Vitamin D-induced LL-37 modulates innate immune responses of human primary macrophages during DENV-2 infection

Running title: Immunomodulatory properties of LL-37 in macrophages infected with DENV-2

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ABSTRACT

Epidemics of dengue, an acute and potentially severe disease caused by mosquito-borne dengue virus (DENV), pose a major challenge to clinicians and health care services across the sub(tropics). Severe disease onset is associated with a dysregulated inflammatory response to the virus and there are currently no drugs to alleviate disease symptoms. LL-37 is a potent antimicrobial peptide with a wide range of immunoregulatory properties. In this study, we assessed the effect of LL-37 on DENV-2-induced responses in human monocyte-derived macrophages (MDMs). We show that simultaneous exposure of exogenous LL-37 and DENV-2 resulted in reduced replication of the virus in MDMs, while the addition of LL-37 post-exposure to DENV-2 did not. Interestingly, the latter condition reduced the production of IL-6 and increased the expression of genes involved in virus sensing and antiviral response. Finally, we demonstrate that low endogenous levels and limited production of LL-37 in MDMs in response to DENV-2 infection can be increased by differentiating MDMs in the presence of Vitamin D (VitD3). Taken together, this study demonstrates that in addition to its antimicrobial properties, LL-37 has immunomodulatory properties in the curse of DENV infection and its production can be increased by VitD3.

KEYWORDS

Dengue virus, macrophages, innate immune response, vitamin D, LL-37, inflammation

INTRODUCTION

Dengue is a mosquito-borne infectious disease threat causing a major health problem worldwide. Dengue and severe dengue can be caused by one of the four related but antigenically different serotypes of dengue virus (DENV 1-4) [1]. Annually, an estimated 390 million individuals acquire DENV infection of which approximately 0.5 - 1 million individuals develop severe dengue, and over half of the global population is at risk for DENV infection. The precise mechanisms underlying progression to severe dengue are not fully understood, and these are likely to be a combination of virus, mosquito and host factors [2,3]. However, clinical observations and *in vitro* studies have pointed out to dysregulated inflammation as a common characteristic preluding severe dengue [4,5]. Despite the high clinical impact, there are no specific antiviral therapies for DENV-infected patients, and the approved vaccine is not fully protective [6]. Therefore, antiviral therapies should be able to target virus replication and/or be able to modulate the inflammatory response leading to severe dengue onset.

DENV is an enveloped virus with a single-stranded RNA genome of positive polarity (ssRNA+) of approximately 11kb in length. DENV genomic RNA has one open reading frame that codifies for a single polyprotein which is further processed by cellular and viral proteases [7]. Upon infection and translation of the viral RNA, the action of these proteases leads to the production of three structural proteins (capsid, pre-membrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). These non-structural proteins are involved in the regulation of viral protein synthesis, in viral replication and the evasion of immune responses [8]. Immune sentinels, such as macrophages, sense DENV particles and replication through the expression of an array of pattern recognition receptors (PRRs) including retinoic acid-inducible gene-I-like receptors (RLRs) such as RIG-I and MDA5 [9], and Toll-like receptors (TLRs) [9,10]. Recognition of molecular patterns PRRs ultimately leads to the production of proinflammatory cytokines and antiviral type I interferons (IFN I), which play a crucial role in containing the infection [11].

The human cathelicidin antimicrobial peptide (CAMP, LL-37) is a product of the proteolytic cleavage of the cationic 18kDa protein (hCAP-18) encoded by the CAMP gene [12]. Similar

to other antimicrobial peptides such as beta-defensins and due to its amphipathic structure, LL-37 binds and disturbs the membrane of microbes thereby inducing the lysis of the cells [13]. LL-37 has been shown to have antiviral activity against several enveloped viruses including Influenza A virus (IAV) [14], human respiratory syncytial virus (RSV) [15], Venezuelan equine encephalitis virus (VEEV) [16] and DENV [17]. Previously, it has been shown that simultaneous treatment of Vero E6 cells with LL-37 and DENV-2 led to decreased viral infection and replication [18]. Furthermore, Castañeda-Sanchez et al. (2016) reported that DENV infection of THP1 cells induced both mRNA and protein expression of human beta-defensin 1 (HBD1) and cathelicidin LL-37 [19], while DENV-2 infection in human HaCat keratinocytes also induced LL-37 production and showed antiviral activity against DENV-2 [20]. These studies support the hypothesis that the LL-37 peptide plays an important role in the control of DENV-2 replication and spread. Yet, little is known about its role in DENV-infected human primary macrophages.

In addition to its antimicrobial potential, LL-37 has been shown to exhibit wide immunomodulatory effects *in vitro* and *in vivo*. LL-37 can dampen or enhance the immune responses depending on the cell type and the nature of the agonist recognized by different PRRs. For example, LL-37 inhibits activation of p38 and extracellular signal-regulated kinases (ERK) leading to a decreased production of TNF-α after LPS stimulation in mouse macrophages [21]. Furthermore, LL-37 can bind directly to LPS interfering with its downstream inflammatory signaling [22]. On the other hand, LL-37 upregulates the expression of type I interferons (IFN-I), TNF-α, and IL-6 in dendritic cells (DCs). This mechanism appears to be mediated by the cationic nature of LL-37, which forms complexes with extracellular nucleic acids, both self-RNA and DNA, thus promoting an enhancement of TLR7/8 and TLR9 activation [23]. However, the immunoregulatory effect of LL-37 in DENV primary cell targets has not been established yet.

Here, we sought to determine whether LL-37 alters DENV infectivity and induced immune responses in human primary macrophages infected with DENV-2. To this end, we assessed the antiviral effect of LL-37 in monocyte-derived macrophages (MDMs) and the effect of LL-37 on the expression of PRRs involved in DENV sensing, SOCSs, IFN-I, and IFN-stimulated genes (ISGs). We show that LL-37 limits DENV-2 replication in MDMs and modulates the

expression of some proinflammatory cytokines, TLRs, and increases the expression of some ISGs.

MATERIALS AND METHODS

Cell lines and reagents

Aedes albopictus mosquito C6/36-HT cell line was obtained from the ATCC and maintained in Leibovitz L-15 medium (L-15) (Sigma-Aldrich, USA) supplemented with 2% FBS (Sigma-Aldrich, USA) at 34°C without CO_2. BHK-21 cells were purchased from ATCC and maintained in D-MEM medium (Sigma-Aldrich, USA) supplemented with 2% FBS (Sigma-Aldrich, USA) at 37°C with 5% CO2. Synthetic peptide LL-37 used in this study was purchased from RyD systems (USA). For some assays LPS (Invivogen, USA) was used at a concentration of 20ng/ml for 24 hours.

Virus stocks

DENV-2 strain New Guinea C (NGC) was provided by the Center for Disease Control and Prevention (CDC, USA) and used in all experiments. Viral stocks were obtained by infecting a confluent monolayer of C6/36-HT seeded in a 75cc flask with a multiplicity of infection (MOI) of 0.05, as previously described [24]. The supernatant was aliquoted and stored at -70°C for future use.

