-PE-Cy7, CD16-APC-Cy7, CD4-PE, CD8-APC-Cy7, CD32-PE, and CD14-PerCP Cy5.5, CD3-PacBlue, and all isotype controls were purchased from BD Biosciences (San Jose, CA). CD11c-APC was obtained from Invitrogen (Carlsbad, CA). Anti-NS3 mAb was generated previously in the Harris laboratory (P.R. Beatty and E. Harris, unpublished results).

Flow Cytometry

Samples were analyzed on a Becton Dickinson FACSAria flow cytometer (San Jose, CA). Cryovials were removed from liquid nitrogen, rapidly thawed in a 37°C water bath, and transferred to a 15-mL tube containing Hank's Buffered Saline Solution HBSS (Cambrex Bio Science, Walkersville, MD). The tubes were centrifuged for 8 minutes at 300 × *g*, and the cell pellet was resuspended in 1mL PBS (Cambrex Bio Science). One μL of fluorescent live/dead marker (Molecular Probes) was added to 1×10^6 cells and mixed well. After a 30-minute incubation on ice, cells were washed with PBS and incubated for 20 minues on ice with blocking buffer consisting of 10 μg/mL human IgG (Sigma Chemical Co., St. Louis, MO) in staining buffer (3% heat inactivated fetal calf serum [Atlantic Biologicals, Atlanta, GA] in PBS, pH 7.4–7.6). Then 250,000–300,000 cells were transferred into 12 × 75 mm tubes (Evergreen Scientific, Los Angeles, CA), fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences), and incubated at 4°C for 30 minutes with conjugated mAb 2H2-Alexa-488 at a final dilution of 1:600. Cells were washed twice in Perm/Wash buffer (BD Biosciences) and resuspended in 75μL of staining buffer containing either mAb cocktail 1 (CD86-PE, CD83-APC, CD209-PerCP Cy5.5, CCR7 PECy7, and CD16-APC) or cocktail 2 (CD4-PECy7, CD8-APC-Cy7, CD32-PE, CD14-PerCP Cy5.5, and CD11c-APC). Anti-NS3 mAb was conjugated to phycoerythrin using the Zenon IgG labeling kit (Invitrogen). Samples were stained with cocktail 1 and, if sufficient cells were present, were also stained with cocktail 2.

Samples were read in a FACSAria flow cytometer within 3 hours of staining. Isotype controls were included in each run. In addition, a sample from a patient hospitalized with suspected dengue but who was negative for DEN infection by serology and virus isolation was included in the experiments staining with cocktail 1 and cocktail 2. There were not sufficient cells available from dengue-negative patients for inclusion in the experiments staining for NS3. Compensation was optimized using BD CompBeads (BD Biosciences). The percentage of live cells expressing each surface marker contained in an individual cocktail was first determined. Analysis of live cells was performed by first gating on CD86-positive and CD86-negative cells (cocktail 1) or CD32-positive and CD32-negative cells (cocktail 2) and studying the subpopulations within each gate to determine which were positive for DEN antigen. Staining with anti-NS3 antibody was performed on the subset of samples with sufficient PBMCs to specifically identify replicating virus. The NS3-PE antibody was included in a cocktail containing 2H2-APC and CD14-perCPCy5.5 and analyzed on the FACSAria flow cytometer. Only those samples containing a sufficient number of viable PBMCs (>40% viability as determined by live-dead marker) were analyzed.

Statistical Analysis

The student's *t* test and JMP software (version 5.0.1.2; SAS Institute) was used to analyze comparisons of the expression of intracellular DEN antigen as well as of cellular markers on the surface of PBMCs obtained from children with confirmed acute DF versus DHF. The expression of these markers in primary versus secondary DEN dengue infection was also compared in a similar manner.

