Dengue-2 and yellow fever 17DD viruses infect human dendritic cells, resulting in an induction of activation markers, cytokines and chemokines and secretion of different TNF-α and IFN-α profiles

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Flaviviruses cause severe acute febrile and haemorrhagic infections, including dengue and yellow fever and the pathogenesis of these infections is caused by an exacerbated immune response. Dendritic cells (DCs) are targets for dengue virus (DENV) and yellow fever virus (YF) replication and are the first cell population to interact with these viruses during a natural infection, which leads to an induction of protective immunity in humans. We studied the infectivity of DENV2 (strain 16681), a YF vaccine (YF17DD) and a chimeric YF17D/DENV2 vaccine in monocyte-derived DCs in vitro with regard to cell maturation, activation and cytokine production. Higher viral antigen positive cell frequencies were observed for DENV2 when compared with both vaccine viruses. Flavivirus-infected cultures exhibited dendritic cell activation and maturation molecules. CD38 expression on DCs was enhanced for both DENV2 and YF17DD, whereas OX40L expression was decreased as compared to mock-stimulated cells, suggesting that a T helper 1 profile is favoured. Tumor necrosis factor (TNF)-α production in cell cultures was significantly higher in DENV2-infected cultures than in cultures infected with YF17DD or YF17D/DENV. In contrast, the vaccines induced higher IFN-α levels than DENV2. The differential cytokine production indicates that DENV2 results in TNF induction, which discriminates it from vaccine viruses that preferentially stimulate interferon expression. These differential response profiles may influence the pathogenic infection outcome.

Key words*:* cytokines - dendritic cells - dengue virus - yellow fever vaccine - flavivirus

Flaviviruses are arthropod-borne viruses that may cause severe acute infectious diseases, such as dengue fever and yellow fever (YF). Dengue fever is the most important arthropod-borne emerging viral disease in tropical countries due to high morbidity and increased risk of mortality (Gubler 2002). The YF17DD vaccine induces long-lasting immunity, similar to wild viruses (Monath 2001, Halstead 2007). For dengue fever, a chimeric YF/dengue vaccine (ChimeriVax™ technology) is currently being tested in clinical trials (Guy et al. 2010). Both dengue and YF wild viruses can induce a broad spectrum of clinical manifestations from asymptomatic to severe clinical features, the latter of which is characterised by haemorrhaic manifestations and shock syndrome, which are associated with vascular permeability and leakage. Cytokines play a key role in the generation of these physiopathological processes (Geisbert & Jahrling 2004). Moreover, the occurrence of rare fatal cases and adverse clinical manifestations has been associated with the YF vaccine (Vasconcelos et al. 2001, Silva et al. 2010).

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Several cytokines have been associated with disease severity in patients. For dengue fever, tumor necrosis factor (TNF)-α (Hober et al. 1993, Braga et al. 2001), interleukin (IL)-10 (Green et al. 1999, Azeredo et al. 2001), IL-6 (Nguyen et al. 2004), macrophage inhibitory factor (Chen et al. 2006, Assuncao-Miranda et al. 2010) and interferon (IFN)-γ (Bozza et al. 2008), among others (Srikiatkhachorn & Green 2010), have been reported. Chemokines also have an important role in pathogenesis. For example, IP-10/CXCL-10 was found to be elevated in dengue haemorrhagic fever (DHF) (Fink et al. 2007) and MCP-1/CCL-2 (Lee et al. 2006), while MIP-1β/CCL-4 is associated with a good prognosis (Bozza et al. 2008). Elevated serum levels of IL-6, IL-8, TNF-α, MCP-1, IL-1 receptor agonist (Ra) and IL-10 were observed in fatal YF cases when compared with non-fatal cases (Bae et al. 2008). Finally, in vaccinated individuals, high levels of IP-10, IL-1β, TNF-α, IFN-γ and IL-10 were detected (Querec et al. 2009, Silva et al. 2011).

Monocytes, macrophages and dendritic cells (DCs) are the main targets for viruses involved in vascular permeability induction (Schnittler & Feldmann 2003, Clyde et al. 2006). Mononuclear phagocytes become activated by virus, which induces synthesis and release of cytokines (e.g., TNF-α, IL-6, IL-1β, IFN-α/β and IL-10) and chemokines (e.g., IL-8, MIP-1α and MCP-1). These factors produced by in vitro infected cells are known to induce alterations in the endothelium, which leads to the imbalance of fluid between the intra and extra-vascu-

lar areas of tissues and ultimately culminates in shock (Srikiatkhachorn 2009). Coagulation features that occur during dengue fever may be linked to mononuclear phagocyte and endothelial cell activation because proinflammatory cytokines interfere with the activation of coagulation factors (Suharti et al. 2002).

After virus infection, DCs become activated and mature. During maturation processes, DCs usually upregulate co-stimulatory molecules, such as CD80, CD86, CD83, CD40, CD38 and OX40L (Quah & O'Neill 2005). These molecules may be involved in the polarisation of T helper (Th) cells into either Th-1 or Th-2 subsets. CD38, IFN-γ and IL-12p70 are associated with Th-1 responses (Frasca et al. 2006). On the other hand, OX40L expression on DCs contributes to Th-2 polarisation (Delespesse et al. 1999). Mature DCs are able to activate T lymphocytes and drive the type of the immune response (Lanzavecchia & Sallusto 2004). It is believed that T cell activation in patients with severe dengue leads to T cell responses with less avidity for serotype-specific responses than mild dengue, resulting in a pathologic cytokine storm with limited antiviral responses (Duangchinda et al. 2010).

The aim of this investigation was to study the innate immune response induced in vitro by distinct flaviviruses. We selected a pathogenic strain of dengue virus (DENV) 2 (16681) originated from a DHF SE Asian case and the Brazilian YF17DD, which is known for its protective properties with minimal deleterious effects. A chimeric YF17D/DENV2 vaccine virus (Caufour et al. 2001) was assayed as well. We report for the first time that flaviviruses induce the expression of the DC maturation marker CD38 and downregulate OX40L, suggesting that a Th-1 response has been generated. Additionally, differential TNF-α and IFN-α expression by DCs infected with DENV and vaccine YF17DD or 17D/DENV2 viruses are discussed, with regard to their possible influence on pathogenicity and virus clearance.