Virus titration

Virus titration was performed by quantifying plaque-forming units (PFU) by plaque assay in BHK-21 cells and by the quantification of the genome equivalent copies (GECs) using RT-qPCR as described briefly below.

For PFUs calculation, 5×10^4 BHK-21 cells seeded in 24-well plates, were infected with 10-fold serial dilutions of the virus in 250 µL of the medium. After 2 h of adsorption, the virus was removed and washed once with PBS and D-MEM medium containing 1.5% m/v carboxymethylcellulose sodium salt (medium viscosity, Sigma-Aldrich, USA) and 2% FBS, 4 mM L-glutamine and 10 units/ml Penicillin/0,1 mg/ml Streptomycin, was added to the cells. After 5 days of culture at 37°C with 5% CO2, the medium was removed, and cells were incubated with 4% m/v crystal violet solution and 3.5% v/v formaldehyde (Merck,

Germany) for 30 min. After fixing and staining, cells were washed once with PBS, and the plaque count was performed manually to obtain PFU/mL.

For GECs quantification, total RNA was purified from supernatants of DENV-2-infected and LL-37-treated MDMs, using Trizol reagent (Thermo Scientific, USA). RNA was reversed transcribed (cDNA) using Revertaid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA), using 50ng of RNA. cDNA was used for amplification using specific primers depicted in supplemental Table 1 and Maxima SYBR Green qPCR master mix (Thermo Scientific, USA). Samples were analyzed in CFX96™ Real-Time PCR Detection System (Biorad, USA). Calculation of DENV RNA copies was based on a standard curve of Ct values of 10-fold serial dilutions of a plasmid encoding full genome of DENV-2 of 7797 nt of length and a concentration of 1ng/ml.

Macrophage differentiation

To obtain monocyte-derived macrophages (MDMs), peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained from Buffy coats donated by the blood bank "Escuela de Microbiología, UdeA, Medellín, Colombia". PBMCs were separated using a density gradient centrifugation with lymphoprep (Stem cell technologies, USA), centrifuged at 800 g at room temperature for 20 min. Then, 5 x 10^5 CD14+ cells were seeded in 24-well plates (Corning Incorporated Life Science, USA), in RPMI-1640 medium supplemented with 0.5% of inactivated autologous serum and cultured at 37°C with 5% CO2 to allow enrichment of monocytes. After 3 hours of adherence, non-adherent cells were removed by extensive washing with pre-warmed PBS supplemented with 0.5% FBS. Adherent cells were differentiated to macrophages for 6 days in RPMI-1640 supplemented with 10% FBS at 37°C with 5% CO2. Culture medium with 10% FBS was replenished every 48 hours. MDMs purity was always above 90%, measured by the presence of contaminant cell populations including CD19+, CD3+, and CD56+.

Macrophage differentiation in the presence of Vitamin D3

To obtain MDMs differentiated in the presence of VitD3 (D3-MDMs), 1α,25-dihydroxyvitamin D3 (calcitriol, Sigma-Aldrich, USA) was added to primary monocytes at a final concentration of 0.1 nM, immediately after their purification. Fresh medium with 0.1 nM of Calcitriol was replenished every 48 hours. To evaluate the effect of calcitriol in D3-

MDMs cultures, VDR and cytochrome P450 family 24 subfamily A member 1 (CYP24A1) mRNAs were quantified by RT-qPCR.

MDMs and D3-MDMs infection with DENV-2

MDMs and D3-MDMs monolayers cultures in 24-well plates, were challenged with DENV-2 at an MOI of 5 diluted in 300 µl of RPMI-1640 supplemented with 2% FBS. After 2 hours, cells were washed with PBS, and the medium was replenished with RPMI-1640 containing 10% FBS and cultured at 37°C with 5% CO2. At 24 hours post-infection, cell monolayers were harvested and the percentage of DENV E positive cells was quantified by flow cytometry. Cell culture supernatants were used for the quantification of viral copy numbers by RT-qPCR and viral titration by plaque assay.

Treatment of MDMs with LL-37

After 6 days of differentiation, MDMs were treated with LL-37 using two different conditions. MDMs were infected with DENV-2 at an MOI of 5 for 2 hours in the presence of increasing concentrations of LL-37 (0.25 µM to 5µM) as previously described [18]. At 2 hours post-infection, the inoculum was washed, and cells were cultured in the absence of LL-37 for 24 hours. This is referred as simultaneous treatment (ST). Also, MDMs were infected with DENV-2 at an MOI of 5 and after 2 hours of infection, the inoculum was removed and a medium containing 5µM of LL-37 was added for another 22 hours. This is referred to as post-treatment (PT) conditions. DENV-2 infection was determined by the assessment of the percentage of E-positive cells by flow cytometry. In addition, DENV-2 genome load and production of infectious particles were determined by the quantification of the intracellular viral RNA copies and the viral titer in the supernatant, only for ST condition.

Flow cytometry assays

The percentage of DENV-2-infected cells was determined through the detection of viral E protein using flow cytometry. Briefly, infected MDMs were fixed using a Fixation/Permeabilization buffer (eBioscience, USA) and stained with the monoclonal antibody anti-Flavivirus envelope (E) protein (clone 4G2, Millipore, Germany). Next, secondary fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Thermo

Scientific, USA) was used for detection of E protein. Cells were analyzed on a FACScan flow cytometer (BD Biosciences, USA) using the FACSDiva software.

LL-37 and cytokine production

Antimicrobial peptide LL-37 levels were quantified in supernatants from mock and DENV-2-infected MDMs and D3-MDMs, using the human cathelicidin antimicrobial peptide (CAMP) ELISA kit (MyBioSource, USA). Quantification of IL-6 and TNF-α were assessed in supernatants from DENV-2-infected MDMs treated with LL-37 (ST and PT conditions) at 24 hpi, using an ELISA assay (BD OptEIA, BD Biosciences, USA). Results from all ELISAs were analyzed in a spectrophotometer (Dynex Technologies, USA) using a wavelength of 450 nm.

Quantification of gene expression

Total RNA was purified from mock and DENV-2-infected MDMs and treated with LL-37 (ST and PT conditions) using Trizol reagent (Thermo Scientific, USA). cDNA synthesis was done as described above and used for quantification of IFN-β, PKR, OAS TLR3, TLR4, TLR9, RIG-I, SOCS-1, and Ubiquitin mRNA, through RT-qPCR and Maxima SYBR Green (Thermo Scientific, USA). The expression of mRNA from CAMP and Ubiquitin were also evaluated in MDMs and D3-MDMs infected with DENV-2 at 2, 8, and 24 hours post-infection (hpi). Specific primers were used for amplification (Supplemental Table 1), whereas the specificity of the amplification product was determined by a melting curve. The relative quantification was expressed by the ΔΔCt method: (1.8)−ΔCt, in which 1.8 is the mean efficiency of the PCR, and ΔΔCt is the difference between the critical threshold values of two independent replicates of each sample and the constitutive gene ubiquitin.