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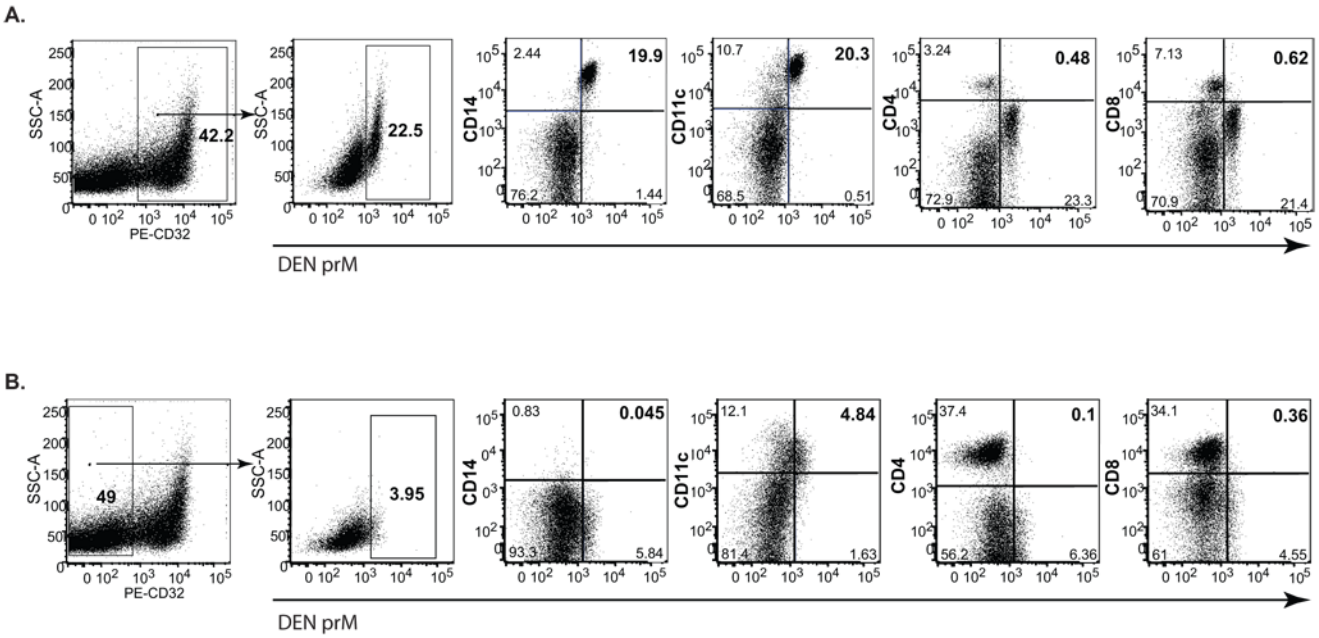

Figure 1.

DEN antigen detection in $CD32^+$ and $CD32^-$ populations of PBMCs. Samples were stained with a cocktail containing the conjugated mAbs CD32-PE, CD14-PerCPCy5.5, CD11c-APC, CD4-PECy7, CD8-APC-Cy7, 2H2-Alexa488 and a live-dead marker. Samples were first gated on $CD32^+$ cells and $CD32^-$ cells, and populations within those gates were examined for the presence of DEN antigen (prM) using mAb 2H2. DEN antigen was found primarily in $CD32^+$ PBMCs. *(A)* Representative sample of the prM-positive populations within the $CD32^+$ gate. The majority of DEN antigen found in $CD32^+$ gate is present in $CD14^+$ and $CD11c^+$ cells. *(B)* Representative sample of the prM-positive populations within the $CD32^-$ gate. The majority of DEN antigen in the $CD32^-$ gate is present in $CD11c^+$ cells.