MATERIALS AND METHODS

Virus strain, cell cultures, virus stock preparation and titration - DENV2 (strain Thailand/16681/1984) was provided by Dr SB Halstead (Naval Medical Research Centre, USA). YF strain 17DD is the live attenuated virus used in the YF vaccine manufactured by Bio-Manguinhos, Oswaldo Cruz Foundation, Rio de Janeiro (RJ), Brazil (Post et al. 1991). For virus stock preparation, Vero cells (CCL 81, ATCC) were maintained in 199 medium with Earle's salts buffered with sodium bicarbonate and supplemented with 5% foetal bovine serum (FCS) and antibiotics (Gibco, Invitrogen). Viral stock was prepared by flavivirus infection of a Vero cell monolayer in T175 flasks. When cytopathic effects were observed seven days later, the supernatant was harvested and supplemented with 30% FCS and aliquots were frozen at -70ºC. Virus stock was titrated by serial dilution cultures in microtitre plates and detected by immunofluorescence as previously described (Miagostovich et al. 1993). An uninfected flask was also maintained and the supernatant was collected to be used as a mock inoculum. A mock infection was included in each experiment.

A set of experiments was performed using the chimeric virus YF/DENV2 strain 44/3. This virus contains the pre-M and E genes from DENV2 strain New Guinea C, with the carboxyl end of protein E (E261-E495 amino acids) that belongs to the Brazilian strain 44/2 (Caufour et al. 2001). This chimeric virus elicits a protective immune response against DENV challenge both in mice and Rhesus monkeys, which includes neutralising antibodies (Caufour et al. 2001, Galler et al. 2005).

Virus titre was calculated as 50% tissue culture infectious dose ($TCID_{50}$/mL). However, when the virus was compared with the chimeric virus 44/3 (Fig. 1), all three viruses were titrated by the plaque-forming assay as described before (Galler et al. 2005).

Human monocyte-derived DCs (MoDCs) - Human peripheral blood was obtained from University Hospital Clementino Fraga Filho, Federal University of Rio de Janeiro (UFRJ), RJ. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors' buffy coats by centrifugation on a density gradient (400 *g* for 30 min in Ficoll-Paque Plus, Amersham Biosciences Corp), as described previously (Reis et al. 2007). Cells were resuspended at 10^6 cells/mL in RPMI 1640 supplemented with 200 mM glutamine, 100 U/mL penicillin and 10 mg/mL streptomycin (Gibco, Invitrogen) and incubated at 37ºC under a humid atmosphere with 5% CO2. The cells where then allowed to adhere to uncoated polystyrene flasks (150 cm^3) for 90 min to enrich for monocytes. Non-adherent cells were gently removed by washing and the adherent cells were detached by mechanical cell harvesting with cell scrapers in cold cell culture medium. Cell viability was verified in the cultures by Trypan blue exclusion and was ≥ 95%. Enriched monocytes were suspended in RPMI 1640 medium supplemented with 10% FCS, 500 U/mL human rGM-CSF and 500 U/mL rIL-4 (Peprotech) and seeded at 1 x 10^6 cells/mL on 24-well plates (Nunc, Thermo Fisher Scientific). Cytokines were added every other day for six days. The appropriate phenotype of DC differentiation was confirmed before each experiment by flow cytometry.

Infection of human MoDCs by flaviviruses - After a six-day cell differentiation period, MoDCs were incubated with YF17DD, a dose equivalent to that used for DENV2 (2.5 x 10^5 $TCID_{50}$/mL), a dose five times more concentrated or cell culture supernatant as mock treatment diluted inoculum (500 μL). This compensation for the infectivity of different flavivirus has also been previously reported (Youn et al. 2010). For experiments comparing the YF17DD, DENV2 and YF17D/DENV2 viruses, the multiplicity of infection (MOI) was 4 (4x10^6 plaque-forming unit/10^6 cells).

After three-hour incubation for adsorption at 37ºC under humid atmosphere with 5% CO_2, culture supernatants were replaced with medium containing 10% FCS and further incubated for 24-120 h. Each different parameter was set up in triplicate wells. After infection, supernatants were collected at defined intervals and stored at -70ºC until cytokine measurement and cells were recovered for viral antigen and surface marker determination by flow cytometry.

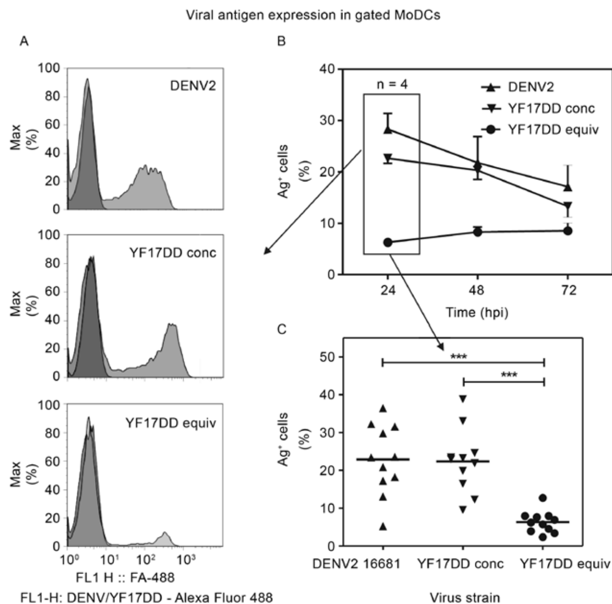

Fig. 1: monocyte-derived dendritic cells (MoDCs) infection with dengue virus 2 (DENV2) infection and yellow fever (YF) 17DD. MoDCs were infected with DENV2 or YF17DD at equivalent multiplicity of infections (MOIs) [2.5 x 10^5 50% tissue culture infectious dose (TCID)/mL] or YF17DD at a higher MOI (1.25 x 10^6 $TCID_{50}$/mL) or mock-infected. Cells were labelled with anti-DENV or anti-YF monoclonal antibodies using triplicates for each peripheral blood mononuclear cells (PBMC) donor. A: flow cytometry patterns for viral antigens on gated MoDCs 24 h after in vitro infection. Histograms demonstrate cell distribution by Alexa Fluor-488 fluorescence intensity (FL1-H: level of fluorescence). Cells were cultured with mock (dark grey) or DENV2 or YF17DD (light grey) at concentrated (conc) or equivalent (equiv) doses; B: viral antigen detection kinetics on infected MoDCs (data from four PBMC donors) [hpi: hours post-infection; X axis: time after infection (hours); Y axis: percentage of viral antigen positive cells (Ag^+)]; C: intracellular viral antigen detection 24 h after infection representing 11 cell donors (horizontal lines: distribution mean; X axis: viral inoculum; Y axis: percentage of viral Ag^+ cells. Asterisks mean: $p < 0.0001$ in one-way analysis of variance and Tukey's multiple comparison test).

