Innate immune response to dengue and chikungunya virus (co-)infections

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Introduction and scope of this thesis

Epidemiology of dengue virus and chikungunya virus (co-)infections

RNA viruses transmitted by mosquitoes are currently responsible for the most prevalent and debilitating arthropod-borne (arbo)viral diseases worldwide. Dengue and chikungunya are two arboviral diseases that have caught world's attention because of the increase in frequency and morbidity of epidemics in recent decennia. The etiologic agents of these diseases are dengue virus (DENV) and chikungunya virus (CHIKV) both transmitted to humans by the mosquitoes of *Aedes* spp., in particular *Aedes aegypti* and *Aedes albopictus*. Trends of urbanization and increased mobilization of people and goods is contributing to increasing number of infections in (sub)tropical regions and the spread to new geographical areas (Fig. 1). [1-3]

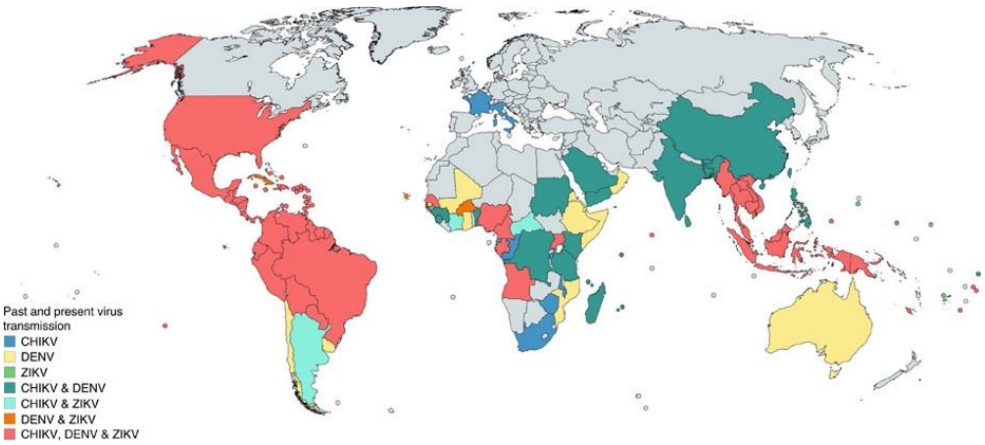

Figure 1. Global burden of dengue and chikungunya. The map shows countries where autochthonous cases of DENV, CHIKV and ZIKV infections and different combinations of co-infections have been reported until May 2017. Reproduced from[4] with permission from Nature Publishing Group.

It has been estimated that the four serotypes of DENV (DENV1-4) infect approximately 390 million individuals per year worldwide, making dengue currently the most prevalent arboviral human disease.[5] The earliest records describing a dengue-like disease date back to the first century AD in China. In 1780, an epidemic describing dengue-like disease was reported in Africa, Asia and North America. However, it was not until 1943, that the etiologic agent was identified and over the last fifty years, the global incidence has increased 30-fold. [6] The virus is now endemic in 100 countries throughout the territories

in East Africa, Southeast Asia, the Western Pacific, and America. More recently sporadic autochthonous infections have also been reported in the Eastern Mediterranean region.[7] Not only the incidence but also the number of individuals with severe dengue increased. Severe dengue was first recognized during epidemics in Philippines and Thailand and until 1970 it was only observed in 9 countries. Today, it is reported as the leading cause of hospitalization of children in Asian and Latin American countries.

CHIKV was first isolated in 1953 during an epidemic in Tanzania among the Makonde tribe.[8] Its name derives from a Makonde word that translates to 'disease that bends up the joints' and refers to the posture of infected persons experiencing severe joint pain. Until the beginning of the 21st century CHIKV epidemics were small and mainly restricted to the African continent. In 2005, CHIKV caused a large outbreak in La Reunion Island that was characterized by a high incidence rate. The virus then spread to India, where in 2006, around 1.5 million individuals were infected.[9] In 2007, the virus crossed the European border and ever since has caused autochthonous infections in Italy, Croatia, France, Spain and Portugal. In 2013, the first cases of CHIKV were reported in South America in the Island of St. Martin. Thereafter, CHIKV spread through South and Central America and currently CHIKV is endemic in 45 countries worldwide.[10]

The growing overlap in the geographical distribution of the viruses and their vector (Fig. 1) in recent years has led to an upsurge of human cases of DENV/CHIKV co-infections.[11-29] The rise of DENV/CHIKV co-infections reporting can be also attributed to the increase in molecular diagnostics and epidemiological surveillance in regions where multiple arboviruses such as DENV, CHIKV and most recently Zika virus (ZIKV) co-circulate. In 2015, the World Health Organization and Pan American Health Organization announced that the co-circulation of the three arboviral diseases poses a new challenge for public health in the Americas. To aid the prevention and control of arboviral disease a document was published to be used as a tool for the diagnosis and care of patients with suspected arboviral diseases. [30,31]

