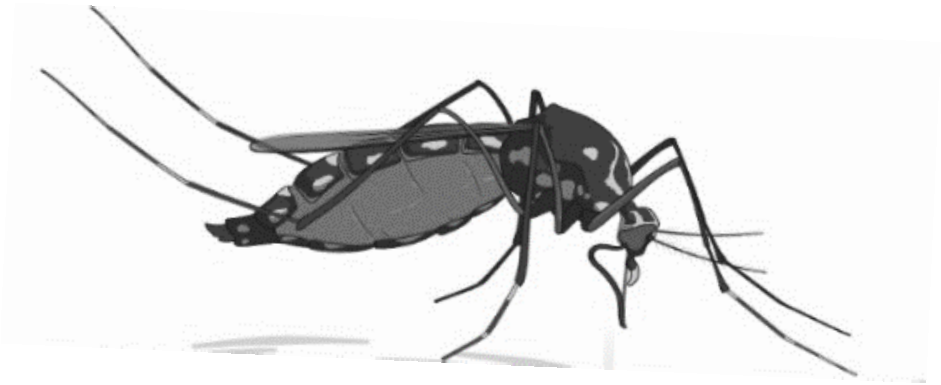
Innate immune response during DENV-2 infection of monocytes/macrophages

PhD candidate Jorge Andrés Castillo

CONTENT



CHAPTER 1

Introduction and scope of the thesis

1. Epidemiology of dengue virus infection

Dengue disease is caused by infection with any of the four different serotypes of dengue virus (DENV-1-4) [1]. All DENV serotypes are transmitted to humans by mosquitos of the genus *Aedes*, mainly *Ae. Aegypti* and *Ae. albopictus* [2,3]. DENV is the most frequent transmitted arbovirus (arthropod-borne virus) in the world **(Figure 1)**. Before 1970, only 9 countries suffered from the burden of dengue epidemics [4]. Nowadays, the disease caused by DENV is endemic in over 125 tropical and subtropical countries around the world, including territories of the Americas, Africa, South-East Asia and the Eastern Mediterranean [5]. DENV causes an estimated 390 million annual infections, of which approximately 100 million are symptomatic causing 10,000 deaths [5,6].

Until 1970, the transmission of the 4 serotypes of DENV was only reported in Asia [4]. However, the expansion of the mosquito vectors together with climate change, globalization and continuing urbanization have led to a great increase in the transmission of the virus, and managed to overlap areas where both the vector and the virus circulate [7]. Consequently, dengue outbreaks have occurred in new areas, like for example in the Madeira islands of Portugal in 2012 and in the state of Florida of the United States in 2013 [5]. Also, imported cases in Europe occur constantly and sporadic local transmission has been reported in France, Croatia and Italy [8–10]. Given these facts, dengue prevalence and distribution is predicted to increase by 50% by 2090 [6], and highlight the importance of dengue surveillance and research for global public health.

In recent years, the number of cases has increased rapidly. Only in the Americas and South-Asia, the reported cases exceeded 1.2 million in 2008, whereas in 2015 the reported cases went up to 3.2 million [5]. The Americas is one the regions that suffer the most from the burden of dengue, not only due to the high number of DENV transmissions, but also because of the low capacity of the health care system. In 2015, 2.35 million of cases were reported in the Americas alone, causing 10,200 cases of severe dengue and 1,181 deaths [5]. Therefore, the best strategy for mitigating the burden of the disease caused by DENV infection, is the effort towards the development of a safe vaccine and/or new therapeutic approaches.

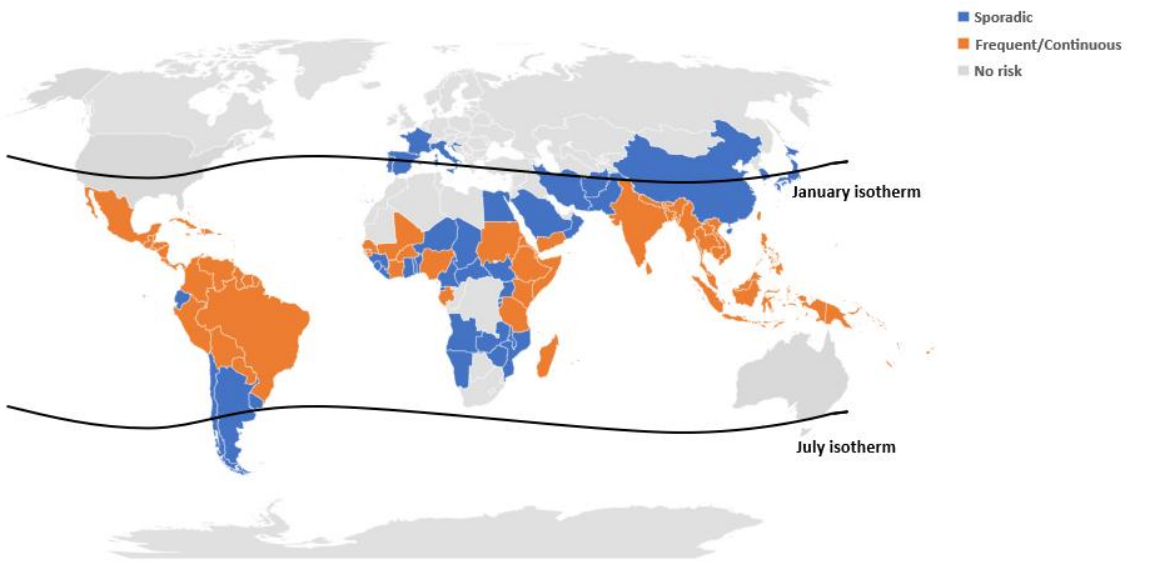


Figure 1. Global distribution of dengue

The map depicts the distribution of dengue and countries at risk for DENV outbreaks, while different colors show the recurrence of outbreaks according to data from the World Health Organization (WHO) [5]. Figure designed with Biorender.

2. Virus structure and replication cycle

DENV belongs to the family *Flaviviridae* and the genus Flavivirus. It is an enveloped virus with an icosahedral structure and a diameter of approximately 50 nm [11]. The genome of DENV consists of an 11kb long, single-stranded RNA (ssRNA) that is referred to as positive-sense RNA as it serves as viral mRNA. The 5´end of the RNA genome comprises an untranslated region (5´UTR) together with a CAP structure (7MeGppp2´OMe), whereas in the 3´end there is also located an UTR (3´UTR) but lacks a Poly (A) tail [12]. Both UTRs are highly conserved and structured because these regions mediate circularization of the RNA genome and replication by the viral polymerase [12]. The RNA has only one open reading frame which codifies for one single polyprotein of approximately 3.391 amino acids. The action of cellular and viral proteases leads to the production of three structural proteins: capsid (C), envelope (E) and pre-membrane (prM); and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) **(Figure 2)** [11].

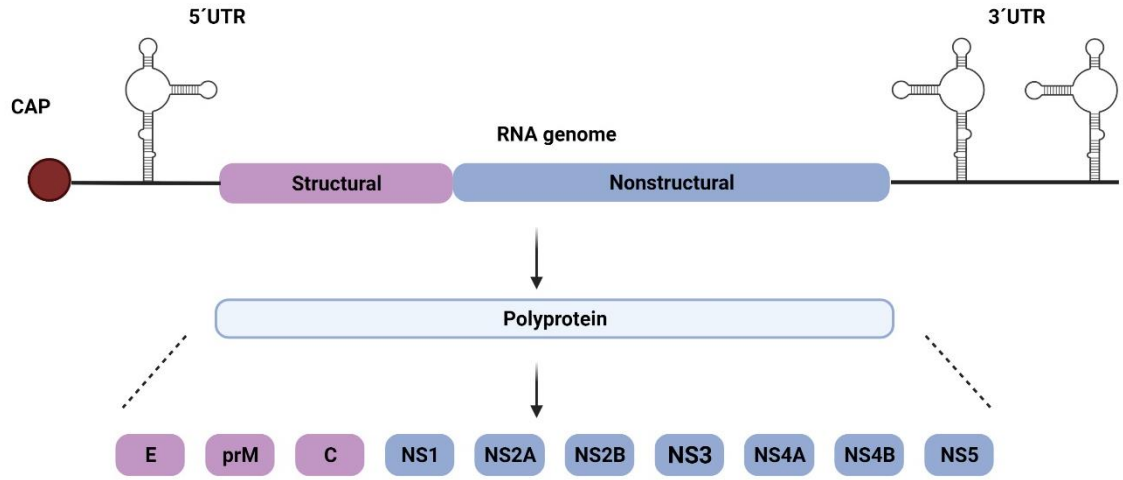


Figure 2. DENV genome and proteins

DENV genome consists of a single-stranded RNA of positive polarity (ssRNA+) with a type I CAP structure in the 5´ end and no poly-A tail in the 3´end. In both 5´and 3´ end, secondary structures are formed from untranslated regions (UTRs) that are neccesary for genome circularization and replication. The translation of the ssRNA+ gives rise to a single polyprotein that is further processed by cellular and viral protease, leading to the expression of 3 structural proteins: envelope (E), pre-membrane (prM) and capsid (C); and 7 non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Figure designed with Biorender.

The viral replication cycle **(Figure 3)** starts with binding to the target cell via the envelope E glycoprotein and cellular receptors, such as heparan sulfate glycosaminoglycans and mannose receptor (MR) in monocytes/macrophages and other types of cells [13,14], and DC-SIGN (Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) in dendritic cells (DCs) [15]. Following binding of the viral particles to cellular receptors, DENV enters the cell via receptor-mediated endocytosis, a pathway that transports the virus to the endosomes [16]. Then, acidification of the endosomes promotes conformational changes of the viral E protein allowing the fusion of the viral and endosome membranes [16]. This process results in the release of the viral capsid into the cytoplasm, carrying the viral RNA genome used for translation and replication [17].

Viral translation and replication occur in the endoplasmic reticulum (ER) which function as a scaffold for the development of structures used by the virus for replication. Recently, it was described that these structures inside ER resemble "vesicular packets" in which viral

non-structural proteins and genome are hidden from the cellular innate immune system and are also concentrated promoting high levels of viral replication [18,19]. Once the polyprotein is translated, NS2B and NS3 protease are crucial for its processing, leading to the production of single NS proteins. During replication, NS5 protein is essential as it is responsible for replicating the viral genome and mediates the process of the CAP synthesis and loading into viral RNA. CAP synthesis is mediated by the activity of the methyltransferase (MTase) domain of NS5 along with the activity of the RNA-triphosphatase domain of NS3 [20,21]. After translation and replication, the virus is assembled inside the ER, where C proteins interact with viral RNA to form the nucleocapsids. This process leads to the formation of an immature virus that migrates across the Golgi apparatus where the host furin enzyme completes the maturation process. This maturation leads to the conformational change of prM, which inhibits the fusion of the immature virus inside the cell, into M to form a fully infectious DENV particle. Finally, the mature virus particles are liberated by exocytosis [22].

Extensive research has described the function of some non-structural proteins in viral pathogenesis. For example, NS4B and NS5 are essential for subversion of host antiviral response by inhibiting IFN-I signaling [23–25]. Similarly, NS3 has been shown to block sensing of DENV components thus leading to a decreased IFN-I response [26]. On the other hand, most evidence has shown that NS1 protein shapes a replication complex along with NS4A, NS4B and NS3, in which NS5 and the viral RNA genome are loaded, allowing the generation of new viral RNA molecules [27]. Furthermore, NS1 is secreted by infected cells early during viral infection in the host, with a conformational structure different from NS1 protein found inside the cell (soluble hexameric NS1), which has allowed the development of diagnostic tool by the detection of NS1 in blood [27,28].

3. DENV infection and course of the disease

Symptomatic DENV infection in humans gives rise to a self-limiting syndrome clinically apparently to a flu-type disease, which resolves with appropriate supportive care and has only two phases: a febrile phase and a recovery phase (dengue). However, in a small proportion of patients, dengue can evolve into a more severe and life-threatening disease that includes several hemorrhages and organ impairment, referred to as dengue with

warning signs and severe dengue according to new guidelines from the World Health Organization (WHO) [3].

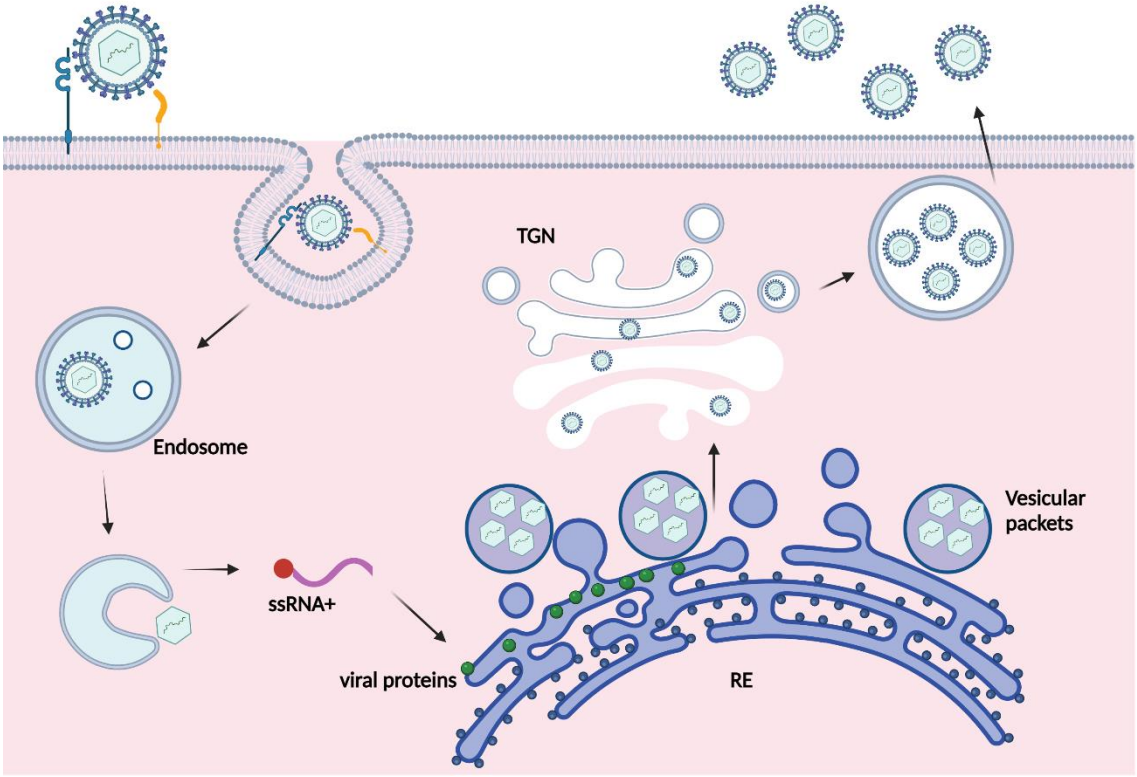


Figure 3. DENV replication cycle

DENV replication starts with viral attachment to the cell via heparan sulfate glycosaminoglycans mannose receptor or DC-SIGN. Following binding, viral particles enter the cell via receptor-mediated endocytosis, which transports the virus into the early endosomes of the cell. Maturation of endosomes and decrease in the pH leads to a conformational change of the viral E protein which mediates the fusion of the viral and endosome membranes. This process allows the liberation of the capsid into the cytoplasm and the following uncoating of the viral RNA genome, which is translated in the RE of the cell. After translation of viral proteins, viral replication occurs within the RE in viral-induced structures called vesicular packets, in which the viral genome and proteins are accumulated and hidden from the innate immune sensing system. Once viral replication has taken place, viral assembly occurs in the ER, followed by a maturation of the viral particles via the trans-Golgi network (TGN), and finally, mature and infectious viral particles are released via exocytosis. Figure adapted from [12,22] and designed with Biorender.

Severe dengue disease is presented in three phases: febrile, critical and recovery phases. After an incubation period of 3 to 7 days, the febrile phase suddenly starts with a high fever

(≥ 38.5 C°) accompanied by a variety of symptoms including headache, muscle pain arthralgias, leukopenia and thrombocytopenia. In some cases, small hemorrhages can also be found, although these are not serious and do not risk patient´s recovery [3,29]. This phase can last for 3 up to 7 days, after which the disease is resolved without further complications, or evolve into a critical phase, but only in patients that develop dengue with warning signs or severe dengue. Symptoms observed in the critical phase of these two forms of the disease include severe abdominal pain, persistent vomiting, liquid accumulation such as pleural and pericardial effusion, respiratory distress, severe thrombocytopenia and severe bleeding **(Figure 4)** [3,29]. If necessary supportive care is not given on time, the life of patients with severe dengue is at risk, resulting in death in several cases. After 48 to 72 hours of appropriate medical care, altered vascular permeability and patient´s symptoms improve, leading to the recovery phase.

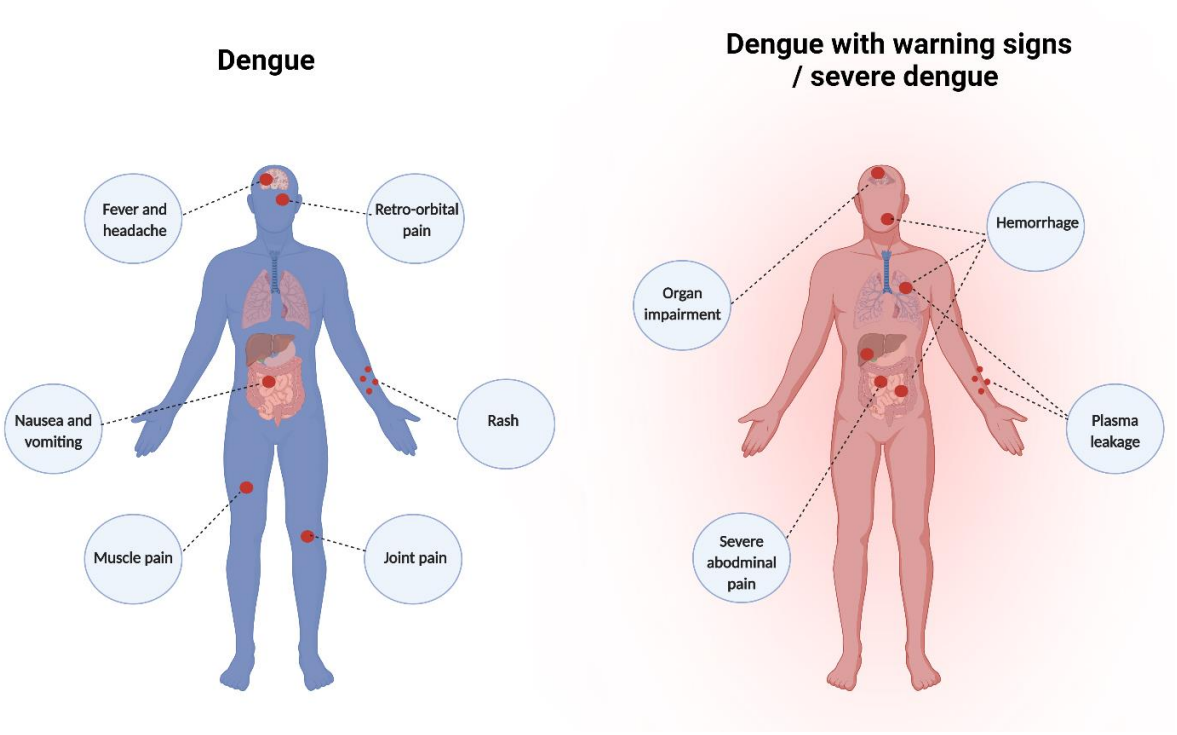


Figure 4. Signs and symptoms observed in dengue, dengue with warning signs and severe dengue

Patients infected with DENV can develop a mild flu-type syndrome called dengue, characterized by fever muscle pain, joint pain, among others. A small proportion of patients can develop dengue with warning signs or severe dengue in which disseminated hemorrhages and organ impairment is seen.

Consequence of plasma leakage, a sudden drop in blood pressure threatens patient´s lives. Figure designed with Biorender.

4. Immunopathogenesis of dengue virus infection

Nowadays, it is not possible to predict or explain why some people develop severe dengue while other people develop a mild dengue disease. However, it has been found consistently that secondary infections represent a significant higher risk for developing severe disease [30,31]. This is explained by increased viral entry into target cells mediated by heterologous non-neutralizing antibodies. In fact, children with sub-neutralizing antibodies from immune mothers develop severe dengue during primary DENV infections [32,33]. On the other hand, long-lived homologous immunity is acquired upon infection with any of the four different serotypes of DENV (DENV-1-4) [34]. The pathogenesis of DENV infection is complex and involves viral virulence factors, mosquito factors and mechanisms mediated by the immune response. In the following paragraphs I will show the most recent evidence regarding these particular aspects of DENV pathogenesis

4.1. DENV tropism

Multiple *in-vitro, ex-vivo* and *post-mortem* studies have shown that the virus infects or interacts with various cellular populations [35,36] **(Figure 5).** During the feeding of Aedes sp. mosquitoes, DENV is inoculated into skin and into the bloodstream of the human host, where the virus targets several cells that are present in the epidermis and dermis [35,36]. Keratinocytes, fibroblasts and Langerhans cells present at such sites can facilitate DENV replication [37–41]. In these cells, the IFN-I/III response and the production of antimicrobial peptides are particularly important for controlling DENV replication [38,42]. Also, at the site of the mosquito bite, immature DCs sense and take up virus particles via MR and DC-SIGN receptors [14,43]. Infected DCs migrate to regional lymphatic nodes as a process of their differentiation and promote the infection of new target cells like monocytes and macrophages, amplifying viral replication and facilitating the dissemination of the virus via lymphatic system [36]. Infection of monocytes further promote the dissemination of the virus to different tissues, such as liver, spleen and bone marrow. At these sites, DENV can infect other type of cells like hepatocytes, Kupffer cells, splenic macrophages,

megakaryocytes and also endothelial cells [35,44]. Infection of these cells will eventually contribute to the pathogenesis of dengue disease, evidenced by alteration of the hepatic and coagulation system, decrease in platelet numbers, amongst others [35] **(Figure 5)**.

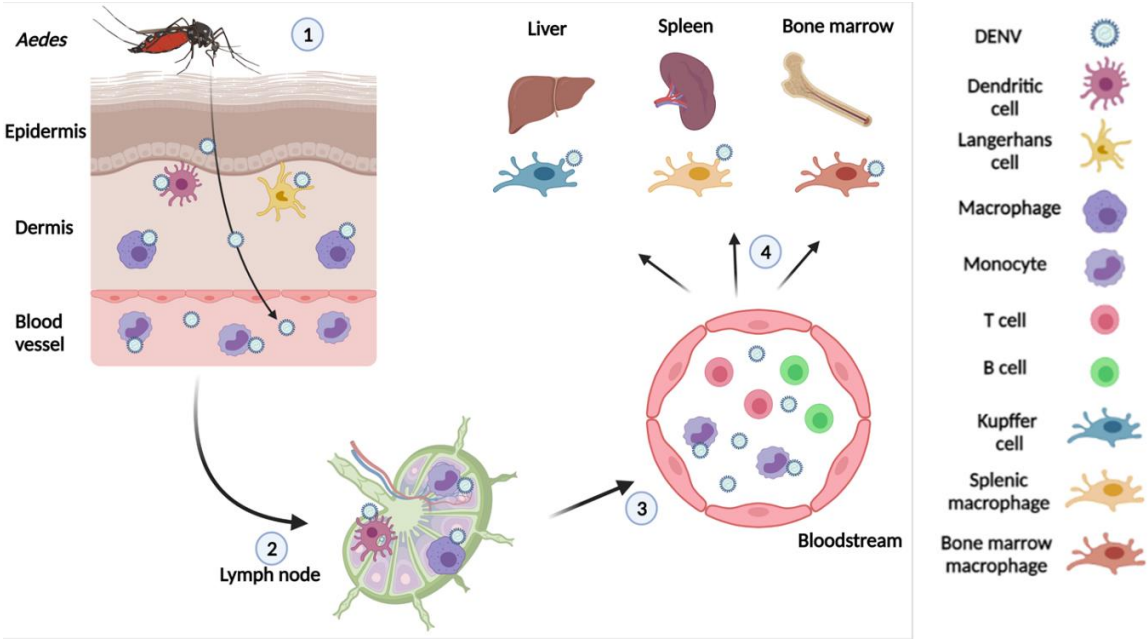


Figure 5. Infection and dissemination of DENV in the host

Infected mosquito inoculates DENV during blood feeding epidermis and dermis of the skin. Here, Langerhans cells, DCs and macrophages uptake the virus. Also, monocytes present in small blood vessels get infected with DENV during mosquito blood-feeding. As a consequence of the maturation process, DCs migrate to regional lymph nodes taking the virus into the lymphatic system. In blood, DENV targets mainly monocytes, producing high titers of the virus in blood. Through blood stream DENV reaches other tissues like liver, spleen and bone marrow in which various cells get infected including Kuppfer cells, splenic macrophages and bone marrow macrophages. Figure designed with Biorender.

4.2. Increased production of cytokines

The infection and/or activation of immune cells leads to the production of high concentrations of cytokines in blood. These cytokines are typically produced during the viremic phase and can their production is sustained until the critical phase, in which severe dengue is developed [45]. A great variety of studies have reported high levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, transforming growth factor β (TGF-β), IFN-γ,

macrophage inflammatory protein 1β (MIP-1β), and monocyte chemoattractant protein 1 (MCP-1) [46–48]. High levels of some of these cytokines, especially TNF-α, IL-1β, IL-6 and MIP-1β, have been associated with the development of hemorrhagic manifestations observed during severe dengue and dengue with alarm signs [48,49]. The effect of these cytokines in the pathogenesis of severe dengue may be mediated by their direct effect on endothelial cells. For example, various cytokines that are increased in DENV-infected patients can activate and modify endothelial cell-to-cell junctions, thus promoting an increase in endothelial permeability [50,51]. Even though the activation of innate immune cells and subsequent production of inflammatory mediators have a considerable impact on the endothelial tissue, it is considered that such response is necessary for the early control of DENV replication and to restrict the dissemination of the virus during the first days of infection. The pathogenic role of the proinflammatory mediators may be related to a sustained and uncontrolled production of different cytokines and chemokines, that ultimately promote functional alterations in endothelial cells in latter stages of DENV infection, as it is observed in the critical phase of severe dengue patients.

4.3. Activation of endothelial cells and increased permeability

The hallmark of severe dengue is the increase in endothelial permeability and endothelium dysfunction. This phenomenon is evidenced by early signs of plasma leakage in DENV infected patients, such as hemoconcentration, pleural effusion, ascites, hematuria and other types of hemorrhages [35]. The endothelium dysfunction is mainly mediated by a reorganization of the endothelial junctions rather than a direct cytotoxic effect of the virus in the endothelial cells [52].

Endothelial integrity and vascular homeostasis are maintained by the proper conformation and architecture of endothelial cells, and by cell-cell adhesion molecules. There are two types of cell binding namely adherens and tight junctions, that provide different functions to the cells including the association with the actin cytoskeleton and the reorganization of cytoskeleton for shaping cell to cell binding [53]. Further, the molecules involved in these endothelial junctions, like E-cadherin, occludin, claudin and ZO-1 (Zonula occludens-1), are responsible for shaping endothelial architecture and mediate endothelial permeability during the inflammatory response, allowing the migration of leukocytes to the sites of

infection [54]. Some studies have described the events that might explain the increase in endothelial permeability observed during DENV infection. Plasma of DENV infected patients was found to change the expression of VE-cadherin and ZO-1 in HUVECs cultures, mediated by the presence of proinflammatory cytokines like IP-10, MCP-1, IL-9, since blocking of these molecules reverted the effect observed in HUVECs [55]. Also, infection of the endothelial cell line EA.hy926 (HUVECs cell line fused with lung carcinoma cells A549) with DENV-2 disrupts the organization of the actin cytoskeleton and decreases the expression of VE-cadherin and ZO-1. This mechanism induced by DENV-2 replication also promotes an alteration of the distribution of endothelial junction molecules, therefore, leading to increased permeability [56]. Also, soluble viral NS1 is recognized by endothelial cells via TLR4, mediating the expression of proinflammatory cytokines, increasing endothelial permeability [57,58], and disrupting glycocalyx layer conformation [59]. In conclusion, the mechanisms responsible for the activation of endothelial cells and increased permeability during DENV infection are complex but involve in a great manner the immune response.

4.4. Adaptive immune response

Few days after onset of symptoms during DENV infection, B cell-mediated responses are initiated and plasmablasts and specific antibodies titers increase. Neutralizing antibodies of both IgM and IgG isotypes contribute to viral clearance [60]. Despite these physiological functions, during secondary infections with a different DENV serotype, non-neutralizing antibodies produced in the first infection binds to the new DENV serotype and facilitate viral uptake by Fc receptor bearing cells like monocytes and macrophages [31,61]. Similarly, sub-neutralizing levels of antibodies resulting from waning immunity [62] can also bind to heterotypic infecting serotypes and enhance viral replication. This mechanism is referred to as antibody-dependent enhancement (ADE) of infection, as ADE results in enhancement of viral replication and inflammatory response [63,64].

Intriguingly, antibodies generated by the humoral immune response directed against DENV, also show cross reactivity towards self-antigens. For example, IgM antibodies produced during DENV infection and directed against viral E and NS1 proteins cross-react

with self-antigens of platelets, thus explaining the thrombocytopenia observed during DENV infection [65]. Further, IgM antibodies from acute DENV infected patients cross-react *in vitro* with self-antigens of endothelial cells as well, causing cellular damage and apoptosis via a caspase-dependent pathway [66,67]. These mechanisms could contribute in part to the pathogenesis and increased endothelial permeability observed during DENV infection.

During primary infections, initiation of T cell mediated immunity is responsible for viral clearance. CD8+ T cells are involved in direct killing of DENV-infected cells and produce cytokines such as IFN-γ which reduces the production of DENV particles by infected cells including macrophages [60]. Nevertheless, during heterologous secondary DENV infections, memory CD8+ T cells specific for DENV serotypes from previous infections, have low affinity for the different and newly infecting DENV serotype, and exhibit weak cytotoxic effector response but high production of inflammatory cytokines [68]. Despite the important role of polyfunctional dengue-specific T cell responses in the control of DENV infection [69], this phenomenon could be contributing to severe dengue in secondary infections

5. Role of monocytes/macrophages in DENV infection

5.1. General remarks of monocytes

Monocytes are responsible for the early response against invading pathogens, contributing to the control of initial viral replication and limiting viral spread to other tissues, until the proper development of adaptive immune response [70]. They sense viruses through an array of receptors both extra and intracellularly, and produce high quantities of cytokines and chemokines that contribute to the elimination of the virus [71].

Monocytes are functionally and phenotypically a heterologous cell population. Classification of monocytes is based on their expression of several molecules such as CD14, which along with TLR4 recognize LPS, and CD16, low-affinity FcγRII that recognizes IgG and is implicated in recognition of immune complexes [72]. These subpopulations are the classical (CD14+ CD16-[CM]), the intermediate (CD14+ CD16+ [IM]) and the non-classical monocytes (CD14dim CD16+ [NM]) [73]. CM and IM are characterized by the expression of

high levels of the chemokine receptors CCR2 and CCR5, while expressing low levels of CX3CR1 [74]. On the other hand, NM shows high expression of CX3CR1 but low expression of CCR2 and CCR5 [75]. Regarding their effector functions, CM produce large quantities of various effector molecules both with inflammatory and anti-inflammatory effects. They have a high phagocytosis capacity and play a key role in initiating inflammation. On the other hand, IM has a higher inflammatory potential capable of migrating to the site of infection, where they will contribute to systemic inflammation [72]. NM has the greater capacity of migrating and patrolling the vasculature and they are responsible for sensing and responding with cytokine production against viral invaders [75,76] **(Figure 6).**

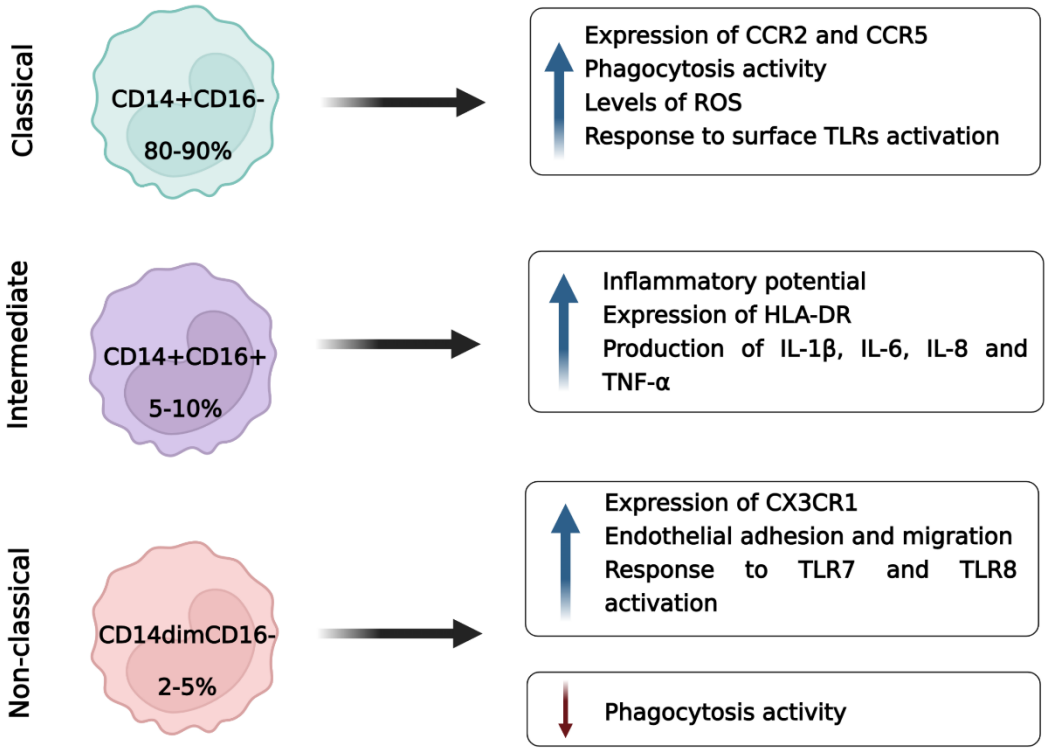


Figure 6. Human monocyte subpopulations in blood

Three different monocyte subpopulations can be found in peripheral blood in human. These are classified in classical, intermediate and non-classical monocytes, based on the expression of CD14 and CD16. In the figure, their different functions are depicted. Figure adapted from [79-83] and designed with Biorender.

During homeostasis, CM represents the majority of the monocyte population (80-90%) while IM and NM represent a small proportion of monocytes (5% and 2-5%, respectively) [77] **(Figure 6)**. Nevertheless, these proportions can vary considerably in some diseases or inflammatory conditions. For example, alterations in the number and function of monocyte subpopulations have been observed in Systemic Lupus Erythematosus, rheumatoid arthritis and coronary diseases [78–80]. Furthermore, alterations of monocyte subpopulations frequency have been associated with the pathogenesis of viral infections such as the pandemic SARS-CoV-2, Hepatitis C virus, human immunodeficiency virus 1 (HIV-1) and Zika virus [81–84].

5.2. General remarks of monocyte-derived macrophages

Although monocytes are important for controlling invading pathogens, their life span in the bloodstream is very short, and most of these cells undergo spontaneous apoptosis within 48 hours. Only a small fraction of circulating monocytes that reach specific tissues remain alive for up to 168 hours or even more before they begin their process of differentiation to macrophages [85]. Once monocytes have reached tissues, they differentiate into tissue specific macrophages, including Kupffer cells, splenic macrophages, alveolar macrophages, among others [86]. Cytokines like M-CSF and GM-CSF are essential for differentiation to macrophages and prolong the survival of these cells. Tissue macrophages share common functions as they are the first line of defense against invading pathogens. Similar to monocytes, once activated, they produce high amounts of chemokines and cytokines, but they show higher phagocytosis activity [87]. After recognition of microbes, macrophages show high plasticity and polarization towards different profiles depending on the surrounding stimuli [88].

Hence, macrophages can polarize to the M1 profile during inflammatory settings mediated by TLRs activation and IFN-γ action. The polarization of macrophages towards an M1 profile is largely mediated by the cytokines produced by Th1 CD4+ T cells. On the other hand, when macrophages are present in sites where tissue regeneration and anti-inflammatory response must be triggered, they polarize to M2 macrophages. Unlike M1 macrophages, M2 profile is mediated mainly by IL-4 produced by Th2 of CD4+ T cells [89].

Functionally, M1 and M2 macrophages are different. M1 profile produces high levels of proinflammatory cytokines such as IL-1β, IL-12, IL-23 y TNF-α and shows high microbicidal activity, a consequence of an increased expression of inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) and lysosomal enzymes [90]. On the other hand, M2 macrophages express high levels of arginase 1 (ARG-1) and anti-inflammatory cytokines such as IL-10 and TGF-β. They can regulate the activity DCs and T cells while contributing to fibrosis and regeneration of tissues [90].

Using *in vitro* studies, the plasticity and polarization of macrophages have been evaluated under infection with several microbes, including viruses. Experimentally, polarization of monocyte-derived macrophages (MDMs) into M1 and M2 profiles is possible by the addition of LPS plus IFN-y and IL-4, respectively. These different types of MDMs (M1, M2, and M0 [non-polarized MDMs]) resemble the majority of biological functions and markers that can be found in tissue macrophages [91–93].

5.3. Monocytes during DENV infection

Ex vivo and *in vitro* studies have shown that monocytes are the main permissive cells for DENV infection and replication among peripheral blood mononuclear cells (PBMCs), with different levels of infection depending on the strategy used [14,31,94–97]. During the febrile stage of patients suffering from dengue, CD14+ monocytes are the cell population that expresses the higher levels of viral antigens like E and NS1 proteins (49%), while infection of lymphocytes is barely detectable (2%) [97]. Likewise, *in vitro* challenge of PBMCs from healthy individuals with DENV-2 results in DENV replication on monocytes but not in other types of cells such as CD3+ and CD19+ lymphocytes [96]. Although there are significant differences in the frequency of DENV infection in monocytes using *ex vivo* approaches that analyze DENV-infected patients directly compared to *in vitro* studies, it is clear that monocytes represent the main target cell for DENV infection among PBMCs. Finally, it is important to note that in the context of natural DENV infection, levels of viral replication in monocytes could result enhanced by ADE infection mediated by the expression of Fcγ receptors (FcγR) and the presence of sub-neutralizing antibodies. In fact, DENV is growth better in cultures of monocytes obtained from dengue-immune individuals [98], and a significant increase in DENV infection of monocytes *in vitro* can be achieved using

antibodies in sub-neutralization concentrations, enhancing monocyte infection up to 20-30% [99]. Monocytes are key in this type of infection due to their high basal expression of FcγR [100]. Furthermore, they promote rapid differentiation of B lymphocytes into antibody-producing plasmablasts when they are infected *in vitro* with DENV-2 [100]. This phenomenon may be related to the quick increase of serum antibodies that have been observed in DENV-infected patients [101,102], which could be promoting the infection of monocytes via FcγR even in primary infections, and in higher proportions in secondary infections.

5.4. Monocyte subsets and their role during DENV infection

In the case of DENV infection, few studies have scrutinized the role of the different monocyte subsets in pathogenesis. However, consistently we and others have reported alterations of monocyte subsets in DENV infected patients, observing an increase of IM and decrease of CM frequencies compared to healthy controls [100,103–105]. Interestingly, Kwissa et al. described that infection of monocytes with DENV-2 *in vitro* upregulated expression of CD16 and promoted differentiation into CD14+CD16+ monocytes, which in turn mediated differentiation of resting B cells to plasmablasts with increased secretion of IgG and IgM [100]. This mechanism depended on the B cell activation factor, proliferation-inducing ligand (APRIL) and IL-10 [100]. Likewise, Naranjo et al. showed that NM of DENV infected patients had increased activation and differentiation markers such as CD64, CD86 and TNF-α. Moreover, NM showed decreased expression levels of markers associated with endothelial surveillance including CX3CR1 and high CCR2 and CCR5 [103]. Nevertheless, both studies failed to associate these findings with severe dengue. On the other hand, by studying a pediatric cohort, our group recently described that TLR2 sustained expression in CM, but not in other subsets, was associated with severe dengue [105]. Also, detection of expression of viral NS3 protein in monocytes was predominantly higher in CM and IM, but low levels were found in NM, suggesting different susceptibilities of monocyte subsets to DENV infection. Altogether these findings strongly suggest that each monocyte subset may contribute differently to DENV pathogenesis, as they have differential expression of molecules and biological functions, hence contributing differently to the immune-mediated damage caused by DENV replication.

5.5. Sensing of pathogens by innate immune cells including monocytes/macrophages

Monocytes and macrophages are responsible for recognition and response against invading pathogens, providing the innate immunity necessary for the first line of defense and protecting the host. This response limits early replication and restricts viral dissemination across the body [106]. Monocytes and macrophages express an array of pattern recognition receptors (PRRs) for sensing pathogens including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), DNA sensors, scavenger receptors and other types of receptors involved in the activation of the inflammasome such as NLR family pyrin domain containing 3 (NLRP3), NLRP2 and NLRP1 [107,108]. Among these PRRs, TLRs have a pivotal role due to the variety of pathogen-associated molecular patterns (PAMPs) that can recognize and the different effector responses that can elicit.

TLRs consist of a family of type I transmembrane receptors located extracellularly in the membrane and intracellularly within endosomes. These receptors are characterized by an extracellular (or cytoplasmatic for the case of intracellular TLRs) leucine-rich repeat (LRR) domain and an intracellular (or endosomal for the case of intracellular TLRs) Toll/IL-1 receptor (TIR) domain [109]. In humans, 10 members of the TLR family have been described so far, which are expressed in tissues involved in immune function including blood, epithelium, endothelium, gastrointestinal tract, lung, among others [110]. For most TLRs, their corresponding agonist has been described. In this way, TLR2 is necessary for the recognition of a variety of PAMPs present in gram-positive bacteria like lipoproteins and lipoteichoic acids; TLR3 is implicated in the detection of viral replication products such as dsRNA; TLR4 is mainly activated by bacterial lipopolysaccharide; TLR5 detects flagellin present in bacteria; both TLR7 and TLR8 sense ssRNA which is the genome of some viruses; and finally, TLR9 is required for response to unmethylated CpG DNA [111]. TLR1 and TLR6 are required for the recognition of some PAMPs by TLR2, and form heterodimers with this last one. In the case of TLR10, it is not clear which agonist is responsible for its activation [112]. However, recent reports have demonstrated that TLR10 can also form heterodimers similar to TLR1/TLR2 and TLR6/TLR2, but unlike these last two, TLR10 activation leads to an anti-inflammatory response [113]. The cases of TLR2 and TLR4 are interesting as they function as a complex with different co-receptors. As mentioned before TLR2 is associated

as a heterodimer with other TLRs, but also, both TLR2 and TLR4 are associated with the co-receptor CD14 and the lipopolysaccharide-binding protein (LBP) [114], which promotes loading of different ligands onto these receptors. Similar to CD14, CD36 has been recently shown as a TLR2 co-receptors that mediates binding of ligands onto TLR2 [115].

After TLRs activation, signaling pathways are initiated to induce the expression of genes involved in host defense, including inflammatory cytokines, type I interferons (IFN-I), major histocompatibility complex (MHC) and co-stimulatory molecules. Ligand recognition leads to the recruitment of TIR domain-containing adaptor molecules such as MyD88 (Myeloid differentiation primary response 88), TRIF (TIR-domain-containing adapter-inducing interferon-β) and TRAM (TRIF-related adaptor molecule) [106]. The recruitment of such adaptor molecules leads to the activation of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), the transcription factors IRFs, or the activation of MAP kinases like p38, JNKs and ERK1/2 inducing the activity of the transcription factor AP-1 [116]. Because of the complexity of the different signaling pathways, activation of different TLRs has been categorized into MyD88-dependent and independent responses. MyD88-dependent pathways usually involves extracellular TLRs.

Analysis of TLR expression have revealed that professional phagocytes express the greatest variety of TLRs including monocytes and macrophages [108,117]. Of all TLRs, TLR2 expression is particularly high compared to other TLRs. In fact, Shirk et al. showed that TLR2 bright cells correspond to circulating whole blood monocytes in humans and two species of macaques [118], suggesting that TLR2 is highly and constitutively expressed in monocytes and can be used as a marker for their identification. Within monocytes population there is also some difference regarding TLRs expression as some studies have shown NM monocytes have higher expression of TLR7 and TLR8, mediating the recognition of RNA genome from viruses and subsequent production of TNF-α, IL-1β and CCL3 [76].

5.6. Sensing of DENV infection by monocytes/macrophages

Monocytes and macrophages can sense DENV specifically through an array of PRRs that include TLRs, RIG-I and MDA5, and CLRs including CLEC5A and MR. Silencing of RIG-I or MDA-5 increases the replication of DENV-1 in cells lines derived from hepatocytes, indicating an important role of these PRRs in sensing DENV replication [119]. Also,

silencing of TLR3 increased susceptibility and viral replication in macrophages [119]. Similarly, TLR3 has been shown as an important innate immune sensor for restricting DENV replication in cultured hepatoma cells [120] and U937 cells [121], through the expression of IFN-β. Activation of TLR7 and TLR8, which can sense ssRNA produced during DENV replication cycle, reduces viral replication and induced an inflammatory response without clear signs of exacerbated disease in rhesus macaques [122]. These findings highlight the critical role of intracellular TLRs in DENV recognition and mediating innate immune response mainly by the expression of IFN-I. However, recent reports have found that other types of TLRs can sense DENV components, namely TLR4, which can recognize soluble NS1 and induce inflammatory gene expression in endothelial cells [57,58], and TLR2, which we recently showed that recognize DENV particles and mediate immune response of PBMCs [105]. Finally, CLEC5A has a critical role in the induction of proinflammatory response after DENV-2 recognition. In human macrophages, DENV-2 interacts with CLEC5A which does not result in internalization of the virus but induces inflammasome activation, production of IL-1β [123] and expression of other inflammatory cytokines [124]. Interestingly, Lo et al. suggested that MR and DC-SIGN in macrophages serve as initial receptors for DENV binding into the cell, while CLEC5A is subsequently activated by DENV particles and activates downstream signaling [125].

5.7. Activation of monocytes/macrophages after DENV sensing

After activation of PRRs induced by DENV replication, monocytes/macrophages are activated. For example, monocytes from DENV-infected patients show higher expression of ICAM-1, TLR2, TLR4 and TLR8 compared to healthy controls, suggesting higher activation levels during DENV infection [104]. Also, monocytes from hospitalized children infected with DENV show high expression levels of CD32, CD86 and CD11c expression, associated as well with severe disease [126]. The activation of monocytes and macrophages, evidenced by these studies, leads to expression of effector molecules that mediate immunopathogenesis. Several studies have shown that monocytes and macrophages infected in vitro with DENV for 48 hours produce high quantities of TNF-α, IL-1β, IL-6, IL-12, IL-10, IL-8, MCP-1 and MIP-1β in response to viral replication [100,127–130]. The production of most of these cytokines may be related to replication of DENV, since it has been showed that production of TNF-α decreased when cells are stimulated with heat-

inactivated virus [131]. However, other studies have shown that UV-inactivated DENV can still induce the production of TNF-α, IL-1β and IL-6 by PBMCs, although not in the same levels as the replicative virus [105]. Thus, it is still unclear whether recognition by TLRs of viral particles is sufficient for cytokine production or whether viral replication is necessary for sufficient stimulation and subsequent expression of cytokines. Supporting this last hypothesis, it is interesting to note that NS1 soluble protein can activate mouse macrophages and human PBMCs via TLR4, mediating the expression of TNF-α and IL-6 after 3 hours of stimulation [132]. Furthermore, NS1 can activate human monocytes and induce the production of IL-10 [133], however in this study the receptor involved in NS1 recognition was not evaluated.

6. RNA interference as a host pathway for restricting viral infections

RNA interference (RNAi) comprises a series of RNA-mediated regulatory pathways first described as an innate defense mechanism against microbial infections in prokaryotes [134,135]. Similarly, RNAi in eukaryotes plays an essential role in both defense against microbes and in regulating of the expression of a wide array of cellular programs [135]. It is estimated that about 6000 genome sequences encode for microRNAs (miRNAs), which control the gene of proteins involved in cell cycle division, cell metabolism, cell death programs, tissue development, immune response, among several others [136].

6.1. MiRNA synthesis and mode of action

MiRNAs are small non-coding RNAs of 20 to 25 nt in length [137,138]. This regulation is mediated by the binding of the miRNAs into target sequences present in the 3´UTR of mRNAs [139]. The transcription of miRNAs in eucaryotes is done by the RNA polymerase II or III, which leads to a primary transcript or pri-miRNA of 200 nt of length approximately. This pri-miRNA folds into a self-double stranded RNA structure, with an important number of non-paired nucleotides allowing to shape a stem-loop that bears single or clustered hairpins and overhangs in both the 3´ and 5´ends [140]. Then, the primary transcript pri-miRNA is recognized by a microprocessor complex formed by the subunit DGCR8 (DiGeorge syndrome critical region 8) that contains an RNA-binding domain, and the subunit Drosha that cleaves the RNA structure and results in the formation a stem-loop pre-

miRNA [141]. The pre-miRNA structure is exported from the nucleus to the cytoplasm and recognized by a second RNase II known as Dicer and its cofactor TRBP (transactivating region RNA-binding protein), which together remove the stem-loop from the pre-miRNA and gives rise to an intermediary miRNA duplex that has the mature miRNA sequence and its complementary RNA [142,143].

For the recognition of target mRNAs by mature miRNAs, the miRNA duplex is loaded into the Argonaute protein (AGO) to form the RNA-induced silencing complex (RISC) [142]. Ago aids the removal of one of the strands of the miRNA duplex, leaving the mature miRNA sequence ready for targeting the complementary mRNAs sequences. The first 6 nucleotides of the miRNA sequence are essential for the resulting efficacy of silencing after mRNA recognition, as they are defined as the "seed sequence" [144]. Depending on the degree of sequence matching between miRNAs and target mRNAs, the fate of mRNAs can result in expression decay or endonucleolytic cleavage and degradation of the mRNAs [145] **(Figure 7)**. In mammals, more than 90% of interactions between miRNAs and target mRNAs results in expression decay, while degradation of miRNAs is common in plants. However, the precise mechanisms that tune and mediate mRNA repression and translation decay by miRNAs are not fully understood in mammals [146].