Detection of viral antigen and specific co-stimulatory markers by flow cytometry in human MoDCs - Cells were harvested by vigorous pipetting using cold wash buffer [phosphate-buffered saline (PBS) pH 7.4, 1% bovine serum albumin (BSA) (Sigma) and 0.1% sodium azide] and were resuspended at 1 x 10^6 cells/microtube. The cells were then centrifuged (260 *g*, 7 min) and washed once more with wash buffer. Single or double cell labelling of infected and uninfected cultures was performed. For surface labelling, the cells were blocked with 5% heat-inactivated human plasma in wash buffer for 30 min at 4ºC and further incubated for 30 min with anti-human CD1a-PE (Iotest Immunotech, Beckman Coulter Inc, Brea, CA, USA), DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN)-FITC (R&D Systems), CD14-PE (DakoCytomation), HLA-DR,DP,DQ-FITC (Dako Cytomation), CD80-FITC (Iotest Immunotech), CD38-FITC (Caltag, Invitrogen) or OX40L-PE (BD Biosciences Pharmingen). Matching isotype antibodies were used as negative controls. The cells were washed and subsequently fixed with 2% paraformaldehyde. Intracellular staining was performed according to previously described methods (Neves-Souza et al. 2005), with slight modifications. Briefly, the cells were fixed with cold 2% paraformaldehyde in PBS for 20 min and, after centrifugation, the membranes were permeabilised with 0.15% saponin in wash buffer. The cells were blocked with 5% heat-inactivated human plasma in wash buffer for 30 min at 4ºC. Primary monoclonal antibody incubation was performed with anti-Dengue Complex (Chemicon, Millipore, Billerica, MA, USA), anti-Yellow Fever (Biogenesis, AbD Serotec) or an isotype-matched antibody diluted in wash buffer containing 0.15% saponin for 60 min at 4ºC. The cells were washed once with wash buffer and further incubated with secondary anti-mouse labelled with Alexa Fluor-488 (Molecular Probes, Invitrogen) for 30 min at 4ºC. The cells were acquired (10,000 events for DC-SIGN positive gate) on a FACS-Calibur flow cytometer (BD Biosciences). Analysis was performed using FlowJo (TreeStar Inc, Ashland, OR, USA) or Summit (Dako Automation).

Enzyme-linked immunosorbent assay (ELISA) for TNF-α - ELISA plates (MaxiSorp, Nunc) were coated overnight at room temperature (RT) with 1 µg/mL at 100 µL/well of rabbit anti-human TNF-α antibody provided in the Human TNF-α ELISA Development Kit (Peprotech). The plates were washed with 0.05% Tween 20 (Sigma) in PBS and were incubated with 1% BSA in PBS at 250 µL/well for 90 min at RT. The samples and standards were added in duplicate at 100 µL/well and the plates were incubated overnight at 4ºC. Subsequent incubation with 0.25 µg/mL at 100 µL/well of biotinylated detection antibody for TNF-α was performed for 120 min at RT. This procedure was followed by a 30-min incubation with streptavidin-peroxidase (Streptavidin, Zymed, Invitrogen) at 1:2,000 dilution with 100 µL/well. Tetramethylbenzidine (KPL Inc) was added and the plates were read at 630 nm after 15-min incubation.

Cytokine detection in cell culture supernatant by multiplex microbead immunoassay - A multiplex biometric immunoassay, which contains fluorescent microspheres conjugated with a monoclonal antibody specific for a target protein, was employed for cytokine and chemokine measurement according to the manufacturer's instructions (Upstate, Millipore) as described previously (Reis et al. 2007). Measured cytokines including the following: IFN-α, IL-6, IL-1Ra, IL-10, MCP-1/CCL-2, MIP-1β/CCL-4, IP-10/CXCL-10 and RANTES/CCL-5. Briefly, cell culture supernatants (50 µL) were incubated overnight at 4ºC with pooled antibody-coupled beads in bottom filter plates (Millipore). The complexes were washed and incubated with pooled biotinylated detection antibodies for 90 min at RT. Then, streptavidin-phycoerythrin was added for 30 min at RT prior to assessing cytokine concentration titres. A broad range (1.95-8,000 pg/mL) of concentrated human recombinant cytokines, provided by the vendor, was used to establish standard curves to maximise assay sensitivity and dynamic range. Cytokine levels were determined with a multiplex array reader from Luminex Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). The analyte concentration

was calculated by the software provided by the manufacturer (Bio-Plex Manager Software), which provided a regression analysis to derive the equation for cytokine concentration prediction in cell culture samples.

Ethics - Procedures performed in this work were approved by the Ethical Committee of the Fiocruz, Brazilian Health Ministry (recognised by the Brazilian National Ethics Committee) (111/00 and CAAE-0064 .0.011.000-07).

Statistical analyses - Data were first tested for normality with Prism version 4.0 for Windows (GraphPad Software). Flow cytometry data exhibited normal distributions, while normality was not detected for cytokine and chemokine production. To determine whether there were significant differences in viral antigen expression, data values were subjected to one-way analysis of variance followed by Tukey's multiple comparison test. Data from cytokine and chemokine assessments were submitted to a Wilcoxon signed rank test.

RESULTS

DENV2 and YF17DD infection kinetics in human MoDCs - Considering that DCs have been described as targets for DENV and YF vaccines (Barba-Spaeth et al. 2005, Neves-Souza et al. 2005) we investigated these host-virus interactions in detail. MoDCs that originated from healthy human PBMC donors exhibited characteristic downregulation of CD14 (1.8 ± 0.9%) and upregulation of DC-SIGN (75 ± 5%) and CD1a (54 ± 6%). These cells expressed low levels of CD80 and less HLA-DR,P,Q than MoDCs that were stimulated with bacterial lipopolysaccharides and human IFN-γ (data not shown). The MoDCs exhibit characteristics of immature DCs, which have the ability to perform endocytosis and capture antigens and are more susceptible to virus infection (Steinman & Nussenzweig 2002).

MoDC cultures were incubated with YF17DD vaccine virus at a dose either equivalent to that used for DENV2 (2.5 x 10^5 $TCID_{50}$/mL) or with a dose five times more concentrated. Fig. 2A shows histograms obtained by flow cytometry analysis after intracellular viral antigen labelling, representing data from cells incubated with the three different inocula at 24 h after infection. Fig. 1B shows percentages of viral antigen-positive cell (Ag^+) rates from 24-72 h post-infection (hpi). Both viruses were able to infect MoDCs, but higher inoculum doses of YF17DD were required to generate similar frequencies of infected cells compared with DENV2. The peak infection rate was detected at 24 hpi, although significant percentages were still detected at 48-72 hpi. Fig. 2C shows viral antigen detection in MoDCs originated from 11 different PBMC donors, confirming that DENV2 is significantly more infectious than YF17DD when the same MOI is used ($p < 0.0001$). Cells cultured with mock or heat-inactivated inocula contained less than 0.5% positive labelled cells PBMC donors in all assays (data not shown).