The viruses

Dengue virus

Virion structure

DENV is a member of the *Flaviviridae* family, genus *Flavivirus*. As other flaviviruses (eg. West Nile virus/WNV, Zika virus/ZIKV), the virus particle comprises a host–derived lipid envelope in which two viral glycoproteins are embedded: the envelope glycoprotein E and the (pre)membrane protein (pr)M. The ectodomain of the E glycoprotein is constituted by three structural domains (DI, DII, and DIII). DI is the hinge region, DII contains the fusion loop and the putative receptor domain is localized on DIII. The envelope encapsulates a single-strand positive-sensed ~11 kb RNA genome which together with multiple copies of the capsid protein C form a nucleocapsid. Immature virions have a spiky surface: each spike representing three prM/E heterodimers (Fig. 2). In contrast, mature virions, contain 90 dimers of the E protein that are arranged flat over the viral envelope thereby giving the particle a smooth appearance. The DENV RNA genome has a single open reading frame (ORF) encoding for three structural proteins: C, (pr)M, and E; and seven non-structural proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5.[32,33]

Replicative cycle

The viral E glycoprotein mediates host cell binding and delivery of the genome into the cell cytoplasm. Although several attachment factors, such as DC-SIGN, mannose receptor, glycosaminoglycans (heparin sulfate) and immunomodulatory proteins (TIM/TAM receptors) have been described, so far, no specific entry receptor has been identified.[34] The virus enters the cell by hijacking clathrin-mediated endocytosis. Subsequent acidification of the endosomal compartments triggers conformational changes that cause dissociation of the E protein dimers and exposure of the fusion loop.[35] The fusion loop inserts in the target membrane and subsequent E1 trimerization drives fusion of the viral envelope with that of the endosome and ultimately the release of the viral genome. The viral RNA is subsequently translated by ribosomes located in close proximity of the membranes of the endoplasmic reticulum into a single polyprotein. The polyprotein is cleaved co- and post-translationally by viral and cellular proteases to produce ten viral proteins (Fig. 2). The non-structural proteins form a replication complex that produces viral RNA copies. RNA replication occurs within virus-induced ER membrane invaginations named vesicle packets (VPs) and is driven by the RNA-dependent

polymerase activity of NS5. Assembly of new, immature virions takes place in the lumen of the ER. Virions mature during egress via acidic compartments of the trans-Golgi network. There, a host-derived protease, furin, cleaves the prM protein to M and the small pr peptide that remains attached to the surface of the E protein until the virions are secreted to the extracellular milieu. The peptide is released from the particle due to the change to a neutral pH environment upon secretion. Hereafter, the virus becomes fusion competent. Importantly, DENV maturation is generally inefficient and results in secretion of a heterogeneous population of mature, partially mature and immature particles that have different infectious properties.[36-38]

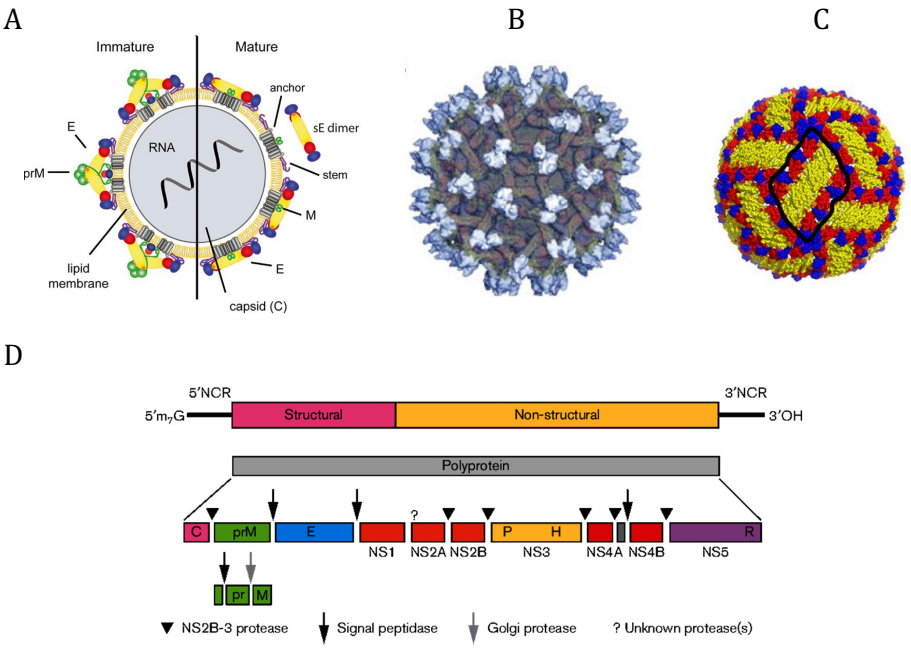

Figure 2. Structure of DENV. (A) Conformation of the structural proteins in an immature and a mature DENV virion. prM and M are shown in green and the three functional ectodomains of the E protein are depicted in red (DI), yellow (DII) and blue (DIII). (B) 3D model of an immature virion. (C) 3D model of a fully mature virion. (D) Schematic representation of the genome of DENV. Arrows indicate the cleavage site of proteases and the signal peptidase. Panels A, B and C are adapted from [39] and D from [40].