6.2. Role of miRNAs in viral infections

MiRNAs can be encoded either from the host or viruses genomes. The antiviral activity of miRNAs has been extensively described in various infections of plants, fungi, and mammals [147]. On the other hand, miRNAs encoded by viruses can target host proteins and repress the immune response or other pathways for favoring viral replication [148]. The interplay between miRNA regulation between the host and some viruses can explain some features of viral pathogenesis, as miRNAs regulation can result in direct repression of viral genomes, repression of host protein essential for viral replication, or may regulate pathways involved in immune response [149]. The cellular miR-32 has two different target sequences in the primate foamy virus (PFV) genome, resulting in the inhibition of viral replication [150]. Similarly, miR-24, miR-93 inhibit vesicular stomatitis virus (VSV) [151], miR-29a inhibit HIV-1 replication [152], and miR-323, miR-491 and miR-654 inhibit H1N1 Influenza A

replication [153]. These results demonstrate that a wide number of host miRNAs can directly target sequences in genomes of various viruses.

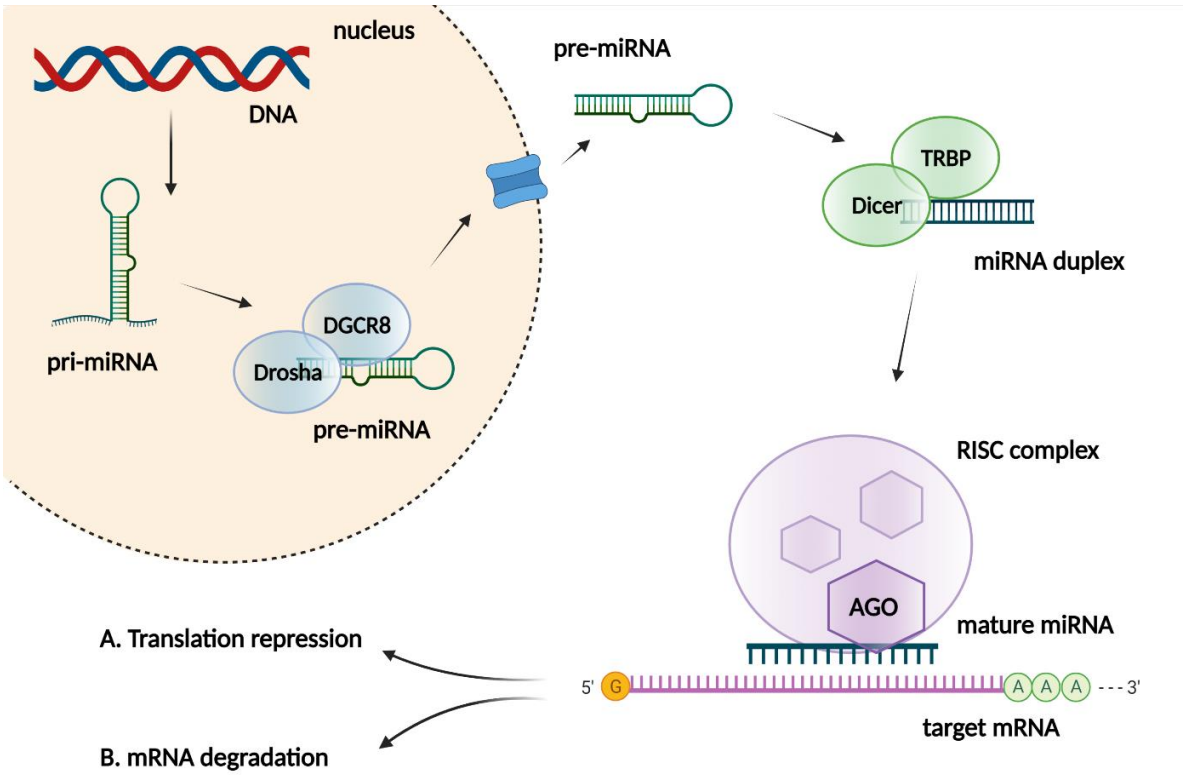


Figure 7. miRNA biogenesis

MiRNAs are encoded in DNA and are transcribed by the RNA polymerase II, leading to the synthesis of the primary transcript pri-miRNA. In the nucleus, the pri-miRNA is processed by the RNAse Drosha and the RNA-binding protein DGCR8, which cleaves part of the RNA structure and results in a pre-miRNA with a stem-loop. The pre-miRNA is exported to the cytoplasm and recognized by Dicer and TRBP, which remove the stem-loop and form a miRNA duplex that is still not functional. The miRNA duplex is loaded into the AGO protein to form the RISC complex and allow the removal of one of the strands of the miRNA duplex. The leading strand of the mature miRNA targets the "seed sequence" in the mRNA, which depending on the degree of complementary, will lead to the repression of the mRNA translation or the degradation of the mRNA. Figure designed with Biorender.

Other miRNAs can modulate viral replication indirectly. MiR-155 and miR-29b, induced by IFN-I activity, can modulate the replication of VSV and Japanese encephalitis virus (JEV) by

regulating the expression of other types of host proteins [154–156]. MiRNA let-7c modulates the oxidative stress in Huh7 cells during DENV-2 infection, and its over-expression significantly reduced viral replication [157]. Furthermore, miR30e* inhibits the expression of IκBα leading to hyperactivation of NF-κB. This leads to an increased expression of IFN-β and downstream interferon-stimulated genes (ISGs) such as OAS1, MxA, and IFITM1, significantly decreasing DENV replication in Hela and U937 cells [158]. High expression of these miRNAs could explain resistance or low susceptibility of some cells to certain viral infections. For example, in human primary MDMs, bystander cells resistant to DENV-2 infection express high levels of miR-3614 leading to a repression of the adenosine deaminase acting on RNA 1 (ADAR1) protein expression [159]. Interestingly, ADAR1 was identified as a host factor that promoted infectivity at early time points of DENV replication [159]. Overall, these results suggest that miRNAs could represent an important pathway that limits viral replication by targeting host factors that are essential for replication.

On the other hand, various miRNAs can be exploited by different viruses to evade the innate immune response or to enhance viral replication. Expression of miR-146a is highly over-expressed early during DENV replication, which targets TRAF6, therefore, reducing IFN-I expression levels in human primary monocytes [160]. A similar event occurs in fibroblasts during Chikungunya virus (CHIKV) infection, in which the virus exploits miR-146a expression to favor viral replication [161]. Increased expression of miRNAs can also be associated with pathogenesis. Increased expression of miR-155 has been found in patients infected with hepatitis C virus (HCV) that develop hepatocellular carcinoma (HCC) [162]. Altogether, this information shows that miRNA plays an important role in the pathogenesis of viral infections, which implies that targeting or controlling the expression of miRNAs could be a valuable tool for improving DENV pathogenesis or preventing the development of severe dengue. However, miRNA-based therapies for controlling DENV replication are not under research, even though *in vitro* studies show their potential as therapy candidates.

7. Strategies to mitigate dengue burden

7.1. Current prevention strategies

To date, there is one approved vaccine, Dengvaxia, which has been used in 11 countries since 2015 [163]. Studies, however, reported a higher risk of developing severe dengue in seronegative individuals upon vaccination that receive the vaccine, especially in children younger than 5 years old [164,165]. Therefore, the WHO currently advises that Dengvaxia should be only administered to people above 9 years of age that have pre-existing immunity against DENV and only in endemic countries [166]. Thus, most efforts have been focused on the development of a safe and effective vaccine. Although several candidates are being studied at the moment, this is a challenging task due mainly to the need of protection against four different serotypes.

Given the challenges for developing an effective vaccine, the most effective method for preventing DENV infection nowadays is the control of the mosquito. Current methods for control of *Aedes* are based on insecticides and community management of mosquito habitats. Although the insecticide programs vary among different countries, most programs use toxic insecticides such as pyrethroids, organophosphates and carbamates that inhibit the larvae growth [167]. However, there is a current global concern regarding the use of insecticides due to the spread of insecticide resistance among Aedes mosquitoes that could reduce the efficiency of insecticide-based programs [168]. Community management appears to be promising. The WHO publishes and distributes continuously fact sheets that explain the required activities for controlling urban reproduction of the mosquito, including reduction of containers and habitats favorable for the oviposition of Aedes mosquitoes [3]. Although other strategies such as sterile insect techniques, genetic manipulation or the use of Wolbachia (Aedes-infecting bacteria that inhibits DENV replication in the mosquito and decreases transmission to the human) are promising [169,170], they are still in research stage and their effect in the transmission of DENV is yet to be determined.

7.2. Current therapeutical strategies

There are some non-specific treatments aimed to prevent the development of a severe form of the disease and for ameliorating the symptoms. Ambulatory patients that are sent home are encouraged to take oral hydration solutions like fruit juice and other fluids to prevent electrolytes loss and dehydration. Similarly, the indication of paracetamol is the most common treatment given by clinicians [3]. For patients that develop dengue with warning

signs and severe dengue, a more rigorous management should be done as they are usually admitted in a hospital. This includes fluid therapy for maintaining hematocrit and blood pressure, and transfusions of plasma and platelets when necessary [3,171]. In conclusion, the treatment of patients suffering from dengue is non-specific and is mainly aimed to prevent liquid loss and hypovolemic shock.

7.3. Under investigation: Antiviral and immunomodulatory compounds

Several studies have contributed to the development of specific antivirals against DENV replication, but few of them have advanced into evaluation on animal models or being tested in clinical trials [172]. These antivirals have been developed using high-throughput screening in replication assays and structure-based docking studies, including inhibitors for viral entry, capsid inhibitors, NS4B inhibitors, viral protease inhibitors, nucleoside analog inhibitors, and other non-specific inhibitors targeting host proteins [173]. Most of these inhibitors have good performance during *in vitro* assays but show high cytotoxicity in AGE129 mice or low efficacy in clinical trials, like NITD-008 and Balapiravir, which were some of the more promising [174,175]. Recent research has shown that NS4B inhibitors are the most frequent direct-acting DENV inhibitors identified with different screenings [172]. Further, new technologies that use proteomics and transcriptomics have provided attractive antiviral targets that comprise viral or host proteins that interact within viral replication complexes [172]. More research is needed to further development of these drugs.

Due to the important role that the inflammatory response plays in the pathogenesis of dengue, some therapeutical trials have tested drugs that can modulate the disease but have not shown promising results. For example, the anti-inflammatory drug prednisolone did not change hematological or virological parameters [176]. Similar results were observed with lovastatin, a drug that limits cholesterol synthesis and can modulate the immune response, which showed no evidence of clinical manifestations of amelioration or decrease in DENV viremia [177]. Despite the lack of immunomodulatory drugs available to treat DENV infection, the pursuit for antivirals that can limit viral replication while also modulate the inflammatory response is highly valuable. In this context, Vitamin D is very relevant, since this hormone shows broad immunomodulatory properties in several immune cells, and also wide antiviral activity.

7.4. Vitamin D

Vitamin D (VitD3) is a pleiotropic hormone acquired in the body through the exposition of the skin to the sunlight or by the uptake in some foods [178]. In blood, VitD3 can be found in its inactive form as calcidiol (25-hydroxyvitamin D), also known as VitD2, and transported throughout the body bound to the specific plasma carrier protein: vitamin D binding protein (DBP). This intermediate hormone is biologically inactive until it suffers a series of chemical changes first in the liver, mediated by cytochrome P450 2R1 (CYP2R1), and then in the kidney, mediated by the hydroxylase CYP27B1, which converts calcidiol into the active form calcitriol (1-alpha,25-dihydroxyvitamin D) [179]. Calcitriol, also referred to as VitD3, is important for the metabolism of calcium and phosphorus, promoting the intestinal absorption of these molecules present in the food. VitD3 is also important for calcium reabsorption by the kidney. Furthermore, VitD3 contributes to the development and maintenance of bones [178,180].

The biological actions of VitD3 involve the regulation of the transcription of several genes, which is dependent on the interaction of the hormone with the vitamin D receptor (VDR) present in the nuclei of numerous cells [181]. The complex VitD3-VDR associates with the nuclear receptor retinoid X receptor (RXR) and together act as a transcription factor that will increase the expression of genes that have Vitamin D response elements (VDREs) [182,183]. Various immune cells including T cells, DCs and macrophages constitutively express the enzymes necessary for transforming the inactive VitD2 into the active VitD3, suggesting that this hormone have a significant effect on these cells and therefore in the immune response.

Apart from its metabolic functions, VitD3 has wide immunoregulatory properties. VitD3 can modulate the polarization of T CD4+ cells by restricting the development of the Th1 profile and decreasing the production of IL-2 and IFN-γ. This process is highly dependent on the upregulation of IL-4 production promoting the development of a Th2 profile [184]. In DCs, VitD3 decreases the expression of co-stimulatory molecules like CD40, CD80 and CD86, and increases the production of IL-10, modulating the immune response of these cells during antigen presentation [185]. Furthermore, in macrophages, the active VitD3 can decrease antigen presentation by reducing the expression of class-II major

histocompatibility molecules (MHC-II) [186]. Also, VitD3 reduces the expression of TLR2, TLR4 and TLR9 and production of some inflammatory cytokines such as TNF-α, IL-1β and IL-6 [187,188]. VitD3 can also enhance the microbicidal activity of neutrophils, monocytes and macrophages by increasing the expression of antimicrobial peptides such as beta-defensins and cathelicidin LL-37 [189] **(Figure 8)**.

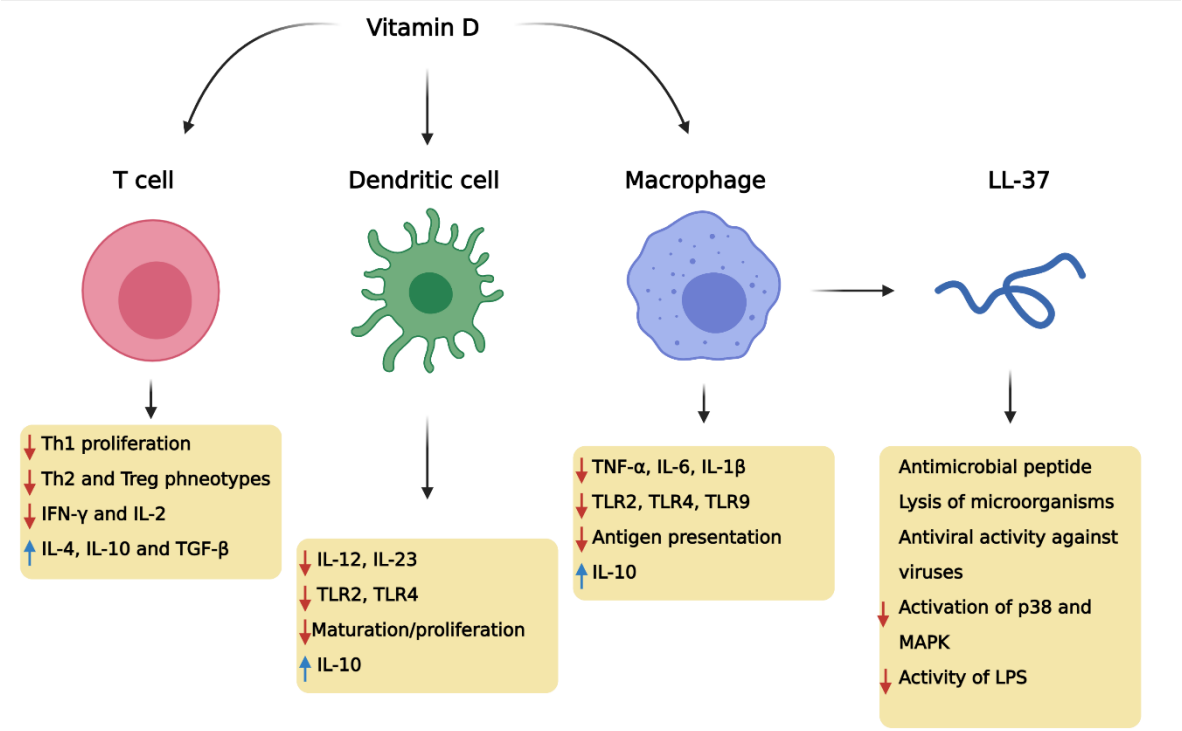


Figure 8. Immunomodulatory effects of VitD3 in immune cells

The Bioactive form of Vitamin D, 1α,25-dihydroxyvitamin D3 (VitD3), have wide immunomodulatory properties in T lymphocytes, dendritic cells (DCs) and macrophages. VitD3 can shape the polarization of CD4+ T cells by reducing Th1 proliferation, decreasing the production of IFN-γ and IL-2, promoting the polarization of Th2 and Treg phenotypes and increasing the production of IL-4, IL-10 and TGF-β. In DCs VitD3 decreases TLR2 and TLR4 expression, maturation and proliferation, while increases the production of the regulatory IL-10. Macrophages respond to VitD3 by reducing pro-inflammatory cytokines production, reducing TLR expression and decreasing antigen presentation. Also, macrophages produce LL-37 in response to VitD3, which has other immunomodulatory properties of its own. Apart from its antimicrobial properties, LL-37 reduce the activity of some agonists like LPS by directly binding to it or by reducing the activation of activation of p38 and MAPK. Figure designed with Biorender.

7.5. VitD3 in viral infections

VitD3 has shown also broad antiviral activity against various viruses. VitD3 limits the replication of Hepatitis C virus (HCV) in Huh7.5 hepatoma cells, which is associated with an increase of IFN-β and MxA expression [190]. Similarly, VitD3 decreases viral load of herpes simplex virus 1 (HSV-1) in supernatant of Hela cells, accompanied by downregulation of TLR2 and TLR9 mRNAs [191]. Also, VitD3 is an important modulator of the proinflammatory response in airway epithelial cells after infection with respiratory viruses, as VitD3 increases the expression of IkBa (NF-kB inhibitor) during respiratory syncytial virus (RSV) infection [192], and modulates the expression of some chemokines during infection with rhinovirus [193]. During DENV infection, VitD3 has also shown antiviral activity in hepatic HuH-7 cells, in monocytic U937 cell line [194], and in human primary MDMs [195,196]. Interestingly, Arboleda-Alzate et al. demonstrated that MDMs differentiated in the presence of VitD3 are less susceptible to DENV infection due to downregulation of MR expression [195]. Importantly, in these studies, the antiviral effect of VitD3 was accompanied by a decreased production of proinflammatory cytokines including TNF-α, IL-1β, IL-6 [194–196]. Despite the potential of VitD3 as a therapeutic alternative, few studies have evaluated the antiviral and immunomodulatory potential of VitD3 in DENV infected patients. Yet, the rs2228570 polymorphism in the start codon, and the 3'UTR polymorphisms (rs1544410, rs7975232 and rs731236) in the VDR gene have been associated with severe dengue [197]. Interestingly, these polymorphisms affect VDR functionality, suggesting that VitD3 may have an important role during DENV infection.

7.6. Antimicrobial peptide LL-37 and it's antiviral and immunoregulatory activity

As mentioned above, one of the main functions of VitD3 is the induction of antimicrobial peptides. Production of antimicrobial peptides such as defensins and LL-37 is observed during inflammatory conditions [198]. However, genes encoding some of these peptides such as beta-defensin 2 and LL-37, have been shown to harbor several VDREs [199]. Therefore, it is well known that expression of *CAMP* gene, which codifies for LL-37, and subsequent production of LL-37 peptide is strongly over-expressed by VitD3 treatment [200,201].

LL-37, also known as human cathelicidin antimicrobial peptide, is a product of the proteolytic cleavage of the cationic 18kDa protein (hCAP-18) encoded by the *CAMP* gene [202]. Expression of this antimicrobial peptide is found in several types of cells including epithelial cells, cells of the respiratory tract, genitals, among others [198]. In addition to epithelial tissues, expression of LL-37 is induced in several cells of the immune system such as neutrophils, NK cells, DCs, monocytes, and macrophages. Similar to other antimicrobial peptides such as beta-defensins, LL-37 first binds to the membrane of microbes and due to its amphipathic structure, insertion of the peptide follows that disturbs the membrane and induces leakage of cell contents [198,203,204]. LL-37 acts as a primary defense system against some microbes, as it is able to kill bacteria, fungi, can destroy bacterial biofilms, and also limit the replication of some viruses.

An antiviral effect of LL37 has been described against Influenza A virus (IAV) [205], Venezuelan equine encephalitis virus [206], Rhinovirus, RSV [207], Hepatitis C virus [208], Zika virus (ZIKV) [209] and Human immunodeficiency virus [210]. Further, treatment of HaCat and Vero E6 cell lines with exogenous LL-37 during DENV infection resulted in decreased proportion of infected cells, viral genome load in supernatants and decreased viral progeny [42,211]. Also, Jadhav et al. recently demonstrated that exogenous LL37 limit the replication of all DENV serotypes in U937 DC-SIGN cells [212]. Also, human keratinocytes HaCat, macrophage type THP-1 cells and neutrophils express higher levels of LL-37 after DENV infection [42,213].

Besides its antimicrobial activity, LL-37 shows broad immunoregulatory effects. LL-37 can decrease the inflammatory response in mouse macrophages by blocking the activation of p38 and extracellular signal-reduced kinases (ERK). This inhibition leads to a decreased production of TNF-α after LPS stimulation [214]. Furthermore, LL-37 can bind directly to LPS interfering with its downstream inflammatory signaling [215]. LL-37 can also enhance and shape the quality of immune response since this peptide induce the migration of neutrophils, monocytes/macrophages, eosinophils, and mast cells while also prolonging the lifespan of neutrophils [216]. Similarly, the shape of the immune response of monocytes can be modulated by LL-37, by increasing the expression of chemokines like CXCL1, CXCL8, CCL2 and CCL7 [217] and anti-inflammatory cytokines like IL-10 and IL-19 [218]. On the other hand, LL-37 has been shown to promote the inflammatory response by

mediating an increased activation of some TLRs. Specifically, LL-37 can form complexes with extracellular nucleic acids, both self-RNA and DNA, which promote an enhancement of TLR7/8 and TLR9 activation in dendritic cells (DCs). This process ultimately leads to an upregulation of type I interferon (IFN-I), TNF-α, and IL-6 expression [219,220]. It appears that the nature of the agonist would predict the outcome of LL-37 immunomodulation- However, this has not been studied during DENV infection.

8. Scope of the thesis

The pathogenesis of DENV infection is characterized by a dysregulated inflammatory response and a transient increase in endothelial permeability. A sustained and dysregulated inflammatory response ultimately targets endothelial cells, which is responsible for endothelial dysfunction. Monocytes and macrophages play an essential role during innate immune response against DENV infection. Nevertheless, as they are the main target for DENV replication, once activated, monocytes/macrophages produce high levels of soluble factors that contribute to systemic inflammation. This thesis aimed to dissect the role of some innate immune sensors in DENV sensing and induction of inflammatory response of monocytes/macrophages and how this response can be modulated by immunomodulators such as VitD3 and LL-37.

Previous work in our group revealed that TLR2 in PBMCs senses DENV, controlling the release of inflammatory mediators that activate vascular endothelial cells (ECs). Further, in a pediatric cohort of DENV-infected patients, elevated expression of TLR2 on the surface of CM but not on IM and NM was associated with severe dengue. This suggested that DENV TLR2 could have a differential role in viral replication and inflammatory response among monocyte subsets. In **chapter 2**, we sought to determine the function of TLR2 axis in monocytes subsets and its impact on DENV-2 replication and inflammatory response by separating monocyte subsets from PBMCs and challenging them with DENV-2 under TLR2 inhibition conditions. We also investigated the involvement of TLR2 co-receptors, such as CD14 and CD36, in the immune response and subsequent ECs activation triggered by DENV-2 and TLR2 interaction. Finally, a systematic block of key PRRs using specific inhibitors, delineated the contribution of other PRRs like TLR3 and TLR8 in the activation

of monocytes initiated by TLR2. Overall, this study provided evidence that TLR2 axis can function as a receptor for DENV in monocytes and has a dual role depending on each monocyte subset.

Despite the high burden of dengue disease, limited resources are available to treat patients suffering from DENV infection. Therefore, compounds that limit DENV replication and modulate the dysregulated inflammatory response, could serve as promising therapeutics to mitigate disease symptoms. VitD3 has drawn the attention of the field due to its broad antiviral and immunomodulatory properties. In **chapter 3**, we described the effect of the differentiation of macrophages in the presence of VitD3 in innate immune response after infection with DENV-2. We delineated the effect of VitD3 in the expression of TLRs and RIG-I, ROS production, expression of IFN-I and IFN-I stimulated genes, and lastly in the expression of anti-inflammatory SOCS-1.

One of the many genes that VitD3 upregulates is *CAMP*, which encodes for the antimicrobial peptide LL-37. VitD3 enhance the microbicidal activity of neutrophils, monocytes and macrophages by increasing the expression of LL-37. In **chapter 4**, we dissected the antiviral and immunomodulatory effects of LL-37 during DENV-2 infection of macrophages. In addition, we assessed if macrophages differentiation in the presence of VitD3 could increase the baseline levels of LL-37 produced by macrophages after DENV-2 infection.

VitD3 can regulate the expression of several genes that harbors VDRE. Regulation of miRNAs by VitD3 could significantly impact the immune response of monocytes/macrophages since they can regulate the expression of a wide array of messenger RNAs via post-transcriptional repression or degradation. In **chapter 5,** we assessed the effect of VitD3 in the expression of miRNAs during DENV-2 infection in MDMs. Since we found a set of differentially expressed miRNAs under VitD3 treatment, we inhibited the expression of some selected miRNAs, for simulating the effect observed with VitD3 treatment, and measured the production of some pro-inflammatory cytokines, some PRRs, SOCS-1, IFN-I, and some ISGs in MDMs during DENV-2 infection.

Finally, in **chapter 6**, I have summarized and discussed the key results of this thesis.

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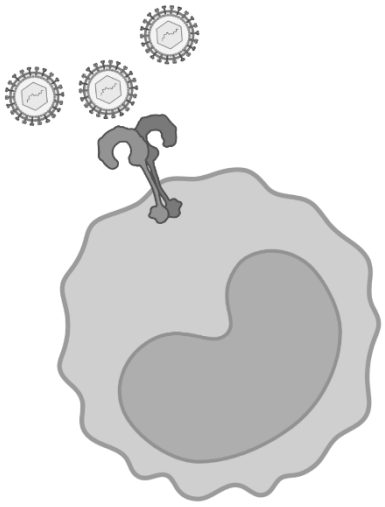
CHAPTER 2



TLR2 in CD16- monocytes drives dengue virus infection and inflammatory response

Jorge Andrés Castillo[a,b], Vinit Upasan[a], Peter van Duinkerken[a], Bram ter Ellen[a], Denise van de Pol[a], Silvio Urcuqui-Inchima[b], Jolanda M Smit[a], Izabela A Rodenhuis-Zybert[a,*]

a. Department of Medical Microbiology and infection Prevention, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands Grupo de Inmunovirología.

b. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No 52-21, Medellín, (Antioquia), Colombia

* Corresponding Author: i.a.rodenhuis-zybert@umcg.nl

ABSTRACT

Systemic inflammation leading to loss of microvascular endothelium integrity is the hallmark of the pathogenesis of dengue virus (DENV) infection. Our previous work identified Toll-like receptor 2 (TLR2) on monocytes as a key sensor of DENV infection in peripheral blood mononuclear cells and demonstrated that in DENV-infected patients, high TLR2 expression specifically on classical monocytes is predictive of severe dengue. Here, we scrutinize the function of TLR2 axis during DENV-2 infection in purified blood monocytes by blocking the activation of TLR2, its coreceptors and intracellular pattern-recognition receptors known to sense DENV. The data identified TLR2, CD14, CD36 and TLR3 as the main contributors to DENV-2 infection-induced immune response. Engagement of TLR2, CD14 and CD36 during DENV-2 infection facilitated viral entry and led to subsequent activation of TLR3 and the release of NF-κB-dependent inflammatory and antiviral response. Also, blocking of TLR2 prevented the activation and increased permeability of endothelial cells. Finally, by using sorted monocyte subsets, we found that only in classical, but not intermediate and nonclassical monocytes, DENV-2 infection relies on TLR2 axis, clarifying why in DENV-2-infected patients sustained high expression of TLR2 only in classical subset is associated with subsequent development of severe. Altogether, our data provides evidence for the dual function of TLR2 axis in DENV-2 replication in monocyte subsets and its contribution to systemic inflammatory and antiviral response.

KEYWORDS

Dengue virus, innate immunity, TLR2, endothelial permeability, monocyte subsets

INTRODUCTION

Infection caused by one of the four dengue virus serotypes (DENV1-4) can cause dengue disease, which is characterized by systemic inflammation and a transient increase in endothelial permeability [1,2]. The infection can present itself in a wide spectrum of clinical manifestations ranging from undifferentiated fever (dengue) accompanied by headache, myalgia, arthralgia, erythema, among other mild symptoms, to a more life-treating clinical manifestations such as hemorrhages, decrease in platelet count and organ impairment (dengue with warning signs and severe dengue) [3,4]. There is no specific treatment nor a fully protective vaccine available to prevent the disease [5]. Precise mechanisms leading to severe disease development are not fully understood. However, it is accepted that high viral titers in blood and an imbalance in the production of inflammatory mediators can lead to plasma leakage and other hemorrhagic symptoms.

DENV belongs to family Flaviviridae and genus Flavivirus. Viral infection of target cells occurs by receptor mediated endocytosis via heparan sulfate glycosaminoglycans, MR [6,7] or DC-SIGN [8]. The genome of DENV consist of a single stranded (ss) RNA of positive polarity (RNA+) approximately 11kb long, which codifies for one single polyprotein that consists of 3.391 amino acids [9]. After uncoating and translation of the viral RNA, cellular and viral proteases cut the polyprotein to synthetize three structural proteins: capsid (C), pre-membrane (prM) and envelope (E) glycoprotein; and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, involved in the regulation of viral protein synthesis, in viral replication and in the subversion of host antiviral response [10]. After translation and replication, viral particles are packed inside the RE as an immature virus, followed by a migration across the Golgi apparatus where the host furin enzyme completes the maturation process. Finally, the mature virus particles are liberated by exocytosis [11].

Blood monocytes are an important target cell for viral replication and their activation contributes to systemic inflammation in the course of DENV infection [12–17], which ultimately help containing viral dissemination. However, an exacerbated production of inflammatory modulators produced by monocytes after DENV infection can also mediate loss of endothelial integrity [18,19]. Monocyte activation upon DENV infection is mediated by the expression of a wide array of PRRs that recognize pathogen-associated molecular

patterns (PAMPs) that are part of the viral particle or produced during viral replication [20,21]. Among these PRRs, several Toll-like receptors (TLRs) has been shown to sense DENV. Endosomal TLR3 and TLR7/8 can recognize viral single-stranded and double stranded RNA structures respectively, that prime host cells to produce type I interferons (IFN-I) [22,23]. Moreover, we have recently uncovered that TLR2 together with its coreceptor CD14, both expressed on monocytes, senses DENV infection and the released inflammatory mediators activate endothelial cells (ECs) [24]. In addition, TLR2 engagement appeared to facilitate DENV Infection of monocytes, although no effect was found on replication [24].

Monocytes are a heterogeneous cell population that is canonically divided into three subsets (classical [CM], intermediate [IM] and non-classical monocytes [NM]) based on the expression of CD14 (LPS co-receptor CD14), and CD16 (low affinity IgG FcγRII receptor) [25]. These subsets show different functional properties and distribution, in which CM represent 80-90% of total monocytes, while IM and NM count for 5 and 2.5% respectively [26,27]. We and others have shown that DENV infected patients exhibit a decrease of CM and an expansion of IM and NM, suggesting a differential role for monocyte subsets in pathogenesis [17,24,28]. Interestingly, CM and IM predominantly expressed NS3 viral antigen compared to a low proportion of NM positive for NS3, suggesting a differential susceptibility to DENV infection among monocyte subsets [24]. Further, sustained, relatively highest expression of TLR2 on CM of DENV-infected patients was associated with subsequent development of severe disease [24]. Since TLRs and chemokine receptor expression and functionality are different among monocytes subsets [29,30], DENV driven activation of TLR2 could lead to different responses in monocytes subsets that could differentially impact endothelial cells and thus contribute to pathogenesis. This study aimed to evaluate the expression and function of TLR2 axis in monocyte subsets and its impact on DENV-2 replication and inflammatory response.

MATERIALS AND METHODS

Cells

The mosquito C6/36 cell line was obtained from the American Type Culture Collection (ATCC) and cultured in MEM (Sigma Aldrich, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, USA), 7.5% sodium bicarbonate, non-essential amino acids 4 mM L-glutamine, 10 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma Aldrich, USA), at 28°C with 5% CO_2. Baby hamster kidney cells (BHK-21) clone 15, were kindly gifted from Suzanne Kaptain (Leuven University) and maintained in D-MEM (Sigma Aldrich, USA), supplemented with 10% v/v FBS, 4 mM L-glutamine, 10 cfu/mL penicillin, and 0.1 mg/mL streptomycin at 37°C with 5% CO_2. Primary human umbilical vein endothelial cells (HUVECs) (Lonza, Switzerland) were cultured using EBM-2 media supplemented with EGM-2 endothelial growth SingleQuot kit supplement & growth factors (Lonza, Switzerland) at 37°C with 5% CO_2. HEK-Blue™ hTLR2 cells (InvivoGen, USA) were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), 100 µg/mL Normocin and HEK-Blue selection antibiotic according to the manufacturer's instructions. Green monkey kidney BS-C-1 cells (ATCC CCL-26, USA) were cultured in DMEM supplemented with 10% FBS, 25 mM HEPES, penicillin (100 U/ml), and streptomycin (100 U/ml) at 37°C with 5% CO_2.

Antibodies and Inhibitors

Conjugated antibodies against CD14-eFluor 450 (clone 61D3), CD16-APC (clone eBioCB16 (CB16)), CD16-PE (clone B73.1), CD56-APC (clone CMSSB), CD36-PerCP-CY5 (NL07), TLR1-PE (clone GD2.F4), TLR2-PE (clone TL2.1), IgG1-PE isotype control (clone P3.6.2.8.1), IgG1-APC (P3.6.2.8.1) and IgG1-eFluor 450 isotype control (clone P3.6.2.8.1) were purchased from Thermo Scientific (USA). Conjugated antibodies against HLA-DR-FITC (clone L243), CD19-APC (clone HIB19), CD3-APC (clone UCHT1), TLR6-PE (clone TLR6.127), TLR10-PE (clone 3C10C5), E-selectin-PE (clone HCD62E), ICAM-1-FITC (clone HCD54), VCAM-1-APC (clone STA) and IgG1-FITC isotype control (clone MOPC-21) were purchased from Biolegend (USA). The small molecule C29 was obtained from MedChem Express (USA). TLR2 axis was blocked using the neutralizing antibodies against TLR2 (Invivogen, USA), TLR1 (Invivogen, USA), TLR6 (Invivogen, USA), CD14 (Invivogen, USA), TLR10 (Thermo Scientific, USA), CD36 (Stem cell technologies, Canada) and the chemical inhibitor C29 (MedChem Express, USA). NF-κB inhibitor Bay 11-7082 and TLR8 inhibitor CU-CPT9a were

purchased from Invivogen (USA). TLR3/dsRNA complex inhibitor was purchased from Sigma-Aldrich-Merck (Germany).

Virus stocks

DENV-2 16681 strain viral stocks were generated by infecting C6/36 monolayers in 225-cm2 tissue culture flasks using a multiplicity of infection (MOI) of 0.05 diluted in 10 ml of MEM supplemented with 2% FBS. After 2 hours of infection, fresh 2% FBS was added to the cells to complete 20 ml and cultured further for 7 days at 28°C with 5% CO_2. Supernatants from infected cells was obtained and centrifugated at 800g for 10 minutes for removal of cellular debris. Supernatants was aliquoted and stored at -70°C until further use. Viral titration was performed by the quantification of plaque-forming units (PFU) and genome equivalent copies (GECs) using plaque assay and RT-qPCR respectively, as described previously [31].

Viral titration

For the assessment of PFU/ml titer $1.3x10^5$ of BHK-21 cells were seeded in 12 well plates in D-MEM supplemented with 10% FBS and incubated at 37°C with 5% CO_2 overnight. Then, cells were infected with 10-fold serial dilutions of the virus diluted in 600 µL of medium for 2 hours. After infection, 1.5 ml of plaque medium consisting of 1% agarose (Low melting point, Lonza, Switzerland), 4% FBS, 3% NaCO3, 1% HEPES (Sigma-Aldrich-Merck, Germany) and 10 cfu/mL penicillin, 0.1 mg/mL streptomycin, was added on top. Cells were cultured for 7 days at 37°C with 5% CO_2 and then agarose plug was removed, and cells fixed with 10% for formaldehyde (Sigma-Aldrich-Merck, Germany) for 30 min. After fixation, cells were stained with 1% crystal violet (Sigma-Aldrich, USA) and plaques were counted manually to obtain PFU/ml.

For the assessment of GECs titer, viral RNA was purified from supernatants using QIAamp Viral RNA mini kit (Qiagen, Germany) following manufacturer's instructions. Then, cDNA was synthesized using the reverse primer 5´TGC AGC AAC ACC ATC TCA TTG 3´ and Omniscript retrotranscriptase (Qiagen, Germany) following the manufacturer's instructions. cDNA was used for qPCR reaction using Hot Goldstar Taq DNA polymerase (Qiagen, Germany), forward primer 5´ACA GGT TAT GGC ACT GTC ACA AT 3´, and reverse primer depicted above. Detection of newly synthetized DNA was done using a FAM/TAMRA probe 5´AGT GCT CTC CAA GAA CGG GCC TCG 3´ (Eurogentec, The

Netherlands) and analyzed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The number of genome-equivalent copies (GEC) was based on a standard curve of Ct values of 10-fold serial dilutions of a plasmid encoding the full genome of DENV 2 of known length and concentration, as previously described [32].

Isolation of PBMCs

Monocytes were purified from PBMCs obtained from buffy coats of healthy and anonymous donors (Sanquin Blood Bank, Groningen, The Netherlands) with written consent following the declaration of Helsinki as previously described [33]. In brief, PBMCs were separated using a density gradient centrifugation with Ficoll-Plaque Plus (GE Healthcare, USA) centrifuged at 450g at room temperature for 30 min. Next, cells were washed three times with RPMI-1640 medium (Sigma-Aldrich, USA) at 250g for 10 min for platelet removal. PBMCs were suspended in FBS, counted, and cryopreserved at -196°C in 10% DMSO. PBMCs were thawed with warm FBS and used either for infections, for monocyte purification or for monocyte depletion.

Monocyte enrichment and depletion

Monocytes were purified through negative selection from PBMCs using biotinylated antibodies against CD2, CD3, CD7, CD19, CD20, CD56, CD57, CD123, CD235a and streptavidin-coated magnetic beads according to the instructions of the Magnisort Human pan-monocyte enrichment kit (Thermo, USA). After negative selection from $1x10^7$ PBMCs, $4x10^5$ of monocytes were cultured in 48-well plates (Corning Incorporated Life Science, USA) in RPMI-1640 medium supplemented with 0.5% of FBS at 37°C with 5% CO_2 to allow enrichment of monocytes through plastic adherence. After 2 hours of adherence, remaining non-adherent cells were washed with warm RPMI-1640 medium and cultured further with 10% supplemented FBS media at 37°C with 5% CO_2. Purity of monocyte culture was assessed by measuring the presence of non-adherent CD3, CD19 and CD56 positive cells. Only monocyte cultures with purity above 90% were used in this study.

Monocyte were depleted from PBMCs (MoΔPBMCs) using Magnisort Human CD14 positive selection kit based on biotinylated CD14 antibody and streptavidin-coated magnetic beads (Thermo, USA). Efficiency of monocyte depletion was assessed by the

detection of CD14 positive cells. MoΔPBMCs were used when depletion efficiency depletion was above 80%.

Monocyte subsets sorting

1.5x10^8 PBMCs were thawed with warm FBS and stained with anti-CD3-APC, anti-CD19-APC, anti-CD56-APC, anti-HLA-DR-FITC, anti-CD14-eFluor 450 and anti-CD16-PE. CD14+CD16- (classical) and CD14+CD16+ (intermediate and non-classical) were electromagnetically sorted using the Sony SH800S sorter (Sony, Japan). Purity of monocyte subsets cultures were above 96%, while efficiency of recovery was above 85% for CD14+CD16- monocytes and above 70% for CD14+CD16+ monocytes. Next, 1.5x10^5 monocytes of each subset were cultured in 96 well plates for 2 hours in RPMI-1640 supplemented with 0.5% FBS. After 2 hours of adherence media was removed and cultures were replenished with RPMI-16040 with 10% FBS and cultured at 37°C with 5% CO_2.

DENV-2 infections in the absence or presence of inhibitors

4x10^5 Monocytes, 1.5x10^5 monocyte subsets, or 5x10^5 PBMCs and MoΔPBMCs were exposed to DENV-2 16681 strain at an MOI of 10 prepared in RPMI-1640 supplemented with 2% FBS. After 2 hours, virus inoculum was removed, and cells washed with warm media. After adding fresh 10% FBS RPMI-1640 medium the culture was continued at 37°C with 5% CO_2.

In experiments assessing contribution of TLR2 axis in DENV-2 infection and immune response, cells were treated with different inhibitors for TLR2 axis for 2 hours prior to infection and during attachment period. Anti-TLR2 (7.5 ug/ml), anti-TLR1 (7.5 ug/ml), anti-TLR6 (7.5 ug/ml), anti-TLR10 (3 ug/ml), anti-CD14 (3 ug/ml), anti-CD36 (2.5 ug/ml) or control isotypes at same concentration were added individually diluted in 150µl of media. C29 (100 nM), Bay 11-7082 (2µM), CU-CPT9a (50µM) and TLR3 (10µM) inhibitors prepared in DMSO was also added individually and vehicle DMSO stimulation was used as control. After 2 hours of treatment all different inhibitors were washed and were added again during 2 hours of infection. After infection, virus inoculum and inhibitors were washed and added again, and RPMI-1640 supplemented with 10% FBS were added to cells and cultured at 37°C with 5%CO_2 until required. Stimulation of total monocytes with PAM3CSK4 (PAM3, 100 ng/ml), PAM2CSK4 (PAM2, 100 ng/ml), Poly I:C (25µg/ml) or ssRNA40 (1µg/ml) (Invivogen, USA) agonists were done in the presence or absence of different Inhibitors and

treated as positive controls for evaluating the efficiency and specificity of treatments. At the indicted time points post-infection, cells were collected and subjected to flow cytometric analysis to assess their viability and frequency of infection. Collected cell-free supernatants were snap frozen and stored at -80°C for future use. Supernatants were used for viral RNA purification, quantification of viral copy number by qPCR, viral titration by plaque assay and for endothelial assays.

Endothelial activation assay

Supernatants from mock and DENV-2-infected monocytes, PBMCs and MoΔPBMCs were used to stimulate confluent HUVECs monolayers cultured in 24 well plates. Supernatants were mixed with EBM-2 media in a 1:1 ratio and added directly to HUVECs for 6 hours. LPS (1 ug/ml) (Invivogen, USA) was added as a positive control. Also, DENV-2 at a MOI of 10 was added directly to HUVECs for 6 hours in order to control non-specific activation induced by the virus and not by the inflammatory soluble factors present in supernatants. After stimulation, HUVECs activation was assessed by measuring the increase in the expression of E-selectin, ICAM-1 and VCAM-1 determined by flow cytometry.

Flow cytometry assays

Flow cytometry was used to analyze the DENV-2 infected cell mass of total monocytes, PBMCs and MoΔPBMCs, changes in TLR2 axis expression in monocyte subsets, and activation of HUVECs. The infected cell mass in monocyte subsets was not possible due to low numbers in cultures. DENV-2 infection was evaluated through the intracellular staining of viral E protein as described previously [24]. Briefly, cells were recovered from cultures and fixed using 4% of paraformaldehyde. Next, cells were permeabilizated using PBS with 0.5% of tween 20 for 15 minutes. Following washing steps, cells were stained with the monoclonal antibody, 4G2 (Millipore, Germany) for 30 min, followed by 30 min staining with goat anti-mouse IgG-Alexa Fluor 647 (Thermo Scientific, USA). Expression of TLR2 axis in monocyte subsets, was analyzed in thawed PBMCs using anti-TLR2-PE, anti-TLR1-PE, anti-TLR6-PE, anti-TLR10-PE, anti-CD36-anti-CD14-eFluor 450, anti-CD16-APC, anti-PerCP-CY5 and anti-HLA-DR-FITC. After 25 minutes of staining, cells were fixed with 4% of paraformaldehyde and analyzed. For activation of HUVECs, cells were stained with anti-E-selectin-PE, anti-ICAM-1-FITC and anti-VCAM-1-APC for 25 minutes. After staining,

cells were washed, fixed with 4% of paraformaldehyde and analyzed. Isotype-matched controls were used as negative controls and for determination of positive cells. Cell viability was determined by staining the cells with the fixable viability dye eFluor 780 and normalized against un-treated control cells. All data acquisition was done using BD FACSVerse (BS Biosciences, USA) system and analyzed using FlowJo software (BD Biosciences, USA).

Quantification of cytokine production

Cytokine levels of IL-1β, TNF-α, IL-6, IL-8, IL-10, IL-12p70, IP-10, GM-CSF, IFN-α2, IFN-β, IFN-λ1, IFN-λ2/3 and IFN-γ were quantified using the human anti-virus response panel (13-plex, LEGENDplex, Biolegend, USA). Data was collected using BD FACSVerse (BS Biosciences, USA) system and analyzed using LGENDplex v8.0 (Biolegend, USA).

Endothelial permeability assays

Supernatants from DENV-2 infected monocytes were used in an *in vitro* model of endothelial permeability as previously described [18,34]. Briefly, $5x10^4$ HUVECs In a 24-well plate transwell plate containing inserts with 0.4µm pores (Corning Inc, USA), which were pre-coated with collagen I (Corning Inc, USA) at a final concentration of 10 ug/cm2 at 37°C overnight. Cells were cultured in 250 ul of EGM-2 MV medium for 3 days at 37°C 5% CO_2 for obtaining confluency. Culture medium was changed every 48 hours. HUVECs were stimulated with supernatants from monocytes diluted with EGM-2 MV medium in a 1:1 ratio. As positive control TNF-α at a concentration of 100 ng/ml was added to cells. Trans-endothelial electrical resistance (TEER) was measured using Millicell ERS-2 Voltohmmeter (Sigma-Aldrich-Merck, Germany), every 2 hours until 12 hours after stimulation and at 24 hours after stimulation. Endothelial permeability was expressed as relative TEER which represents a ratio of resistance values (Ohms, Ω) between test and control as follows: (Ω experimental condition – Ω transwell without HUVECs) / (Ω non-treated HUVECs – Ω transwell without cells).

Additionally, endothelial permeability was assessed by measuring the migration of Dextran-FITC from the upper chamber to the lower chamber. For this, 22 hours after stimulation, 200ug/ml of Dextran-FITC (Sigma-Aldrich, USA) was added to the upper chamber and incubated for 2.5 hours at 37°C 5% CO_2. FITC fluorescence in the lower

chamber was measured using GloMax microplate reader (Promega, USA) with excitation wavelength of 485 nm and emission wavelength of 535 nm. Concentration of Dextran-FITC was calculated by performing a standard curve using 12 standards points of 2-fold dilutions starting from 200 ug/ml.

Cell-based ELISA

Flat-bottom 96-well plates were coated with poly-L-lysine (0.1%) for 2 hours. HEK-Blue™ hTLR2 cells were plated at the density of $2x10^4$ cells/well in 100ul medium and incubated for 2.5 days. On the day of infection, cells were incubated for 2 hours with DENV-2. To remove surface-bound DENV virions, cells were washed with PBS. After the incubation, cells were washed, fixed, permeabilized and blocked for 2 hours with PBS supplemented with 5% FBS. Cells were then stained with mouse anti-flavivirus envelope protein antibody 4G2 for 1 hour followed by a secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase (HRP). Finally, antigen-antibody complexes were detected by the addition of HRP substrate [o-phenylenediamine dihydrochloride (OPD) tablets dissolved in phosphate citrate buffer]. After incubation for 20 minutes at room temperature, reaction was stopped using 0.5N H_2SO_4. Plates were read using microplate reader at 492 nm.

Statistical analysis

GraphPad Prism version 6 (GraphPad Software, USA) software was used to determine differences significance between treated monocytes and isotype control treated monocytes. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Blocking TLR2 on monocytes abrogates DENV-2-induced immune response and inhibits viral replication

Within peripheral blood mononuclear cells (PBMCs), monocytes are not only an important target for DENV replication but also key drivers of inflammatory response during *in vitro* and *in vivo* infections [13,14]. Also, in our infection model, depletion of monocytes from PBMCs prior to DENV-2 infection with an MOI of 10, led to a reduction of 64% in % E positive cells, 84% of genomic equivalent copies (GECs) and 98% in PFU titer (Supplemental

Figure 1A, B and C). Further, monocyte depletion completely abrogated activation of ECs induced by the supernatants of infected PBMCs (Supplemental Figure 1D). These data support the evidence that monocytes are essential for DENV-2 replication and induction of systemic inflammatory response.

We have previously shown that DENV-2 activates TLR2 in PBMCs inducing the production of proinflammatory cytokines [24]. As we observed that monocytes are important for DENV-2 replication and inflammatory response of PBMCs, we scrutinized the role of TLR2 in monocytes during DENV-2 infection. Purity of monocyte cultures was above 90% repeatedly (Supplemental Figure 2). For assessing the role of TLR2, this receptor was inhibited using blocking antibody or the chemical inhibitor C29 [35]. Blocking of TLR2 with antibody or C29 did not decreased monocyte viability under mock or DENV-2 infection conditions (Supplemental Figure 3A). Also, inhibitors efficiently blocked TLR2 function as we observed a decreased activation of ECs by supernatants of PAM3-stimulated monocytes (Supplemental Figure 3B).

Having stablished the optimal conditions for treatments, we infected monocytes with DENV-2 at an MOI of 10 and at 48 hours post-infection (hpi), and evaluated the induced immune response according to the scheme depicted in Figure 1A. ECs activation was significantly decreased by supernatants of monocytes treated with α-TLR2 or C29, compared to the high activation of ECs induced supernatants of monocytes treated with control isotype or infected alone (Figure 1B), suggesting that TLR2 inhibition decreases inflammatory response of monocytes. Activation of ECs could lead to increased endothelial permeability, hallmark of severe dengue [1]. Thus, the role of TLR2 in endothelial permeability was evaluated by stimulating ECs with DENV-2 infected monocytes supernatants and measuring TEER and Dextran-FITC migration across transwells (Figure 1A). Supernatants of DENV-2 infected monocytes induced a significant decrease of ECs TEER and increased migration of Dextran-FITC, effect that was completely abrogated when TLR2 was blocked on monocytes before infection (Figure 1C and D). Importantly, direct infection of ECs with DENV-2 did not activate the cells at 6 hpi nor increased the endothelial permeability (Supplemental Figure 4), suggesting that soluble factors produced after TLR2 activation are mediating activation and permeability of ECs.

