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Effect of high doses of vitamin D supplementation on dengue virus replication, Toll-like receptor expression, and cytokine profiles on dendritic cells

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Abstract

Dengue, caused by dengue virus (DENV) infection, is a public health problem worldwide. Although DENV pathogenesis has not yet been fully elucidated, the inflammatory response is a hallmark feature in severe DENV infection. Although vitamin D (vitD) can promote the innate immune response against virus infection, no studies have evaluated the effects of vitD on DENV infection, dendritic cells (DCs), and inflammatory response regulation. This study aimed to assess the impact of oral vitD supplementation on DENV-2 infection, Toll-like receptor (TLR) expression, and both pro- and anti-inflammatory cytokine production in monocyte-derived DCs (MDDCs). To accomplish this, 20 healthy donors were randomly divided into two groups and received either 1000 or 4000 international units (IU)/day of vitD for 10 days. During pre- and post-vitD supplementation, peripheral blood samples were taken to obtain MDDCs, which were challenged with DENV-2. We found that MDDCs from donors who received 4000 IU/day of vitD were less susceptible to DENV-2 infection than MDDCs from donors who received 1000 IU/day of vitD. Moreover, these cells showed decreased mRNA expression of TLR3, 7, and 9; downregulation of IL-12/IL-8 production; and increased IL-10 secretion in response to DENV-2 infection. In conclusion, the administration of 4000 IU/day of vitD decreased DENV-2 infection. Our findings support a possible role of vitD in improving the innate immune response against DENV. However, further studies are necessary to determine the role of vitD on DENV replication and its innate immune response modulation in MDDCs.

Keywords Inflammation · Toll-like receptors · Antiviral activity · Double-stranded RNA · Vitamin D receptor

Introduction

Dengue is a global public health concern and the most prevalent arthropod-borne disease in humans and is caused by dengue virus (DENV) infection. It is estimated that 3.9 billion people are at risk for DENV infection in more than 100 countries. Annually, the disease manifests itself in 96 million cases out of a total of 390 million infections [1]. DENV infection can lead to a broad spectrum of clinical presentations, known as dengue with or without warning signs, to severe and fatal forms of the disease, known as severe dengue (SD) [2]. Although it is not clear why some patients develop dengue and others SD, it is known that the physiopathology of SD is multifactorial, and there exists a variety of host and viral factors that contribute to the severity of symptoms and disease progression, including human genetics, age, virulence, viral load, and DENV serotype [2]. Cells infected with DENV exhibit a critical immune response that plays a determinant

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role in DENV immunopathogenesis [3]. In fact, it has been speculated that an adverse effect of inflammation triggered in response to DENV infection contributes to plasma leakage in patients, a significant pathological hallmark of SD [4]. Generally, plasma leakage can be caused by cytokine storms induced by the activation of primary target cells of DENV infection, such as dendritic cells (DCs), monocytes, and macrophages, which are susceptible to viral replication. Indeed, it has been reported that DENV-infected DCs can induce endothelial permeability and plasma leakage through the production of soluble factors like cytokines and metalloproteinases [5, 6]. Furthermore, we previously reported that, in the DCs of DENV patients, the profile of Toll-like receptor (TLR) expression, specifically TLR3 and TLR9, changed depending on disease severity [7]. Interestingly, Lai et al. [8] reported that DENV infection induces the release of mitochondrial DNA (mtDNA) and activates the TLR9 signaling pathway, resulting in interferon production. Hsu et al. [9] also reported that DENV NS1 induces type I interferon production in human DCs through the TLR3 signaling pathway, the IFN regulation factor (IRF)-3, and NF-κB, resulting in the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-8, and IL-12, which affect endothelial integrity [10, 11]. In agreement with these observations, George et al. [12] recently reported that DENV-infected DCs expressed high levels of inflammatory mediators and activator molecules in a TLR2/MyD88 pathway-dependent manner. Owing to this, studies investigating therapeutic alternatives to prevent DENV or to avoid SD are essential.

Previous studies indicated that vitamin D (vitD) modulates the inflammatory immune response to viral infections [13], including DENV [14–16]. Another study has associated vitD serum levels, vitD supplementation, and genetic variants in the vitamin D receptor (VDR) with DENV infection outcomes [17]. Recently, decreased mannose receptor expression levels, pro-inflammatory cytokines production, and susceptibility to DENV infection when monocytes differentiated into macrophages in the presence of vitD were reported [15]. Similar effects were observed in monocyte-derived macrophages from healthy donors who take vitD supplements [14]. Although it was recently demonstrated that DCs can convert inactive 25-hydroxy vitamin D3 into calcitriol (active form) via 25-hydroxyvitamin D3 1-α-hydroxylase (CYP27B1) [18, 19], no studies have evaluated the effects of vitD on DENV infection and regulation of the inflammatory response in DCs, which are primary target cells of DENV. Therefore, this study aimed to evaluate the roles of vitD on DENV-2 infection and replication and regulation of TLR expression and pro- and anti-inflammatory cytokines in infected monocyte-derived DCs (MDDCs).

Materials and methods

Ethics statement

The protocols for healthy donors' enrollment and sample collection were approved by the Committee of Bioethics Research of the Universidad de Antioquia (Medellin, Colombia). All donors included in the study signed an informed consent form after a clear explanation of the procedure according to the principles expressed in the Declaration of Helsinki.

