CHAPTER 5

Summarizing discussion and concluding remarks

GENERAL SUMMARY

With an estimated 390 million human infections every year, dengue virus (DENV) is the most frequently transmitted arbovirus in tropical and subtropical areas of the world [1]. Although the majority of human infections are clinically inapparent or manifest as an acute flu-like illness, approximately 500,000 to 1 million of infected individuals develop severe symptoms [2,3]. The severe manifestations are triggered by aberrant immune responses that lead to excessive inflammation and increased permeability of microvascular endothelium [4]. If untreated, plasma leakage and hemorrhages caused by endothelial cells (ECs) dysfunction, may ultimately lead to life-threatening hypovolemic shock and organ failure [5].

Innate immune cells that encounter DENV in the early stages of infection are responsible for infection-induced inflammation [6]. Of these, monocytes and macrophages play a dual role as they also represent DENV primary target cells and, thus play a critical role in the process of systemic inflammation and disease progression [6–8]. In this thesis, we characterized the mechanisms by which monocytes and macrophages sense and respond to DENV infection. Furthermore, we evaluated how natural, host-intrinsic immunomodulatory compounds such as Vitamin D (VitD3) and LL-37 influence the innate immune response of macrophages to DENV infection.

In **chapter 2,** we dissected the mechanisms underlying TLR2-mediated activation of DENV-infected monocytes. Using targeted blocking of various TLRs in purified monocytes, we demonstrated that recognition of DENV by TLR2 and its coreceptors CD14 and CD36 induces NF-κB-dependent inflammatory and antiviral responses. This TLR2-mediated activation in turn activates endothelial cells (ECs) and leads to the loss of their integrity. Functional analysis revealed that the key function of surface-expressed TLR2 axis relies on its ability to facilitate DENV internalization and active infection. In addition, intracellular activation of TLR3, but not TLR8, was found to contribute to TLR2-initiated responses. These results highlight the dual role of the TLR2 axis during DENV replication in monocytes: on the one hand, TLR2 mediates cell activation and immune responses, and on the other hand, it also mediates viral entry and replication.

Previous work of our group demonstrated that a sustained high expression of TLR2 on Classical monocytes (CM) of acutely DENV-infected patients is associated with the subsequent development of severe disease [9]. Therefore, in **chapter 2,** we continued our research by dissecting the function of TLR2 during DENV infection in different monocyte subsets. Analysis of the steady state expression of TLR2 and its co-receptors on different monocyte subsets showed that only the expression of the co-receptors differed among monocyte subsets. CM and Intermediate monocytes (IM) expressed higher levels of CD14 and CD36 than Non-classical monocytes (NM), while TLR10 expression was higher in NM. By infecting sorted CD16- (CM) and CD16+ (NM and IM) monocyte subsets, we found that CM produce significantly higher levels of infectious virus than NM and IM together. Furthermore, TLR2 blocking prior to DENV infection affected the virus production in CM, whereas it had no effect on the infection in IM and NM. Altogether, findings described in **chapter 2** provided strong evidence for the central role of CM-expressed TLR2 axis in DENV infection and inflammation, and thereby shed a light on a distinct role of this receptor and monocyte subsets in disease pathogenesis.

Therapeutic strategies that prevent or regulate the aberrant immune response to DENV infection might mitigate dengue disease burden. We and others have previously found that VitD3 has antiviral and immunomodulatory effect against DENV infection [10,11]. In **chapter 3,** we studied the regulation of innate immune responses to DENV infection in monocyte-derived macrophages (MDMs) differentiated in the presence or absence of VitD3 (D3-MDMs). We observed that D3-MDMs expressed lower levels of several PRRs including *RIG-I, TLR3, TLR7* and *TLR9.* Furthermore, in response to DENV infection, D3-MDMs produced lower quantities of IL-6 and TNF-α when compared to their MDMs counterpart. Downregulation of *TLR9* in D3-MDMs was associated with a lower production of reactive oxygen species in response to DENV infection. Also, we found that although VitD3 differentiation of D3-MDMs did not modulate expression of *IFN-I*, higher expression levels of *PKR* and *OAS1* were seen during DENV infection. Importantly, the observed effects were independent of reduced infection, highlighting the intrinsic differences between D3-MDMs and MDMs Taken together, our study demonstrates that the presence of VitD3 during monocyte differentiation modulates the innate immune response of macrophages during DENV infection.

VitD3 is known to enhance the expression of various antimicrobial and immunomodulatory peptides including LL-37 [12,13]. In **chapter 4**, we evaluated the baseline gene expression and production of LL-37 in MDMs that were differentiated in the absence or presence of VitD3. Notably, irrespectively of DENV 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. We further evaluated the antiviral and immunomodulatory properties of exogenous LL-37 in DENV-infected MDMs. We found that simultaneous exposure of MDMs to LL-37 and DENV inhibits viral replication and results in a reduced production of TNF-α and IL-6. Interestingly, addition of LL-37 2 hours after DENV infection had no effect on infection, yet decreased the production of IL-6, suggesting that LL-37 can directly modulate the immune response. Indeed, further analysis showed that LL-37 added after DENV infection increases expression of *TLR4, TLR9, PKR, OAS1* and *SOCS-1*. In summary, the results presented in **chapter 4**, demonstrate that LL-37 has both antiviral and immunoregulatory properties in MDMs and its production can be boosted by treatment with VitD3 prior to DENV infection.

Altogether, the studies presented in this thesis can be summarized in two key messages. First, TLR2 on CM plays an important role in DENV pathogenesis. Second, VitD3 and LL-37 show both antiviral and immunomodulatory effects against DENV infection in human primary cells and show potential as therapeutic candidates. Below I will discuss the relevance of these findings. In part I, I will focus on the implications of TLR2 function in DENV pathogenesis. In part II, I will debate the feasibility of exploiting VitD3 and LL-37 as therapeutic strategies to prevent and/or mitigate the development of severe dengue.