Except when mentioned, the assays described below were performed using YF17DD at the higher MOI, which induced similar rates of cell infection when compared to DENV2.

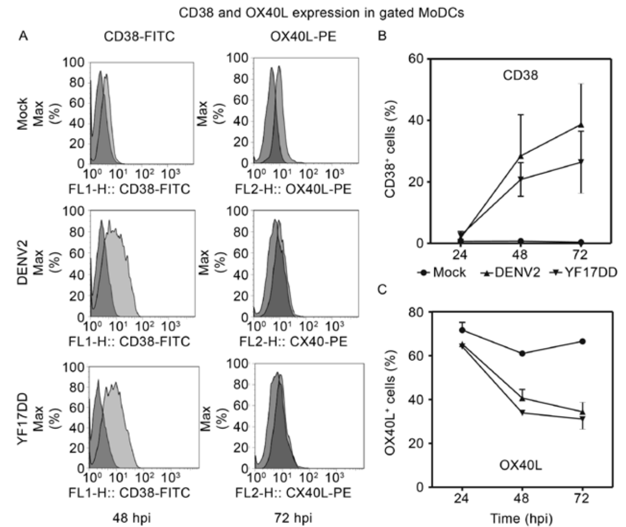

Fig. 2: CD38 and OX40L maturation markers expression during dengue virus 2 (DENV2) and yellow fever (YF) 17DD monocyte-derived dendritic cells (MoDCs) infection. MoDCs were infected with DENV2 [2.5 x 10^5 50% tissue culture infectious dose (TCID)/mL] or YF17DD (1.25 x 10^6 $TCID_{50}$/mL) or mock-infected. Cells were labelled with anti-CD38-FITC and anti-OX40L-PE monoclonal antibodies. A: flow cytometry patterns on gated MoDCs for CD38 at 48 h (left panel) and OX40L at 72 h (right panel) after in vitro incubation with mock, DENV2 or YF17DD. Overlapping histograms from labelled cells (light gray for CD38 or OX40L) and isotype control labelling (dark gray). FL1-H: level of fluorescence; hpi: hours post-infection; B: CD38 or OX40L (C) expression on MoDCs incubated with DENV2, YF17DD or mock. Data were calculated by statistically reducing the number of positive cells on isotype control from each sample (histogram by Overton Subtraction Method, Summit, Dako Automation). Assays were performed in triplicates for each of two peripheral blood mononuclear cells donors.

CD38 and OX40L activation/maturation markers are regulated during MoDCs infection with DENV2 and YF17DD - DCs interact with pathogens or exogenous molecules and undergo activation and maturation processes, resulting in reduced antigen processing capacity and increased expression of co-stimulatory and adhesion molecules. These molecules may induce tissue migration, antigen-specific immunological responses and Th polarisation (Quah & O'Neill 2005, Wallet et al. 2005). CD38 is involved in chemotaxis and calcium mobilisation and binds to CD31 on endothelial cells, facilitating DC migration through endothelium (Frasca et al. 2006). It may participate in the Th-1 response, which involves lymphocytes producing IL-12 and IFN-α. On the other hand, OX40L expression on DCs (Ohshima et al. 1997) may contribute to the Th-2 polarisation by enhancing IL-4 and IL-13 induction and suppressing IFN-α after it binds to OX40 on T cells (Ohshima et al. 1998, Delespesse et al. 1999).

MoDCs were cultured with DENV2, YF17DD or mock supernatants. Fig. 3A shows that CD38 was weakly expressed on infected cells but increased when fluorescence intensity virus was present. DC activation occurs between 48-72 hpi (Fig. 3B). MoDCs express OX40L early in infection, but this molecule was downregulated at 48 and 72 hpi (Fig. 3C). This shift is shown in the histograms from flow cytometry data (Fig. 3A), whereas the mock-infected cells remain unchanged and express OX40L.

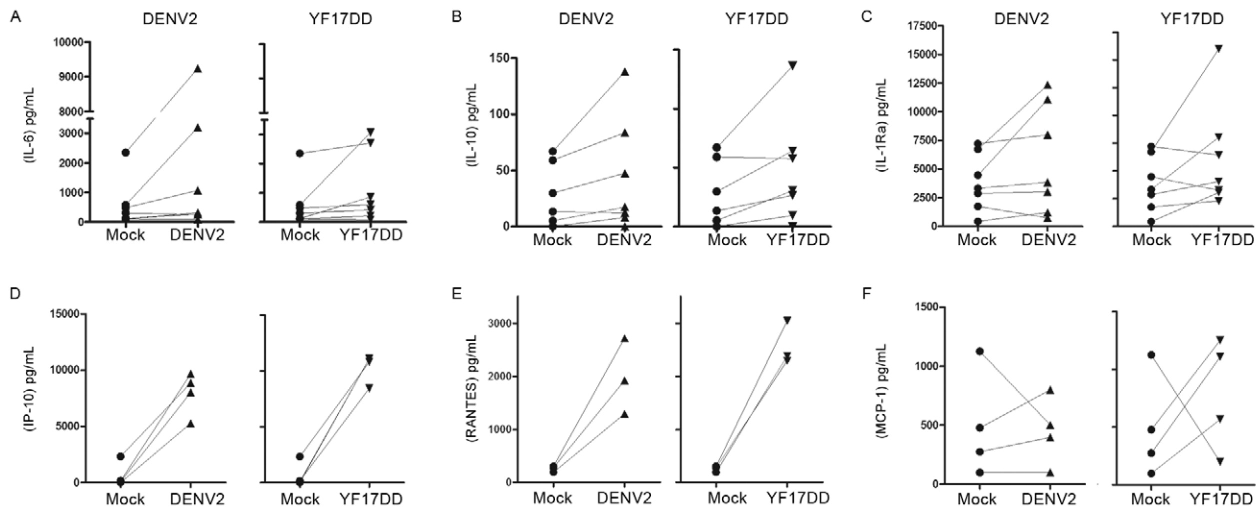

Fig. 3: multiple cytokine and chemokine induction by dengue virus 2 (DENV2) or yellow fever (YF) 17DD after monocyte-derived dendritic cells (MoDCs) infection. MoDCs were infected with DENV2 [2.5 x 10^5 50% tissue culture infectious dose (TCID)/mL], YF17DD (1.25 x 10^6 $TCID_{50}$/mL) or mock-infected. Supernatants were collected 48 h after infection and analyzed by immunofluorescent multiplex-bead assay. A-F: lines represent paired production for each donor of interleukin (IL)-6 (A), IL-10 (B), IL-1 receptor agonist (Ra) (C), CXCL-10/IP-10 (D), CCL-5/RANTES (E) and CCL-2/MCP-1 (F) induced after mock virus infection. Three-seven peripheral blood mononuclear cells donors were used.

