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# Vitamin D-mediated attenuation of miR-155 in human macrophages infected with dengue virus: Implications for the cytokine response

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ABSTRACT

Clinical manifestations of dengue disease rely on complex interactions between dengue virus (DENV) and host factors that drive altered immune responses, including excessive inflammation. We have recently established that vitamin D can modulate DENV-induced cytokine responses and restrict infection in human macrophages. Cytokine responses are finely regulated by several homeostatic mechanisms, including microRNAs (miRNAs) that can rapidly target specific genes involved in the control of immune signaling pathways. However, the modulation of miRNAs by vitamin D during DENV infection is still unknown. Here, using a qPCR miRNA array we profiled immune-related miRNAs induced by DENV infection in human monocyte-derived macrophages (MDM) differentiated in absence or presence of vitamin D ($D_3$-MDM). We found several miRNAs differentially expressed in both MDM and $D_3$-MDM upon DENV infection. Interestingly, from these, a set of 11 miRNAs were attenuated in $D_3$-MDM as compared to MDM. Gene set enrichment analysis of the predicted mRNA targets of these attenuated miRNAs suggested a predominant role of miR-155-5p in the TLR-induced cytokine responses. Indeed, validation of miR-155-5p attenuation in $D_3$-MDM was linked to increased expression of its target gene SOCS-1, a key component for TLR4 signaling regulation. Likewise, TLR4 activation with LPS further corroborated the same miR-155-5p/SOCS-1 negative correlation observed in $D_3$-MDM upon DENV exposure. Moreover, $D_3$-MDM differentiation induced down-regulation of surface TLR4 that was linked to less TLR4/NF-κB-derived secretion of IL-1β. These data suggest a key role of vitamin D in the control of inflammatory cytokine responses during DENV infection of human macrophages *via* the TLR4/NF-κB/miR-155-5p/SOCS-1 axis.

## 1. Introduction

Infection with dengue virus (DENV) causes approximately 100 million symptomatic cases and 25,000 deaths per year, embodying a huge economic and disease burden for global health systems (Shepard et al., 2016). It is estimated that around 50% of the human population is now at risk of infection in tropical and subtropical areas where the viral vector, *Aedes aegypti*, distributes (Aguiar et al., 2016; Murray et al., 2013; Scott, 2016). DENV belongs to the *Flaviviridae* family and is an enveloped virus with single-stranded positive-sense RNA genome from which four eco-epidemiologically relevant serotypes (DENV 1–4) have been identified (Murray et al., 2013; Mustafa et al., 2014). Infection with any of these serotypes can result in an acute, self-limiting febrile illness known as dengue fever (DF) with or without warning signs that can progress to severe dengue, characterized by hemodynamic compromise that can lead to organ failure, hypovolemic shock and eventually death (Martina et al., 2009).

The pathogenesis of dengue disease depends on complex interactions between virus and host factors; however, the magnitude and duration of pro-inflammatory cytokine responses play a critical role in the progression of the disease. Antigen-presenting cells, including dendritic cells, macrophages and monocytes, can recognize DENV antigens through several pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) 2, 3 and 4, promoting activation of several signaling pathways that trigger pro-inflammatory and antiviral responses to contain dissemination of the virus (Costa et al., 2013; Modhiran et al., 2017; Puerta-Guardo et al., 2013). However, the prolonged production of TNF-α, IL-1β, IL-18, and IL-6 can cause capillary permeability and endothelial dysfunction (Martina, 2014). Thus, fine-tuning of these pathways is required to avoid disruption of the homeostatic mechanisms that control immune responses upon pathogen infection.

One of the regulatory mechanisms that control immune activation and cytokine secretion are miRNAs (Baltimore et al., 2008; O'Neill

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et al., 2011). These are small (18–25 nt) non-coding RNAs sequences acting primarily as translational repressors of messenger RNA (mRNA) by interacting with their 3′untranslated region (He and Hannon, 2004). Under pathological conditions, alterations in the expression and function of certain miRNAs have been associated with excessive pro-inflammatory responses (Virtue et al., 2012). Indeed, we have proposed miRNAs as factors regulating the extent and timing of PRR responses during DENV infection (Arboleda and Urcuqui-Inchima, 2016; Urcuqui-Inchima et al., 2017). In line with this, variations in the miRNAs profile and increased secretion of pro-inflammatory mediators have been associated with clinical manifestations of dengue and *ex vivo* infection of peripheral blood mononuclear cells (Qi et al., 2013; Tambyah et al., 2015).

Vitamin D is widely recognized to modulate immune responses to microbial infection (Borella et al., 2014). Its immunomodulatory effect *via* vitamin D receptor (VDR) can directly affect the expression of hundreds of genes related to the sensing of pathogens and control of immune signaling pathways, including miRNAs (Beckett et al., 2014; Giangreco and Nonn, 2013; Hewison, 2010). Recently, we demonstrated that human monocyte-derived macrophage (MDM) differentiation in presence of vitamin D ($D_3$-MDM) contributed with reduction of the cytokine response induced by DENV and less infection *via* down-regulation of mannose receptor, a critical receptor for viral binding and immune activation (Arboleda Alzate et al., 2017). We hypothesized that alternatively, vitamin D could contribute to control DENV-induced cytokine responses *via* modulation of miRNAs (Arboleda and Urcuqui-Inchima, 2016). Therefore, in this study, we profiled immune-related miRNAs in MDM and $D_3$-MDM after DENV infection and analyzed their contribution to cytokine response control. Furthermore, transcriptional validation of miRNA-155 attenuation in $D_3$-MDM after DENV infection and LPS stimulation was observed concomitantly with decreased TLR4-derived cytokine responses, suggesting a key role of vitamin D and miR-155 interplay in the control of TLR4-derived pro-inflammatory responses.

## 2. Material and methods

### 2.1. Ethics statement

Sample collection protocols and written informed consent were approved by the Committee of Bioethics Research of the Sede de Investigación Universitaria, Universidad de Antioquia (Medellín – Colombia).

### 2.2. Blood donors

This study was conducted in accordance with the principles expressed in the declaration of Helsinki. Venous peripheral blood samples were obtained in Medellin-Colombia from an equal proportion of healthy women and men between 20 and 35 years old that were not vaccinated against yellow fever virus and were seronegative for the DENV NS1 antigen and DENV IgM/IgG, as determined by Platelia Dengue NS1 Antigen kit (Bio-Rad Laboratories, Marnes La Coquette, France). All our experiments were performed from cells of at least 3 different healthy donors.