Study subjects

Following the strategy described previously [14], we enrolled 20 healthy donors (11 women and nine men) without vaccination history against the yellow fever virus. All participants were seronegative for DENV NS1 antigen and DENV IgM/IgG antibodies as assessed using the *Dengue Duo kit* (SD BioLine, Abbott Laboratories, Chicago, IL, USA) and were residents of Medellin, Colombia, during the years 2015–2016. Pregnant or lactating women, subjects medicated with anticonvulsants or glucocorticoids, individuals with chronic diseases such as diabetes, and patients with a recent history of liver or kidney disease or history of intestinal malabsorption were excluded from the study. Study participants were randomly grouped into two groups to receive daily oral vitD supplementation for 10 days using either dosing scheme: (i) 10 donors received 1000 IU/day of vitD3 (Farma D, Colombia), and (ii) 10 donors received 4000 IU/day of vitD3. These doses and the time of vitD supplementation were based on previous reports, showing the therapeutic schemes that allow increasing serum vitD concentrations [20, 21].

Blood sample collections

Peripheral blood samples were collected from each healthy volunteer before vitD supplementation (VD0) and after 11 days, 1 day after vitD supplementation (VD11). Autologous serum was also obtained at both time points to be used during the differentiation process, and the resulting DCs were termed VD0-untreated MDDCs and VD11-treated MDDCs, respectively. After the first blood sample, the participants started taking daily doses of vitD for 10 days. Study participants reported no incidents during the supplementation period. The autologous serum was used to determine the levels of calcidiol [25(OH)D] before and after vitD supplementation using an enzyme-linked fluorescent assay (ELFA, *VIDAS*® 25 OH Vitamin D TOTAL, Biomerieux S.A.). Additionally, peripheral blood mononuclear cells (PBMCs)

were isolated by Ficoll Histopaque-1077 (Sigma-Aldrich, MO, USA) according to the manufacturer's instructions. Subject demographics and total 25(OH)D levels before and after vitD3 supplementation are shown in Table 1.

Monocyte purification and differentiation

Monocyte purification was performed as previously described [14]. Briefly, PBMCs were isolated using a Ficoll Histopaque-1077 (Sigma-Aldrich) gradient at 650 g for 30 min. Platelets were removed by washing with PBS (Sigma-Aldrich) three times at 250 g for 10 min. Afterward, the percentage of CD14+ cells (monocytes) was determined by staining 1×10^6 of PBMCs with 1 µL of anti-CD14 antibodies (eBiosciences) for 30 min. Monocytes were isolated from PBMCs (VD0 and VD11) by plastic adherence as previously described [22]. Briefly, 5×10^5 CD14+ cells from total PBMCs were seeded in 24-well plates for 2.5 h in 500 µL of RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.5% heat-inactivated fetal bovine serum at 37 °C and 5% CO_2. Non-adhering cells were removed by washing twice with PBS, and CD14-positive cells were cultured in 1.0 mL of differentiation medium (DM) [RPMI-1640 supplemented with 750 U/mL granulocytes/macrophages colony-stimulating factor (GM-CSF) and 500 U/mL recombinant human IL-4 (Peprotech SA, Mexico)] with 10% of autologous serum and incubated for 6 days at 37 °C and 5% CO_2. Half of the medium was replaced every 2 days, and differentiation was performed for 6 days, obtaining approximately 4.2×10^5 MDDCs from 5×10^5 $CD14^+$ cells. Subsequently, the MDDCs were collected for flow cytometry phenotyping. The MDDCs were evaluated for both morphology and phenotype.

Phenotyping MDDC markers by flow cytometry

The expression of both surface and intracellular markers on MDDCs was evaluated by flow cytometry. FITC-labeled anti-human HLA-DR (clone TU36), PE-Cy5-labeled anti-human CD11c (clone B-Iy6), and PE-Cy5-labeled anti-human CD80 (clone L307.4) antibodies were used (all are from BD Biosciences). PE-Cy7-labeled anti-human CD83 (clone HB15e) and CD86 Horizon-V450 (clone 2331-FUN-1) antibodies were from eBiosciences.

Table 1 Demographic data and total 25(OH)D levels before and after supplementation with VitD for 10 days

	1000 IU/day ($n=10$)	4000 IU/day ($n=10$)
Sex (men: women)	5:5	4:6
Age (years)	29.6 ± 6.5	31.6 ± 8.2
25(OH)D levels before supplementation (ng/mL)	26.8 ± 6.4	28.0 ± 3.8
25(OH)D levels after supplementation (ng/mL)	29.0 ± 5.9	35.5 ± 6.0

(Mean ± standard deviation)

DENV stocks and titration

DENV-2 New Guinea C (NGC) strain was obtained from the Center for Disease Control and Prevention (CDC, Ft. Collins, CO, USA) and was propagated in C6/36 HT cells obtained from ATCC as described previously [7]. The supernatants were obtained by centrifugation for 5 min at 1800 rpm to pellet cellular debris, aliquoted, and stored at − 70 °C until further use. Virus titration was performed by flow cytometry with the monoclonal antibody 4G2 (Millipore, Darmstadt, Germany) to detect DENV E protein and the goat anti-mouse IgG-FITC antibody (Invitrogen, Life Technologies, CA, USA), as previously described [23].

DENV-2 infection of MDDCs

After 6 days of differentiation, MDDCs obtained from VD0 and VD11 (VD0-untreated MDDCs and VD11-treated MDDCs, respectively) samples were washed with warm 1 × PBS before being challenged with wild-type DENV-2, at MOI 5, in a medium supplemented with 10% autologous serum and incubated for 2 h at 37 °C and 5% CO_2. Afterward, the cells were washed with warm 1 × PBS to remove unbound viruses and resuspended in DM supplemented with 20% autologous serum and cultured for 48 hpi at 37 °C and 5% CO_2. The cells were harvested, and the percentage of infected cells and TLR expression were assessed by flow cytometry. Approximately 1×10^6 cells were conserved in RNAlater Stabilization Reagent (Qiagen, Hilden, Germany) and stored at − 70 °C for future RNA extraction; the supernatants were stored at − 70 °C for future cytokine quantification.

