Dengue Virus Serotype-2 Impairs Proliferation of Healthy Donors' T Lymphocytes

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Key Words

T lymphocytes · Dengue virus · Cell proliferation · NF-AT · NF-κB · Intracellular Ca²⁺

Abstract

Objectives: T lymphocytes are not infected by dengue virus (DENV), nevertheless it is possible that exposure to DENV may affect their function. T lymphocytes from DENV-infected individuals are impaired in their proliferative capacity, although this effect has been attributed to altered function of antigen-presenting cells rather than to an intrinsic defect on T lymphocytes. Here we analyzed whether T lymphocytes from healthy donors became impaired in their proliferative capacity following in vitro exposure to DENV serotype-2 (DENV-2), as well as the possible mechanisms for this. **Methods:** Isolated CD4⁺ and CD8⁺ T lymphocytes from healthy donors were in vitro exposed to DENV-2, before polyclonal activation, cell proliferation, IL-2 synthesis. IL-2Rα expression, nuclear translocation of NF-AT and NF-κB, and intracellular calcium flux were assessed. **Results:** In vitro exposure of both CD4⁺ and CD8⁺ T lymphocytes from healthy donors to DENV-2 impairs cell proliferation, IL-2 synthesis, and IL-2Rα (CD25) cell membrane expression. Signalling wise, exposure to DENV-2 impairs the nuclear translocation of NF-AT, downstream of intracellular calcium mobilization, as well as that

of NF-κB. **Conclusion:** In the course of a dengue infection, direct exposure of T lymphocytes to DENV could affect cell-mediated immune responses.

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Introduction

Dengue is caused by any of the four dengue virus (DENV) serotypes – 1, 2, 3 or 4. The World Health Organization estimates that these viruses cause 100–500 million infections each year in over 100 countries, resulting in 20,000 deaths annually [1]. The more severe forms of this viral infection are dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2, 3]. The mechanisms that lead to these clinical outcomes are unknown, however there is growing evidence that the pathophysiology of dengue is mediated by host immune response [2, 4]. Several lines of evidence indicate that anti-dengue antibodies [5–7], cytokines [8–10], soluble immune mediators [11–13] and cross-reactive memory T lymphocytes [14–16], along with high dengue viral loads [17], contribute to the progression to DHF/DSS and death [18, 19].

T lymphocytes play both effector and regulatory roles in the innate and adaptive immune responses, and several studies have shown that T lymphocytes are involved in the development of DHF/DSS [20]. In particular, cross-

reactive memory T lymphocytes have been associated with changes in the vasculature, leading to vascular leakage [21–23], and activation of T lymphocytes during viremia, which is more evident in DHF, is reflected in the overproduction of cytokines [24]. Furthermore, CD4+ and CD8+ T-lymphocyte clones that proliferate in response to DENV antigens in a serotype cross-reactive fashion have been isolated from dengue patients [25–27], thus suggesting that dengue serotype cross-reactive T lymphocytes may contribute to dengue pathogenesis.

On the other hand, the role of activated T lymphocytes in the pathology of DENV infection contrasts with the observed drop in the absolute numbers of T lymphocytes during the acute phase of DENV infection [28, 29]. For example, a study that compared the cellular immune status of patients with DHF, dengue fever, and non-dengue viral infections showed a reduction of total CD3+, CD4+ and CD8+ T lymphocytes in DHF but not in the other two groups [30]. In addition, T lymphocytes isolated from acute DENV-infected patients are impaired in their proliferative response to in vitro mitogenic stimulation [31].

Several cell types have been identified as targets for DENV [32], mainly monocytes [33, 34], macrophages [35, 36], dendritic cells [37, 38], Langerhans' cells [39], and B cells [40]. Attempts to determine if DENV infects T lymphocytes have been made and results have been contradictory [41–43].