Next, we evaluated the TLR2-driven inflammatory mediators responsible for ECs dysfunction by the quantification of cytokines in monocytes supernatants. Inhibition of TLR2 significantly decreased production of inflammatory cytokines, namely IL-1β, TNF-α, IL-6, IP-10, IL-8 and IL-10 (Figure 1E). Besides NF-κB activation, TLR2 engagement can activate IRFs and induce IFN-I expression [36]. Therefore, IFN-I and IFN-III were measured after TLR2 inhibition in monocytes. Interestingly, inhibition of TLR2 in monocytes before DENV-2 infection significantly decreased the production of antiviral IFNs, namely IFN-α2, IFN-β, IFN-λ1 and IFN-λ2/3 (Figure 1F). Altogether these results suggest that TLR2 drives DENV-2 induced immune response in monocytes.

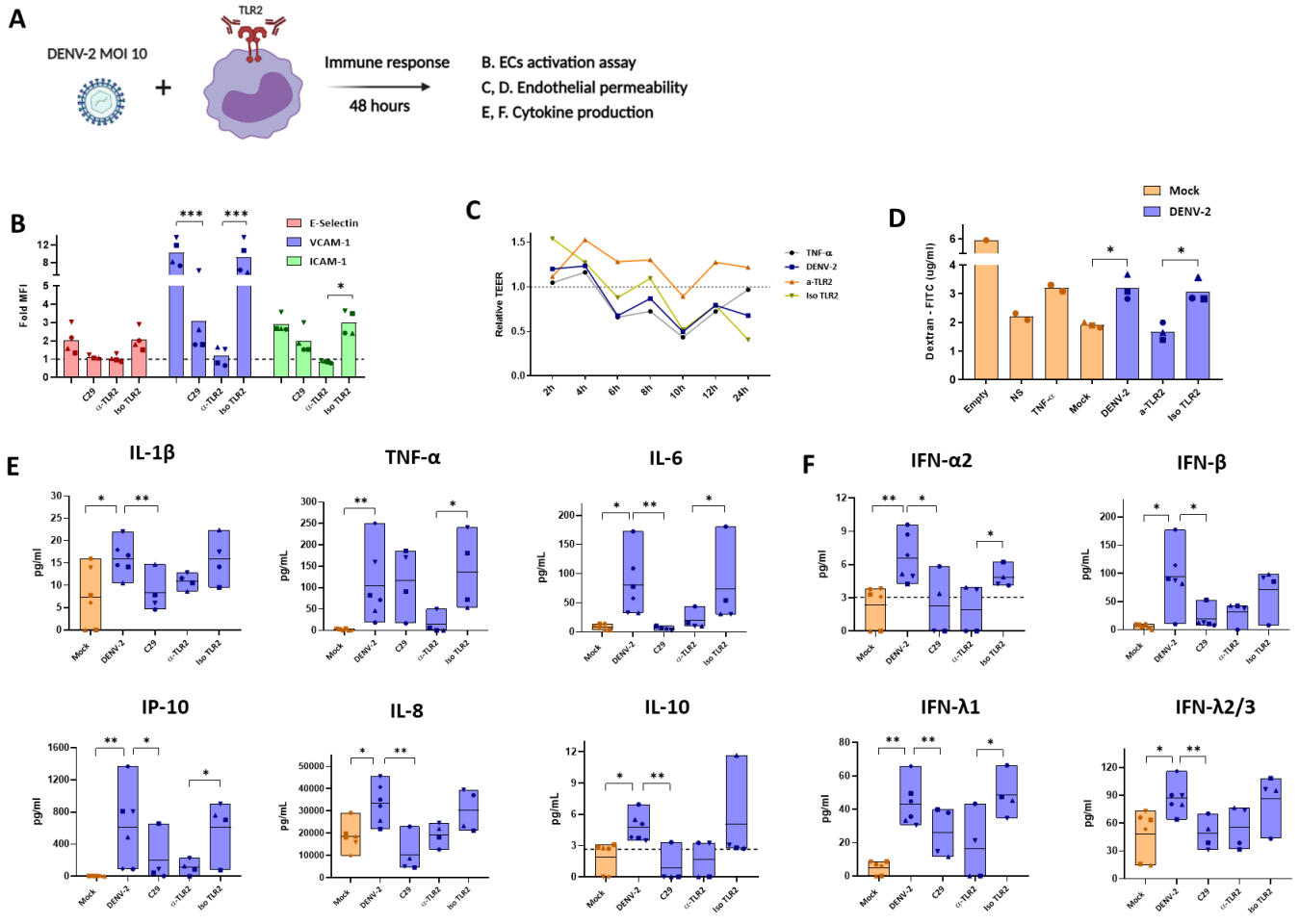


Figure 1. TLR2 blocking inhibits inflammatory and antiviral response in DENV-2 infected monocytes.

(A) Representative scheme showing experimental strategy. (B) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with α-TLR2, matching control isotype or C29, and infected with DENV-2 for 48 hours.

Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with supernatants from mock-infected monocytes (n=4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05). HUVECs were stimulated with supernatants from monocytes treated with α-TLR2, matching control isotype or C29, and Infected with DENV-2 for 48 hours, and (C) transendothelial electrical resistance (TEER) was measured every 2 hours. (D) Dextran-FITC migration across transwell was measured at 24 hours after stimulation. Data of TEER was normalized against control HUVECs stimulated with supernatants from mock-infected monocytes, and Dextran-FITC concentration was obtained using a standard curve (n= 3 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (* p < 0.05). (D, E) Concentration of cytokines (pg/ml) produced by monocytes treated with α-TLR2, matching control isotype or C29, and Infected with DENV-2 for 48 hours (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05).

Our previous work suggested that TLR2 sensing facilitated DENV infection of PBMCs, yet TLR2 had no detectable effect on virus production [24]. Here we sought to clarify these discrepancies by blocking TLR2 in monocytes prior to DENV-2 infection (Figure 2A). To do this, we first took advantage of the different mode of action of TLR2 blocking antibody and C29 Inhibitor, which bind to the extracellular domain and to the intracellular TIR domain of TLR2 [35], respectively. Blocking TLR2 with specific antibody or C29 inhibitor in DENV-2 infected monocytes significantly decreased infected cells mass (Figure 2B and Supplementary Figure 5) GECs (Figure 2C) and PFU titers (Figure 2D) in supernatants, compared to isotype control or DENV-2 infected alone monocytes. Notably, blocking of TLR2 with specific antibody was more efficient in decreasing DENV replication compared to C29 inhibitor (Figure 2A, B and C).

Then, we evaluated if TLR2 facilitates binding or internalization of DENV-2. For this, we optimized a cell-based ELISA for determining the amount of bound and internalized DENV-2 virions in HEK-TLR2 cells (Figure 2E). Blocking of TLR2 In HEK-TLR2 cells prior to challenge with DENV-2, resulted in decreased DENV binding and internalization as indicated by their lower OD values compared to challenge with DENV-2 alone or with its corresponding isotype (Figure 2F). Altogether, our results suggest that TLR2 mediates DENV-2 binding and drives replication in monocytes.

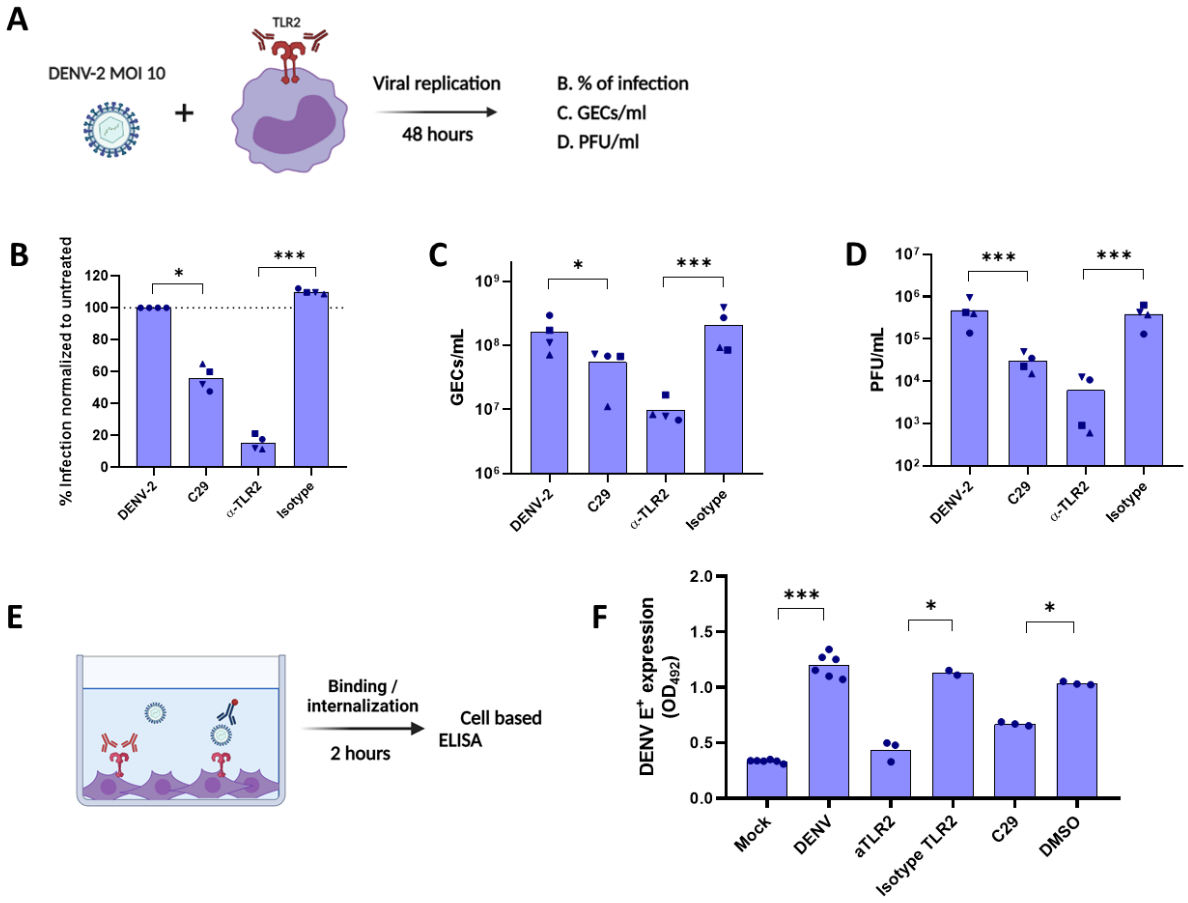


Figure 2. TLR2 blocking inhibits DENV-2 replication in monocytes.

(A) Representative scheme showing experimental strategy. (B) Percentage of E+ cells, (C) genome equivalent copies (GEC) and (D) infectious titer (plaque-forming unit, PFU) in supernatants of monocytes treated with C29, α-TLR2 or matching control isotype, prior to infection with DENV-2 at MOI=10 for 48 hours (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$). (E) Representative scheme showing experimental strategy. (F) Cell based ELISA of BHK-21 cells treated with C29, α-TLR2 or matching control isotype, prior to infection with DENV-2 at MOI=5 for 2 hours (n=3 different donors). Kruskal Wallis test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$).

Activation of TLR2 by DENV-2 induces NF-κB mediated response in monocytes

TLR2 activation by ligands like PAM2 or zymosan leads to the activation of NF-κB transcription factor and subsequent expression of proinflammatory genes [37]. To test whether TLR2 activation by monocytes leads to activation of NF-κB or a different signaling

pathway, the activation of NF-κB was evaluated under TLR2 blocking conditions. By measuring phosphorylated p65 using flow cytometry, a significant decrease in the activation of NF-κB was observed under TLR2 blocking conditions (Figure 3A). Further, the inflammatory response of monocytes under TLR2 inhibition and NF-κB inhibition (using Bay11) was compared. Viability of monocytes under Bay11 treatment is shown in Supplemental Figure 6A. Efficiency of inhibition NF-κB by Bay11 was evaluated by assessing the expression of TNF-α and the induced activation of ECs by supernatants after stimulation with PAM3 (Supplemental Figure 6B and C).

Similar to TLR2 blocking conditions, inhibition of NF-κB decreased the activation of ECs induced by DENV-2 infected monocytes (Figure 3B) and decreased the production of the inflammatory cytokines TNF-α, IL-6 and IL-10 (Figure 3C). Interestingly, NF-κB inhibition decreased the production of antiviral IFNs such as IFN-α2, IFN-β and IFN-λ1 (Figure 3D) by monocytes under DENV-2 infection, suggesting that NF-κB induces the expression of IFN-I/III. Furthermore, activation of ECs induced by DENV-2 infected monocytes supernatants was lower when Bay11 and α-TLR2 were combined than Bay11 used alone (Figure 3B). Similar results were observed with production of TNF-α and IFN-β (Figure 3C and D) suggesting that TLR2 activation by DENV-2 induces the activation of other signaling pathways different to NF-κB. Altogether, these results show that inflammatory and antiviral response to DENV-2 infection depend on TLR2-mediated NF-κB activation.

TLR3 contributes to TLR2-initiated NF-κB response to DENV-2 infection in monocytes

Monocytes can sense DENV infection through an array of PRRs that recognize viral components present on the viral particle itself or produced during replication. TLR3 and TLR8 can sense dsRNA or ssRNA, respectively, produced during viral replication cycle [23,38]. We sought to elucidate the contribution of these TLRs in the immune response initiated by TLR2 in DENV-2 infected monocytes. For this, monocytes were treated with TLR3 and TLR8 inhibitors (iTLR3, iTLR8) and inflammatory response was compared to that observed in TLR2 blocking conditions. Optimal non-toxic concentration of iTLR3 and iTLR8 were determined by assessing the expression of IFN-β and activation of ECs induced by

supernatants of monocytes stimulated with Poly I:C or ssRNA40 (Supplemental Figure 7B and C).

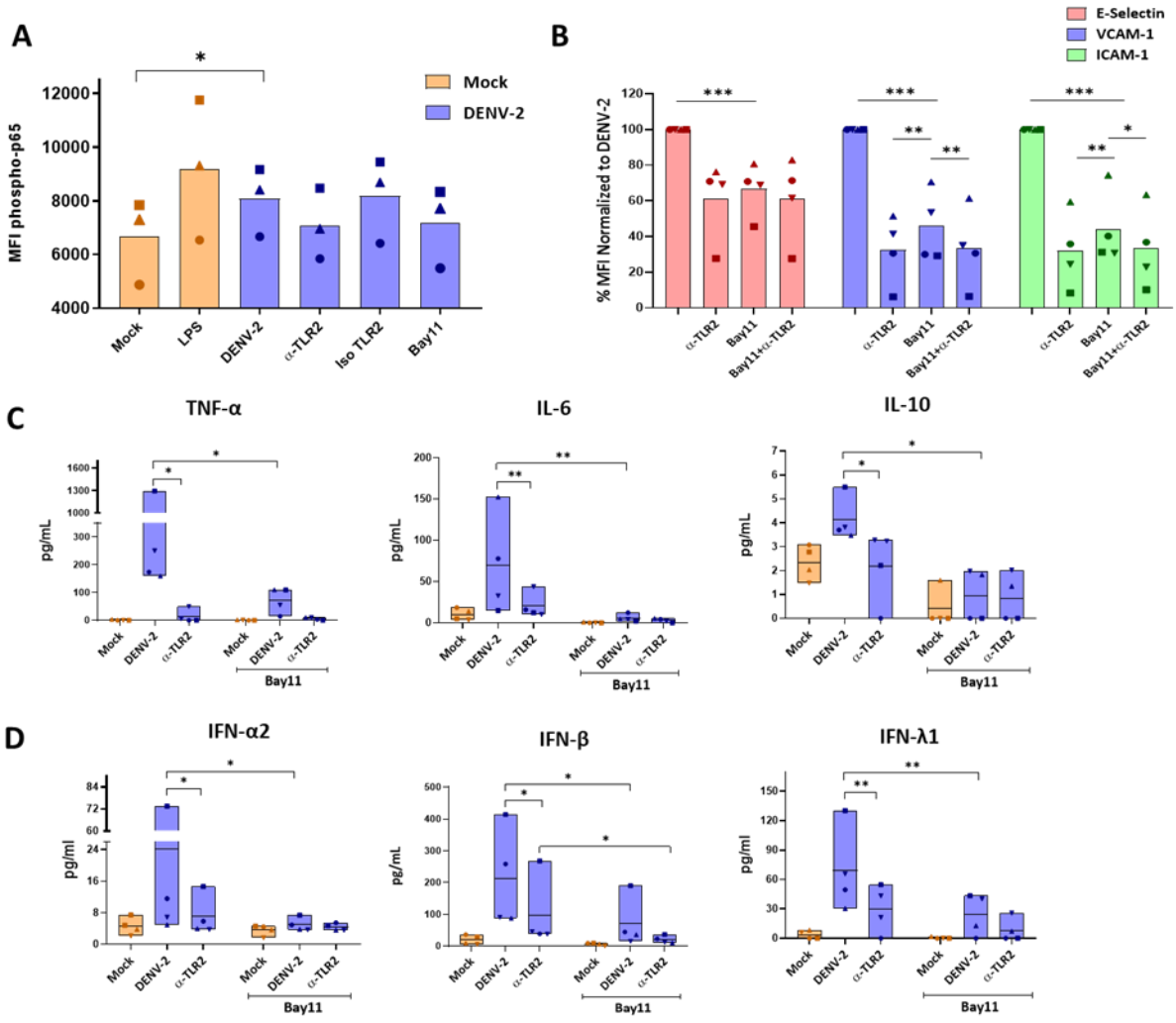


Figure 3. TLR2 activation by DENV-2 induces NF-κB dependent response in monocytes.

(A) MFI of phosphorylated p65 subunit in monocytes treated with α-TLR2, matching control isotype or Bay11 (2μM) and infected with DENV-2 for 4 hours. Expression of phosphorylated p65 was assessed by flow cytometry and (n=3 different donors). Kruskal Wallis test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$). (B) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with α-TLR2 or/and Bay11(2μM), and infected with DENV-2 for 48 hours. Expression was assessed by flow cytometry and data was normalized against HUVECs stimulated with supernatants from DENV-2 infected monocytes alone (n=4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$). (C, D) Concentration of cytokines (pg/ml) produced by monocytes treated with α-TLR2 or/and

Bay11(2μM), and infected with DENV-2 for 48 hours (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05).

Inhibition of TLR3 decreased the inflammatory response of monocytes as supernatants of monocytes treated with iTLR3 showed decreased activation of ECs (Figure 4A) and low production of IL-1β and IL-8 (Figure 4C). As expected, production of IFN-α2, IFN-β and IFN-λ1 decreased after inhibition of TLR3 in DENV-2 infected monocytes (Figure 4D). On the contrary, inhibition of TLR8 did not have any effect on the inflammatory response of monocytes or production of cytokines (Figure 4B, C and D). There was a trend to an increase in immune response of monocytes with iTLR8 treatment, but it was only significant for the case of IL-10 (Figure 4C). Activation of ECs by monocytes supernatants, and production of IL-1β, IL-8, IL-10, IFN-β, and IFN-λ1 were lower when iTLR3 and α-TLR2 were combined (Figure 4A, C and D). Together these results suggest that TLR3 contributes to TLR2 signaling initiated by DENV-2 activation during infection in monocytes.

Blocking CD14 or CD36 decreases DENV-2 replication and immune response in monocytes which are differentially expressed among monocyte subsets

TLR2 complex signals by the association with other co-receptors such as TLR1, TLR6 and CD14 which promotes binding of ligands to TLR2 and subsequent signaling [39]. Like CD14, CD36 can also associate with TLR2 promoting loading of ligands onto TLR2 [40]. Also, TLR10 is known to form heterodimers with TLR2, similar to TLR1 and TLR6, but unlike these last two, its activation leads to an anti-inflammatory response [41]. Therefore, we tested the effect of TLR2 co-receptors in DENV-2 replication and immune response of monocytes. For this, TLR2 axis in monocytes was blocked with specific antibodies and then infected with DENV-2 for 24 hours. Efficiency of TLR2 axis blocking was evaluated by assessing the inhibition of PAM3/2 induced responses for the case of TLR1 and TLR6 and enhancement of PAM3 induced responses for TLR10 (Supplementary Figure 8B, C and D). Blocking TLR1, TLR6 or TLR10 did not alter infection (Figure 5A) or DENV-2 replication in monocytes (Figure 5B, C). On the other hand, blocking CD14 or CD36 decreased monocyte infected cell mass (Figure 5A) and decreased viral titer (Figure 5C), compared to respective isotypes controls. Inhibition of CD14 also decreased GECs in supernatants (Figure 5B). In a

similar way, blocking CD14 or CD36 decreased inflammatory response of DENV-2 infected monocytes, as ECs activation induced by supernatants was lower compared to that induced by monocytes treated with control isotypes (Figure 5E). However, inhibition of CD36 did not alter production of the cytokines evaluated in this study by monocytes (Figure 5F and G). Finally, blocking TLR1, TLR6 or TLR10 did not alter the immune response of the infected monocytes (Figure 5D, F and G). Altogether, these data indicate that CD14 and CD36, but not TLR1, TLR6 or TLR10, are important for TLR2 mediated infection of DENV-2 in monocytes and contribute to subsequent immune response.

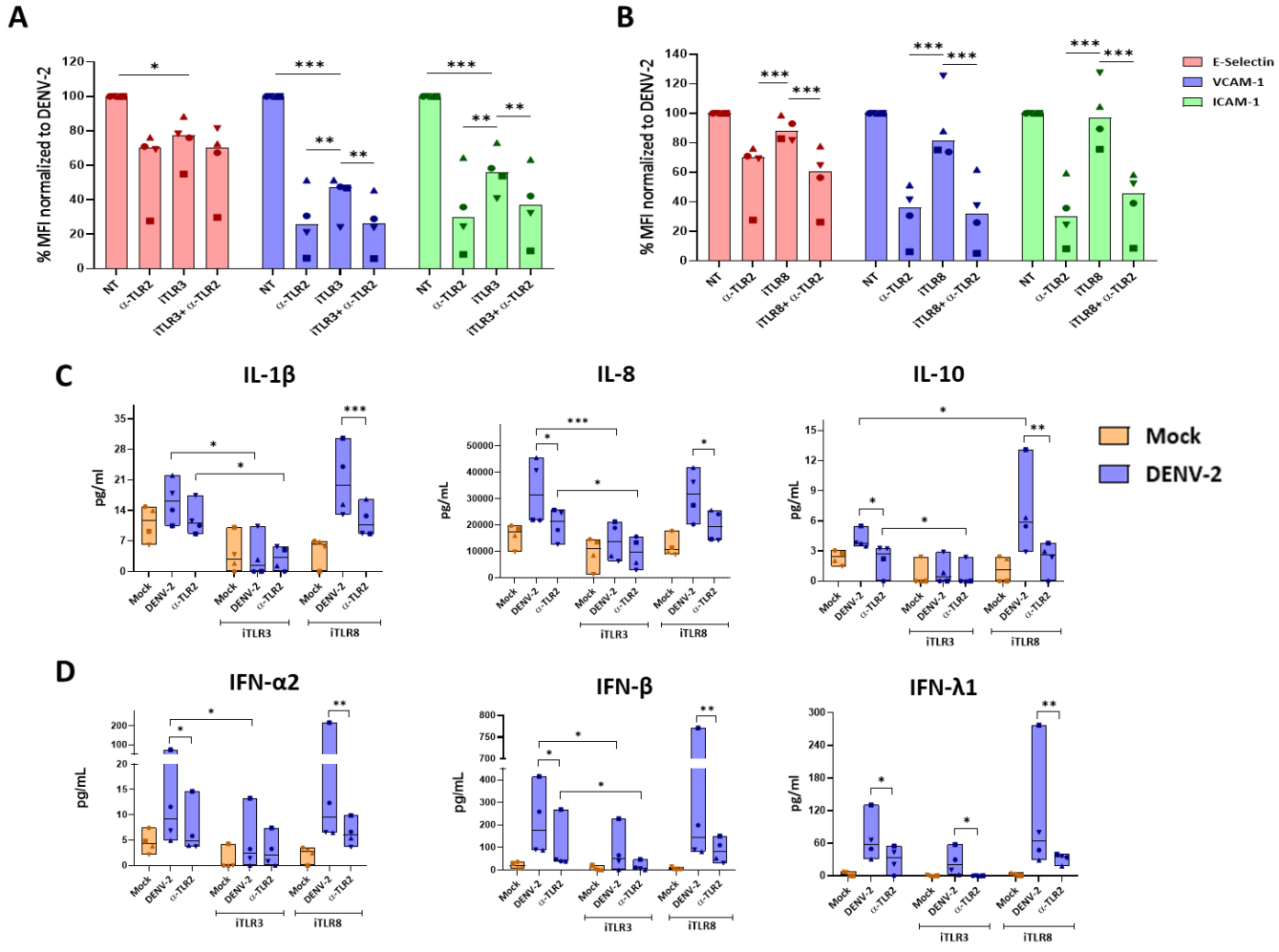


Figure 4. TLR3 contributes to TLR2 mediated immune response.

(A, B) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with α-TLR2, iTLR3 (50 μM) and/or iTLR8 (CU-CPT9a,

10 µM), and infected with DENV-2 for 48 hours. Expression was assessed by flow cytometry and data was normalized against HUVECs stimulated with supernatants from DENV-2 infected monocytes alone (n=4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05). (C-D) Concentration of cytokines produced by monocytes treated with α-TLR2, iTLR3 (50 µM) and/or iTLR8 (CU-CPT9a, 10 µM), and infected with DENV-2 for 48 hours (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05).

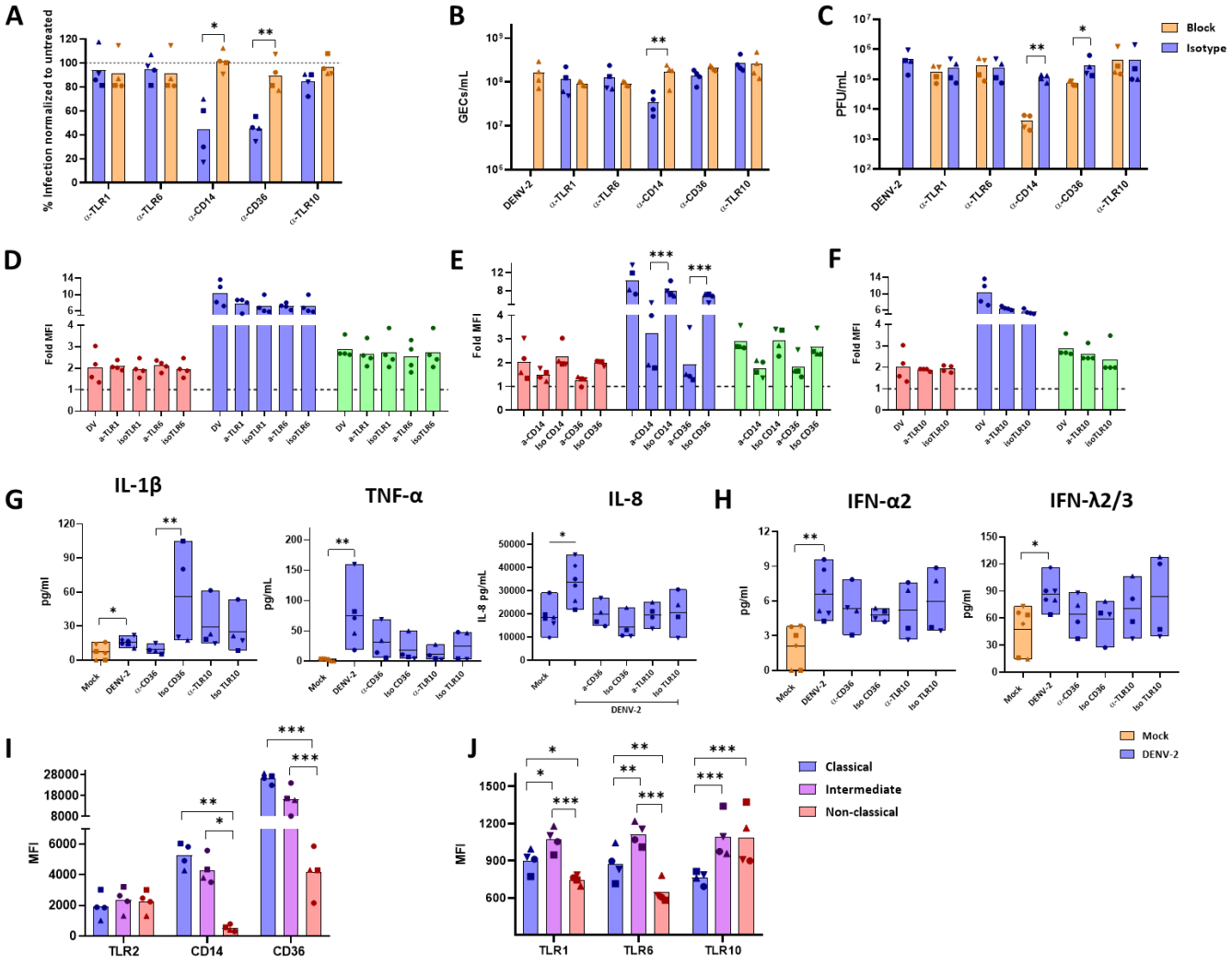


Figure 5. Blocking TLR2 co-receptors CD14 or CD36, but not TLR6 and TLR10, decreases DENV-2 replication and immune response.

(A) Percentage of E+ cells, (B) GECs and (C) infectious titer (plaque-forming unit, PFU) in supernatants in monocytes treated with α-TLR1, α-TLR6, α-TLR10, α-CD14, α-CD36 or matching control isotypes, and infected with DENV-2 for 48 hours (n= 4 different donors). Two-way ANOVA

with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05). MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with α-TLR1, α-TLR6 (D), α-CD14, α-CD36 (E), α-TLR10 (F) or matching control isotypes, and infected with DENV-2 for 48 hours. Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with supernatants from mock-infected monocytes (n=4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05). (G, H) Concentration of cytokines (pg/ml) produced by monocytes treated with α-TLR1, α-TLR6, α-TLR10, CD14, CD36 or matching control isotypes, and infected with DENV-2 for 48 hours (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05). MFI of TLR2, CD14, CD36 (I), TLR1, TLR6 and TLR10 (J) expression in classical, intermediate and non-classical monocyte subsets (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***p < 0.001, *p < 0.05).

Monocytes are a heterogeneous cell population that comprises three subsets (classical [CM], intermediate [IM] and non-classical monocytes [NM]), which express different receptors and have with different functional properties [26,27]. To test whether TLR2 and TLR2 co-receptors could have a differential role in DENV infection of monocyte subsets, we first evaluated expression of TLR2 axis in basal conditions by flow cytometry. Gating strategy of TLR2 axis expression analysis in monocyte subsets is showed in Supplementary Figure 9. Expression of TLR2 was similar among monocytes subsets. On the contrary, expression of CD14, CD36, TLR1 and TLR6 were higher in CM and IM compared to NM. Interestingly, expression of TLR10 was higher on NM and IM compared to CM subset (Figure 5H and I). In conclusion, TLR2 axis is differentially expressed among monocyte subset in basal conditions.

TLR2 mediates DENV-2 infection and inflammatory response only in CD16- monocytes

We have previously shown that CM of DENV infected patients express higher levels of TLR2 compared to other subsets, which was associated with development of severe dengue [24]. To test if TLR2 has a differential role in the induction of immune response of monocyte subsets, we separated CD16- and CD16+ monocytes by electromagnetic sorting, cultured them under TLR2 blocking conditions and then infected them with DENV-2. After 48 hours of infection, immune response were evaluated (Figure 6A). Gating strategy for separation

of monocyte subsets, morphology of cells after culture and purity are shown in Supplemental Figure 10.

By measuring TEER in ECs, we observed that inflammatory mediators produced by DENV-2 infected CD16- and CD16+ monocytes Increased endothelial permeability, which was completely abrogated by TLR2 blocking in CD16- monocytes (Figure 6A). However, no effect in endothelial permeability was observed with TLR2 blocking in CD16+ monocytes (Figure 6B). CD16- produced higher levels of inflammatory cytokines, including IL-1β, IL-6, TNF-α, IL-8 and IL-10 (Figure 6C), compared to CD16+ monocytes. In the same way CD16- monocytes produced higher levels of antiviral IFNs such as IFN-α2, IFN-β, IFN-λ1 and IFN-λ2/3 (Figure 6D), compared to CD16+ monocytes. Also, TLR2 blocking decreased Inflammatory and antiviral response of monocytes but only on the case of CD16- monocytes, whereas inhibition of TLR2 had no effect on DENV-2 infected CD16+ monocytes (Figure 6C and D). To check the specificity of TLR2-driven responses observed among monocyte subsets, the cytokine response of monocytes CD16- and CD16+ monocytes stimulated with PAM3 for 24 hours was compared. Contrary to the effect observed with DENV-2 infection on monocyte subsets, after PAM3 stimulation CD16+ monocytes produced higher levels IL-1β, IL-6, TNF-α, GM-CSF, IL-10, IFN-α, IFN-β and IFN-λ1 (Supplementary Figure 11), suggesting activation of more than one PRR during DENV infection.

We also showed previously, that in DENV-infected patients, CM and IM predominantly expressed NS3 viral antigen compared to low proportion of NS3 positive NM, suggesting a differential role of TLR2 for DENV infection in monocytes subsets [24]. To test this hypothesis, we challenged sorted monocyte subsets with DENV-2 under TLR2 blocking conditions (Figure 7A). After blocking TLR2, a significant decrease in GECs and viral titer in supernatants was observed in DENV-2 infected CD16- monocytes, whereas no effect was observed in DENV-2 replication in CD16+ monocytes (Figure 7B and C). In line with the increased immune activation of CD16- monocytes after DENV-2 infection, higher levels of viral infectious particles were produced by CD16- monocytes without blocking conditions, suggesting a differential susceptibility of monocyte subsets to DENV Infection (Figure 7B and C). Altogether, these results indicate that TLR2 drives DENV-2 infection and immune response in CD16- monocytes, whereas infection of CD16+ monocytes by DENV-2 may be mediated by different receptors.

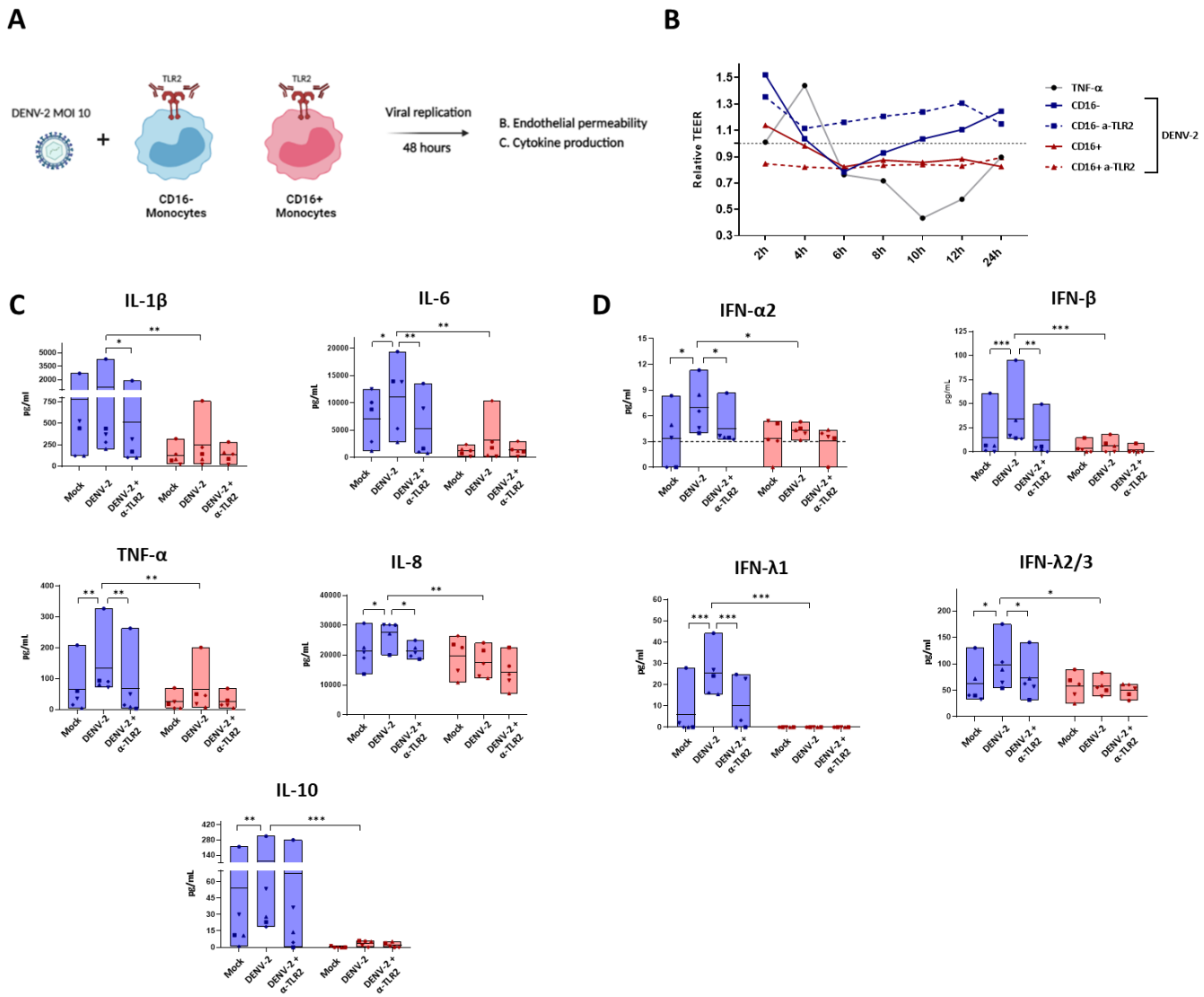


Figure 6. Blocking TLR2 decreases inflammatory and antiviral response induced by DENV-2 in CD16- monocytes but not in CD16+ monocytes.

(A) Representative scheme showing experimental strategy. (B) TEER of HUVECs after stimulation with supernatants from monocytes subsets treated with α-TLR2 or matching control isotype and infected with DENV-2 for 48 hours. Data of TEER was normalized against control HUVECs stimulated with supernatants from each mock-infected monocyte subset (n= 3 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (* $p < 0.05$). (B-J) Concentration (pg/ml) of cytokines released by monocytes treated with α-TLR2, matching control isotype or C29 prior to infection with DENV-2 (n= 4 different donors). Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$).

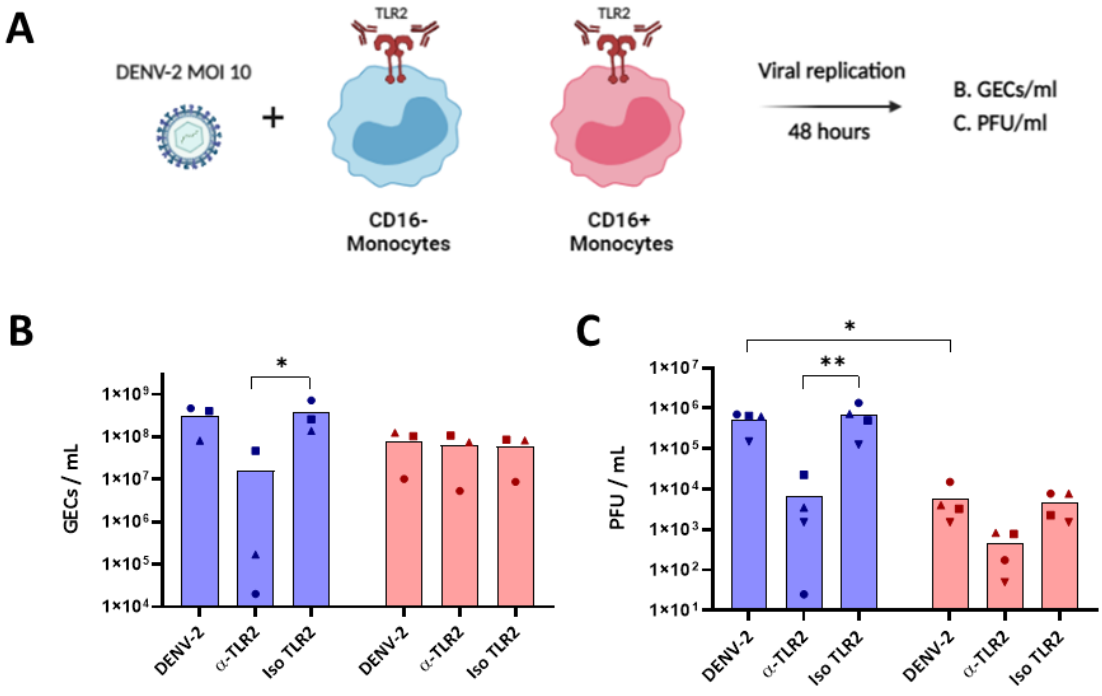


Figure 7. Blocking TLR2 decreases DENV-2 replication only in CD16- monocytes but not in CD16+ monocytes.

(A) Representative scheme showing experimental strategy. (B) GECs (n=3 different donors) and (C) infectious titer (plaque-forming unit, PFU) (n=4 different donors) in supernatants of monocytes subsets treated with α-TLR2 or matching control isotype prior to infection with DENV-2 at an MOI of 10 for 48 hours. Two-way ANOVA with a Dunnet post-hoc test was used to determine significance with a 95% confidence interval (***$p < 0.001$, *$p < 0.05$).

DISCUSSION

By separating monocyte subsets from PBMCs, we demonstrated that DENV-2 infection relies on TLR2 axis only in CM, while infection of IM and NM is mediated by other type of receptors. This resulted in broader immune response of CM after DENV-2 infection, evidenced by higher production of inflammatory cytokines and antiviral IFN-I/III. Functional analysis revealed that CD14 and CD36 together with TLR2, mediate immune response of monocytes and subsequent EC activation, which could explain differential usage of TLR2 axis by subsets since TLR2 co-receptors were differently expressed among them. Finally, TLR2-mediated inflammatory and antiviral response depended on NF-κB, although TLR3 contributed to the initial response triggered by DENV-2 and TLR2 axis

interaction. Altogether, our data provides evidence into the dual role of TLR2 axis for DENV-2 replication in monocyte subsets and its contribution to inflammatory and antiviral response.

Using blocking antibodies and the chemical inhibitor C29, we showed that TLR2 controls immune response of monocytes upon DENV-2 infection, which altered homeostasis of ECs. Activation and increased permeability of ECs evidenced endothelial dysfunction which is the hallmark of severe dengue [1]. Our study showed that ECs dysfunction was controlled by TLR2 in total and CM, highlighting its role in pathogenesis of severe manifestations. ECs dysfunction driven by TLR2 may be caused by the inflammatory mediators we found increased in monocytes cultures after DENV infection, as it has been described elsewhere using DENV-infected patient´s serum [42] and supernatants from DENV-infected monocytes [18,19]. Importantly, TLR2 mediated immune response, including production of antiviral IFN-I, were mainly dependent on NF-κB activation. Other studies have shown that DENV-2 activates signaling pathways involving NF-κB leading to TNF-α, iNOS and macrophage inhibitory factor expression [43,44]. These results highlight the role of NF-κB mediating inflammatory response during DENV infection, which points out this molecule as candidate for therapeutical target in DENV infected patients.

Notably, we found that TLR2 also mediated production of IFN-I and IFN-III upon DENV-2 infection. Even though TLR2 was originally thought to elicit proinflammatory but not IFN-I response, recent studies have demonstrated that viral ligands can activate TLR2 and induce production of IFN-I [45], which is mediated by TRAM signaling [36]. Our study highlights the role of TLR2 in the antiviral response against DENV infection, especially with production of IFN-III which to our knowledge little is known in DENV pathogenesis. Furthermore, we found that TLR3 contributed to the immune response initiated by TLR2 and DENV-2 interaction. This sequential engagement of TLRs could be mediating IFN-I/III expression by monocytes upon DENV infection, since TLR3 senses DENV replication and prime host cells to produce IFN-I [22,23]. On the other hand, TLR8, which senses ssRNA produced during DENV infection and mediate immune response of DCs [46], did not have a role in sensing DENV in monocytes. This finding was different from reported previously using TLR7/TLR8 agonist in macaques [46]. Probably this was due to an important role of TLR7 in sensing DENV which could not be evaluated in this study. In the same line, we

could not test the contribution of RIG-I/MDA5 in TLR2-mediated immune response by DENV-2 which is a limitation of our study. Similar results were observed with TLR10, an anti-inflammatory TLR that can form heterodimers with TLR2, but did not regulate immune response of DENV-2 infected monocytes [41]. However, TLR10 expression was higher in NM, and may be important for regulation of immune response that were not possible to be found with this study. Overall, a critical role of TLR2 and TLR3 for induction of immune response during DENV-2 infection, including inflammatory cytokines and antiviral IFN-I/III, is described here. Sequential activation of TLR2 and TLR3 may be important for monocyte defense against DENV replication, as it has been described for herpes simplex virus (HSV) infection [47].

In monocytes and macrophages, DENV-2 entry is mediated by heparan sulfate glycosaminoglycans and mannose receptor [6,7], whereas DC-SIGN is important for infection of dendritic cells (DCs) [8]. We have provided evidence showing TLR2 is an important DENV receptor in PBMCs [24] and monocytes (this study). Inhibition of TLR2 with antibodies or chemical inhibitor C29 strongly inhibited DENV-2 replication in monocytes. Further, TLR2 co-receptors CD14 and CD36 contributed to viral infection mediated by TLR2, as it has been described for other type of TLR2 ligands in which CD14 and CD36 promote their loading onto TLR2 [40,48]. In fact, LPS suppressed DENV infection of primary human monocytes/macrophages when it was added to the culture before infection [49]. However, Chen et al. [49] failed to replicate the results using specific α-CD14 antibodies, suggesting that other receptors close to CD14 also mediate DENV infection, as we described in this study. Growing evidence have shown that upon stimulation with bacterial and viral ligands, TLR2 receptor is internalized and transported to endosomal compartments mediated by clathrin [36,50,51]. Here, we also showed that TLR2 mediated DENV-2 binding into target cell. In fact, in a previous study we showed that sensing of DENV by TLR2 is controlled by clathrin-mediated endocytosis (CME) [24]. Altogether, these data suggest that TLR2 could be controlling CME of DENV-2 particles as the initial step of viral replication cycle. Even though, is reasonable to propose this mechanism, more studies are needed to prove TLR2 function as a receptor, such as microscopy studies and experiments involving non-susceptible TLR2-transfected cells.

Monocytes subsets (CM, IM and NM) express different receptors and have different functional properties [26,27]. CM are major producers of proinflammatory factors, NM migrate and patrol to vasculature to respond against viral invaders [29,30], while IM show the highest inflammatory potential and can migrate to the site of infection contributing to systemic inflammation [30,52]. In line with these results, we found that CD16+ monocytes (IM and NM) produced higher levels of inflammatory cytokines and some interferons in response to TLR2 agonist. Consequently, expansion of certain subsets has been associated with some diseases [53–55] and pathogenesis of some viral infections [56–59]. DENV-2 infected patients develop an increase in IM and decrease in CM numbers [17,24,28,60], yet this has not been associated with disease severity. However, in a pediatric cohort, we reported that TLR2 sustained expression in CM, but not in other subsets, was associated with severe dengue [24]. Since we found that TLR2-mediated infection of DENV occurs only in CM but not in IM and NM, the role of monocyte subsets in pathogenesis could rely on susceptibility to DENV-2 infection and subsequent inflammatory response. In fact, we found that CM are more susceptible to DENV-2 infection compared to IM and NM, which is explained by differential expression of TLR2 co-receptors. Expansion of IM observed in DENV-2 infected patients could result from CM differentiation after viral infection, as CM can differentiate into other monocyte subsets *in vivo* [61]. Further studies are needed to describe in detail intrinsic differences in monocyte subsets for DENV resistance. Overall, pharmacological target of TLR2 in CM may serve as a promising therapeutic strategy for fighting DENV infection, as it could inhibit initial viral replication and subsequent inflammatory response that targets ECs.

TLR2 is known for recognition of bacterial ligands and subsequent inflammatory response mediated by MyD88 and NF-κB [37]. Increasing evidence have demonstrated that TLR2 can recognize viral PAMPs either present in viral particles or produced intracellularly during viral replication. Thus, TLR2 can be activated by yellow fever vaccine YF-17D virus [62] by hemagglutinin protein from measles virus [63], by envelop gp proteins from human cytomegalovirus [64], by varicella zoster virus [65], by core and NS3 proteins from hepatitis C virus (HCV) [66], among others. Whether this activation controls viral spread or is detrimental to the host is subject of discussion. HCV chronic infected patients overexpress TLR2 which is correlated with increased TNF-α levels in serum [67,68]. Also, TLR2-

mediated inflammatory response induced by HSV contributes to encephalitis in mice [47,69]. On the other hand, activation of TLR2 by vaccinia virus promotes survival and clonal expansion of CD8 T cells [70], and induce proper NK cells activation contributing to control of infection *in vivo* [71]. Therefore, balanced immune response given by TLR2 activation may depend on the type of virus and infected cell. Our data suggest that TLR2 activation in monocytes may be detrimental to the host during DENV infection. Inflammatory response of monocytes mediated by TLR2 during DENV infection activated and increased the permeability of ECs. Further, the association of sustained TLR2 expression in CM with severe dengue in patients [24], supports our claims.

In conclusion, this study provided insights into the mechanisms of TLR2 activation and signaling in monocytes during DENV-2 infection. By separating monocyte subsets from PBMCs, we described a dual role of TLR2 axis for DENV-2 replication that is different in each monocyte subsets and explains higher susceptibility of CD16- (CM) compared to CD16+ (IM and NM). Immune response triggered by TLR2 activation by DENV-2 were controlled by NF-κB, which ultimately led to activation and increased permeability of ECs. Consequently, targeting TLR2 early in the course of DENV infection could mitigate the pathogenesis of severe disease.