Quantification of DENV-2 infection by flow cytometry

DENV-2 infection of MDDCs was measured by flow cytometry for 48 hpi. For this, the MDDCs were harvested and mixed with fixation/permeabilization buffer (eBioscience). Incubation with 4G2 antibodies (Millipore, Darmstadt, Germany) was then performed according to the manufacturer's instructions, followed by incubation with the secondary goat anti-mouse IgG-FITC antibody. Unstained cells and mock-infected cells plus secondary antibodies served as controls. All acquisitions were performed using the FACS Canto II ™ flow cytometer (BD Biosciences, San Jose, CA, USA). Infected cells were reported as the percentage of positive

MDDCs for DENV E antigen over the total number of cells analyzed.

Quantification of DENV-2 genomic RNA by real-time PCR

Total RNA from DENV-2-infected MDDCs was extracted using an RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Next, the cDNA was synthesized from 109 ng RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Both the positive- and negative-strand viral RNAs were measured by qPCR using primers for the DENV-2 capsid gene as previously reported [24, 25] (forward: 5′CAA TAT GCT GAA ACG CGA GAG AAA 3′ and reverse: 5′ CCC CAT CTA TTC AGA ATC CCT GCT 3′) and using the Maxima SYBR Green qPCR master mix kit. The calculation of viral RNA copies was performed based on a standard curve, as previously reported [25].

Quantification of TLR expression by flow cytometry

Flow cytometry was used to evaluate the expression of TLR2, TLR3, TLR4, and TLR9 in MDDCs as previously reported [7]. Briefly, to assess TLR2 and TLR4 abundance, DENV-2-infected MDDCs were surface-stained with anti-TLR2-PE (clone TL2.1) and anti-TLR4-PE antibodies (clone HTA125) for 25 min. For TLR3 and TLR9 staining, infected MDDCs were treated with fixation/permeabilization buffer and stained with anti-TLR3-PE (clone TLR3.7) and anti-TLR9-PE (clone eB72-1665) antibodies (eBiosciences) for 25 min following the manufacturer's recommendations. For each experiment, unstained cells, conjugated isotype antibodies, and fluorescence minus one were included as controls. The expression is shown as the mean fluorescent intensity (MFI) of the overall cell sub-population after subtraction of the isotype control.

RNA isolation, cDNA synthesis, and analysis of TLR mRNA by real-time PCR

The mRNA quantification for TLR2, TLR3, TLR4, TLR7, and TLR9 was performed in DENV-2-infected MDDCs by qPCR as previously reported [14]. Briefly, total RNA was prepared using the RNeasy mini kit. Next, the cDNA was synthesized from 100 ng of RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The primers used to quantify TLR mRNAs are shown in Supplementary 1, and the Maxima SYBR Green qPCR master mix kit was used. The relevant transcripts of each target gene were reported as fold change (ΔΔCt) after being normalized to the unstimulated control and the housekeeping gene β-actin.

Quantification of cytokine production by ELISA

DENV-infected MDDC culture supernatants were tested 48 hpi for the production of TNF-α, IL-6, IL-8, IL-12 (p70), and IL-10 using an ELISA kit (BD Biosciences) according to the manufacturer's instructions.

Statistical analyses

To establish differences between related samples, the paired *t* test or the Wilcoxon rank test was applied depending on the normality test. To determine the statistical differences between groups, the Kruskal–Wallis test was used, with a confidence level of 95%, followed by Dunn's multiple comparison test. Significant results are defined as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). All data were plotted and analyzed using GraphPad Prism 5.03 (GraphPad Software Inc. San Diego, CA, USA). The http://jaspar.genereg.net/cgibin/jaspardb.pl link, an open-access database of transcription factor binding profiles, was used to find putative response elements to vitD.

Results

Morphology and phenotype of MDDCs

After 6 days of differentiation, the obtained VD0-untreated MDDCs and VD11-treated MDDCs (VD0 and VD11, respectively) were semi-adherent cells with irregular morphology and dendrites (Supplementary 2A), as previously reported [26, 27]. MDDCs expressed high levels of CD11c and HLA-DR and expressed low levels of CD80, CD86, and CD83 (Supplementary 2B). The purity of the cultures was higher than 90% (Supplementary 3C). Furthermore, CD14 expression was downregulated on the MDDCs; taken together, these results were consistent with typical MDDC phenotypes reported previously [22]. These results suggested that the MDDCs obtained presented an immature phenotype.

High doses of vitD3 significantly increased serum calcidiol levels

To assess the impact of vitD3 supplementation on serum vitD levels, serum 25-(OH) D (calcidiol) levels were quantified in volunteers, specifically before and after 10 days of supplementation. Serum calcidiol levels before supplementation were similar between the two groups. In the vitD1000 group before supplementation, out of these

10 healthy subjects, 1/10 was found deficient (< 20 ng/mL), 5/10 had insufficiency (20–29 ng/mL), and 4/10 had sufficiency of vitD (≥ 30 ng/mL) according to what was previously reported [28] (Fig. 1a). In the vitD4000 group of healthy subjects, 1/10 were deficient, 7/10 were insufficient, and 2/10 were sufficient for vitD (Fig. 1b). However, as expected, after 10 days of oral vitD3 supplementation (1000 IU/day), a significant increase in serum calcidiol levels was observed (Fig. 1a); it was noted that donors with insufficiency failed to reverse their status. By contrast, oral supplementation at 4000 IU/day increased calcidiol serum levels (Fig. 1b) and reversed the vitD deficiency status in 80% of donors within 10 days of treatment. The increase in serum calcidiol levels following supplementation was statistically significant in both groups (Fig. 1a, b).