Here we have analyzed the consequences of the in vitro exposure of T lymphocytes isolated from healthy donors to DENV-2. We found that DENV-2 reduces the proliferative capacity of T lymphocytes in response to concanavalin A (ConA), by impairing the activation of NF-AT, and NF- κ B. Impairment of these two transcription factors correlated with lower IL-2 synthesis and lower IL-2R α expression by T lymphocytes. Activation-induced calcium influx was not impaired. Together these findings suggest that DENV-2 directly inhibits T-lymphocyte activation by interfering with cell activation pathways downstream of calcium mobilization and that alterations in cell-mediated immunity during the course of DENV infections cannot be exclusively attributed to the 'classical' DENV cell targets, i.e. monocytes and dendritic cells.

Materials and Methods

Dengue Virus

DENV-2, strain New Guinea, was a kind gift of InDRE (Mexico). Virus was propagated in *Aedes albopictus* C6/36 cells. When cell cultures reached about 80% of the cytopathic effect,

cells were lysed by freezing and thawing and, after centrifugation at 4,000 g for 10 min, the supernatants were collected. DENV-2 was purified by centrifugation of 2 vol (8 ml/tube) of supernatant over 1 vol (4 ml/tube) of 30% sucrose in PBS at 38,000 rpm in a SW40Ti rotor (Beckman) for 3.5 h. The DENV-2-containing pellet was recovered and washed with PBS by centrifugation at 38,000 rpm in a SW40Ti rotor for 1 h. Viral titer was assessed by the cytopathic effect on Vero cells and expressed as plaque-forming units/ml (pfu/ml). The titer of DENV-2 stock was adjusted to 1.5×10^6 pfu/ml.

Cell Isolation and Cell Proliferation

Peripheral blood mononuclear cells were obtained by differential centrifugation over Ficoll-Paque (Invitrogen) from buffy coats from healthy donors. CD3+, CD4+ and CD8+ T lymphocytes were purified by a magnetic-based negative selection approach (human pan T-cell isolation kit II, CD4+ T-cell isolation kit II, and human CD8+ T-cell isolation kit II, respectively, from Miltenyi Biotec). CD4+ and CD8+ T-lymphocyte purity was assessed by flow cytometry by using FITC-labelled anti-human CD4, FITC-labelled anti-human CD8, and PerCP-labelled anti-human CD3 (BD Biosciences). T-cell proliferation in response to ConA (Sigma) was assessed in carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) loaded cells stimulated with ConA (5 μ g/ml). Briefly, prior to stimulation, 1×10^7 cells/ml were incubated in 5 μ M CFSE for 8 min at 37°, after which the excess of CFSE was quenched by adding an equal volume of FBS for 5 min. Cells were washed, adjusted to a cell concentration of 1×10^6 /ml, stimulated, and then cultured in serum-free AIM-V medium in U-bottomed 96-well culture plates (Corning). In order to assess the effect of DENV-2 on T-cell proliferation, T lymphocytes were incubated in the presence of DENV-2 at a multiplicity of infection (MOI) of 10 for 2 h before the addition of ConA. Cells were incubated at 37° for 5 days and the diminution of CFSE mean fluorescent intensity, as indicative of cell proliferation, was analyzed by flow cytometry. Results are expressed as the percentage of cells with low CFSE mean fluorescent intensity.

IL-2 Synthesis

The amount of IL-2 was assessed in the culture supernatants of 24 h activated CD3+ T lymphocytes by ELISA. Briefly, 1×10^6 cells/ml/well in 12-well plates were (a) left unstimulated, (b) incubated in the presence of DENV-2, (c) preincubated with DENV-2 and then stimulated with 5 μ g/ml of ConA, or (d) stimulated with 5 μ g/ml of ConA alone. After 24 h of incubation at 37°, 100 μ l of the culture supernatants (in triplicate) were collected and diluted 1:2 in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 0.2 g/l NaN₃, pH 9.6), transferred to MaxiSorb ELISA plates (Nunc) and incubated overnight at 37°. Next, the plates were washed 3 times with 0.5% Tween-20 in PBS (PBS-T) blocked with 3% non-fat powder milk in PBS-T for 1 h at 37°, and then incubated with 1 μ g/ml of anti-IL-2 monoclonal antibody (200 μ l/well) (Sigma) for 2 h. The plates were then washed with PBS-T and 200 μ l of PO-labelled secondary antibody (1 μ g/ml) was added. Plates were further incubated for 2 h, washed 3 times with PBS-T, and finally 100 μ l of freshly prepared peroxidase substrate (Sigma) was added. After 30 min, the enzymatic reaction was stopped by adding 30 μ l of 12 N H₂SO₄. The optical density, indicative of the amount of IL-2 in the supernatants, was read at 492 nm in an ELISA plate reader (Lab Systems).

