The American Journal of Pathology, Vol. 182, No. 6, June 2013

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

REVIEW

Inflammatory and Innate Immune Responses in Dengue Infection

Protection versus Disease Induction

Vivian Vasconcelos Costa,*† Caio Tavares Fagundes,*† Danielle G. Souza,*† and Mauro Martins Teixeira*

From the Department of Biochemistry and Immunology and the Laboratory of Microorganism/Host Interaction,† Department of Microbiology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil*

CME Accreditation Statement: This activity ("ASIP 2013 AJP CME Program in Pathogenesis") has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education (ACCME) through the joint sponsorship of the American Society for Clinical Pathology (ASCP) and the American Society for Investigative Pathology (ASIP). ASCP is accredited by the ACCME to provide continuing medical education for physicians.

The ASCP designates this journal-based CME activity ("ASIP 2013 AJP CME Program in Pathogenesis") for a maximum of 48 AMA PRA Category 1 Credit(s)™. Physicians should only claim credit commensurate with the extent of their participation in the activity.

CME Disclosures: The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose.

Accepted for publication February 5, 2013.

Address correspondence to Mauro Martins Teixeira, Ph.D., Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av Antônio Carlos, 6627 − Pampulha, 31270-901, Belo Horizonte, MG, Brazil. E-mail: mmtex@icb.ufmg.br.

Dengue disease is a mosquito-borne viral disease of expanding geographical range and incidence. Infection by one of the four serotypes of dengue virus induces a spectrum of disease manifestations, ranging from asymptomatic to life-threatening Dengue hemorrhagic fever/dengue shock syndrome. Many efforts have been made to elucidate several aspects of dengue virus−induced disease, but the pathogenesis of disease is complex and remains unclear. Understanding the mechanisms involved in the early stages of infection is crucial to determine and develop safe therapeutics to prevent the severe outcomes of disease without interfering with control of infection. In this review, we discuss the dual role of the innate and inflammatory pathways activated during dengue disease in mediating both protection and exacerbation of disease. We show that some mediators involved in each of these responses differ substantially, suggesting that interfering in disease-associated immune pathways may represent a potential therapeutic opportunity for the treatment of severe dengue. *(Am J Pathol 2013, 182: 1950−1961; http://dx.doi.org/10.1016/j.ajpath.2013.02.027)*

Dengue is a spectrum of disease caused by one of the four serotypes of the most prevalent arthropod-borne virus that affect humans today, dengue viruses (DENV). The four DENV serotypes (DENV-1 to -4) belong to the *Flaviviridae* family and circulate concomitantly in different regions of the world, covering approximately 100 countries in tropical and subtropical areas of the globe, with potential for further spread. Worldwide, it is estimated that 2.5 billion people are at risk of infection, with occurrence of >50 million infections each year, including 50,000 hospitalizations of patients with severe cases, mainly among children, with the case fatality rate exceeding 5% in some areas.[1,2]

Most DENV infections are clinically unapparent. The traditional World Health Organization classification includes two major entities: dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS).[3] DF is defined as a self-limited though debilitating febrile illness, accompanied by a combination of nonspecific symptoms, including headache, retro-orbital pain, myalgia, and occasionally hemorrhagic manifestations.[3−5] The case definition for DHF lists the presence of four criteria: fever, hemorrhagic manifestations, thrombocytopenia (platelets counts of

Supported by grants from the Instituto Nacional de Ciência e Tecnologia em Dengue and PRONEX in Dengue funded by Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), and the Brazilian Ministry of Health.

Current address of C.T.F., Trinity College Dublin, Dublin, Ireland.

http://dx.doi.org/10.1016/j.ajpath.2013.02.027

100,000 cells/mm^3), and evidence of plasma leakage (pleural effusion, ascites, hemoconcentration $\geq$20%, or hypoproteinemia). In turn, DHF is divided into four grades (I to IV), where grades III and IV are defined as DSS with hypotensive shock or narrow pulse pressure plus clinical signs of shock.[3] A revised World Health Organization classification of dengue disease severity was performed recently and proved to have much more sensitivity and specificity to identify cases in need of heightened care. However, it is no longer as specific for a particular pathogenic entity as was the traditional scheme, suggesting that its utility in pathophysiologic and epidemiologic studies needs further investigation in future research.[3]

Treatment of DF and DHF/DSS infection is largely supportive.[6] The large number of infected individuals, the lack of clinical or laboratory markers that indicate which patients will develop severe disease, and the lack of specific treatment place an enormous burden on health systems of low-income countries. Much interest has been placed in understanding inflammatory and immune pathways that are unleashed during host response to infection. It has been suggested that mechanisms of protection against infection may differ substantially from those associated with disease progression, indicating that this difference may be exploited for the development of novel therapeutic strategies against dengue infection.[5,7,8] We describe some of the mechanisms associated with protection against infection and those associated with severe disease evolution in DENV infection.

Pathophysiologic Aspects of Dengue Disease

A common major technical barrier in understanding the pathogenesis of DENV infection is the absence of a suitable animal model that mimics dengue disease, especially the severe forms of disease (DHF/DSS).[5,9] However, several animal models of DENV infection have been described, are currently available, and have been extensively reviewed elsewhere.[5,9,10] Table 1 summarizes the features and applicability of the most common mouse models described. As in any other area of biomedical research, these models have advantages and disadvantages but clearly contribute to the understanding of dengue infection, including immunopathogenesis and preclinical testing of antiviral drugs and vaccines. It is very unlikely that a single experimental model will take into account all aspects of this complex human disease. Therefore, knowledge in the field must come from adequate interpretation of data from current animal models, *in vitro* experiments with immune cells, and clinical and epidemiologic studies.