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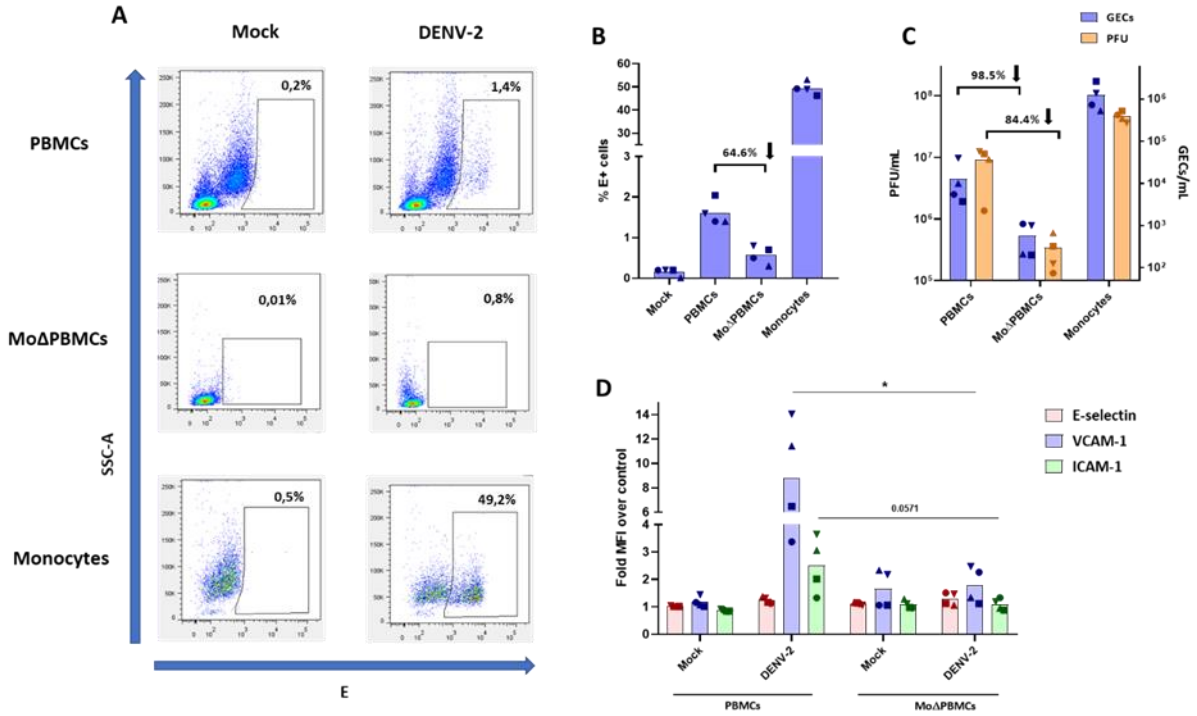
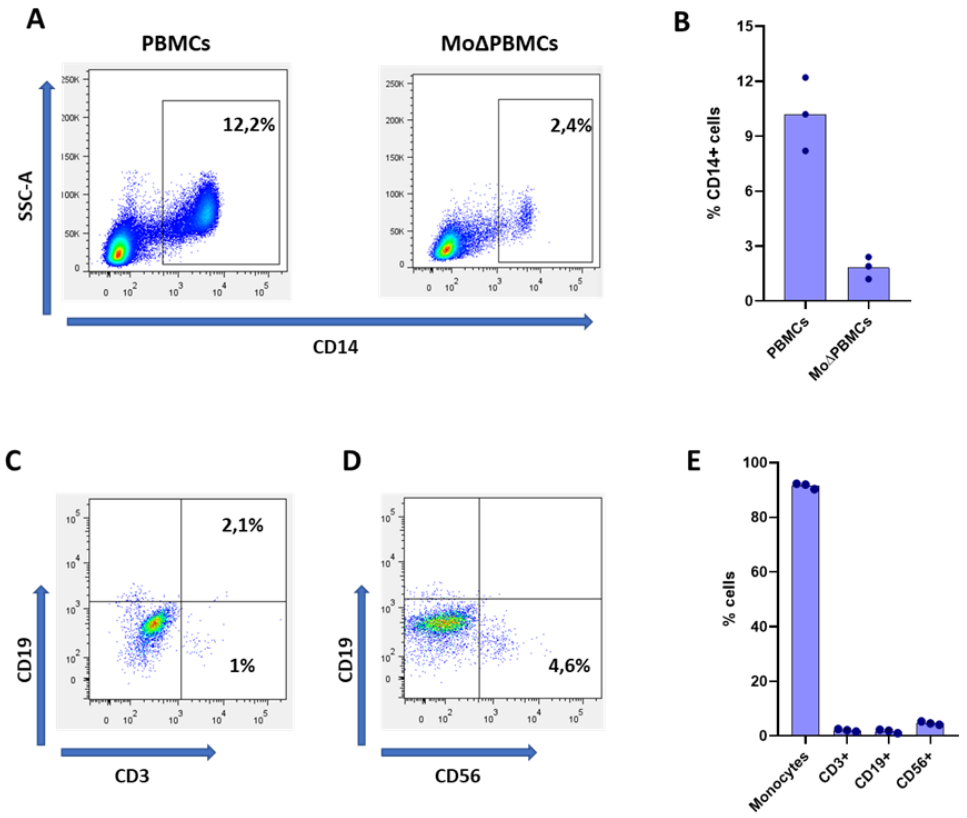
SUPPLEMANTARY MATERIAL



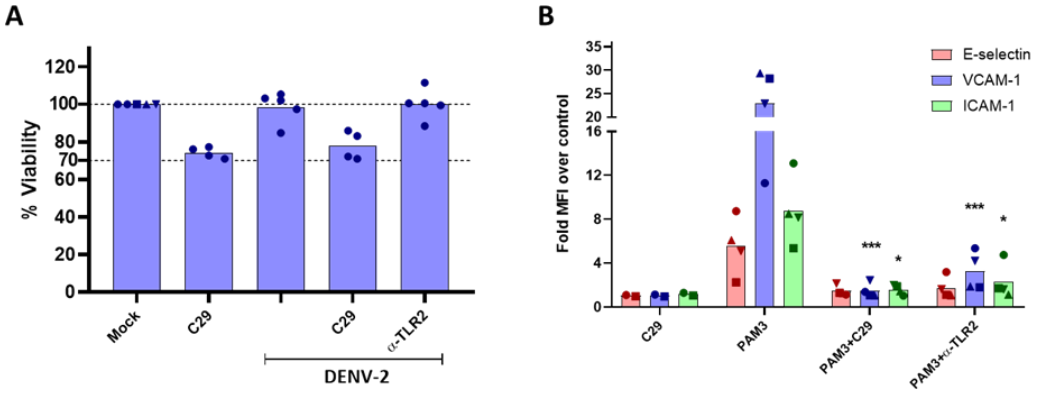
Supplementary Figure 1. Depletion of monocytes from PBMCs reduces DENV-2 replication and induced inflammatory response.

(A) Representative scatter plots of mock and DENV-2 infected PBMCs, MoΔPBMCs and monocytes with MOI=10 for 48 hours, assessed by the detection of viral E+ cells using flow cytometry. (B) Percentage of E+ cells, (C) genome equivalent copies (GEC) and infectious titer (plaque-forming unit, PFU) assessed in PBMCs, MoΔPBMCs and Monocytes after 48 hours of infection with DENV-2 at an MOI=10 (n= 4 different donors). (D) MFI of E-Selectin, VCAM-1 and ICAM-1 expression determined in HUVECs after 6 hours of stimulation with supernatants from mock and DENV-2 infected PBMCs and MoΔPBMCs. Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=4 different donors). Mann-Whitney test was used to determine significance with a 95% confidence interval (*$p<0.05$).



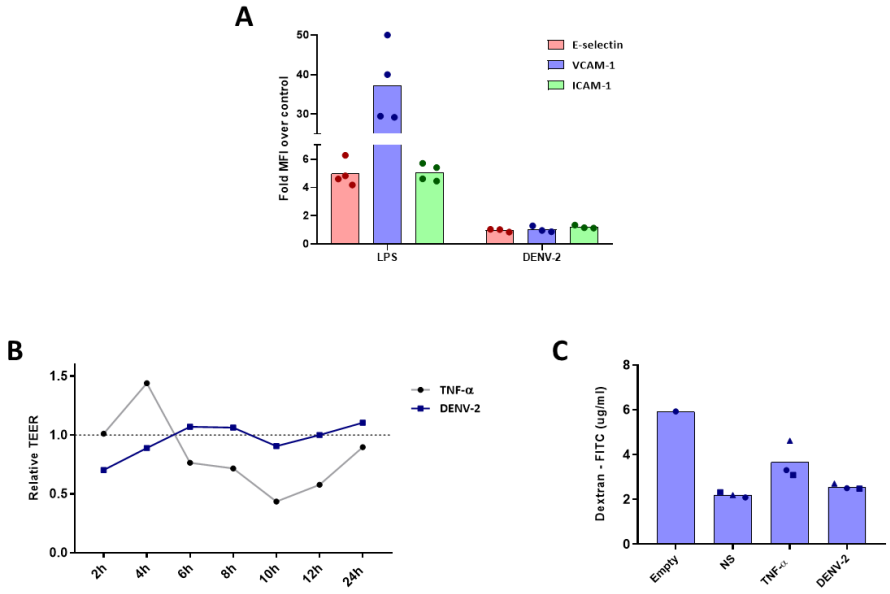
Supplemental Figure 2. Purification and depletion of monocytes from PBMCs.

(A) Representative scatter plots of monocyte depleted PBMCs (MoΔPBMCs), which efficiency was assessed by determination of CD14+ cells using flow cytometry. (B) Efficiency of monocyte depletion using positive selection (n=3 different donors). (C, D) Representative scatter plots of purified monocytes, which efficiency was assessed by determination of CD19+, CD3+ and CD56+ cells using flow cytometry. (E) Efficiency of monocyte purification using negative selection (n= 3 different donors).



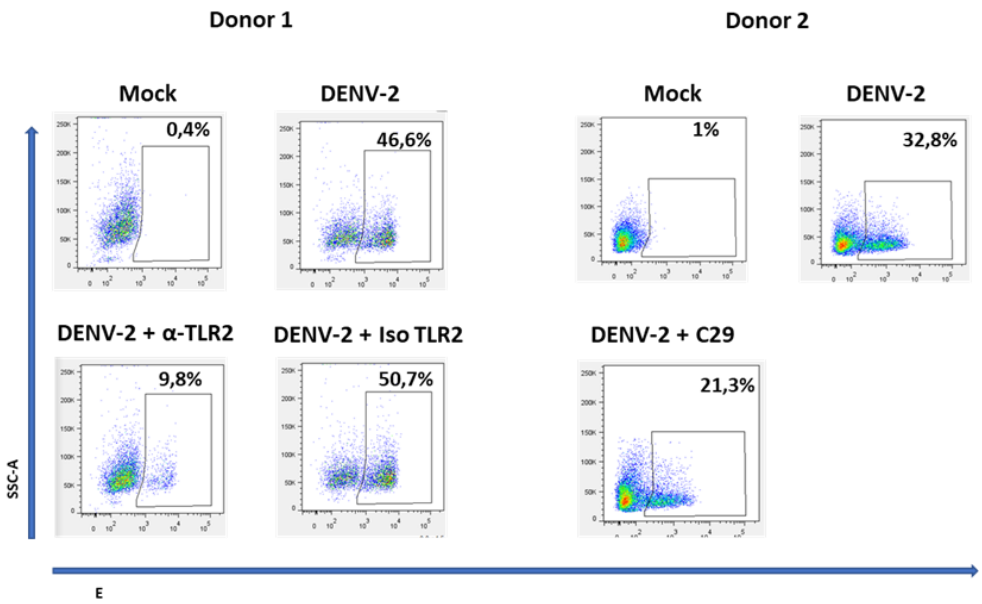
Supplemental Figure 3. TLR2 antibody and C29 inhibitor are not cytotoxic and efficiently block TLR2 function

(A) Monocyte viability after treatment with C29 (100nM) and α-TLR2 (7.5µg/ml) in mock and DENV-2 infected monocytes. Viability was determined with MTS assay and normalized against untreated and mock infected monocytes. (n=4 individual donors). (B) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with PAM3 (100 ng/ml), C29 (100nM), or α-TLR2 (7.5µg/ml). Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=4 different donors).



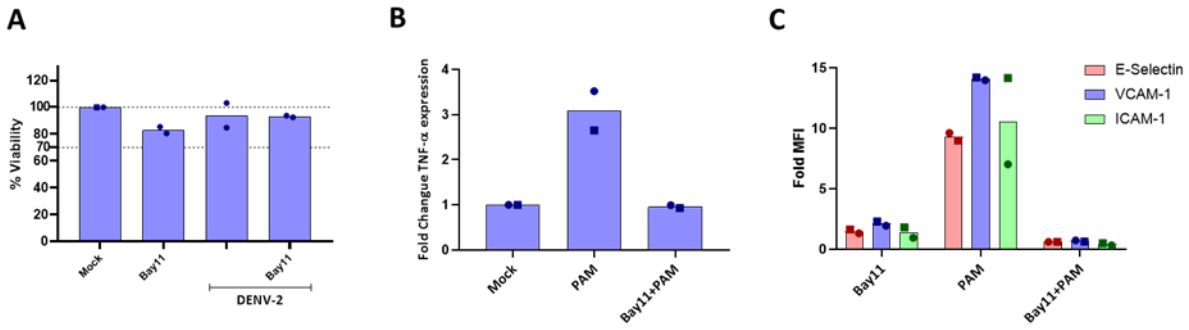
Supplemental Figure 4. Challenge with DENV-2 does not activate HUVECs or induce increased permeability

(A) MFI of E-Selectin, VCAM-1 and ICAM-1 expression determined in HUVECs after stimulation with LPS (1µg/ml) or DENV-2 with an MOI of 5 for 6 hours. Expression was assessed by flow cytometry and data was normalized against unstimulated HUVECs (n=3). (B) Transendothelial electrical resistance (TEER) of HUVECs after stimulation with DENV-2 at an MOI of 5. (D) Dextran-FITC migration across transwell was measured at 24 hours after stimulation with DENV-2 at an MOI of 5. Data of TEER was normalized against unstimulated HUVECs, and Dextran-FITC concentration was obtained using a standard curve (n= 3).



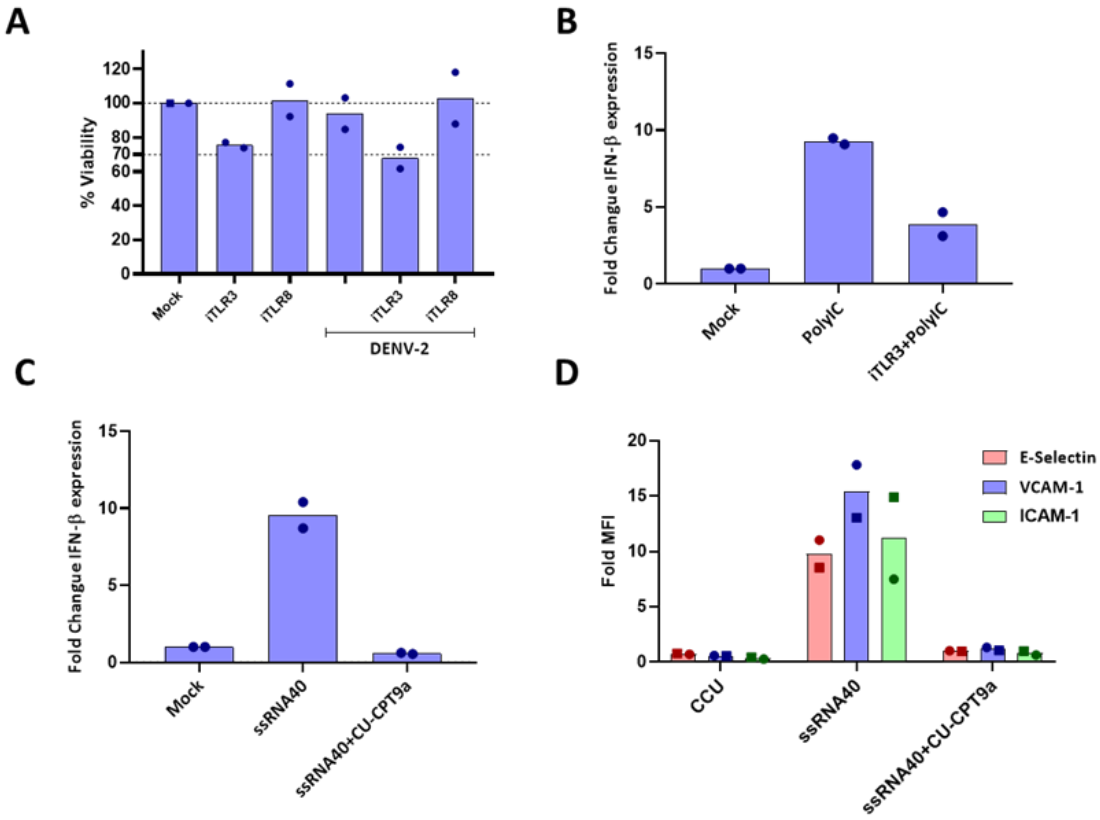
Supplemental Figure 5. Blocking TLR2 decreases DENV-2 infection in monocytes.

Representative scatter plots of mock and DENV-2 infected Monocytes from two of four donors assessed by the detection of viral E+ cells using flow cytometry.



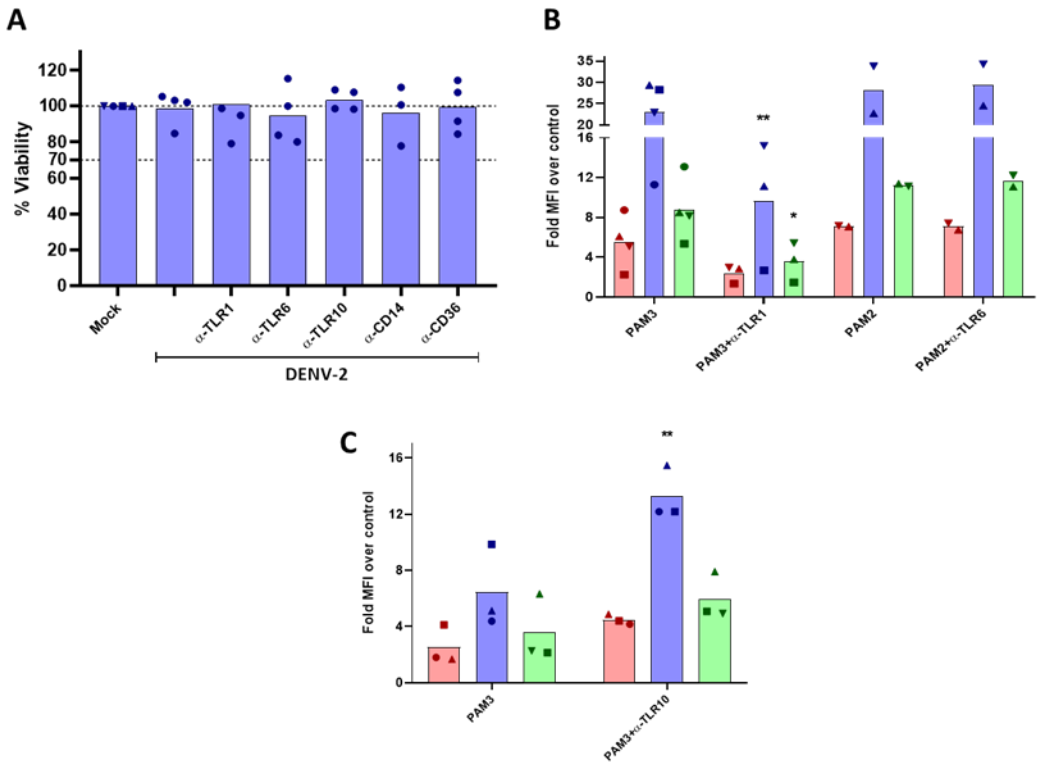
Supplemental Figure 6. Viability and inhibition of PAM responses under Bay11 treatment

(A) Monocyte viability after treatment with Bay11 (2μM) in mock and DENV-2 infected monocytes. Viability was determined with MTS assay and normalized against non-treated and mock infected monocytes. (n=2 individual donors). (B) mRNA expression of TNF-α in monocytes after treatment with Bay11 (2μM) and PAM3 (100ng/ml) for 12 hours. Data was normalized with expression of constitutive gene GADPH and expression in mock-infected monocytes (n=2 different donors). (C) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated Bay11 (2μM) and PAM3 (100ng/ml) for 24 hours. Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=2 different donors).



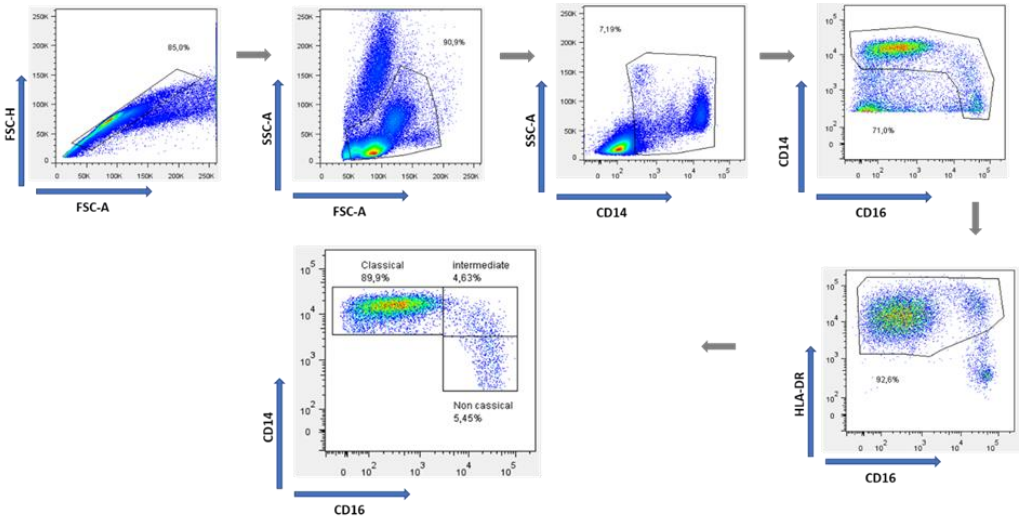
Supplemental Figure 7. Viability and inhibition of PAM responses under iTLR3 and iTLR8 treatment

(A) Monocyte viability after treatment with iTLR3 (50µM) and iTLR8 (CU-CPT9a, 10µM) in mock and DENV-2 infected monocytes. Viability was determined with MTS assay and normalized against non-treated and mock infected monocytes. (n=2 individual donors). (B, C) mRNA expression of IFN-β in monocytes after treatment with iTLR3 (50µM) or iTLR8 (CU-CPT9a, 10µM), and Poly I:C (25µg/ml) or ssRNA40 (1µg/ml) for 12 hours. Data was normalized with expression of constitutive gene GADPH and expression in mock-infected monocytes (n=2 different donors). (D) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with iTLR8 (CU-CPT9a, 10µM) and ssRNA40 (1µg/ml) for 24 hours. Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=2 different donors).



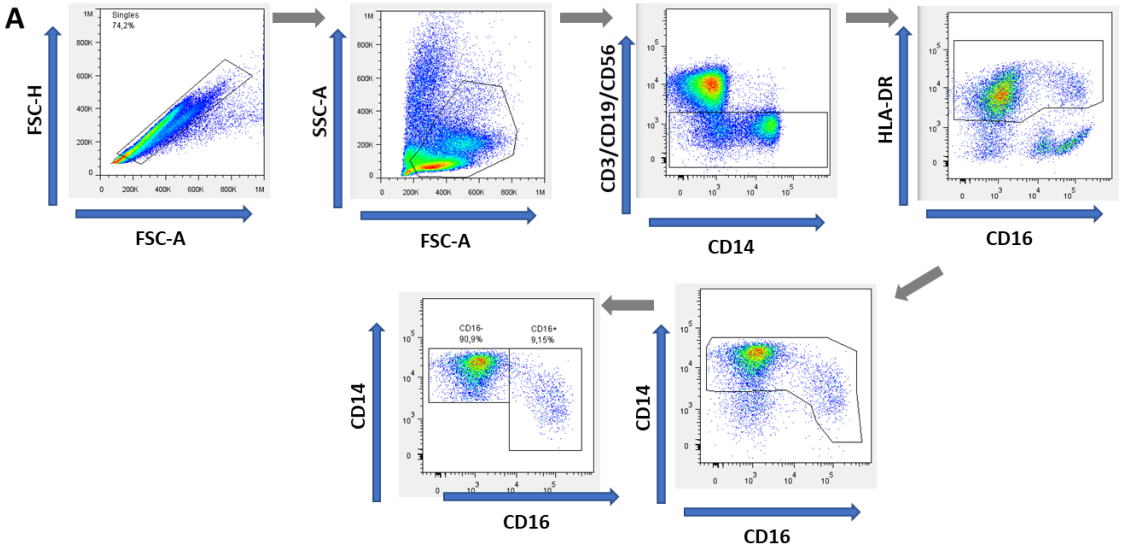
Supplemental Figure 8. Viability and inhibition of PAM responses under α-TLR1, α-TLR6 and α-TLR10

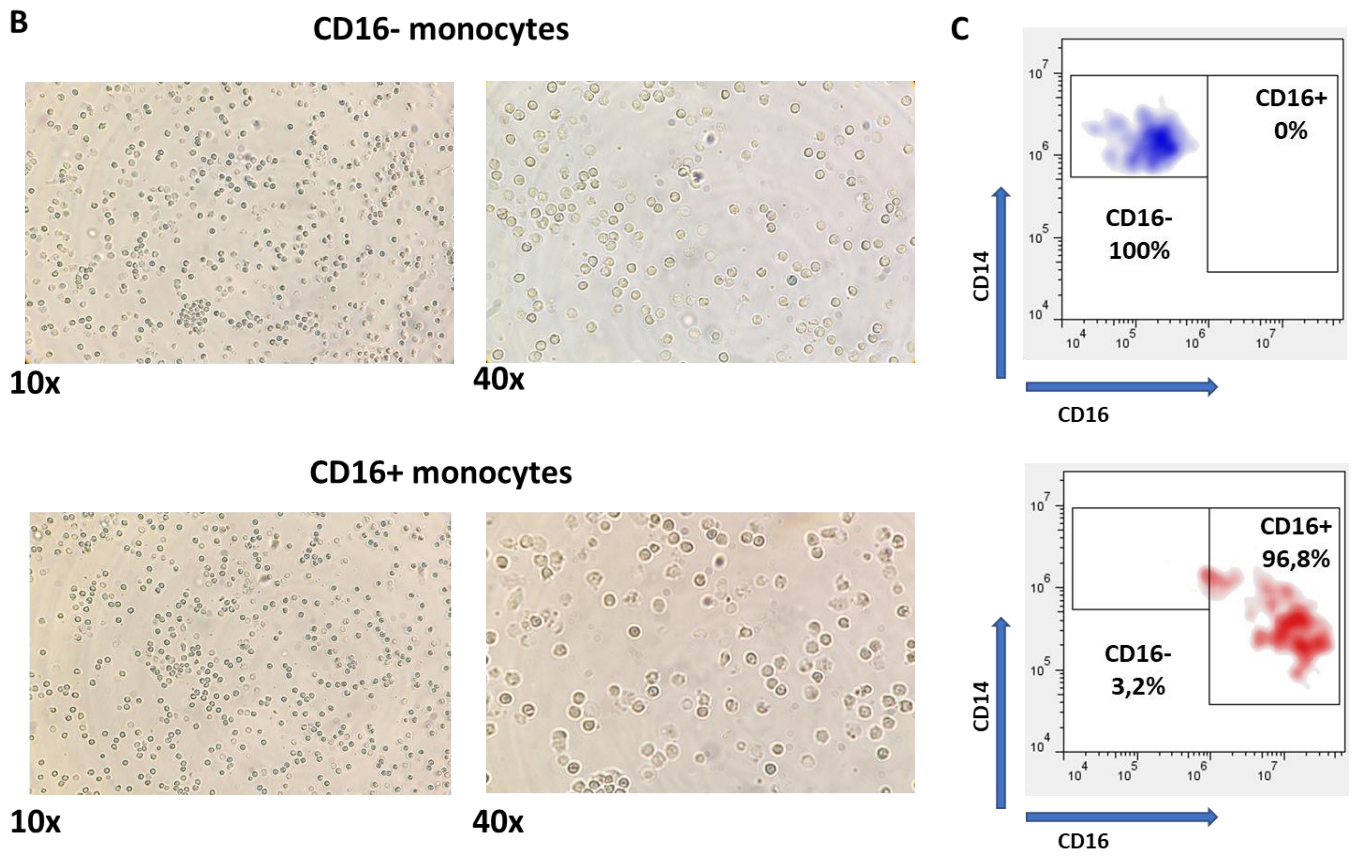
(A) Monocyte viability after treatment with α-TLR1 (7.5µg/ml), α-TLR6 (7.5µg/ml), α-TLR10 (3µg/ml), α-CD14 (3µg/ml) or α-CD36 (2.5µg/ml) in DENV-2 infected monocytes. Viability was determined with MTS assay and normalized against non-treated and mock infected monocytes. (n=4 different donors). (B) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with PAM2 (100ng/ml) and α-TLR1 (7.5µg/ml) or α-TLR6 (7.5µg/ml). Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=3 different donors). (C) MFI of E-Selectin, VCAM-1 and ICAM-1 expression in HUVECs after stimulation for 6 hours with supernatants from monocytes treated with PAM3 (10ng/ml) and α-TLR10 (3µg/ml). Expression was assessed by flow cytometry and data was normalized against control HUVECs stimulated with RPMI alone (n=3 different donors).



Supplemental Figure 9. Gating strategy for evaluation of the expression of TLR2 axis in monocyte subsets

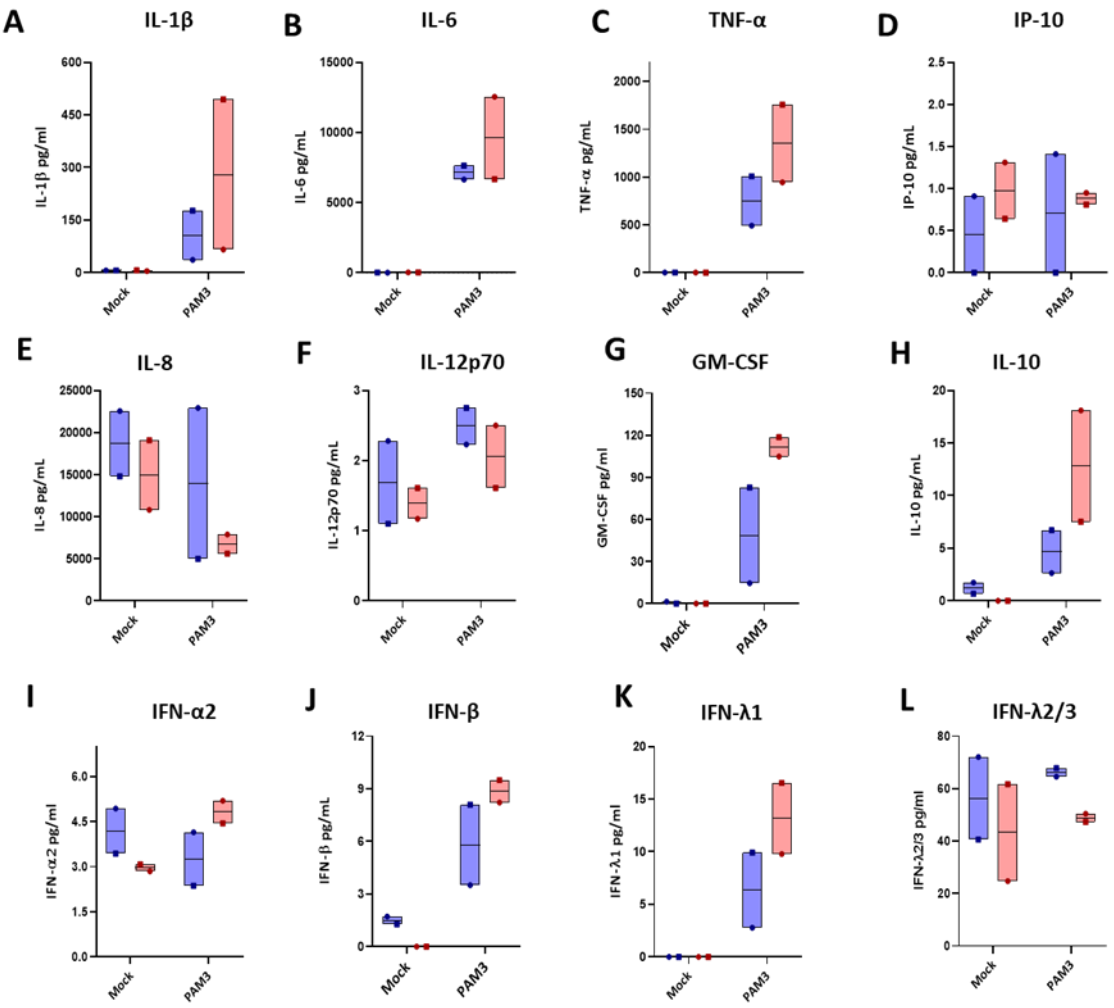
Representative scatter plots of PBMCs stained with anti-CD14-eFluor 450 and anti-CD16-APC, anti-HLA-DR-FITC. Monocyte subsets were gated first using singles inclusion, based on morphology and then based on CD14, CD16 and HLA-DR expression. Classical (CD14+CD16-), intermediate (CD14+CD16+) and non-classical monocytes (CD14dimCD16+) were further analyzed for TLR2, CD14, CD36, TLR1, TLR6 and TLR10 expression.





Supplemental Figure 10. Gating strategy for monocyte subsets sorting

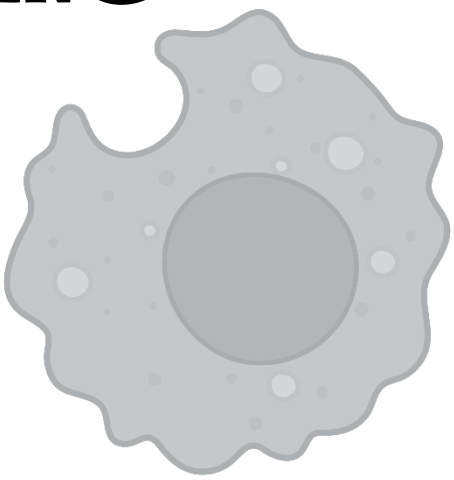
(A) Representative scatter plots of PBMCs stained with anti-CD14-eFluor 450 and anti-CD16-APC, anti-HLA-DR-FITC. Monocyte subsets were gated first using singles inclusion, based on morphology and then based on CD14, CD16 and HLA-DR expression. Classical (CD16-), and intermediate/non-classical monocytes (CD16+) were further analyzed. (B) Morphology of monocyte subsets examined under light microscope with 40X and 10X. (C) Representative scatter plots of monocytes subsets which purity was assessed by determination of CD14+ and CD16+ cells using flow cytometry.



Supplemental Figure 11. Production of cytokines by monocytes subsets after PAM3 stimulation

(A-L) Concentration of cytokines produced by monocytes subsets treated with PAM3 (100ng/ml) for 24 hours (n= 2 different donors).

CHAPTER 3



Regulation of innate immune response in macrophages differentiated in the presence of vitamin D and infected with dengue virus 2

Jorge Andrés Castillo[a,c], Diana M Giraldo[a], Juan C Hernandez[b], Jolanda M Smit[c], Izabela A Rodenhuis-Zybert[c] and Silvio Urcuqui-Inchima[a,*]

a. Grupo de Inmunovirología. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No 52-21, Medellín, (Antioquia), Colombia

b. Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, (Antioquia), Colombia

c. Department of Medical Microbiology and infection Prevention, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands

* Corresponding Author: silvio.urcuqui@udea.edu.co

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ABSTRACT

A dysregulated or exacerbated inflammatory response is thought to be the key driver of the pathogenesis of severe disease caused by the mosquito-borne dengue virus (DENV). Compounds that restrict virus replication and modulate the inflammatory response could thus serve as promising therapeutics mitigating the disease pathogenesis. We and others have previously shown that macrophages, which are important cellular targets for DENV replication, differentiated in the presence of bioactive vitamin D (VitD3) are less permissive to viral replication, and produce lower levels of pro-inflammatory cytokines. Therefore, we here evaluated the extent and kinetics of innate immune response of DENV-2 infected monocytes differentiated into macrophages in the presence (D3-MDMs) or absence of VitD3 (MDMs). We found that D3-MDMs expressed lower levels of RIG I, Toll-like receptor (TLR)3, and TLR7, as well as higher levels of SOCS-1 in response to DENV-2 infection. D3-MDMs produced lower levels of reactive oxygen species, related to a lower expression of TLR9. Moreover, although VitD3 treatment did not modulate either the expression of IFN-α or IFN-β, higher expression of protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase 1 (OAS1) mRNA were found in D3-MDMs. Importantly, the observed effects were independent of reduced infection, highlighting the intrinsic differences between D3-MDMs and MDMs. Taken together, our results suggest that differentiation of MDMs in the presence of VitD3 modulates innate immunity in response to DENV-2 infection.

KEYWORDS

Dengue, vitamin D, macrophages, innate immunity, Toll-like receptors, type I interferon

INTRODUCTION

With an estimated 390 million infections every year, the dengue virus (DENV) is one of the most frequently transmitted arboviruses in tropical and subtropical countries [1]. Although close to 75% of DENV infection cases are asymptomatic, the clinically apparent infections can range from a mild flu-like condition (dengue) to a life-threatening (severe dengue) disease characterized by hemorrhages and hypovolemic shock [2]. To date, predicting the disease outcome is impossible and there is no specific treatment available. In addition, Denvaxia, the only available vaccine approved in 11 countries, is only partially effective [3]. The availability of an effective therapeutic strategy is, therefore, an important goal, considering that almost 50% of the human population lives in zones with a risk of transmission [1]. The mechanism underlying the aberrant inflammatory response is not fully understood, however, the infection-induced excessive production of proinflammatory cytokines is known to promote endothelial permeability and damage to endothelial integrity, thought to be responsible for severe disease [4]. Consequently, ameliorating exacerbated inflammation is considered a key aim of therapeutic strategies.

Immune sentinels such as monocytes, dendritic cells (DCs), and macrophages sense DENV through an array of PRRs including retinoic acid-inducible gene-I-like receptors (RLRs) such as RIG-I and MDA5 [5], and Toll-like receptors (TLRs) [6,7]. Recognition of molecular patterns expressed in DENV virus or replication intermediates by 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. Importantly, DENV can also replicate in immune cells and has evolved mechanisms to alter or evade innate immune response (reviewed in [8]. Indeed, aberrant inflammation characterized by inflammatory cytokines predominating over the antiviral response is thought to lead to endothelial permeability underlying severe disease [9,10].

Given the importance of the inflammatory response in the pathogenesis of DENV infection, the use of immune-regulatory compounds such as vitamin D (VitD3) could be useful as an alternative therapeutic strategy for DENV-infected patients. In fact, this hormone shows several immunomodulatory effects in various cells of the immune system, including antigen-presenting cells [11]. Furthermore, VitD3 has antiviral activity that impairs the

replication of several viruses including the hepatitis C virus (HCV), the human immunodeficiency virus (HIV), and the respiratory syncytial virus (RSV) and DENV [12–14]. *In vitro* studies have shown that VitD3 decreases DENV infection and replication and the production of proinflammatory cytokines [15,16]. We have previously demonstrated that monocyte-derived macrophages (MDMs) differentiated in the presence of VitD3 (D3-MDMs) are more resistant to DENV-2 infection, due to a decreased expression of mannose receptor (MR) and thus reduced viral uptake [17]. Likewise, the production of proinflammatory cytokines in response to DENV infection was decreased in D3-MDMs [17]. In addition, we reported a decrease in the susceptibility to DENV-2 infection and the production of proinflammatory cytokines by MDMs obtained from healthy individuals who received a high-dose oral supplementation of VitD3 for 10 days [18]. Indeed, VitD3 has been shown to downregulate the expression of several TLRs in monocytes and thus contribute to the amelioration of the inflammatory response triggered by IFN-γ, LPS, or lipoteichoic acid [19,20]. Also, it has been shown that VitD3 suppressed the inflammatory response of LPS in mice by up-regulating the expression of the suppressor of cytokine signaling 1 (SOCS-1) [21], an important regulator of inflammatory response [22]. This body of evidence demonstrates that VitD3 has wide immunomodulatory and antiviral activities against DENV infection.

Consequently, the present study aimed to evaluate the effect of VitD3 on the regulation of the innate immune response during DENV infection. To do so, we assessed temporal changes in the expression of the most studied PRRs, SOCS-1, IFN I, and IFN-stimulated genes (ISGs) in MDMs and D3-MDMs infected with DENV-2. Altogether, the results of this study increase our insights into the mechanisms underlying the antiviral and anti-inflammatory effects of VitD3 in DENV-2 infected macrophages.

MATERIALS AND METHODS

Ethics statement

The protocols for individual enrollment and sample collection were approved by the Committee of Bioethics Research of Sede de Investigación Universitaria, Universidad de

Antioquia (Medellín, Colombia), and inclusion was preceded by a signed informed consent form, according to the principles expressed in the Declaration of Helsinki.

Cells and reagents

The mosquito C6/36 HT cell line was obtained from the American Type Culture Collection (ATCC) and cultured in Leibovitz L-15 medium (Sigma Aldrich, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, USA), 4 mM L-glutamine, 10 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma Aldrich, USA), at 34°C in an atmosphere without CO_2. BHK-21 cells, obtained from the ATCC, were maintained in D-MEM (Sigma Aldrich, USA), supplemented with 10% v/v FBS, 4 mM L-glutamine, 10 CFU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C with 5% CO_2, and used for plaque assays. Conjugated antibodies against CD14 (clone M5E2), TLR3 (clone TLR3.7), TLR4 (clone HTA 125), and TLR9 (clone eB72-1665) were purchased from eBioscience (USA).

Blood samples from healthy donors

Venous peripheral blood samples were obtained from healthy individuals aged 20–40 years who had not been previously vaccinated against yellow fever virus and were seronegative for the DENV NS1 antigen and DENV IgM/IgG, as determined by the SD BIOLINE Dengue Duo rapid test (Standard Diagnostics). All our experiments were performed with cells from at least six healthy donors.

Monocyte isolation and monocyte-derived macrophage differentiation (MDMs)

To obtain MDMs, peripheral blood mononuclear cells (PBMCs) were obtained from 50 mL of peripheral blood from healthy individuals with 2% v/v ethylenediaminetetraacetic acid, as described previously [17,18]. Briefly, the PBMCs were separated using density gradient centrifugation with Lymphoprep (STEMCELL technologies, USA) at 800 × g at room temperature for 20 min and then washed three times with phosphate-buffered saline (PBS) at 250 × g for platelet removal. The PBMCs were suspended in RPMI-1640 medium (Sigma Aldrich, USA) supplemented with 0.5% autologous heat-inactivated serum (30 min at 56°C). Monocytes were then obtained from the PBMCs by plastic adherence, as described previously [17]. Briefly, 1 × 105 cells were stained with anti-CD14-FITC (clone M5E2, BD Biosciences, USA) and analyzed by flow cytometry. The percentage of CD14+ cells was used

to seed 5 × 105 CD14+ cells into 24-well plates (Corning Incorporated Life Sciences, USA) in RPMI-1640 medium supplemented with 0.5% inactivated autologous serum and cultured at 37°C with 5% CO_2 to allow enrichment of monocytes through plastic adherence. After 3 h of adherence, non-adherent cells were removed by extensive washing with pre-warmed PBS supplemented with 0.5% FBS. Adherent cells were then cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C with 5% CO_2 for 6 days to obtain MDMs. Fresh medium with 10% FBS was replenished every 48 h. The purity of MDMs and D3-MDMs was repeatedly above 90%, as measured by the presence of contaminant cell populations, including CD19+, CD3+, and CD56+ (Supp. Fig1A, 1B and 1C) in monocytes before differentiation, and measuring CD68+ cells after 6 days of differentiation (Supp. Fig1D).

D3-MDMs differentiation in the presence of Vitamin D3

Monocytes were differentiated for 6 days in the presence of 1α,25-dihydroxyvitamin D3 (VitD3; Sigma Aldrich, USA), at a concentration of 0.1 nM which represents the physiological and therapeutical concentration [23,24], and as we have previously described [17,18]. VitD3 was replenished with fresh medium every 48 h. To evaluate the effect of VitD3 on D3-MDMs cultures, vitamin D receptor (VDR) and cytochrome P450 family 24 subfamily A member 1 (CYP24A1) gene expression were evaluated using quantitative polymerase chain reaction (qPCR). The biological activity VitD3 was determined by the transcriptional induction of VitD3 signaling targets, such as VDR and CYP24A1 [17]. The purity of D3-MDMs was repeatedly above 90%, as measured by the presence of contaminant cell populations, including CD19+, CD3+, and CD56+ (non-myeloid cells).

Virus stocks and titration

DENV-2 New Guinea C was provided by the Centers for Disease Control and Prevention (CDC, USA). Viral stocks were obtained by inoculating a monolayer of C6/36 HT cells in a 75-cm2 tissue culture flask with the virus at a multiplicity of infection (MOI) of 0.05 diluted in L-15 supplemented with 2% FBS. After 3 h of adsorption, fresh L-15 medium supplemented with 2% FBS was added, and the cells were cultured for 5 days at 34°C without CO_2. The supernatant was obtained by centrifugation at 1000 × g for 5 min to remove cellular debris and then aliquoted and stored at −70°C until use. Virus titration was performed by quantification of plaque-forming units (PFU) using a plaque assay. A total of

5 × 104 BHK-21 cells/well were cultured in 24-well plates in D-MEM supplemented with 2% v/v FBS, 4 mM L-glutamine, 10 U/mL penicillin, and 0.1 mg/mL streptomycin and incubated at 37°C with 5% CO_2 overnight. Next, cells 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, washed once with PBS and plaque medium of D-MEM containing 1.5% m/v carboxymethylcellulose sodium salt (medium viscosity, Sigma-Aldrich, USA), 3% NaCO3, and 1% HEPES (Sigma-Aldrich, USA), and 10 CFU/mL penicillin, 0.1 mg/mL streptomycin, and 1% of FBS were added to the cells. Then, the cells were incubated at 37°C with 5% CO_2 for 5 days. Next, the plaque medium was removed, and the cells were washed twice with PBS and stained with 4% m/v crystal violet solution and 3.5% v/v formaldehyde (Merck, Germany) for 30 min. After staining, cells were washed once with PBS, and the plaque count was performed manually to obtain PFU/mL.

MDMs and D3-MDMs infection with DENV

Both MDMs and D3-MDMs monolayers were challenged with DENV-2 at an MOI of 5, diluted in 300 µl of RPMI-1640 medium supplemented with 2% FBS. Two hours post-infection (hpi), cells were washed with PBS, and the medium was replenished with RPMI 10% FBS and then cultured at 37°C 5% CO_2 .At 2, 8, and 24 hpi, monolayers were harvested, and either the percentage of infection was determined by flow cytometry, or total RNA extraction was carried out and used for viral RNA quantification, while the supernatants were used for viral titration by plaque assay and for quantification of cytokine production.

Flow cytometry assays

Flow cytometry was used to assess the frequency of DENV-infected cells, expression levels of VDR and, changes of TLR3/4/9 expression, as previously described [17,18]. Also, flow cytometry was used for assessing the production of ROS production was evaluated by the detection of dihydrorhodamine 123 (DHR 123; Sigma Aldrich, USA). In detail, DENV infection was evaluated through the intracellular detection of DENV E antigen at the indicated time points and the cells were fixed using a fixation/permeabilization buffer (eBioscience, USA). Following washing steps with PBS, cells were stained with the monoclonal antibody, 4G2 (Millipore, Germany) for 40 min, followed by 40 min staining with goat anti-mouse IgG-FITC (Thermo Scientific, USA). Expression of TLR3, TLR4, and

TLR9 in DENV-2-infected and mock-infected cells was evaluated at 2, 8, and 24 hpi as we described in [25]. All data acquisition and analysis were done using the BD FACScan system and FACSDiva software, respectively.

Quantitation of viral RNA copy number

Total RNA was purified from DENV-2-infected and mock-infected MDMs and D3-MDMs using TRIzol reagent (Thermo Scientific, USA) following the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Then, cDNA was synthesized using random primers from a standard concentration of 50 ng of RNA and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. Viral RNA copy quantification with cDNA was carried out using the specific primers depicted in supplemental Table 1, as described previously [26]. qPCR was performed with Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) and analyzed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The calculation of viral RNA copies was based on a standard curve of Ct values of 10-fold serial dilutions of a plasmid encoding the full genome of DENV-2 of known length and concentration, as previously described [27].

Cytokine production

The levels of IL 6, TNF-α, and IL 10 were assessed in supernatants from MDMs and D3-MDMs infected with DENV-2 at 2, 8, and 24 hpi, using an ELISA assay (BD OptEIA, BD Biosciences, USA), following the manufacturer's recommendations.

Quantification of gene expression

The mRNA quantification of TLR3, TLR4, TLR7, TLR8, TLR9, RIG I IFN-α, IFN-β, protein kinase R (PKR), 2′-5′-oligoadenylate synthetase 1 (OAS1), VDR, CYP24A1, SOCS-1, and ubiquitin was performed in DENV-2-infected and mock-infected MDMs and D3-MDMs using qPCR. Briefly, cDNA was synthesized from total RNA using random primers, a standard concentration of 50 ng of RNA, and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Then, qPCR was performed using Maxima SYBR Green (Thermo Scientific, USA), following the manufacturer's instructions with specific

primers for each gene (S1 Table). The specificity of the amplification product was determined by melting curve analysis. The relative quantification of each mRNA was normalized to the constitutive gene, ubiquitin, and mock-treated MDMs and D3-MDMs from each time point evaluated (e.g. 2 hours mock MDM vs 2 hours DENV-2-infected MDMs), using the ΔΔCt method and reported as the fold change.

Detection of ROS production

For quantification of ROS production, MDMs and D3-MDMs were infected with DENV-2 for 2, 8, and 24 h, or stimulated with zymosan at a final concentration of 10 μg/mL for 4 h. Thereafter, cells were stained with DHR 123 (Sigma Aldrich, USA) at a final concentration of 3.5 μg/mL for 1 h at 37°C in the dark. Then, the analysis was performed by flow cytometry using a FACScan flow cytometer and the FACSDiva software. The production of ROS was inhibited by treatment with N-acetylcysteine (NAC) for 1 h at a final concentration of 10 mM. Treated and non-treated cells were then infected with DENV-2 or treated with zymosan. ROS production and TLR9 expression were measured as described above.