The ratio of percentages of CD38 and OX40L-expressing cells was calculated for each PBMC donor. In the presence of DENV2, there was an increase from 0.49 ± 0.01 at 24 hpi to 1.90 ± 0.34 at 48 hpi. A similar pattern was observed for the YF17DD-infected cultures, with a ratio of 0.50 ± 0.15 at 24 hpi and one of 1.55 ± 0.03 at 72 hpi. For mock-infected cells this ratio remained below 0.5 (data from 2 cell donors). We therefore observed a change in the CD38 and OX40L balance during infection by both flaviviruses.

Several cytokines and chemokines are present in supernatants of virus-infected MoDCs - Cytokines and chemokines play important roles in dengue physiopathology (Bozza et al. 2008, Noisakran & Perng 2008). Since DCs are one of the main sources for these molecules early in viral infection, we investigated the production of several of them after MoDC infection by DENV2 and YF17DD from 24-120 hpi. We searched for the presence of inflammatory cytokines, chemokines and antiviral molecules that could drive T cell polarisation, either inducing vascular permeability or controlling infection. We detected TNF-α, IFN-α, IL-6, IL-1Ra, IL-10, MIP-1β/CCL-4, MCP-1/CCL-2, IP-10/CXCL-10 and RANTES/CCL-5 in cell culture supernatants. A mock-infected MoDc culture supernatant was also assayed.

Cytokines were detected after infection by both flaviviruses. IL-6 and IL-10 (Fig. 4A, B) were detected in cultured PBMC from seven donors at significant levels ($p < 0.5$, in a Wilcoxon signed rank test). These factors are known to modulate the immune response (Sabatte et al. 2007). IL-1Ra, the antagonist of the pro-inflammatory cytokine IL-1β and chemotactic factor IP-10 were also increased in most of the donors tested (Fig. 4C, D), although with borderline significance ($0.0624 < p < 0.0782$; in a Wilcoxon test). RANTES and MCP-1 are both chemokines related to severity of infection (Lee et al. 2006, Pulendran et al. 2008) and were only slightly increased after infection (Fig. 4E, F). However, low donor numbers did not allow us to perform reliable statistical analysis between the two viruses. MIP-1β/CCL-4, which is correlated with non-severe disease, was detected in mock cultures from seven PBMC donors and, apparently, its production was not altered after infection with either virus (data not shown). In vivo*,* other cells may be responsible for its synthesis and release.

Significant TNF-α amounts were already detected at 24 hpi in the presence of either virus when compared to the mock culture (data not shown), with a peak at 48 hpi and a decline thereafter in most PBMC donors tested (Fig. 5). DENV2 induced the highest TNF-α levels (255 ± 75 pg/mL), which were significantly higher when compared with those produced by YF17DD (121 ± 31 pg/mL) in eight different PBMC donors assayed at 48 hpi ($p = 0.0156$ in the Wilcoxon test).

In Fig. 6, IFN-α is plotted against viral Ag^+ cells during the course of infection in four different PBMC donors. At 24 hpi, IFN-α is already detected in YF17DD-infected MoDCs and at 48 hpi in DENV2-infected MoDCs. Virus load decreases as the IFN-α levels increase. MoDCs from seven donors were assayed side-by-side at 48 h with both viruses and those infected with YF17DD produced significantly higher IFN-α levels (393 ± 139 pg/mL) than those infected with DENV2 (72 ± 29 pg/mL; $p = 0.0156$ in a Wilcoxon test).

When the ratio of TNF-α and IFN-α levels for each cell donor is calculated, significantly higher indexes were obtained for DENV2 (13 ± 6) than for YF17DD (1.1 ± 0.3; $p = 0.0156$ in a Wilcoxon test).

As mentioned, data shown in Fig. 4B were generated with higher doses of YF17DD than DENV2 inoculum dose to obtain viral Ag^+ cells at the same rates. When

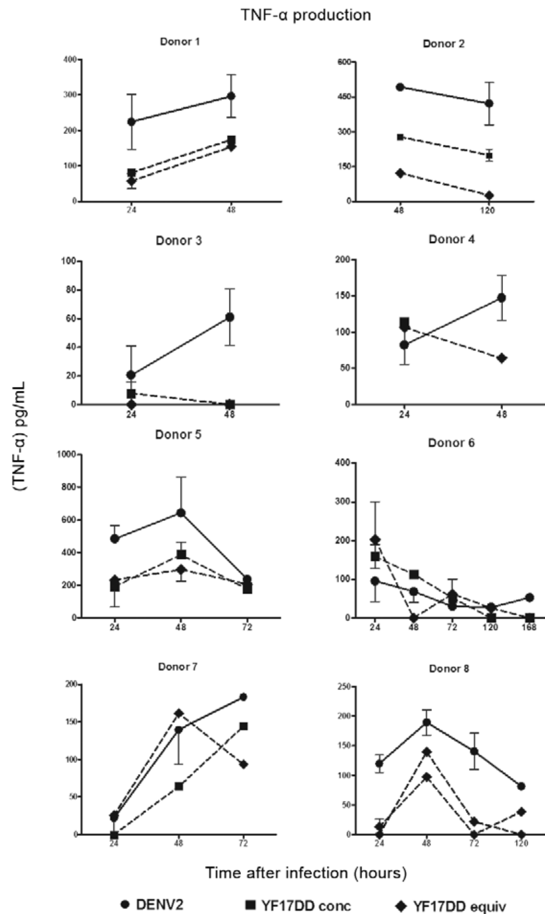

Fig. 4: tumor necrosis factor (TNF)-α induction by dengue virus 2 (DENV2) or yellow fever (YF) 17DD after monocyte-derived dendritic cells (MoDCs). MoDCs were infected with DENV2 or YF17DD equivalent (equiv) doses [2.5 x 10^5 50% tissue culture infectious dose (TCID)/mL] and YF17DD at concentrated (conc) dose (1.25 x 10^6 $TCID_{50}$/mL). Cultures were incubated from 24-168 h. TNF-α cell culture supernatant content was determined by enzyme-linked immunosorbent assay. Individual data for eight different peripheral blood mononuclear cells donors are shown. Mock-infected cells were included for each donor, but no detectable TNF-α was recorded (data not shown). Mean and standard error from on-three replicates were plotted.