Virus tropism

The initial targets of DENV at the site of the mosquito bite are skin-resident dendritic cells (Langerhans cells), macrophages, mast cells and keratinocytes.[41-43] Inflammatory responses induced in the skin lead to the recruitment of monocytes, which become infected and as a consequence contribute to virus amplification and dissemination.[44] In blood, circulating mononuclear cells, specifically monocytes and dendritic cells (DCs), are main targets of viral replication, however viral antigens have also been detected in B[45,46] and T cells[47-49] and platelets[50,51]. Additionally, DENV infection has been observed in hepatocytes and endothelial cells *in vitro* and in patients' material.[41,52-54]

Chikungunya virus

Virion structure

CHIKV belongs to the *Alphavirus* genus of the *Togaviridae* family. The CHIKV genome consists of a positive single-stranded RNA (11,8 kb) with two separate ORFs. The 5´ORF encodes for 4 non-structural proteins (nsp1-4) and the 3´ORF for 5 structural proteins (C, E3, E2, 6K and E1). The genome is packaged together with 240 units of the C protein to form the nucleocapsid, which is surrounded by a host-cell derived envelope. Within the viral envelope, 240 units of the transmembrane glycoproteins E1 and E2 are inserted. These proteins are assembled into 80 spikes: a single spike consisting of three E2/E1 heterodimers (Fig. 3).

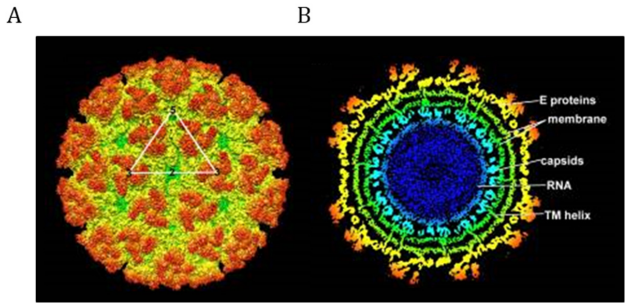

Figure 3. Structure of CHIKV. (A) Cryo-EM structure of CHIKV. (B) Cross-section of CHIKV showing the different components of the virus. Adapted from [55].

Replicative cycle

The replicative cycle of CHIKV starts with the interaction of the E2 glycoprotein with a plasma membrane receptor expressed on the target cell. To date, several attachment factors, such as glycosaminoglycans (GAGs), prohibitin and phosphatidylserine-mediated virus entry-enhancing receptors (PVEERs) have been described.[56] Recently, Mxra8 was identified as an attachment factor and receptor for CHIKV as well as for other arthritogenic alphaviruses.[57] Cell entry occurs mainly via clathrin-mediated endocytosis, however other pathways have also been observed depending on the cell type used.[58] Membrane fusion occurs following acidification of the endosomal compartment to pH values below 6.2 or 5.9, depending on the virus strain used.[59] The low pH destabilizes the E2/E1 dimer resulting in exposure of the fusion loop and its interaction with the limiting membrane. E1 trimerization is required for merging of the viral and endosomal membranes. Immediately after the release of the nucleocapsids, the viral mRNA is translated and subsequent processing of the non-structural proteins nsP1-nsP4 generates the replication complex (RC). The RC produces a full-length negative-strand RNA intermediate that serves as the template for both genomic and subgenomic RNAs. The structural proteins are translated from the subgenomic 26S RNA as a single polyprotein precursor. Processing of the polyprotein by an autoproteolytic serine protease produces the capsid. The remaining of the polyprotein is translocated to the endoplasmic reticulum where it is further processed to form E1/pE2 heterodimers. Upon further modification, the E1/pE2 heterodimers are transported via the Golgi to the plasma membrane. Capsid proteins pack the viral RNA in the cytoplasm and newly formed nucleocapsids line-up at the plasma membrane where the assembly of progeny virions takes place.[60]

Virus tropism

During infected-mosquito probing, CHIKV-containing saliva is spread in the dermis, where dermal fibroblasts represent the main targets of infection followed by keratinocytes and resident dendritic cells, including Langerhans cells.[61,62] In parallel, as the mosquito feeds, CHIKV is also introduced directly into the bloodstream. There, circulating immune cells, mainly monocytes and B cells become infected.[63,64] Although the susceptibility of monocytes to CHIKV infection is relatively low, infection of these cells is thought to be crucial for viral dissemination to peripheral tissues (muscle, joints, skin and eye).[65,66] Interestingly, despite the restricted tropism *in vivo*, CHIKV is able to infect a variety of cells *in vitro.*[61]

The diseases

Dengue

Disease presentation

Out of the estimated 390 million DENV infections per year, 75% are asymptomatic meaning most individuals do not realize they are infected with DENV. The remaining 25% (approximately 96 million cases per year) develop acute and relatively mild disease termed dengue or dengue fever (Fig. 4).[5] Dengue fever is characterized by high fever, headache, severe muscle and joint pain, abdominal pain, nausea, and leukopenia all of which self-resolve within one week. However, in approximately half a million cases per year when the fever subsides, the condition exacerbates to severe dengue (critical phase).[67] According to the guidelines published in 2009 by the World Health Organization (WHO) clinical dengue is divided into three categories: dengue, dengue with warning signs, and severe dengue.[7] The warning signs include severe abdominal pain or persistent vomiting, rash and liver enlargement. If not treated promptly, serious complications such as vascular leakage and organ impairment, might lead to death. In areas where early and adequate medical care are available the mortality rate is less than 1%, however, lack of supportive treatment in marginalized areas raises this percentage to 20%.[68] The old classification (dating from 1997), groups symptomatic infections into: undifferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS)[69]; and many clinicians and scientists still use this classification in case descriptions.