Supplementation with 4000 IU/day of vitD significantly decreased the frequency of MDDCs positive for the DENV E antigen

To assess the efficacy of oral vitD3 supplementation on DENV-2 infection of MDDCs, the percentage of infected cells was determined by flow cytometry before and after supplementation (Fig. 2). In the vitD4000 group, a significant decrease ($p = 0.037$) was found in the percentage of positive MDDCs for DENV E antigen after 10 days of supplementation compared with day 0 (Fig. 2a). By contrast,

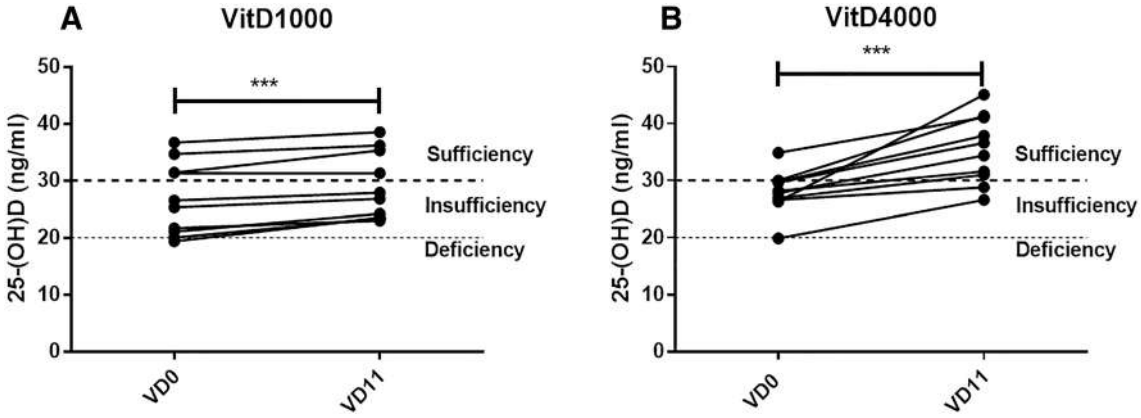

Fig. 1 Supplementation with VitD for 10 days increases serum calcidiol levels. In **a** the differences between serum levels of calcidiol at day 0 and at day 11, in the VitD1000 group of individuals, are shown. In **b** the differences between serum levels of calcidiol at day 0 and day 11, in the VitD4000 group of individuals, are shown. Differences were calculated using a paired *t* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

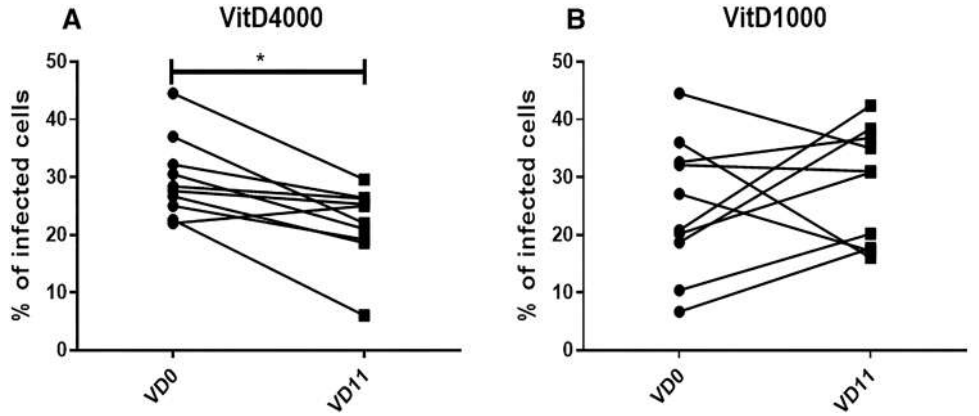

Fig. 2 Supplementation with 4000 U/daily of VitD3 decreases the % of E (+) antigen cells. The monocyte-derived MDDCs obtained at day 0 and day 11 were infected with DENV-2 and 48 hpi the cells were harvested, and the percentage of cells positive for the 4G2 antibody was determined by flow cytometry, using as control of infected and infected MDDC staining permeabilized, labeled only with the secondary antibody (IgG2a-FITC anti-mouse). In **a** the percentage of MDDCs infected on days 0 and 11 in the VitD4000 group of individuals is shown ($p = 0.0042$). In **b** the percentage of MDDCs infected by DENV-2 on days 0 and 11 in the VitD1000 group of individuals is shown ($p = 0.4185$). Differences were calculated using a paired *t* test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

in the vitD1000 group, there was no statistically significant change ($p = 0.4185$) in the percentage of infected MDDCs (Fig. 2b). These results suggested that high levels of vitD might modulate MDDC permissiveness to DENV-2 infection; thus, sufficient levels of vitD could be associated with decreased frequency of infected cells.

Efficacy of vitD3 supplementation on DENV-2 replication

To determine the effect of vitD3 supplementation on viral replication, the number of DENV-2 RNA copies was determined. Although we observed a slight tendency of decreases and increases in the number of copies of positive-sense RNA in the vitD4000 and vitD1000 groups, respectively, the data were not statistically significant (Fig. 3a, b). Similar results were observed for the number of copies of negative-sense RNA in the vitD4000 and vitD1000 groups (Fig. 3c, d).

Supplementation with 4000 IU/day of vitD3 decreases TLR expression in DENV-2-infected MDDCs

The immunomodulatory activity of vitD is related to its ability to regulate the expression of innate immune response receptors, such as TLRs. First, we proceeded to evaluate the TLR expression of MDDCs differentiated from monocytes obtained from healthy individuals who had not received an oral supplement of vitamin D3 and challenged with DENV-2 (Supplementary 3A). DENV-2 infection significantly induced the upregulation of TLR3 and TLR9 in MDDCs (Supplementary 3B and 3C), as