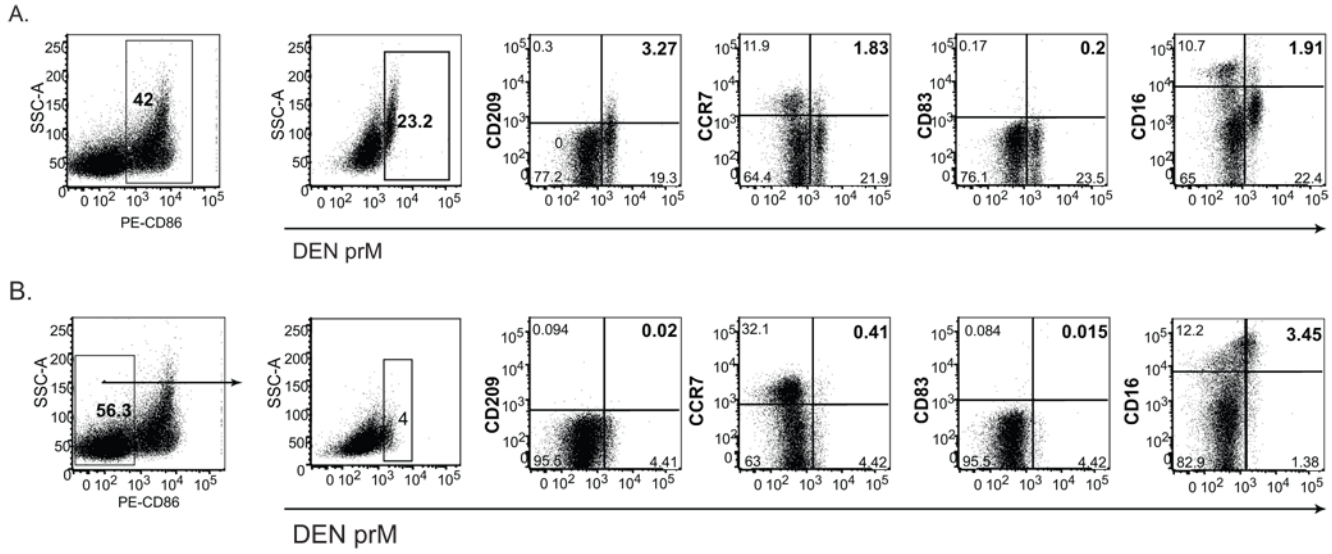

Figure 2.

DEN antigen detection in CD86^{+} and CD86^{-} populations of PBMCs. Samples were stained with a cocktail containing the conjugated mAbs CD86-PE, CD209-PerCPCy5.5, CD83-APC, CCR7-PECy7, CD16-APC-Cy7, 2H2-Alexa488, and a live-dead marker. Samples were first gated on CD86^{+} cells and CD86^{-} cells, and populations within those gates were studied for the presence of DEN antigen (prM) using mAb 2H2. DEN antigen was found primarily in CD86^{+} PBMCs. *(A)* Representative sample of the prM-positive populations within the CD86^{+} gate. Only a small percentage of CD86^{+} cells containing DEN antigen expressed CD209, CCR7, or CD16. *(B)* Representative sample of the prM-positive populations within the CD86^{-} gate. The majority of DEN antigen in the CD86^{-} gate is present in CD16^{+} cells.