PART I: Key role of TLR2 in monocytes in shaping innate immune responses to DENV infection

1. Role of TLR2 in DENV infection

A number of viruses have been shown to activate TLR2 including yellow fever vaccine YF-17D virus [14] measles virus [15], human cytomegalovirus (CMV) [16], varicella zoster virus [17], hepatitis C virus (HCV) [18] human immunodeficiency virus 1 (HIV-1) [19] herpes

simplex virus (HSV) [20], vaccinia virus (VV) [21], and recently, SARS-CoV-2 [22] and DENV [9]. In addition, we showed in **chapter 2,** that recognition of DENV by TLR2 mediates activation of NF-κB and production of inflammatory and antiviral interferons that affects ECs barrier function.

Activation of TLR2 during viral infections has proven both advantageous and disadvantageous to the host. For example, TLR2 activation by VV and CMV induce the production of IFN-β [23], which restricts viral replication in an auto- and paracrine manner [24]. In addition, TLR2 activation in NK cells is important for cell activation and control of murine CMV and VV infections [25,26]. Furthermore, previous studies have shown that TLR2 activation is important for the development and shaping of adaptive immunity. In an *in vitro* model of bacterial infection, stimulation of MoDC with a TLR2 agonist promoted Th2/Th17 T cell differentiation [27]. Similarly, activation of TLR2 in CD8 T cells by VV promotes survival and clonal expansion of these cells [28].

In contrast with an advantageous effect of TLR2 activation for the host, other studies have shown that this contributes to the pathogenesis of certain viral infections. For example, TLR2 activation by HSV-1 mediates an inflammatory cytokine response in murine DCs, thereby contributing to encephalitis [29,30]. Likewise, in patients with chronic hepatitis caused by HCV infection, an increased expression of TLR2 in PBMCs correlated with increased levels of proinflammatory cytokines and alanine aminotransferase in serum [31,32]. Furthermore, in PBMCs, TLR2 has been shown to sense SARS-CoV-2 envelope protein thereby contributing to COVID-19 disease severity [33]. Therefore, it appears that function of a protective or detrimental role of TLR2 on viral infections may depend on the cell type it is expressed and the biology of the pathogen.

In **chapter 2**, we also disclosed a dual role for TLR2 on CM during DENV infection and its contribution to disease pathogenesis **(Figure 1)**. The TLR2 axis was found to facilitate binding of DENV particles and internalization into CM thereby promoting viral replication and release of infectious particles. On the other hand, TLR2 activated NF-κB and induced the production of proinflammatory cytokines, which in turn induced ECs dysfunction. Additionally, activation of TLR2 in the course of infection triggered the production of IFN-I/III by monocytes during DENV infection, which suggests that TLR2-mediated signaling

may contribute to an antiviral state. Whether TLR2-induced antiviral interferons contribute to containment of DENV replication and viremia remains to be studied, for instance using humanized mice [34].

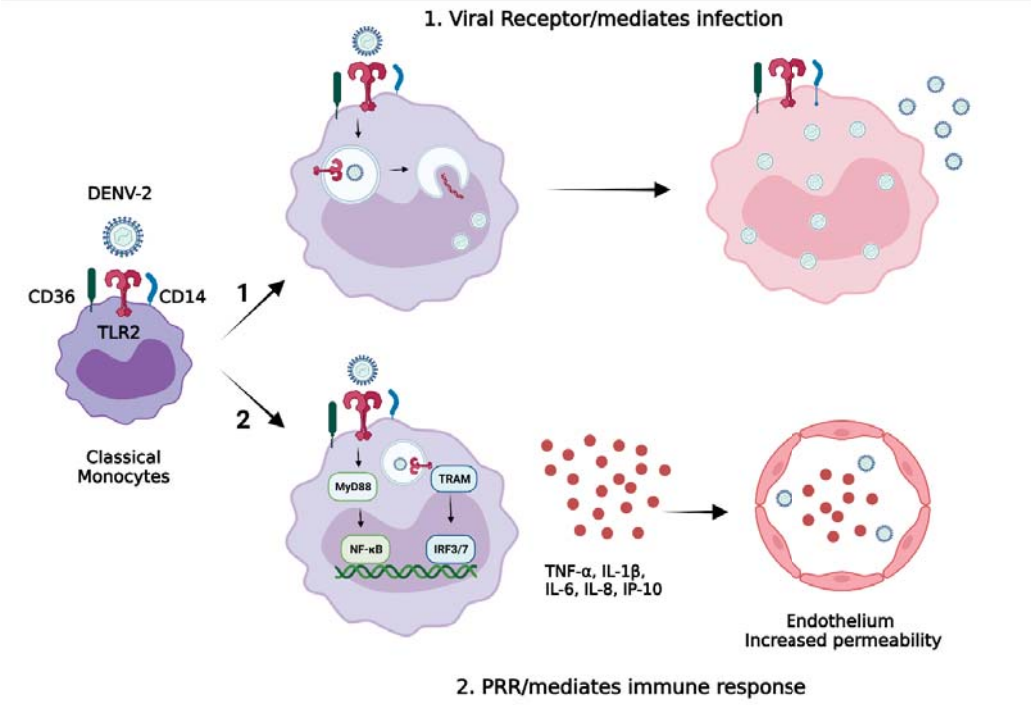

Figure 1. Dual role of TLR2 during DENV infection in monocytes

DENV 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 replication and production of newly synthesized infectious particles. 2. On the other hand, TLR2 activation induced by DENV 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 line with our results, other studies have also shown that TLR2 contributes to DENV pathogenesis. Interestingly, TLR2 expressed on neutrophils and macrophages together with CLEC5A have been shown to underly the release of inflammatory mediators in response to extracellular vesicles produced by DENV-infected platelets [35]. Furthermore, the

simultaneous blockade of TLR2 and CLEC5A in STAT1KO mice reduced inflammation and mitigated DV-induced systemic vascular permeability and lethality [35]. Also, an *in vivo* study using BL/6 mice revealed that TLR2 activation by DENV in DCs promoted Th2 polarized humoral responses, which increased infectivity of secondary DENV infection via antibody-dependent enhancement (ADE) [36].

Future studies will be needed to demonstrate how TLR2 on human immune cells contributes to the pathogenesis of primary and secondary dengue infections. However, the currently available body of evidence suggests that TLR2-mediated recognition of DENV, by either mediating infection and/or modulating innate and adaptive immunity represents a trojan horse for the host defense and thereby may contribute to severe disease development.