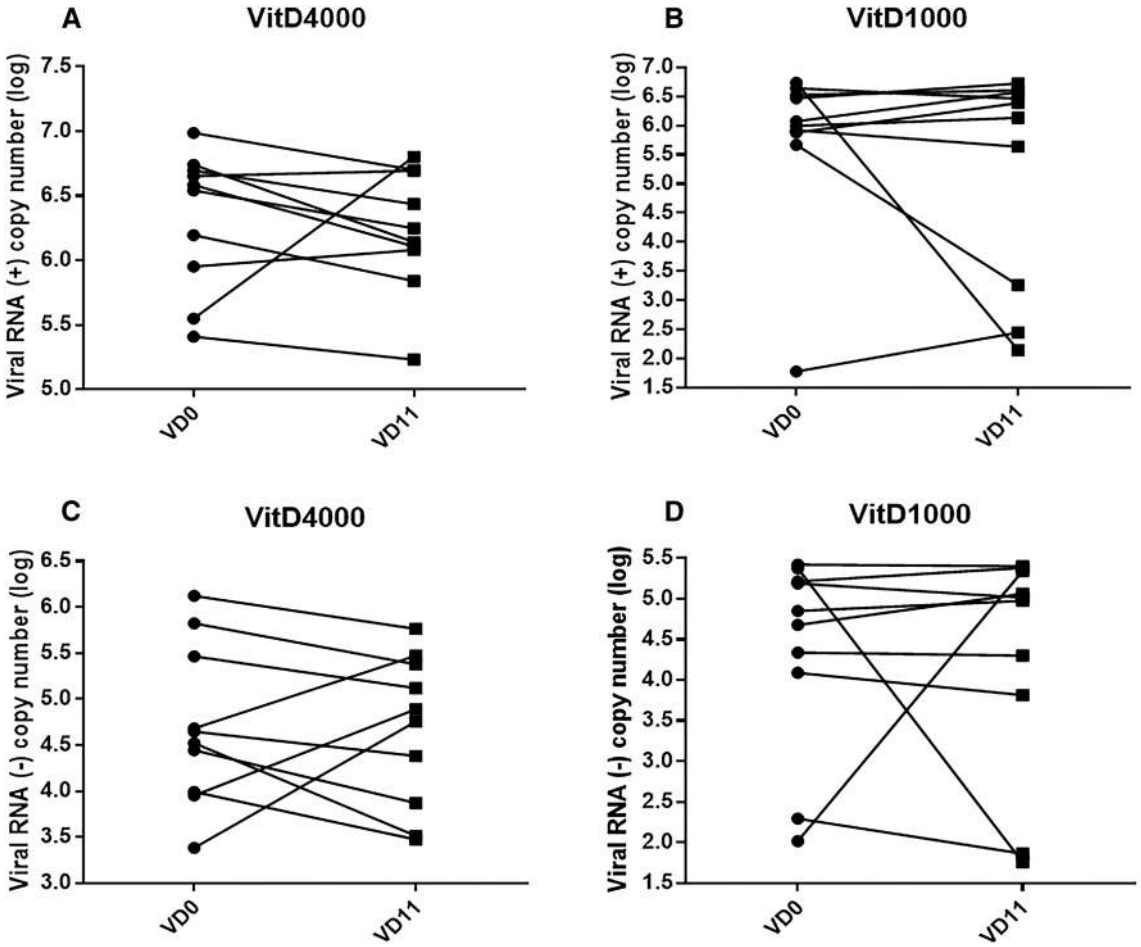

Fig. 3 VitD3 supplementation does not affect the replication of DENV-2 in MDDCs. The MDDCs from monocytes obtained both on 0 and day 11 were infected with DENV-2 (MOI 5) and 48 hpi the cells were harvested and RNA was used to quantify the number of copies of positive and negative polarity viral RNA by qPCR. In **a**, the copy number of viral RNA of positive polarity in MDDCs of the VitD4000 group, $p = 0.23$ is shown. In **b**, the number of viral RNA copies of positive polarity in MDDCs of the VitD1000 group, $p = 0.6953$, is plotted. In **c** the number of viral RNA copies of negative polarity in MDDCs of the VitD4000 group, $p = 0.43$ is shown. In **d** we show the number of viral RNA copies of negative polarity in MDDCs of the VitD1000 group, $p = 0.82$ is presented. Differences were calculated using a Wilcoxon test

we previously reported in DCs of infected patients [7]. Furthermore, there was a tendency of increased expression of TLR2 and TLR4 in DENV-2-infected MDDCs (Supplementary 3D and 3E).

Next, to determine the effect of vitD on the modulation of TLR expression in DENV-infected MDDCs, the expression of these receptors was evaluated by flow cytometry and RT-qPCR in both the vitD4000 and vitD1000 groups. Interestingly, a significant decrease in the MFI of TLR9 was found in MDDCs from donors of both groups (vitD4000 and vitD1000) after 10 days of treatment (VD11) and infected with DENV-2 compared to expression on day 0 (VD0) (Fig. 4a, b, respectively), but no significant changes for either TLR2 or TLR3 expression were observed (data not shown). Nevertheless, a statistically significant decrease in the mRNA levels of TLR3 (Fig. 4c; $p < 0.0147$), TLR7 (Fig. 4d; $p < 0.0020$), and TLR9 (Fig. 4e; $p < 0.0137$) was found in MDDCs on day 10 vs. day 0. In the vitD1000 group, no significant changes in the mRNA levels of TLR2, TLR3, TLR7, and TLR9 were observed (data not shown).