Statistical analysis

Comparisons between MDMs and D3-MDMs were undertaken using a two-way analysis of variance (ANOVA) along with a Bonferroni post-hoc test. A value of $p < 0.05$ was considered statistically significant. The calculation of these parameters was carried out using GraphPad Prism version 6 (GraphPad Software, USA) software.

RESULTS

VitD3 modulates the expression of RIG I, TLR7, TLR3 and TLR4 in D3-MDMs infected with DENV-2

We have previously shown that differentiation of macrophages in the presence of VitD3 confers partial resistance to DENV-2 infection, due to a decrease in the expression of MR, accompanied by a reduced inflammatory response of D3-MDMs [17]. Here, as was previously reported [17], we confirmed not only that VitD3 decreases the percentage of DENV-infected D3-MDMs (Supp. Fig2A and 2B), but also significantly decreased DENV replication according to the viral RNA copy number and viral load, determined by qPCR

and plaque assay, respectively (Supp. Fig. 2C and 2D). Further, the reduced inflammatory response of D3-MDMs during DENV-2 infection was confirmed by the observation of lower levels of IL-6 and TNF- α produced by D3-MDM (Supp. Fig. 3A and 3B).

We then sought to test if VitD3 differentially regulates innate immune response by modulating the expression of PRRs known to sense DENV infection. Specifically, we quantified the mRNA expression of RIG-I, TLR7, TLR8, and the mRNA and protein level of TLR3 and TLR4 in response to DENV-2 infection, in D3-MDMs and MDMs infected at 2, 8, and 24 h. The expression mRNA levels of the tested genes did not change over time in mock-treated MDMs and D3-MDMs, at 2, 8, and 24 h (Supp. Fig 4), and these results were used to normalize the data from DENV-2 infected samples. The successful effect of VitD3 in the differentiation of D3-MDMs was confirmed by the increase in the transcription of the target gene CYP24A1 (Supp. Fig 5A), and VitD3 receptor (VDR) at the protein level (Supp. Fig 5B and 5C), in both DENV-2 infected and mock-infected cells.

DENV-2 infection in both MDMs and D3-MDMs led to an increase in RIG-I mRNA expression, compared to mock-treated cells, with the highest expression measured at 24 hpi; although at this time point, we observed no significant differences in RIG-I mRNA expression between MDMs and D3-MDMs (Fig. 1A). However, a significant decrease in the induction of RIG-I mRNA expression was noted at 8 hpi in D3-MDMs as compared to MDMs (Fig. 1A). Expression levels of TLR7 mRNA remained at baseline levels at 2 and 8 hpi in both MDMs and D3-MDMs infected, whereas a 3-fold increase in TLR7 mRNA expression occurred only in infected-MDMs at 24 hpi and was not observed in infected-D3-MDMs (Fig. 1B). Expression levels of TLR8 mRNA did not change among DENV-2 infected MDMs and D3-MDMs (Supp. Fig 5D). On the other hand, the expression of TLR3 mRNA was significantly downregulated in D3-MDMs at 8 and 24 hpi compared with MDMs in response to DENV-2 infection (Fig. 1C). In the same way, a significant decrease in TLR3 protein levels in D3-MDMs compared with MDMs at 2 and 8 hpi (Fig. 1D and Supp. Fig 5E) was observed, suggesting early downregulation of TLR3 expression in infected-D3-MDM. The highest expression of TLR4 mRNA was observed at 8 hpi in both D3-MDMs and MDMs, although no significant differences were observed (Fig. 1E). However, we did observe a significant increase in TLR4 mRNA expression at 24 hpi in DENV-2-infected D3-MDMs. Likewise, a significant increase in TLR4 protein level was found in D3-MDMs compared

with MDMs at 24 hpi (Fig. 1F and Supp. Fig 5F). At early time points of infection (2 and 8 h), VitD3 had no effect on the TLR4 expression.

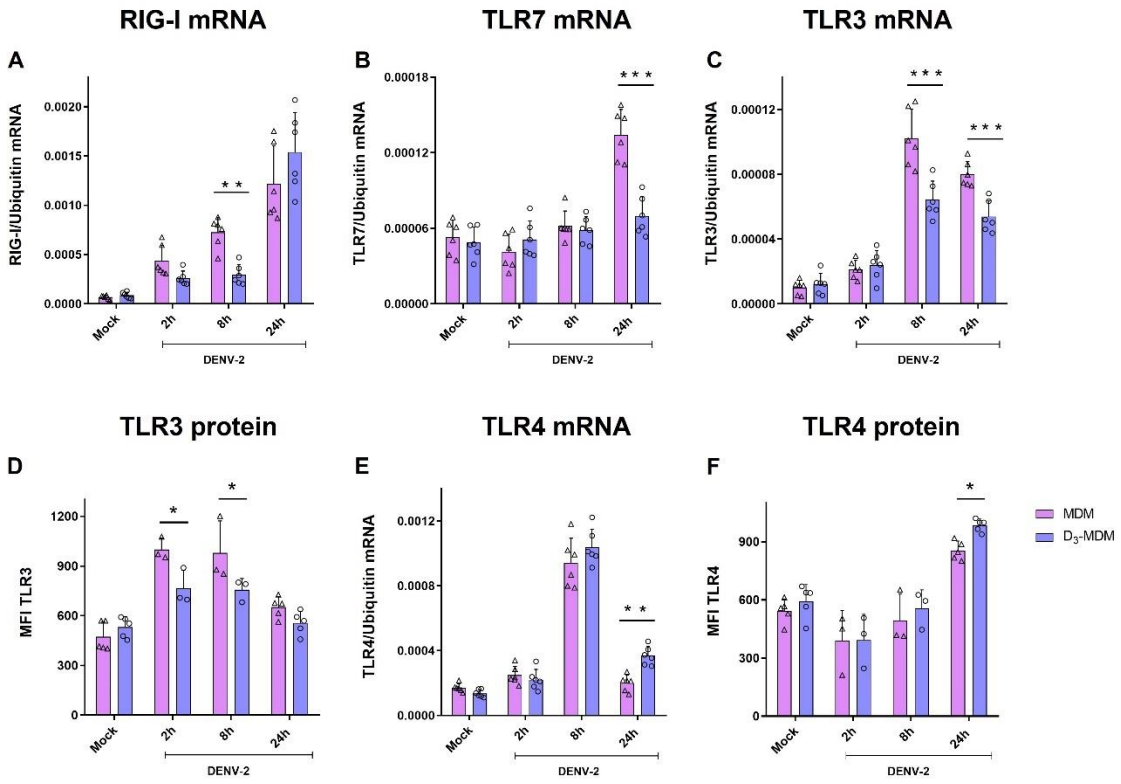


Fig 1. VitD3 modulates the expression of TLRs in D3-MDMs

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with an MOI of 5 for 2, 8, or 24 h. Expression of RIG I (A), TLR7 (B), TLR3 (C), and TLR4 (E) was measured by qPCR in MDMs and D3-MDMs using the gene encoding ubiquitin as a housekeeper gene. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs from each time point. Expression of TLR3 (D) and TLR4 (F) protein was measured by flow cytometry and expressed as mean fluorescence intensity. Six different donors were analyzed. Differences were identified using a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

To test if the observed regulation of TLRs was related to a direct effect of VitD3 rather than restricted infection of DENV-2 in D3-MDMs, we infected MDMs and D3-MDMs with different MOIs of DENV-2. As shown in Figure 2A, VitD3 decreased the percentage of infected cells, regardless of the MOI used. An additional analysis was done to confirm these

results, and the % of suppression of infection was calculated with each MOI (Fig 2B). We observed that suppression of infection induced by VitD3 was similar among different MOIs, excep for MOI of 1, in which VitD3 appeard to induce a stronger restriction of DENV-2 infection (Fig 2B). Then, we compared the expression of TLR3 and TLR7 mRNA in MDMs and D3-MDMs infected with different DENV-2 MOIs and observed that VitD3 regulated the expression of these TLRs with several of the MOI used (Fig 3A, 3B). Notably, when we stratified the data based on the matched percentage of infected cells (e.g., MDMs MOI 3 vs. D3-MDMs MOI 5), D3-MDMs still showed a significant decrease in TLRs mRNA expression induced by VitD3 (Fig 3D and 3E). These findings suggest that regulation of TLR3 and TLR7 expression by VitD3 in D3-MDMs is a result of the direct effect of the hormone rather than a consequence of reduced levels of viral replication. We suggest that the downregulation of TLR expression by VitD3 could be contributing to the regulation of the inflammatory response, in addition to a decreased C-type lectin activation that was previously reported by us in [17].

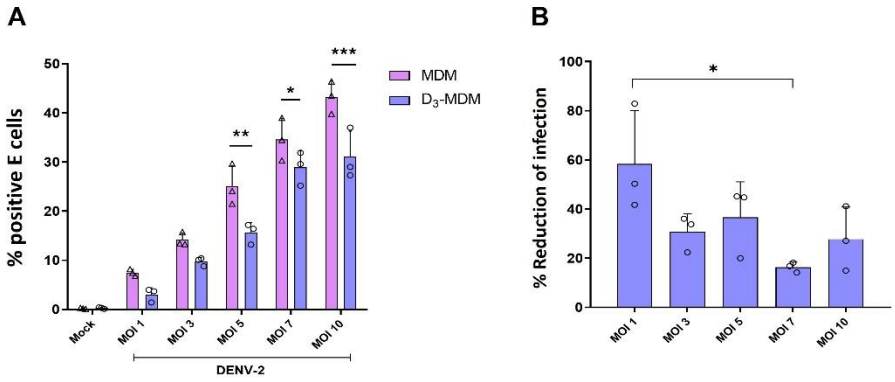


Fig 2. Reduction of E-positive cells in D3-MDMs infected with different MOI of DENV-2

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with different MOI for 24 h. Infection was evaluated through flow cytometry and expressed as % E-positive cells. Three different donors were analyzed. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

VitD3 decreases the expression of TLR9 and the production of ROS in D3-MDMs infected with DENV-2

TLR9 is known to recognize unmethylated CpG motifs in DNA and cannot recognize viral RNA components. However, previously we reported that myeloid DCs of dengue infected patients have an increased expression of TLR9 compared to healthy controls [25]. In addition, upregulation and activation of TLR9 in DENV-2-infected Mo-DCs were described recently [28]. In line with this study, we found an upregulation of TLR9 mRNA in MDMs after DENV-2 infection at 8 and 24 hpi (Fig 4A), and an increase in TLR9 protein expression at 2, 8, and 24 hpi quantified by flow cytometry (Fig. 4B). Interestingly, we observed significant downregulation in TLR9 mRNA and protein expression in D3-MDMs at 8 and 24 hpi (Fig 4A, 4B and Supp. Fig 5G). We also compared the expression levels of TLR9 mRNA in matched DENV-2-infected MDMs and D3-MDMs, as described above, and found that the decrease in the expression of this TLR was a direct effect of VitD3 (Fig 3C, 3F), suggesting that VitD3 may counteract the effect of DENV-2 induction on TLR9 expression.

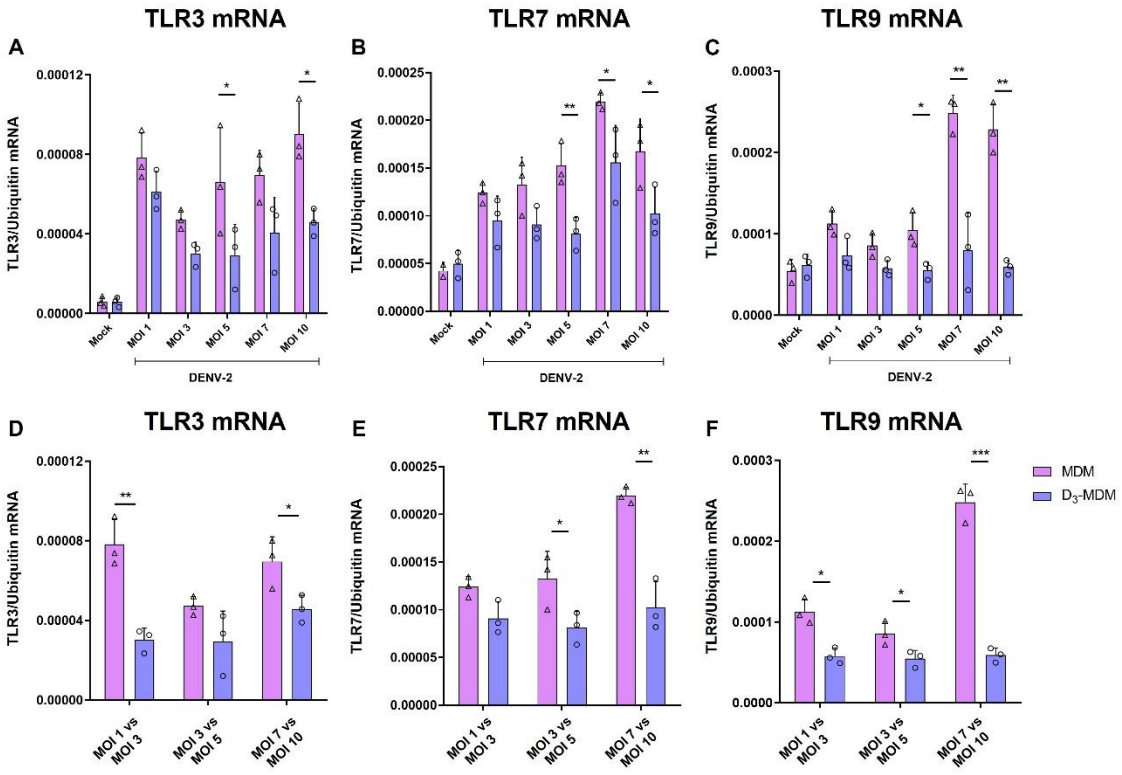


Fig 3. Regulation of TLR expression is independent of the MOI used

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with different MOI for 24 h. Expression of TLR3 (A and D), TLR7 (B and E),

and TLR9 (C and F) were measured by qPCR in MDMs and D3-MDMs using ubiquitin as housekeeping. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs. Three different donors were analyzed. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***p < 0.001, **p < 0.01, *p < 0.05).

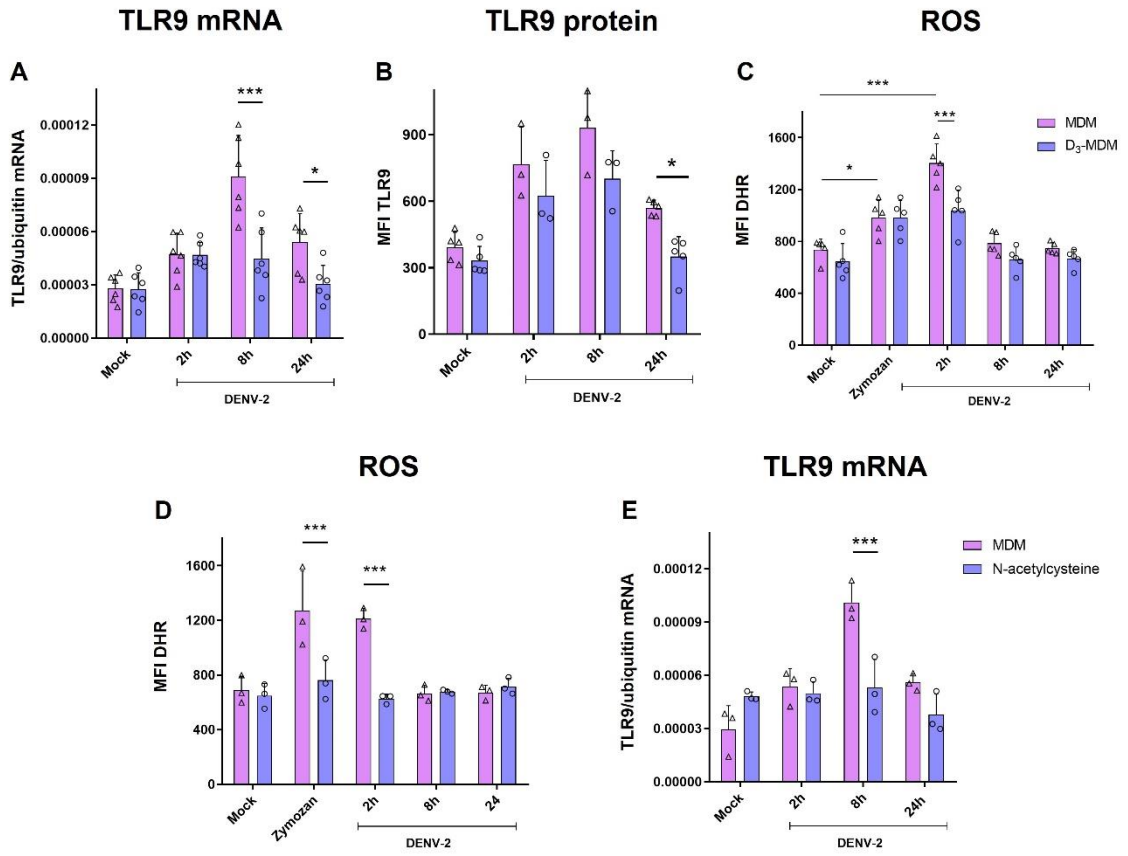


Fig 4. VitD3 downregulates TLR9 expression and ROS production in D3-MDMs

MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with an MOI of 5 for 2, 8, or 24 h. Expression of TLR9 (A) was measured by qPCR in MDMs and D3-MDMs using the gene encoding ubiquitin as a housekeeper gene. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs from each time point. Expression at the protein level of TLR9 (B) was measured by flow cytometry and expressed as mean fluorescence intensity. The production of ROS was measured by flow cytometry through DHR 123 in MDMs and D3-MDMs infected with DENV-2 with an MOI of 5 for 2, 8, and 24 h, or stimulated with 10 μg/mL of zymosan (C). The MDMs were pre-treated with N-acetylcysteine at 10 mM for 1 h and then infected with DENV-2 with an MOI of 5 for 2, 8, and 24 h or treated with 10 μg/mL of zymosan. Production of ROS was measured by flow cytometry through DHR 123) (D) and the TLR9 expression was measured by qPCR (E). Six different donors (A, B, and C) or three different donors (D and E) were analyzed. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***p < 0.001, **p < 0.01, *p < 0.05).

Considering that the upregulation of TLR9 expression in Mo-DCs was dependent on ROS production [28], the production of total ROS in MDMs and D3-MDMs after DENV-2 infection was evaluated at 2, 8, and 24 hpi by staining with DHR 123. At 2 hpi a significant increase in the production of ROS by both MDMs and D3-MDMs in response to DENV-2 infection was observed, yet levels produced by MDMs were significantly higher than those observed in D3-MDMs (Fig. 4C). At later time points, the production of ROS in DENV-2-infected MDMs and D3-MDMs was restored to the levels detected in mock-infected cells (Fig. 4C). To test whether a direct relationship exists between ROS production and the upregulation of TLR9 mRNA, we proceeded to inhibit the production of ROS, using the ROS scavenger, NAC. Using 10 mM of NAC, a significantly decrease ROS production in MDMs treated with zymosan, used as a positive control, was observed, and also in DENV-2 infected MDMs at 2 hpi, obtaining a level of ROS similar to mock-infected cells (Fig 4D). Interestingly, when TLR9 mRNA expression was quantified in MDMs infected with DENV-2 and treated with 10 mM of NAC, a significant decrease in TLR9 was observed at all time points evaluated, compared with non-treated and DENV-2-infected MDMs (Fig. 4E).

Altogether, these results suggest that DENV-2 infection induces early production of ROS in MDMs that, in turn, upregulates the expression of TLR9. Conversely, VitD3 downregulates both the early production of ROS in DENV-2-infected D3-MDMs and both mRNA and protein level of TLR9.

VitD3 upregulates mRNA expression of SOCS-1, PKR, and OAS1 in DENV-2-infected D3-MDMs

SOCS family of proteins are important in the regulation of the inflammatory response upon activation of TLRs [22]. Thus, the expression of SOCS-1 mRNA in DENV-2-infected D3-MDMs and MDMs was compared at 2, 8, and 24 hpi. As shown in Fig 5A, at 2 hpi, there were no differences in expression levels of SOCS-1 mRNA between D3-MDMs and MDMs; however, we observed an increase in SOCS-1 in DENV-2-infected D3-MDMs at 8 and 24 hpi compared with MDMs.

Finally, regulation of other antiviral mechanisms by VitD3, such as regulation of the IFN-I response, was explored. Previously, it was reported that VitD3 can enhance the activity of IFN-I, which was evidenced by an increase in ISG expression [29]. The mRNA levels of IFN-

α, IFN-β, PKR, and OAS1 were quantified in D3-MDMs and MDMs infected with DENV-2 at 2, 8, and 24 hpi. The expression of tested genes did not change over time in mock-treated D3-MDMs (S2 Fig). There was a significant increase in the expression of both IFN-α and IFN-β mRNA in MDMs in response to DENV-2 infection, especially at 24 hpi (Fig 5B and 5C). However, IFN-α mRNA expression was downregulated in DENV-2-infected D3-MDMs at 24 hpi compared with MDMs (Fig. 5B), possibly due to lower levels of viral replication in D3-MDMs. Conversely, there was no difference in IFN-β mRNA expression in D3-MDMs compared with MDMs infected with DENV-2 (Fig 5C). Although D3-MDMs showed a lower expression of IFN-α and no increase in IFN-β, a significantly higher expression of both PKR and OAS1 mRNA was found in DENV-2-infected D3-MDMs compared with MDMs at 24 hpi (Fig 5D and 5E).

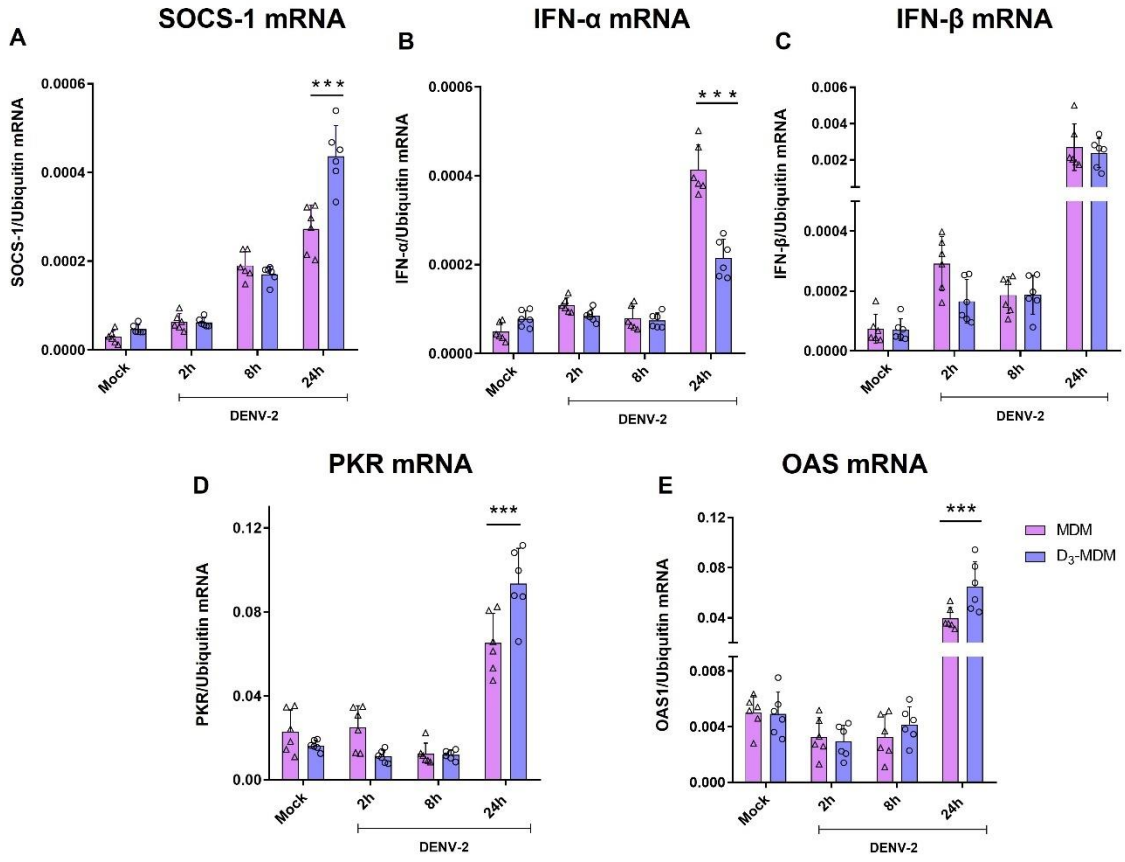


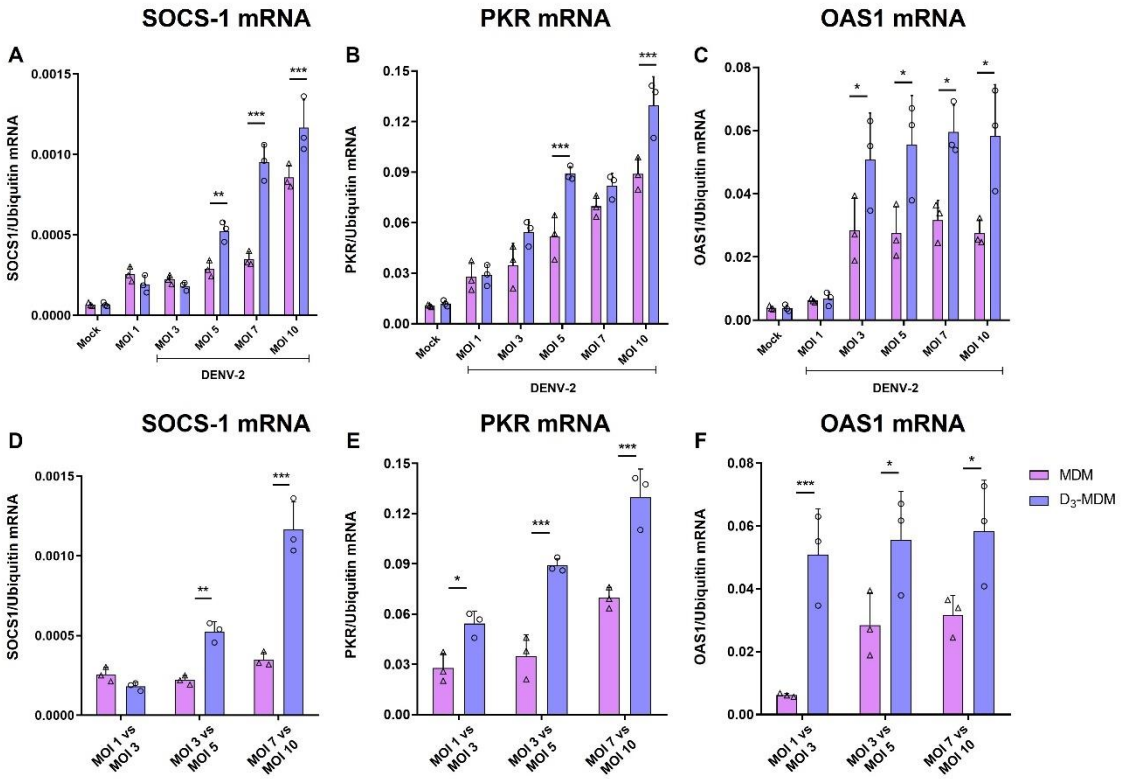
Fig 5. VitD3 upregulates the expression of SOCS-1 and ISGs in D3-MDMs

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 for 2, 8, or 24 h. Expression of SOCS-1 (A), IFN α (B), IFN β (C), PKR (D), and OAS1 (E) were measured by qPCR in MDMs and D3-MDMs using the gene encoding ubiquitin as a housekeeper gene. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs from each time point. Six different donors were analyzed. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***$p < 0.001$, **$p < 0.01$).

To check if the noted differences between MDMs and D3-MDMs were due to different levels of infection in these cells, we evaluated the expression of SOCS-1, PKR, and OAS1 mRNA in MDMs and D3-MDMs infected with different DENV-2 MOIs, as described in Figure 3. As shown in Figures 6A, B, and C, regulation of SOCS-1, PKR, and OAS1 mRNA expression induced by VitD3 were MOI-independent. Also, the expression levels of SOCS-1, PKR, and OAS1 mRNA were higher in D3-MDMs than in MDMs when matching infection rate conditions were done (e.g., MDMs MOI 3 vs. D3-MDMs MOI, Fig 6D, E, and F), suggesting a direct effect of VitD3 on the regulation of these genes in D3-MDMs. Taken together, our results may suggest that VitD3 can increase the expression of ISGs such as PKR and OAS1 in D3-MDMs after DENV-2 infection, which could constitute an additional antiviral mechanism of VitD3 in addition to the downregulation of MR reported by us previously [17].

Fig 6. Regulation of SOCS-1 and ISGs expression is independent of the MOI used

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with different MOIs for 24 h. Expression of SOCS-1 (A and D), PKR (B and E), and OAS1 (C and F) were measured by qPCR in MDMs and D3-MDMs using ubiquitin as a housekeeper. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs. Three different donors were analyzed. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).



Discussion

Compounds exhibiting both anti-inflammatory and antiviral properties are considered promising therapeutic options to prevent severe dengue. In recent years, VitD3 has drawn the attention of the field due to its wide antiviral and immunomodulatory properties [14,16,30,31]. Indeed, we have recently shown that D3-MDMs express lower levels of MR, which conferred partial resistance to DENV-2 infection [17]. In the present study, we observed that the presence of VitD3 during the differentiation of macrophages downregulated the expression of major PRRs and inflammatory pathways while increasing the expression of ISGs (Fig 7).

Here we observed that VitD3 significantly reduced the expression of TLR3, TLR7, and TLR9 in DENV-2-infected D3-MDMs. However, different to TLR3 and TLR9, reduced expression of TLR7 could not be confirmed at protein level, which is a limitation of our study. Regardless, regulation of TLRs was observed at both early and late time points of infection, suggesting that VitD3-mediated responses counteract the effect of DENV infection on TLR

expression [32,33]. To note, we found discrepancies between kinetics of mRNA and protein expression of TLR3, noticing a faster increase in protein expression than mRNA levels. Unfortunately, these findings could not be dissected further in this study. However, such discrepancies could be explained by post-transcriptional mechanisms including regulation by microRNAs [34] that can occur early after viral recognition; or by the existence of pre-formed TLR3 complexes that could be recruited to endosomes rapidly after DENV recognition, as it has been described for TLR2 complexes and recruitment to lipid rafts after recognition with other types of ligands [35].

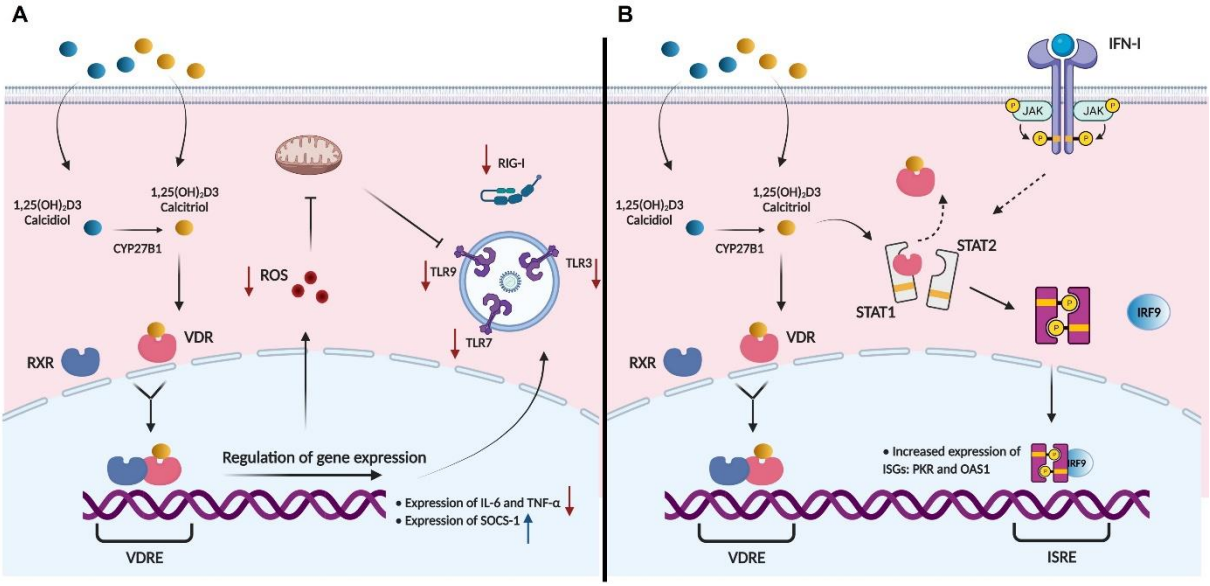


Fig 7. Hypothetical model of VitD3 effects in D3-MDMs during DENV-2 infection

(A) VitD3 can be found as inactive form 25(OH)2D2 (calcidiol), as active form 1,25(OH)2D3 (calcitriol) or be converted from inactive to active form within the cell by the action of CYP7B1. VitD3 interacts with the vitamin D receptor (VDR) present in the nuclei and associates then with the nuclear receptor retinoid X receptor (RXR). Together, they act as transcription factor that will increase the expression of genes that have vitamin D response elements (VDREs). Genomic effects of VitD3 during DENV-2 infection include decreased expression of IL-6, TNF-α, RIG-I, TLR3, TLR7 and decreased production of reactive oxygen species (ROS). Since ROS can induce mitochondrial damage and then increase activation and expression of TLR9, VitD3 reduced TLR9 expression indirectly. (B) As a non-genomic effect, VitD3 can bind to VDR that is initially bound to STAT1, and indirectly enhance the activity of JAK-STAT pathway and IFN-I activity, thus increasing expression of PKR and OAS1.

Other studies have shown that VitD3 modulate the expression of various TLRs as we observed in our study. For instance, PBMCs obtained from patients with systemic lupus

erythematosus that were treated with 50 nM of VitD3 express lower levels of TLR3, TLR7, and TLR9 compared to non-treated PBMCs [36], as observed in this study. Culturing human corneal epithelial cells with 100 nM of VitD3 for 24h leads to a reduced expression of TLR3 along with a reduced response to poly-IC stimulation [37]. On the other hand, in a recent study in which DENV-2-infected U937 cells were treated with 0.1 or 1 μM of VitD3, no effect on TLR3 expression was observed [15]. The observed differences could be due to different cell types and concentrations of VitD3; however, overall, most studies show downregulation of TLR3, TLR7, and TLR9 by VitD3 like we found. Whether regulation of TLR3 by VitD3 is dose-dependent, or if different baseline levels of VitD3 in DENV- infected patients are important for host response and control of infection remains to be studied.

Some TLRs have been implicated in DENV pathogenesis. For instance, recognition of DENV infection by TLR4 and TLR2 has been shown to mediate endothelial activation and permeability [38,39]. Increased TLR2/4 expression was also observed on monocytes of acute infected patients [40]. While we did not measure TLR2 expression and it is a limitation of our study, we found that DENV-2 infection led to an upregulation of TLR4 in D3-MDMs and MDMs. Notably, however, the increase was significantly higher in D3-MDMs than in MDMs. This was unexpected, as VitD3 has been shown to downregulate the expression of TLR4 in monocytes [20], keratinocytes [41], and PBMCs [42], which is accompanied by a decreased response to stimulation with LPS. However, the authors in these studies used higher concentrations of VitD3 (ranging from 0.1–1 μM) than we used (0.1 nM), and these high concentrations are not within the conventional and safe treatment in humans. Although the overall effect of VitD3 on TLR4 expression is likely to be cell type and concentration-dependent, the higher surface expression of TLR4 in D3-MDMs found in our study could be potentially worrisome. Importantly, however, we have previously shown that TLR4 agonist LPS did not induce TNF-α production in D3-MDMs [17], suggesting that signaling downstream TLR4 is affected by VitD3. Future studies should elucidate how VitD3 regulates the function of the TLR4 and TLR2 axis in macrophages in the presence and absence of DENV infection.

TLR9 recognizes CpG DNA, which is not produced by DENV replication, yet the expression of this TLR is upregulated in DCs infected with DENV-2 *in vitro* [28] and DCs obtained from DENV-infected patients [25]. We observed upregulation in TLR9 expression in MDMs

induced by DENV-2 infection both at the mRNA and protein level. Intriguingly, this expression was found decreased in D3-MDMs. This data is in line with other studies in which VitD3 was shown to decrease TLR9 expression in HeLa cells infected with Herpes simplex virus-1 [43], in monocytes [44], in murine plasmacytoid DCs [45], and in DENV-2 infected Mo-DCs [46]. We also found that VitD3 reduced the production of ROS at 2 hpi in D3-MDMs after infection with DENV-2. Interestingly, when ROS production was inhibited with the ROS scavenger, NAC, in DENV-2-infected MDMs, a reduction in ROS production and TLR9 mRNA was observed, suggesting a direct upregulation of TLR9 expression by ROS production. To our knowledge, this is the first report that demonstrates VitD3 induced regulation of ROS production and therefore TLR9 downregulation in DENV-2 infected macrophages. Although similar results were observed in Mo-DCs, in which upregulation of TLR9 expression was driven by DENV-2 induction of ROS [28], TLR9 expression kinetics were different compared to our results. Further studies are needed to better understand this process and its relevance in the context of DENV infection.

Other studies have shown that VitD3 can protect from the effect of oxidative stress induced by ROS, especially H2O2, including apoptosis and inflammation in epithelial [47] and endothelial cells [48]. A novel mechanism by which VitD3 exerts its antioxidant functions involves activation of the Nrf2 pathway in mice [49] and diabetic rats [50]. Furthermore, VitD3 derivatives protect human keratinocytes from DNA damage and oxidative stress by upregulating Nrf2 and various antioxidant genes [51]. Whether VitD3 decreases the oxidative response of D3-MDMs to DENV-2 infection by upregulating antioxidant genes driven by Nrf2, remains to be tested.

Following DENV-2 infection, SOCS-1 was upregulated in both D3-MDMs and MDMs, but this increase was higher in D3-MDMs. In neutrophils treated with VitD3 and infected with Streptococcus pneumoniae, there is an increased expression of SOCS-1 and SOCS-3 compared with non-treated neutrophils [52]. Further, mice treated with a synthetic analog of VitD3 upregulated the expression of SOCS-1 in a miR-155-dependent mechanism [21]. Accordingly, our results may imply that upregulation of SOCS-1 and downregulation of TLRs in DENV-2 infected D3-MDMs could be contributing to the decreased inflammatory response, in addition to a decreased C-type lectin activation that was reported by us previously [17].

Finally, we did not observe any upregulation in IFN-I expression induced by VitD3 in DENV-2-infected D3-MDMs. In contrast, we observed the downregulation of IFN α expression at 24 hpi, possibly due to a lower degree of replication of DENV-2. This suggests that the antiviral effect of VitD3 against DENV-2 in D3-MDMs is independent of IFN I expression. Few studies have evaluated the regulation of antiviral response induced by VitD3 in viral infections or other types of infectious diseases. Treatment of Huh7.5 hepatoma cells with the active form of VitD3 restricted HCV replication and induced upregulation of IFN β and the MxA antiviral gene [12]. Here, we observed a significant upregulation in PKR and OAS1 expression at 24 hpi in D3-MDMs. Our results are in line with a study by Jadhav NJ et al, in which treatment with VitD3 of DENV-2 infected U937-DC-SIGN upregulated OAS1, OAS2, and OAS3 expression [15]. Altogether, previous reports and our results, indicate that VitD3 enhances the activity of IFN I rather than increasing its expression. VitD3 can promote the function of IFN I by fast non-genomic action. VDRs can interact directly with STAT1 and, therefore, somehow restrict the function of the JAK-STAT signaling pathway [53]. Indeed, VitD3 can compete with STAT1 for its natural receptor, VDR, and enhance the activity of IFN I by promoting the function of the JAK-STAT signaling pathway [29]. However, future studies are needed to explore the interaction between VDR and STAT1 in DENV-2-infected MDMs and its implications in VitD3 enhancement of IFN I activity.

If the data observed in this study could be extended for other serotypes should be addressed in a future study. However, as most of the effects of VitD3 occurred at cellular level, it is likely that same results can be observed during infection with other DENV serotypes. Importantly, we found that regulation of immune response by VitD3 was a direct effect of the hormone rather than a reduced effect of decreased DENV-2 infection in D3-MDMs. However, this data should be extended in future analysis as well, since we did not evaluate DENV-2 replication levels in matched-infected samples, which could be different due to intrinsic differences of D3-MDMs and MDMs.

In conclusion, our study demonstrates the immunoregulatory effects of VitD3 in DENV-2-infected D3-MDMs. The VitD3-mediated modulation of components of the innate immune response, such as expression of TLRs, production of ROS, and expression of SOCS-1 during

DENV-2 infection, thus contributing to the anti-inflammatory effects of this hormone (Fig 7).

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STATEMENT OF ETHICS

The protocols for individual's enrollment and sample collection were approved by the Committee of Bioethics Research of Sede de Investigación Universitaria, Universidad de Antioquia (Medellín, Colombia) and inclusion was preceded by a signed informed consent form, according to the principles expressed in the Declaration of Helsinki.

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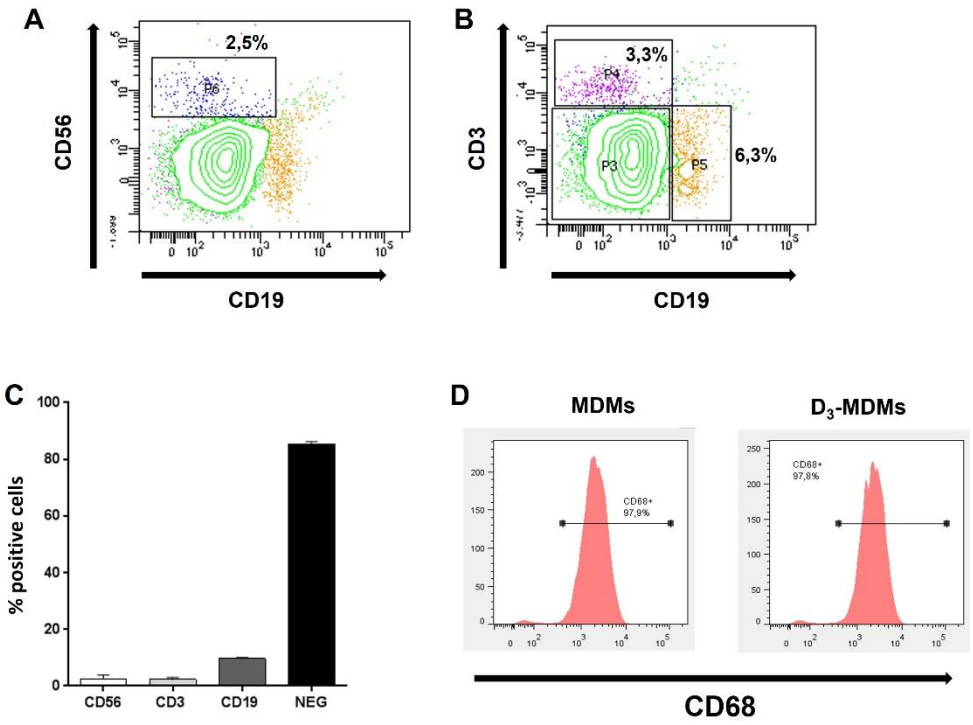
SUPPLEMENTARY DATA

Supplemental table 1. Primers used in the study

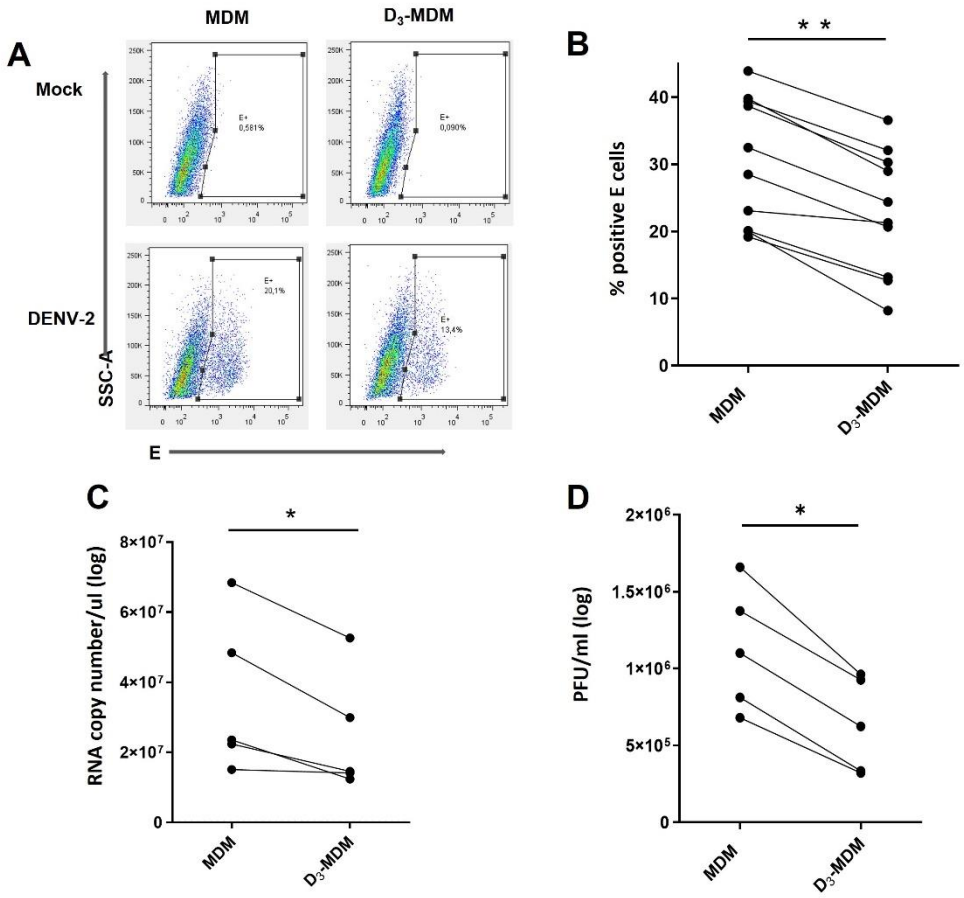
Gene	Sequence 3´---5´
TLR3	Fw: GTCAGATTTAAACATTCCTCTTCGC
	Rv: ATTGGGTCTGGGAACATTTCTCTTC
TLR4	Fw: GCCCTGCGTGGAGGTGGTTCCTA
	Rv: AGCTGCCTAAATGCCTCAGGGGAT
TLR7	Fw: TTACCTGGATGGAAACCAGCTACT
	Rv: TCAAGGCTGAGAAGCTGTAAGCTA
TLR9	Fw: TTATGGACTTCCTGCTGGAGGTGC
	Rv: CTGCGTTTTGTCGAAGACCA
DDX58 **(RIG-I)**	Fw: AGGAAAACTGGCCCAAAACT
	Rv: TTTCCCCTTTTGTCCTTGTG
IFNA1 **(IFN-α)**	Fw: CAGAGTCACCCATCTCAGCA
	Rv: CACCACCAGGACCATCAGTA
IFNB1 **(IFN-β)**	Fw: CGCCGCATTGACCATCTA
	Rv: GACATTAGCCAGGAGGTTTCTCA
EIF2AK2 **(PKR)**	Fw: GGTACAGGTTCTACTAAACA
	Rv: GAAAACTTGGCCAAATCCACC
OAS1	Fw: GTGTGTCCAAGGTGGTAAAGG
	Rv: CTGCTCAAACTTCACGGAA
VDR	Fw: TGCTATGCACTGTGAAGGCGT
	Rv: AGTGGCGTCGGTTGTCCTT
CYP24A1	Fw: CGCAAATACGACATCCAGGC
	Rv: AATACCACCATCTGAGGCGT

SOCS1	Fw: CACTTCCGCACATTCCGTTC
	Rv: CACGCTAAGGGCGAAAAAGC
DENV-2	Fw: CAATATGCTGAAACGCGAGAGAAA
	Rv: CCCCATCTATTCAGAATCCCTGCT
UBE2D2 **(ubiquitin-conjugating enzyme E2D2**	Fw: CCCTTCAAACCACCTAAGGTTGC
	Rv: GTGCTGGAGACCACTGTGATCG



Supplemental Figure 1. Purity of monocyte and monocyte-derived macrophages (MDMs) cultures

Monocytes were purified from PBMCs, and purity was assessed by the quantification of contaminant CD19+, CD3+ and CD56+ (A, B and C). In D, a representative histogram with CD68 expression levels of monocyte-derived macrophages (MDMs) and D3-MDMs after 6 days of differentiation is shown. Figure in C represent 3 individual experiments.