2. Distinct role of monocyte subsets during DENV infection

2.1. Different susceptibility of monocytes subsets to DENV infection

The distinctive transcriptomic programs of monocyte subsets are likely to underly differences in susceptibility and permissiveness for viral infections [37]. Indeed, CD16+ monocytes (IM and NM) are more susceptible to HIV-1 infection than their CD16- counterpart (CM) [38]. Also, Michlmayr et al. reported IM to be the main target of infection for the closely related to DENV, Zika virus (ZIKV) [39]. On the other hand, the observations made in **chapter 2** suggest that CM are the primary contributors to DENV viremia. This may indicate that ZIKV and DENV differentially infect different monocyte subsets. Importantly however, Michlmayr et al. did not assess the relative permissiveness of monocyte subsets like it has been done for HIV-1 [38] or DENV (**chapter 2**)**.** It is thus possible that the reported preferential targeting of IM by ZIKV is a result of the ability of DENV-infected CM to differentiate into IM, as explained above. Consistent with this concept, both DENV and ZIKV infections are associated with simultaneous decrease of CM and increase in IM numbers [9,39,40]. Moreover, DENV NS3 is found predominantly in CM and IM, with no marginal detection in NM [9]. Therefore, we propose that CM subset, which accounts for approximately 90% of all monocytes prior to infection, represents the most frequent and most susceptible/permissive subset to DENV infection, but during infection it differentiates into IM subset. In the study presented in **chapter 2**, we couldn't separate enough IM and

NM to assess their individual susceptibility and permissiveness. However, we did learn that together IM and NM produce less infectious virions than CM, and that their infection does not rely on TLR2 axis. Future studies will be needed to establish individual susceptibility and permissiveness of IM and NM to DENV infection and elucidate what host factors facilitate or impede DENV infection in these cells. This knowledge will allow to design specific interventions targeted at ameliorate viral load and disease pathogenesis

2.2. Distribution of monocyte subsets during DENV infection

Upon DENV infection the distribution of monocyte subsets changes considerably. We and others have consistently shown that frequencies of IM are higher in DENV-infected patients compared to healthy controls, while frequencies of CM are decreased [8,9,40,41]. Also, IM and NM show increased expression of activation markers and increased production of proinflammatory cytokines during DENV infection [8,40]. Whether and how this altered distribution and activation of monocyte subsets is linked to disease pathogenesis is subject of investigation. In a collaborative study with Institute Pasteur in Cambodia, our group has recently found that CM frequency in acute DENV-infected children correlates with disease progression. Additionally, a sustained high TLR2 expression on CM of these patients, was associated with severe dengue [9], suggesting that CM play a key role in DENV pathogenesis. In **chapter 2** we elaborated on these findings and found that CM are more susceptible/permissive to DENV infection and produced a higher inflammatory response when compared to IM and NM, highlighting a differential role of monocyte subsets in DENV pathogenesis.

Changes in monocyte subsets during DENV infection could be explained by the role of CM as the main target for viral infection. Lower numbers of CM may be a result of increased cell death of this subset caused by viral replication. Supporting this hypothesis, it has been found that lifespan of CM is shorter than IM and NM (1 day vs 4 and 7 days) [42], suggesting that CM are more prone to cell death. More studies are needed to explore test this hypothesis. Conversely, expansion of IM may be the consequence of CM differentiation into IM after DENV infection mediated by TLR2 **(Figure 2)**. Indeed, 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 [42]. Also, Fenutria et al. showed that *in vitro* infection of PBMCs with ZIKV or DENV increases the proportion of IM and NM [43]. However, in our

PBMC infection model, DENV did not lead to increased IM proportions but rather of NM [9]. Nevertheless, active DENV infection upregulated the expression of CD16 in a TLR2-dependent manner, suggesting that TLR2 plays a key role in CM differentiation to other monocyte subsets. Further studies are needed to study 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

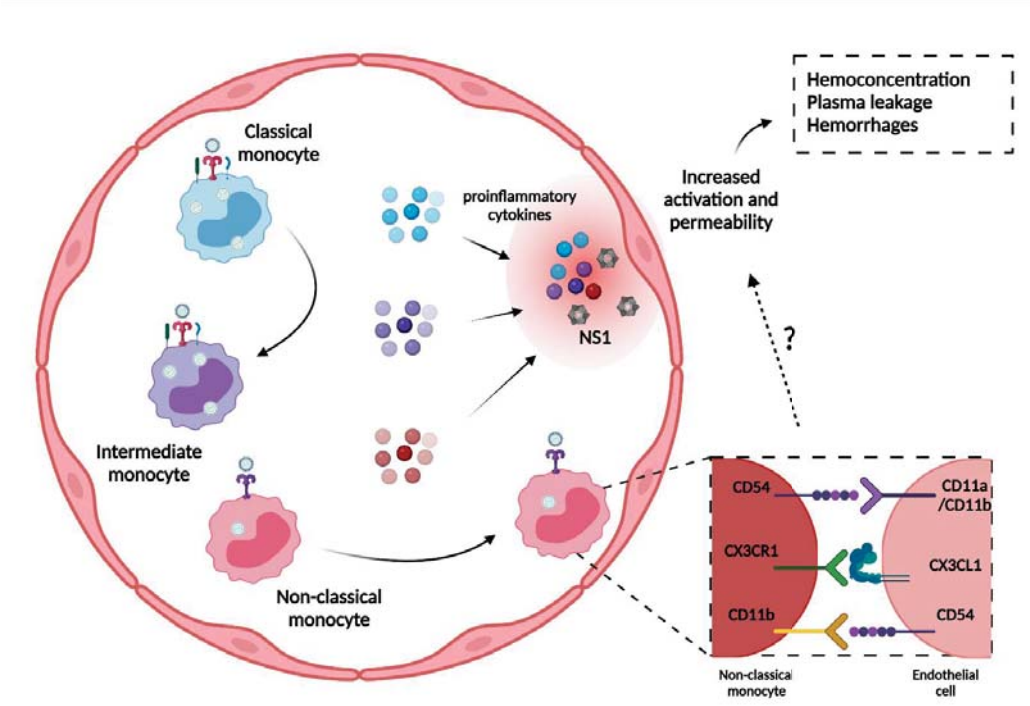

Figure 2. Model of the role of monocyte in DENV pathogenesis

Classical (CM) monocytes sense 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 in intermediate (IM) and non-classical monocytes (NM) is mediated by other type of receptors, leading to lower levels of replication and inflammatory response compared to other subsets. Inflammatory mediators produced by monocytes together with inter alia 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 [40]. Whether interaction of DENV-infected NM with ECs contribute to endothelial permeability remains to be explored. Figure designed with Biorender