IL-2R α Expression

Expression of IL-2R α (CD25) was evaluated by flow cytometry. CD3+ T lymphocytes were (a) left unstimulated, (b) incubated in the presence of DENV-2, (c) preincubated with DENV-2 and then stimulated with 1 ng/ml PMA + 200 ng/ml ionomycin, or (d) 1 ng/ml PMA + 200 ng/ml ionomycin. 48 h after mitogen stimulation, the cells were harvested, washed with PBS, and simultaneously stained with PE-conjugated anti-human CD25 mAb (BD Biosciences) (1 μ g/ml) and with PerCP-conjugated anti-human CD3 (BD Biosciences) (1 μ g/ml) at 4° for 30 min. The cells were then washed with 0.01% BSA, 0.01% NaN₃ in PBS and analyzed by flow cytometry (Becton-Dickinson). The results are expressed as the percentage of CD25+ cells in the CD3+ population.

Transcription Factor Activation Assays

Nuclear translocation of NF-AT and p65 NF- κ B was assessed by confocal microscopy (LSM5 Pascal; Zeiss), for which CD4+ and CD8+ T lymphocytes were independently incubated in the presence of DENV-2 at a MOI of 10 for 2 h and then with ConA for 16 h, when indicated. Cells were then fixed and permeabilized (Cytofix/Cytoperm; BD Biosciences) and stained with anti-NF-AT mAb (Santa Cruz Biotechnology) or anti-p65 NF- κ B rabbit polyclonal antibody (Santa Cruz Biotechnology) at a final concentration of 1 μ g/ml, followed by FITC-labelled secondary antibody. Cells were spun down on glass slips by using a cytospin and then mounted with DAPI-containing Vectashield (Vector) and analyzed. The percentage of cells showing nuclear translocation of NF-AT or NF- κ B was calculated from several microscopic fields from two independent experiments.

Calcium Flux

CD3+ T lymphocytes (1 $\times$ 10⁷/ml) were loaded with Fluo-4 (Invitrogen) at a concentration of 3 μ M for 30 min at 37°. After washing with PBS the cells were adjusted at a concentration of 1 $\times$ 10⁶ cells/ml in RPMI-1640 medium supplemented with 2 mM CaCl₂. Mean fluorescent intensity was measured by flow cytometry (FACScan; Becton-Dickinson) and recorded over 30 s (base level) and then ConA at a final concentration of 5 μ g/ml was added and the mean fluorescent intensity (as indicative of intracellular calcium concentration) was recorded over a period of approximately 400 s. Raw data (CellQuest software; BD Biosciences) was analyzed by FlowJo version 8.7 software (Tree Star, Inc.).

DENV-2 Virus Attachment to T Lymphocytes

In order to assess DENV-2 virus interaction with T lymphocytes, freshly isolated T lymphocytes were cultured in serum-free AIM-V medium in the presence of DENV-2 at a MOI of 1 for 30 min, 60 min, 1 h, and 2 h. Cells were fixed with 1% paraformaldehyde and then stained with anti-E (DENV envelope protein) mouse mAb (Santa Cruz Biotechnology) (0.5 μ g/ml in 1% powdered milk-PBS), followed by anti-mouse IgG-FITC (Santa Cruz Biotechnology). T lymphocytes with no DENV-2, stained under the same protocol, were used as a negative control. Cells were mounted in DAPI-Vectashield (Vector) and observed in a LSM5 Pascal confocal microscope (Zeiss). A 2-hour incubation with DENV-2 proved to be the optimal time to analyze virus attachment to T lymphocytes. In addition, in order to quantify DENV-2 attachment to T lymphocytes, fixed cells treated as described were analyzed by flow cytometry (BD Biosciences). In the latter case, the percentage of CD3+ cells that harbor DENV-2 envelope (E) pro-

tein was assessed by dual staining, i.e. anti-E mAb, followed by anti-mouse IgG-FITC + anti-CD3-PE. Data was analyzed using CellQuest software (BD Biosciences).