### 2.3. Monocyte-derived Macrophage differentiation in presence of Vitamin D

Total blood from healthy volunteer donors was mixed with EDTA 4% *v*/v and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque (Sigma-Aldrich) gradient at 650 x g during 30 min as described in (Eligini et al., 2013). Platelet depletion was performed by washing with PBS (Sigma-Aldrich) three times at 250 x g for 10 min. Monocytes were obtained from total PBMCs by plastic adherence as described in (Eligini et al., 2013). Briefly, $5 \times 10^5$ CD14+ cells from total PBMCs were plated in 24 well plastic plates and were allowed to adhere during 4 h in 1640 RPMI medium (Sigma-Aldrich) supplemented with 0.5% of heat-inactivated human serum pool (HSP) at 37 °C and 5% of $CO_2$. Non-adherent cells were removed by washing twice with PBS, and MDM differentiation was allowed for 144 h in RPMI medium 10% HSP at 37 °C and 5% of $CO_2$. Additionally, MDMs were differentiated in presence of 1,25 di-hydroxyvitamin $D_3$ to obtain $D_3$-MDM, as previously described (Arboleda Alzate et al., 2017). In brief, 1,25 di-hydroxyvitamin $D_3$ (Sigma-Aldrich) was added to the culture media at a final concentration of 0.1 nM and replenished every 48 h. Kinetics and concentration of the vitamin D dose were determined previously on the basis of cytotoxicity levels, transcriptional induction of vitamin D signaling targets and modulation of immune responses (Arboleda Alzate et al., 2017; Fitch et al., 2016; Sadeghi et al., 2006). For each experiment, two equal fractions of PBMCs were used from the same donor to differentiate between MDM and $D_3$-MDM. For indicated experiments, cells were pre-treated with the IKK2 inhibitor, SC514 (100 μM) (Sigma Aldrich) and its respective dimethyl sulfoxide (DMSO) vehicle control (Mock) during 1 h previous to stimulation. After this, cells were stimulated with LPS (100 ng/mL) and incubated overnight before sample collection.

### 2.4. DENV stocks

All experiments reported in this study were performed using DENV-2 New Guinea C (NGC) strain that was provided by the Center for Disease Control (CDC, CO, USA) and was propagated in C6/36 HT cells. In brief, monolayers of C6/36 HT cells were grown in 75-$cm^2$ tissue culture flasks and inoculated with DENV-2 at MOI of 0.05 in 1 mL of L-15 medium supplemented with 2% of heat-inactivated Fetal Bovine Serum (FBS). After 3 h, 10 mL of L15 medium containing 2% FBS was added, and the cells were cultured for 5 days at 34 °C without $CO_2$. The supernatants were obtained by centrifugation for 5 min at 250 x g to remove cellular debris and were stored at −70 °C. Virus titration was performed by flow cytometry as described (Lambeth et al., 2005). Briefly, C6/36 HT cells were seeded in 12-well plates and cultured overnight at 34 °C without $CO_2$. The cells were infected with 10-fold serial dilutions of the virus and harvested at 24 h post-infection (hpi). Indirect intracellular staining of DENV E protein with the monoclonal antibody 4G2 (Millipore, Darmstadt, Germany) and the secondary antibody goat anti-mouse IgG-FITC (Invitrogen, Life Technologies, CA, USA) was performed as described later below. The cells were analyzed by FACScanto flow cytometry using the FACSdiva software. The percentage of infected cells in each sample and the total number of cells seeded per well were used to calculate the final titer of the virus. Isolation of viral RNA from cell lysates and supernatants was performed according to manufacturer's instructions using the RNeasy mini kit and the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), respectively. The number of genome equivalent copies (GEc) was determined by RT-qPCR using DENV-2 specific primers (forward: 5′CAATATGCTG AAACGCGAGAGAAA 3′, and reverse: 5′ CCCCATCTATTCAGAATCCCT GCT 3′). The calculation of the GEc was performed based on a standard curve, as previously reported (Sachs et al., 2011; Shu et al., 2003).

### 2.5. DENV infections

MDM and $D_3$-MDM monolayers were washed with warm PBS and were infected with DENV2 at MOI 10 in 300 μL of RPMI medium and 2% HSP per well. Three hpi, the cells were washed with warm PBS to remove unbound virus and were incubated at 37 °C with 5% $CO_2$ in RPMI medium and 10% PSH. 24 hpi, supernatants and macrophage monolayers were used for sample collection.

### 2.6. miRNA PCR array assay

Total RNA (> 200 nt) and miRNA-enriched fraction were obtained with miRNeasy Mini kit following manufacturer's instructions (Qiagen,

Valencia, CA, USA). RNA samples from four groups of macrophages (mock-infected MDM and $D_3$-MDM, and DENV-infected MDM and $D_3$-MDM) obtained from 3 different donors were pooled, and cDNA synthesis was performed using the miScript HiSpec buffer as recommended for mature miRNAs profiling (Qiagen, Valencia, CA, USA). Resulting cDNA was used for real-time PCR profiling of miRNA using Immunopathology miscript miRNA PCR array from Qiagen (Cat MIHS-104Z). This kit allows specific profiling and quantification of 84 mature immune related-miRNAs using SYBR green real-time PCR. In brief, for each macrophage group, PCR mix was prepared and was loaded onto miScript miRNA PCR 96 well plates. Reaction and cycling conditions were set as recommended by manufacturer's instructions and data quantification was performed using the $\Delta\Delta C_T$ method relative to the control assay wells (including primer control, alternative normalizer, snoRNA/snRNA, reverse transcription control and positive PCR control). Differential expression of miRNAs in DENV-infected MDM and $D_3$-MDM was calculated with the online provided miscript miRNA PCR array data sheets templates. Calculations were relative to their respective mock-infected MDM and $D_3$-MDM and considered significant only when fold changes (FC) cutoffs were $\geq |3|$ and $p$-values $\leq .05$ as recommended by the manufacturer.

### 2.7. miRNA clustering, miRNA-mRNA target predictions and pathway analysis

The miRNA expression profiles were visualized plotting a heat-map of the centered and normalized data (z-score), which represents units of standard deviation from the normalized mean of zero, for each gene across the experimental groups. For clustering of miRNA profiles, we used cluster3 by implementing k-means clustering to mean-centered expression values. Candidate miRNA–target relationships were assessed using validated experimental targets deposited in miRTarBase, TarBase, and MiRecords. Additionally, to identify potential miRNA-mRNA targets in a macrophage context, we used the dataset GSE36952 deposited in the GEO database to discard mRNAs not expressed in macrophages (Beyer et al., 2012). mRNAs abundance that fell below the 25th percentile (determined as counts per million) was not considered as relevant for miRNA-mRNA interaction. Jaccard index was used to find the similarity in targets between the differentially expressed genes. Gene Ontology Enrichment Analysis (GO enrichment) was performed with BiNGO Cytoscape plugin, using a hypergeometric test with a Benjamini and Hochberg False Discovery Rate correction. A $p$-value cut-off of 0.05 was used to identify enriched processes. Additionally, the ClueGO Cytoscape plugin was used to group and analyze the gene ontology and KEGG enrichments. Networks were visualized and analyzed with Cytoscape (Shannon et al., 2003).