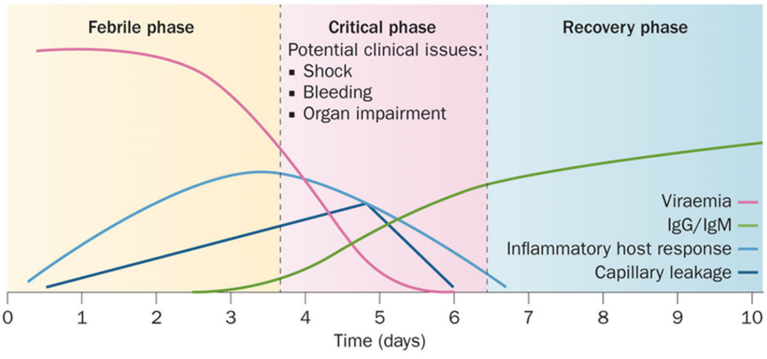

Figure 4. Clinical course of DENV infection. About 4-8 days after the bite of an infected mosquito, the virus is detectable in the human blood. The febrile phase lasts for 4-6 days. Viremia levels peak usually around day 1-3 after disease onset. The decrease in virus titers coincides with

high levels of inflammatory mediators represented by inter alia IFN-γ, TNF-α, IL-6, MCP-1 and IL-8 and precedes the critical phase of the infection. During this phase the patient's condition either improves or worsens leading to severe dengue. Finally, about 7 days post-illness, high antibodies titers can be detected and the severe symptoms subside. Adapted from [70].

(Immuno-)pathogenesis

The hallmark of severe disease is plasma leakage caused by the increase in permeability of vascular endothelium. The pathological, yet transient loss of endothelial integrity is thought to be a result of exacerbated inflammation triggered by DENV infection. Indeed, IL-6, IL-8, IL-10, IFN-γ, migration inhibitory factor (MIF), monocyte chemoattractant protein 1 (MCP-1/CCL-2), tumor necrosis factor (TNF)-α, chemokine ligand 3 (CCL-3), C-X-C motif chemokine (CXCL)-8, CXCL-10 and vascular endothelial growth factor (VEGF) are repeatedly found to be elevated in patients with severe dengue.[71] The risk of severe disease is increased in secondary heterologous infections (infections with a different DENV serotype than the one that caused the first infection) and primary infections of infants born from dengue immune mothers. High viremia titers and high levels of inflammatory and vasoactive soluble immune modulators (cytokine storm) preluding severe disease symptoms are attributed to two main immune-pathological features of dengue: antibody-dependent enhancement (ADE) of infection and original antigenic sin. [72-74] ADE of infection postulates that pre-existing cross-reactive antibodies from a primary infection opsonize the virus and facilitate successful virus internalization via the Fc receptors. Consequently, enhanced infection of phagocytic cells higher viremia and excessive production of immune soluble factors is seen.[75] This process is further fueled via original antigenic sin of T and B cells, a phenomenon in which the immune response is skewed to the primary antigen leading to expansion of T and B cells with lower affinity for the newly infecting virus serotype and therefore is less effective in clearing the virus.

Thus, for the containment of the virus spread, it is crucial that the host mounts a rapid, effective but also controlled innate immune response.

Chikungunya

Disease presentation

The vast majority of CHIKV infections are symptomatic, the incidence of asymptomatic cases range between 3 to 25%.[2] The incubation period in the human host following an infected mosquito bite lasts 2 to 4 days. (Fig. 5). Thereafter, the virus in detectable in the blood (up to 10^8 viral particles per mL of blood).[60] The peak of viremia coincides with the acute symptoms of chikungunya fever (CHIKF), which very much resemble the acute symptoms of dengue including fever, headache, rash, myalgia and intense arthralgia. CHIKF is an acute, self-limiting illness and most patients recover within 5 to 7 days. However, in 30 to 40% of cases, recurring musculoskeletal pain affecting the peripheral joints persist for months to years following CHIKV infection and thereby has a profound impact on the quality of life of infected individuals. Sporadically (1 in 1,000 cases) and mainly in newborns, elderly or patients with underlying medical conditions, CHIKV infection leads to potentially fatal complications such as encephalitis, hepatitis, renal failure and myocarditis.[76]