Vitamin D supplementation alters the production of both pro- and anti-inflammatory cytokines in MDDCs infected with DENV-2

The release of pro- and anti-inflammatory cytokines in DENV infection has been previously reported in MDDCs [29]. On the basis of these observations and because of our aforementioned results demonstrating that TLR expression is downregulated in MDDCs of donors who received vitD3 and infected with DENV, we proceeded to quantify the production of pro- and anti-inflammatory cytokines in culture supernatants. In both the vitD4000 and vitD1000 groups, cytokine production was determined on VD0 (day 0) and VD11 (day 11). There was no significant difference in cytokine production in MDDCs without infection when comparing VD0 versus VD11 in both groups (Fig. 5). There was a significant increase in the production of IL-6 (Fig. 5a) and TNF-α (Fig. 5b) in infected MDDCs with (vitD1000 and vitD4000 groups) or without vitD3 (VD0) compared with uninfected MDDCs, suggesting that vitD3 does not affect the secretion of these cytokines. An important finding of the current study was that the production of IL-8 significantly decreased in MDDCs from the vitD1000 group after 10 days

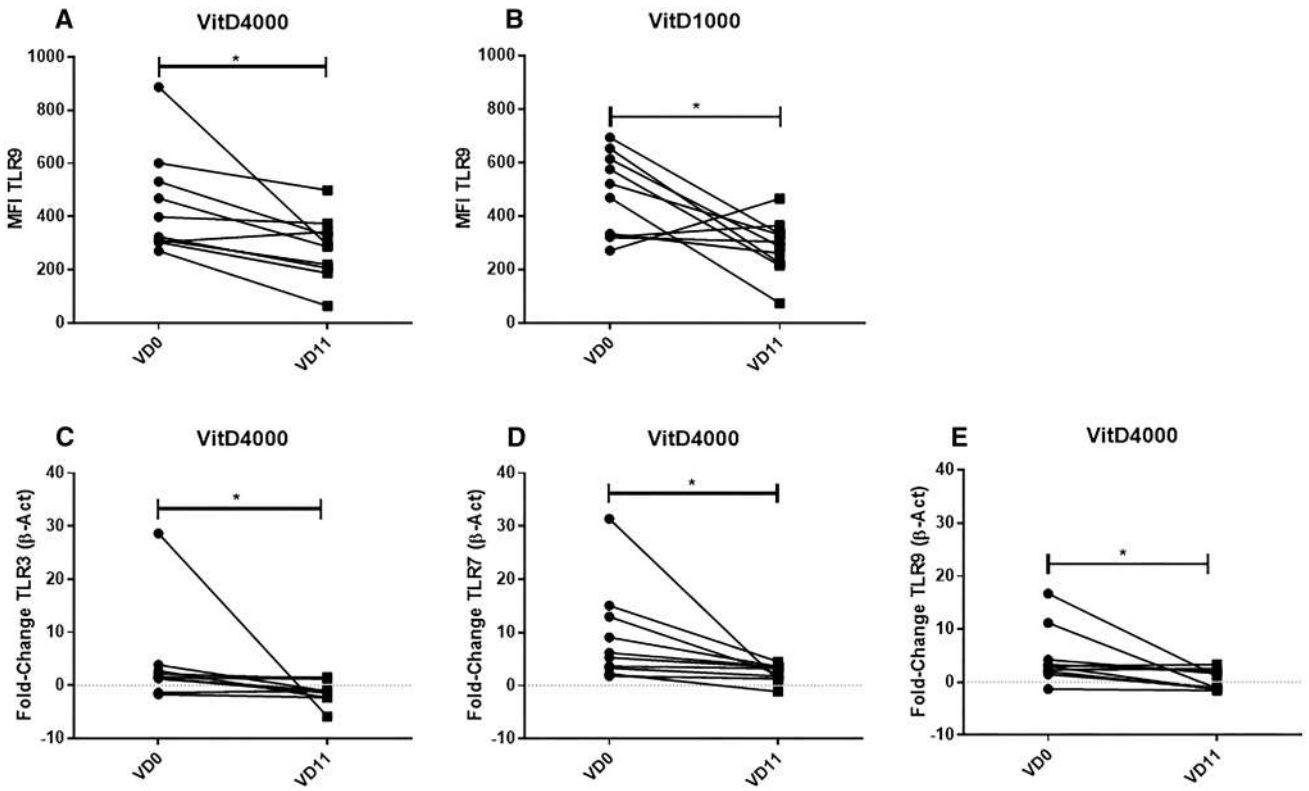

Fig. 4 DENV-2-infected MDDCs from VitD4000 individuals express lower TLR levels. The MDDCs obtained at day 0 and day 11 of the VitD4000 and VitD1000 individuals were infected with DENV-2 and 48 hpi the cells were harvested and the density of expression of TLR9 (**a**, **b**) were determined by flow cytometry (protein expression); TLR3 (**c**), TLR7 (**d**) and TLR9 (**e**) were determined by qPCR (mRNA expression). Differences were calculated using a Wilcoxon test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