### 2.8. Flow cytometry analysis

Surface staining using anti-TLR4 PE (eBiosciences) was performed on MDMs and $D_3$-MDM. Briefly, cells were removed from plates and incubated in presence of 2 μL of anti-TLR4 antibody during 20 min at room temperature in a final volume of 100 μL, as recommended by the manufacturer. Next, cells were washed with PBS and stored at 4 °C until reading time. For each experiment, unstained and isotype controls were included to set positive events gate. The percentage of TLR4 + cells was expressed as the number of TLR4 positive cells over the total number of cells analyzed. Samples were read on a FACScanto flow cytometer (BD Biosciences), and their data were analyzed using the FACSdiva (BD Biosciences) and Flowjo (LLC) software.

### 2.9. Gene expression measurement

miR-155 expression was validated using independent samples by RT-qPCR. Total RNA was isolated by RNeasy Mini Isolation Kit (Qiagen, Valencia, CA, USA) and cDNA synthesis was performed using the RevertAid Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Wilmington, DE, USA) using 100 ng of total RNA and miR-155-specific steem-loop reverse transcription primers (Thermofisher, Cat 4427975). The small ribosomal 18S RNA was used as constitutive gene control. Analysis of melting curves was used to discard the presence of unspecific amplification. miR-155 expression levels were calculated using a regression fit in the linear phase of the PCR amplification curve, and the data was obtained with Bio-Rad CFX software. Duplicate assays were performed for each sample, and relative transcript units (RTU) and fold-change values were calculated in relation to 18S expression levels and mock-infected cells by using the ΔCt and the ΔΔCt method, respectively. Similarly, SOCS1 mRNA level and the constitutive gene β2M were measured by quantitative real-time PCR. For this, RNA isolation was obtained as described above and cDNA synthesis was performed using the RevertAid Minus First Strand cDNA Synthesis Kit and random primers (Thermo Scientific). Quantitative real-time PCR reactions were performed using the following primers. For SOCS1, forward: 5′- TTGCCTGGAACCATGTGG -3′ and reverse: 5′ GGTCCTGGCCTCCA GATACAG -3′. For β2M, forward 5′-GAGTATGCCTGCCGTGTG-3′ and reverse 5′-AATCCAAATGCGGCATCT-3′. Data was obtained with Bio-Rad CFX software, and expression levels were calculated as described above.

### 2.10. Cytokine response

TNF-α and IL-1β concentration levels in mock or DENV infected MDM and $D_3$-MDM culture supernatants were measured by ELISA (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

### 2.11. Statistical analysis

Statistical graphs and comparisons were made using the software GraphPad Prism 5 (GraphPad Prism, CA, USA). Comparisons between MDM and $D_3$-MDM were performed using the non-parametric Mann-Whitney test and the Wilcoxon signed rank test. The critical value for statistical significance used for the analysis in the present study was $p < .05$, denoted as * and $p < .01$ denoted as **.

## 3. Results

### 3.1. Differential expression of immune-related miRNAs in MDM and $D_3$-MDM infected to DENV-2

Vitamin D activity has been linked to miRNA expression (Giangreco and Nonn, 2013; Li et al., 2014), therefore, here we sought to identify the participation of this interplay during DENV infection of $D_3$-MDM. To this aim, we profiled miRNAs expression of MDM and $D_3$-MDM after exposure to DENV-2 (MOI 10) by using a miScript PCR array system. Samples were collected at 24 hpi, time in which we previously observed downregulation of DENV-induced pro-inflammatory cytokines and reduced infection in $D_3$-MDM (Arboleda Alzate et al., 2017). Expression values of every miRNA in DENV-2-infected MDM and $D_3$-MDM were obtained as explained in the methods section. A heat map was generated to identify distinguishable miRNAs expression profile between mock-infected MDM and $D_3$-MDM *versus* DENV-2-infected MDM and $D_3$-MDM (MDM + DENV and $D_3$-MDM + DENV), respectively. Fig. 1A shows the distinctive expression profile of miRNAs in both mock and DENV infected cells. From all analyzed miRNAs, a total of 15 miRNAs were differentially expressed in MDM + DENV, and 12 miRNAs were differentially regulated in $D_3$-MDM + DENV. From these, 8 were equally regulated in both MDM and $D_3$-MDM (Fig. 1B). All these miRNAs were grouped into clusters according to their expression patterns relative to the respective mock control cells (CT) and the number of their validated targets (Fig. 1C and Supplementary Table 1). Three clusters were identified: (i) DENV-responsive miRNAs (miR-195-5p,