2.3. Role of monocyte subsets in DENV-induced inflammation and endothelial permeability

Induction of endothelial permeability, the hallmark of severe disease development is likely to involve multiple mechanisms. To date, multiple host cell-derived mediators and viral NS1 protein have been suggested to either directly or indirectly affect ECs barrier function in an acute phase of disease (**Figure 2**) [44–46]. Within blood cells, monocyte-derived responses to DENV are well known to drive EC dysfunction [7,47]. However, the individual contributions of each monocyte subset to EC activation and/or permeability in the course of infection remain poorly understood.

In general, monocyte subsets vary in their ability to secrete the highly inflammatory cytokines IL-6, IL-1β and TNF-α. For example, most studies have shown that after LPS stimulation IM and NM are the main producers of IL-6, IL-1β and TNF-α, while CM are the highest producers of chemokines like IL-8, CCL-2, CCL-3 and the anti-inflammatory IL-10 [37,48–50]. This inflammatory potential is also seen in some diseases like sepsis and lupus [50]. Therefore, IM and NM are usually referred as "inflammatory monocytes" in literature. In line with these observations, in **chapter 2,** we found a profound difference in the responsiveness of monocyte subsets to DENV and TLR2 agonist, PAM3CSK4. The TLR2 agonist triggered inflammatory response primarily in CD16+ monocytes (IM and NM) evidenced by increased production of IL-6, IL-8, IL-1β, TNF-α, IFN-α, IFN-β, IFN-λ1 and IFN-λ2/3 compared to CD16- monocytes (CM). In contrast, DENV infection prompted TLR2 (and TLR3) dependent inflammatory responses predominantly in CM (**Figure 2**). Altogether, our results evidence that the inflammatory response of CM during DENV infection is dependent of TLR2 axis and plays a key role in the pathogenesis of severe disease.

Notably, our results in **chapter 2** showed that the levels of proinflammatory cytokines released by individual monocytes subsets did not necessarily translate to a lack of their ability to induce inflammation in ECs. In depth analysis of individually *in vitro* infected CD16- and CD16+ monocytes indicated that responses of both subsets induce ECs permeability. Nonetheless, they do so via TLR2-dependent and TLR2-independent

mechanisms and to a lower extent than DENV-infected (unsorted) monocyte cultures. Based on these results, we propose a monocyte crosstalk model in which a TLR2-indepenent mechanism in CD16+ monocytes trigger production of inflammatory mediators that exert enhancing or additive effect on TLR2-mediated activation of CM. This crosstalk would ultimately drive ECs activation and permeability (**Figure 2**), similar to that reported by Pan et al, which showed NS1-mediated recruitment of MMP-9 close to ECs and facilitated its enzymatic activity [51]. More studies will be needed to elucidate the role of other cytokines, DAMPS and cell contact mediated crosstalk between the individual subsets during DENV infection and ECs permeability.

2.4. Future perspectives on the function of monocyte subsets in DENV pathogenesis

Monocyte subsets appear to have different roles in DENV pathogenesis. Unfortunately, in **chapter 2** we did not manage to distinguish between the contribution of IM and NM in infection and immune responses, due to the low number of these subsets in peripheral blood. Fortunately, multidimensional single cell techniques have recently been developed and can be used to delineate function of each of the subsets in the course of DENV infection, even under the low numbers of IM and NM observed in humans in steady state and in disease. In fact, latest insights from single-cell RNA sequencing (scRNAseq) and mass cytometry (CyTOF) data have underscored differences in expression and functionality among subsets [37,52,53]. For example, Villani et al. suggested that within IM, there are two set of different expressed genes that would suggest further heterogenicity [52]. Furthermore, analysis of additional markers such as the carbohydrate residue 6-sulfo LacNAc (SLAN), has also improve distinction between IM and NM, in a better way than only based in CD14 and CD16 expression [54].

To date only a few and relatively small number of studies have used single cell omic techniques for *ex vivo* analysis of immune signature of PBMCs isolated from dengue patients. One of the largest *ex vivo* studies reported an association of CD163 upregulation on the surface of IM with progression to severe dengue [55]. Others noted that genes involved in inflammatory response and mitochondrial function are upregulated in monocytes of acutely infected DENV-1 patients [56]. CyTOF, on the other hand, has been used to study the innate immune profile in PBMCs infected in vitro with DENV and ZIKV, showing differences in expression of CD86, CD38, CXCL8 and CXCL10 expression among

monocyte subsets [43]. It will be interesting to learn from larger deep phenotyping studies in different patient cohorts how different immune signatures correlate with disease severity. In addition, mechanistic studies will be necessary to assess monocyte subsets function in the course of dengue disease. I believe, that combining detection of immune and virus-specific markers antigens under different infection conditions will allow to achieve this goal and elucidate responses in infected vs bystander cells. Taken together, recently developed strategies can be used to increase our understanding of the monocyte subsets function during DENV infection, and therefore placing the foundations for the development of new therapeutic strategies.