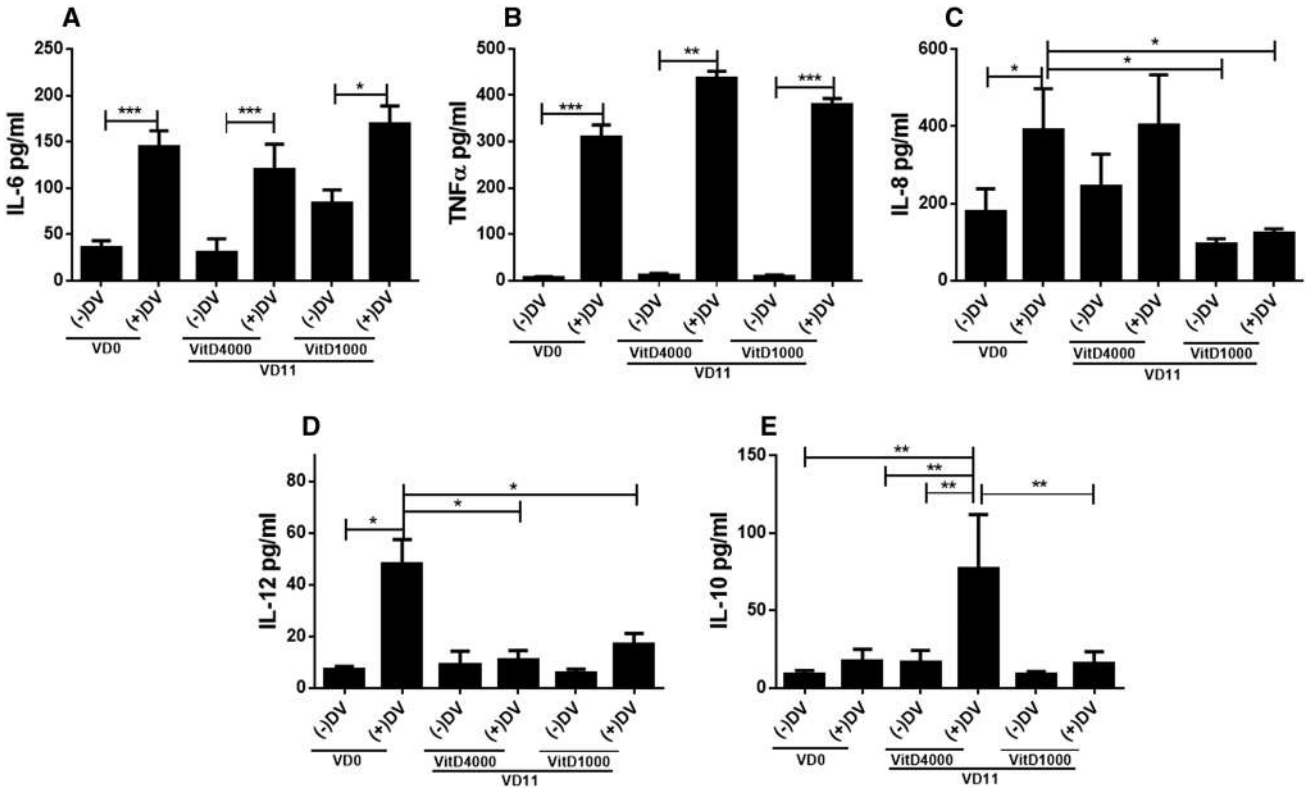

Fig. 5 VitD3 supplementation modulates the secretion of pro- and anti-inflammatory cytokines in MDDCs infected with DENV-2. The culture supernatants of MDDCs infected [(+) DV] or not (−) DV] with DENV-2 at day 0 (control) and at day 11, obtained from VitD4000 and VitD1000 individuals, were collected 48 hpi and were tested for IL-6 (**a**), TNFα (**b**), IL-8 (**c**), IL-12 (**d**) and IL-10 (**e**). To determine the statistical differences between the groups a Kruskal–Wallis test was used followed by Dunn's multiple comparison test; the error bars show the median and interquartile range. $*p<0.05$, $**p<0.01$, $***p<0.001$

of vitD3 supplementation (VD11) vs. VD0 MDDCs, both of which were challenged with DENV-2 (Fig. 5c). Interestingly, IL-12 production was significantly decreased in DENV-2-infected MDDCs in both the vitD1000 and vitD4000 groups after 10 days of supplementation (VD11) compared with VD0 (Fig. 5d). Moreover, a significant increase in IL-10 production was found in DENV-2-infected MDDC cultures of donors supplemented with 4000 IU vitD3 (Fig. 5e).

Discussion

As previously reported [30], we found that supplementation of 4000 IU/day of vitD3 led to the reversal of vitD insufficiency/deficiency status of most subjects in this study but not in donors receiving 1000 IU/day. These findings contrasted with previous reports suggesting that a 1000 IU/day dose of vitD3 is sufficient to achieve and maintain optimal serum vitD levels [31], implying that there may be socio-cultural, environmental, and genetic factors that contribute in establishing the levels of response to vitD supplementation. This is important because vitD3 functions in the modulation of the immune response, and its deficiency has been associated with susceptibility to several infectious diseases [31], such as hepatitis virus-associated diseases [32]. Furthermore, a recent study has implicated the antiviral effect of vitD against HIV-1, and the increased risk or severity of HIV infection is linked to a deficiency of vitD [33].

It has been reported that vitD oral supplementation improved overall clinical symptoms of dengue that has been associated with VDR genetic variants [17, 34, 35]. In this study, we observed that MDDCs obtained from healthy donors who received high doses of vitD and differentiated in the presence of autologous serum were more resistant to DENV-2 infection. Although there was a significant reduction in the percentage of DENV-2-infected MDDCs, we only observed a slight decrease in the number of copies of both positive- and negative-sense viral RNAs. These results were in line with our previous findings showing that MDM (monocyte-derived macrophages) differentiated in the presence of vitD3 or macrophages differentiated from monocytes obtained from individuals supplemented with high doses of vitD restricted DENV infection and pro-inflammatory cytokine production [14, 15]. The effects of

oral supplementation with high doses of vitD have also been reported in other viral infections, such as HIV-1, showing attenuated viral replication [36]. Bergman et al. [37] found that symptoms were significantly reduced among participants with frequent respiratory tract infections who received 4000 IU/day of vitD3.

On the other hand, there was no change in the percentage of DENV-2-infected MDDCs obtained from donors who received a low dose of vitD (1000 IU/day; $p = 0.4185$), suggesting that vitD3 supplementation can promote inhibition of DENV infection in a dose-dependent manner. However, the mechanism involved is still unclear. Because some studies suggested that vitD induced the expression of some antimicrobial peptides, such as defensins or cathelicidin [38, 39], the anti-DENV activity might be associated with these peptides [40, 41]. Alternatively, an effect on the expression of the receptor could be expected, as we reported previously in MDMs [15], in which its expression and functionality were reported to decrease in MDDCs treated with vitD in vitro [42]. Puerta-Guardo et al. [16] also found that vitD treatment of monocytes or hepatic cell lines resulted in a significant decrease in the percentage of infected cells.