The pathogenesis of DENV-induced disease involves a complex interplay of viral and host factors in the context of the history of the patient. Risk factors for severe disease include age, viral serotype and genotype, and the genetic background of the host.[2,11,12] Differences in virulence among viral strains and especially among virus genotypes have been found to play an important role in disease outcome. For example, DENV-3 genotype III, which includes isolates from East Africa, South Asia, and Latin America, has been associated with an increase in DHF/DSS in these regions.[13,14] These studies indicate that certain viruses may cause more severe disease in the population. However, mechanisms explaining the higher proportions of severe dengue disease manifestation during infection with these viral genotypes are still obscure. They have been reviewed elsewhere.[15]

Most dengue cases reported are asymptomatic, and apparent disease due to dengue infection represents <10% of all symptomatic reported cases.[4] The incidence of severe disease (DHF/DSS) varies significantly between primary and secondary DENV infections,[4] and observational studies demonstrate that DHF/DSS occurs predominantly either in individuals with secondary heterologous DENV infections or in infants with primary DENV infections born from dengue immune mothers.[6,9,16] Epidemiologic and serologic studies performed in Thailand and Cuba are good examples of the importance of heterologous secondary infections as a risk factor for DHF/DSS development and fatal cases. For example, Thai data of DENV infections in children <15 years of age demonstrated that just 0.18% of primary infected children manifested DHF/DSS, whereas 2% of secondary infected children developed severe disease manifestation.[4] Similarly, Guzmán et al[17] found that during a 1997 outbreak in Santiago de Cuba, there were an adjusted 5208 laboratory-confirmed dengue cases, with 202 of the 205 DHF/DSS cases (98.5%) and 4608 of the 5003 dengue fever cases (92.1%) from secondary dengue infections. Only 3% of 13,116 primary infections cases caused apparent symptoms.[17] The sensitizing infection for DHF/DSS cases in the DENV-2 epidemic in 1997 was the DENV-1 serotype virus, which was transmitted in 1977 to 1979.[18] These data suggest that a prior dengue infection may set the stage for a more deadly infection by a different serotype in a few years' time.

Several theories have been raised to explain the observation that more severe disease occurs in the context of secondary infections. Most theories suggest that the immune status of the patient is related to disease progression. One of them, the antibody-dependent enhancement theory, postulates that after an initial period of cross-reactive protection, antibodies from a primary infection remain cross-reactive with other DENV serotypes but have waned to non-neutralizing levels. These nonneutralizing antibodies could then lead to viral internalization via the Fc receptors and increase virus replication into phagocytic cells, what is accompanied by massive release of soluble factors that could account for the increased vascular permeability and hemostatic disorder found in severe cases.[6,16] In accordance, Dejnirattisai et al[19] have demonstrated that although most monoclonal antibodies against anti−E protein neutralized DENV effectively, a large proportion of the human antibody repertoire directed against the precursor membrane protein, which is present on immature virus particles, failed to do so, leading to enhanced viral infection and replication in Fc

Table 1 Features of Mouse Models of Dengue Virus Infection Used for Preclinical Studies

Mice strains	Features	Advantages	Disadvantages	Applicability
Mouse-human chimeras (humanized mice) SCID mice, NOD/SCID mice, *RAG2*$^{-/-}$ mice, and NSG mice	Mice reconstituted with human hematopoietic stem cells (from umbilical cord blood or fetal cells) or a variety of dengue virus—susceptible tumor cells infected with relevant clinical isolates of dengue virus	Mice develop functional human immune system, including some level of adaptive immunity; infection of human cells lineages; study of human response to infection.	Only mild disease manifestation (DF)	Study of DF pathogenesis, human cell virus tropism, virulence screening of dengue virus (including differences in genotypes), and vaccine studies evaluating virus attenuation *in vivo*
Immunocompromised mice AG129 (mice lacking both IFN-α/β and IFN-γ receptors)	Mice infected with clinical isolates of dengue virus or with mosquito cell/mouse adapted dengue virus isolates; higher viremia	Support replication of relevant dengue virus clinical isolates; mice develop marked viremia (viremia model), antibody-dependent enhancement model	Absence of critical components of host antiviral system; inadequate for studies evaluating immune responses to infection and severe disease pathogenesis; occurrence of paralysis and lethal encephalitis	Investigate tissue and cellular tropism of dengue virus, antiviral drugs screening
Immunocompetent mice BALB/c and C57BL/6j strains	Mice with intact immune system infected with mouse-adapted dengue virus isolate	Mice develop the major clinical manifestations of human severe dengue virus infection (pain, thrombocytopenia, hemoconcentration, increased vascular permeability, hypotension, increased levels of cytokines, and chemokines, tissue hemorrhage, viremia, and recovery of viral load in target organs of infection)	Single strains of dengue virus 2 or 3 that were adapted by multiple passages in mice; modification of the virus to the murine host may involve pathologic mechanisms different to that of the original virus in humans; severe disease occurs in primary infection	Study of severe dengue disease pathogenesis, cell virus tropism, and host inflammatory and immune responses to infection

Reviewed in Fagundes et al,[5] Rothman,[9] and Zompi and Harris.[10]
DF, dengue fever; NOD, nonobese diabetic; NSG, nonobese several combined immunodeficiency γ; SCID, severe combined immunodeficiency.

receptor—bearing cells. Experimental infections in mice have also brought support to this theory,[20,21] and non-neutralizing antibodies also drive higher virus loads in nonhuman primates.[22,23] Therefore, subneutralizing levels of antibodies may contribute to the development of severe dengue diseases during secondary infections. A distinct but complementary theory of immunopathology involves reactivation of cross-reactive memory B and T cells specific for the previous rather than the current DENV infection, resulting in delayed viral clearance and/or increased cytokine secretion along with increased apoptosis of both infected and uninfected bystander cells.[9] There is immunologic evidence that this phenomenon of original antigenic sin may occur during secondary DENV infections.[9,24,25]