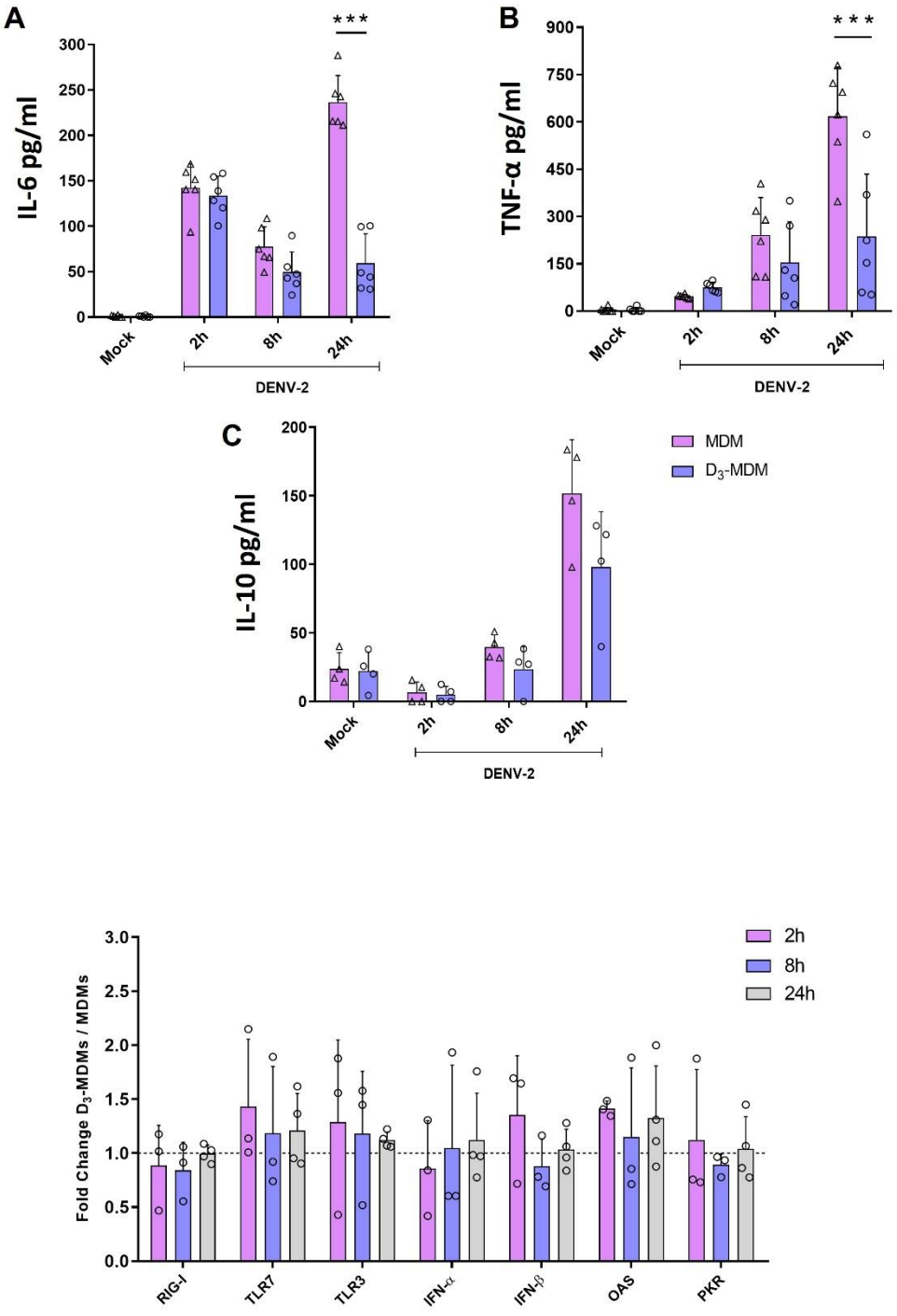


Supplemental Figure 2. VitD3 reduces DENV 2 infection and replication in D3 MDMs

MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3 MDMs) and infected with DENV 2 with an MOI of 5 for 24 h. Infection was evaluated through flow cytometry and expressed as % E-positive cells. In A, a representative dot-plot of an individual experiment is shown, while B shows a summary of the results. Replication of DENV 2 was evaluated by quantification of intracellular viral RNA copy number using qPCR (C) and by quantification of viral titer in the supernatant using a plaque assay (D). Figures represent 10 individual experiments from different donors (B), while five individual experiments are shown in C. Differences were obtained with a Wilcoxon test using a 95% confidence interval (**p < 0.01, *p < 0.05).

Supplemental Figure 3. VitD3 reduces the production of IL-6, TNF-α, and IL-10 in D3-MDMs

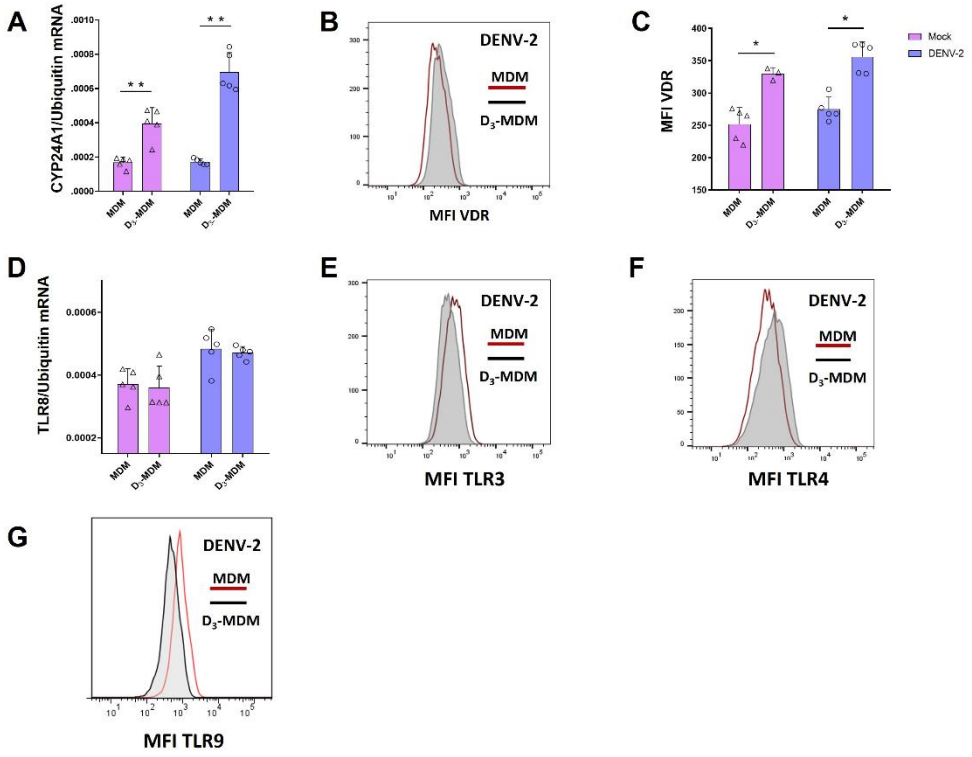
MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with a MOI of 5 for 2, 8, or 24h. Production of IL-6 (A), TNF-α (B), and IL-10 (C) was measured by ELISA in MDMs and D3-MDMs. Figures represent six individual experiments from different donors (A and B), while four individual experiments are shown in C. Differences were obtained using a two-way ANOVA with a Bonferroni post-hoc test using a 95% confidence interval (*** p < 0 .001)



Supplemental Figure 4. Expression of TLRs, IFN-I, ISGs and SOCS-1 at 2 8 and 24 hours in mock D3 MDMs

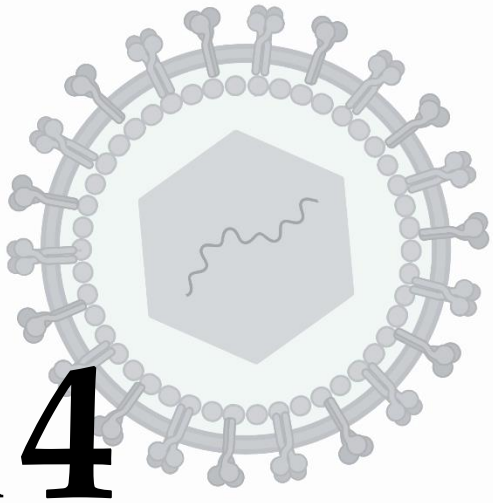
MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3 MDMs) and then expression of RIG-I, TLR7, TLR3, IFN-α, IFN-β, PKR and OAS1 were measured by qPCR in MDMs and D3 MDMs using ubiquitin-conjugating enzyme E2D2 as housekeeping. Data is expressed as fold change relative to mock treated MDMs. Figures represent three individual experiments from

different donors. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (***p < 0.001, **p < 0.01, *p < 0.05).



Supplemental Figure 5. VitD3 regulates the expression of CYP24A1, VDR, TLR3 and TLR4 in D3 MDMs, but not TLR8

MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3 MDMs) and then infected with DENV 2 with a MOI of 5 for 24 h. Expression of CYP24A1 (A) and TLR8 (D) were measured by qPCR in MDMs and D3 MDMs both infected and mock-infected with DENV 2, using the gene encoding ubiquitin-conjugating enzyme E2D2 as a housekeeper gene. Data is expressed as relative expression normalized to the expression level of the housekeeper gene. Expression at the protein level of VDR (B), TLR3 (E) TLR4 (F) and TLR9 (G) was measured by flow cytometry and expressed as mean fluorescence intensity. Figures represent five individual experiments from different donors. Differences were obtained with a two-way ANOVA with a Bonferroni post-hoc test and a 95% confidence interval (**p < 0.01, *p < 0.05).



CHAPTER 4

LL-37 modulates innate immune response of human primary macrophages during DENV-2 infection

Jorge Andrés Castillo[a,b], Diana M Giraldo[a], Jolanda M Smit[b], Izabela A Rodenhuis-Zybert[b] and Silvio Urcuqui-Inchima[a,*]

a. Grupo de Inmunovirología. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No 52-21, Medellín, (Antioquia), Colombia

b. Department of Medical Microbiology and infection Prevention, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands

* Corresponding Author: silvio.urcuqui@udea.edu.co

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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 addition of LL-37 post-exposure to DENV-2 did not. Interestingly, the latter condition reduced the production of IL-6 and increased 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 boosted by VitD3.

KEYWORDS

Dengue virus, innate immune response, vitamin D, LL-37, Toll-like receptors

Chapter 4

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 of length. [7]. Upon infection and translation of the viral RNA, the action of 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) [8]. Immune sentinels, such as macrophages, sense DENV particles and replication through the expression of an array of 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]. A recent study demonstrated 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 response 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 form 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 response 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) at 34°C without CO_2. BHK-21 cells were purchased from ATCC and maintained in D-MEM medium (Sigma-Aldrich, USA) at 37°C with 5% CO_2. Synthetic peptide LL-37 used in this study was purchased from RyD systems (USA).

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 C6/36 HT with a multiplicity of infection (MOI) of 0.05. The supernatant was aliquoted and stored at -70°C for future use.

Virus titration

Virus titration was performed by the quantification of 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, washed once with PBS and D-MEM medium containing 1.5% m/v carboxymethylcellulose sodium salt (medium viscosity, Sigma-Aldrich, USA was added to the cells. After 5 days of culture at 37°C with 5% CO_2, 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 known length and concentration.

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, $5x10^5$ 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% CO_2 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% CO_2. 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 and was present during differentiation for 6 days. Fresh medium with the same concentration 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% CO_2. At the indicated times 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 (5μM to 250 nM) 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 medium containing 5μM of LL-37 was added for another 22 hours. This is referred 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 and treated with LL-37 (ST and PT conditions)

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, OAS1 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: $(1.8)^{-\Delta Ct}$, 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 mRNA CAMP 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 are upregulated in DENV-2-infected macrophages, the expression of CAMP mRNA (LL-37 gene) and production of LL-37, HBD2 and HBD3 were quantified. 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 1A).

Furthermore, we did not detect any significant difference in the production of LL-37 peptide between DENV-2-infected MDMs and mock-infected MDMs (Fig 1B). To verify whether the low levels of LL-37 produced in response to DENV-2 infection was not due to 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 1A), 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 (Supplemental Fig 1A). Taken together, these results indicate that exposure to DENV-2 does not induce expression of CAMP mRNA and subsequent production of LL-37 peptide in MDMs.

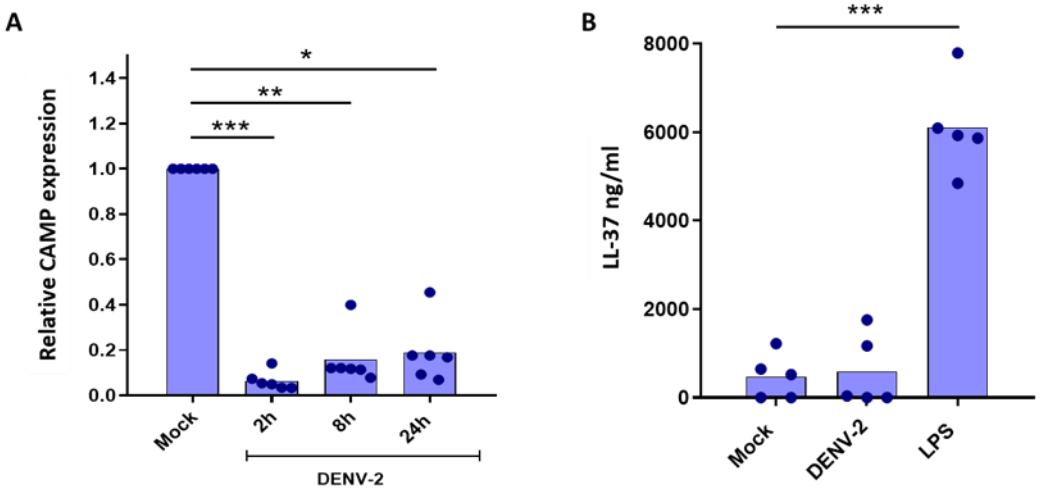

Figure 1. DENV-2 infection decreases the expression of CAMP and LL-37 in MDMs

(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 (***$p<0.001$, **$p<0.01$, *$p<0.05$).

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

Before testing the ability of LL-37 to modulate the immune response 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 (250nM 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 250nM (Fig 2B). This data allowed us to calculate a dose-response curve which showed the IC50 at 0.4 nM, and the IC90 at 1.3 μM (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 2C) and the infectious titer (PFU) by approximately 3 logs (Fig 2D), 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 2E). 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.

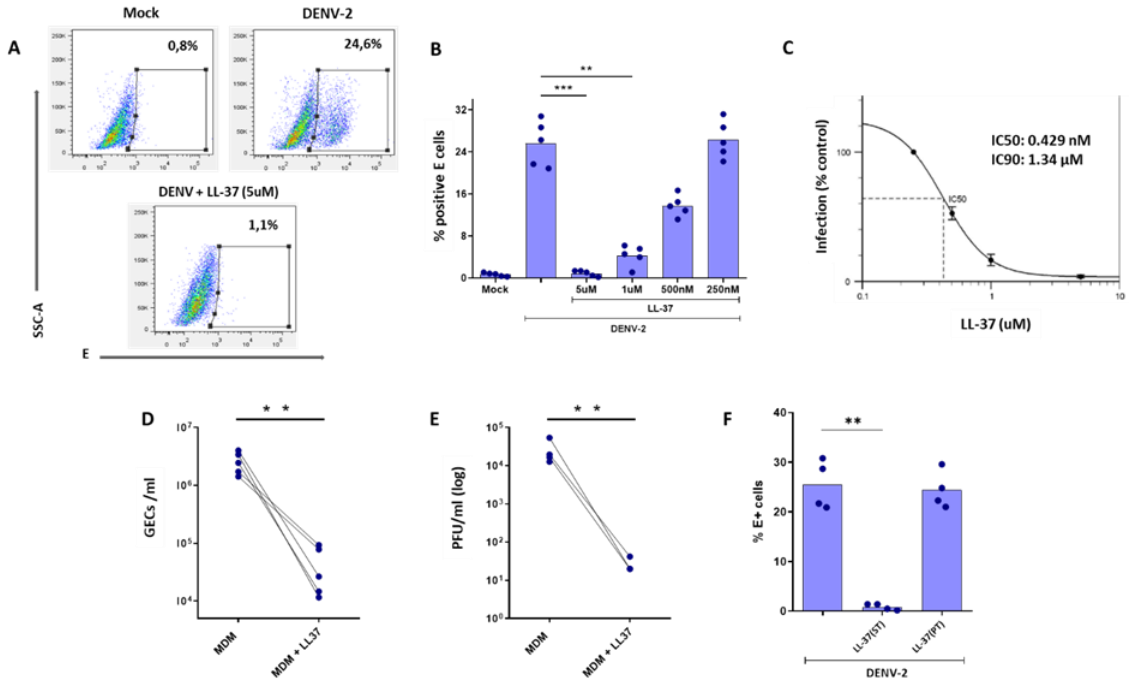

Figure 2. LL-37 restricts DENV-2 infection and replication in MDMs

(A, B) MDMs were infected with DENV-2 and treated simultaneously with LL-37 using different concentrations (5 µM, 1 µM, 0.5 µM and 0.25 µM). 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).

The antimicrobial peptide LL-37 regulates the expression of TLRs, RIG-I, IFN-β, PKR, OAS1, 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 response as well. Therefore, to distinguish the immunomodulatory effect of LL-37 from its antiviral effect, we assessed the regulation of immune response 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 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 of 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), that play a crucial role containing infection by inducing the expression of antiviral genes such as protein kinase K (PKR) and 2´5 Oligoadenylate synthase 1 (OAS1) [24]. 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 OAS1 (Fig 3G) mRNAs. Presence of LL-37 under PT condition during DENV-2 infection of MDMs resulted in an increase in the expression of PKR and OAS1 mRNA (Fig 4F and 4G), but expression levels of IFN-β mRNA was not changed (Fig 3E). We also tested 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 [25]. 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 induction of well-balanced immune response.

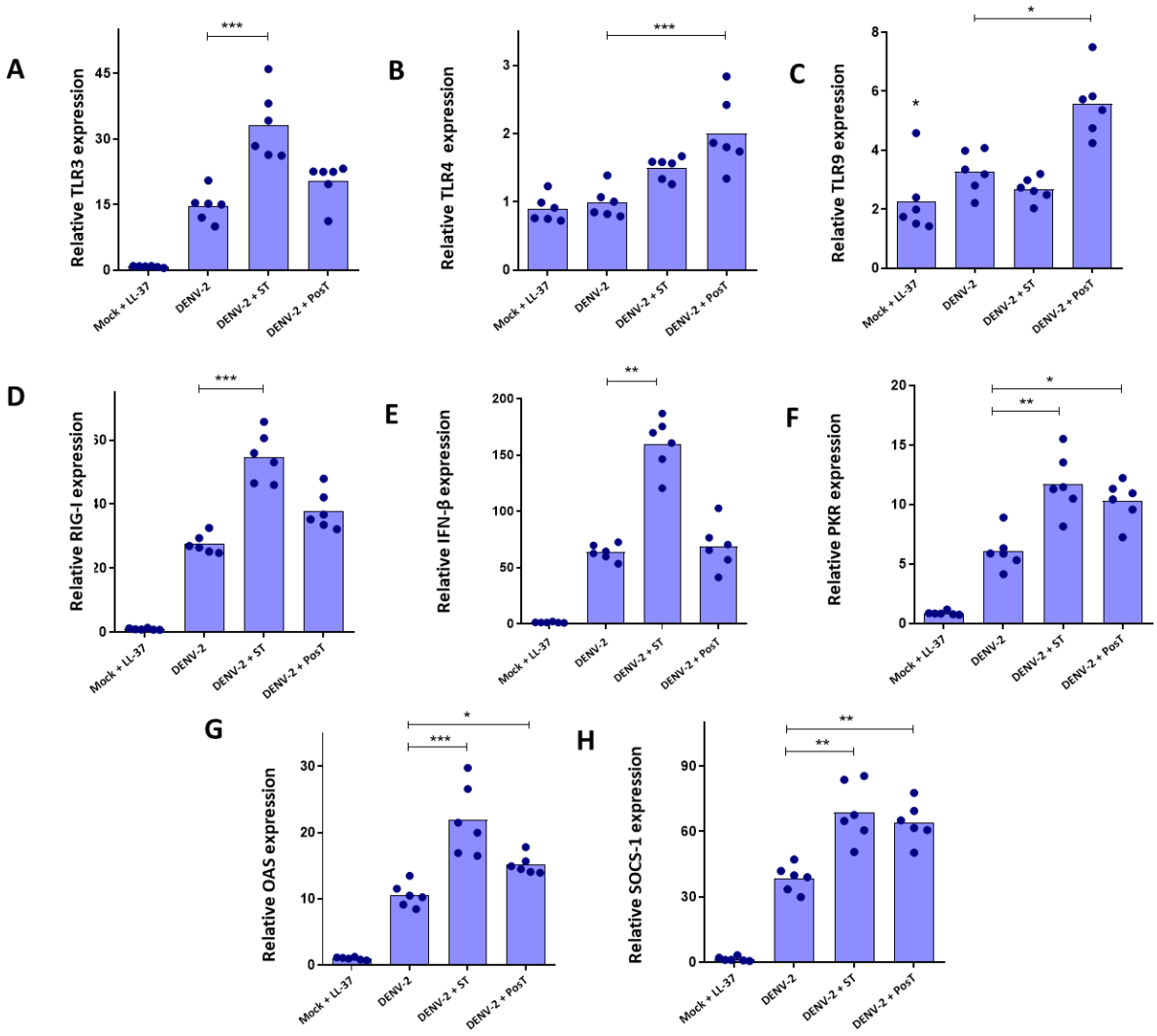

Figure 3. LL-37 modulates the expression of PKR, OAS, TLRs and SOCS-1 in MDMs infected with DENV-2

(A-H) MDMs were infected with DENV-2 and treated simultaneously with 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).

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

Activation of PRRs during DENV infection leads to the production of inflammatory mediators [26]. To test if regulation of PRRs and SOCS-1 expression by LL-37 altered DENV-2 induced inflammatory response, 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.

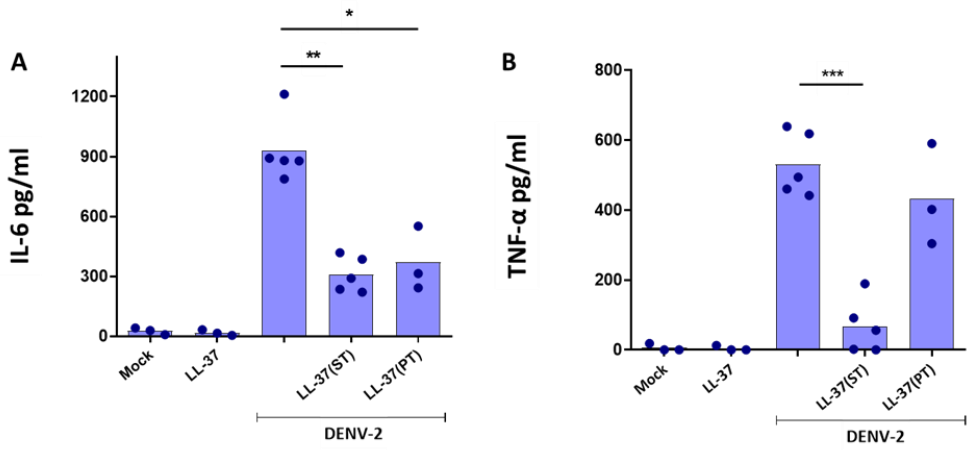

Figure 4. LL-37 Reduces de production of pro-inflammatory cytokines

(A, B) MDMs were infected with DENV-2 and treated simultaneously with 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).

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 [27]. 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 [28]. 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) [29]. 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 boosted 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.

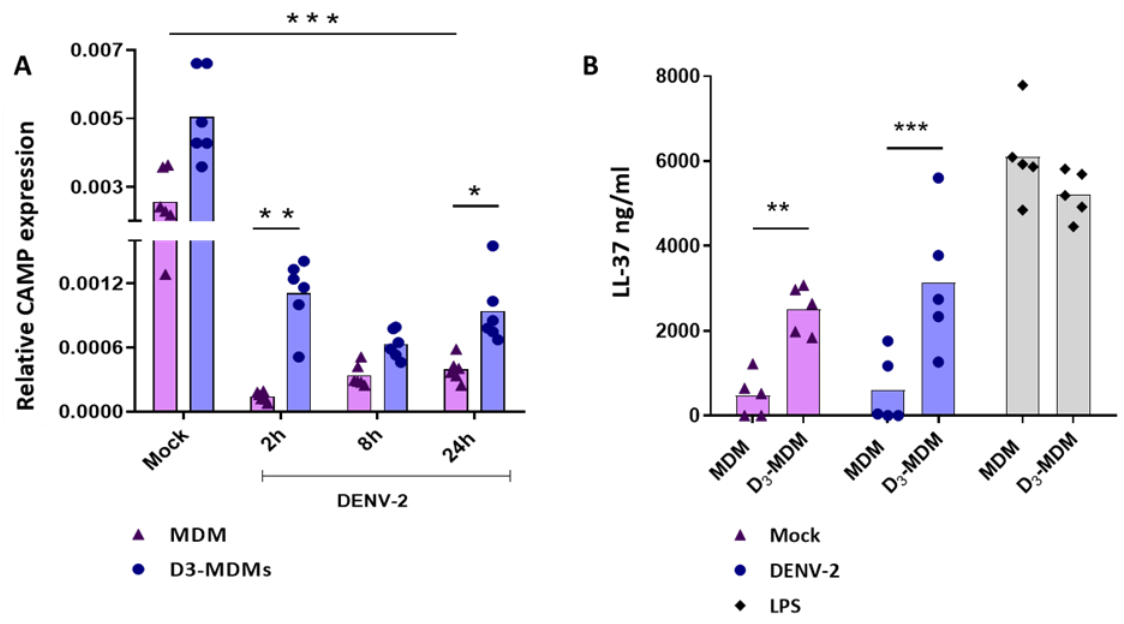

Figure 5. VitD3 up-regulates the expression of CAMP and LL-37 D3-MDMs

(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).

DISCUSSION

Herein we evaluated the effect of the peptide LL-37 on DENV-2 infection-induced innate immune response 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 response including lower gene expression of PRRs and 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 led to 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 [30]. Likewise, in DCs [31], keratinocytes [32] and in tissue mast cells [33] LL-37 increased the expression and activation of TLR9 induced by CpG-DNA or self-DNA, a process mediated by its directly binding to these agonists, hence increasing their uptake and processing by endosomes. Interestingly, in dendritic cells (DCs), DENV-2 infection induces the release of mitochondrial DNA that in turn upregulates TLR9 expression [34]. 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. Although the mechanism behind LL-37 antiviral activity remains elusive, based on published findings [18] and on our data, LL-37 is likely to inhibit viral entry into target cells. Therefore, the presence of LL-37 would result in reduced levels of dsRNA available for TLR3 and RIG-I activation and subsequent IFN-I expression. Notably however, 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 [35]. 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) [36] and in human astrocytes infected with ZIKV [37]. Whether this mechanism could explain the increase in the expression of TLR3 and RIG-I in our experimental model remains to be explored.

Despite the increase of IFN-β expression by LL-37 treatment under ST conditions, induction of ISGS such as PKR and OAS1 was induced by LL-37 under 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 type of cells like mast cells and A549 [38,39]. In our model, 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 response 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 [40] and in lungs of IAV-infected mice [41]. 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 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 [21,22,42–44]. However, in human vascular cells LL-37 enhanced the expression of IL-6 in human vascular cells after stimulation with poly I:C, which was mediated by TLR3 recognition [45]. 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 [14,16,37,46,47]. 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]. By adding LL-37 simultaneously with DENV-2 or after the initial period of infection (PT), we found that LL-37 is likely to inhibit the first stages of viral infection. However, the main mode of action of LL-37 is the disruption of the external membrane of microbes [48], 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. However, DENV-2 increased 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. Although, CAMP mRNA expression was down-regulated in DENV-2-infected MDMs, low levels of expression of this gene may difficult analysis. Whether DENV-2 actively down-regulates LL-37 expression or whether MDMs have low baseline levels of expression of LL-37 remains to be studied. However, we observed that differentiation of MDMs in the presence of VitD3 (D3-MDM) boosted 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 [49]. 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 [46,50]. In addition, we have previously shown that D3-MDMs exhibit lower expression of mannose receptor, and thereby are less susceptible to DENV-2 infection [29]. The ability of D3-MDMs to produce more LL-37 represent thus an additional paracrine defense mechanism against DENV infection. 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 boosts 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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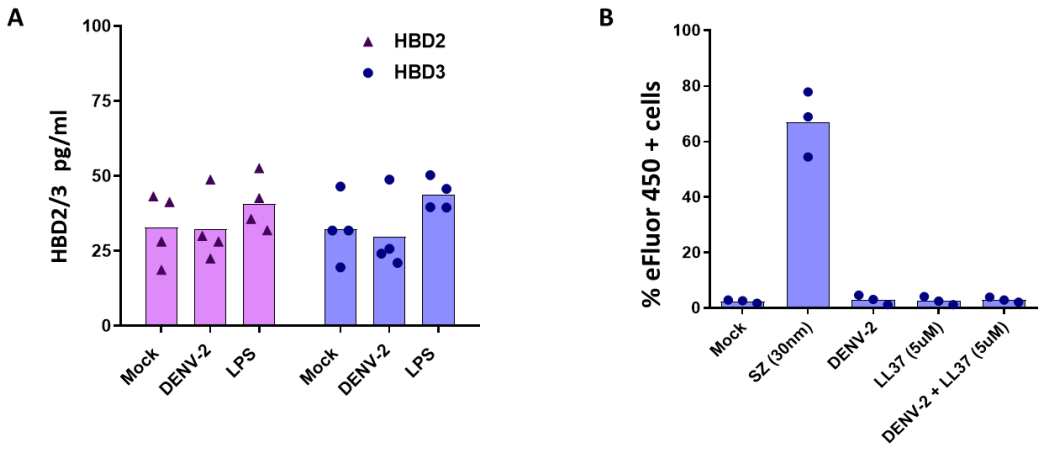
Chapter 4

SUPPLEMENTAL MATERIAL

Supplemental table 1. Primers used in the study

Gene	Sequence 3´---5´
TLR3	Fw: GTCAGATTTAAACATTCCTCTTCGC
	Rv: ATTGGGTCTGGGAACATTTCTCTTC
TLR4	Fw: GCCCTGCGTGGAGGTGGTTCCTA
	Rv: AGCTGCCTAAATGCCTCAGGGGAT
TLR9	Fw: TTATGGACTTCCTGCTGGAGGTGC
	Rv: CTGCGTTTTGTCGAAGACCA
RIG-I	Fw: AGGAAAACTGGCCCAAAACT
	Rv: TTTCCCCTTTTGTCCTTGTG
IFN-β	Fw: CGCCGCATTGACCATCTA
	Rv: GACATTAGCCAGGAGGTTTCTCA
PKR	Fw: GGTACAGGTTCTACTAAACA
	Rv: GAAAACTTGGCCAAATCCACC
OAS	Fw: GTGTGTCCAAGGTGGTAAAGG
	Rv: CTGCTCAAACTTCACGGAA
VDR	Fw: TGCTATGCACTGTGAAGGCGT
	Rv: AGTGGCGTCGGTTGTCCTT
CYP24A1	Fw: CGCAAATACGACATCCAGGC
	Rv: AATACCACCATCTGAGGCGT
SOCS-1	Fw: CACTTCCGCACATTCCGTTC

	Rv: CACGCTAAGGGCGAAAAAGC
DENV-2	Fw: CAATATGCTGAAACGCGAGAGAAA
	Rv: CCCCATCTATTCAGAATCCCTGCT
CAMP	Fw: GGATGCTAACCTCTACCGC
	Rv: AGGGTCACTGTCCCCATACA
Ubiquitin	Fw: CCCTTCAAACCACCTAAGGTTGC
	Rv: GTGCTGGAGACCACTGTGATCG



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.

CHAPTER 5

Inhibition of inflammatory miRNAs expression by Vitamin D modulates the inflammatory response of macrophages during DENV-2 infection

Jorge Andrés Castillo, and Silvio Urcuqui-Inchima*

a. Grupo de Inmunovirología. Departamento de Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No 52-21, Medellín, (Antioquia), Colombia

* Corresponding Author: silvio.urcuqui@udea.edu.co

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ABSTRACT

Dengue disease caused by dengue virus (DENV) infection is the most common vector-borne viral disease transmitted worldwide. Nowadays, no specific treatment is available to fight dengue symptoms. Vitamin D (VitD3) has arisen as a novel therapeutical candidate, as we and others, have demonstrated its antiviral and immunomodulatory properties during DENV infection. MicroRNAs (miRNAs) are small non-coding RNAs responsible for regulating several cell processes including antiviral defense. Here, we evaluated the effect of VitD3 supplementation in the regulation of miRNAs expression in monocyte-derived macrophages (MDMs). For this, 1000 o 4000 IU of VitD3 supplementation was given to healthy individuals and after 10 days, miRNA expression was evaluated using qPCR array in MDMs obtained from these individuals and challenged with DENV-2. Our results showed that supplementation with VitD3, differentially regulated the expression of several miRNAs in MDMs obtained after finishing treatment and infected *in vitro* with DENV-2. This included the downregulation of the inflammatory miR-182-5p, miR-130a-3p, miR125b, among others. We further confirmed the regulation of some miRNAs *in vitro* and found that differentiation of MDMs in the presence of VitD3 decreased the expression of the inflammation-linked miRNAs miR-182-5p, miR-130a-3p, miR125b, miR146a and miR-155-5p. Also, we inhibited the expression of selected miRNAs (miR-182-5p, miR-130a-3p, miR-125b-5p and miR-155-5p), as observed with VitD3 treatment. Our results showed that inhibition of miRNA expression led to decreased of TNF-α and TLR9 expression, while increased expression of SOCS-1, IFN-β and OAS1 was observed. Notably, inhibition of miRNAs did not affect DENV replication levels. In conclusion, our results suggest that VitD3 immunomodulatory effects involve the regulation of some inflammation-linked miRNAs.

KEYWORDS

MicroRNAs, inflammation, dengue virus, vitamin D, innate immunity, Type I intereferon

Chapter 5

INTRODUCTION

Dengue disease is caused by the transmission of the arthropod-borne dengue virus (DENV) and is considered a significant health problem in developing countries worldwide [1]. The World Health Organization (OMS) estimates that about 50% of the world population lives in areas where the DENV transmitter mosquito Aedes spp. is present, including territories of the Americas, Africa, South-East Asia and the Eastern Mediterranean [2]. This transmission of DENV causes an estimated of 390 million annual infections, of which approximately 100 million are symptomatic causing 10,000 deaths [2,3]

An exacerbated and sustained inflammatory response is a hallmark of DENV pathogenesis [4,5]. Therefore, the development of new therapeutics that can modulate the inflammatory response while also restrict DENV replication is needed. Vitamin D (VitD3) is a pleiotropic hormone that has a wide immunoregulatory effect [6]. Indeed, VitD3 downregulates the expression of several Toll-like receptors (TLRs) in monocytes and thus contributes to the amelioration of the inflammatory response triggered by IFN-γ, LPS, or lipoteichoic acid [7,8]. This suggests that VitD3 could protect from exacerbated inflammatory responses. We and others have previously shown that VitD3 decreases DENV infection and replication and the production of proinflammatory cytokines *in vitro* [9–12]. In addition, we reported a decreased susceptibility to DENV-2 infection and production of proinflammatory cytokines by MDMs and monocyte-derived dendritic cells (MoDCs) obtained from healthy individuals who received a high-dose oral supplementation of VitD3 for 10 days [13,14]. This body of evidence demonstrates that VitD3 has a wide immunomodulatory and antiviral effect against DENV infection. However, the specific mechanisms by which VitD3 regulates the inflammatory response during DENV infection remains unclear.

VitD3 can regulate the expression of several genes that harbors Vitamin D response elements [15,16]. One of these sets of regulated genes could be those encoding microRNAs (miRNAs), which are small no codifying RNAs of 20-25 nucleotides of length that post-transcriptionally regulates the expression of a great number of genes involved in the regulation of cell development, cell division, metabolism, cell death and in the pathogenesis of viral infections [17]. It has been shown that MoDCs treated *in vitro* with VitD3 produce lower levels of IL-23 and lower expression of miR-155-5p, while levels of miR-378 are higher

[18]. Likewise, VitD3 treatment in murine macrophages reduces the expression of miR-155-5p, which in turn increases the activity of SOCS-1 leading to a decreased inflammatory response after TLR4 activation [19]. Similarly, VitD3 treatment in human adipocytes reduces the expression of miR-146a-5p, miR-150 and miR-155-5p during the stimulation with TNF-α [20]. These results suggest that VitD3 can regulate the expression of some miRNAs that are over-expressed in various inflammatory conditions.

Therefore, this study aimed to evaluate the regulation of miRNAs expression by VitD3 during DENV-2 infection. To do so, we did a comparative miRNA expression profile of DENV-2 infected monocyte-derived macrophages (MDMs), obtained from healthy individuals that took 1000 or 4000 UI/daily of VitD3 for 10 days. Also, we confirmed the modulation of the expression of some inflammation-linked miRNAs by VitD3 *in vitro,* using MDMs differentiated in the presence of VitD3 and infected with DENV-2. We further determined the role of these miRNAs in the inflammatory response by inhibiting their expression in MDMs and assessing the expression of pro-inflammatory cytokines, PRRs, SOCS-1, IFN-I, and IFN-stimulated genes (ISGs). Altogether our results showed that VitD3 treatment can modulate the expression of inflammatory miRNAs *in vivo* and *in vitro*, which can be associated with decreased expression of TNF-α and TLR9 and increased expression of SOCS-1, IFN-β and OAS1.

MATERIALS AND METHODS

Ethics statement

The Committee of Bioethics Research of Sede de Investigación Universitaria, Universidad de Antioquia (Medellín, Colombia), approved the protocols for individual enrollment and sample collection, which was preceded by a signed informed consent form, according to the principles expressed in the Declaration of Helsinki.

Study subjects

We followed the same strategy described in [21]. Briefly, 10 healthy volunteers were randomly grouped into two groups to receive 1000 IU/day or 4000 IU/day of VitD (cholecalciferol) for 10 days. An additional 5 healthy donors did not receive VitD

supplement and were used as controls. Volunteers agreed to participate in this study and signed a written informed consent before participation.

Cells and reagents

The mosquito C6/36 HT cell line was obtained from the American Type Culture Collection (ATCC) and cultured in Leibovitz L-15 medium (Sigma Aldrich, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Thermo Scientific, USA), 4 mM L-glutamine, 10 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma Aldrich, USA), at 34°C in an atmosphere without CO_2. BHK-21 cells, obtained from the ATCC, were maintained in D-MEM (Sigma Aldrich, USA), supplemented with 10% v/v FBS, 4 mM L-glutamine, 10 CFU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C with 5% CO_2, and used for plaque assays. A conjugated antibody against CD14 (clone M5E2) was purchased from eBioscience (USA).

Virus stocks and titration

DENV-2 New Guinea C was provided by the Centers for Disease Control and Prevention (CDC, USA). Viral stocks were obtained by inoculating a monolayer of C6/36 HT cells in a 75-cm2 tissue culture flask with the virus at a multiplicity of infection (MOI) of 0.05 diluted in L-15 supplemented with 2% FBS. After 3 h of adsorption, fresh L-15 medium supplemented with 2% FBS was added, and the cells were cultured for 5 days at 34°C without CO_2. The supernatant was obtained by centrifugation at 1000 × g for 5 min to remove cellular debris and then aliquoted and stored at −70°C until use. Virus titration was performed by the quantification of plaque-forming units (PFU) using a plaque assay. A total of 5 × 104 BHK-21 cells/well were cultured in 24-well plates in D-MEM supplemented with 2% v/v FBS, 4 mM L-glutamine, 10 U/mL penicillin, and 0.1 mg/mL streptomycin and incubated at 37°C with 5% CO_2 overnight. Next, cells 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, washed once with PBS and plaque medium of D-MEM containing 1.5% m/v carboxymethylcellulose sodium salt (medium viscosity, Sigma-Aldrich, USA), 3% $NaCO_3$, and 1% HEPES (Sigma-Aldrich, USA), and 10 CFU/mL penicillin, 0.1 mg/mL streptomycin, and 1% of FBS were added to the cells. Then, the cells were incubated at 37°C with 5% CO_2 for 5 days. Next, the plaque medium was removed, and the cells were washed twice with

PBS and stained with 4% m/v crystal violet solution and 3.5% v/v formaldehyde (Merck, Germany) for 30 min. After staining, cells were washed once with PBS, and the plaque count was performed manually to obtain PFU/mL.

Blood samples from healthy donors

Venous peripheral blood samples were obtained from healthy individuals aged 20–40 years who had not been previously vaccinated against yellow fever virus and were seronegative for the DENV NS1 antigen and DENV IgM/IgG, as determined by the SD BIOLINE Dengue Duo rapid test (Standard Diagnostics). All our experiments were performed with cells from at least six healthy donors.

Monocyte isolation and monocyte-derived macrophage differentiation (MDMs)

To obtain MDMs, peripheral blood mononuclear cells (PBMCs) were obtained from 50 mL of peripheral blood from healthy individuals with 2% v/v ethylenediaminetetraacetic acid, as described previously [17,18]. Briefly, the PBMCs were separated using density gradient centrifugation with Lymphoprep (STEMCELL technologies, USA) at 800 × g at room temperature for 20 min and then washed three times with phosphate-buffered saline (PBS) at 250 × g for platelet removal. The PBMCs were suspended in RPMI-1640 medium (Sigma Aldrich, USA) supplemented with 0.5% autologous heat-inactivated serum (30 min at 56°C). Monocytes were then obtained from the PBMCs by plastic adherence, as described previously [11]. Briefly, 1 × 105 cells were stained with anti-CD14-FITC (clone M5E2, BD Biosciences, USA) and analyzed by flow cytometry. The percentage of CD14+ cells was used to seed 5 × 105 CD14+ cells into 24-well plates (Corning Incorporated Life Sciences, USA) in RPMI-1640 medium supplemented with 0.5% inactivated autologous serum and cultured at 37°C with 5% CO_2 to allow enrichment of monocytes through plastic adherence. After 3 h of adherence, non-adherent cells were removed by extensive washing with pre-warmed PBS supplemented with 0.5% FBS. Adherent cells were then cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C with 5% CO_2 for 6 days to obtain MDMs. Fresh medium with 10% FBS was replenished every 48 h.

D3-MDMs differentiation in the presence of VitD3

Monocytes were differentiated for 6 days in the presence of 1α,25-dihydroxyvitamin D3 (VitD3; Sigma Aldrich, USA), at a concentration of 0.1 nM which represents the physiological and therapeutical concentration [22,23], as we have described previously [11,12]. The biological activity VitD3 was determined by us previously by the quantification of the induction of expression of VitD3 signaling targets, such as VDR and cytochrome P450 family 24 subfamily A member 1 (CYP24A1) [11,12]. The purity of D3-MDMs was repeatedly above 90%, as measured by the presence of contaminant cell populations, including CD19+, CD3+, and CD56+ (non-myeloid cells), and it can be observed in Supplemental Figure 1 of **Chapter 3**.

MDMs and D3-MDMs infection with DENV

Both MDMs and D3-MDMs monolayers were challenged with DENV-2 at an MOI of 5, diluted in 300 µl of RPMI-1640 medium supplemented with 2% FBS. Two hours post-infection (hpi), cells were washed with PBS, and the medium was replenished with RPMI 10% FBS and then cultured at 37°C 5% CO_2. At 2, 8, and 24 hpi, monolayers were harvested, and either the percentage of infection was determined by flow cytometry, or total RNA extraction was carried out and used for viral RNA quantification. In contrast the supernatants were used for viral titration by plaque assay and for quantification of cytokine production.

Expression profiling of miRNAs

Total RNA was isolated using a Direct-zol RNA miniprep kit (Zymo Research) as described by the manufacturer. Total RNA quantity was determined by the absorbance at 260 nm and quality by the integrity of the 28S and 18s RNA in a 1% Agarose gel RNA samples from different donors were pooled, and cDNA synthesis was performed using the miScript HiSpec Kit (Qiagen, Valencia, CA, USA).Global miRNA profiling of Control (CT), Volunteers supplemented with 1000 IU (VD1000) and volunteers supplemented with 4000 IU (VD4000) were performed with the Immunopathology miScript miRNA PCR array from Qiagen (Cat MIHS104Z) for 84 mature miRNAs. The miRNA RT-qPCR analyses were performed as described by the manufacturer and run on the h Bio-Rad CFX PCR System. Finally, raw data from the array was retrieved and imported into miScript miRNA PCR array data sheets templates (Qiagen, USA). miRNAs expression was relative to control and

considered significant only when fold changes (FC) cutoffs were ≥ 1.5. Finally, the significant miRNA was visualized plotting a heat-map of the fold change respect to the control.

Quantification of miRNA expression

Total RNA was obtained from MDMs and D3-MDMs by using the kit Direct-zol RNA miniprep (Zymo Research, USA) following manufacturer´s instructions. The RNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). MicroRNA cDNA was synthesized from 1µg total RNA samples using specific miRNA stem-loop primers and TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). miRNA qPCR analysis was performed in a 15 µl reaction (TaqMan™ Gene Expression Master Mix for miRNAs; Thermo Fisher Scientific, USA), and run on a Bio-Rad CFX PCR System using the following cycle conditions: 95°C for 10 mins followed by 40 cycles of 95°C for 15 secs and 60°C for 1 min. The TaqMan Assays for miRNAs used were: miR-182-5p (Assay ID # 000597), miR-146a-5p (Assay ID # 000468), miR-130a-3p (Assay ID # A25576), miR-125b-5p (Assay ID # 000449), miR-155-5p (Assay ID # 002623), and RNU48 (Assay ID # 001006). RNU48 was used as a reference gene to normalize the miRNA. Relative quantification of miRNA expression was evaluated using the 2-ΔΔCT method. Cutoffs for significant changes were set at p-value ≤ 0.05.

Inhibition of miRNAs expression

To inhibit the expression of some selected miRNAs, MDMs were transfected with synthetic miR-182-5p, miR-130a-3p, miR-125b-5p and miR-155-5p antisense at a final concentration of 50 nM/well (Ambion, TX, USA), using DharmaFect (Thermo Scientific, NH, USA) according to manufacturer's instructions. At 24 h post-transfection, cells were infected with DENV-2 following the procedure described above. At 24 hpi, cell monolayers were harvested and the percentage of infection was measured by flow cytometry. The cell supernatants were used to quantify the viral RNA copies by RT-qPCR and viral titer by plaque assay.

Flow cytometry

Flow cytometry was used to assess the frequency of DENV-infected cells [17,18]. In detail, DENV infection was evaluated through the intracellular detection of DENV E antigen at 24 hpi and the cells were fixed using a fixation/permeabilization buffer (eBioscience, USA). Following washing steps with PBS, cells were stained with the monoclonal antibody, 4G2 (Millipore, Germany) for 40 min, followed by 40 min staining with goat anti-mouse IgG-FITC (Thermo Scientific, USA). Data acquisition and analysis were done using the BD FACScan system and FACSDiva software.

Quantitation of viral RNA copies

Total RNA was purified from DENV-2-infected and mock-infected MDMs and D3-MDMs using TRIzol reagent (Thermo Scientific, USA) following the manufacturer's instructions. The RNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA). Then, cDNA was synthesized using random primers from a standard concentration of 50 ng of RNA and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer's instructions. Viral RNA copies quantification with cDNA was carried out using the specific primers depicted in supplemental Table 1, as described previously [24]. qPCR was performed with Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) and analyzed with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The calculation of viral RNA copies was based on a standard curve of Ct values of 10-fold serial dilutions of a plasmid encoding the complete genome of DENV-2 of known length and concentration, as previously described [25].

Cytokine production

The levels of IL-6 and TNF-α were assessed in supernatants from MDMs and D3-MDMs infected with DENV-2 at 2, 8 and 24 hpi, using an ELISA assay (BD OptEIA, BD Biosciences, USA), following the manufacturer's recommendations.

Quantification of gene expression

The mRNA quantification of TLR3, TLR4, TLR9, RIG-I, IFN-α, IFN-β, protein kinase R (PKR), 2′-5′-oligoadenylate synthetase 1 (OAS1), CAMP, SOCS-1, and ubiquitin was performed in DENV-2-infected and mock-infected MDMs and D3-MDMs using qPCR.

Briefly, cDNA was synthesized from total RNA using random primers, a standard concentration of 50 ng of RNA, and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Then, qPCR was performed using Maxima SYBR Green (Thermo Scientific, USA), following the manufacturer's instructions with specific primers for each gene (Supplemental Table 1). The specificity of the amplification product was determined by melting curve analysis. The relative quantification of each mRNA was normalized to the constitutive gene, ubiquitin, and mock-treated MDMs and D3-MDMs from each time point evaluated (e.g. 2 hours mock MDM vs 2 hours DENV-2-infected MDMs), using the ΔΔCt method and reported as the fold change.

Statistical analysis

Comparisons between MDMs and D3-MDMs were undertaken using two-way variance analysis (ANOVA) and a Bonferroni test. A value of $p < 0.05$ was considered statistically significant. The calculation of these parameters was carried out using GraphPad Prism version 6 (GraphPad Software, USA) software.

RESULTS

VitD modulates the expression of miRNAs in DENV-2 infected MDMs

Earlier studies did by us showed that MDMs obtained from healthy donors that received a high supplement of VitD3 (4000IU/daily of cholecalciferol) for 10 days, exhibit lower DENV-2 infection and production of proinflammatory cytokines *in vitro* [21]. To explore the role of miRNAs as possible modulators of gene expression during VitD treatment, we performed a comparative miRNA expression profiling in MDMs obtained from healthy donors that took a VitD3 supplementation with 1000 or 4000 UI/daily for 10 days, and challenged *in vitro* with DENV-2 for 24 hours. From the 168 mature miRNAs profiled, 29 miRNAs were differentially expressed (fold change ≥ 2) in MDMs from donors supplemented with 1000 IU/daily, and 34 miRNAs were differentially expressed in MDMs from donors supplemented with 4000 IU/daily (Fig 1A). When we compared the miRNAs profile of the two doses of VitD, we found an overlap with few miRNAs, including miR-143-3p, miR-182-5p, miR-130a-3p and miR-125b-5p (Fig 1B). In addition, when we

compared the amount of differentially regulated miRNAs with both VitD doses, we found that the number of regulated miRNA were higher using a supplement of 4000 IU/daily compared to 1000 IU/daily supplement (Fig 1C).