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

In contrast to TLR specific ligands, microbes represent complex PAMPs that can be sensed by different PRRs expressed in a host cell. For instance, HSV-1 activates TLR2 and TLR9 in DCs in a sequential fashion and this combined activation increased the production of INF-α [29]. In a similar fashion, we observed in **chapter 2** that in addition to TLR2, a TLR3 block also reduced production of inflammatory cytokines (IL-1β, IL-8) and antiviral IFNs (IFN-α2, IFN-β, IFN-λ1) in DENV-infected monocytes. Since TLR2 inhibition abrogated not only immune responses but also DENV infection, we concluded that sequential activation of TLR2 and TLR3 occurs in infected cells. Although in our monocyte-based model blocking TLR3 did not affect DENV infection, sequential activation of TLR2 and TLR3 may be important for defense against DENV replication, as it has been described for HSV-1 infection in DCs [29]. 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 replication through induction of IFN-β and IFN-λ2/3 [57]; and silencing of TLR3 in mouse macrophages made the cells more susceptible to DENV replication [58]. Overall, these data show the pivotal role of TLR3 activation for 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 also appears to be important for controlling flavivirus replication. Studies have shown that administration of TLR7/8 agonist in rhesus macaques was found to reduce

DENV replication [59], that *knock-out* of TLR7 in mice results in increased viral load of Japanese encephalitis virus (JEV) in the brain [60], and that TLR7 and TLR8 recognize DENV infection in HEK cells [61]. In **chapter 2,** we focused on the study of TLR8 using the specific inhibitor CU-CPT9a and found that TLR8 does not mediate immune responses of monocytes during DENV infection. Furthermore, in **chapter 3**, we observed that *TLR8* mRNA expression was not regulated in macrophages during DENV infection, suggesting that TLR8 is not activated by the virus. Our findings appear to be different from previous reports. However, most studies so far have evaluated the role of TLR7 and TLR8 in sensing DENV using agonists that activate both receptors simultaneously. Therefore, it is reasonable to conclude that differences between previous studies and our data, are due to the difficulty of evaluating TLR7 and TLR8 activation individually. In fact, recent crystallographic studies have showed that successive uridine-containing ssRNAs have high affinity to TLR7 [62,63], whereas uridine and guanosine-rich ssRNAs have high affinity for TLR8 [64], suggesting that these receptors sense different types of agonists. On the other hand, TLRs recognition and signaling is redundant, and the dispensable function of TLR8 that we observed in DENV-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 [60]. 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. Also, it will be important to evaluate the expression of TLR7 and TLR8 in human monocyte subsets using specific, non-cross-reactive antibodies.

In summary, activation of TLRs during DENV infection of monocytes/macrophages shape immune responses during DENV infection **(Figure 3)**. 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 any inhibitory effect. In other types of cells including hepatocytes, RIG-I and MDA5 are important for DENV control [58]. It is probable that in monocytes RLRs also contribute to TLR2-initiated immune responses, but this should be evaluated in future studies.

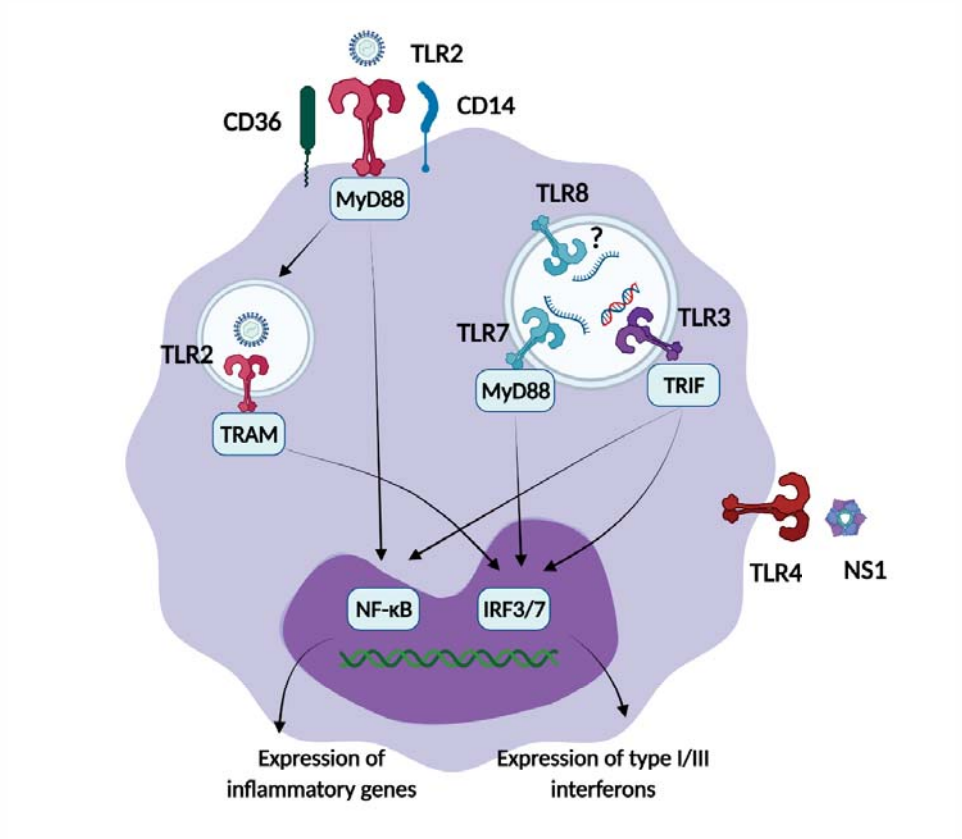

Figure 3. Activation of TLRs during DENV infection of monocytes/macrophages

CM 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 that are recognized by TLR3, TLR7 and TLR8 within endosomes. dsRNA and ssRNA are sensed by TLR3 and TLR7 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 and endothelial cells senses viral NS1, leading to the expression of proinflammatory cytokines. Figure designed with Biorender.

4. Blocking TLR2 as a therapy to mitigate DENV pathogenesis

So far, we have shown that TLR2 has a key role in DENV sensing and subsequent inflammatory response. Further our study done in **chapter 2** evidenced that TLR2 activation

in monocytes drives endothelial activation and increased permeability thereby contributing to pathogenesis. Collectively, prevention of TLR2 activation during DENV infection could be a promising strategy for fighting dengue disease.

As specified in **chapter 1,** TLRs have a vital role in sensing microbes and are key intermediaries in the generation of the inflammatory response. A dysregulated activation of TLRs can lead to a harmful inflammatory response for the host. In consequence, TLRs are attractive targets of the innate immune system for modulation in favor of patients suffering for inflammatory diseases, such as dengue. Currently, several drug formulations are being evaluated against all TLRs [65]. Recently developed TLR2 antagonists such as OPN-305 is being evaluated in phase 2 clinical trials for treatment of Myelodysplastic syndrome, pancreas tumor and other oncogenic diseases [66,67]. OPN-305 is a humanized IgG4 monoclonal antibody against TLR2 that can block its activation in human monocytes from 14 to 90 days *in vivo* [67]. Data of these studies suggest that the use of OPN-305 is a promising strategy for prevention of DENV replication in classical monocytes and at the same time for modulation of the inflammatory response mediated by TLR2 activation. Future studies should evaluate the effect of blocking TLR2 with OPN-305 in DENV pathogenesis using humanized mice model [68]. Nevertheless, is important to note that TLR2 plays an essential role in the activation of innate and shaping the adaptive immunity in various microbial infections. In addition, it is expressed on monocytes, macrophages and neutrophils in many tissues. Consequently, dose, timing, and specificity for TLR2 targeting on CM should be considered with caution.