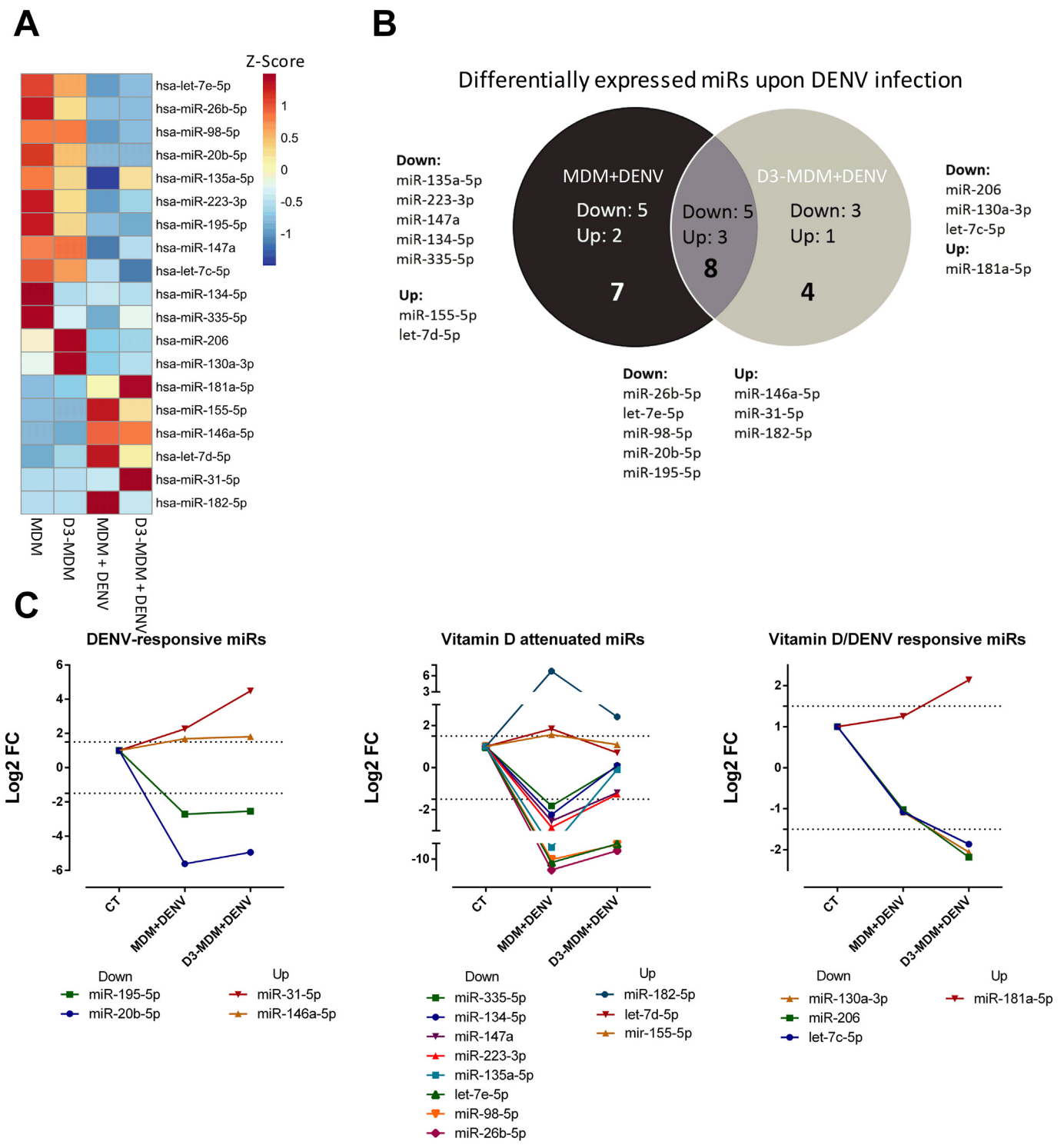


**Fig. 1.** Exposure to DENV induces a differential expression profile of immune-related miRNAs in MDM and D3-MDM. Monocyte-derived macrophages were differentiated in absence (MDM) or presence ($D_3$-MDM) of vitamin D (0.1 nM) and infected with MOI 10 of DENV-2. Sample collection for PCR array was performed at 24hpi. A) The heat map is showing distinctive miRNAs profiles in mock-infected MDM and D3-MDM *versus* DENV-infected MDM and $D_3$-MDM. B) Venn diagram is showing common miRNAs between DENV-infected MDM and DENV-infected $D_3$-MDM. C) Clusters of differentially DENV-induced regulated miRNAs in MDM and $D_3$-MDM relative to mock-treated macrophages (CT). Y-axis represents log2-fold change.

miR-20b-5p, miR-31-5p, miR-146a-5p) that are similarly expressed after DENV exposure in both MDM and $D_3$-MDM. (ii) Vitamin D attenuated miRNAs (miR-335-5p, miR-134-5p, miR-147a, miR-223-3p, miR-135a-5p, Let-7e-5p, miR-98-5p, miR-26b-5p, miR-182a-5p, Let-7d-5p, miR-155-5p), which are a set of DENV-responsive miRNAs in MDM and $D_3$-MDM but that were attenuated by at least 2 fold-change values in $D_3$-MDM. And, (iii) vitamin D/DENV responsive miRNAs (miR-181a-5p, miR-130a-3p, miR-206 and let7c-5p), which are a cluster of miRNAs that were regulated only in DENV-2-infected $D_3$-MDM. These findings revealed a set of immune-related miRNAs in human macrophages that are responsive to DENV-2 infection, and that can be modulated by vitamin D.