However, although in smaller rates, primary DENV infection may lead to severe disease manifestation.[4] This can be exemplified by the DENV-3 introduction in Rio de Janeiro, Brazil, in 2002, which was responsible for one of the most severe epidemics in the state's history in terms of the highest number of reported cases, the severity of clinical manifestations, and the number of confirmed deaths.[26] During this epidemic, 56% of the confirmed DENV-3 non-fatal cases analyzed were due to primary infection. Furthermore, 54% of the lethal cases analyzed were classified as primary DENV-3 infections,[26] demonstrating that primary infections may cause severe disease. To explain findings such as these, a theory claims that severe dengue disease manifestation does not necessarily depend on a secondary infection but rather on a combination of viral load, strain virulence, and host immune response.[27] Hence, field studies have noted higher levels of viremia in DHF patients, which supports the assertion that increased viral replication is associated with more severe disease.[28—31] In addition, there is some correlation among higher viral titers or the infecting virus serotype/genotype with the occurrence of DHF/DSS, independently of whether the infection is primary or secondary.[28,32,33] These findings suggest that evolution to severe disease during primary DENV infections is favored by

elevated viral replication, which could potentially lead to enhanced production of mediators of inflammation.

Of note, there seems to be a consensus among these theories: whether involving primary or secondary infections, an exacerbated host response is believed to play a direct role in the pathogenesis of severe dengue disease.[9,27,34] It has been suggested that DHF/DSS manifests in the context of amplified production of cytokines—a cytokine storm—that ultimately targets the vascular endothelium and eventually leads to transient increase in vascular permeability, occurrence of hemorrhagic manifestations, hemoconcentration, and, in some cases, development of intractable shock, resulting in death.[9,27,34]

Therefore, it seems that excessive inflammation contributes to the pathogenesis of severe dengue disease.[11,35] Hence, although elevated levels of proinflammatory cytokines and chemokines occur in patients with DF, most of them are found in higher levels in patients experiencing severe dengue disease.[36–39] For example, heightened levels of tumor necrosis factor (TNF)-α, IL-6, IL-8, IL-10, chemokine ligand (CCL)-2, CCL-3, CXCL-8, CXCL-10, and interferon (IFN)-γ have been reported in patients with severe dengue disease.[9,39–44] Many of these cytokines have already been linked to disease severity, including hepatic dysfunction, hypotension, thrombocytopenia, and hemorrhagic shock.[9] However, it is not clearly understood how this massive cytokine production is induced and eventually controlled. Nevertheless, the latter findings suggest that severe dengue disease relies on uncontrolled immune cell activation, increased proinflammatory mediator production, and consequent endothelial cell dysfunction unleashed by the virus and several immunopathologic mechanisms.

Innate Immune and Inflammatory Pathways Activated during Dengue Disease: Protective versus Pathologic Mediators

Pattern Recognition Receptors and Other Molecules Involved in DENV Sensing

Langerhans cells, dermal cells, and interstitial dendritic cells have long been proposed as initial targets for DENV infection at the site of the mosquito bite.[39,45] Viral antigens have also been detected on monocytes, lymphocytes, Kupffer cells, alveolar macrophages, and endothelial cells of DENV-infected humans.[46] Although the ability of these cells to support DENV replication *in vivo* and the mechanisms involved in viral tropism to them are yet to be demonstrated, they are thought to mount the initial antiviral responses at the first stages of DENV infection. Host pattern recognition receptors (PRRs) in these cells are responsible for sensing viral proteins or nucleic acids. The endosomal Toll-like receptors (TLRs)[47] and the cytoplasmic receptor family of DExD/H box RNA helicases [eg, retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5)][48] are the two most important families of sensors present in mammalian cells that are implicated in detecting viral nucleic acids. On viral recognition by these molecules, there is activation of two important families of transcriptional factors: the interferon regulatory factors and the NF-κB. Both of these signaling cascades activate the production of IFN-α/β and inflammatory cytokines that leads to activation of dendritic cells and establishment of an antiviral response.[49]

Recently, several studies have demonstrated the importance of TLR3 in the recognition and restriction of DENV replication in different cell lineages.[50–52] TLR7 was also demonstrated to be essential for DENV-induced production of type I IFN in plasmacytoid dendritic cells.[53] In addition, administration of TLR3 and TLR7/8 agonists to rhesus macaques enhanced antiviral mechanisms via increasing inflammatory and humoral responses, leading to decreased DENV replication during primary infection.[54] The involvement of RIG-I and MDA5 in DENV sensing has been recognized through gene expression analysis.[55] Interaction of DENV with these receptors results in the activation of a macromolecular signaling complex that stimulates interferon regulatory factor 3 and NF-κB, which in turn induces IFN-β promoter. In addition, experiments using single and double RIG-I/MDA5−deficient mouse fibroblasts showed that DENV triggers both of these responses.[56] Also, early detection of antibody-enhanced DENV infection via RNA sensors, such as the RIG-I, by tissue-resident mast cells culminated in production of type I interferons and rapid production of chemokines, including CCL4, CCL5, and CXCL10.[57] In accordance, DENV infection of human brain microvascular endothelial cells promoted an increase in the production of type I IFN and proinflammatory cytokines, which were abolished after RIG-I silencing,[58] and mice deficient for the RIG-I/MDA5 adaptor IPS-1 present delayed production of type I IFN in lymphoid tissues and increased viral loads in the early phases of primary infection.[59] Finally, Nasirudeen et al[51] found that the RIG-I, MDA5, and TLR3 sensors act synergistically to induce IFN-β production and impair DENV replication *in vitro*. Therefore, RIG-I, MDA5, and TLR3 sensors are the major PRRs involved in recognition and mounting of innate responses to DENV infection.