ConA Binding to T Lymphocytes

Purified T lymphocytes were incubated in the presence of DENV-2 for 2 h at 37° in 5% CO₂ atmosphere. After this, cells were washed with PBS or left unwashed and labelled with ConA-biotin (5 μ g/ml) (Sigma) for 40 min followed by Streptavidin-FITC (Sigma) for 60 min with a washing step in between. Finally, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. The percentage of ConA-labelled cells was assessed by flow cytometry (BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Results

CD4+ and CD8+ T Lymphocytes Are Susceptible to DENV-2-Dependent Inhibition of ConA-Induced Cell Proliferation

CD4+ and CD8+ T lymphocytes were independently isolated from the same blood donors. Cell proliferation was quantified after 5 days in culture. Figure 1a shows that T lymphocytes incubated in medium alone or in the presence of DENV-2 did not proliferate, whereas stimulation with ConA induced a significant cell proliferation. A 2-hour incubation of T lymphocytes with DENV-2 before mitogen addition diminished the cell proliferation as compared to cells treated with the mitogen in the absence of DENV-2. These experiments show that both CD4+ and CD8+ T lymphocytes are impaired in their proliferation capacity if they were previously exposed to DENV-2. Figure 2b shows a representative example of the purity of CD4+ and CD8+ T lymphocytes used for these experiments.

DENV-2 Inhibits the Synthesis of IL-2 in Mitogen-Stimulated T Lymphocytes

IL-2 synthesis is a hallmark of T-cell activation and plays an important role in T-cell proliferation. It has been previously shown that several viruses such as human herpesvirus 6 and hepatitis C inhibit IL-2 synthesis and T-lymphocyte proliferation [44, 45]. In order to explore if a similar mechanism is at play in T lymphocytes exposed to DENV-2, we evaluated the production of IL-2 in the supernatants of mitogen-activated T lymphocytes as well as in T lymphocytes exposed to DENV-2 prior to mitogen stimulation. Figure 2 shows that the synthesis of IL-2 was impaired in T lymphocytes exposed to DENV-2 before ConA stimulation as compared with the synthesis of IL-2 in T lymphocytes stimulated with ConA in the absence of DENV-2.

Fig. 1. CD4+ and CD8+ T lymphocytes are susceptible to DENV-2-dependent inhibition of ConA-induced cell proliferation. CD4+ and CD8+ T lymphocytes were independently isolated from the same blood donors. T lymphocytes were labelled with CFSE and then split into four culture conditions: medium alone, ConA, DENV-2, and DENV-2 for 2 h and then ConA. Cells were incubated at 37° for 5 days and the CFSE dilution (cell proliferation) was assessed by flow cytometry. **a** Representative CFSE dilution profile for CD4+ and CD8+ T-lymphocyte proliferation. **b** Purity of CD3+ CD4+, and CD3+ CD8+ T lymphocytes. Results are representative of three independent experiments.

Fig. 2. DENV-2 impairs ConA-induced synthesis of IL-2 by T lymphocytes. T lymphocytes were cultured at 37° for 24 h in the presence of medium alone, DENV-2 from C6/36 supernatant, ConA or DENV-2 + ConA. After this time, cell supernatants were harvested and assayed for IL-2 content. Results are expressed as pg/ml $\pm$ SD. Four independent experiments were carried out. * $p < 0.01$ (Student's t test).

DENV-2 Inhibits the Expression of IL-2Ra

Concomitant to the synthesis of IL-2, activated T lymphocytes express the IL-2Ra receptor (CD25) on their cell membrane, thus engaging themselves in a feedback loop of cell activation and proliferation [46]. Therefore, we wanted to evaluate if DENV-2 is able to interfere with this process. In preliminary experiments (data not shown) we observed that PMA/ionomycin performs better than ConA for IL-2Ra expression. Figure 3 shows that DENV-2 interferes with IL-2Ra (CD25) expression in PMA/ionomycin-activated T lymphocytes. In the absence of PMA/ionomycin, DENV-2 by itself did not induce any significant change in the expression of CD25.