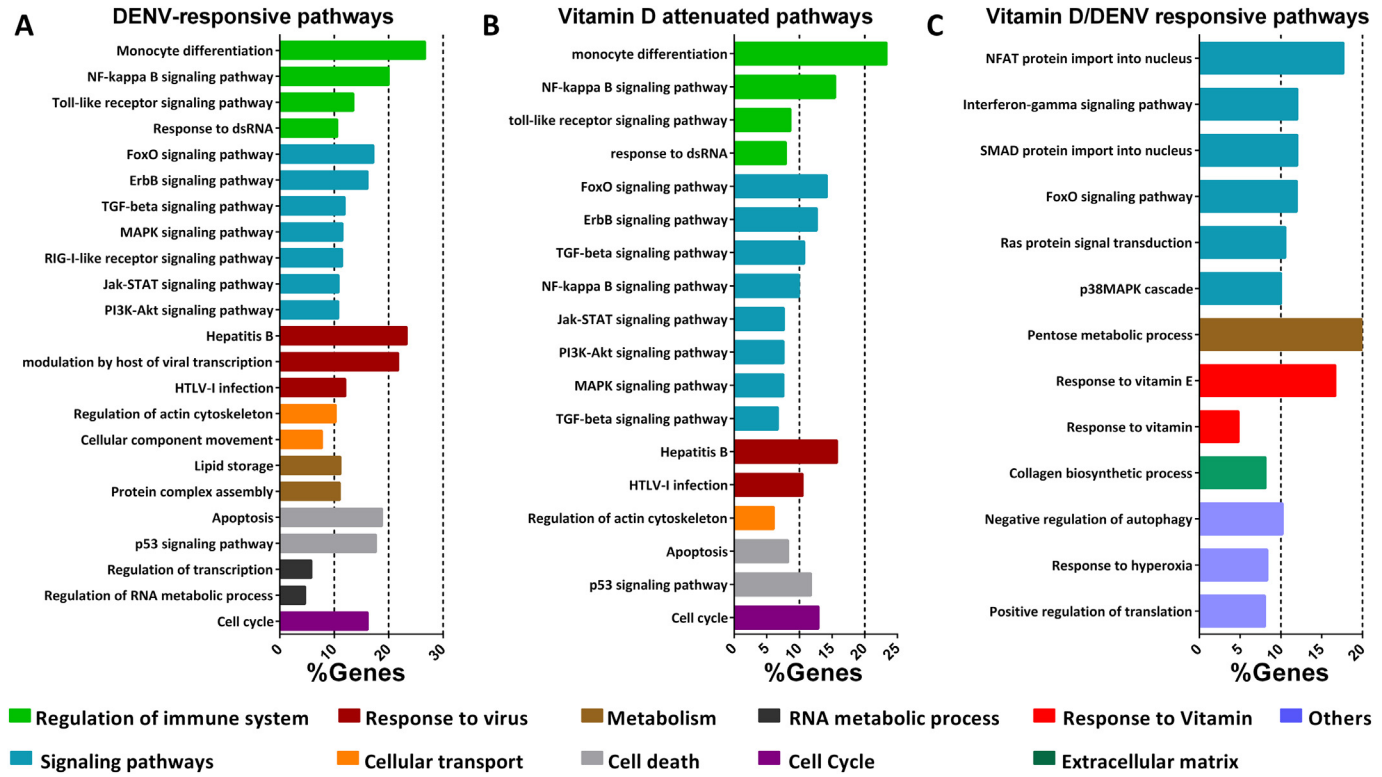


**Fig. 2.** Gene-term enrichment analysis of the predicted mRNAs regulated by miRNAs. A) Pathways altered by miRNAs in the DENV-responsive cluster. B) Pathways altered by the miRNAs in the Vitamin D-attenuated cluster. C) Pathways altered by miRNAs in the Vitamin D/DENV responsive cluster. Bars represent the percentage of genes present in the data set compared to the total number of genes present in each selected pathway. Each color represents a major module.

### *3.2. Identification of biological pathways targeted by DENV-2-responsive and vitamin D attenuated miRNAs clusters*

To elucidate the biological role of the identified miRNAs and their predicted targets during DENV-2 infection of human macrophages, the miRNAs from every cluster were included in a gene-term enrichment analysis using clueGO to query Gene Ontology (GO) categories, Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome and Wiki pathways. The analysis identified biological pathways for the predicted target genes of the differentially expressed miRNAs in each cluster. As shown in Fig. 2A–B and Supplementary Table 2, predicted target genes for both DENV-responsive and vitamin D attenuated miRNAs clusters shared common target pathways that were predominantly associated with monocyte/macrophage differentiation, regulation of immune activation, mainly *via* TLR pathways, cell signaling adaptor molecules and NF-κB-mediated activation. In addition, response to the virus, cellular transport, RNA metabolic process, cell death, metabolism and cell cycle were identified for these clusters. Moreover, predicted target genes for vitamin D/DENV responsive cluster of miRNAs were related to immune activation, cellular homeostasis, metabolism, extracellular matrix and response to vitamins, such as vitamin E, suggesting that responses to vitamins present an overlap in many genes (Fig. 2C and Supplementary Table 2). These observations highlight the potential effect of vitamin D on the immune-related miRNAs altered during DENV-2 infection and their participation in the control of immune responses.

### *3.3. miR-155 is relevant to the immune pathways altered by vitamin D and DENV infection in MDMs*

To identify potential targets of miRNAs involved in DENV-induced immune activation pathways and their crosstalk with vitamin D, miRNAs from every cluster were also mapped to experimentally validated targets. To this end, miRNA and target gene interactions (MTIs) were identified from information deposited in the online databases miRTarBase, TarBase and miRecords (Chou et al., 2016; Sethupathy et al., 2006; Xiao et al., 2009). Only MTIs validated experimentally by reporter assay were selected for this study and identified targets were filtered by selecting those that are known to be expressed in human macrophages (Beyer et al., 2012). The validated targets were used in combination with miRNAs from each cluster to generate interaction networks and visualize miRNA-target mRNA relationships at a broad level. In general, we found a network including 403 MTIs between 19 miRNAs and 340 target mRNAs (Fig. 3A and B). Interestingly miRNAs from DENV-responsive and vitamin D attenuated clusters sub-networked together with every miRNA affecting at least 2 target mRNAs, and approximately, one-third of these mRNAs were co-deregulated by 2 or more miRNAs (Fig. 3A-B and Supplementary Table 3). This indicates a complex inter-connected network regarding both target multiplicity and miRNA cooperativeness that can be modulated by vitamin D activity. The number and the overlap among these predicted mRNA targets for each miRNA are represented in Fig. 3A. Only the miRNA let-7d-5p showed no overlap with other targets. In contrast, miR-155-5p, a DENV infection responsive miRNA that was also attenuated by vitamin D, showed to target approximately 140 different genes and target overlapping with almost all the miRNAs in the network. In addition, the rest of the miRNAs in the network (miR-223-3p, miR-26b-5p, miR-98-5p, miR135-5p, miR-20-5p, miR-146-5p, miR-31-5p, miR-335-5p, let-7e-5p, miR-195-5p, miR-134-5p and miR-147a) appeared to target only from 4 to 40 genes with a low index of overlapping with other miRNAs. Furthermore, miRNAs from the vitamin D/DENV responsive cluster showed low overlapping, and no connection with either DENV responsive or vitamin D attenuated miRNAs (Fig. 3B). These data suggest an important role of cellular miRNAs and vitamin D interplay during macrophage response to DENV-2 infection and predominant participation of miR-155-5p along these immune-related pathways.

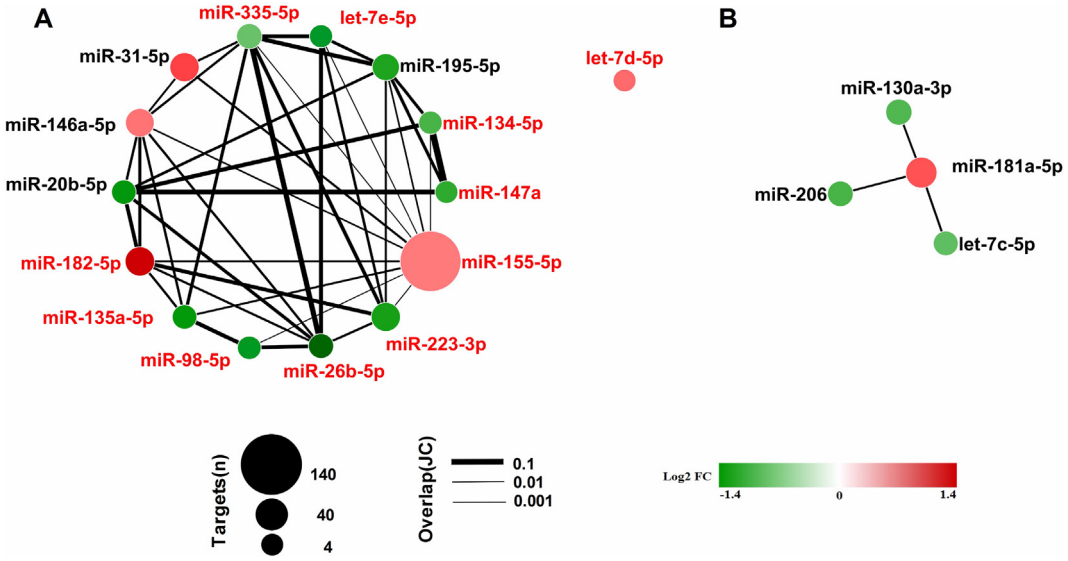


**Fig. 3.** Differentially regulated miRNA-mRNA network. A) DENV-responsive miRNAs (green) and Vitamin D- attenuated miRNAs (red) sub-network. B) Vitamin D/DENV responsive miRNAs sub-network. Connecting solid lines represent miRNA-mRNA interaction and edge width denotes the overlap between miRNAs measured by the Jaccard coefficient (JC). Node size represents the number of targets for each miRNA and is colored based on the log2 fold change expression of each miRNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.4. DENV-induced expression of miR-155-5p is attenuated in $D_3$-MDM