Statistical analysis

Results were analyzed using the statistical software GraphPad Prism version 6 (USA). Comparisons between MDMs and D3-MDMs were done with a two-way ANOVA analysis along with a Bonferroni post-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

DENV-2 infection in MDMs decreases production of LL-37 peptide and CAMP mRNA expression

LL-37 and human beta-defensins (HBD) are antimicrobial peptides produced by various cell types upon DENV infection [18–20]. To test whether expression of these peptides is upregulated in DENV-2-infected macrophages, the expression of CAMP mRNA (LL-37 gene) and production of LL-37, HBD2, and HBD3 were quantified. As reported previously, DENV-2 activately replicated in MDMs as evidenced by the proportion of infected cells (Fig 1A), viral load in supernatant and production of infectious particles (Fig 1B). Unexpectedly, a significant decrease of CAMP mRNA expression was observed in DENV-2-infected MDMs compared to mock-infected cells, from 2 up to 24 hours post-infection (hpi) (Fig 1C). Furthermore, we did not detect any significant difference in the production of peptide LL-37 between DENV-2-infected MDMs and mock-infected MDMs (Fig 1D). To verify whether the low levels of LL-37 produced in response to DENV-2 infection was not due to the low responsiveness of MDMs, we treated cells with LPS for 24 hours. LPS significantly enhanced the production of LL-37 in comparison to mock or DENV-2-infected MDMs (Fig 1D), indicating that exposure of MDMs to DENV-2 does not induce LL-37 production. Furthermore, DENV-2 infection or LPS stimulation did not increase the production of HBD2 and HBD3 by MDMs, when compared to mock-treated cells (see Supplementary figure 1A). Taken together, these results indicate that exposure to DENV-2 does not induce expression of CAMP mRNA and subsequent production of peptide LL-37 in MDMs.

The peptide LL-37 inhibits DENV-2 infection and replication in MDMs

Before testing the ability of LL-37 to modulate the immune responses induced by DENV-2 infection, we sought to establish its effect in DENV-2 replication in human primary cells. For this, MDMs were infected with DENV-2 and treated simultaneously (ST) with increasing concentrations of synthetic LL-37 (0.25μM to 5μM) which were based on previous studies [17,18], and viral infection was evaluated at 24 hpi. Importantly, the highest concentration of LL-37 (5μM) had no effect on the viability of MDMs either alone or in combination with DENV-2 (Supplementary Fig 1B). As observed in Fig 2A and 2B, LL-37 treatment significantly decreased the percentage of cells expressing DENV-2 envelope

protein (E) in a dose-dependent manner. The highest inhibitory effect of LL-37 was observed at 5μM, while the inhibitory effect was diminished at 0.25 μM (Fig 2B). This data allowed us to calculate a dose-response curve which showed the IC50 at 0.4μM, and the IC90 at 1.3μM (Fig 2C). To confirm the antiviral effect of LL-37 during DENV-2 infection, we next assessed the production of new virions. Indeed, 5μM of LL-37 decreased the genomic equivalent copies (GECs) titer by approximately 2 logs (Fig 2D) and the infectious titer (PFU) by approximately 3 logs (Fig 2E), when compared to infected MDMs without LL-37 treatment. These results indicate that LL-37 inhibits DENV-2 infectivity in MDMs.

Alagarasu et al. suggested that LL-37 may block DENV entry into target cells since *in silico* analysis predicted LL-37 binding to E viral protein [18]. To verify this hypothesis, we compared the extent of inhibition of DENV-2 infection by LL-37 when it was added ST with the viral inoculum, or when it was added after removal of the virus inoculum at 2 hours of infection as a post-treatment (PT). Following this approach, ST condition led to the highest effect in the inhibition of DENV-2 infection (% of DENV-2 E+ cells), whereas no effect was observed under PT condition (Fig 2F). Altogether, these results suggest that LL-37 restricts the early steps of DENV-2 infection and has no effect on post-entry steps of viral replication.

The antimicrobial peptide LL-37 regulates the expression of TLRs, RIG-I, IFN-β, PKR, OAS, and SOCS-1 mRNAs in DENV-2-infected MDMs

LL-37 has been shown to have wide immunoregulatory functions [12]. However, reduced infection as observed in DENV-2-infected MDMs in the presence of ST with LL-37 is likely to reduce inflammatory responses as well. Therefore, to distinguish the immunomodulatory effect of LL-37 from its antiviral effect, we assessed the regulation of immune responses in the absence and presence of LL-37 in DENV-2-infected MDMs under ST and PT conditions.

Macrophages sense DENV infection through an array of pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and retinoic acid-inducible gene-I-like receptors (RLRs) such as RIG-I [9,10]. Thus, first, we sought to test if LL-37 differentially regulates PRRs that are involved in DENV infection. Specifically, mRNA levels of TLR3, TLR4, TLR9, and RIG-I were quantified by RT-qPCR in DENV-2-infected MDMs and treated with LL-37 under ST and PT conditions (Fig 3A-3D). Treatment of MDMs with LL-37 alone did not alter

the gene expression of any tested gene compared to mock-infected MDMs, except for TLR9 mRNA, where a significant upregulation was observed compared to untreated MDMs (Fig 3C). DENV-2 infection significantly upregulated the expression of TLR3, TLR9 and RIG-I mRNA (Fig 3A, 3C and 3D), while ST with LL-37 only significantly increased the expression of TLR3 mRNA (Fig 3A) and RIG-I (Fig 3D). In contrast, mRNA expression levels of TLR3 and RIG-I were similar in DENV-2-infected MDMs in the absence and presence of LL-37 under PT conditions (Fig 3A and 3D). On the other hand, LL-37 significantly increased the mRNA expression TLR4 (Fig 3B) and TLR9 (Fig 3C) under PT condition, suggesting that LL-37 modulates the expression of these genes independently of viral replication.

Activation of PRRs by DENV also leads to expression of type I interferons (IFN-I), which play a crucial role in containing infection by inducing the expression of antiviral genes such as protein kinase K (PKR) and 2´5 Oligoadenylate synthase 1 (OAS1) [25]. To test whether LL-37 regulates antiviral IFN-I response, mRNA levels of IFN-β, PKR, and OAS1 were determined in DENV-2-infected MDMs and treated with LL-37 under ST and PT conditions. In mock-infected MDMs, LL-37 did not change expression levels of these genes (Fig 3E-H). Conversely, in DENV-2-infected MDMs addition of LL-37 under ST condition significantly increased the expression levels of IFN-β (Fig 3E), PKR (Fig 3F) and OAS (Fig 3G) mRNAs. PT with LL-37 resulted in an increase in the expression of PKR and OAS mRNA (Fig 3F and 3G) in DENV-2-infected MDMs, but expression levels of IFN-β mRNA were not changed compared to DENV-2 alone (Fig 3E). We also tested the modulation of SOCS-1 mRNA by LL-37 given the importance of the protein encoded by this gene in the negative feedback of proinflammatory cytokine signaling [26]. Similar to that observed with PKR and OAS, treatment with LL-37 under ST and PT conditions induced a significant increase in the mRNA levels of SOCS-1 in DENV-2-infected MDMs (Fig 3H), suggesting that LL-37 might contribute to the regulation of the inflammatory response through SOCS-1 upregulation. Taken together, our results suggest that immunoregulatory LL-37 specifically upregulates the transcription of several PRRs, interferon-stimulated genes (ISGs) and SOCS-1, which are known to be associated with the induction of well-balanced immune responses.