In **chapter 2** we described that TLR2 is particularly important for DENV replication and subsequent inflammatory response in CM subset. Specific delivery of TLR2 inhibitors to CM could improve the effect of TLR2 blockage. In recent years nanoparticle-based technology has been developed for improving the targeting of specific cells in certain types of treatments. For example, nanoparticle medicine that target macrophages present specifically in atherosclerotic plaques is currently under research [69]. This strategy can suppress pro-atherogenic activity of macrophages and thus modulate the inflammation and development of atherosclerotic plaques [69]. Nanoparticle medicine could be a useful tool for targeting TLR2 inhibitors specifically in CM during DENV infection.

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

1. General remarks of current therapies for DENV infection

Currently, a specific antiviral treatment for patients suffering of dengue disease is not available [70]. Furthermore, a fully protective vaccine is still awaited, as Dengvaxia, the only approved vaccine for use in humans, is recommended only for adults and children above 9 years old living in countries where DENV is endemic [71,72]. Therefore, there is a need for new therapies that can prevent severe dengue either by limiting viral replication and/or regulating the inflammatory response.

An optimal nutritional status is essential for both shaping the immune response and restoring the numbers of cells involved in innate and adaptive defense [73]. Among these nutrients, VitD3 status has emerged as a risk factor for severe forms of various infectious diseases [74]. For example, VitD3 deficiency among COVID-19 patients has been associated with development of acute respiratory failure and ICU treatment [75–77]. Whether low levels of VitD3 prior to or during DENV infection contribute to the development of severe disease remains elusive. In the next sections, I will discuss the associations of VitD3 levels with progression to severe dengue and how our studies contributed to the current knowledge on the mechanisms of VitD3 that shape innate immune responses during DENV infection.

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

Apart from its function in calcium metabolism, VitD3 has wide immunoregulatory effects [74] (summarized in Figure 5 of **chapter 1**). We and others have previously shown that 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 [11,78–81]. However, VitD3 also decreases DENV infection by downregulating the expression of the viral receptor MR [11]. Hence, it was unclear whether the reduction of inflammatory responses was due

to its immunoregulatory functions or due to a lower DENV replication in treated cells. In **chapter 3** we addressed 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 between these two types of macrophages rather than the level of infection in these cells. As discussed in detail in **chapter 3,** VitD3 decreased production of ROS which was associated with a downregulation of *TLR9* expression **(Figure 4).** 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.

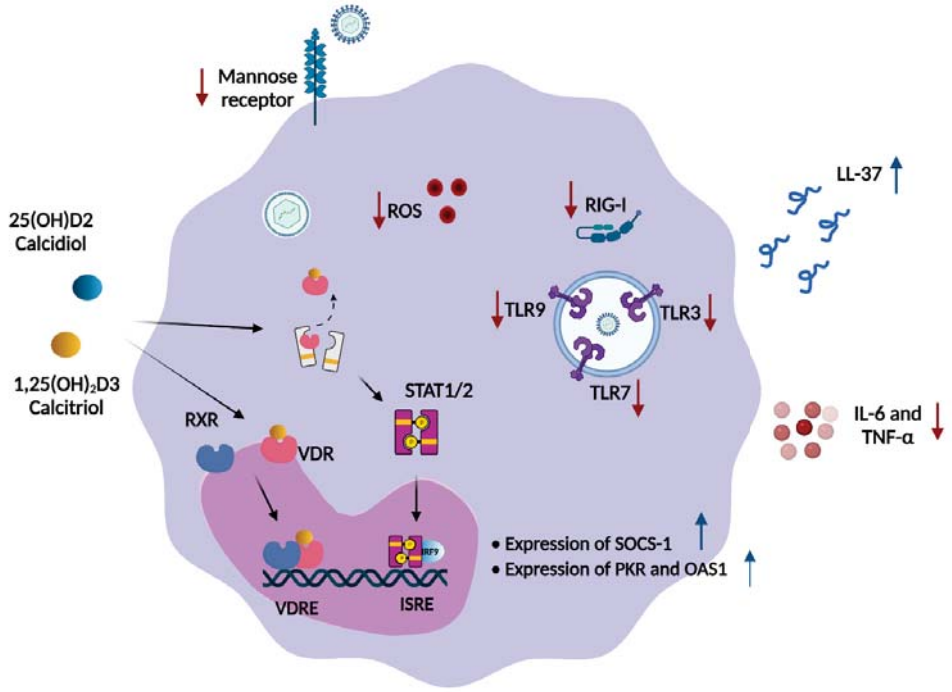

Figure 4. Effects of VitD3 in macrophages during DENV infection

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 [82,83]. Genomic effects during DENV 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 [84], 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 infection. Figure designed with Biorender.

In addition to its immunomodulatory effect, VitD3 has been shown to increase the expression of antimicrobial peptides (AMPs), including induction of CAMP expression and LL-37 production [12,13], which has potent antiviral properties. For example, VitD3 has been shown to restrict Rhinovirus replication in primary bronchial epithelial cells, by increasing LL-37 expression [85,86]. LL-37 has also been shown to restrict DENV infection in human keratynocytes (HaCaT) and in DC-SIGN expressing U937 cells [87,88]. In **chapter** 4 we demonstrated that differentiation of macrophages in the presence of VitD3 boosted the baseline expression levels of *CAMP* and production of LL-37 peptide in mock and DENV-infected cells. Additionally, exogenous LL-37 showed antiviral activity against DENV, highlighting the role of LL-37 during innate immune defense against DENV infection. These results show the potential use of VitD3 in therapeutics as it also increases the expression of LL-37 peptide during DENV infection.