YF17DD was inoculated at DENV2 equivalent MOIs (thus resulting in lower infection rates) (Fig. 2), IFN-α levels were 2-10 times higher in YF17DD cultures than those in DENV2. Therefore, the TNF-α vs. IFN-α cytokine imbalance has a different pattern in the two flavivirus studied irrespective of the MOI used.

YF17D/DENV2 vaccine virus infection compared with DENV2 or YF17DD infections - To investigate whether a chimeric dengue target vaccine virus could induce similar responses to the YF17DD vaccine virus, we performed pilot experiments infecting MoDCs with the YF17D/DENV2 vaccine virus. Cell infection rates were lower for the chimeric virus compared with the other two viruses (DENV2 16681 strain and YF17DD) (Fig. 6). IFN-α levels detected in experiments with this chimeric virus were slightly higher than those produced by DENV2, but these differences were not statistically significant (Wilcoxon signed rank test). However, TNF-α levels were significantly higher after DENV2 infection when compared with YF17D/DENV2 infection ($p = 0.0313$). Therefore, the chimeric vaccine virus apparently displayed a similar cytokine response profile to YF17DD, even though it had lower replication rates.

DISCUSSION

During either a natural infection or a vaccination, the entry site of flaviviruses into the vertebrate host is the dermis. Langerhans cells were characterised as permissive cells for DENV in vivo replication (Wu et al. 2000). Indeed, DCs have a crucial role in initiating host defence mechanisms; through antigen presentation and cytokine production, they can define the fate of the immunological response (Blanco et al. 2008). Both DENV and YF vaccine viruses are known to infect DCs and monocytes in vitro (Wu et al. 2000, Barba-Spaeth et al. 2005, Reis et al. 2007). We aimed to characterise the similarities and differences between flavivirus infections with distinct virulence patterns that elicit long lasting in vivo immunity (Monath 2001, Halstead 2007).

DC infections with the YF Brazilian vaccine strain 17DD and with YF17D/DENV2 (44/3) chimeric virus were reported here for the first time. The YF17DD vaccine virus infects cells at inoculum doses equivalent to those used for DENV2 (16681 strain) but showed reduced expression of viral antigens within DCs at all time points tested when compared to DENV. Similar results were described for the YF vaccine strain 204, which is poorly infectious compared with DENV (Deauvieau et al. 2007). Other authors used higher MOIs for DENV to obtain the same relative infection level as West Nile virus because these viruses replicate at different rates. They then studied NS1 antigenic expression by flow cytometry analysis (Youn et al. 2010).

DENV sequences from YF17D/DENV2 (44/3) studied here are from the NGC virus. Although our DENV2 originated from strain 16681, NGC and 16681 are very similar with regard to their genome and they belong to the same genotype - SE Asian strains - that characteristically induce the most severe forms of dengue fever (Leitmeyer et al. 1999). We do not intend to compare the virus structures but instead the virulence (pathogenic vs. vaccine).

Virulent YF wild-type or Asibi strains have not been reported to infect DCs, but a few studies compared these viruses with YF17DD during infection in Kupffer or endothelial cells (Khaiboullina et al. 2005, Woodson et al. 2011). Both viruses infect these cells, but the resulting cytokine production is quite different. The virulent YF strains produce a much more intense pro-inflammatory cytokine response (including TNF-α, IL-6, IL-8 and RANTES/CCL5) than the YF vaccine, which produces more IL-10.

During infection by viruses, DCs may undergo several phenotypic changes to become activated and capable of antigen presentation to T lymphocytes. Among these changes is the upregulation of surface markers and soluble molecules related to the polarisation of the T effector cell response. YF17DD immunisation leads to natural killer (NK) cell and monocyte activation (Martins et al. 2008). With respect to DENV and YF17D/