miR-155-5p clustered as a Vitamin D attenuated miRNA. Thus, we aimed to explore whether vitamin D and miR-155-5p interplay could participate in the downregulation of the cytokine response induced by DENV in $D_3$-MDM reported by us (Arboleda Alzate et al., 2017). First, we aimed to specifically validate by RT-qPCR the miR-155-5p expression in DENV-infected MDM and $D_3$-MDM. For this, MDM and $D_3$-MDM were infected with DENV-2 at MOI 10 and 24 hpi the percentage of infection was measured by intracellular detection by FACS of DENV E protein labeled with anti-4G2 antibody. As we reported previously (Arboleda Alzate et al., 2017) we found that at 24 hpi the average of infected MDMs was around 9.5% whereas for $D_3$-MDMs was around 4% (data not shown). Next, the cells were lysed, and RT-qPCR for miR-155-5p was performed as described in the methods section. In support of our observations from the PCR array assay, exposure to DENV-2 induced a significant increase in the transcriptional activity of miR-155-5p in MDM (fold change = 3) (Fig. 4A). Conversely, in $D_3$-MDM a decrease in the miR-155-5p levels was observed (fold change = 0.2) and was statistically significant as compared with that observed in MDM ($p < .05$). Targeting of SOCS1 mRNA by miR-155 has been shown to be altered by vitamin D signaling (Chen et al., 2013a); therefore, to functionally verify our findings, we also quantified mRNA levels of SOCS-1. In MDM, the SOCS-1 mRNA levels remained unaltered after exposure to DENV2 (fold change = 1) (Fig. 4B). However, in $D_3$-MDM, we found a significant increase of SOCS-1 mRNA levels in response to DENV2 infection (fold change = 5) ($p < .05$), likely due to the attenuation of miR155-5p in $D_3$-MDM (Fig. 4B). These data provide evidence that DENV-responsive miR-155-5p is attenuated by vitamin D activity in human macrophages and may play a role in the regulation of immune responses of these cells.

We previously reported a reduction of DENV infection in $D_3$-MDM (Arboleda Alzate et al., 2017). To confirm the attenuation of miR-155 in $D_3$-MDM as an intrinsic host factor and not as a result of the viral antigen decrease, we aimed to evaluate miR-155 and SOCS1 dynamics after stimulation of TLR4. This receptor is well known to induce miR-155 and to be equally modulated by the regulatory loop of this miRNA (Bala et al., 2011; Ceppi et al., 2009; Swaminathan et al., 2012). First, we validated by RT-qPCR the mRNA levels of miR-155-5p after TLR4 stimulation with LPS (100 ng/mL) in MDM and $D_3$-MDM. Fig. 4C shows

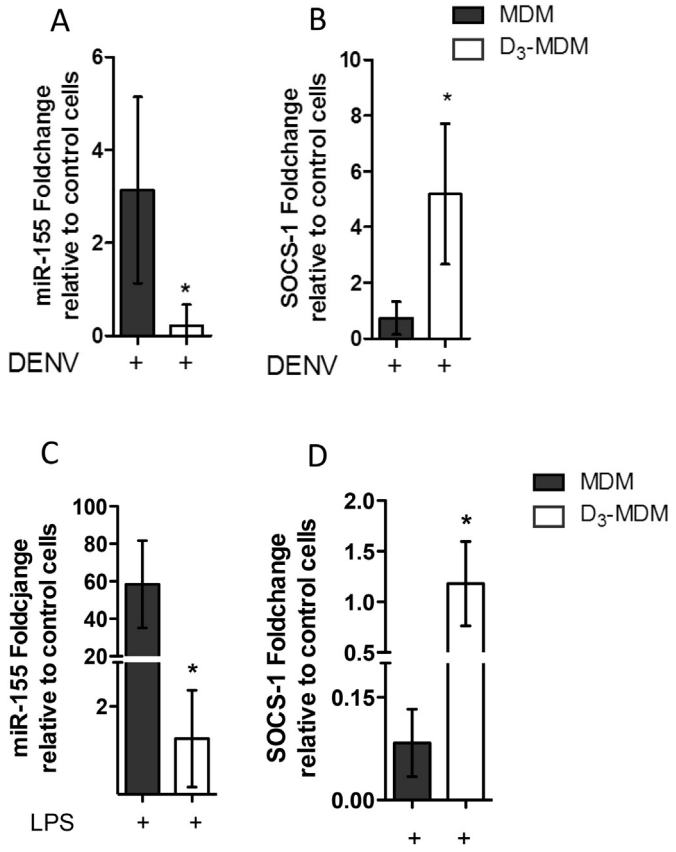


**Fig. 4.** RT-qPCR Validation of DENV and LPS-induced attenuation of miR-155 in $D_3$-MDM. Monocyte-derived macrophages were differentiated in absence (MDM) and presence ($D_3$-MDM) of vitamin D (0.1 nM) and exposed to DENV-2 (MOI 10) (A and B) or LPS (100 ng/mL) (C and D). Samples were collected 24hpi, and RT-qPCR for miR-155 and SOCS-1 were performed. Fold-change values were calculated in relation to mock-infected MDM and $D_3$-MDM as described in the methods section. Bars represent mean ± SD of data from 3 different donors. Mann-Whitney test; $*p < .05$, $**p < .01$. n.s = non-significant.