Interestingly, treatment of MDMs with LL-37 after the initial 2 hours of infection (**chapter 4),** 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. In fact, Alagarasu et al. proposed an interaction between LL-37 and DENV E protein by bioinformatic modeling (*in-silico* docking) [89]. However, the precise mechanism by which LL-37 inhibits DENV replication remained unanswered. Future studies including time-of addition experiments are needed to delineate the mechanisms involved in the antiviral activity of LL-37.

The antiviral effect of LL-37 against DENV infection suggest that AMPs may have an important role during DENV infection, especially in cells present at the first site of infection such as keratinocytes, macrophages and DCs. Yet, in **chapter 4** we found that macrophages differentiated with FBS (with depreciable levels of VitD3) produce insignificant levels of HBD2, HBD3 and LL-37 in response to DENV infection. Furthermore, DENV infection appeared to reduce *CAMP* expression in macrophages. These results contrast with findings observed in different human cell types like HaCaT, THP1 and neutrophils, which produce several AMPs including HBD-1, HBD-3 and LL-37 in response to DENV infection [90,91].

The reason behind these differences may be rooted in the cell-type dependent expression or cell-type specific signaling pathways necessary for AMPs induction. Indeed, keratinocytes and neutrophils show higher basal levels of LL37 expression compared to macrophages [92]. Importantly, the lack of LL-37 production was specific to DENV infection as LPS treatment induced a significant production of LL-37, as also reported by Duits et al [93]. This finding together with the observed decreased *CAMP* expression following DENV infection, suggest that viral replication may actively antagonize LL-37 expression. Interestingly, a similar observation has recently been made for human metapneumovirus (hMPV), which causes common respiratory tract infections in young children [94]. hMPV suppressed basal and VitD3 induced *CAMP* expression in MDMs through downregulation of C/EBPα, a transcription factor critical for CAMP expression [94]. Whether a similar mechanism is triggered by DENV infection and at which step of viral replication the antagonism is activated remains to be studied. Here, assessment of the expression of LL-37 after stimulation with inactivated DENV, and evaluation of LL-37 expression in infected vs bystander non-infected MDMs will provide valuable first clues for the mechanism of LL-37 regulation during DENV infection. Altogether, the currently available data signify the multifold advantageous function of VitD3 in the course of DENV infection, as it has immunomodulatory properties and induce LL-37 expression.

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 in 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 [95–97]. 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, these 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 [97]. Evidence of a protective role of VitD3 came from clinical studies done in Pakistan [98] and Mexico [99] that showed that VitD3 supplementation during acute

DENV infection improved the patients' clinical condition by increasing the platelet count and ultimately prevented the development to severe dengue. It is important to note, that these clinical trials were not controlled, and nutritional status or VitD3 levels before and after supplementation were not assessed. Therefore, there is a lack of knowledge regarding the clinical effect of VitD3 supplementation in the protection or improvement of dengue disease. I would recommend follow-up studies in which VitD3 serostatus can be determined, and thus associate clinical improvement with VitD3 levels. In conclusion, preliminary results from clinical studies are optimistic and suggest that VitD3 could be an effective therapy for preventing the development of severe dengue. Yet large and controlled clinical trials are needed to may confirm this notion.

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

In order to design controlled clinical trial few matters need to be solved first. It remains elusive if a potential VitD3 treatment should be administered either as a treatment or as a prophylaxis. For discussing this issue, the stages of dengue disease must be taken into account. After an incubation period of 3 up to 7 days, dengue patients develop an initial acute febrile phase [100], 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 [101,102]. Currently, there is no biomarker that predicts the development of severe disease. Therefore, the best window for VitD3 supplementation in DENV-infected patients should be in the febrile phase, and practically at the moment of hospital admission. Also, several studies have shown that high doses of VitD can increase serum levels of calcitriol within 24 hours and return to physiological range by 72 hours [103,104]. This indicates that oral supplementation with VitD could have a rapid effect in DENV-infected patients.

In addition to solve the issue of timing of VitD3 supplementation, the right dose of VitD3 needs to be considered. The bioactive VitD3 (calcitriol) serostatus of an individual is generally measured by the quantification of the serum levels of its inactive and transported form VitD2 (also known as calcidiol). According to the Endocrine Society, sufficient levels of VitD2 include concentrations above 30 ng/ml, levels go from 21 to 29 ng/ml are considered insufficient, while values below 20 ng/ml are considered as vitamin D

deficiency [105,106]. Physiological levels of bioactive VitD3 vary between 0.1 to 0.5 nM [107,108]. Based on data from several studies, we are currently in a global pandemic of VitD2 deficiency in the general population, that is largely owed to our behavior including limited sunlight exposure and improper diet [105,106]. For fighting this VitD2 deficiency, clinicians usually recommend Vitamin D oral supplementation with 1000 to 5000 international units (IU) of inactive precursors such as cholecalciferol or ergocalciferol (VitD), which can surge VitD3 serum levels to 0.25-0.75nM [109]. We observed **in chapter 3** that physiological concentrations of VitD3 (0.1nM) had antiviral and immunoregulatory functions during DENV infection *in vitro*. Thus, conventional therapy directed to reach physiological levels of VitD3 may be sufficient for treating DENV-infected patients. In conclusion, future research is required to investigate if conventional treatment with VitD either as a prophylaxis or in DENV-infected patients could potentially shape the inflammatory response and prevent the development of severe dengue.

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

As detailed in **chapter 1,** due to its mode of action LL-37 displays broad spectrum antimicrobial/antiviral properties including towards DENV. Furthermore, by showing striking inhibitory activity against *in vitro* formation and development of biofilms caused by many clinically important bacteria [110–114], LL-37 has emerged as a novel alternative therapeutic strategy to combat antibiotic bacterial resistance. However, testing applicability of LL-37 has proven difficulty, due to high costs of treatments, susceptibility to proteolytic degradation and high toxicity in human cells [112]. Accordingly, to our knowledge, only two studies have evaluated the clinical potential of LL-37 treatment. One first study assessed the effect of LL-37 on the clearance of venous leg ulcers (VLU) in 34 patients. The authors observed that topical treatment with 0.5 and 1.6 mg/ml of LL-37 was well tolerated and resulted in increased healing rates of the VLU compared to placebo [115]. However, a second trial that assessed the effect of intra-tumoral LL-37 treatment in melanoma patients reported side-effect dermatological toxicity in a 63-year old woman [116]. Notably, a recent study demonstrated that the limitations of using LL-37 as a therapeutic can be circumvented by an engineering and shorter LL-37, with improved biostability and ultimately enhanced antiviral activity against Ebola virus infection, compared to the native LL-37 [117]. Whether

engineered or native LL-37 represent a feasible treatment alternative for fighting viral infections and more specifically DENV infection, remains unanswered. These data warrant future *in vitro* and *in vivo* which assess the potential and safety of LL-37 peptide against viral infections.