LL-37 decreases the production of TNF-α and IL-6 during DENV-2 infection of MDMs

Activation of PRRs during DENV infection leads to the production of inflammatory mediators [27]. To test if regulation of PRRs and SOCS-1 expression by LL-37 altered DENV-2 induced inflammatory responses, we assessed the production of IL-6 and TNF-α during DENV-2 infection in MDMs treated with LL-37 under ST and PT conditions. In the absence of LL-37, DENV-2 induced the production of high levels of IL-6 and TNF-α. However, as shown in Fig 4A, we found a significant decrease in the production of IL-6 in DENV-2-infected MDMs with both treatments (ST and PT), compared to DENV-2-infected MDMs without treatment. Considering that PT condition didn't affect viral replication, these results suggest that regulation of IL-6 production by LL-37 is independent of viral replication. In contrast, TNF-α production by DENV-2-infected MDMs was only significantly decreased under ST condition (Fig 4B), suggesting that the production of this cytokine is dependent of DENV-2 replication and is not modulated by LL-37. Mock-infected MDMs, with or without LL-37 treatment, did not produce high levels of IL-6 nor TNF-α (Fig 4A and B). Altogether, these results suggest that in addition to its antiviral properties, the LL-37 peptide has immunomodulatory properties in DENV-2-infected MDMs, as it can downregulate the production of IL-6.

MDMs differentiated in the presence of Vitamin D3 show increased expression of CAMP mRNA and LL-37 peptide under DENV-2 infection

Vitamin D3 (VitD3) is a pleiotropic hormone, which is known for its immunomodulatory effects in immune cells [28]. Importantly, CAMP expression and subsequent production of LL-37 is under control of the Vitamin D receptor (VDR) and is overexpressed in response to VitD3 treatment [29]. To test this in the context of DENV-2 infection, we exploited our previously established model of MDMs differentiated in the presence of VitD3 (D3-MDMs) [30]. The expression of CAMP mRNA and LL-37 peptide were quantified in mock and DENV-2-infected D3-MDM, and then compared to those found in MDMs. As expected, higher levels of CAMP mRNA and LL-37 were observed in mock-infected D3-MDMs compared to MDMs (Fig 5A and B), indicating that differentiation of MDMs in the presence of VitD3 promotes expression of CAMP. Notably, differentiation of MDMs with VitD3 recovered the expression of CAMP mRNA levels at 2 and 24 hpi (Fig 5A) and the production

of LL-37 peptide at 24 hpi (Fig 5B) in DENV-2-infected D3-MDMs. Interestingly, this effect was DENV-2-specific, as MDMs and D3-MDMs responded to LPS with the same high levels of LL-37 to stimulation. Altogether, these results suggest that differentiation of D3-MDMs with VitD3 increases the baseline production of LL-37 in response to DENV-2 infection.

DISCUSSION

Herein we evaluated the effect of the peptide LL-37 on DENV-2 infection-induced innate immune responses in human monocyte-derived macrophages (MDMs). Our data showed that while DENV-2 infection does not induce expression of LL-37 in MDMs, exogenous administration of LL-37 can modulate the infection outcome. Simultaneous exposure of MDMs to LL-37 and DENV-2 led to reduced infection and innate immune responses including lower production of inflammatory cytokines. Notably, administration of LL-37 after initiation of DENV-2 infection had no effect on virus replication, yet significantly increased expression of ISGs, SOCS-1 and decreased production of IL-6, indicating direct immunomodulatory properties of LL-37. In addition, we demonstrated that differentiation of MDMs in the presence of VitD3 increases the baseline levels of LL-37 produced after DENV-2 infection.

We found that LL-37 has immunoregulatory properties during DENV-2 infection. Expression of TLR4 and TLR9 was upregulated by LL-37 under PT condition, suggesting a modulation of these TLRs independent of viral replication. These observations are in line with other studies which have assessed the effect of LL-37 on TLR-mediated cellular responses. For instance, treatment of HT29 cells with LL-37 and LPS induced a synergic increase in the expression of TLR4 compared to stimulation with LPS alone [31]. Likewise, in DCs [32], keratinocytes [33] and tissue mast cells [34] LL-37 increased the expression and activation of TLR9 induced by CpG-DNA or self-DNA, a process mediated by its direct binding to these agonists, hence increasing their uptake and processing by endosomes. Interestingly, in DCs, during DENV-2 infection there is a release of mitochondrial DNA that in turn upregulates TLR9 expression [35]. Therefore, it will be interesting to test whether the increase of TLR9 expression induced by LL-37 during DENV-2 infection of MDMs is

initiated by the direct binding of LL-37 to mitochondrial DNA. Further studies are needed to test this hypothesis.

DENV-2 infection of MDMs in the presence of LL-37 under ST conditions resulted in decreased viral infection but at the same time increased expression of TLR3, RIG-I and IFN-β. LL-37 has been shown to bind to dsRNA structures and enhance the activation of some TLRs [23]. In fact, LL-37 can form structures with the synthetic RNA analog poly(I:C) and increase TLR3 activation and signaling in human bronchial epithelial cells [36]. Further, LL-37 increased the expression of IFN-β in human keratinocytes, which was associated with decreased replication of human Herpes simplex virus 1 (HSV-1) [37] and in human astrocytes infected with ZIKV [38]. Whether this mechanism could explain the increase in the expression of TLR3 and RIG-I in our experimental model remains to be explored.

Despite LL-37 only increasing the expression of IFN-β expression under ST conditions, LL-37 induced the expression of ISGs, such as PKR and OAS1, under both ST and PT conditions. To our knowledge, little is known about the modulation of the IFN-I signaling pathway by LL-37. Recently Jadhav et al. reported that even though LL-37 restricted DENV-2 replication, expression levels of OAS2 and OAS3 in U937-DC-SIGN were high, suggesting that either their expression is independent of viral replication or that LL-37 increased the expression of ISGs [17]. Contrary to our results, induction of ISGs such as PKR and OAS have been demonstrated to limit DENV replication in other types of cells [39,40]. In our model, the addition of LL-37 after initial DENV-2 infection increased ISGs while DENV-2 replication was not affected. This could be explained by subversion of innate immune responses by DENV-2 in MDMs, as it has been reported previously (reviewed in [11]), or by insufficient protein levels of PKR and OAS1 produced by MDMs that we could not measure. More experiments are needed to determine how LL-37 modulates the expression of ISGs and how this contributes to the inhibition of DENV replication.

Infection of MDMs in the presence of LL-37 decreased the production of IL-6 and TNF-α in DENV-2-infected MDMs. Our results are in line with a previous study that showed how LL-37 treatment decreased the production of IL-6 and TNF-α by IAV-infected monocytes [41] and in lungs of IAV-infected mice [42]. Interestingly, data presented here indicate that the hampered production of TNF-α was due to decreased viral replication in the presence of

LL-37 rather than an immunomodulatory function of the peptide. In contrast, reduced production of IL-6 occurred irrespectively of DENV-2 infection, suggesting that LL-37 directly modulates IL-6 expression. The mechanism underlying the immunomodulatory function is not clear. Several studies have shown that LL-37 regulates the production of chemokines, such as RANTES, IP-10, and IL-8, and decreased the activation of NF-kB, p38 and ERK, after stimulation with LPS and dsRNA [21,22,43–45]. However, in human vascular cells, LL-37 enhanced the expression of IL-6 after stimulation with poly I:C, which was mediated by TLR3 recognition [46]. Therefore, whether binding of LL-37 with PAMPs such as LPS or dsRNA inhibits or promotes their recognition by TLRs remains unknown and should be further studied. Probably the mechanism underlying this effect is cell-dependent since enhancement of immune response by LL-37 via binding with dsRNA and recognition by TLRs occurs also in DCs [23].