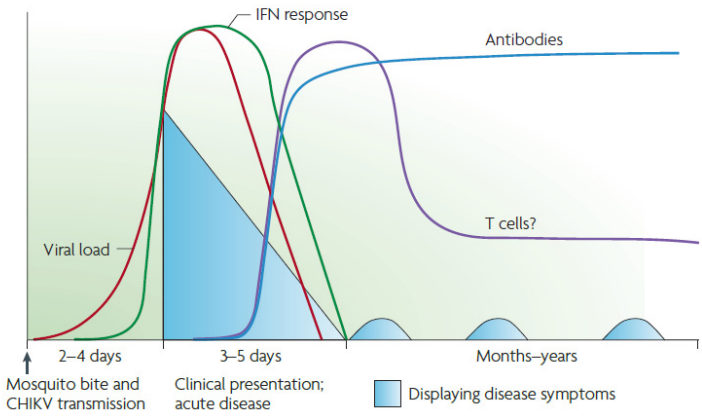

Figure 5. Time course of CHIKV infection. During the incubation period (2-4 days), the viral load rapidly increases and peaks at day 0-2 after disease onset. The febrile phase usually lasts for 3-5 days. The recovery phase coincides with high IFN response, antibody production and T cell activation.[60]

(Immuno-)pathogenesis

The immune-pathogenesis of CHIKV infection leading to more severe or chronic disease presentations remains poorly understood. Several soluble innate immune factors in

patients with acute chikungunya have been associated with disease severity and persistent symptoms. In the acute phase, CHIKF patients present higher levels of many typical inflammatory cytokines and chemokines including IL-1β, IL-2R, IL-5, IL-6, IL-7, IL-10, IL-15, IFN-α, MCP-1, CXCL9, CXCL10, when compared to healthy controls.[77] Additionally, high viremia has been shown to correlate with increased levels of IL-6, IL-12, IL-15, CXCL10, MCP-1, IFN-α, and IL-1Rα.[78] The hallmarks of CHIKV-induced arthritis include local joint inflammation, infiltration of monocytes/macrophages into de synovial cavity and bone destruction.[79] Indeed, in patients with chronic persistent arthralgia levels of IL-6, IL-8, granulocyte-macrophage colony–stimulating factor (GM-CSF) and MCP-1 in synovial fluid and tissue are significantly elevated.[80] Furthermore, in some cases of CHIKV-induced chronic arthritis and other viral arthropathies, virus RNA and viral antigens have been detected in the affected joint tissues months after the acute infection.[81-83] The ability of CHIKV to persist in infected cells has ever since been corroborated by studies in several animal models.[63,65,80,81] In addition, the interaction of activated and/or infected monocytes and macrophages with synoviocytes and osteoblasts may contribute to the development of the arthritis-associated bone erosion.[84,85] Consequently, extensive monocyte/macrophage infiltration in joints and persistent infection of macrophages are thought to play key role in the pathogenesis of CHIKV-induced arthritis.[79,86]

The infiltration of inflammatory monocytes into synovial tissue seems to be the central event underlying CHIKV-mediated arthralgia and arthritis.[87] The excessive production of immune mediators involved in activation and migration of monocytes, such *inter alia* MCP-1, could thus explain the events of CHIKV pathogenesis.

DENV/CHIKV co-infections

The reported incidence of co-infections is variable and ranges from 0.1 to 43%[88], depending on the country, season and diagnostic tools used to identify infections. Most of the case reports come from South and West India, where in 2010 co-circulation of DENV and CHIKV with high morbidity was observed. Nonetheless, the effect of co-infections on disease presentation remains elusive. Few studies describe the symptoms that are specifically observed in co-infected patients although there is little overlap between

different cohorts.[11,12,22,89] For instance, in a study conducted in India, Gandhi and colleagues reported that co-infection was associated with more severe clinical disease, based on an increased requirement of mechanical ventilation and blood transfusion, when compared to mono-infections.[22] In another Indian cohort where the highest incident of co-infections was as high as 43%, only co-infected patients (however not all of them, 16.2%) presented with diarrhea.[12] In contrast, a cohort study in Gabon, detected no clinical manifestations specifically associated with co-infection.[11] Clearly, the effect of co-infection on the clinical presentation of patients is variable and requires further investigation.

Innate immune system during DENV and CHIKV infections

Innate immunity

Host cells can sense invading pathogens through a set of specialized pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs).[90] PRRs recognize a specific type of PAMP, e. g. glycoproteins or nucleic acids. PRRs have different expression levels among cell types, through which pathogens induce tissue-specific responses [90]. There are many families of PRRs, however, the most studied regarding RNA virus recognition are the toll-like receptors (TLRs) and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Fig. 6).[91] TLRs are transmembrane proteins that are localized either on the plasma membrane (TLR1, TLR2, TLR4, TLR5, TLR6) or reside inside endosomal compartments (TLR3, TLR7, TLR8, TLR9, TLR11 and TLR13). Of the 10 TLRs expressed in human cells, 6 have been related to antiviral immunity.[92] TLR3, TLR7, TLR8 and TLR9 recognize viral nucleotides while TLR2 and TLR4 detect viral glycoproteins. The RLR family comprises the RNA helicases MDA5 (melanoma differentiation-associated gene 5) and RIG-1 (retinoic acid-inducible gene 1). These are cytoplasmic receptors that are expressed in low levels in many cell types.[93,94] Increase in their expression is often induced by viral infection and interferon (IFN) exposure.[95] RIG-1 specifically recognizes single and double-stranded RNA (ssRNA/dsRNA) of the viral genome, replication intermediates of RNA viruses, and 5′ triphosphate dsRNA. The ligands of MDA5 include dsRNA fragments longer than 7kb.[91,96]