Activation of NF-AT and NF- κ B Is Impaired by DENV-2

By using a confocal microscopy approach, we evaluated the effect that the in vitro exposure of DENV-2 has on the activation status of NF-AT and NF- κ B on ConA-stimulated T lymphocytes. As shown, the nuclear translocation of NF-AT (fig. 4a) and NF- κ B (fig. 4b) was impaired when T lymphocytes had been exposed to DENV-2 prior to ConA stimulation.

Intracytoplasmic Calcium Fluxes in T Lymphocytes Are Not Impaired by Pre-Exposure to DENV-2

T-cell activation is largely dependent on intracellular calcium fluxes [47] and calcium flux is a key factor for activation of NF-AT [48, 49]. After finding that T lymphocytes that had been exposed to DENV-2 are impaired in their proliferative response to ConA, in the synthesis of IL-2, in the expression of IL-2Ra, and in the activation of NF-AT, we wondered whether these effects were due to impairment in the ability of T lymphocytes to mobilize calcium. Basal intracellular calcium concentration in T lymphocytes maintained in culture medium alone or pre-exposed to DENV-2 was recorded for 30 s and after stimulation with ConA, calcium fluxes were followed over a period of about 400 s. Figure 5 shows that DENV-2 did not inhibit ConA-induced mobilization of intracellular calcium. Moreover, a discreet but consistent increase in intracellular calcium concentration was observed in DENV-2 pre-exposed T lymphocytes upon ConA stimulation, as compared to that in unexposed T lymphocytes. However, the time-course response was very similar in both cases.

DENV-2 Attachment to T Lymphocytes

DENV-2 attachment to T lymphocytes was assessed by staining DENV-2 envelope protein with anti-E moAb followed by FITC-labelled secondary antibody and confocal

Fig. 3. DENV-2 impairs activation-induced expression of IL-2R α (CD25) in T lymphocytes. T lymphocytes were cultured at 37° for 48 h in the presence of medium alone, DENV-2 from C6/36 supernatant, PMA/ionomycin or DENV-2 + PMA/ionomycin. After this time, cells were harvested and stained with anti-CD3-PerCP and anti-CD25-PE monoclonal antibodies. CD25 expression on CD3+ cells was determined by flow cytometry. **a** Representative histograms of CD25 expression in the CD3+ cell population. **b** Percentage of CD25+ cells in the CD3+ population $\pm$ SD (n = 4). * p < 0.01 (Student's t test).

microscopy (fig. 6a). In order to quantify DENV-2 attachment to T lymphocytes, purified T lymphocytes were exposed to DENV-2 and then double stained for CD3 and DENV-2 envelope (E) protein expression. DENV-2 does bind to the cell membrane of T lymphocytes. The percentage of CD3+ T lymphocytes harboring viral particles was only about 2%, as assessed by flow cytometry (fig. 6b).

Binding of ConA to T Lymphocytes Is Not Impaired by Pre-Exposure to DENV-2

In order to test the possibility that the observed DENV-2 inhibitory effects on ConA-induced T-lymphocyte activity was due to DENV-2-mediated inhibition of ConA binding to the cognate receptor of T lymphocytes, purified T lymphocytes were labelled with ConA-biotin followed by Streptavidin-FITC after appropriate culture conditions. Data showed that preincubation of T lymphocytes with DENV-2 did not inhibit binding of ConA, and that ConA binding to T lymphocytes proceeds just as well whether DENV-2 is washed away or not, previous to ConA-biotin/Streptavidin-FITC labeling (fig. 7).