A dose-dependent antiviral effect of LL-37 against DENV-2 replication was observed in MDMs, as it has been described for other viruses [14,16,38,47,48]. Also, treatment of HaCat cells and Vero E6 cells with exogenous LL-37 decreased DENV replication [18,20]. In agreement with these results, Jadhav et al. described antiviral effects of LL-37 against all serotypes of DENV in U937-DC-SIGN cells, using higher concentrations of LL-37 [17]. Adding LL-37 after the two first hours of DENV-2 infection did not alter viral replication. These results together with previously published findings [18] suggest that LL-37 is likely to inhibit viral entry into target cells. Future studies using labeled-DENV particles and analysis of cell entry via microscopy would be valuable for dissecting LL-37 antiviral mechanism. However, the main mode of action of LL-37 is the disruption of the external membrane of microbes [49], which would suggest LL-37 has virucidal activity. The precise mechanism by which LL-37 inhibits DENV replication remains a subject for a future study.

DENV-2 infection in MDMs did not lead to a significant expression of CAMP or production of LL-37 peptide. This contrast to LPS stimulation, which induced significant levels of the LL-37 peptide and has been shown to induce CAMP expression in other studies [50,51]. However, DENV-2 increases the expression of LL-37 in the human keratinocyte HaCat cell line [20], in human macrophage-like cell line THP-1, and in neutrophils [19]. Different induction of LL-37 expression by these cells and MDMs suggest different pathways for its expression dependent on cell type. Our results suggest that DENV-2 is actively down-

regulating CAMP mRNA expression in MDMs. Of note, a previous study found that human Metapneumovirus infection downregulated mRNA expression of CAMP in MDMs [52], in agreement with our results. Furthermore, we found that CHIKV infection also downregulated the mRNA expression of CAMP in both monocytes and MDMs (submitted manuscript). Supposing that DENV antagonizes LL-37 expression, it would represent an additional evasion mechanism of innate immunity that may impact DENV pathogenesis. Future studies should address this issue.

However, we observed that differentiation of MDMs in the presence of VitD3 (D3-MDM) increased expression levels of CAMP mRNA and LL-37 peptide in mock and DENV-2-infected MDMs, as it has been previously reported in primary immune cells [53]. Likewise, other studies have shown that VitD3 treatment of human bronchial epithelial cells increases the expression of LL-37 which inhibits replication of Rhinovirus and RSV [47,54]. In addition, we have previously shown that D3-MDMs exhibit lower expression of mannose receptor, and thereby are less susceptible to DENV-2 infection [30]. The ability of D3-MDMs to produce more LL-37 may be an additional paracrine defense mechanism against DENV infection, that should be tested in further studies. Altogether, these results support the use of VitD3 as a therapeutic agent that induces the expression of the antiviral and immunomodulatory peptide LL-37 during DENV-2 infection.

Taken together, we showed that LL-37 has antiviral and immunomodulatory properties against DENV-2 infection in human primary macrophages. In addition, we demonstrated that VitD3 increases expression levels of CAMP and LL-37 in D3-MDMs infected with DENV-2, suggesting that induction of this peptide could represent an additional antiviral mechanism of VitD3 against DENV. In conclusion, our data advocate future considerations of LL-37 utilization as a therapeutic compound to control DENV-2 infection and contribute to the amelioration of the inflammatory response

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AUTHORSHIP CONTRIBUTION STATEMENT

JAC and DMG: conceptualized, executed experiments and interpreted the data, wrote original draft, and edited revisions. JMS: reviewed and edited the manuscript. IAR-Z: interpreted the data, reviewed and edited the manuscript. SUI: Conceptualized, analyzed data, provided resources, wrote original draft, reviewed and edited the manuscript, supervised JAC and DMG, was responsible for project administration.

DECLARATION OF COMPETING INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

Figure 1. DENV-2 infection decreases the expression of CAMP and LL-37 in MDMs

(A) MDMs were infected with DENV-2 at an MOI of 5 and harvested at 2, 8 or 24 hours post-infection. Expression of CAMP was measured by RT-qPCR using ubiquitin as housekeeping gene. Data (n=6) is expressed as fold change normalized to the expression of both the housekeeping gene and CAMP in mock-infected MDMs. (B) Levels of LL-37 in supernatants in mock, DENV-2 (MOI=5) exposed and LPS (20ng/ml) stimulated MDMs were measured by ELISA (n=5). Figures represent individual experiments from different donors. Differences were obtained with a Kruskal-Wallis test using a 95% confidence (***$p<0.001$, **$p<0.01$, *$p<0.05$).

Figure 2. LL-37 restricts DENV-2 infection and replication in MDMs

(A, B) MDMs were infected with DENV-2 and treated simultaneously with LL-37 using different concentrations (0.25μM, 0.5μM, 1μM and 5μM). 24 hours later, DENV-2 infection was evaluated by the staining of viral envelope protein and detected by flow cytometry (n=5). (C) Dose response curve was done using data from B. (D, E) MDMs were infected with DENV-2 and treated simultaneously (ST) with LL-37 (5μM) and 24 hours later viral replication was evaluated by the quantification of viral RNA copies using RT-qPCR (GECs), and by the quantification of viral titer in supernatant through plaque assay (PFU) (n=5). (F).

MDMs were infected with DENV-2 with and treated simultaneously (ST) with LL-37, or infected first for two hours with DENV-2, and LL-37 was added to cells post-infection (PT). At 24 hpi, DENV-2 infection was evaluated by the staining of viral envelope protein detected by flow cytometry (n=4). Figures represent individual experiments from different donors. Differences were obtained with a Kruskal-Wallis test for B, while Mann-Whitney test was used for C and D with a 95% confidence (***p<0.001, **p<0.01, *p<0.05).

Figure 3. LL-37 modulates the expression of PKR, OAS, TLRs and SOCS-1 in MDMs infected with DENV-2

(A-H) MDMs were infected with DENV-2 and treated simultaneously with 5µM of LL-37 (ST), or infected first for two hours with DENV-2, and LL-37 was added to cells post-infection for 24 hours (PT). 24 hours-post infection expression of TLR3, TLR4, TLR9, RIG-I, IFN-β, PKR, OAS1 and SOCS-1 were measured by RT-qPCR using ubiquitin as housekeeping. Data (n=6) is expressed as fold change normalized to the expression of both the housekeeping gene and the expression of the interest gene of mock-infected MDMs. Figures represent individual experiments from different donors. Differences were obtained with a Kruskal-Wallis test using a 95% confidence (***p<0.001, **p<0.01, *p<0.05).