Activation of these PRRs by DENV is thought to induce a strong type I IFN response during human natural infections. IFN-α is present at high levels for long periods in pediatric patients after defervescence period.[60] In addition, it was demonstrated in mice that this IFNAR-dependent control of DENV primary infection involved both STAT1-dependent and STAT1-independent mechanism.[61] The STAT1-dependent pathway was demonstrated to be involved in the control of initial steps of infection; however, the STAT1-independent pathway was suggested to be involved in the later antiviral mechanisms that control virus propagation and disease control.[61] Interestingly, DENV has evolved mechanisms to subvert antiviral response induced by type I IFNs. Hence, expression of NS2A, NS4A, and NS4B in cells was

capable of enhancing replication of IFN-sensitive viruses in the presence of exogenous IFN-α/β stimulation, indicating that these proteins may block the effects of IFN-α/β before infection.[62] These studies have demonstrated that NS4B blocks the JAK/STAT signaling induced by IFN-α/β through inhibition of STAT1 phosphorylation and nuclear translocation.[62] These data highlight the crucial importance of type I IFN system in host response to DENV infection.

Despite the role of DENV-sensing receptors on the development of protective responses on infection, it seems that some other molecules involved in recognition of DENV may unleash pathological host responses. For example, Chen et al[63] noted that the C-type lectin domain family 5 member A (CLEC5A) directly interacts with the dengue virion, resulting in stimulation of the release of proinflammatory cytokines. Importantly, blockade of the CLEC5A−DENV interaction suppressed the secretion of proinflammatory cytokines after primary infection *in vitro* and *in vivo*. Moreover, treatment of DENV-infected mice with anti-CLEC5A monoclonal antibodies inhibited DENV-induced plasma leakage and vital-organ hemorrhaging, culminating in reduced mortality after infection by approximately 50% in STAT1-deficient mice, without interfering with host response to infection.[63] Hence, CLEC5A blockade did not interfere with the production of type I IFNs by infected cells.[63] These findings support the concept that DENV recognition by CLEC5A induces proinflammatory cytokine release and disease exacerbation during infection. However, it remains to be shown whether CLEC5A activation affects disease evolution in patients. A study evaluating single-nucleotide polymorphisms in CLEC5A could not find any association between genetic variants of this molecule and manifestation of DF or DHF in patients.[64] In addition, CLEC5A expression seems to be diminished in dengue patients.[65] Although these findings do not exclude the possibility that CLEC5A activation may favor severe disease evolution during DENV infection in humans, more extensive clinical data are required to confirm the importance of CLEC5A in dengue pathogenesis. Future studies looking for enhanced CLEC5A activation in dengue patients, for example, evaluating DAP12 phosphorylation, along with an association with proinflammatory cytokine production and severe disease manifestation may help to answer this question.

DENV-antibody complex recognition by host cells during antibody-dependent enhancement−mediated disease also seems to unleash pathologic responses during infection. It has been reported that ligation between DENV-antibody complexes and FcR not only down-regulates TLR gene expression but also up-regulates negative regulators of the NF-κB pathway, resulting in suppression of innate responses and increased viral production.[66] Importantly, the same phenomenon was seen in peripheral blood mononuclear cells of secondary DHF/DSS patients but not in peripheral blood mononuclear cells of DF patients. These results were supported by experiments with anti-FcγRI or anti-FcγRIIa antibodies, which reduced viral production, up-regulated IFN-β synthesis, and increased gene expression in the TLR-dependent signaling pathway.[66] Therefore, recognition of DENV−antibody complexes impairs host protective responses to infection, favoring viral replication and disease exacerbation.

Cytokines, Chemokines, and Leukocyte Populations

As stated, in addition to type I IFNs, PRR activation on DENV infection mediates proinflammatory cytokine production. For example, several studies have demonstrated enhanced levels of TNF-α, IL-6, IL-8, CCL2, CCL3, CXCL10, and IFN-γ in primary and secondary DENV infection of humans[9,38−44,60] or mice.[20,21,67−69] Some of these cytokines may play essential protective roles during infection. This is the case with IFN-γ. Using murine immunocompetent models of primary DENV infection, we have demonstrated that production of IL-12 and IL-18 proinflammatory cytokines precedes IFN-γ release, and optimal IFN-γ production relies on the combined action of these two cytokines.[68,69] For instance, Pacsa et al[70] have reported that DF patients present elevated levels of IL-12, whereas patients experiencing DHF grades III and IV had nondetectable levels of this cytokine.[70] In our experimental settings, we demonstrated that IFN-γ production is essential for host resistance to primary DENV infection in part by controlling nitric oxide synthase 2−mediated nitric oxide production,[68,69] a virustatic radical previously found to inhibit DENV replication.[71] Importantly, Gunther et al[72] have demonstrated in a human challenge model of DENV infection that only sustained IFN-γ production was associated with protection against fever and viremia during the acute phase of illness.[72] These data establish IFN-γ as essential for host control of DENV replication and resistance to infection. The correlation between increased IFN-γ production and higher survival rates in DHF patients[41] also supports this idea.

On the contrary, other proinflammatory cytokines seem to play a pathologic role during host response to DENV infection. For example, increased levels of TNF-α have been associated with severity of dengue manifestation in humans.[40] T cells isolated from hospitalized patients with more severe disease evolution produced higher amounts of TNF-α after *ex vivo* stimulation with DENV antigens.[73] Findings in murine experimental models indicated that TNF-α blockade resulted in prevention of primary[74,75] and secondary[20] infection−induced lethality, and TNF-α action has been implicated in increased vascular permeability after infection in experimental settings.[12] Altogether, these findings suggest that severe disease may occur due to enhanced TNF-α production during host response to infection.

Another cytokine found to have a deleterious role during DENV infection is the macrophage migration inhibitory factor (MIF). MIF concentrations are positively correlated with severity in DENV infection.[76,77] In addition, experimental primary infection demonstrated that clinical disease was less severe in $MIF^{-/-}$ mice, and they exhibited a significant delay in lethality.[76] This reduction in all parameters of severity of DENV infection in $MIF^{-/-}$ mice correlated with reduced proinflammatory cytokine levels (such as TNF-α) and lower viral

loads at the initial phases of infection.[76] Therefore, elevated production of the proinflammatory cytokines TNF-α and MIF during host response to DENV infection seems to play a deleterious role, favoring manifestation of more severe disease.