Alternatively, VitD3 treatment appears to be a solution for increasing LL-37 levels. I am certain 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 [118]. Also, supplementation with 500,000 IU of VitD3 in critically ill ventilator-dependent adults increased mRNA expression of LL-37 and serum levels of LL-37 that correlated with increased phagocytosis of alveolar macrophages [119]. Based on our *in vitro* data of **chapter 4,** differentiation of MDMs in the presence of VitD3 increased expression LL-37 levels by 5-fold during DENV infection (590ng/ml in MDMs vs 3200ng/ml in D3-MDMs). Importantly, with the IC50 of LL-37 at the level of 2,246 ng/ml, the concentration of LL-37 induced by VitD3 in our model could potentially restrict DENV replication. Whether the levels of LL-37 expression obtained with VitD3 treatment during *in vitro* DENV infection can be achieved in a clinical setting with VitD3 supplementation remains to be explored. Taken together, boosting the levels of LL-37 in serum and tissues through either direct administration of modified LL-37 peptide or by VitD3 supplementation, could be useful strategies to inhibit DENV replication and regulate the inflammatory response in patients after early onset of symptoms **(Figure 5)**.

CONCLUDING REMARKS

The studies presented in this thesis underscore the importance of innate immune responses in control and pathogenesis of DENV infection. Activation of monocytes/macrophages can ultimately lead to ECs dysfunction, underlying the development of severe dengue. Our novel data disclosed the specific role of TLR2 in CM facilitating DENV infection and induction of inflammatory responses, which were associated with vascular dysfunction and severe disease pathogenesis. In future studies, I would focus on single cell omics technologies combined with immunophenotyping in order to scrutinize the TLR2-driven

immune responses in infected and bystander monocyte subsets of DENV-infected patients. Hence, the identification of intrinsic mechanisms differentiating TLR2 function in CM, IM and NM would be possible, and may allow the development of new therapies targeted directly to specific monocyte subsets. To that end, *in vitro* and *ex vivo* studies in PBMCs isolated from dengue patients should be considered fundamental. In addition, the use of humanized mouse models will be indispensable for assessing the potential of specific TLR2 inhibition in limiting DENV replication and/or mitigating disease pathogenesis.

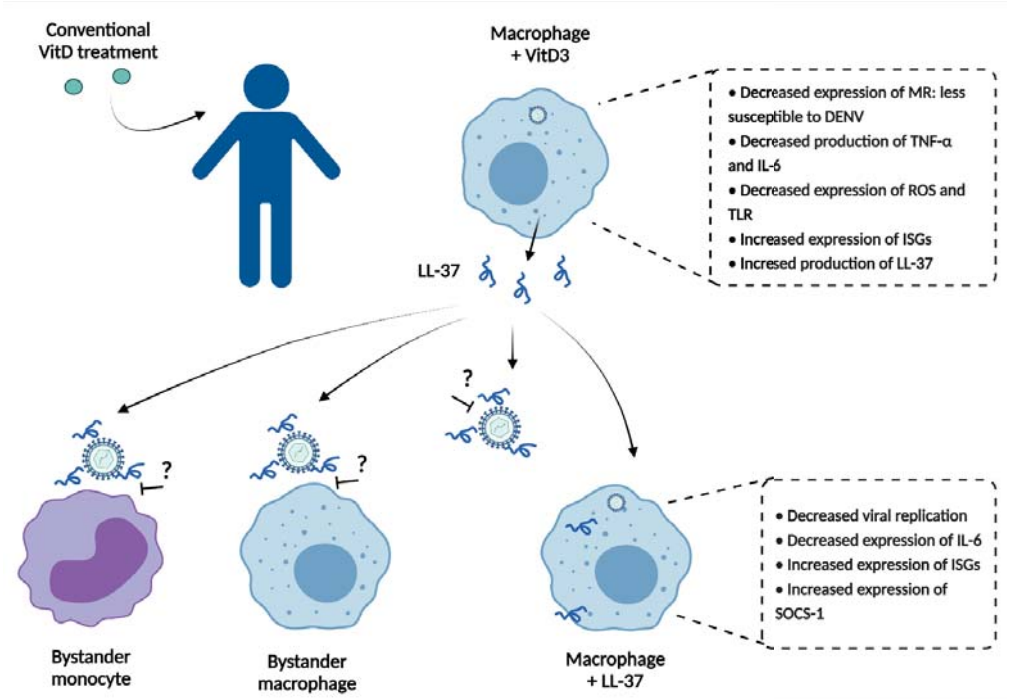

Figure 5. Potential effects of VitD treatment in DENV-infected patients

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. Furthermore, increased production of LL-37 by macrophages with high levels of VitD3 could potentially restrict DENV replication, either by directly disturbing the viral particles or by inhibiting viral entry into bystander target cells like monocytes and macrophages. Figure designed with Biorender.

In addition, our studies detailed the immunomodulatory properties of VitD3, including the regulation of innate immune response and the induction of LL-37 expression in macrophages. These findings warrant future investigations for dissecting the effects of VitD3 insufficiency and deficiency in disease pathogenesis, and for assessing the possibility of VitD or LL-37 supplementation in DENV-infected patients. Supplementation of VitD during DENV infection could be a useful tool for ameliorating the symptoms caused by the inflammatory response and for preventing the development of severe dengue. Finally, I am certain that detailed knowledge of the innate immune mechanisms driving pathological inflammation in the course of DENV infection would open the floodgates for the development of specific therapeutics preventing the development of severe dengue.

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