Figure 4. LL-37 Reduces de production of pro-inflammatory cytokines

(A, B) MDMs were infected with DENV-2 and treated simultaneously with 5µM of LL-37 (ST), or infected first for two hours with DENV-2, and LL-37 was added to cells post-infection for 24 hours (PT). Production of IL-6 and TNF-α were measured by ELISA (n=5). Figures represent individual experiments from different donors. Differences were obtained with a Kruskal-Wallis test using a 95% confidence (***p<0.001, **p<0.01, *p<0.05).

Figure 5. VitD3 up-regulates the expression of CAMP and LL-37 D3-MDMs

(A) MDMs were differentiated in presence of VitD3 (0.1nM) for 6 days (D3-MDMs) and then infected with DENV-2 for 2, 8 or 24 hours. Expression of CAMP was measured by qRT-PCR

in MDMs and D3-MDMs using ubiquitin as housekeeping (n=6). (B) Production of LL-37 was measured by ELISA in supernatants of mock and DENV-2-infected MDMs and D3-MDMs. An additional control was used by stimulating MDMs and D3-MDMs with 20 ng/ml of LPS for 24 hours and LL-37 was measured by ELISA as well (n=5). Figures represent individual experiments from different donors. Differences were obtained with a two-way Anova with a Bonferroni post-test using a 95% confidence was used for statistical analysis (***$p<0.001$, **$p<0.01$, *$p<0.05$).

Supplementary figure 1. DENV-2 infection does not induce HBD production by MDMs and LL-37 does not induce a loss on the viability of MDMs

(A) MDMs were infected with DENV-2 for 24 hours and production of HBD2 and HBD3 were measured by ELISA (n=4). (B) MDMs were either treated with Sulfasalazine (30nM), LL-37 (5μM), DENV-2 MOI 5, or with a combination of LL-37 and DENV-2. At 24 hpi, the viability of cells was measured through the staining of eFluor 450, which only stains cells with a loss of the integrity of cell membrane, using flow cytometry (n=3). Figures represent individual experiments from different donors.

Fig. 1

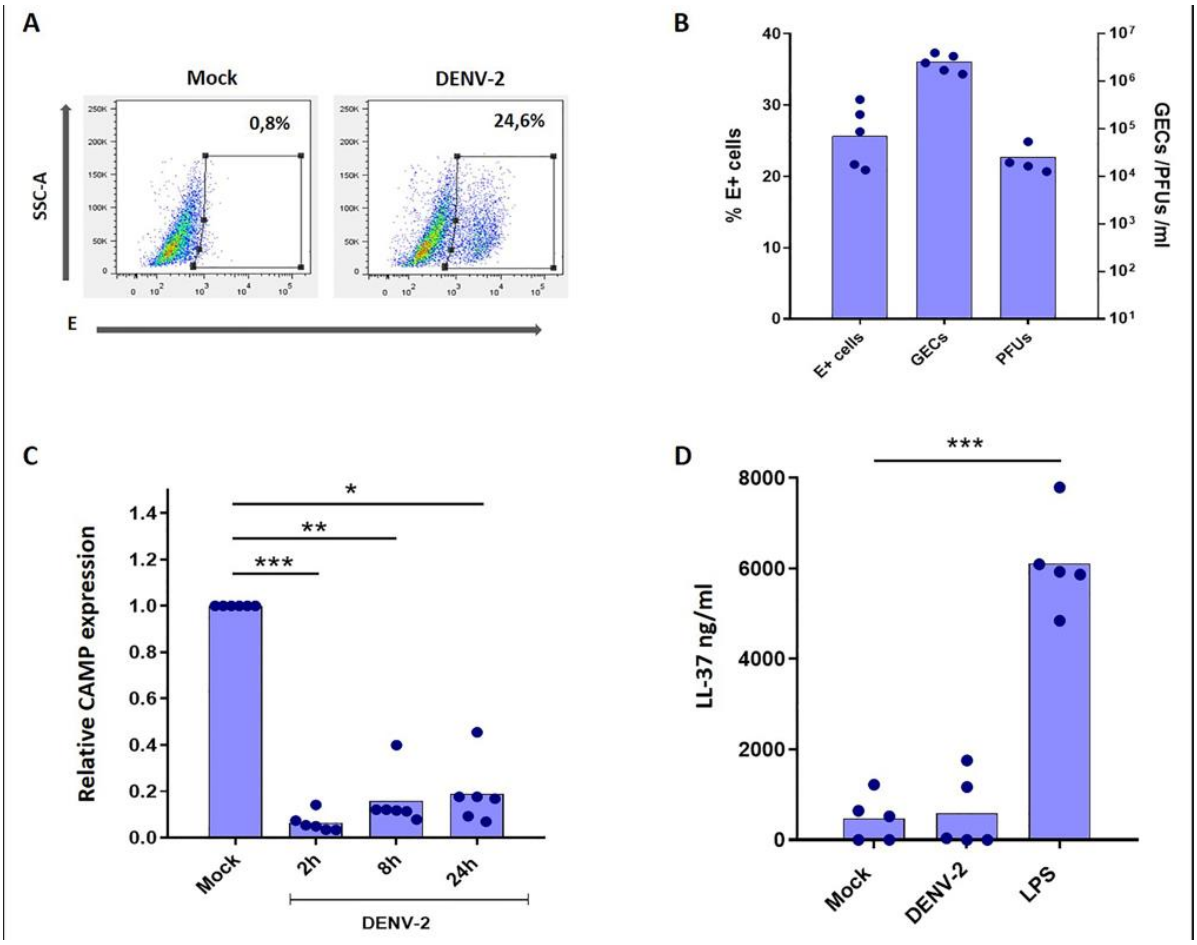

A
Mock
DENV-2
0,8%
24,6%
SSC-A
E
B
% E+ cells
GECs /PFUs /ml
E+ cells
GECs
PFUs
C
Relative CAMP expression
*
**
***
Mock
2h
8h
24h
DENV-2
D
***
LL-37 ng/ml
Mock
DENV-2
LPS