Notwithstanding that DCs are primary targets and major players in early immune responses to DENV [43], the effect of vitD on the innate immune response in MDDCs during DENV infection has not been studied. Consequently, our study is the first to explore the impact of vitD on DENV infection on these cell populations. Here we examined the effect of vitD3 on TLR expression in MDDCs infected with DENV-2. An oral supplement of 4000 IU/day of vitD3 significantly decreased TLR9 protein levels and the mRNA abundance of TLR3, TLR7, and TLR9. However, 1000 IU/day of vitD only decreased the TLR9 protein level in MDDCs infected with DENV. Similar results were recently reported [44]; TLR9 expression is downregulated in monocytes exposed to vitD3 with a functional consequence because these downregulations were associated with lower production of IL-6. Although the underlying mechanisms are not clear, using computational methods (http://jaspar.genereg.net/), we found that these TLRs presented multiple vitD-response elements (VDRE) in their gene sequence (Supplementary 4), suggesting a possible direct regulation of vitD in the expression of these TLRs. This is very interesting because it has been reported that extensive TLR stimulation leads to the activation of the inflammatory process in response to DENV infection [11, 45]. The downregulation of intracellular TLR (TLR3, TLR7, and TLR9) expression observed becomes more critical considering that it was previously reported that these TLRs are important in the detection of DENV and subsequent activation of the inflammatory response receptors [46–48]. However, it was recently reported that TLR9 activation, through mtDNA, contributes to DENV-induced immune activation [8]. Interestingly, like us [7], other authors recently have associated alterations in TLR9 regulation with the severity of dengue disease. It has been found downregulation of TLR9 expression in the period of defervescence in patients with severe dengue [49]. This suggests a possible role of TLR9 in the immunopathology of the disease, therefore the action of vitD observed in this study could regulate the inflammatory response in infection through TLR9 signaling. However, in order to determine the involvement of TLRs in the disease pathology, to study the dynamic of TLR expression is needed.

Given that high levels of IL-10 and low IL-12 production have been documented as common features in regulatory DCs [50], we examined the secretion of these two cytokines by MDDCs. We found that DENV-infected MDDCs differentiated from monocytes obtained from subjects supplemented with 1000 or 4000 IU/day of vitD3 significantly decreased the production of IL-12 in a dose-independent manner. Bartels and colleagues [18] also observed in vitro that LPS-stimulated MDDCs exposed to different concentrations of vitD decreased the production of IL-12. This finding is relevant because previously an increase in plasma concentrations of IL-12 in patients with dengue was shown [51], which suggests that low production of this cytokine might promote a beneficial role in reducing the ability of DCs to induce a pro-inflammatory profile, as reported in other diseases [52]. By contrast, in DENV-infected MDDCs differentiated from monocytes obtained from healthy donors who received 4000 IU/day, IL-10 production was significantly increased. It could be through VDREs because, in monocytes, it was reported that the promoter region of the IL-10 gene contains two conserved VDR binding sites [53]. The fact that we have observed an increase in IL-10 is interesting because Ho et al. [29] reported that DCs infected with DENV induced the production of TNF-α and IFN-α, but not IL-12 or IL-10.

High levels of IL-10 have been associated with the induction of the tolerogenic profile of DCs, decreasing the activation of T lymphocytes with a pro-inflammatory profile (Th1) [54]. Because IL-10 has potent immunoregulatory properties in viral infections, we speculated that IL-10 produced by MDDCs may contribute to the control of SD by suppressing the production of vasoactive pro-inflammatory cytokines related to endothelial damage, such as IL-1β, IL-6, and TNFα produced after DENV infection, which has been proposed by others in monocytes [55]. Because in our model we observed that DENV infection of MDDCs (before vitD3 supplementation) suppressed IL-10 production, we purported that it can limit the ability of DCs to promote differentiation and proliferation of CD4+ T cells. After vitD treatment, DCs recovered this ability because these cells secreted high levels of IL-10. In addition, it has been reported that high IL-10 production inhibits the expression of some pro-inflammatory

cytokines, leading to further suppression of the ability of effector cells to prolong the inflammatory response [56], thus avoiding the progression of dengue to SD. However, high levels of serum IL-10 have been proposed as a marker of SD infection given the increased levels of this cytokine that has been found in the bloodstream of patients with dengue hemorrhagic fever (DHF) [57, 58]. Other studies reported that the timing of IL-10 production is dynamic and varies throughout the illness. There are peaking levels of IL-10 that have been observed around defervescence of DHF patients, but no peaks were observed in patients with dengue fever [59], suggesting that regulation of IL-10 expression by DENV might be bidirectional depending on the time of quantification. Nevertheless, the high levels of this cytokine found in the plasma of patients with SD may be the result of the participation of other factors involved in the modulation of the immune system and the increase in the production of IL-10 or compensatory response of pro-inflammatory factors generated during DENV infection.

Moreover, in MDDCs obtained from vitD1000 donors, a significant decrease in IL-8 secretion was also observed after DENV-2 infection, an important finding considering that this cytokine is elevated in the serum of patients with DENV [51]. Nevertheless, the effect of vitD on IL-8 secretion is controversial because some studies suggest that vitD decreases serum IL-8 concentrations [60]. A previous study in monocytes reported that vitD increased the secretion of IL-8 in response to stimulation with TLR ligands [26, 61]; hence, additional studies are needed to elucidate the role of vitD in regulating IL-8 production in DENV infection.

In conclusion, the findings of our study indicated that DENV-infected MDDCs obtained from healthy donors supplemented with high doses of vitD highly expressed anti-inflammatory cytokines (IL-10) and lower levels of pro-inflammatory cytokine IL-12 because of the down-regulation of TLR expression. In addition, in these cells, lower levels of DENV infection were found. These results suggested that increased vitD3 supplementation might be a way of lowering the pro-inflammatory response observed in patients with SD and could be used as a therapeutic strategy to control dengue progression.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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