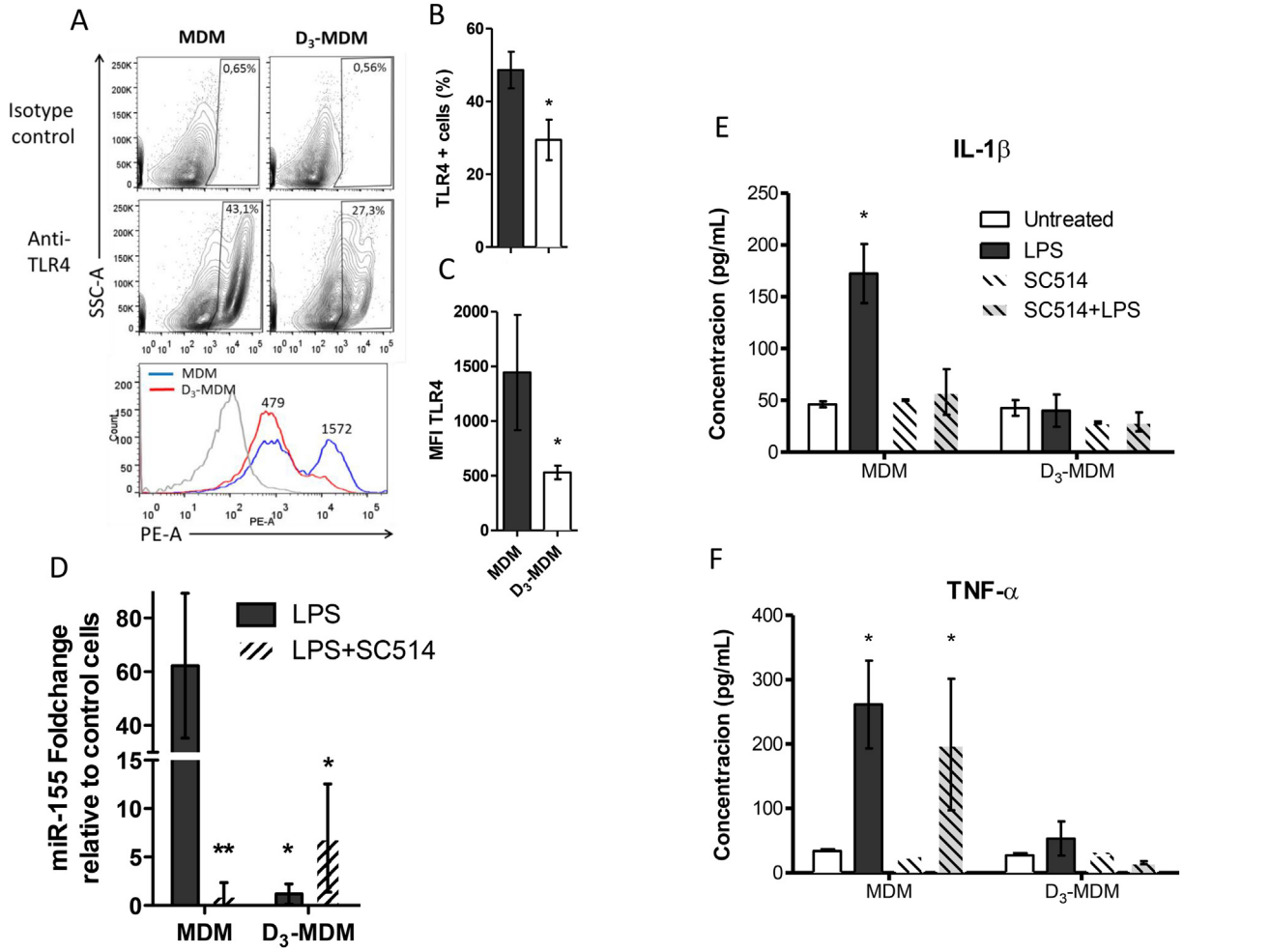


**Fig. 5.** TLR4 expression and TLR4/NF-κB-derived activation in MDM and $D_3$-MDM. MDM and $D_3$-MDM were obtained, and FACS measurement of surface TLR4 was performed. A) Contour plots for gating strategy, isotype controls and geometric Mean fluorescence intensity (MFI) histograms for TLR4 in MDM and $D_3$-MDM. B) Percentage of TLR4+ cells. C) MFI of TLR4. MDM and $D_3$-MDM were pre-treated with SC-514 (100uM) and stimulated with LPS (100 ng/mL), and RT-qPCR was performed for D) miR-155 as described in methods. Culture supernatants were collected and the concentration levels of E) IL-1β and F) TNF-α were measured by ELISA. Bars represent mean ± SD of data from 3 different donors. Mann-Whitney test; $*p < .05$, $**p < .01$, n.s = non-significant.

that, similarly to DENV-induced miR-155-5p, LPS induced a significant increase in the miR-155-5p mRNA levels only in MDM (fold change = 60) but not in $D_3$-MDM (fold change = 1) ($p < .05$). Additionally, we found that LPS induced a decrease in the mRNA levels of SOCS-1 in MDM (fold change = 0.1) (Fig. 4D). In contrast, for $D_3$-MDM, mRNA levels of SOCS-1 were increased (fold change ~1.2) and were statistically significant as compared to that in MDM ($p < .05$). All these data show an overall functional attenuation of miR155-5p after TLR4 stimulation of $D_3$-MDM.

### *3.5. Attenuation of miR-155 in $D_3$-MDM is linked to downregulated TLR4 expression and reduced NF-kB-derived cytokine response*

The miR-155 expression can be up-regulated in response to TLR4-derived activation (Chen et al., 2013a). To further investigate a potential mechanism supporting the attenuation of miR-155 in $D_3$-MDM, we aimed to evaluate the profile of surface TLR4 expression in MDM and $D_3$-MDM by flow cytometry. Fig. 5A–C shows the gating strategy for TLR4 measurement, the percentage of TLR4+ cells and the mean fluorescence intensity (MFI) of TLR4, respectively. We found that both the percentage of TLR4+ cells and MFI were significantly lower in $D_3$-MDM as compared to MDM, suggesting that less engagement of TLR4 could be responsible for the attenuation of miR-155 in $D_3$-MDM. Indeed, TLR4-derived regulation of miR-155 occurs *via* activation of the nuclear factor NF-κB that controls the B-cell integration cluster (BIC) containing the primary transcript of miR-155 (Kluiver et al., 2005). To verify the role of TLR4/NF-κB axis in the attenuation of miR-155 gene, we pre-treated cells with SC-514, a selective inhibitor of IKK-β that is an activation subunit of the NF-kB complex. Accordingly, LPS induced an increase of miR-155-5p levels in MDM (fold change ~60) (Fig. 5D). However, in SC-514 pre-treated MDM, LPS did not alter the miR-155 levels (fold change = 1) ($p < .01$) and were comparable with those observed in $D_3$-MDM. These results suggest that in MDM and $D_3$-MDM, the expression of miR-155-5p is mainly dependent on the NF-kB-derived transcription.