Fig. 2

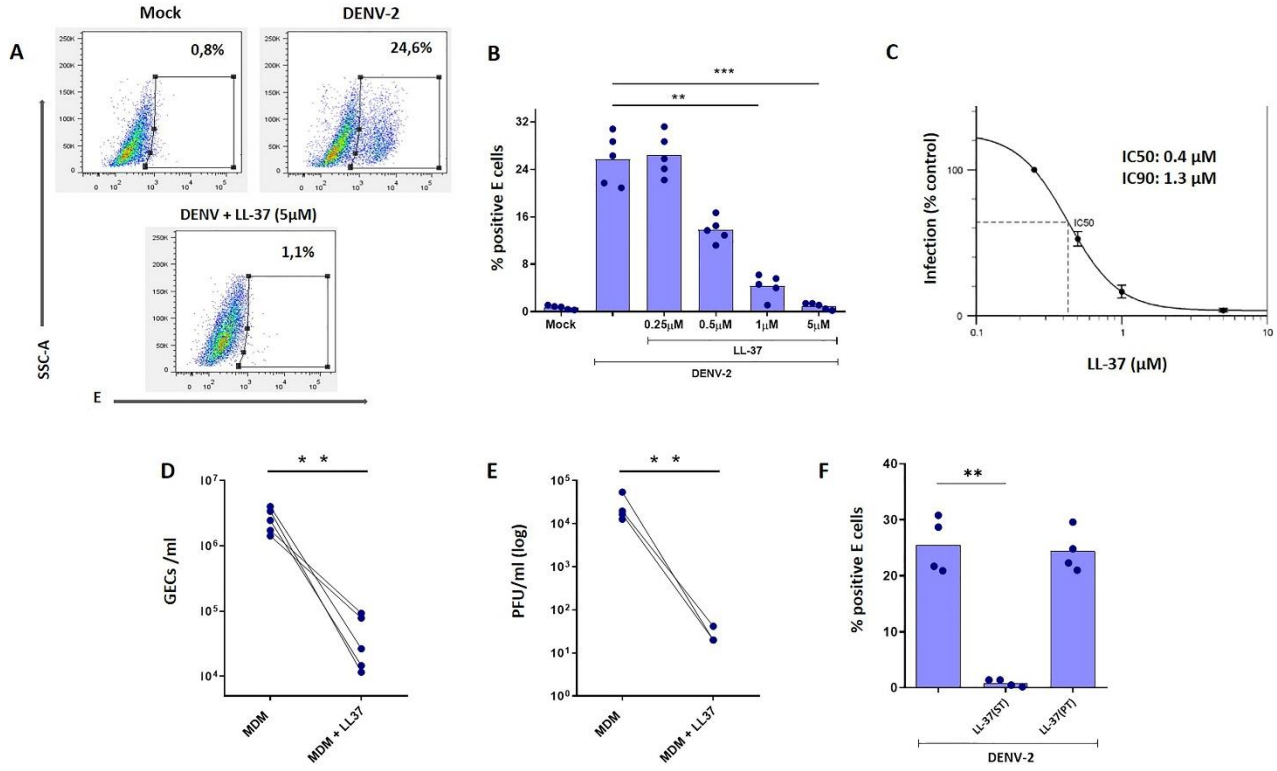

A
Mock
DENV-2
0,8%
24,6%
DENV + LL-37 (5µM)
1,1%
SSC-A
E
B
% positive E cells
***
**
Mock
0.25µM
0.5µM
1µM
5µM
LL-37
DENV-2
C
Infection (% control)
IC50: 0.4 µM
IC90: 1.3 µM
IC50
LL-37 (µM)
D
GECs /ml
* *
MDM
MDM + LL37
E
PFU/ml (log)
* *
MDM
MDM + LL37
F
% positive E cells
**
LL-37(ST)
LL-37(PT)
DENV-2

Fig. 3

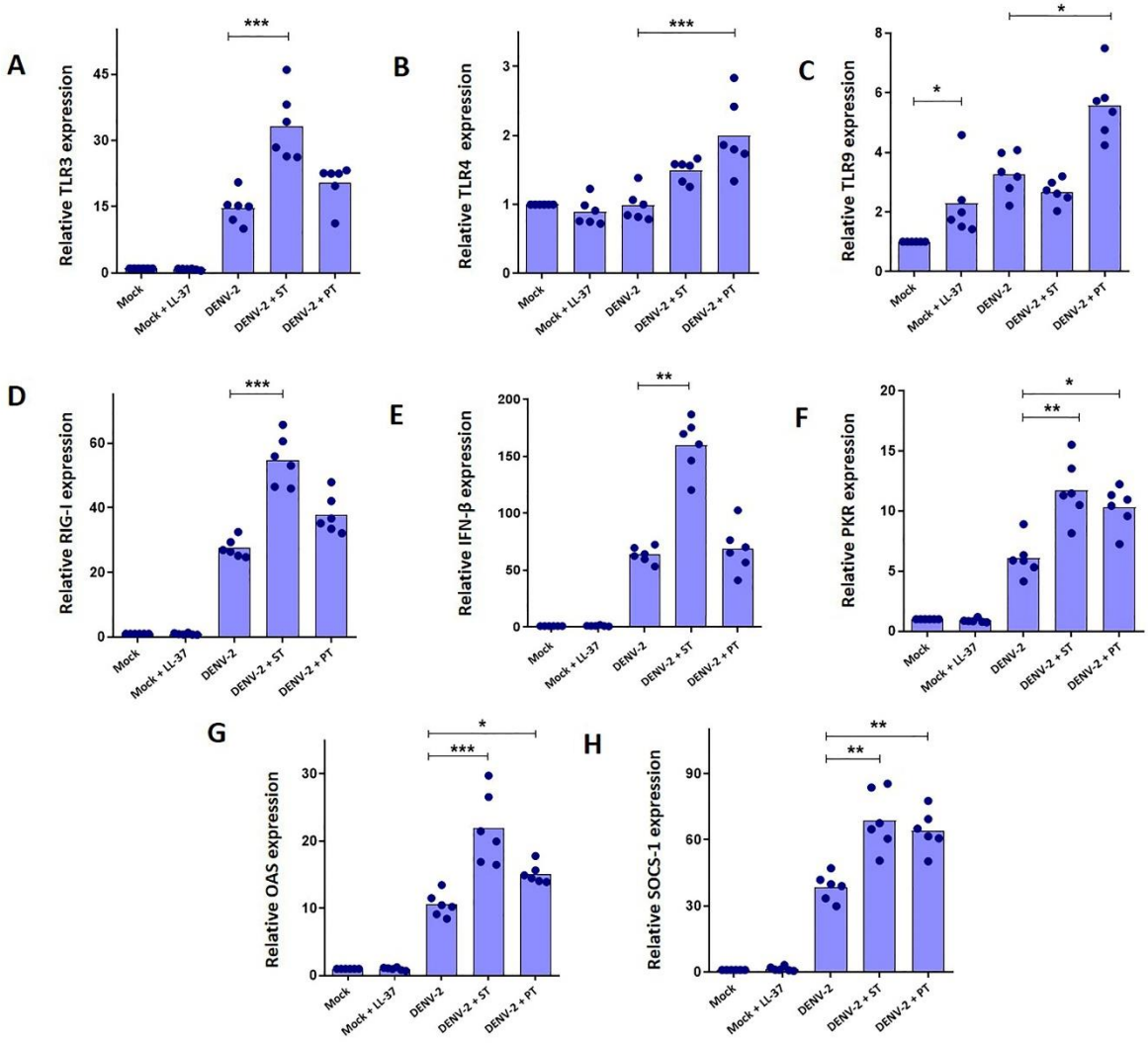
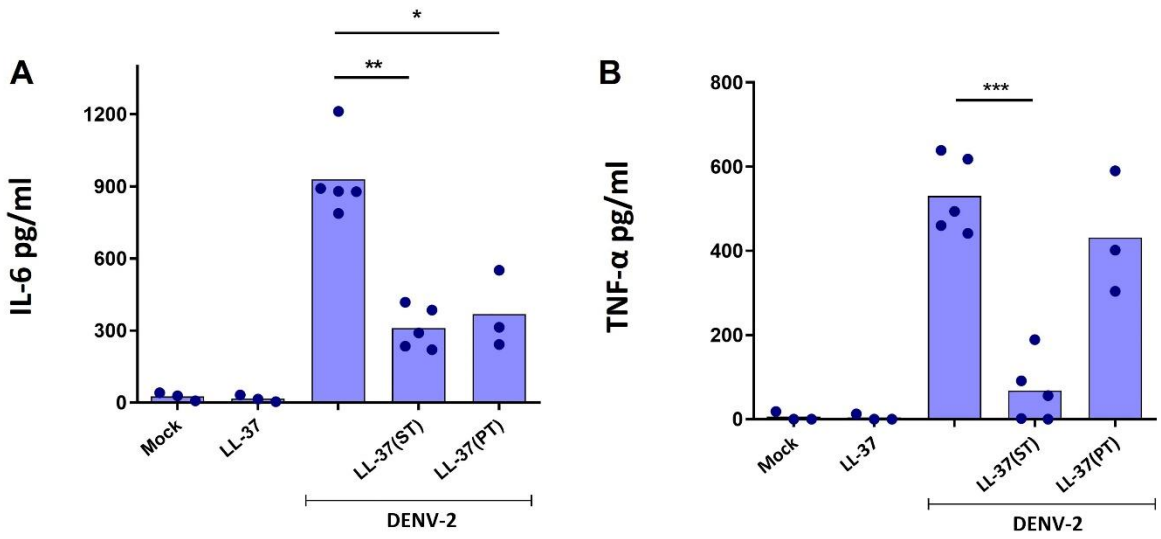
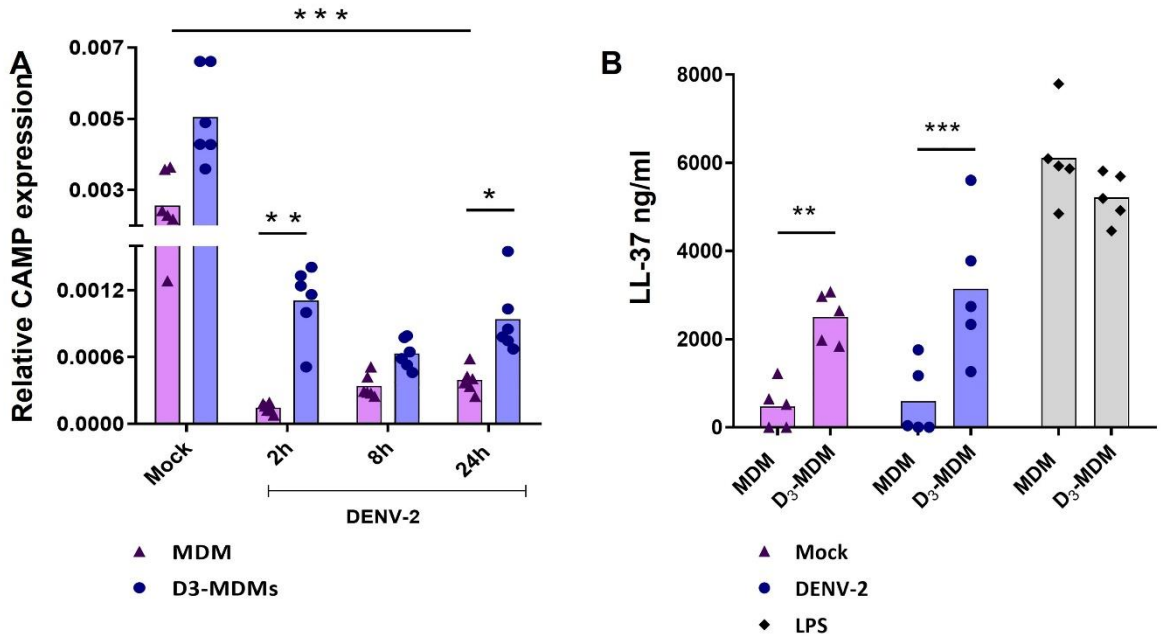