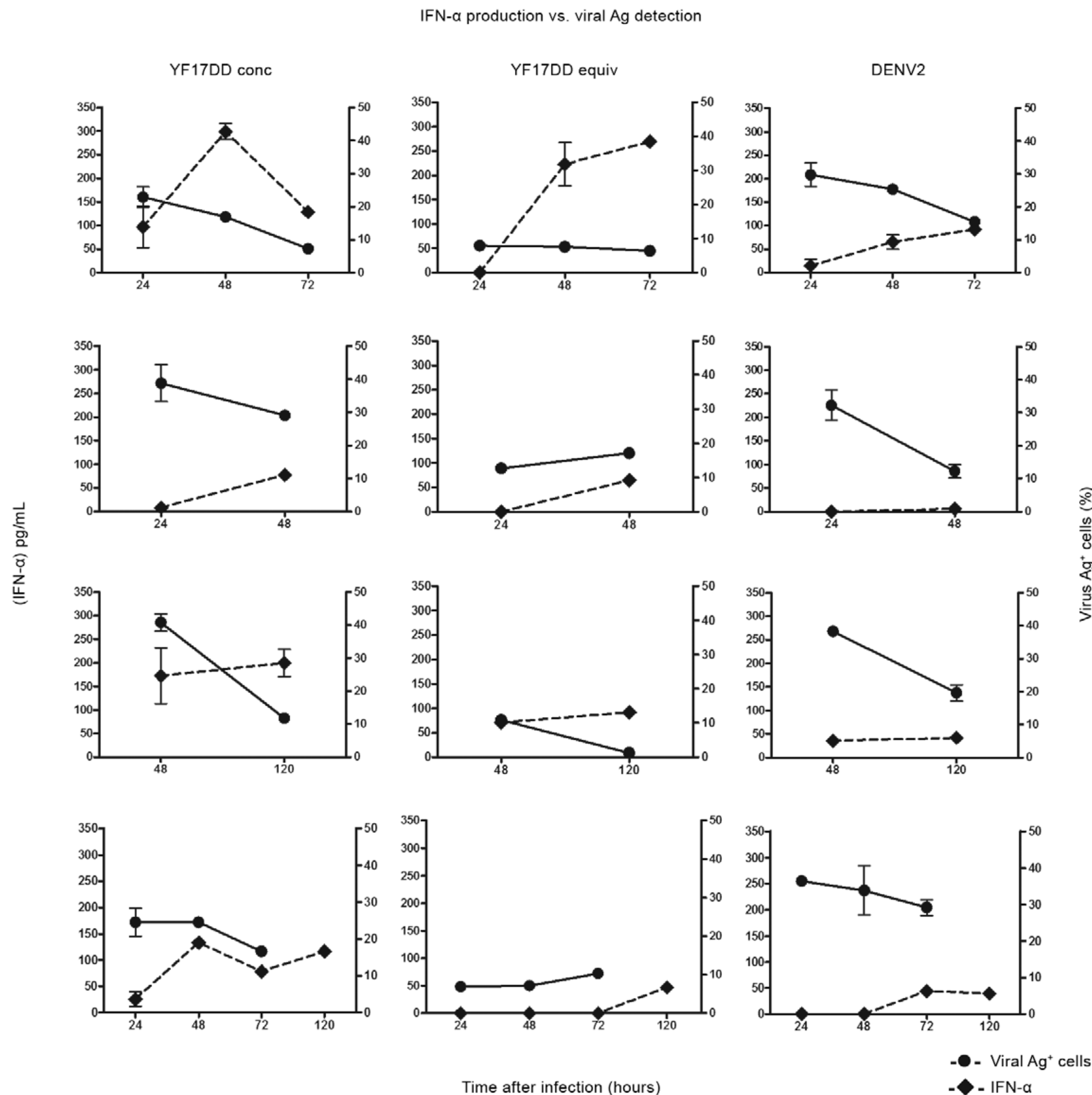

Fig. 5: viral antigen positive cells (Ag+) vs. interferon (IFN)-α production by monocyte-derived dendritic cells (MoDCs) after dengue virus 2 (DENV2) or yellow fever (YF) 17DD infection. MoDCs were infected with DENV2 or YF17DD equivalent (equi) doses [2.5 x 10^5 50% tissue culture infectious dose (TCID)/mL] or YF17DD at concentrated (conc) dose (1.25 x 10^6 $TCID_{50}$/mL). Cultures were incubated from 24-120 h. X axis represents time after infection. Left Y axis, the percentage of viral Ag+ cells obtained after flow cytometry analysis. Right Y axis, the IFN-α cell supernatant content determined by enzyme-linked immunosorbent assay. Individual data for four different peripheral blood mononuclear cells donors are shown. Mock-infected cells were included for each donor, but no detectable IFN-α was recorded (data not shown). Mean and standard error from one-three replicates were plotted.

DENV chimeric viruses, earlier reports showed that activation molecules, such as CD80, CD86 and CD83, can be upregulated after DC infection (Libraty et al. 2001, Deauvieau et al. 2007, Sun et al. 2009). Co-stimulation molecules, such as CD38, are present in circulating monocytes but are poorly expressed after their differentiation into immature DCs. During the maturation process, CD38 is re-expressed on DCs (Fedele et al. 2004). In the present study, we observed that flavivirus infection favours CD38 expression on DCs. This molecule is involved in cytoplasmatic calcium release, chemotaxis and IFN-γ production, indicating a role for CD38 in Th-1 polarisation (Frasca et al. 2006).

OX40L expression is a fundamental requirement for optimal induction of primary and memory Th-2 responses in vivo. It binds to OX40 on T lymphocytes and stimulates the appropriate expansion and/or survival of T cells, leading to IL-4 and IL-13 production (Jenkins et al. 2007, Blazquez & Berin 2008). Here we observed that the immature DCs expressed OX40L in culture and that this expression was downregulated as infection progressed in both viral infections. These data are informative with respect to Th-1/Th-2 axis determination by surface markers on DCs after flavivirus infection. CD38 upregulation and OX40L downregulation indicate that both viruses favour a Th-1 type response during the

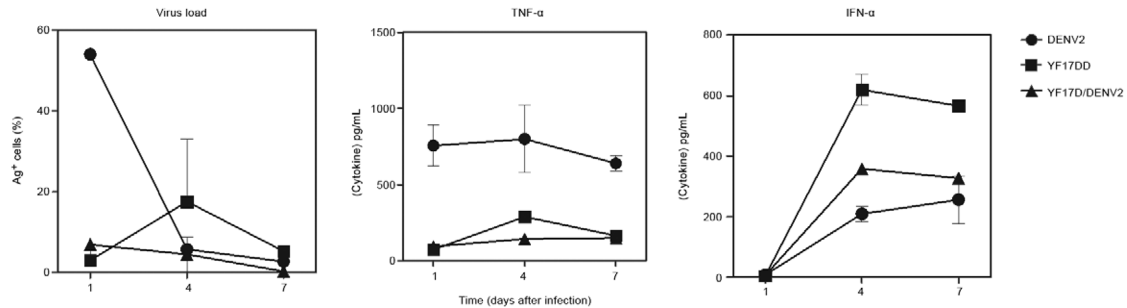

Fig. 6: viral antigen positive cells (Ag+), tumor necrosis factor (TNF)-α or interferon (IFN)-α production by monocyte-derived dendritic cells (MoDCs) after yellow fever (YF) 17DD/dengue virus 2 (DENV2) infections are compared to DENV2 or YF17DD infection. MoDCs were infected with YF17D/DENV2, DENV2 or YF17DD at multiplicity of infection 4 (4 x 10^6 plaque-forming unit/10^6 cells). Cultures were incubated from one-seven days (X axis: time after infection; Y axis: percentage of viral Ag+ cells obtained after flow cytometry analysis). TNF-α or IFN-α cell supernatant content are determined by enzyme-linked immunosorbent assay. Mean and standard error from duplicates were plotted. Mock-infected cells were included for each donor, but no detectable TNF-α or IFN-α was recorded and viral Ag+ cells were present at < 2% (data not shown). Data plotted were obtained from one representative peripheral blood mononuclear cells donor out of two independent performed experiments.

early immune response, which likely plays a role in virus clearance, although a Th-2 response may be present as well during infections by both viruses (Bozza et al. 2008, Querec et al. 2009).

Indeed, Th-1/Th-2 mixed cytokine patterns, in which an early Th-1 profile gives rise to a late Th-2-predominant pattern, likely occur during the course of a dengue fever infection (Chaturvedi et al. 2000, Mustafa et al. 2001, Nguyen et al. 2004). The difference in timing of IFN-γ peaks in plasma have influences the severity of disease. On the other hand, reports of an association between low T-bet mRNA expression and high IL-10 levels may indicate a Th-2 role in the pathogenesis of dengue hemorrhagic fever (Chen et al. 2005). YF17DD vaccination leads to a mixed pro/anti-inflammatory cytokine profile, including TNF+ monocytes, IFN-γ+ NK cells and IL-10+ cells (Silva et al. 2011).