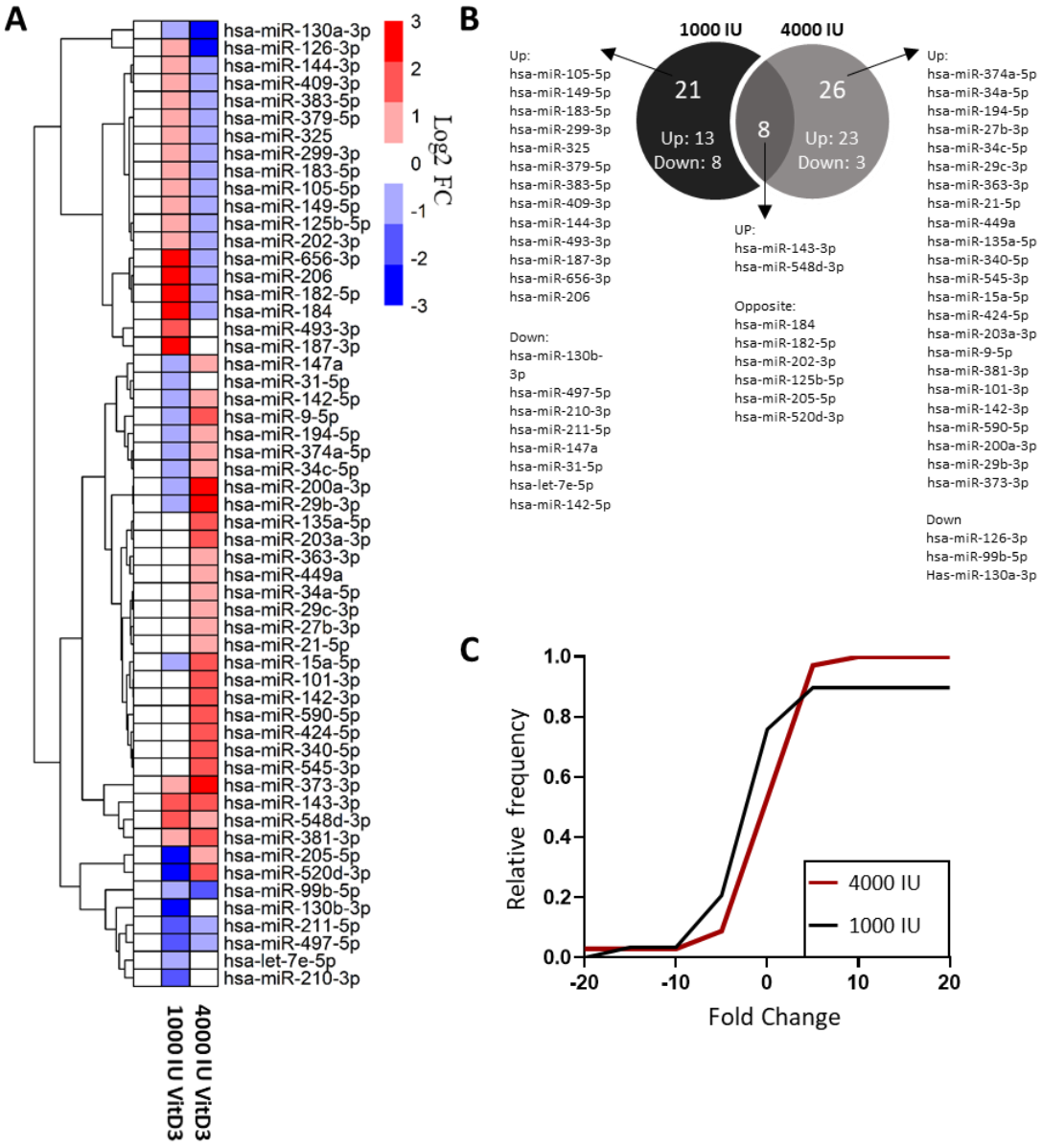

Figure 1. Different doses of VitD induce specific miRNA profiles in macrophages infected with DENV-2

Heatmap of Z-score normalized differentially expressed miRNAs of DENV-2 infected MDMs without VitD treatment and with two different vitD doses (VD1000 and VD 4000). Down-regulated and up-regulated genes with absolute values of fold-change > 1.5 and FDR < 0.05 (A). The Venn diagram shows common miRNAs between DENV-2 infected MDMs treated with 1000 IU or 4000 IU

of vitD (B). The relative frequency comparing the number and magnitude of differentially expressed miRNAs in DENV-2 infected MDMs corresponding to each vitD treatment is shown in (C).

From this set of differentially expressed miRNAs, we confirmed *in vitro* the regulation of the expression of miR-182-5p, miR-130a-3p and miR-125b-5p by VitD3 during DENV infection, since their expression has been associated with an inflammatory response in various diseases [26–28]. In addition, we included miR-146a-5p since its overexpressed early during DENV replication and mediates IFN-I evading, while previous reports have shown that VitD3 regulates its expression [20,29]. It was also included the analysis of miR-155-5p because we and others have previously described that VitD3 can regulate its expression, and is also associated with inflammation [19,30]. For this confirmation, monocytes were differentiated to macrophages in the absence (MDMs) or presence of 0.1nM of VitD3 (D3-MDMs) and expression of miR-182-5p, miR-130a-3p, miR-125b-5p, miR-146a-5p and miR-155-5p were compared during DENV-2 infection at 2, 8 and 24 hpi.

In the absence of VitD3, DENV-2 induced different kinetics of miRNA expression. MiR-182-5p expression increased at 8 and 24 hpi, while expression of miR-146a-5p and miR-155-5p significantly increased at 2 and 24 hpi compared to mock MDMs (Figure 2A, C and E). On the other hand, expression of miR-130a-3p in DENV-2 infected MDMs decreased at 24 hpi compared to mock MDMs (Figure 2B). Notably expression of miR-182-5p, miR-130a-3p and miR-146a-5p was significantly reduced in DENV-2 infected D3-MDMs compared to that observed in MDMs during DENV-2 infection (Figure 2A, B and C). We did not observe differences in the expression of miR-125b-5p and miR-155-5p between MDMs and D3-MDMs differentiated in the presence of 0.1nM of VitD3, during DENV-2 infection (Fig 2D and E). Since it has been reported that VitD3 can regulate the expression of these last two miRNAs [19,31], we differentiated D3-MDMs with increasing concentrations of VitD3 and compared the expression of miR-125b-5p and miR-155-5p to that observed in MDMs under DENV-2 infection. We found that miR-125b-5p and miR-155-5p were downregulated in D3-MDMs with VitD3 doses of 10nM and 1nM, respectively (Fig 2F and G). These results suggest that VitD3 modulates miR-125b-5p and miR-155-5p at different concentrations. In summary, data obtained using qPCR array analysis is consistent with *in vitro* experiments, and indicates that VitD3 downregulates the expression of some inflammatory-linked

miRNAs, including miR-182-5p, miR-130-3p, miR-146a-5p, miR-125b-5p and miR-155-5p in DENV-2-infected D3-MDMs.

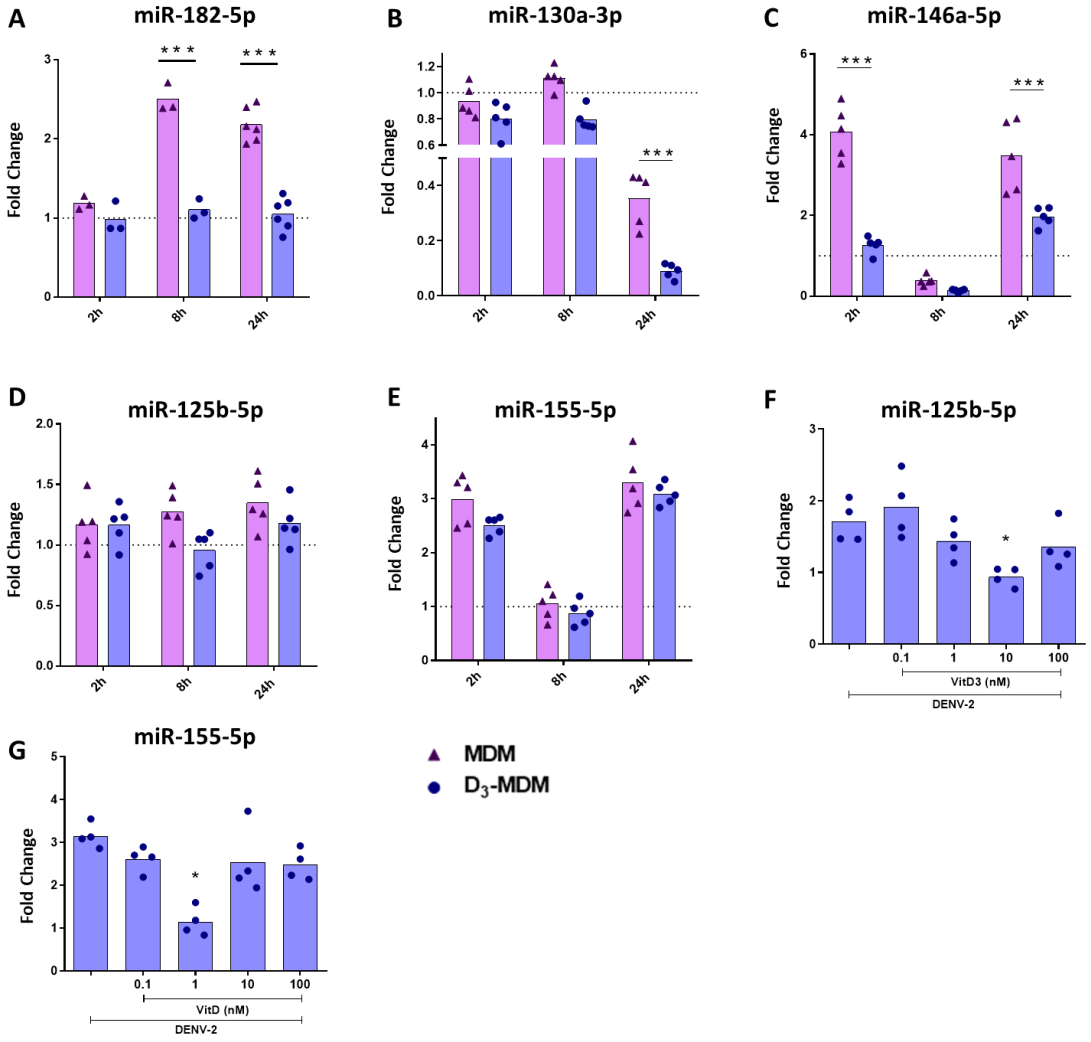

Figure 2. Differentiation of MDMs in the presence of VitD3 decreases the expression of miR-182-5p, miR-130a-3p, miR-146a-5p, miR-125b-5p and miR-155-5p during DENV-2 infection

The MDMs were differentiated in the presence of VitD3 (0.1 nM) for 6 days (D3-MDMs) and then infected with DENV-2 with an MOI of 5 for 2, 8, or 24 h. Expression of miR-182-5p (A), miR-130a-3p (B), miR-146a-5p (C), miR-125b-5p (D) and miR-155-5p (E) was measured by qPCR in MDMs and D3-MDMs using RNU48 as a housekeeper gene. Data are expressed as fold change relative to mock-treated MDMs and D3-MDMs from each time point. The MDMs were also differentiated with increasing concentrations of VitD3 from 0.1 to 100nM and then infected with DENV-2 for 24 h. Expression of miR-125b-5p (F) and miR-155-5p (G) was measured by qPCR in D3-MDMs using RNU48 as a housekeeper gene. Figures represent five individual experiments from different donors Differences were identified using a two-way ANOVA with a Bonferroni test for A-E and using a

Kruskal-Wallis test for F and G. In both cases a 95% confidence interval was used (***p < 0.001, **p < 0.01, *p < 0.05).

Inhibition of miR-182-5p, miR-130a-3p or miR-125b-5p in DENV-2 infected-MDMs lead to lower production of TNF-α

VitD3 decreased the expression of miR-182-5p, miR-130-3p, miR-125b-5p and miR-155-5p, which have been associated with an inflammatory response in some diseases [20,26–28]. Furthermore, bioinformatic analysis showed that VitD3 regulates the expression of genes involved in stress and immune response in MDMs infected *in vitro* with DENV-2, by inducing specific miRNAs [32]. Thus, to test whether the inhibition of miR-182-5p, miR-130a-3p, miR-125b-5p or miR-155-5p by VitD3 modulated the inflammatory response of MDMs after DENV-2 infection, we transfected MDMs with anti-sense oligonucleotides of each of these miRNAs, inducing in this way, a miRNA duplex and therefore inhibiting their activity. After 24 of transfection, MDMs were infected with DENV-2 for 24 hours, and the production of TNF-α and IL-6 was quantified. The inhibition of each miRNA was first confirmed through qPCR. The inhibition of each miRNA resulted in a 10-fold decrease in expression (Supplemental Fig 1A). Importantly, the inhibition of tested miRNAs did not affect MDMs viability under DENV-2 infection conditions (Supplemental Fig 1B).

While inhibition of miR-182-5p, miR-130a-3p, and miR-125b-5p significantly decreased the production of TNF-α in DENV-2 infected MDMs compared to MDMs transfected with scramble (miR) control (Fig 3A), the inhibition of miR-155-5p did not affect TNF-α production. Similarly, IL-6 production was not altered by the inhibition of any of the tested miRNAs (Fig 3B). In conclusion, these results suggest that miR-182-5p, miR-130a-3p and miR-25b-5p may contribute to the inflammatory response of DENV-2 infected MDMs as they can regulate TNF-α production.

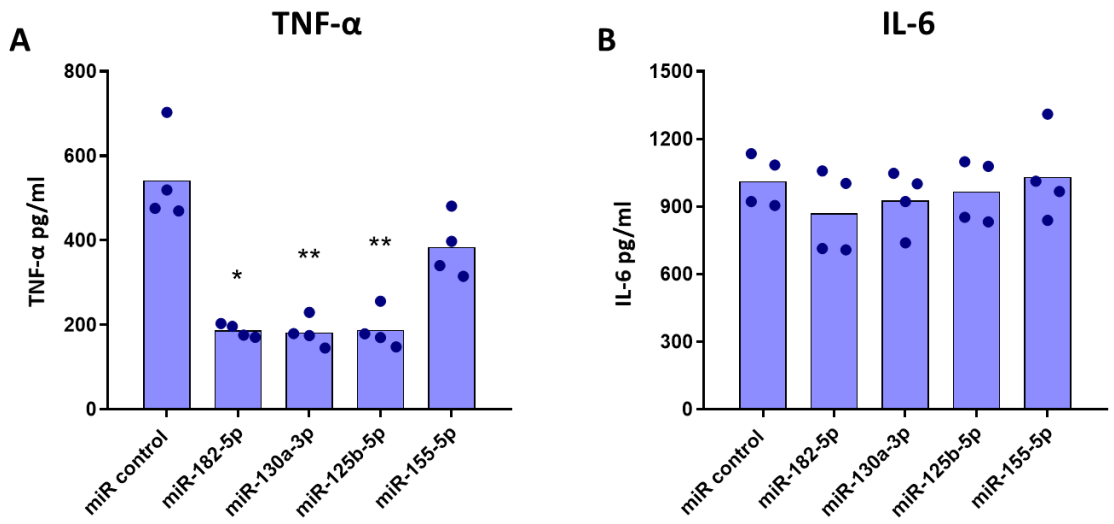

Figure 3. Inhibition of the expression of selected miRNAs decreases the production of TNF-α in DENV-2 infected MDMs

MDMs were transfected either with a miRNA scrambled negative control or with an anti-sense specific miRNA. 24 hours later, cells were infected with DENV-2 at an MOI of 5. At 24, the hpi production of TNF-α (A) and IL-6 (B) was quantified by ELISA. Figures represent four individual experiments from different donors. Differences were identified using a Kruskal-Wallis test with a 95% confidence interval was used (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Inhibition of miRNAs expression in DENV-2 infected MDMs altered TLR9 and SOCS-1 mRNA expression

Macrophages sense DENV infection through an array of PRRs including retinoic acid-inducible gene-I-like receptors (RLRs) such as RIG-I [33,34], and Toll-like receptors (TLRs) [35,36]. Activation of these PRRs can lead to the production of inflammatory cytokines. To test whether regulation of the inflammatory response was mediated by miRNAs inhibition and regulation of PRRs expression, we quantified expression levels of RIG-I, TLR3, TLR4 and TLR9 mRNA under inhibition of selected miRNAs in DENV-2-infected MDMs. Inhibition of miR-182-5p, miR-130a-3p, miR-125b-5p or miR-155-5p, did not regulate expression of RIG-I, TLR3, or TLR4 mRNAs in DENV-2 infected MDMs (Fig 4A, B and C). Yet, inhibition of miR-130a-3p, miR-125b-5p or miR-155-5p decreased expression of TLR9 mRNA in DENV-2 infected MDMs, compared to the levels of infected MDMs transfected with scrambled control (Fig 4D). However, it was only statistically significant for miR-130a-

3p. We further evaluated expression of SOCS-1 under miRNAs inhibition, as the protein encoded by this gene has an important role in the negative feedback of proinflammatory cytokine signaling [37]. Inhibition of miR-182-5p or miR-155-5p significantly increased the expression of SOCS-1 mRNA in DENV-2 infected MDMs, compared to infected MDMs treated with scramble control (Fig 4E). Altogether, these results suggest that miR-130a-3p, miR-125b-5p and miR155-5p regulation network may involve TLR9 in DENV-2 infected MDMs. Also, inhibition of miR-182-5p and miR-155-5p upregulates expression of SOCS-1 which could contribute to the regulation of inflammatory response.

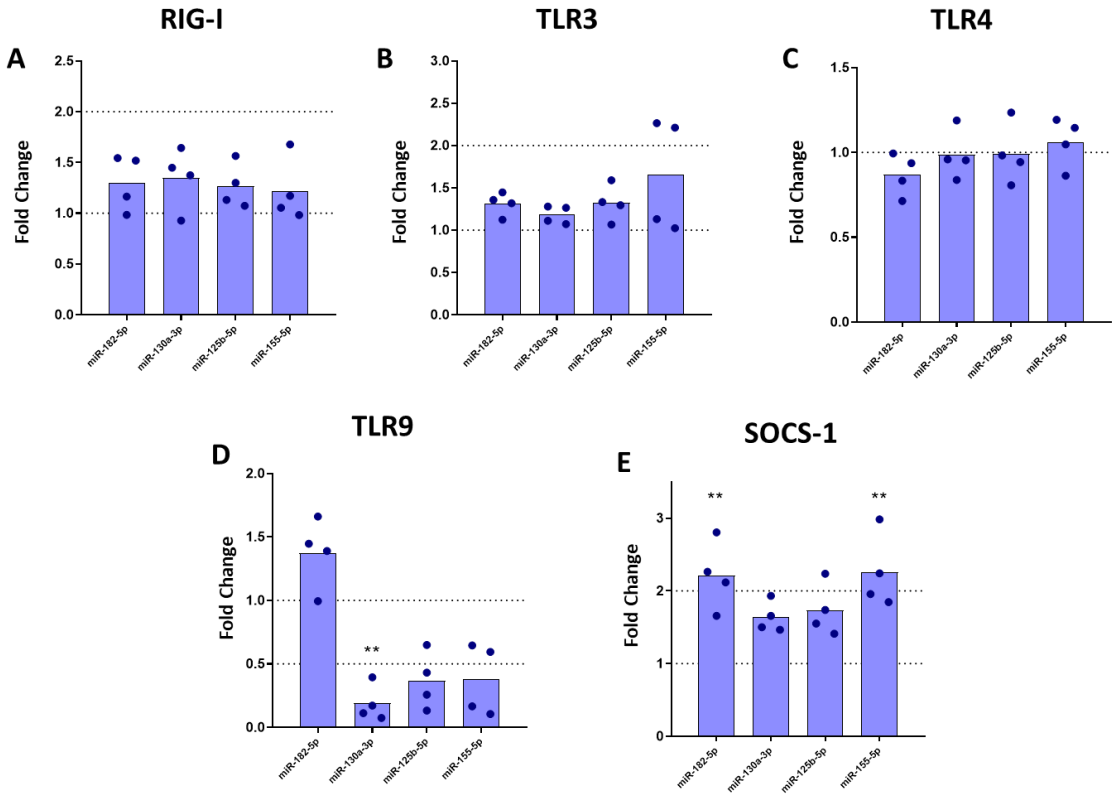

Figure 4. Inhibition of miR-130a-3p, miR-125b-5p and miR-155-5p decreases TLR9 expression, while inhibition of miR-182-5p and miR-155-5p increases the expression of SOCS-1 in DENV-2 infected MDMs

MDMs were transfected either with a miRNA scrambled negative control or with an anti-sense specific miRNA. 24 hours later, cells were infected with DENV-2 at an MOI of 5. At 24 hpi, expression of RIG I (A), TLR3 (B), TLR4 (C), TLR9 (D) and SOCS-1 was measured by qPCR in MDMs using the gene encoding RNU48 as a housekeeper gene. Data are expressed as fold change relative to DENV-2 infected MDMs transfected with miRNA scrambled control. Figures represent four individual experiments from different donors. Differences in expression between samples and miRNA

scrambled transfected controls were identified using a Kruskal-Wallis test with a 95% confidence interval was used (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Inhibition of miR-182-5p, miR-130a-3p, miR-125b-5p or miR-155-5p in DENV-2 infected MDMs increased IFN-I and OAS1 mRNA expression

We have previously shown that VitD3 induces a partial resistance to DENV-2 infection in MDMs via down-regulation of mannose receptor [11,12] and increased expression of LL-37 **(chapter 4)**. To test whether inhibition of inflammatory-linked miRNAs regulated by VitD3 could lead to improved antiviral response, we evaluated the expression of IFN-α, IFN-β, PKR, OAS1 and CAMP mRNAs under miRNAs inhibition in DENV-2 infected MDMs. Inhibition of miR-125b-5p and miR-155-5p expression induced a statistically significant increase of IFN-α mRNA levels in DENV-2 infected MDMs compared to scrambled miRNA transfected MDMs (Fig 5A). Similarly, inhibition of miR-182-5p, miR-130a-3p and miR-155-5p significantly increased IFN-β mRNA expression in DENV-2 infected MDMs compared to MDMs transfected with scrambled miRNA (Fig 5B). While inhibition of miR-182-5p, miR-130a-3p, miR-125b-5p and miR-155-5p did not affect the expression of PKR mRNA (Fig 5C), inhibition of miR-125b-5p and miR-155-5p in DENV-2 infected MDMs increased mRNA expression of OAS1 (Fig 5D). Surprisingly, inhibition of miR-182-5p and miR-155-5p significantly reduced CAMP mRNA expression levels in DENV-2 infected MDMs compared to scrambled control (Fig 5E). Altogether, these results suggest that modulation of some miRNAs may increase the expression of IFN-I and OAS1 mRNA in DENV-2 infected MDMs.

Inhibition of miR-182-5p, miR-130a-3p, miR-125b-5p or miR-155-5p do not affect DENV-2 replication in MDMs

So far, we have shown that inhibition of miRNAs, similar to VitD3 treatment, decreased the production of TNF-α, modulated the expression of some PRRs and increased expression of IFN-I and OAS1. However, DENV-2 replication levels under those miRNA inhibitors are unknown and could explain the regulation of inflammatory response and PRR expression. Therefore, we tested if inhibition of the expression of the miRNAs regulated by VitD3 could

also impact on DENV-2 replication in MDMs. For this, MDMs were transfected with each anti-sense inhibitor of miR-182-5p, miR-130a-3p, miR-125b-5p, and miR-155-5p. After 24 hours of transfection, MDMs were challenged with DENV-2 for 24 hours, and viral replication was evaluated.

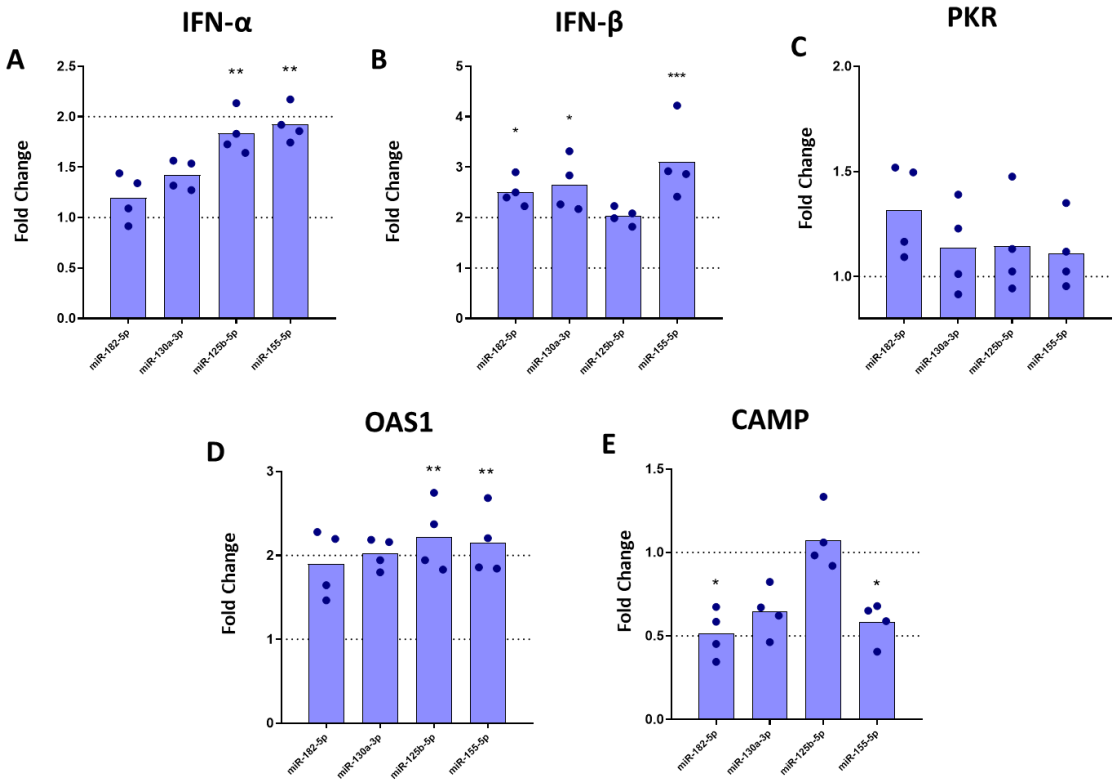

Figure 5. Inhibition of selected miRNAs modulates the expression of IFN-β, OAS1 and CAMP in MDMs during DENV-2 infection

MDMs were transfected with anti-sense specific miRNAs inhibitors or with a miRNA scrambled negative control. 24 hours later, cells were infected with DENV-2 at an MOI of 5 for and additionally 24 hours. Expression of IFN-α (A), IFN-β (B), PKR (C), OAS1 (D) and CAMP was measured by qPCR in MDMs using the gene encoding RNU48 as a housekeeper gene. Data are expressed as fold change relative to mock-treated MDMs transfected with miRNA scrambled control. Figures represent four individual experiments from different donors. Differences in expression between samples and miRNA scrambled transfected controls were identified using a Kruskal-Wallis test with a 95% confidence interval was used (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

Inhibition miR-182-5p, miR-130a-3p, miR-126b-5p and miR-155-5p had no effect in DENV-2 replication, as the percentage of E+ cells (Fig 6A and 6B) and viral titer (PFU/ml; Fig 6C) were not changed, suggesting that effect of miRNAs inhibition on TLR9, CAMP, IFN-I, OAS1 and SOCS-1 expression is independent of viral replication. Altogether, our results show that inhibition of the activity of miR-182-5p, miR-130a-3p, miR-125b-5p, and miR-155-5p do not affect viral replication.

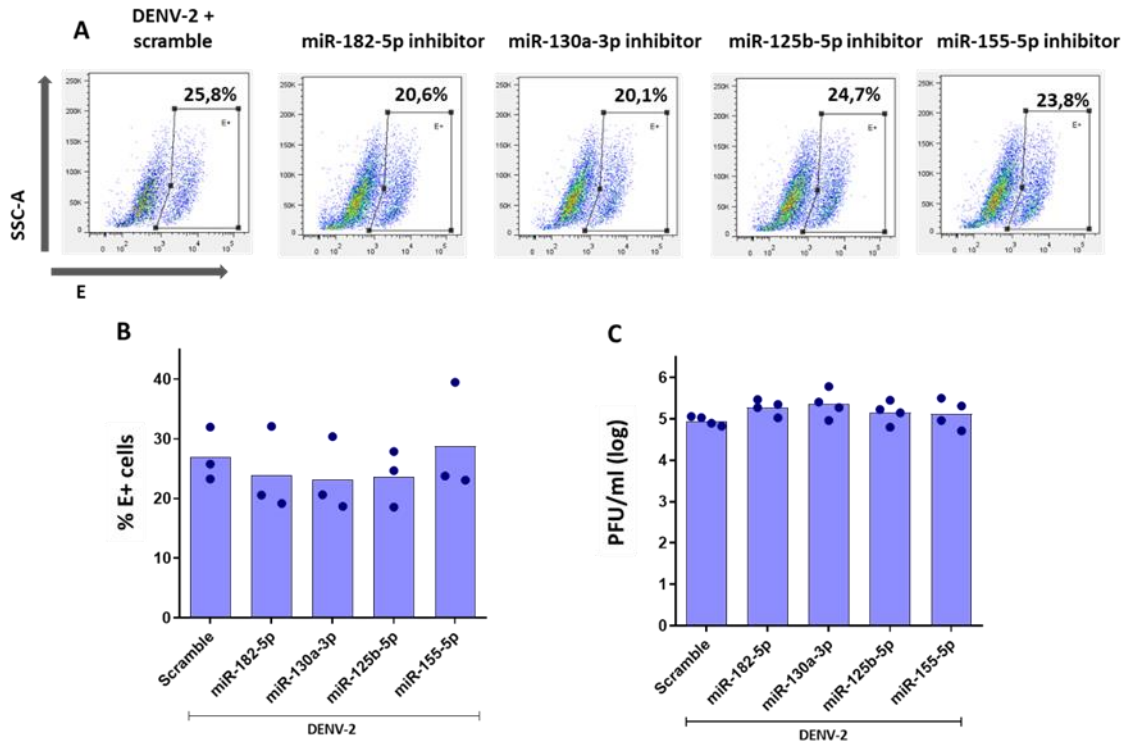

Figure 6. Inhibition of selected miRNAs did not affect DENV-2 replication in MDMs

MDMs were transfected either with a miRNA scrambled negative control or with an anti-sense specific miRNA. 24 hours later, cells were infected with DENV-2 at an MOI of 5. At 24 hpi, expression DENV-2 infection was evaluated by the staining of viral envelope (E) protein and detected by flow cytometry (A, B). Viral replication was evaluated at 24 hpi using plaque assay (C). Figures represent four individual experiments from different donors. Differences were identified using a Kruskal-Wallis test with a 95% confidence interval was used (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

DISCUSSION

MicroRNAs (miRNAs) are small non-coding RNAs that regulate several cellular functions including cell division, metabolism and immune response [17]. In this study we evaluated the regulation of miRNAs expression by VitD3 in MDMs during DENV-2 infection. Our data showed that VitD3 supplement in healthy individuals is able to differentially regulate the expression of several miRNAs, including miR-182-5p, miR-130a-3p and miR-125b-5p, which have been associated with inflammatory response of some diseases. Detailed experiments revealed that inhibition of certain miRNAs that are decreased by VitD3, decreased the production of TNF-α, down-regulated the expression of TLR9 and increased the expression of IFN-I, OAS1 and SOCS-1. Importantly, inhibition of miRNAs expression did not affect DENV-2 replication, suggesting that the effects in the immune response were independent of viral replication. Altogether, our results suggest that some immunomodulatory effects of VitD3 observed in MDMs during DENV-2 infection may be mediated by the regulation of the expression of some miRNAs.

DENV pathogenesis is mediated by an uncontrolled and exacerbated inflammatory response. In recent years, the involvement of miRNAs in the regulation of inflammation and antiviral response have been detailed. For example, stimulation with LPS, Poly (I:C), CpG-ODN or IFN-β induces substantial expression of miR-155 in murine macrophages, which is induced by JNK signaling pathways [38]. Similarly, miR-125b modulates the inflammatory state of macrophages by targeting and restricting the expression of B7-H4 protein, an important inhibitory molecule that suppresses T cell function [27]. In this sense, our results showed that DENV-2 infection in MDMs was accompanied by an increased expression of several inflammatory linked miRNAs including miR-182-5p, miR-130a-3p, miR-146a-5p, miR-125b-5p and miR-155-5p. In line with our results, Wang et al. demonstrated that the leukotriene B4 positively regulates expression of miR-155, miR146b and miR-125b promoting an inflammatory state via suppression of SOCS-1 expression and increasing MyD88 expression [39]. Further, the serine/threonine kinase Akt induced by LPS stimulation in murine macrophages, upregulates the expression of miRNA let-7e, miR-155, miR-181c and miR-125b [40]. The differential expression of some inflammatory miRNAs could contribute to the development of severe dengue, since differential expression of some miRNAs have been observed in patients with severe disease, including miR-6499, miR-122, miR-486, miR-383 and miR-146a [41–43]. Overall, some miRNAs, with special emphasis on

miR-155, miR146 and miR-125b, are involved in the inflammatory response of macrophages, and could be contributing to the exacerbated inflammation observed during DENV infection.

Innate immune response is highly regulated by the activation of NF-κB transcription factor, and its dysregulation can lead to an exacerbated/sustained inflammatory response [44]. MiRNAs have an important role in NF-κB pathway either by regulating its activation indirectly or by being induced by NF-κB signaling [45]. Inflammatory signals in hepatocytes, adipocytes and MoDCs lead to NF-κB activation and increased expression of miR-155 and miR-146 [20,46,47]. These findings suggest that expression of these miRNAs is important for inflammation and clearance of viral infections. However, a fine tuning is probable to occur to compensate the inflammatory response. Mann et al. showed miRNA based regulatory network in which miR-146a repressed the activation of NF-κB induced by miR-155 in mouse macrophages, representing a negative feedback for the resolution of the inflammatory response [48]. In this study, we also observed an increased expression of miR-146a-5p and miR-155-5p in DENV-2 infected MDMs. Surprisingly, during DENV-2 infection the kinetics of expression was the same for the two miRNAs, which may suggest that miR-146a could also represent a negative feedback for the inflammatory response induced by miR-155 during DENV infection. It is also possible that DENV is suppressing this tuning which may contribute to an exacerbated inflammatory response, hallmark of severe dengue. In fact, miR-125b is over-expressed in nasopharyngeal carcinoma promoting NF-κB activation and cell proliferation [49], while miR-155 is over-expressed in mouse macrophages after TNF-α stimulation, promoting NF-κB activation and enhancing TNF-α translation [50]. Overall, these results suggest that miR-125b, miR-146a and miR-155 are involved in NF-κB activation and regulation network during DENV-2 replication, and could contribute to the pathogenesis of DENV infection.

Importantly, we found that VitD3 regulated the expression of various inflammatory-linked miRNAs. Of these, VitD3 was able to downregulate expression miR-182-5p, miR-130a-3p, miR-146a-5p, miR-125b-5p and miR-155-5p during *in vitro* DENV-2 infection of macrophages. Since progression to severe dengue is associated with an exacerbated inflammatory response, we propose that VitD3 has a potential immunomodulatory effect during DENV infection by regulating miRNAs expression. In other diseases with an

inflammatory milieu, VitD3 has also been shown to modulate miRNAs expression. For example, in HUVECs under treatment with serum albumin and glucose (diabetic-like environment) supplementation of VitD3 down-regulate the expression of several miRNAs [51]. Similar results have been observed in human adipocytes stimulated with TNF-α, in which VitD3 reduces the expression of miR-146a-5p, miR-150 and miR-155-5p [20]. Also, during pregnancy, there is a differential expression of miRNAs between women that show insufficient levels of circulating VitD (<25.5 ng/ml) compared to women with sufficient levels of VitD (>31.7 ng/ml) [52]. Altogether, these results show that VitD3 can regulate the expression of various miRNAs involved in inflammatory disorders, including DENV infection.

Suppressor of cytokine signaling proteins (SOCS) are members of a family of intracellular cytokine inducible proteins necessary for the regulation of inflammatory response [53]. In neutrophils treated with VitD3 and infected with *Streptococcus pneumoniae*, there is an increased expression of SOCS-1 and SOCS-3 compared with non-treated neutrophils [52]. Further, we have shown that VitD3 treatment in MDMs increases expression of SOCS-1 during DENV-2 infection [12]. These data suggest that upregulation of SOCS proteins by VitD3 could partially explain its immunomodulatory activity. Here, we detailed the modulation of SOCS-1 by VitD3 and found that inhibition of miR-182-5p and miR-155-5p significantly increased expression of SOCS-1, which are miRNAs downregulated by VitD3. Chen at al. also demonstrated that VitD3 treatment in mice restricted miR-155-5p expression, promoting an increased expression of SOCS-1 and an attenuated inflammatory response to LPS [19]. Interestingly, SOCS-1 3´UTR has target sequences for miR-155-5p, miR-572, miR-221 and miR-150 [54], which explains its downregulation when miR-155-5p is overexpressed. Of note, Chen et al. found that augmented expression of miR-150 and depressed expression of SOCS-1 was associated with severe dengue in patients infected with DENV [54]. However, in our analysis miR-150 was not modulated by VitD3. Altogether, these results demonstrate the inflammatory potential of miR-155-5p and miR-150 during DENV infection by decreasing expression of SOCS-1, which can be modulated further modulated by VitD3 treatment.

Notably, regulation of mRNA levels of TLR9, CAMP, IFN-I, OAS1 and SOCS-1 by miRNAs inhibition could not be confirmed at the protein levels, which is a limitation of our study.

However, the main mode of action of miRNAs is mRNA decay [55], which translates in lower quantities of mRNA as we detected by RT-qPCR. If the mRNA levels correspond to protein levels should be further studied. For example, it could be occurring that in our model, different levels of IFN-I and OAS1 mRNAs and protein levels are being produced, thus explaining why miRNA inhibition did not affect DENV-2 replication even with higher levels of antiviral IFN-I and OAS1 mRNAs. Another possible explanation for DENV-2 replication levels under increased expression of IFN-I is the evasion of this antiviral system by DENV-2. It has been shown that DENV interferes with IFN-I signaling by blocking the activation of STAT1 and STAT2 through the activity of the non-structural proteins NS4B and NS5 [56,57]. This could also explain why we did not observe changes in PKR mRNA expression under miRNA inhibition, even though high levels of IFN-α and IFN-β were seen. We could not answer why miRNA inhibition did not affect DENV-2 replication levels despite increased levels of IFN-I and OAS1, a question that could be addressed in the future.

In conclusion, this study demonstrated that VitD3 regulates the expression of various miRNAs involved in several inflammatory disorders, which could represent a novel immunomodulatory mechanism of VitD3 in addition to other effects we have reported previously, such as PRRs regulation, decrease of ROS production and increased expression of SOCS-1 [11,12,21]. Our data suggest that the mechanisms behind this effect could be regulation of miRNAs expression, since their inhibition had the same effect as VitD3 treatment.

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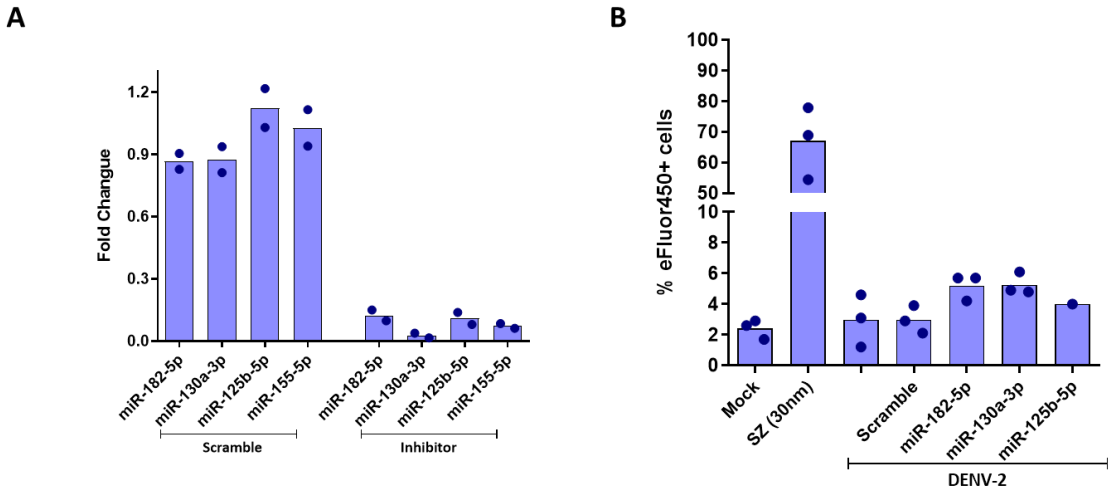
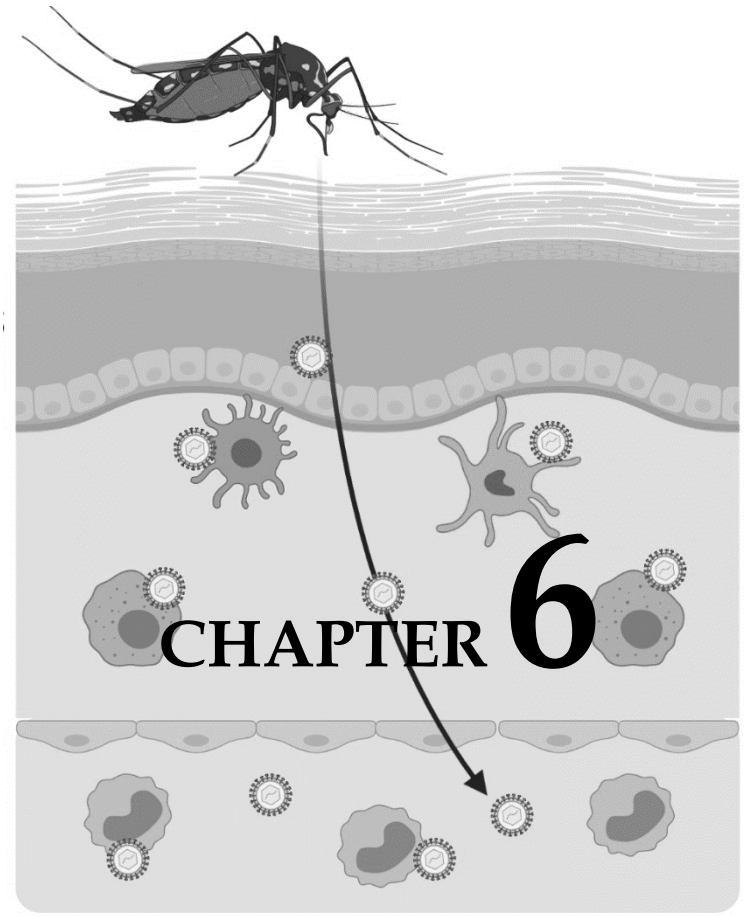
SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Efficiency and effect on the viability of MDMs after inhibition of miRNAs during DENV-2 infection

MDMs were transfected either with a miRNA scrambled negative control or with an anti-sense specific miRNA. 24 hours later, the efficiency inhibition was evaluated by measuring the expression of miR-182-5p, miR-130a-3p, miR-146a-5p, miR-125b-5p and miR-155-5p by qPCR using RNU48 as a housekeeper gene. Data are expressed as fold change relative to mock treated MDMs (A). MDMs viability after miRNAs inhibition was evaluated by measuring the percentage of positive cells for eFour450 staining by flow cytometry in mock, Sulfasalazine (SZ, 30nM) and DENV-2 infected MDMs (B). Figures represent two individual experiments from different donors.



CHAPTER 6

Summarizing discussion and concluding remarks

GENERAL SUMMARY

Dengue disease caused by four serotypes of DENV infection represent a major concern of public health world-wide [1]. Although most infections are clinically inapparent or show an acute flu-type syndrome, approximately 96 million of infected individuals require hospitalization. The severe manifestations are rooted in aberrant inflammatory responses and increased permeability of vascular endothelium.

The extensive activation of innate immune cells that encounter the virus in early stages of viral infection, is responsible for a massive production of inflammatory mediators that eventually target the endothelial cells (ECs), inducing a transient increase in permeability. In this context, monocytes and macrophages have an essential role mediating innate immune responses against DENV, as they harbor high levels of viral replication and produce a vast variety of inflammatory chemokines and cytokines that contribute to systemic inflammation. In this thesis we evaluated to which extent TLR2 and other PRRs that recognize DENV-2 components, mediate the inflammatory response and how these contribute to endothelial permeability. Furthermore, we evaluated how immunomodulatory compounds like Vitamin D (VitD3) and LL-37 influence innate immune response of macrophages to DENV-2 infection.

In **chapter 2** we dissected the mechanisms underlying TLR2-mediated activation in DENV-2 infected monocytes. By using purified monocytes, we demonstrated that recognition of DENV-2 by TLR2 induces an NF-κB-dependent inflammatory and antiviral responses that were able to trigger endothelial inflammatory activation. In addition, TLR2 along with CD14 and CD36 functioned as a viral receptor facilitating DENV-2 entry, highlighting the dual role of TLR2 axis during viral replication in monocytes: on one hand, TLR2 mediated cell activation and immune responses of monocytes, and on the other hand, TLR2 mediated viral entry and replication. Previous work in our group showed elevated expression of TLR2 on CM but not in IM and NM. In **chapter 2,** we dissected the role of TLR2 in different monocytes subsets by sorting them from PBMCs. Different pattern of TL2 co-receptors in monocyte subsets led to a different susceptibility of CD16- (CM) and CD16+ monocytes (IM and NM) to DENV-2 infection, and revealed that TLR2 mediated viral infection only in CD16- monocytes. These findings explain why a sustained and relatively highest expression of

TLR2 only on CM was associated with severe dengue in a pediatric cohort that we studied before. Altogether, the findings described in **chapter 2** provide evidence for the dual role of TLR2 axis in DENV-2 replication in monocytes subsets and its contribution to systemic inflammation and increased endothelial permeability.

Therapeutic drugs that can prevent or regulate the excessive immune response to DENV infection would be highly desirable to mitigate dengue burden. In **chapter 3** we described the regulation of innate immune responses to DENV-2 infection in MDMs differentiated in the presence of VitD3 (D3-MDMs), a pleiotropic hormone that has wide immunoregulatory properties. We found that D3-MDMs expressed lower levels of several PRRs including RIG-I, TLR3, TLR7 and TLR9, while produced lower quantities of IL-6 and TNF-α in response to DENV-2 infection and compared to their MDMs counterpart. Down-regulation of TLR9 in D3-MDMs was associated with lower production of reactive oxygen species in response to DENV-2 infection. Furthermore, we found that although VitD3 treatment did not modulate expression of IFN-I, D3-MDMs expressed higher levels of ISGs PKR and OAS1 during DENV-2 infection, highlighting the intrinsic differences between MDMs and D3-MDMs. Altogether, the findings described in this study show that VitD3 can modulate the innate immune response in macrophages after DENV-2 infection.

VitD3 is known to enhance the expression of various antimicrobial peptides including LL-37. In **chapter 4**, we evaluated the baseline gene expression and production of LL-37 in MDMs that were either differentiated in the absence or presence of VitD3 during DENV-2 infection. Notably, irrespectively of DENV-2 infection, we found very low CAMP (LL-37 gene) expression and no detectable production of LL-37 in MDMs. Yet, differentiation of MDMs in the presence of VitD3 boosted the baseline levels of CAMP expression and LL-37 production. Since LL-37 has wide antiviral and also immunoregulatory properties, we further evaluated the antiviral and immunomodulatory effect of exogenous LL-37 in DENV-2 infected MDMs. We showed that only simultaneous exposure of MDMs to LL-37 and DENV-2 led to reduced infection and viral replication, whereas addition of LL-37 after DENV-2 infection had no effect on viral replication. This difference allowed us to identify direct immunomodulatory effect of LL-37 during DENV-2 infection. Reduced production of IL-6 and increased expression of TLR4, TLR9, PKR, OAS1 and SOCS-1 were due to LL-37 immunomodulation, whereas reduced production of TNF-α and expression of RIG-I and

TLR3 were due to a reduced DENV-2 replication. In summary, the results presented in **chapter 4**, demonstrated that besides its antiviral properties, LL-37 shows immunoregulatory activity in MDMs and its production can be boosted by treatment with VitD3 during DENV-2 infection.

Finally, in **chapter 5** we assessed the role of miRNAs expression in the regulation of innate immune response during treatment with VitD3. Our results showed that supplementation with VitD3, differentially regulated the expression of several miRNAs in MDMs during DENV-2 infection. Furthermore, the inhibition of such miRNAs, as observed with VitD3 treatment, led to decreased production of TNF-α and TLR9 expression, while increased expression of SOCS-1, IFN-β and OAS1 was observed. Notably, inhibition of miRNAs did not affect DENV replication levels. In conclusion, our results suggest that VitD3 immunomodulatory effects involve the regulation of some miRNAs.

Altogether, the studies presented in this thesis can be summarized in two key messages. First, TLR2 has a key role in DENV-2 infection and immune responses of CM underlying the contribution of this monocyte subset in DENV pathogenesis. Secondly, VitD3 and LL-37 have potential as therapeutic candidates as they show both antiviral and immunomodulatory effects against DENV-2 infection in human primary cells. Below I will discuss the relevance of TLR2 in the sensing of DENV and inflammatory response of monocyte subsets and its possible implications for development of severe dengue. Also, I will debate the feasibility of exploiting VitD3 and LL-37 as therapeutic strategies to prevent and/or mitigate the development of severe dengue disease.

PART I: Key roles of monocyte/macrophages in shaping innate immune responses against DENV infection

Monocytes and macrophages represent a key target cell for DENV replication. Further, they encounter the virus in several moments such as site of the mosquito bite [2], in the lymph nodes [3] and in other types of tissues like liver, spleen and bone marrow once DENV disseminates [4,5]. Indeed, as we also showed in **chapter 2,** among peripheral blood mononuclear cells (PBMCs), monocytes represent the main target for DENV-2 replication and induced inflammatory responses, as it has been reported by other studies [6–9]. Owing

to their function as innate sentinels and targets of DENV replication, monocytes and macrophages play key roles not only in the initiation of the innate defense, but also in the exacerbation of the inflammatory response underlying severe dengue pathogenesis. In the following sections I will discuss the current understanding of how TLRs expressed in different monocytes subsets and macrophages contribute to the shaping of innate immune response and its contribution to endothelial dysfunction and eventually progression to severe dengue.

1. Dual role of TLR2 in sensing of DENV

A number of viruses have been shown to activate TLR2 including yellow fever vaccine YF-17D virus [10] measles virus [11], human cytomegalovirus (CMV) [12], varicella zoster virus [13], hepatitis C virus (HCV) [14] human immunodeficiency virus 1 (HIV-1) [15] herpes simplex virus (HSV) [16] and recently DENV [17]. Accordingly, in **chapter 2,** we provided sufficient evidence showing the dual role of TLR2 during DENV infection, and its contribution to pathogenesis on CM **(Figure 1)**. On one hand, TLR2 axis facilitates binding of DENV particles and internalization into monocytes, thus promoting viral replication and release of infectious particles. In addition, recognition of DENV by TLR2 mediates activation of NF-κB and production of inflammatory and antiviral interferons, that ultimately induces ECs dysfunction.

Whether activation of TLR2 controls viral spread or is detrimental to the host is subject of discussion. For example, TLR2 activation by vaccina virus (VV) and CMV induce the production of IFN-β [18], which favored an antiviral state on neighbor cells and aid to restrict viral replication in infected cells[19]. Likewise, we found in **chapter 2** that TLR2 mediated production of IFN-I/III by monocytes during DENV-2 infection, suggesting a protective role for TLR2. In line with these arguments, TLR2 activation in NK cells is important for cell activation and control of murine CMV and VV infections [20,21]. Also, TLR2 activation seems to be important for the proper development of adaptive immune responses. In DCs, TLR2 activation promotes secretion of IL-6, which in turn drives Th2/Th17 T cell differentiation [22]. Similarly, activation of TLR2 in CD8 T cells by VV promotes survival and clonal expansion of these cells [23].