Discussion

There is increasing evidence that the immune system plays a key role in determining the severity of dengue disease [18, 19], in particular the array of cytokines produced during infection [8–10, 24]. The role of T lymphocytes in this process is controversial since on the one hand there is evidence that activation of T lymphocytes leads to pro-inflammatory cytokine synthesis [20–27] and on the other there is evidence that T-lymphocyte proliferation in dengue patients is impaired [28–31] and that patients with acute DENV infection are leukopenic [28, 29]. Additionally, there are contradictory results as to whether T lymphocytes can be infected or not with DENV [41–43]. In our hands, T lymphocytes are not infected with DENV-2 (data not shown).

We show here that isolated CD4+ and CD8+ T lymphocytes from healthy donors are compromised in their proliferative response to a polyclonal stimulus (ConA) if they are preincubated with DENV-2 in vitro. Previously, impairment of T-lymphocyte proliferation had only been observed in dengue patients but this was attributed to a defect in antigen-presenting cells rather than to an intrinsic defect on T lymphocytes [31].

In addition, we show that DENV-2 preincubated T lymphocytes also fail to synthesize IL-2 and to express IL-2R α (CD25) in response to ConA or PMA/ionomycin, respectively. This is in keeping with the previous observation that T cells that are deficient in IL-2 synthesis and IL-2R expression, fail to proliferate in response to adequate stimulation, as in the case of HIV-1, hepatitis C and herpesvirus infections [44, 45, 50, 51].

In order to examine the possible mechanism(s) that could account for the observed DENV-2-induced functional defects on T lymphocytes, we analyzed the nuclear

Fig. 4. DENV-2 impairs activation of NF-AT and NF-κB. Freshly isolated T lymphocytes were cultured in Lab-Tek chambers and treated as indicated. Cells were fixed, permeabilized and stained with the corresponding anti-NF-AT or anti-p65 NF-κB antibodies followed by fluorochrome-labelled secondary antibodies. Cells

were mounted with DAPI-Vectashield and then analyzed by confocal microscopy for intracellular localization of (a) NF-AT and (b) NF-κB in cells treated with DENV-2. Images show representative results from multiple microscope fields out of 2–4 independent experiments.

Fig. 5. DENV-2 does not impair T-lymphocyte calcium mobilization. Freshly isolated T lymphocytes were cultured in medium alone or in the presence of DENV-2 for 2 h, labelled with the calcium indicator Fluo-4 and then washed and suspended in culture medium containing 2 mM CaCl_2 . Mean fluorescence intensity as indicative of intracellular calcium concentration was recorded for 30 s by flow cytometry and at this time ConA was added and intracellular calcium concentration was further recorded for approximately 400 s. **a** Calcium flux (representative raw data) in response to ConA stimulation when sucrose-purified DENV-2 + ConA was used. **b** FlowJo data analysis of calcium flux raw data. Representative experiments out of three independent experiments.

Fig. 6. DENV-2 attachment to T lymphocytes. Freshly isolated T lymphocytes were cultured in Lab-Tek chambers in medium alone or in the presence of DENV-2 at a MOI of 1 for 2 h, cells were then washed, fixed with 1% paraformaldehyde, washed again and stained with anti-E (DENV envelope protein) mouse mAb, followed by anti-mouse IgG-FITC. Cells were mounted with DAPI-Vectashield and then analyzed by confocal microscopy. In addition, T lymphocytes cultured in medium alone or in the presence of DENV-2 at a MOI of 1 for 2 h were stained for DENV-2 envelope protein (anti-E mAb + anti-mouse IgG-FITC) and for CD3 (anti-CD3-PE). **a** Confocal microscope images showing binding of DENV-2 to T lymphocytes. **b** Percentage of T lymphocytes with attached DENV-2 as assessed by flow cytometry. Results are representative from three independent experiments.

translocation of NF-AT and NF- κ B which are transcription factors required for IL-2 and IL-2R α synthesis and expression [52, 53]. Our results showed that the nuclear translocation of NF-AT and NF- κ B was reduced in DENV-2 pretreated T lymphocytes as compared with the nuclear translocation in T lymphocytes stimulated with ConA in the absence of DENV-2 (fig. 4).