The chemokine system also appears to have this dual protective versus pathologic role during DENV infection. It has been shown that CXCL10 production and CXCR3 activation improve host resistance during DENV infection. CXCL10 seems to compete with DENV for their cellular receptors, diminishing viral replication,[41] and, importantly, mice deficient for CXCL10 of CXCR3 had impaired resistance to primary infection, due to a defect in activation of $CD8^+$ T cell and NK cells.[78] On the opposite direction, clinical studies in endemic areas have described a correlation between dengue disease outcome and levels of CCL3, and CCL4[9,42] and CCL2 concentrations are intimately related to hypotension, thrombocytopenia, and hemorrhagic shock.[42] Also, a link between CCL5 and DENV-induced hepatic dysfunction has already been found.[79] Furthermore, data from our group demonstrated that *CCR2*$^{-/-}$ mice showed reduced lethality rates after primary DENV infection, in parallel with diminished liver damage, lower production of IL-6, and attenuation of leukocyte activation.[80] In *CCR4*$^{-/-}$ mice, lethality, hepatic damage, and systemic inflammation were also markedly decreased. Despite differences in disease presentation in CCR-deficient mice, there was no difference in viral load.[80] In conclusion, these data demonstrate that although CXCR3 seemed to mediate protective host responses to DENV infection, activation of CCR2 and CCR4 was mostly associated with the development of disease rather than with protection to infection.

Cellular populations involved in protective or pathologic host responses to DENV infection are still obscure. It is well accepted that some of the activated cell populations during DENV infection may be crucial for control of viral replication, such as $CD8^+$ T cells[81]; although in the context of secondary infections, some clones of these populations may favor severe disease evolution.[9] We have recently reported that invariant natural killer T (iNKT) cells play a critical role in the pathogenesis of severe experimental dengue disease.[82] Our data demonstrated that, on primary DENV challenge of immunocompetent mice, splenic and hepatic iNKT cells became activated. In addition, C57BL/6 mice deficient in iNKT cells (*Ja18*$^{-/-}$) were more resistant to lethal infection than were wild-type animals, and the phenotype was reversed by adoptive transfer of iNKT cells to *Ja18*$^{-/-}$ animals. *Ja18*$^{-/-}$ mice had decreased systemic and local inflammatory responses, less liver injury, diminished vascular leak syndrome, and reduced activation of natural killer cells and neutrophils on infection. Of note, iNKT cell functions were not necessary for control of primary DENV infection after either natural endogenous activation or exogenous activation with the canonical iNKT cell agonist α-galactosylceramide.[82] Whether iNKT cells play such pathological role during clinical DENV settings remains to be evaluated. Nevertheless, these data support the idea that, in a similar way to what is found for viral sensors and soluble mediators, some cellular components activated during DENV infection are related to disease progression rather than to protection.

Protective versus Pathological Pathways during DENV Infection: Their Effects on Vascular Endothelium and Plasma Leakage

Plasma leakage is a hallmark of severe disease manifestation (DHF/DSS) in DENV-infected patients and is thought to contribute to the pathogenesis of disease.[83] Effusions, ascites, and gallbladder wall edema are commonly found in DENV-infected patients around the time of defervescence and correlate with disease severity.[83–88] The endothelium is the primary fluid barrier of the vasculature, and DENV-induced responses that result in edema and hemorrhage are thought to ultimately cause changes in endothelial cell barrier permeability.

Whether blocking vascular leak may result in improvement of DHF/DSS outcome is still unknown. However, one study in a mouse model of infection suggests that this may be true. Groger et al[89] demonstrated that FX06, a 28–amino acid cleavage product of fibrin that interacts with vascular endothelial cadherin, reduced vascular leak and mortality in an animal model of primary dengue infection. Animals treated with FX06 (first treatment on day 3 after infection) had improved survival rates accompanied by reduced capillary leak in the lung and the intestine, a marked reduction in hemoconcentration, and less fibrinogen consumption. Of note, viral loads in serum, liver, and brain at day 7 after infection, the peak of disease, did not differ between groups that received FX06 or controls. The ability of FX06 to preserve endothelial barriers was confirmed in lipopolysaccharide-induced systemic shock, and the authors have evaluated the mechanisms involved in antipermeabilizing effects of FX06 in this model. The protective effects were associated with prevention of thrombin-induced stress fiber formation, myosin light chain phosphorylation, and RhoA activation in endothelial cells.[89] The molecular key for the protective effect of FX06 was the src kinase Fyn, which is associated with vascular endothelial cadherin–containing junctions. After exposure to FX06, Fyn dissociates from vascular endothelial cadherin and associates with p190RhoGAP, an antagonist of RhoA activation.[89] Although these pathways were not demonstrated in the DENV infection model, these data suggest that inhibition of plasma leakage development, via stabilization of endothelial cell-cell contacts, may lead to a better outcome for dengue severe disease, with no major impact on host control of infection. However, there is no evidence of these premises in clinical settings, which represents an interesting and valuable subject for future research.

Of note, the increased vascular permeability during the defervescence phase of dengue illness is usually found without morphologic damage to the capillary endothelium. Because of the paucity of structural damage to the vasculature found in autopsy studies, it has been suggested that

circulating factors are primarily responsible for the occurrence of microvascular permeability during DENV infection.[9,34,83] Therefore, mediators produced by leukocytes during response to infection represent potential players in enhanced plasma extravasation seen in severe cases of DENV infection. Hence, cytokines and other inflammatory mediators act on the endothelium, potentially altering normal fluid barrier functions of endothelial cells.[11,34,90,91] Supporting this hypothesis, Cardier et al[92] have found that serum samples obtained from patients in the acute phase of DENV-infection induce activation of endothelial cells in a mechanism that involves enhanced circulating TNF-α levels.[92] This study did not evaluate any permeabilizing property of the serum samples of DENV-infected patients on the endothelial cell monolayer, but this study supports the concept that production of proinflammatory mediators during infection will ultimately change activation status and function of the endothelium, potentially favoring enhanced plasma leakage.