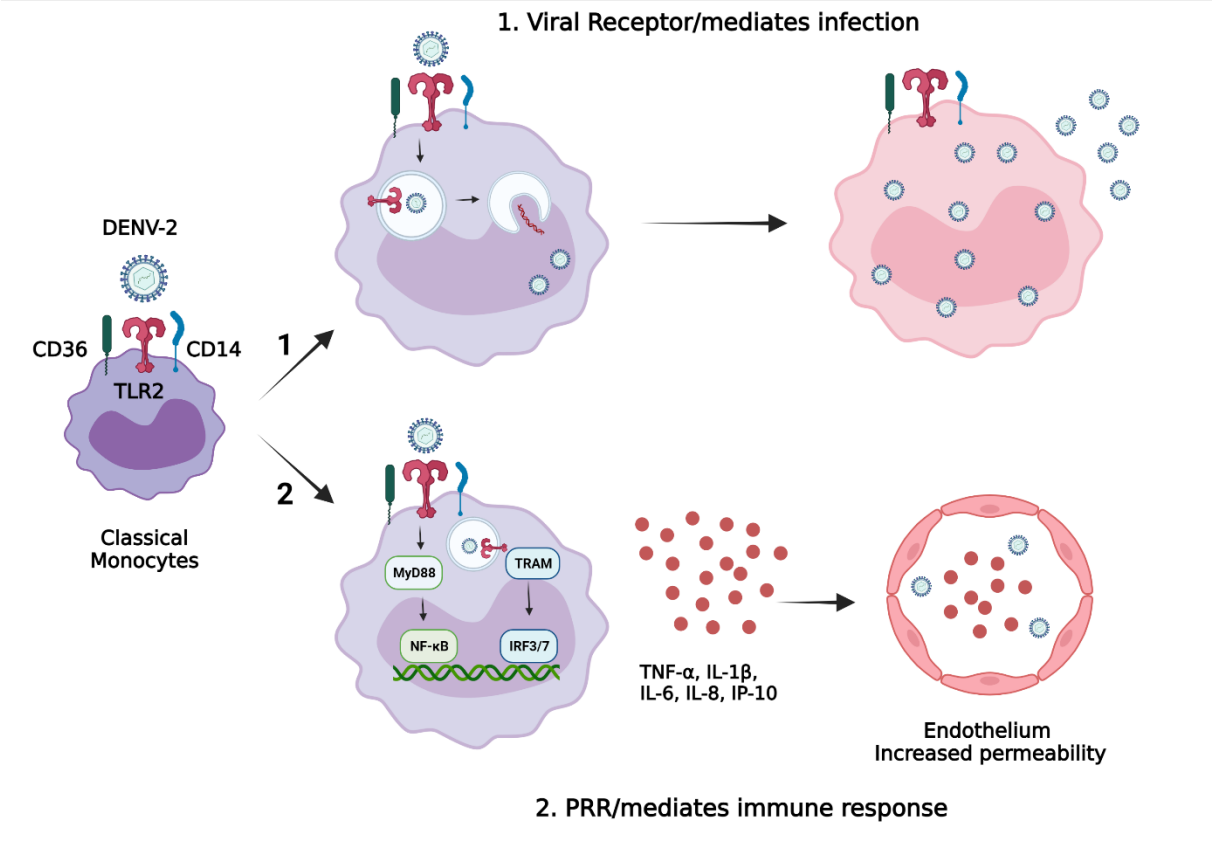


Figure 1. Dual role of TLR2 during DENV infection in monocytes

DENV-2 particles are recognized by TLR2 with the cooperation of CD14 and CD36 co-receptors. 1. On one hand, TLR2 mediates internalization and entry of viral particles into the cell promoting DENV-2 replication and production of newly synthesized infectious particles. 2. On the other hand, TLR2 activation induced by DENV-2 particles, induces activation of signaling pathways involving NF-κB and possibly IRFs, leading to the expression of inflammatory cytokines and antiviral interferons. Production of inflammatory mediators are responsible for the subsequent induction of endothelial dysfunction. Figure designed with Biorender.

In contrast, other studies have shown a detrimental role of TLR2 in some viral infections. For example, patients with chronic hepatitis caused by HCV infection have an increased expression of TLR2 in PBMCs, which is correlated with increased levels of proinflammatory cytokines and ALT in serum [24,25]. Likewise, TLR2 activation by mediates inflammatory cytokine response to HSV-1 in mice [26,27] and in SARS-CoV-2 infected patients [28], contributing to encephalitis and to COVID-19 disease severity, respectively. Specifically,

regarding DENV pathogenesis, George et al, in which was shown that TLR2 facilitated the infection of DCs and mediated the activation of uninfected bystander DCs [29]. TLR2 activation by DENV, promoted Th2 polarized humoral responses which increased infectivity of DENV via antibody dependent enhancement (ADE) [29]. Overall, our results showed in **chapter 2** also suggest that TLR2 activation by DENV-2 contributes to an exacerbated immune response. Therefore, it appears that function of TLR2 may depend on the cell type it is expressed and the biology of the pathogen.

2. Contribution of other TLRs to TLR2-initiated immune responses

Previous studies have shown that some viruses like Herpes simplex virus (HSV) can activate TLR2 and TLR9 in DCs in a sequential fashion. Interestingly, this activation led to higher production of IFN-α [26], suggesting that DCs recognize HSV through multiple TLRs optimizing innate immunity. Of note, we observed in **chapter 2** a reduced production of inflammatory cytokines (IL-1β, IL-8) and antiviral IFNs (IFN-α2, IFN-β, IFN-λ1) in DENV-2 infected monocytes when TLR3 was inhibited. Since TLR2 inhibition also decreased immune responses, sequential activation of TLR2 and TLR3 may be important for monocyte defense against DENV replication, as described HSV infection in DCs [26]. Other studies have also shown that TLR3 is a key PRR for limiting DENV replication. For instance, pre-treatment of hepatoma cells with TLR3 agonist impairs DENV-2 replication through induction of IFN-β and IFN-λ2/3 [30]; and silencing of TLR3 in mouse macrophages made the cells more susceptible to DENV replication [31]. Overall, these data show the pivotal role of TLR3 activation in the production of inflammatory and antiviral mediators during DENV infection. However, the importance of TLR3 activation should be further studied using *in vivo* experiments or directly in DENV infected patients

TLR7/8 appears to be important for controlling Flavivirus replication. Supporting this idea, administration of TLR7/8 agonist in rhesus macaques reduced DENV replication [32], knock-out of TLR7 in mice resulted in increased viral load of Japanese encephalitis virus (JEV) in brain [33], and TLR7 and TLR8 have been shown to recognize DENV-2 infection in HEK cells [34]. Despite this evidence, we showed in **chapter 2** that TLR8 does not mediate immune responses of monocytes during DENV infection, as specific inhibitor CU-CPT9a

did not alter levels of cytokine production. Interestingly, in **chapter 3**, we observed that TLR8 mRNA expression was not regulated in macrophages during DENV-2 infection, suggesting that TLR8 is not activated by the virus. Most studies so far have evaluated the effect of TLR7 and TLR8 activation using agonists that activate both receptors. Therefore, it is reasonable to conclude that differences between previous studies and our data, are due to the use of agonists that activate both TLRs simultaneously. In fact, recent crystallographic studies showed that successive uridine-containing ssRNAs have high affinity to TLR7 [35,36], whereas uridine and guanosine-rich ssRNAs have high affinity for TLR8 [37]. On the other hand, TLRs recognition and signaling are redundant, and the dispensable function of TLR8 that we observed in DENV-2 infected monocytes and macrophages may be explained by a functional TLR7. Indeed, Awais et al. found that JEV infection in TLR7$^{-/-}$ mice resulted in upregulation of TLR8 expression, which was concluded by the authors as a compensatory role [33]. In conclusion, most studies show that TLR7 is important for DENV sensing and induced immune responses, but whether TLR8 is not functional or serves as a compensatory TLR during DENV infection remains to be studied.

In summary, activation of TLRs during DENV infection of monocytes/macrophages shape immune responses during DENV-2 infection **(Figure 2)**. Unfortunately, the role of other types of PRRs could not be tested in our experimental model due to technical difficulties. For example, RIG-I inhibitor MTR did not show optimal effect. In other cells including hepatocytes, RIG-I and MDA5 are important for DENV-1 control [31]. Is probable that in monocytes RLRs also contribute to TLR2-initiated immune responses, but this should be evaluated in future studies.

3. Distinct role of monocyte subsets during DENV infection

3.1. Distribution of monocyte subsets during DENV infection

During inflammatory and viral diseases, the distribution of monocyte subsets can change considerably, suggesting a protective or harmful role during pathogenesis. We and others have consistently shown that frequencies of IM are higher in DENV infected patients compared to healthy controls, while CM are decreased [17,38–40]. Kwissa et al. and Naranjo

et al. described how IM and NM show increased expression of activation markers and increased production of proinflammatory cytokines [38,39]. However, this activation was not associated with severe dengue. We recently showed that TLR2 sustained expression in CM of DENV infected patients, but not in other subsets, was associated with severe dengue [17], suggesting that CM play a key role in DENV pathogenesis. We described in **chapter 2** that CM are more susceptible to DENV-2 infection compared to IM and NM, which is explained by differential expression of TLR2 co-receptors, underlying a differential role of monocyte subsets in DENV pathogenesis.

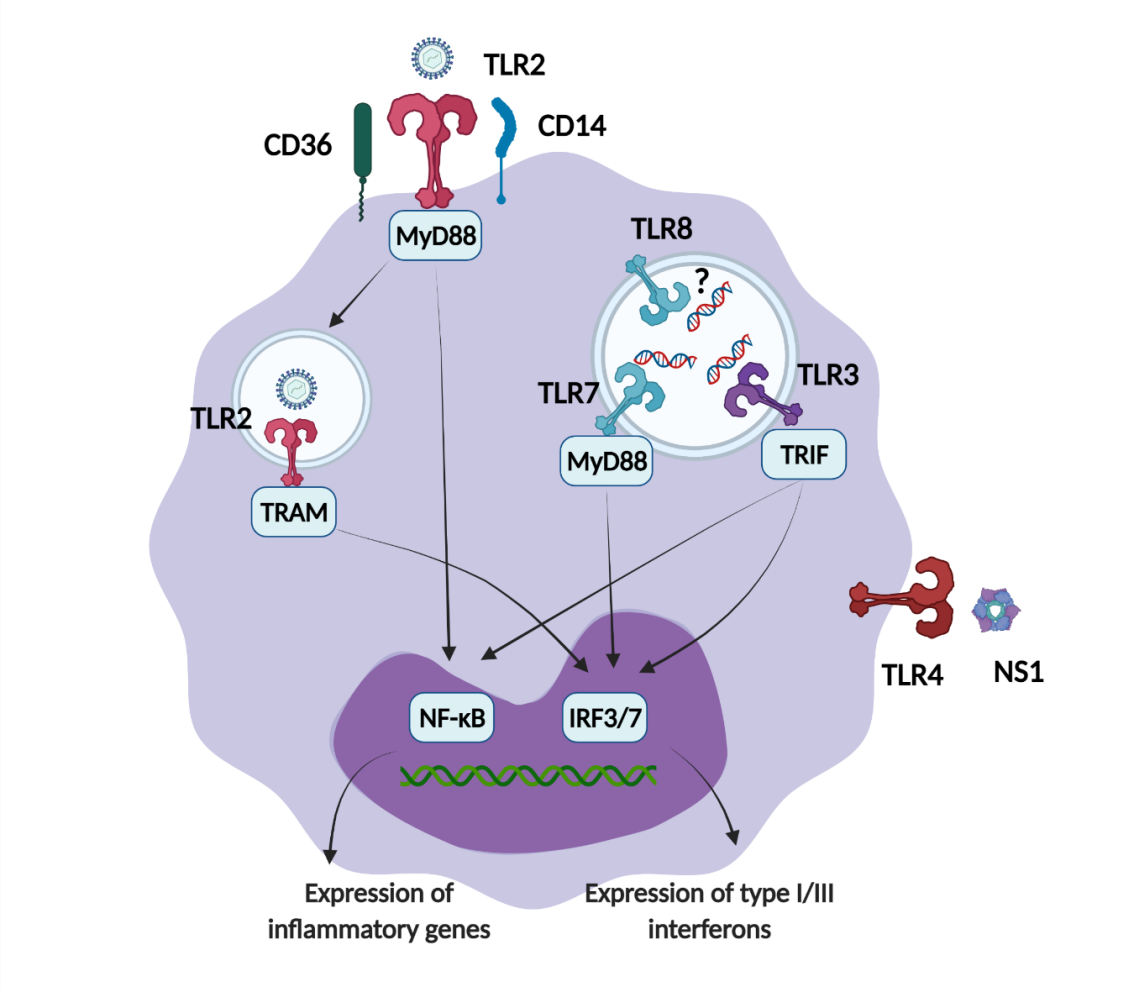


Figure 2. Activation of TLRs during DENV infection of monocytes/macrophages

Monocytes sense DENV via TLR2 receptor, which mediates viral infection and induces the activation of NF-κB and IRF3/7 transcription factors. Conversely, mannose receptor, CLEC5A and heparan-sulfate receptors mediates extracellular sensing and infection of DENV in macrophages. Activation of NF-κB and IRF3/7 transcription factors induced by TLR2 activation leads to the expression of

inflammatory cytokines and type I/III interferons. Also, DENV replication leads to the production of RNA intermediates recognized by TLR3, TLR7 and TLR8 within endosomes. TLR3 and TLR7 sense dsRNA and ssRNA respectively, inducing the expression of inflammatory cytokines and type I/III interferons. Whether TLR8 is functional in monocytes and macrophages during DENV infection, or whether TLR8 is a compensatory TLR in case TLR7 is inhibited, remains to be studied. TLR4 expressed in PBMCs senses viral NS1 (ref), leading to the expression of proinflammatory cytokines.

Differential susceptibility to DENV-2 infection in monocyte subsets could also explain the alteration of their frequencies during *in vivo* DENV infection. Therefore, low numbers of CM observed during DENV infection can be explained by an increased cell death caused by viral replication, since lifespan of CM is shorter than IM and NM (1 day vs 4 and 7 days) [41], suggesting that CM are prompter to cell death. Conversely, IM expansion could be a result of CM differentiation after DENV-2 infection mediated by TLR2, since they are the main target for viral replication among monocyte subsets **(Figure 3)**. Patel et al. showed that CM have the potential to give rise to IM and NM by analyzing monocytes of healthy volunteers grafted into humanized mice [41]. Also, Fenutria et al. showed that infection of PBMCs with ZIKV or DENV increases the proportion of intermediate and non-classical monocytes [42]. Further studies are needed to detail the mechanisms of cell death and differentiation of monocyte subsets during DENV infection that would provide evidence for explaining the variation of monocyte subsets frequencies in infected patients.

3.2. Different susceptibility of monocyte subsets to DENV Infection

The distinctive transcriptomic programs of monocyte subsets are also likely to underly differences in susceptibility and permissiveness for viral infections [43]. During ZIKV infection IM are more susceptible to viral replication [44], while CD16+ monocytes (IM and NM) are more susceptible to HIV-1 infection [45]. On the other hand, in **chapter 2** we found CM as more permissive to DENV-2 infection, compared to IM and NM. Consistent with this data, we previously found that viral NS3 expression was predominantly detected in CM and IM, but not in NM of pediatric DENV infected patients [17]. Such differences between related viruses such as ZIKV and DENV could be explained by the use of different receptors by DENV for entry into monocytes. Importantly, we showed that DENV-2 infection of monocyte subsets was mediated by TLR2 and that differences in susceptibility were explained by differential TLR2 co-receptors expression among subsets. Unfortunately,

during the study described in **chapter 2**, we couldn't separate CM, IM and NM, but rather obtained CD16- (CM) and CD16+ monocytes (IM and NM). However, separation of CM from total monocytes was sufficient for detecting differences in susceptibility to DENV infection among subsets. Therefore, we propose that CM and IM have higher susceptibility to DENV-2 infection, mediated by TLR2 complex, while NM have lower susceptibility to infection, which is mediated by other type of receptors.

3.3. Monocyte subsets and endothelial permeability

Increased amount of evidence has been published which describes the inflammatory potential of each monocyte subset. Although some reports are contradictory, there is a consensus that CM have the highest phagocytic activity and production of ROS, while NM migrate and patrol to vasculature to respond against viral invaders [46,47]. At the same time, IM show the highest inflammatory potential and can migrate to the site of infection contributing to systemic inflammation [47,48]. Some reports have shown that NM also produce the highest levels of TNF-α and IL-1β after agonist stimulation [49] and in diseases like sepsis and lupus [50]. In line with these results, we described in **chapter 2** that CD16+ monocytes (IM and NM) produced higher levels of inflammatory cytokines and some interferons in response to TLR2 agonist. However, under infection with DENV-2, we found that CM produce the highest levels of proinflammatory cytokines and antiviral interferons, including IL-6, IL-8, IL-1β, TNF-α, IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3. Our results suggest that production of inflammatory cytokines and interferons is highly dependent on DENV-2 replication and TLR2 axis expression, and imply that CM play a key role in mediating the exacerbated inflammation that contributes to pathogenesis **(Figure 3).**

Increased production of inflammatory cytokines by CM during DENV-2 could be promoting the migration of immune cells to the site of infection and the activation of endothelium. In depth analysis done in **chapter 2**, showed that both CD16- and CD16+ monocytes subsets have the same potential for inducing endothelial permeability. Interestingly, the effect in ECs permeability was higher using supernatants from total monocytes compared to that observed with individual monocyte subsets. This finding suggest that different soluble factors produced by CD16- and CD16+ monocytes subsets may be exerting enhancing or additive effects in ECs. Similarly, Pan et. recently described

that NS1 recruits and facilitates the enzymatic activity of MMP-9 (matrix metalloproteinase-9) produced by PBMCs and macrophages, which disrupts the junctions between endothelial cells [51]. Therefore, induction of endothelial permeability seems to be a complex process in which various soluble factors and NS1 would contribute. In conclusion, monocyte subsets may have different roles in DENV pathogenesis. While CM and IM may account for viral replication and production of inflammatory mediators, NM may be migrating to the endothelium, and soluble factors produced by all subsets may be promoting endothelial permeability **(Figure 3)**.

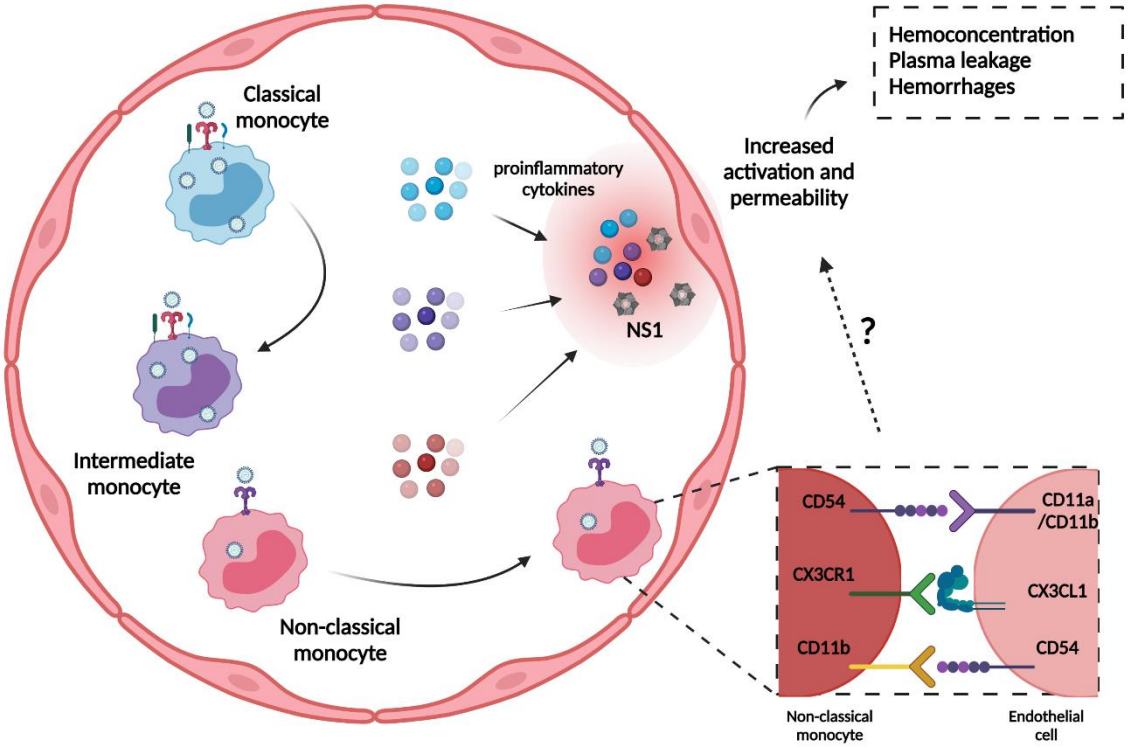


Figure 3. Role of monocyte subsets in DENV pathogenesis

Classical (CM) and intermediate (IM) monocytes senses DENV via TLR2 complex (TLR2, CD14 and CD36) leading to increased viral replication in these subsets and increased production of inflammatory mediators. Activation of CM by TLR2 may be mediating their differentiation into IM, explaining the expansion of this subset in DENV patient´s blood. DENV infection by non-classical monocytes (NM) is mediated by other type of receptors, leading to lower replication and inflammatory response compared to other subsets. Inflammatory mediators produced by all subsets together with viral NS1, may mediate activation and increased permeability of ECs. Further, NM patrol and interact with ECs via CD54, CD11b and CX3CR1 expression, which are overexpressed in

NM of DENV infected patients. Whether interaction of DENV infected NM with ECs contribute to endothelial permeability remains to be explored.

3.4. Fate of monocytes subsets at the site of infection

Fate and mobilization of monocyte subsets is mediated by the expression of chemokine receptors. In this way, CM and IM circulate in blood in steady state and migrate to inflammation sites due to the high expression of CCR2 and CCR5. On the other hand, NM patrol and interact with endothelium in steady state via expression of CXCR1 [52,53]. Interestingly, mouse Ly6C^{high} monocytes (counterpart of CM human monocytes), migrate to the dermis during DENV infection of mice serving as target for viral replication [54]. Whether this phenomenon is occurring in humans has not been studied yet. Nevertheless, it would be important to test if CM monocytes are migrating to the site of DENV infection in dermis, since we showed in **chapter 2** that this subset is more susceptible to DENV infection due to higher levels of TLR2 axis expression. Further studies are needed to test this hypothesis and would provide evidence that explain if CM migrate to different DENV infection sites and serve as precursors for other type of viral target cells.

Inflammation mediates the extravasation of monocytes from blood into inflamed tissues [55] and depending on the microenvironment, they can either maintain their phenotype at the site of infection or differentiate into macrophages or DCs [41]. Whether TLR2 activation influences monocyte differentiation during DENV-2 infection remains to be explored. However, this is a very attractive question, since other studies have shown that TLR2 activation induces a rapid differentiation of monocytes into macrophages and DCs [56], and also TLR2 activation impairs the anti-inflammatory activity of M2-like macrophages [57]. These data suggest TLR2 activation may shape monocyte differentiation in CM leading to an inflammatory M1-like polarization of macrophages, but the role of TLR2 in monocyte subsets migration and subsequent differentiation remains to be stablished.

3.5. New strategies for studying the function of monocyte subsets in DENV pathogenesis

One significant limitation that we encountered in **chapter 2** was the difficulty for studying the three monocyte subsets individually. Due to low number of IM and NM in peripheral

blood, electromagnetic sorting yielded low numbers of these subsets and therefore, we separated and worked with IM and NM (CD16+) together. Even though separation of CM was enough for detecting differences in susceptibility to DENV infection, single-cell RNA sequencing (scRNAseq) has shown that each monocyte subset is genetically and functionally different [43,58,59]. In fact, Villani et al. suggested that within IM, there are two set of different expressed genes that would suggest further heterogenicity [58]. This evidence supports the strengths of scRNAseq for studying the role of monocyte subsets. For example, scRNAseq has been done in PBMCs of patients suffering from dengue and has revealed that CD163 is over-expressed in monocytes before progression to severe dengue [60], and that genes involved in inflammatory response and mitochondrial function are up-regulated in monocytes [61]. However, these analyses have been done in total monocytes, and although valuable information has arisen from this population compared to other cells of PMBCs, scRNAseq could be a useful tool for studying the role of monocyte subsets in patients suffering from dengue.

Another recent application that would allow to make in depth analysis of monocyte subsets is mass cytometry (CyTOF). This technique uses metal-labeled antibodies instead of fluorochrome-labeled, allowing to simultaneously asses up to 42 parameters in a single cell [62]. This would allow to include new markers for monocyte subset differentiation and different activation and effector markers. One of these additional markers could be the carbohydrate residue 6-sulfo LacNAc (SLAN), which has been shown to clearly differentiate IM from NM, better than CD14 and CD16 expression [63]. CyTOF has been used to study the innate immune profile in PBMCs infected in vitro with DENV-2 and ZIKV, showing differences in CD86, CD38, CXCL8 and CXCL10 expression among monocyte subsets [42]. However, in vitro infection can up-regulate the expression of CD16 promoting differentiation of CM into IM, as Fenutria et al also reported in their analysis [42]. Therefore, I believe that CyTOF is a valuable tool for studying monocyte subsets of DENV infected patients, which combined with virus markers, may allow the analysis of overall immune responses of CM, IM and NM in both infected and bystander cells. In conclusion, new strategies are being developed for better analysis and understanding of monocytes subsets during DENV infection, that may allow the development of new therapeutic strategies for fighting severe dengue by targeting monocytes.

4. Role of miRNAs during DENV infection of monocytes/macrophages

MiRNAs post-transcriptionally control gene expression of several genes involved in various cellular programs including defense against viral infections. The expression of some miRNAs can either promote or restrict DENV replication. For example, miRNA-548g binds to stem- loop A (SLA) promoter in the DENV 5'UTR decreasing viral replication [64], while miR-133a targets the cellular protein PTB, which is involved in the formation of DENV replication complex [65]. On the other hand, miR146a increases rapidly after DENV replication repressing TRAF6 and IFN-β expression, thus facilitating viral replication [66]. In chapter 5, we identified a set of differentially expressed miRNAs in DENV-2 infected MDMs that could have a role in pathogenesis. Of these, miR-182, miR130a, miR146a, miR-125b and miR-155 could be of relevance since their expression has been associated with increased inflammatory response in various diseases [67–70]. Similar results have been observed in DENV infected patients. High-throughput RNA sequencing revealed a set of differentially expressed miRNAs present in plasma of DENV infected patients that were associated with the development of severe dengue, including miR-6499, miR-122, miR486, among others [71]. Similarly, miR-150 has been found in higher levels in PBMCs from patients with severe dengue compared to patients of with uncomplicated dengue infection [72], while miR-383 has been found in higher in blood of patients from Singapore diagnosed with severe dengue [73]. Altogether, these results and our data suggest that miRNAs may have an important role in the regulation of the inflammatory response caused by DENV infection and also in the progression to severe dengue.

PART II: Vitamin D and LL-37 as therapeutic alternatives for ameliorating DENV pathogenesis

1. General remarks of current therapies for DENV infection

Currently, a specific antiviral treatment for patients suffering with any of the clinical manifestations of dengue disease is not available [74]. Further, a fully protective vaccine has

not been developed, as Dengvaxia (the only approved vaccine for use in humans) is exclusively administered to adults and children above 9 years old [75,76]. Therefore, the development of new therapies that can limit viral replication and decrease the inflammatory response to prevent severe dengue is needed.

The nutritional status of the host is essential for a proper immune response. Optimal serological levels of a great number of micronutrients is necessary for both shaping the immune response and restoring the numbers of cells involved in innate and adaptive defense [77]. Among these micronutrients, VitD3 status has emerged as a risk factor for severe forms of various infectious diseases [78]. For example, there is a high frequency of VitD3 deficiency in patients suffering from COVID-19, which have been associated with a poor prognosis (development of acute respiratory failure and ICU treatment) [79–81]. This suggest that low levels of VitD3 may contribute to the development of severe dengue. The results presented in **chapter 3 and 4** showed the antiviral and immunomodulatory properties of VitD3 and LL-37 during DENV-2 infection of macrophages. In the following sections, I will discuss the associations of VitD3 levels with progression to severe dengue and the contribution of our studies to the current knowledge on how VitD3 could shape innate immune responses during DENV infection.

2. Possible mechanisms by which Vitamin D shapes immune responses during DENV infection

VitD3 is a pleiotropic hormone involved in calcium metabolism and bone development. Apart from its biological functions, VitD3 has wide immunoregulatory properties in various cells of the immune system, including antigen-presenting cells [78] (summarized in Figure 5 of **chapter 1**). We and others have previously shown that besides its antiviral properties, VitD3 can down-regulate the inflammatory response of cells infected with DENV by decreasing the production of some cytokines and the expression of some PRRs [82–86]. However, with the aforementioned data, it was not possible to associate this immunoregulatory properties to a direct effect of VitD3 or to a lower DENV replication in such treated cells. In **chapter 3** we circumvented this issue and demonstrated that differential expression of various TLRs, PKR, OAS1 and SOCS-1 between MDMs and D3-

MDMs during DENV infection, was due to intrinsic differences and independent to the level of viral infection. As discussed in detail in **chapter 3,** VitD3 immune regulation included decreased production of ROS which in turn downregulated TLR9 expression, and increased expression of OAS1 and PKR explained by a possible non-genomic effect **(Figure 4).**

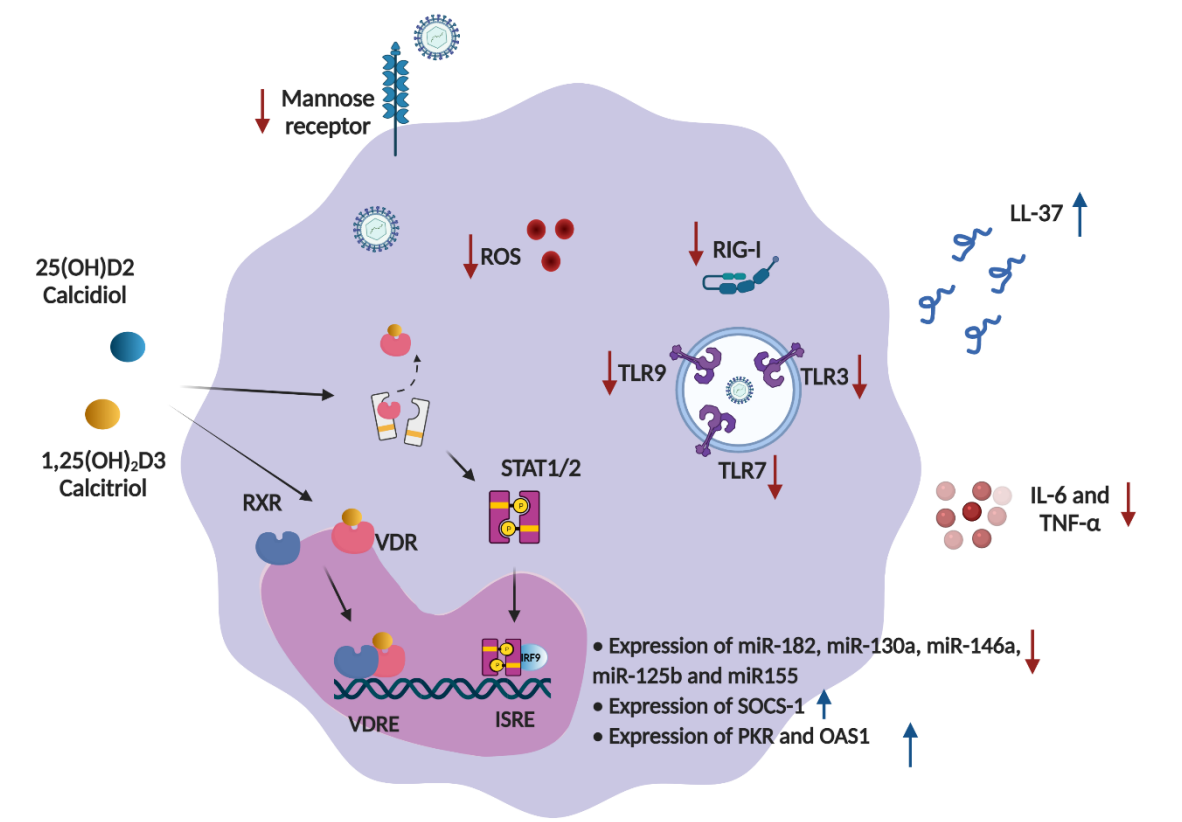


Figure 4. Effects of VitD3 in macrophages during DENV infection

Macrophages can respond to VitD3 via its inactive form 25(OH)D2 (calcidiol) which is converted to its active form 1,25(OH)$_2$D3 (calcitriol) by the action of cellular CYP7B1. VitD3 interacts with the Vitamin D receptor (VDR) which associates with the retinoid X receptor (RXR). Together they act as a transcription factor that binds to vitamin D response elements (VDRE) inducing the expression or repression of several genes [87,88]. Genomic effects during DENV-2 infection included decreased expression of IL-6, TNF-α, RIG-I, TLR3, TLR7, TLR9 and decreased production of ROS. As a non-genomic effect, VitD3 binds to VDR that is initially bound to STAT1 [89], and indirectly enhance the activity of JAK-STAT signaling pathway, thus increasing expression of PKR and OAS1. Finally, VitD3 boosts the expression of CAMP and production of LL-37 during DENV-2 infection.

Additional antiviral features of VitD3 include the induction of antimicrobial peptides. The expression of the CAMP gene, which codifies for the antimicrobial peptide LL-37 is under control of VDR and is strongly over-expressed by VitD3 treatment [90,91]. In fact, among immune cells, macrophages and monocytes produce the highest levels of LL-37 after treatment with VitD3 [92]. In **chapter 4** we showed how VitD3 treatment boosted baseline expression levels of CAMP mRNA and LL-37 peptide in mock and DENV-2 infected D3-MDMs. Also, we described in this chapter that exogenous LL-37 shows antiviral activity against DENV-2 replication, highlighting the role of LL-37 during innate immune defense against DENV infection. Similar to our results, Schögler et al. and Telcian et al. demonstrated that VitD3 reduced Rhinovirus replication in primary bronchial epithelial cells, which was associated with increased expression of LL-37 [93,94]. Further, addition of exogenous LL-37 also restricted Rhinovirus replication [94]. These results evidence the potential of VitD3 use for boosting LL-37 expression during DENV-2 infection, which could by its own restrict viral replication.

As it was mentioned in introductory **chapter 1**, VitD3 can regulate expression of several genes that harbors Vitamin D response elements [87,88]. One of these sets of regulated genes could be those encoding microRNAs (miRNAs), involved in the regulation of various cellular programs [95]. VitD3 has been shown to modulate various miRNAs in HUVECs during inflammatory diabetic-like stimuli (human serum albumin plus glucose) [96], and in adipocytes during TNF-α stimulation [70]. Also, during pregnancy there is a differential expression of miRNAs between women that show insufficient levels of circulating VitD (<25.5 ng/ml) compared to women with sufficient levels of VitD (>31.7 ng/ml) [97]. These results suggest that VitD3 could regulate the expression of some inflammatory miRNAs during DNEV-2 infection. We explored this hypothesis in **chapter 5** and found that VitD3 supplementation *ex vivo* and *in vitro* significantly reduced the expression of several miRNAs, including miR-182, miR-130a, miR-146a, miR-125b and miR-155, which have been associated with the inflammatory response in other types of diseases [67–70]. Taken together, these results suggest that regulation of miRNA expression may explain in part the mechanisms behind the immunomodulatory potential of VitD3.

The significance of miRNA regulation by VitD3 lies in the ability of miRNAs to post-transcriptionally regulate the expression of several mRNAs by inducing their repression or

degradation. In **chapter 3**, we found that VitD3 treatment in MDMs upregulated expression of SOCS-1, an important negative regulator for the inflammatory response after cytokine recognition. Interestingly, SOCS-1 3´UTR has target sequences for miR-155, miR-572, miR-221 and miR-150, which suggest that high expression of these miRNAs result in decreased expression of SOCS-1 and higher inflammatory response [98]. Chen at al. demonstrated that VitD3 treatment in mice restricted miR-155 expression, which in turn promoted increased expression of SOCS-1 and an attenuated inflammatory response to LPS [99]. We explored **chapter 5** whether regulation of miRNAs expression could result in SOCS-1 increased expression during DENV-2 infection and explain as well the immunomodulatory effects observed with VitD3 supplementation. Indeed, we found that inhibition of expression of miR-182 and miR-155, same effect observed under VitD3 treatment, resulted in higher levels of SOCS-1 expression. These results suggest that similar to that observed by Chen et al. in mice [99], during DENV-2 infection high expression of some miRNAs like miR-182, miR-155 and miR-150 can result in down-regulation of SOCS-1 expression and promote an increased inflammatory response. Following this line of thoughts, other investigators also found that augmented expression of miR-150 and depressed expression of SOCS-1 was associated with severe dengue in patients infected with DENV in Taiwan [98]. Overall, these results highlight the antiviral and immunomodulatory effect of VitD3 during DENV infection and support the idea of using VitD3 supplementation as a therapy in patients suffering from dengue.

3. Levels of Vitamin D in DENV infected patients and its correlation with disease severity

Despite the rather clear-cut antiviral and immunomodulatory role of VitD3 during DENV infection *in vitro,* its role during *in vivo* infection remains elusive. Observational studies done in India, Colombia and Ecuador have shown that patients with dengue or severe dengue have higher levels of VitD3 compared to healthy controls [100–102]. Statistical analysis done in these studies suggested that elevated levels of VitD3 predict increased odds for developing severe dengue. Importantly however, and as discussed by authors of one of these studies, the results may have been biased since hemoconcentration often found in

dengue patients increases the serum concentrations of VitD3 and possibly other molecules such as ferritin [102]. Evidence of a protective role of VitD3 came from studies by Zaman et al. and Sanchez-Valdéz et al. who found that VitD3 supplementation to DENV infected patients hospitalized in Pakistan and Mexico, respectively, improved the clinical condition and prevented the development to severe dengue [103,104]. Is important to note, that these clinical trials were not randomized controlled, and nutritional status or VitD3 levels before and after supplementation were not assessed or controlled neither. Therefore, there is a lack of knowledge regarding the clinical effect of VitD3 supplementation in the protection or improvement of dengue disease.

Supporting the protective role of VitD3, a great number of in vitro studies have shown the antiviral effect of VitD3 against DENV replication. In line with previous data reported by us and others [82–85], we showed in **chapter 3** that VitD3 decreased DENV replication in human macrophages, along with a regulation of innate immune responses. Importantly, the regulation of such immune responses by VitD3 were independent of the decreased DENV infection. Our results shed a light into the mechanisms by which VitD3 could be exerting it's antiviral and anti-inflammatory effects during DENV infection. Our data, together with preliminary results of the clinical studies described above, suggest that VitD3 could be an effective therapy for fighting dengue disease and preventing the development of severe dengue. I strongly believe that further large and controlled clinical trials may confirm this hypothesis.

4. Supplementing VitD3 in DENV infected patients: matters to solve

Although *in vitro* data suggest that VitD3 is a promising supplement for fighting the symptoms observed in DENV infected patients, it remains elusive in which moment of the infection should this treatment be administered. After an incubation period of 3 up to 7 days, patients develop an initial acute febrile phase [105], which can last up to 7 days. However, a small proportion of patients develop a critical phase and if not treated properly will eventually lead to death [106,107]. Currently, there is no knowledge that could help to predict the development of the critical phase in patients with severe dengue. Therefore, the window for VitD3 supplementation in DENV infected patients should be the febrile phase,

in order to ameliorate the symptoms observed in this period of the disease and to prevent the development of severe dengue.

In addition to solve if VitD3 supplementation should be done as therapy or as a prophylaxis, we need to solve which is the right dose for VitD3 administration. Some studies have suggested that currently there is a global pandemic of VitD2 deficiency in the general population, due mainly to human conducts such as less sunlight exposure and improper diets [108,109]. Therefore, conventional therapy for reaching physiological levels of VitD3 may be sufficient for treating DENV infected patients. The inactive and transported form calcidiol (VitD2) is the most reliable indicator of functional and bioactive calcitriol (VitD3) status. According to the Endocrine Society, sufficient levels of VitD2 are above 30 ng/ml, insufficient levels go from 21 to 29 ng/ml and deficient levels are below 20 ng/ml [108,109], while physiological levels of bioactive VitD3 go from 0.1 to 0.5 nM [110,111]. VitD3 supplementation is usually done with 1000 to 5000 IU of inactive precursors such as cholecalciferol or ergocalciferol (VitD), which can peak VitD3 levels up to 0.25-0.75nM [112]. In light with this information, the data we showed in **chapter 3** gets more relevance, since we used physiological concentrations of VitD3 (0.1nM) that could be reached with conventional treatment in humans. In conclusion, a conventional treatment with VitD either as prophylaxis or in DENV infected patients going through the febrile stage of the disease, could potentially shape the inflammatory response and prevent the development of severe dengue.

5. Antimicrobial peptides during DENV infection

Human β-Defensins (HBD) and cathelicidins are two major families of AMPs in mammals with different functions including antimicrobial activity, chemotaxis and immunomodulation [113,114]. During DENV infection, AMPs may have an important role in innate immune defense. However, we found in **chapter 4** that macrophages produced very low levels of HBD2, HBD3 and LL-37 in response to DENV infection. Further, DENV-2 replication appeared to reduce CAMP (gene encoding LL-37) mRNA expression. Conversely, human HaCaT cells (Keratinocytes) have been shown to produce HBD-3 and LL-37 peptides after in vitro infection with DENV [115], while monocytic cells line THP1

and human neutrophils produce HBD-1 and LL-37 during DENV infection [116]. Differences observed between the aforementioned studies and our data may rely on cell type differences or signaling pathways necessary for AMPs induction. In fact, higher expression of AMPs by keratinocytes and neutrophils has been reported previously [117]. Nevertheless, we found that LPS stimulated MDMs produced high levels of LL-37, different to that observed with DENV-2 infected MDMs. This finding together with a decreased CAMP expression, suggest that DENV-2 replication may actively antagonize LL-37 expression. In line with this hypothesis, Duits et al. has showed that monocytes, MDM, and monocyte-derived dendritic cells (MoDCs) express HBD-1 and HBD-2 after stimulation with LPS and IFN-γ [118]. Whether DENV actively decreases LL-37 expression in MDMs, differently to that observed in keratinocytes and macrophages, should be studied in future studies.

The antiviral effect of AMPs supports the important role of these peptides during DENV infection. For example, treatment of HaCaT cells with exogenous LL-37, HBD2, and HBD3 inhibits DENV-2 replication [115]. Also, treatment of Vero E6 cells or U937-DC-SIGN cells with exogenous LL-37 decreases DENV infection [119,120]. In line with these results, we found a dose-dependent antiviral effect of exogenous LL-37 against DENV-2 replication in MDMs in **chapter 4**, as described for other viruses [94,121–125]. Interestingly, treatment of MDMs with LL-37 after the initial 2 hours of infection did not influence DENV infection, providing some initial insights that LL-37 may be exerting its antiviral effect in the first steps of viral replication. Alagarasu et al. proposed an interaction between LL-37 and DENV-2 E protein by bioinformatic modeling (in-silico docking) [126]. However, the main mode of action of LL-37 is the disruption of the external membrane of microbes. Therefore, the precise mechanism by which LL-37 inhibits DENV replication remain unanswered. Currently, we are delineating new time-of addition experiments that could explain in detail the antiviral activity of LL-37. In conclusion, evidence shows that LL-37 is important for innate defense against DENV infection, especially in cells present at the first site of infection such as keratinocytes, macrophages and DCs.

6. Is it possible to treat directly with LL-37 rather than VitD3?

To our knowledge, no clinical trials have evaluated the antimicrobial potential of LL-37 against viral diseases, and only a couple of clinical trials have evaluated the potential of LL-37 in the immunomodulation and clearance of some chronic conditions such as melanomas and venous leg ulcers (VLU) [127,128]. Although topical treatment of LL-37 was well tolerated and improved healing of VLU [128], intra-tumoral injections caused side-effect dermatological toxicity [127]. These data highlight the need to develop future clinical trials that can evaluate the safety and effectiveness of LL-37 treatment against viral infections.

Despite this lack of knowledge, I strongly believe that the increase of LL-37 during DENV infection in humans can be easily achieved with conventional supplementation of VitD3. For example, 400,000 IU of cholecalciferol increased 30% of the baseline levels of LL-37 peptide production in a Placebo-Controlled clinical Trial with sepsis patients [129]. Also, supplementation with 500,000 IU of VitD3 in critically ill ventilator-dependent adults increased mRNA expression of LL-37 and serum levels were correlated with increased phagocytosis of alveolar macrophages [130]. In fact, in vitro experiments done in **chapter 4** demonstrated that differentiation of MDMs in the presence of VitD3 increased baseline expression levels more than 500% during DENV-2 infection (590ng/ml in MDMs vs 3200ng/ml in D3-MDMs). Importantly, the concentration of LL-37 induced by VitD3 in our model could potentially restrict DENV replication, since dose-response curve done in DENV-2 infected MDMs showed that the IC50 of LL-37 was 2,246ng/ml which was achieved with D3-MDMs of 4 out of 5 donors. In conclusion, VitD3 supplementation in DENV infected patients early after onset of symptoms could be an useful tool for boosting the baselines levels of LL-37 in serum and tissues, contributing in this way to the early control of viral replication and spread **(Figure 5)**.

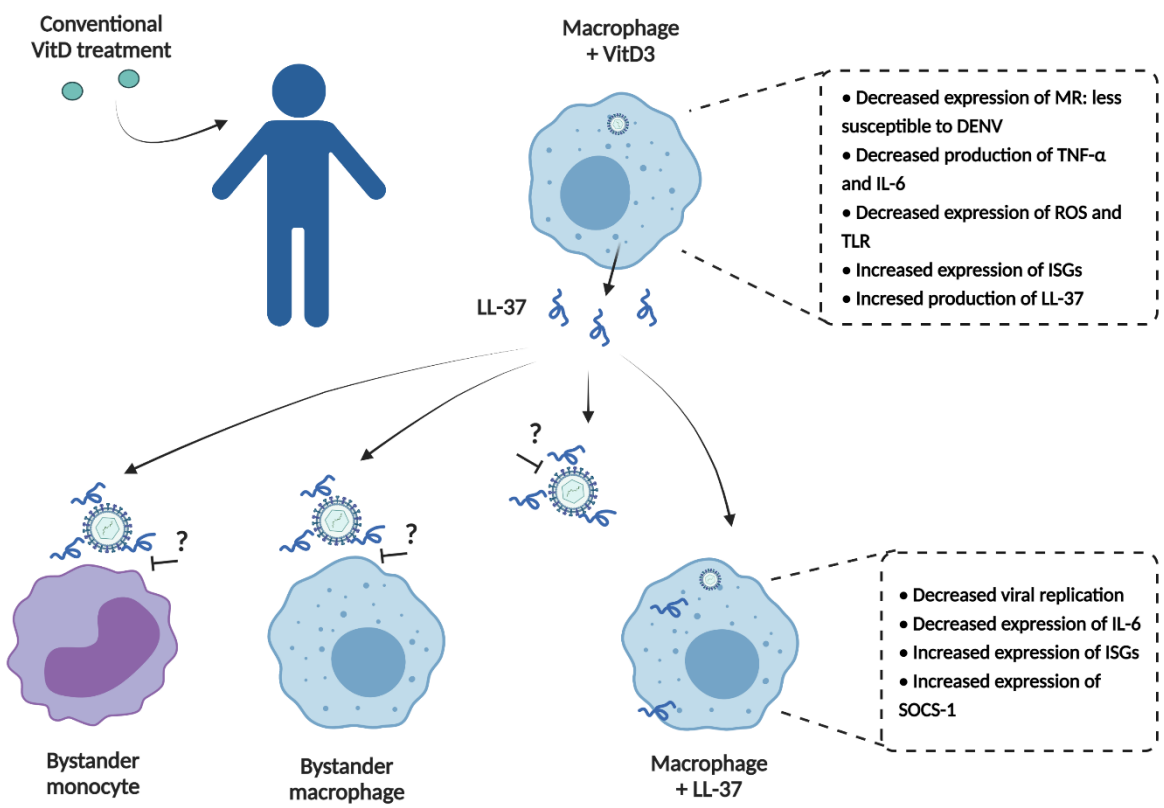


Figure 5. Potential effects of VitD treatment in DENV infected patients

Conventional treatment with VitD aimed to induce sufficient levels of VitD2 in humans, could potentially induce various antiviral and immunoregulatory effects in macrophages during DENV infection. Further, increased production of LL-37 by macrophages with high levels of VitD3 could potentially restrict DENV replication, either by disturbing the viral particles or by inhibiting viral entry into bystander target cells like monocytes and macrophages.

CONCLUDING REMARKS

The data presented in this thesis showed the importance of TLRs in sensing DENV infection, and how they mediate activation and immune response of monocytes/macrophages that eventually leads to ECs dysfunction. First, TLR2 expressed in CM senses DENV, facilitating viral infection, inflammatory response, and ECs dysfunction. Of note, previous data showed that TLR2 represented a prognostic value in a DENV-infected pediatric cohort, suggesting the potential of TLR2 targeting for therapeutics. Secondly, we showed the mechanisms by which VitD3 exerts its immunoregulatory properties in macrophages during DENV infection, which involve the regulation of TLRs and the boosting of antimicrobial LL-37

peptide expression. Altogether, our results place the foundations for the development of future studies that could assess the effect of targeting TLR2 in CM for improving dengue disease in an *in vivo* model. Currently, several drug formulations are being evaluated against all TLRs [131]. Recently developed TLR2 antagonists such as OPN-305 are being evaluated in phase 2 clinical trials for treatment of Myelodysplastic syndrome, pancreas tumor and other inflammatory diseases [132,133]. Recently, nanomedicine technology that can target specific types of cells, for example nanomedicine specifically targeted to macrophages present in atherosclerotic plaques, has been developed [134]. Therefore, combination of this nanomedicine target technology together with TLR2 antagonists could be an useful tool for modulating the capacity of CM as target for DENV replication and for ameliorating the inflammatory response during DENV infection. Finally, I believe there is sufficient evidence that proves the feasibility of treating DENV infected patients with a supplementation of VitD3 that could ameliorate the symptoms caused by the exacerbated inflammatory response and may have a protective role against the progression to severe dengue.

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