In our previous report, we found that the cytokine response induced by DENV was reduced in $D_3$-MDM (Arboleda Alzate et al., 2017). Therefore, we aimed to evaluate whether inhibition of TLR4/NF-κB was linked to the downregulation of the cytokine response in MDM and $D_3$-MDM. For this, we measured by ELISA the cytokine accumulation in culture supernatants 24 h post stimulation with LPS. We found that LPS induced a significant increase of IL-1β and TNF-α secretion in MDM as compared to untreated cells, however in $D_3$-MDM no significant increase was detected for both cytokines (Fig. 5E–F), corroborating our previous data. Also, we found that LPS induction of IL-1β levels was reduced significantly by the SC-514 pre-treatment in MDM and was comparable to that observed in LPS stimulated $D_3$-MDM (Fig. 5F), highlighting the importance of TLR4/NF-κB pathway for the production

of this cytokine in MDM and $D_3$-MDM. In contrast, LPS-induced TNF-α secretion was not affected by SC-514 (Fig. 5F), suggesting that vitamin D effect on TNF-α secretion may not be fully dependent on the NF-κB activation.

## 4. Discussion

Recently we have shown that in $D_3$-MDM the cytokine secretion induced by DENV can be partially restricted *via* downregulation of the mannose receptor, a key trigger of inflammatory responses (Arboleda Alzate et al., 2017). Here, we identified an alternative mechanism by which vitamin D can contribute to the down-tuning of the inflammatory responses induced by DENV. We found that immune-related miRNAs that are responsive to DENV infection can be attenuated by vitamin D. Among these, miR-155 was identified to have a predominant role in the control of TLR/NF-κB-mediated signaling pathways during DENV infection of human macrophages. Indeed, our RT-qPCR validation of miR-155 attenuation after exposure to DENV and LPS in independent experiments was linked to downregulation of surface TLR4 expression and reduced TLR4/NF-κB-derived IL-1β secretion.

Attenuation of miR155 in our profiling approach and independent experiments could be associated to the decreased infection in $D_3$-MDM reported by us (Arboleda Alzate et al., 2017). However, we demonstrated a similar but stronger pattern of miR-155 attenuation in $D_3$-MDM after LPS stimulation, highlighting an intrinsic involvement of the TLR4 pathway in the host cells. TLR4-derived signaling can regulate or be regulated by miRNA-155 *via* targeting of adaptor molecules or by silencing of TLR4 expression, respectively (Bala et al., 2011; Ceppi et al., 2009; Chen et al., 2013a; Wang et al., 2010, 2016). In line with this and as reported before (Puerta-Guardo et al., 2012), we observed a general downregulation of TLR4 and reduced LPS-induced cytokine response in $D_3$-MDM, corroborating the vitamin D mediated effect on the TLR4/ miR-155 axis as a key tuning mechanism controlling excessive inflammatory responses in human macrophages (Arboleda and Urcuqui-Inchima, 2016; Chen et al., 2013a).

The regulatory feedback loop between TLR4 and miR-155 induced by vitamin D relies mainly on the interaction of vitamin D receptor (VDR) and the NF-κB transcription factor. Activated VDR can physically interact and block IKKβ, a subunit required for NF-κB activation, which is, in turn, essential for the transcription of the miR-155 precursor known as BIC (Adli et al., 2010; Chen et al., 2013b; Kluiver et al., 2005; Li et al., 2014). This link between NF-κB and VDR activity not only supports our report on the control of DENV-induced cytokine response (Arboleda Alzate et al., 2017) but also the observation that the attenuation of miR-155 after DENV infection of $D_3$-MDM can be linked to the downregulated TLR4/NF-κB-derived activation. For instance, in response to LPS, inhibition of IKKβ by SC514 in MDM mimicked the downregulation of the TLR4-derived IL-1β response and the miR-155 attenuation observed in $D_3$-MDM. Although we cannot rule out that TLR4 downregulation in $D_3$-MDM is a direct consequence of the vitamin D mediated miR-155 effect, SOCS1 has been shown as a potential target of miR-155 during DENV infection (Chen et al., 2014). In line with this, our data show that SOCS1 expression was significantly upregulated in $D_3$-MDM after both DENV and LPS stimulation. This virtually corroborates the functional effect of vitamin D on miR-155 attenuation and its target gene SOCS1 (Wang et al., 2010), which is extensively known to modulate the TLR4-NF-κB pathway (Kempinska-Podhorodecka et al., 2017; Li et al., 2014; Yoshimura et al., 2007).

A major role of TLR4-derived signaling has been suggested to fuel DENV pathogenesis (Costa et al., 2013). The *de novo* replication of DENV involves the secretion of the non-structural protein 1 (NS1) that has been shown to induce highly altered inflammatory responses and endothelial dysfunction, exclusively *via* activation of TLR4 (Modhiran et al., 2017; Modhiran et al., 2015). Our findings that vitamin D can modulate the TLR4/miR-155-derived signaling during DENV infection may provide insights into diagnostic and therapeutic strategies to avoid progression of dengue disease. Indeed, we recently reported that vitamin D oral supplementation in healthy donors is associated with less susceptibility of monocyte-derived macrophages to DENV infection and reduced pro-inflammatory cytokine response, *in vitro* (Giraldo et al., 2018). In support of this, in patients infected with hepatitis C virus (HCV), vitamin D supplementation has been shown to improve response to treatment, viral control and serum markers associated to the reversal of hepatic fibrogenesis induced by excessive inflammation (Abu-Mouch et al., 2011; Komolmit et al., 2017; Nimer and Mouch, 2012; Yokoyama et al., 2014). Importantly, miR-155 has been identified as a regulator of the TLR3/ NF-κB-derived immune responses induced by HCV; and likewise, high circulating levels of miR-155 have been associated to chronic hepatitis (Bala et al., 2012; Jiang et al., 2014). Moreover, abnormal immune responses and tumor-promoting risk during chronic hepatitis B virus (HBV) infection are associated to high expression of TLR4 and high circulating levels of miR-155, whereas those levels are significantly lower in non-chronic HBV patients and healthy individuals (Wang et al., 2015; Yu et al., 2016).

We additionally identified other miRs that can be modulated by vitamin D upon DENV infection and that can also inhibit viral replication, as the case of Let-7 family miRs (Escalera-Cueto et al., 2015). However further research is required to determine their antiviral and immunoregulatory role in dengue pathogenesis. The present study allows us to propose that vitamin D-mediated effect on the TLR4/NF-κB/ miR-155 axis is a likely mechanism by which vitamin D can limit the excessive inflammation in human macrophages infected to DENV and this could decreased the severity of the disease provokes by the cytokines response. These findings reveal that miR-155 may be a key regulator or biomarker for dengue and highlights the importance of vitamin D as a therapeutic alternative to avoid progression of the disease.

## Competing interests

The authors of this manuscript declare no competing interests.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.12.033.

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