A
Relative TLR3 expression
***
B
Relative TLR4 expression
***
C
Relative TLR9 expression
*
*
D
Relative RIG-I expression
***
E
Relative IFN-β expression
**
F
Relative PKR expression
*
**
G
Relative OAS expression
*
***
H
Relative SOCS-1 expression
**
**
Mock
Mock + LL-37
DENV-2
DENV-2 + ST
DENV-2 + PT

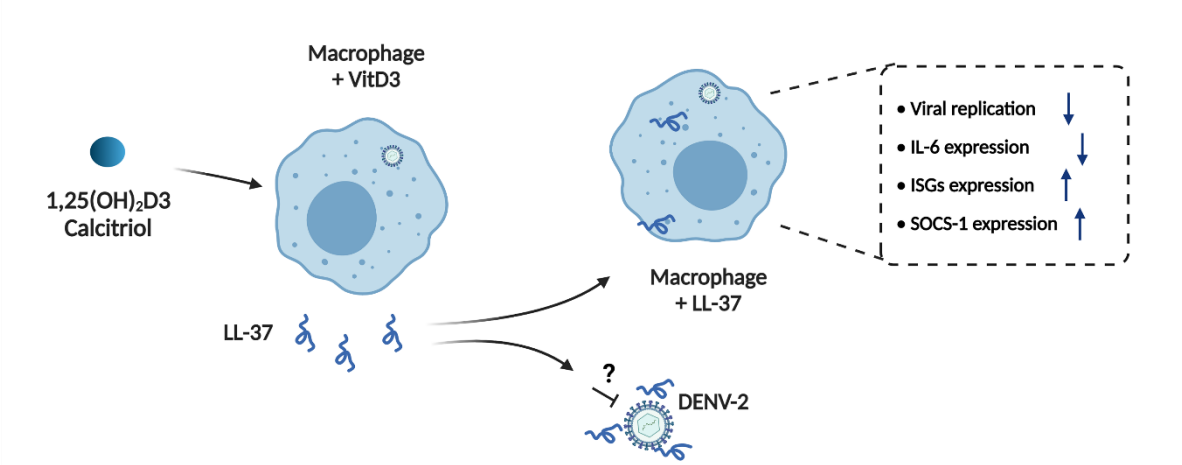
Fig. 4


A
IL-6 pg/ml
1200
900
600
300
0
*
**
Mock
LL-37
LL-37(ST)
LL-37(PT)
DENV-2
B
TNF-α pg/ml
800
600
400
200
0
***
Mock
LL-37
LL-37(ST)
LL-37(PT)
DENV-2

Fg. 5


A
Relative CAMP expression
0.007
0.005
0.003
0.0012
0.0006
0.0000
* * *
* *
*
Mock
2h
8h
24h
DENV-2
MDM
D3-MDMs
B
LL-37 ng/ml
8000
6000
4000
2000
0
**
***
MDM
D3-MDM
MDM
D3-MDM
MDM
D3-MDM
Mock
DENV-2
LPS

Graphical abstract



In this study we found that LL-37 decreases DENV-2 infectivity in human macrophages and modulates the inflammatory response of macrophages upon DENV-2 infection. Also LL-37 expression can be boosted with Vitamin D treatment.