The deleterious role played by MIF during DENV infection seems to involve its permeabilizing effects on the endothelium. *In vitro* studies have found that DENV infection of the human hepatoma cell line induced MIF production and that medium collected from DENV-infected human hepatoma cell line cells enhanced the permeability of the human endothelial cell line, which was reduced in the presence of a MIF inhibitor (ISO-1).[77] Medium from DENV-infected MIF knockdown human hepatoma cell line cells also presented diminished permeabilizing effects in the endothelial cell monolayers. Authors found that MIF interferes in the arrangement of tight junction protein ZO-1 of the human endothelial cell line and that the arrangement was partially recovered when cells were treated with ISO-1 or PI3K/MEK-ERK/JNK signaling pathway inhibitors.[77] Of note, MIF-deficient mice infected with DENV-2 presented reduced hemoconcentration during the disease, suggesting that MIF may mediate increased plasma extravasation during *in vivo* infection.[76] Therefore, elevated production of the proinflammatory cytokine MIF during host response to DENV infection seems to interfere in endothelial barrier function, enhancing plasma extravasation and favoring development of more severe disease.

Chemokines may also be involved in increased vascular permeability found in severe dengue disease. As stated before, CCL2 concentrations are intimately related to hypotension, thrombocytopenia, and hemorrhagic shock in patients.[42] Hence, Lee et al[36] found higher levels of CCL2 in the plasma of DHF patients when compared with DF patients or patients infected with enterovirus-71.[36] In addition, they have reported that exposing monolayers of human umbilical vein endothelial cells to the culture supernatant of DENV 2–infected human monocytes increased the permeability of the cell monolayer, an event partially reversed by CCL2 neutralization. This seemed to involve disruption of endothelial tight junctions through reorganization of protein ZO-1.[36] Although CCL2 correlates with disease severity in patients[42] and mice deficient for the CCL2 receptor CCR2 are protected from DENV-2–induced lethality,[80] there is no *in vivo* evidence that CCL2 may modulate plasma extravasation. Hence, CCR2-deficient mice do not present any improvement in hemoconcentration during the course of DENV-2 infection.[80] Therefore, the data involving endothelial cells monolayers suggest that CCL2 may be involved in DENV–induced plasma leakage, a premise that deserve further investigation in clinical and *in vivo* settings.

Besides the effects of cytokines and chemokines, leading to increased vascular permeability, it was also demonstrated that other molecules produced by DENV-infected leukocytes can also play a role on the vascular endothelium. Luplerdlop et al[93] showed that DENV infection of immature dendritic cells led to overproduction of soluble matrix metalloproteinases 9 and 2 and that these enzymes were capable of enhancing endothelial permeability of human umbilical vein endothelial cell monolayer cells *in vitro* and in a model of vascular permeability *in vivo*.[93] Of note, matrix metalloproteinase 9 production is enhanced in patients with DENV infection and is associated with severity of disease.[94] In another study, Ong et al[95] demonstrated that enhanced levels of high mobility group box 1 (HMGB1) protein was detected after DENV infection of human myelogenous leukemia cells (K562) and also from primary peripheral blood monocytes and that HMGB1 was released from DENV-infected K562 cells into the extracellular milieu. They also demonstrated that application of DENV-infected K562 cell culture supernatants to primary endothelial cells induced vascular permeability *in vitro* in a HMGB1-dependent manner.[95] HMGB1 has also been shown to be elevated in DENV-infected patients, with higher circulating levels in those experiencing secondary DENV infections.[96] Therefore, the elevated levels of HMGB1 and matrix metalloproteinase 9 could potentially mediate the increase in plasma leakage during infection in patients. These two reports suggest that mediators others than cytokines and chemokines may be involved in enhanced plasma extravasation during severe dengue disease.

Although conclusive evidence of DENV infection of endothelial cells in patients is lacking, DENV can clearly infect human endothelial cell lines *in vitro*.[11,58,92] DENV antigens but not genome was detected in endothelial cells in the liver sinusoids and the alveoli in human autopsy samples.[46] In addition, in several recent studies, DENV antigens have been detected in endothelial cells of infected mice.[20,97] These data suggest that DENV may infect endothelial cells and that this cell population represents direct contributors to the immune enhancement, capillary permeability, viremia, and immune targeting of the endothelium during the pathogenesis of the disease. Hence, DENV-infected endothelial cells present high-level transcriptional and secretory responses of chemokines, cytokines, and other inflammatory mediators that are consistent with the immune-enhancing and permeability-inducing responses observed in DENV patients.[58,90]

An example of the importance of endothelial function during severe dengue disease was demonstrated by Srikiatkhachorn et al.[98] They have found that DENV suppressed

soluble vascular endothelial growth factor 2 (VEGFR2) production by endothelial cells but up-regulated surface VEGFR2 expression and promoted response to VEGF stimulation. These *in vitro* results were supported *in vivo*, once plasma viral load in DENV-infected patients correlated with the degree of decline in plasma soluble VEGFR2. Importantly, the severity of plasma leakage in DENV-infected patients inversely correlates with plasma levels of soluble VEGFR2.[98] These results suggest that vascular endothelial growth factor regulates vascular permeability and its activity is controlled by binding to soluble VEGFR2. Dengue virus–induced changes in surface and soluble VEGFR2 expression may be an important mechanism of plasma leakage in DHF,[98] although it is not clear whether such effects are due to direct endothelial cell infection by DENV or involve an indirect mechanism.