Nuclear translocation of NF-AT is largely dependent on intracytoplasmic calcium concentration [48, 49]. Therefore, we evaluated the calcium flux in response to ConA in order to determine if the observed DENV-2-induced impairment in NF-AT activation was related to impaired calcium flux. We found that DENV-2 did not impair calcium flux over a time frame of about 400 s post-

Fig. 7. Binding of ConA to T lymphocytes is not impaired by pre-exposure to DENV-2. Freshly isolated T lymphocytes were incubated for 2 h in medium alone or in the presence of sucrose-purified DENV-2 at a MOI of 1, cells were then washed or left unwashed and stained with ConA-biotin, followed by Streptavidin-FITC. After washing, cells were fixed with 1% paraformaldehyde in PBS and ConA binding was assessed by flow cytometry. Histograms depict the percentage of ConA-positive cells. Results are representative of three independent experiments.

stimulation (fig. 5); on the contrary, a small increase in intracytoplasmic calcium concentration was observed in DENV-2/ConA- as compared to ConA-stimulated T cells.

To the best of our knowledge, calcium fluxes in T lymphocytes exposed to DENV-2 had not been previously explored. However, it has been shown, for instance, that the expression of the Epstein-Barr virus latency-associated membrane protein LMP2A in B lymphocytes diminishes the calcium influx in response to anti-IgM, anti-MHC class II, or anti-CD19 treatments [54], and that herpesvirus K7 protein increases the intracellular calcium concentration by interacting with calcium-modulating cyclophilin ligand [55]. More recently, DENV-2 capsid (C) protein has also been shown to interact with calcium-modulating cyclophilin ligand, thus subverting apoptosis in Huh-7 DENV-2-infected cells [56]. Thus it seems that DENV-2 modifies cell responses by modulating calcium flux, the mechanism of which remains to be analyzed.

There is evidence that T lymphocytes are not infected by DENV, so we wondered how DENV-2 pre-exposure could impair the activation-induced proliferation as well as disrupting associated signalling pathways. In trying to address this we assessed whether DENV-2 is capable of attaching to the cell membrane of T lymphocytes and found that it does, although at a given time only about 2–3% of T lymphocytes harbor attached viral particles, as assessed by confocal microscopy and flow cytometry (fig. 6). Experiments carried out with UV-irradiated DENV-2 virus particles showed that they performed just as well as fully active DENV-2 (data not shown), thus suggesting that a receptor engagement mechanism rather than productive infection may be responsible for the observed DENV-2 biological effects on T lymphocytes.

Since only about 2–3% of T lymphocytes were found to harbor DENV-2, whereas T-lymphocyte proliferation, IL-2R α expression and IL-2 synthesis were reduced by about 50%, we are tempted to speculate that DENV-2 binds with low affinity and high off-rate to a still unknown receptor on T lymphocytes, that this interaction is enough to impair T-lymphocyte activation and that as DENV-2 is released from one such receptor, it is able to sequentially engage the receptors of nearby T lymphocytes, in analogy with the serial engagement model as proposed for the T-cell receptor triggering by peptide-MHC complexes [57, 58].

Finally, since it has been shown that ConA has affinity for DENV-2 envelope (E) protein [59], assessment of

ConA binding to purified T lymphocytes in the presence of DENV-2 was carried out. Results (fig. 7) ruled out the possibility that, under the different culture conditions described here, DENV-2 interferes with ConA binding to these cells and therefore we conclude that the inhibitory effects of DENV-2 on T lymphocytes take place downstream of ConA engagement to its cognate receptor on T-lymphocyte cell membrane. Moreover, IL-2R α expression was assessed by stimulating T lymphocytes with PMA/ionomycin, which in preliminary experiments showed to induce higher levels of expression than ConA. DENV-2 was therefore capable of impairing ConA- as well as PMA/ionomycin-induced responses.

In conclusion, we have shown here that exposure of CD4+ and CD8+ T lymphocytes from healthy donors to DENV-2 impairs the ConA-induced proliferation, and that this effect is dependent on impairment of the synthesis of IL-2 and the expression of IL-2R α by interfering with the activation of NF-AT and NF- κ B downstream of calcium mobilization.

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