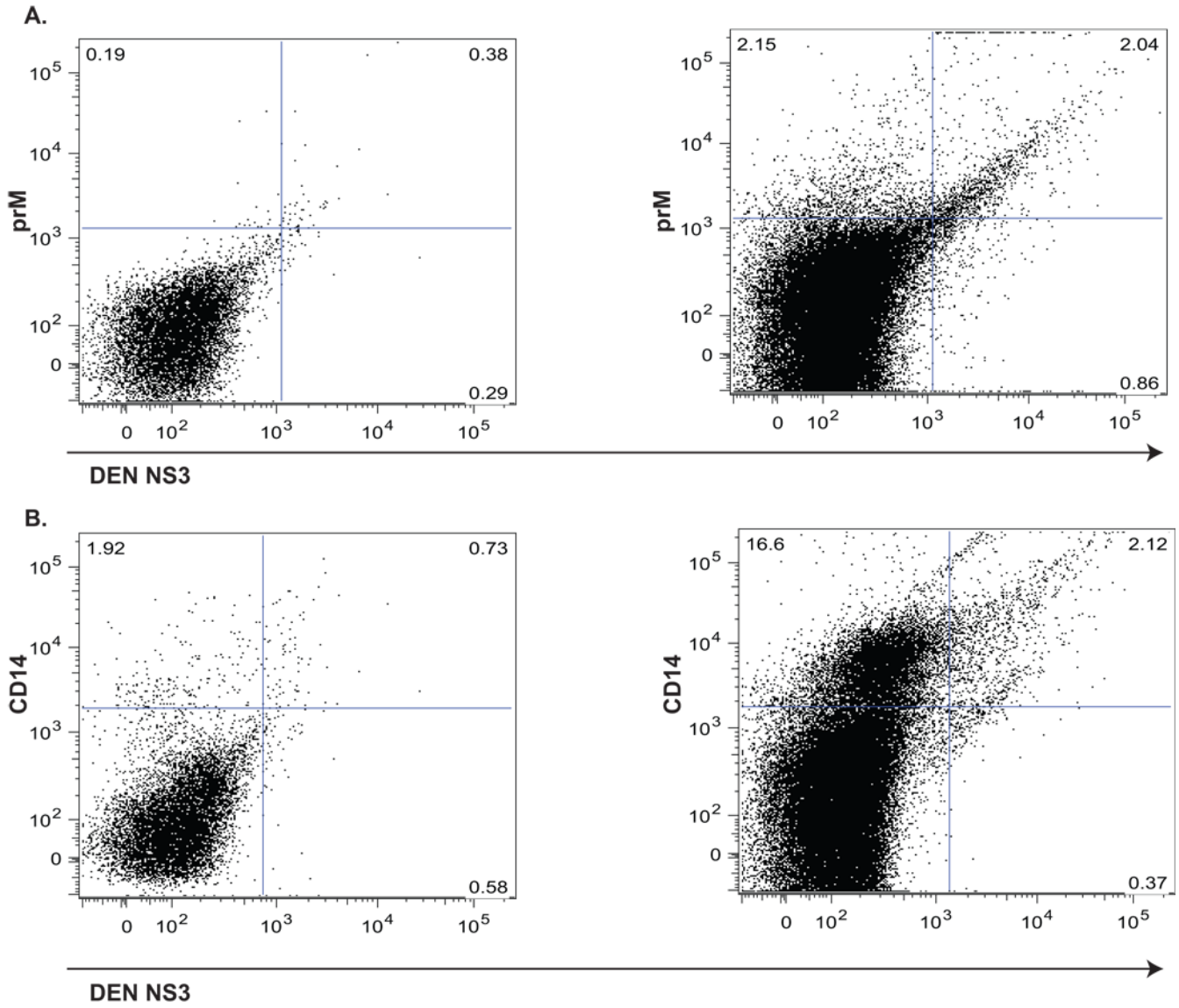

Figure 3.

DEN prM and NS3 detection in PBMCs. Samples were stained with a cocktail containing the conjugated mAbs CD14-PerCPCy5.5, 2H2-APC, and NS3-PE. *(A)* PBMCs from representative patient sample stained for DEN prM and DEN NS3. Total PBMCs stained with anti-DEN prM mAb (vertical axis) and anti-DEN NS3 mAb (horizontal axis) from representative patient sample (right), with isotype control (left). DEN NS3 was detected in approximately 49% of prM^+ cells. *(B)* Representative sample of PBMCs stained for both DEN NS3 and CD14. Total PBMCs stained with anti-CD14 mAb (vertical axis) and anti-DEN NS3 mAb (horizontal axis) from representative patient sample (right), with isotype control (left). DEN $NS3^+$ detected in PBMCs was found predominantly (85%) in $CD14^+$ cells. Extensive analysis of other markers expressed by cells containing detectable NS3 was not performed due the limited availability of samples.