Interestingly, type I IFNs may be important for preventing severe dengue disease also because of their effects on endothelium. DENV replication in endothelial cells is dependent of suppression of early IFN-β responses by DENV.[91] In IFN receptor–deficient mice, there is substantial infection of endothelial cell compartment by mouse-adapted DENV[20,99] and subneutralizing antibodies enhance infection, especially of liver sinusoidal endothelial cells in these mice models of infection.[20] Importantly, in addition to controlling viral replication, it has been found that type I IFN response may mediate protective effects against cytokine-induced increased permeability of endothelial cells.[100] Hence, autocrine type I IFN production suppressed TNF-α–induced hyperpermeability of DENV-infected endothelial cell monolayers. In addition, at later time points of infection of endothelial cells, at a moment when type I IFN production is suppressed, cell monolayers are more susceptible to TNF-α–induced permeability, and addition of exogenous type I IFN prevented this increased permeability of endothelial cell monolayers to macromolecules.[100] Of note, type I IFNs have been reported to stabilize the vascular barrier in several conditions.[101–103] Finally, Gomez and Reich[104] found a proliferative effect of type I IFNs on primary human endothelial cells in a STAT-dependent manner. The authors suggest that through such

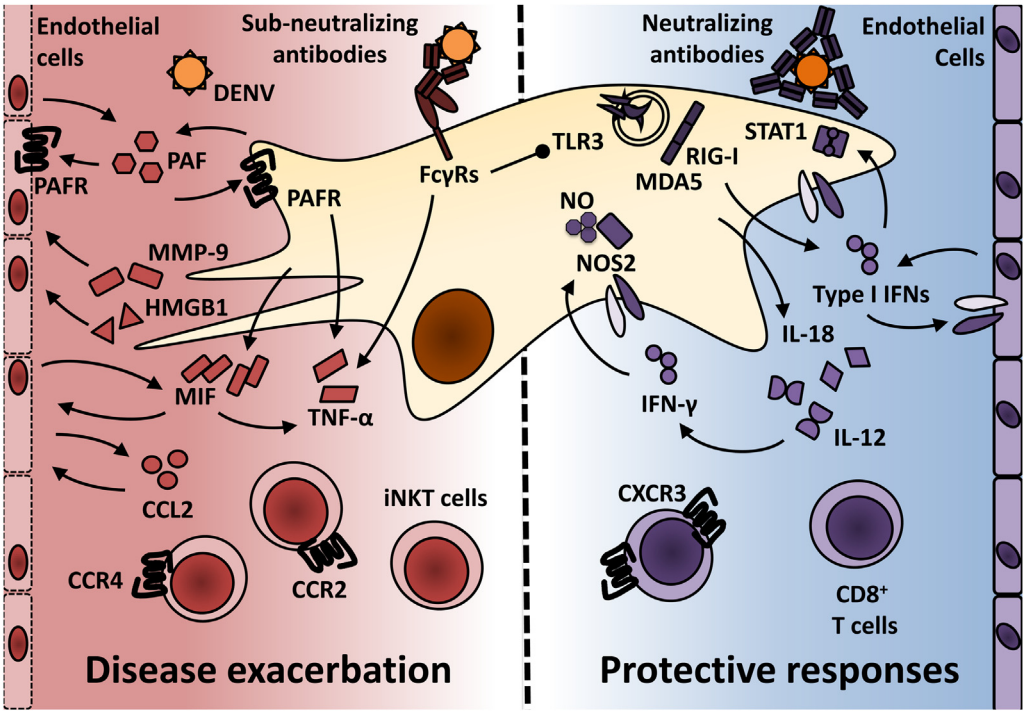

Figure 1 Mechanisms involved in protection and disease during host response to DENV infection. On DENV infection of permissive cells, host immune and endothelial compartments trigger several immune mechanisms and inflammatory pathways to control viral replication (**right**). Some pathways are critical for protection from infection such as that controlled by activation of the receptors TLR3, RIG-I, and MDA5, which mediate type I IFN–mediated antiviral responses (partly dependent on STAT1 activation). In addition, the production of IFN-γ, induced by IL-12 and IL-18, controls the production of the virucide metabolite nitric oxide. Other protective mechanisms unleashed by DENV infection involve the production of neutralizing antibodies (especially in the context of secondary infections), CD8 T-cell activation, and CXCR3-mediated recruitment of leukocytes. However, several mediators produced during host response ultimately leads to exacerbation of disease (**left**). For example, activation of FcγRs by antibody–DENV complexes leads to exacerbated production of proinflammatory mediators. Some of these molecules seem to play negligible roles in the control of infection and, therefore, consist of potential targets for therapeutic intervention. Hence, inhibition of the cytokines TNF-α and MIF; of activation of PAFR, CCR2 receptor, and CCR4 receptor; and of activation of iNKT cells results in reduced disease severity after DENV infection, with no alteration in viral clearance. Importantly, it has been reported that activation of some of these pathways interferes with host antiviral responses, as demonstrated by the inhibition of TLR signaling by the activation of FcγRs by antibody-DENV complexes (**arrow**). As a final target, these molecules with a pathologic role during infection seem to act directly or indirectly in the endothelial vascular barrier, enhancing plasma extravasation (represented by the dashed endothelial cells) during of infection and favoring manifestation of severe dengue disease. This is exemplified in the figure by the effects of matrix metalloproteinase 9, HMGB1, MIF, CCL2, and activation of PAFR on endothelial cells. Interestingly, some of the protective antiviral molecules exert antipermeabilizing effects in the endothelium, as demonstrated for type I IFNs.

proliferative effects on endothelium, IFNs may support the physical barrier between the vascular circulation and the underlying tissue, preventing the occurrence of plasma leakage.[104] Altogether, these data suggest that type I IFNs protect against increased vascular permeability during infection, aside from controlling viral replication. It is possible that other molecules found to be protective against DENV infection may possess antipermeabilizing effects in addition to their antiviral activities, presenting, therefore, a dual mechanism by which they may avoid evolution to severe disease manifestation.