TLR and/or RLR activation can lead to activation of IRF3/7 and NF-κβ transcription factors which together control the expression of type I IFNs and proinflammatory cytokines.[97] The released cytokines bind to their respective receptors in an autocrine or paracrine manner, thereby conveying inflammatory cues. Likewise, binding of IFNs to the interferon receptor (IFNAR) triggers the activation of the Janus activated kinase/signal transducer and activator of transcription (JAK-STAT) signaling pathway that promotes transcription of interferon stimulating genes (ISGs). The products of ISGs control replication in infected cells and protect bystander cells by inducing an antiviral response through different mechanisms.[90] The interplay between the pro-inflammatory and antiviral IFNs shape the effectiveness and duration of the inflammation.

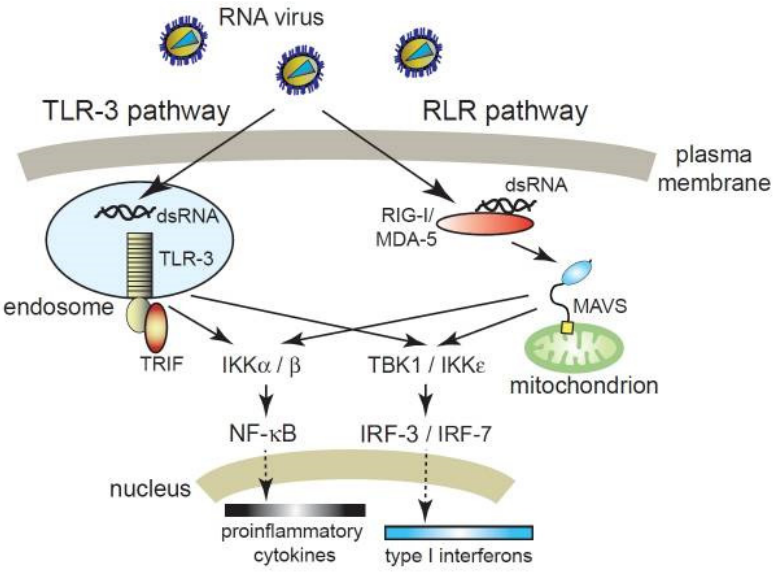

Figure 6. TLR and RLR signaling pathways. PAMP recognition causes dimerization of the TLR followed by recruitment of a TIR-domain-containing adaptor protein. TLR3 utilizes the TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) while other TLRs use the myeloid differentiation primary response gene 88 (Myd88). The corresponding adaptor protein of the RLRs is the IFN-β promoter stimulator 1(IPS-1) (also known as CARD adaptor-inducing IFN-β (CARDIF) and mitochondrial antiviral signaling protein (MAVS)). Downstream of the pathways, kinases are activated; in the RLR pathway, TBK1 and IKKε phosphorylate and activate IRF-3 and IRF-7 while in the TLR-mediated pathway, IRF-7 is phosphorylated by IRAK1 and IKKα. In addition, phosphorylation of inhibitory κB (IκB) by IKKα and IKKβ frees NF-κβ. Finally, nuclear translocation of NF-κβ and IRF3/IRF7 induces expression of proinflammatory cytokines and type I IFNs [91,97]. Adapted from [97].

Innate immune response to DENV infection

The innate response to DENV is initiated by the recognition of viral components via endosomal nucleic acid-receptors: TLR3, TLR7/8, RIG-1 and MDA5[98-102]; and extracellular TLRs: TLR4[103-105]. Studies *in vitro* and *in vivo* have demonstrated that TLR3 and TLR7/8 activation decreases DENV replication *via* the induction of type I IFN and proinflammatory cytokines.[98-100,102] Subsequent studies using RIG-1$^{-/-}$ and MDA5$^{-/-}$ fibroblasts showed that DENV can be recognized by both receptors.[101,102] In addition, TLR4 has been to recognize the soluble form of DENV NS1, which circulates in blood during vireamia. The engagement of NS1by TLR4 in PBMC has been shown to induce a pro-inflammatory response that compromises the integrity of the vascular endothelium and ultimately may lead to vascular leakage.[103,106]

Type I IFNs (α,β and ω) and type III IFN (γ) modulate DENV infection by protecting uninfected cells.[107] Table 1 shows the summary of the ISG products that participate in the control of DENV infection. In addition, Type I IFN prevents DENV-mediated vascular permeability by inhibiting the production of TNF-α.[108]