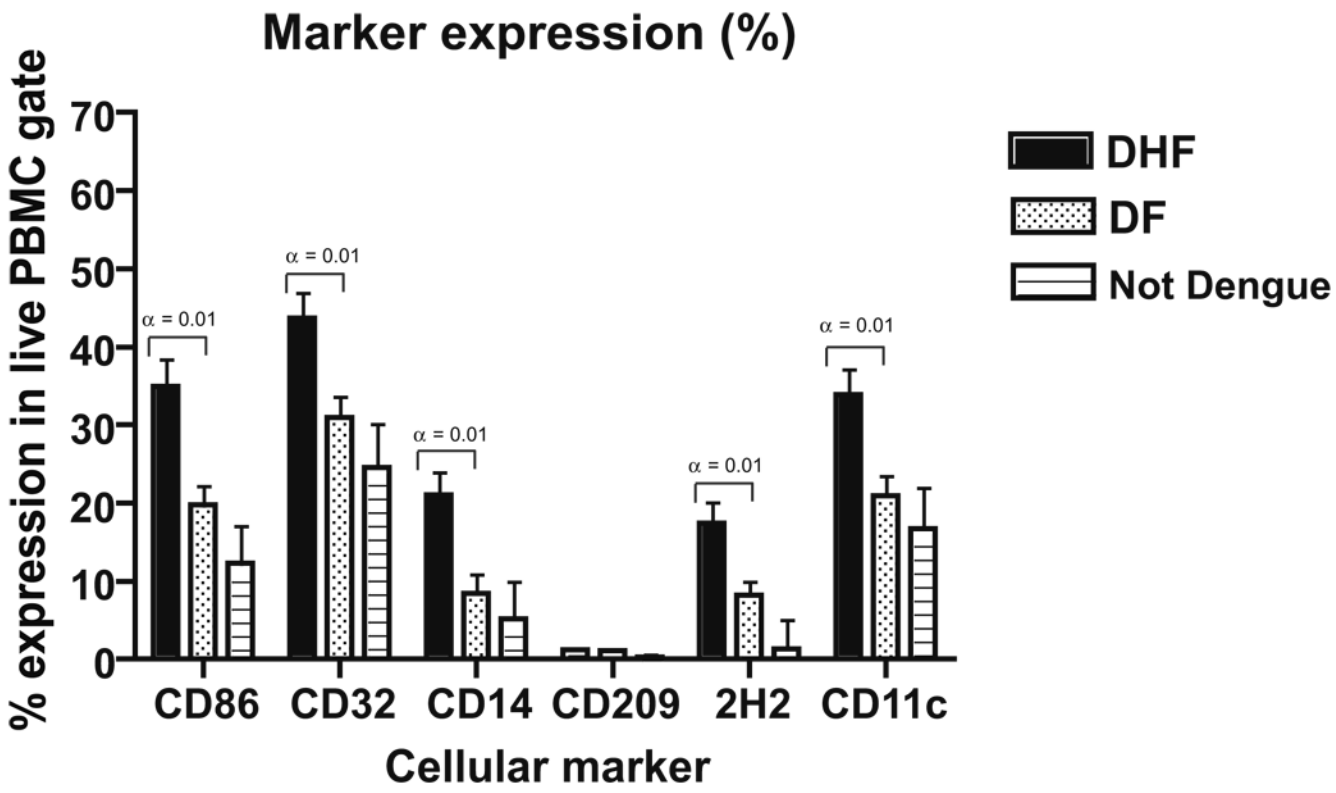

Figure 4.

Comparison of cell populations identified by cellular marker in DHF versus DF patients. Significance was determined by students *t* test and was determined for DHF and DF cases only. PBMCs from DHF patients contained significantly greater populations of $CD86^+$, $CD32^+$ $CD14^+$, and $CD11c^+$ cells than did those from patients diagnosed with DF. In addition, significantly more DEN antigen was detected in the PBMCs of DHF patients as compared to DF cases. Black bars: DHF cases; speckled bars: DF cases; striped bars: dengue-negative controls. Error bars represent the standard error of the mean.

Table 1

Mean percentage of DEN antigen (prM) in live PBMCs stratified by surface marker

	Cocktail 1						
	DEN prM antigen	**CD86$^+$ & prM**	**CD86$^-$ & prM**	**CD209$^+$ & prM[a]**	**CCR7$^+$ & prM[a]**	**CD83$^+$& prM[a]**	**CD16$^+$ & prM[a]**
% of total live PBMCs	11.2±1.8	9.2±1.9[b]	2.0±0.3	1.3±0.2	2.1±0.2	0.5±0.1	6.3±0.4[c]
% of live prM-containing cells	100	82.2	17.8	11.6	18.8	4.5	56.2
	Cocktail 2						
	DEN prM antigen	CD32$^+$ & prM	CD32$^-$ & prM	CD14$^+$ & prM[d]	CD11c$^+$ & prM[d]	CD4$^+$ & prM[d]	CD8$^+$ & prM[d]
% of total live PBMCs	10.5±1.8	7.9±1.7	2.2±0.3	7.2±1.6	9.3±1.6	0.4±0.1	1.0±0.1
% of live prM-containing cells	100	78.2	21.8	71.3	92.1	4.0	9.9

[a] Includes both CD86$^+$ and CD86$^-$ cells positive for the indicated marker and DEN prM.

[b] ± standard error (SE)

[c] 2.4% live cells CD86$^+$/CD16$^+$ cell, 3.9% live cells CD86$^-$/CD16$^+$ cells

[d] Includes both CD32$^+$ and CD32$^-$ cells positive for the indicated marker and DEN prM.