Infection of DCs by DENV induces T cells to produce IL-4, IL-10 and IFN-γ, suggesting a mixed Th cytokine pattern (Ho et al. 2004). DCs infected with YF17DD stimulate CD8+ T cells from YF17DD-immunised or naive donors and induce IFN-γ and present antigen specifically to CD8+ and CD4+ T cells (Barba-Spaeth et al. 2005). In the present study, DC cultures infected with flaviviruses were able to produce a panel of several cytokines and chemokines, which were similar in both viral infections. It is likely that chemokines produced by DCs after flavivirus infections, such as IP-10, RANTES and MCP-1, might be signalling to monocytes, NK cells and Th-1 type lymphocytes, resulting in modulation of endothelial permeability, chemotaxis to the site of inflammation, migration to inflammatory sites and T cell polarisation (Aliberti et al. 2000, Thomsen et al. 2003, Nightingale et al. 2008). These cell subsets are crucial for viral clearance and efficient immunological response generation, but we cannot exclude the possibility that an excessive inflammatory reaction may result in exacerbation of disease severity.

The most striking differences between the DENV2 and YF17DD viruses were found in TNF-α and IFN-α production by DCs. TNF-α levels are significantly increased after DENV2 infection in DC cultures, but only slightly altered in the presence of YF17DD and YF17D/DENV2. On the other hand, these viruses were able to elicit high IFN-α levels that not reached during DENV2 infection.

TNF-α is a pro-inflammatory cytokine that has often been found in patients with acute dengue fever and high levels are associated with haemorrhagic manifestations (Hober et al. 1993, Kubelka et al. 1995, Braga et al. 2001, Chakravarti & Kumaria 2006). Together with IL-1β, TNF-α is known to increase acute phase protein production and act in synergy with other factors to induce microbicidal activity during phagocytosis (Clark 2007). It also has the capacity to induce the upregulation of endothelial adhesion molecules, which in turn signal to chemotactic peptides and lipid mediators and facilitate leukocyte recruitment, which may result in plasma leakage and hypovolemic shock. In our in vitro infection model, we observed markedly higher TNF-α production after infection with pathogenic virus (DENV2) as compared to the non-pathogenic viruses (YF17DD and YF17D/DENV2), providing further evidence that this cytokine plays a role in flavivirus immunopathogenesis. Other authors have also observed TNF-α production in DC or monocyte cultures in the presence of DENV2 and some authors even associate it with the infected cell (Hacker et al. 1998, Querec et al. 2006, Deauvieau et al. 2007, Reis et al. 2007, Ahmad et al. 2008, Nightingale et al. 2008, Levy et al. 2009). A moderate level of TNF-α production may be beneficial to mature cells that may become good antigen presenters. This cytokine was detected in YF vaccinated individuals (Roers et al. 1994, Querec et al. 2006, Deauvieau et al. 2007, Levy et al. 2009, Silva et al. 2011).

IFNs are classically known as antiviral molecules and some reports confirm that flaviviruses are susceptible to these molecules (Roers et al. 1994, Diamond et al. 2000). Indeed, these viruses stimulate IFN-α production (Libraty et al. 2001, Deauvieau et al. 2007, Palmer et al. 2007, Querec et al. 2009), although IFN-α induction was not compared between viruses in previous reports. Earlier studies show that DENV is able to escape IFN

Fig. 7: theoretical model for dendritic cell (DC)-flavivirus interaction. A: during target cell infection, dengue virus (DENV) is sensed/recognized by pattern recognition receptor (1), triggering preferentially pro-inflammatory genes such as for nuclear factor kappa B (NF-κB) (2), resulting in a high tumor necrosis factor (TNF)-α production (3) , while in B interferon (IFN)-α is partially inhibited by DENV proteins, such as NS4, through signal transducer and activator of transcription-2 expression (STAT) inhibition (4). Back to A, DENV infection outcome consists in a high viral load (5) followed by an enhancement of pro-inflammatory gene activation and induction of plasma leakage (6). In B, yellow fever (YF) vaccine virus induces preferentially the IFN pathway activating IRF-7 and IFN-stimulated response element (ISRE) (7) that further induce a high IFN-α production (8), resulting in paracrine or autocrine IFN-associated receptor (IFNAR)-dependent activation (9). In turn, IFN-stimulated genes are expressed (10) such as oligoadenylate synthetase (OAS), interferon (IFN) regulatory factor (IRF)-9, IFN-stimulated gene (ISG)-15, TNF-related apoptosis-inducing ligand (TRAIL), inducible nitric oxide synthase (iNOS), among others, contributing to a low virus load. Besides its antiviral effects on virus replication, a second IFN-α production wave, IFNAR-dependent (11), exert other functions such as: protecting bystander DCs from *de novo* infection (12); activating natural killer cells that are IFN-γ producers and efficient cytotoxic killers for infected-targets (13). IFNs can activate monocytes to produce nitric oxide (NO), another antiviral molecule. RIG-I: retinoic-acid-inducible gene I; TLR: Toll-like receptor.

action by inhibiting steps of IFN type I activation pathways through a decrease in the signal transducer and activator of transcription-2 expression (STAT-2) (Jones et al. 2005). This effect is meditated by the viral non-structural protein 4. Recently, low levels of IFN-α/β production by DENV2-infected MoDCs has been reported as playing a role in the failure of DCs to prime T cells (Rodriguez-Madoz et al. 2010).

In Fig. 7, we outline a theoretical model for flavivirus interactions with DCs. Flavivirus infection may trigger differential signalling pathways that lead to an early clearance of virus. These pathways might include an IFN pathway that activates IFN- regulatory gene (IRF)-7 and IFN-stimulated response element (ISRE), which in turn further induces high IFN-α and nitric oxide production. Alternatively, the blockage of antiviral pathways, such as STAT activation by non-structural viral proteins, may lead to higher virus load and intense stimulation of inflammatory/pathologic mechanisms such as TNF-α. The IFN-α produced by target cells may act on IFN-α receptors on bystander cells to protect them from further infections. Furthermore, IFN-α activates natural killer cells, which have cytotoxic and antiviral functions and produce IFNs that in turn can activate monocytes to exert antiviral functions through nitric oxide production.

Here we observed that DCs infected with YF17DD and YF17D/DENV2 produce more IFN-α than those infected with DENV2. These data suggest that the vaccine viruses may have different virulent properties than DENV2 and that they may have lost (or never acquired) the ability to inhibit the ISRE pathway that was hypothesised to play a role in DENV infection in Fig. 7. YF

vaccination in human subjects studied by microarray analysis showed a response profile related to IFN-based antiviral responses (Scherer et al. 2007, Querec et al. 2009), suggesting a role for IFNs in effective protection from this vaccine. On the other hand, functional genetic studies identified several transcripts for IFN-stimulated genes in patients with dengue fever that were less abundant in adults with dengue shock syndrome, reinforcing the attenuating role of IFNs after infection (Simmons et al. 2007). Vaccine or therapeutic approaches may indeed provide the ability to induce antiviral molecules such as IFNs without inducing or modulating factors involved in vascular permeability.

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