Table 1. Interferon-stimulated gene products active against DENV and CHIKV

ISG products	Virus affected	Mechanism of Action	Ref.
OAS1 and OAS3	DENV CHIKV	Degradation of viral RNA via RNase L activation	109,110
Viperin	DENV CHIKV	Lipid biosynthesis modulation	111,112
IFTM1, IFITM2 and IFITM3	DENV CHIKV	Inhibition of viral entry and fusion	111,113-116
ISG20	DENV	Exonuclease	111
STAT2, IFI6, RIG-1, IL28RA, IRF-7, NAPA, ADM, CD9, IRF-1, HPSE	DENV	Inhibition of viral replication	115
IFI6, MAFK, PAK3, DDX24, IRF9, IFI44L, IFRD1, SC4MOL	DENV	Various mechanisms	117
PKR	CHIKV	Blocks protein translation	118
GADD34	CHIKV	IFN-β Production	119
Tetherin	CHIKV	Blocks viral budding	120
ISG15	CHIKV	Immunoregulation	121
C6orf150, P2RY6, SLC15A3, SLC25A28, IRF-1, HPSE	CHIKV	Unknown mechanism	122

Following virus transmission, the skin resident myeloid DCs (Langerhans cells, dDCs) and macrophages initiate inflammatory responses and recruitment of circulating monocytes to the site of infection. There, the activated monocytes differentiate into monocyte-derived DCs, which fuel the inflammatory cascade characteristic of acute DENV infection. Notably, as myeloid DCs are in general more permissive to DENV infection than monocytes and macrophages, this event increases the pool of highly permissive cells.[44,123] Besides being the initial targets of DENV infection, monocytes, macrophages and DCs are also the first responders of the innate immune system.[41,124,125] The role of monocytes and macrophages in viral clearance was demonstrated in mice deficient in IFN- α/β and -γ receptors (AG129 mice) by depletion with clodronate liposomes.[126] The results showed that in the absence of monocytes/macrophages the viral load is 10-fold higher than in control mice. Furthermore, by knocking-down type I IFN signaling in specific immune cell subsets, it was demonstrated that IFN signaling in macrophages and dendritic cells is critical for the control of DENV infection.[127] Indeed, TLR7-mediated recognition of DENV by plasmacytoid (pDCs)[128] leads to production high amounts of IFN-α, IL-6, TNF-α and increased expression of ISGs.[129,130] Importantly, pDCs are poorly permissive to DENV, thus the mechanism of cytokine induction by these cells is independent of DENV replication. This is an advantage for the host as DENV-replication evasion strategies are not able to block this process. In fact, Décembre et al. revealed that activation of pDCs occurs via cell-to-cell E-glycoprotein-dependent transmission of viral RNA from infected cells to bystander pDCs.[130] The importance of pDCs in the control of virus pathogenesis is strengthened by the observation that a poor pDC response leads to higher viremia and increased risk of severe disease development.[129,131]

Innate immune response to CHIKV infection

Studies in animal models, human primary cells and analysis of patient's material demonstrate the importance of inflammatory and antiviral IFNs in controlling CHIKV infection.[63,66,132-135] Type I IFN expression is strongly induced in CHIKV-infected fibroblasts through stimulation of RLRs.[132,134] Table 1 contains a list of ISGs products with specific activity against CHIKV and their mechanism of action.

Interestingly, IFNAR$^{-/-}$ mice are more sensitive to infection than MAVS$^{-/-}$ mice suggesting that also other innate receptors drive IFN responses in CHIKV infection. Indeed, it was found later that during CHIKV infection hematopoietic cells produce type I IFN upon

activation of endosomal TLR3.[136] Moreover, CHIKV infected TLR3$^{-/-}$ mice presented foot swelling, edema and sustained high viremia, indicating the importance of this receptor in both the inflammatory response and viral clearance. Importantly in the same study, also Myd88$^{-/-}$ infected mice had increased viremia and viral dissemination[132], suggesting that PRRs other than TLR3 such as TLR2, 4, 7/8, TLR7 also contribute to containment of CHIKV.

Balanced inflammatory responses are crucial to contain the infection and mitigate CHIKV pathogenesis. For instance, in the absence of macrophages, CHIKV-infected mice show reduced foot swelling but prolonged viremia.[65] Treatment of CHIKV-infected mice with bindarit, a compound inhibiting MCP-1 expression and thereby also migration of monocyte-derived macrophages to the site of inflammation, prevents monocyte infiltration and joint inflammation.[137,138] Importantly however, knock-out (KO) of the receptor for MCP-1, CCR2, exacerbates CHIKV-mediated musculoskeletal disease, severe neutrophil infiltration and alters various cytokine expression.[135] Likewise, KO of a dendritic cell immunoreceptor (DCIR$^{-/-}$) in mouse bone marrow-derived DCs resulted in elevated levels of IL-6 and IL-10, increased foot swelling and tissue damage following CHIKV infection.[139]

Taken together, multiple PRRs can simultaneously recognize a single pathogen and synergistically activate the antiviral and inflammatory responses. Importantly, however, viruses have evolved to block one or more of these pathways to favour virus particle production.

Innate immune evasion strategies of DENV and CHIKV and the consequences of co-infections

Multiple mechanisms have been identified through which DENV and/or CHIKV evade or manipulate the innate immune antiviral response to create favorable conditions for replication (Fig. 7). DENV utilizes two main strategies as defense against type I IFN production: inhibition of induction and signaling disruption. Through methylation of the viral mRNA cap by DENV NS5, the virus avoids recognition by RIG-1 and MDA5, thereby preventing activation of the signaling cascades downstream of these receptors.[140,141] DENV NS2B/3 functions as a protease that targets the stimulator of interferon genes protein (STING) which is in charge of phosphorylation of the transcription factor IRF-

3.[142-144] Accordingly, cleavage of STING by NS2B/3 results in inhibition of the IFN-β promoter. DENV's second line of defense against type I IFN is inhibition of the JAK/STAT signaling pathway to prevent expression of ISGs. DENV NS2A, NS4A and NS4B block the pathway by inhibiting the phosphorylation of STAT1 and its nuclear translocation.[145] Additionally, DENV NS5 causes degradation of STAT2 via the proteasome.[146,147] Notably, much less effort has been put into unraveling CHIKV evasion strategies, yet the nsP2 protein seems to be the main weapon against the innate antiviral response.[148] NsP2 directly tackles type I IFN signaling through inhibition of the JAK/STAT signaling pathway by blocking nuclear translocation of STAT1.[149] Moreover, it induces host cell transcriptional shutoff by promoting degradation of the catalytic subunit of RNA polymerase II, Rbd1.[150] Additionally, the overall translation of host proteins is affected by nsP2[134], yet the mechanistic details are elusive.

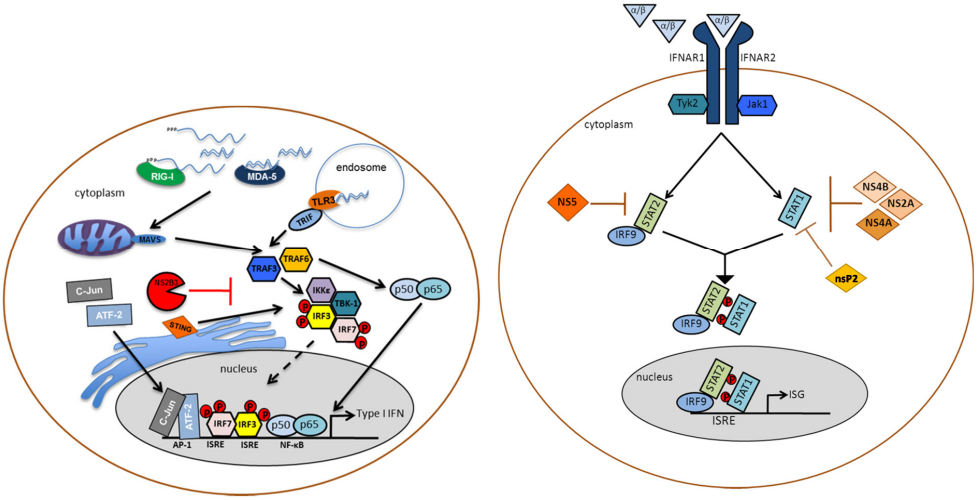

Figure 7. DENV and CHIKV immune evasion strategies. The left panel shows the inhibition of STING signaling by NS2B3 of DENV in the context of other innate recognition pathways. The right panel shows the inhibition of the type I IFN signaling by NS5, NS4B, N2A and NS4A of DENV and nsP2 of CHIKV. Adapted from [151].

Scope of this thesis

The continuing increase in the geographical overlap of DENV and CHIKV and the upsurge of reports of co-infections drove our interest to investigate the effect of concomitant infections on the innate immune responses to these viral infections. Furthermore, whereas the role of peripheral blood immune cells in the pathogenesis of DENV is well-studied, the role of these cells in course of CHIKV is largely unknown. The studies included in this thesis aim to bridge this gap in knowledge by elucidating the phenotype and mechanisms underlying the innate-immune responses triggered by peripheral blood mononuclear cells upon infection.

In **Chapter 2** we describe the dynamics of virus replication and immune response upon DENV and CHIKV co-infection in human peripheral blood mononuclear cells (PBMCs). We use co-infection-matched single virus inoculum controls to stratify data for effects observed solely by the increase in the total number of virions in co-infections. Moreover, by performing co-infections with UV-inactivated viral controls, we define the contribution of virus replication in observed interactions.

In **Chapter 3**, we investigate the mechanism underlying CHIKV infection-mediated production of MCP-1, a chemokine associated with CHIKV-induced arthritis. With use of the established *in vitro* infection model in human PBMC and monocytes we define the source of MCP-1 and identify the prerequisites for its production. Furthermore, as MCP-1 has been linked to enhanced replication of several viruses we also analyzed its role in CHIKV replication.

Blood monocytes are a heterogeneous population that based on the surface expression of CD14 and CD16, has been classified three main subsets: classical (CD14++CD16-, CM), intermediate (CD14++CD16+, IM) and non-classical (CD14+CD16++, NC). Therefore, in **Chapter 4,** we examine the effect of CHIKV infection on monocyte subset distribution with use of an *in vitro* infection model in human PBMCs or primary human monocytes. In addition, we test how priming of monocytes with various TLR agonists modulates CHIKV-mediated monocyte subsets distribution, innate immune responses and virus replication.

In **Chapter 5**, I have summarized and discussed the key results of this thesis.

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