Targeting Mediators Involved in Progression of Disease after DENV Infection

From the findings described, we conclude that in DENV infection, the host cell populations (including both immune and/or endothelial cells) trigger several immune mechanisms and inflammatory pathways to control viral replication. Some pathways are critical for protection from infection, such as the IFN system (including type I IFNs and IFN-γ). On the contrary, several mediators produced during host response ultimately lead to exacerbation of disease (such as TNF-α and MIF) (Figure 1). Whatever the mechanism, these mediators secreted during the infection ultimately target the vascular endothelium, affecting the development of vascular permeability and severe disease manifestation. Some of the latter molecules seem to play negligible roles in the control of infection and are, therefore, potential targets for therapeutic intervention. It is possible that a decrease in the production or action of such molecules may decrease dengue disease severity in humans.

A molecule that seems to be a useful as therapeutic target in the context of the dengue is the platelet-activating factor receptor (PAFR). PAF is released from macrophages obtained from patients previously infected with DENV-1 compared with controls.[105] Importantly, our group has found a novel role for the PAFR in the pathogenesis of severe dengue.[67] Using immunocompetent mice, we found that the course of primary DENV infection was less severe in $PAFR^{-/-}$ mice. Administration of a PAFR antagonist, UK-74,505, after disease manifestation (even 5 days after infection) drastically inhibited the major manifestations of the severe dengue disease, including thrombocytopenia, hemoconcentration, plasma extravasation, hypotension, and lethality. Mechanistically, blockade of PAFR decreased production of TNF-α and other proinflammatory cytokines after infection, suggesting that PAF/PAFR interactions on leukocytes and other cell populations could potentially lead to the cytokine storm and enhanced inflammatory response seen during experimental DENV-2 virus infection.[67] In addition, there was diminished vascular permeability and hypotension in UK-74,505–treated and PAFR-deficient mice infected with DENV.[67] It is well known that PAF exerts potent permeabilizing effects in endothelial cells. Indeed, administration of PAF to rodents and activation of PAFR on endothelial cells induces hypotension and increased vascular permeability.[106,107] Therefore, an action of PAF on PAFR in endothelial cells, promoting increased vascular permeability, may contribute to the changes observed during DENV infection in mice. Of note, the protection afforded by inhibition of PAFR activation during dengue infection occurred without loss of control of viral replication, and there was significant production of IFN-γ in both $PAFR^{-/-}$ and PAFR antagonist–treated mice.[67]

Altogether these studies suggest that PAFR is a disease-associated gene but not necessary for the murine host to control dengue infection. Whether the findings translate into a real therapy for severe cases of dengue still deserves further investigation, but these results represent important proof-of-concept for the idea that interfering with some inflammatory mediators in the context of DENV infection may decrease disease severity without impairing pathogen elimination.

Conclusion

On DENV infection, the host must establish an appropriate inflammatory response to clear the pathogen, thereby eliminating the risk of disease. Several innate and inflammatory pathways activated at the early stages after DENV recognition by host cells are essential for resistance to infection. Some of these protective molecules identified so far include the pathogen-recognition receptors TLR3, RIG-I, and MDA5 and antiviral type I and type II interferons produced on activation of the previous pathogen-recognition receptors. These are only the first molecules proven to provide protection from DENV infection, and several others may have to be identified.

An appropriate inflammatory response requires that the response is controlled in several aspects, including its character (the type of mediators released or cells activated), intensity, location, and duration. Decreased or absent response may lead to parasite growth and disease. Of note, there is now evidence that severe dengue disease manifestation is associated with subversion of protective pathways by both viral and host factors, suggesting that altered host responses are directly involved in dengue disease severity. In addition, it appears that excessive inflammation, whether during primary or secondary cases of infection, contributes to the pathogenesis of severe dengue disease. Therefore, it is possible that a decrease in the production of certain proinflammatory molecules may decrease dengue-induced vascular leakage and disease severity. The biggest challenge will be to determine appropriate targets whose inhibition will result in inhibition of overt inflammation and endothelial barrier permeability without interfering with immune mechanisms involved in DENV clearance. Some potential targets are Fc receptors, as well as some proinflammatory cytokine and chemokines, including CCR2 and CCR4 receptors. Moreover, PAFR blockade represents another novel target to modulate the inflammatory response and vascular leakage in dengue infection without impairment of pathogen clearance. Because antiviral compounds may be developed from drug screening, immune-modulating drugs

that prevent severe disease may represent important adjunct therapy for the treatment of severe dengue.

References

1. Simmons CP, Farrar JJ, Nguyen vV, Wills B: Dengue. N Engl J Med 2012, 366:1423−1432
2. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martinez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW: Dengue: a continuing global threat. Nat Rev Microbiol 2010, 8:S7−S16
3. Narvaez F, Gutierrez G, Perez MA, Elizondo D, Nunez A, Balmaseda A, Harris E: Evaluation of the traditional and revised WHO classifications of Dengue disease severity. PLoS Negl Trop Dis 2011, 5:e1397
4. Kyle JL, Harris E: Global spread and persistence of dengue. Annu Rev Microbiol 2008, 62:71−92
5. Fagundes CT, Costa VV, Cisalpino D, Souza DG, Teixeira MM: Therapeutic opportunities in dengue infection. Drug Devel Res 2011, 72:480−500
6. Murphy BR, Whitehead SS: Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 2011, 29:587−619
7. Srikiatkhachorn A, Green S: Markers of dengue disease severity. Curr Top Microbiol Immunol 2010, 338:67−82
8. Sierra B, Perez AB, Vogt K, Garcia G, Schmolke K, Aguirre E, Alvarez M, Kern F, Kouri G, Volk HD, Guzman MG: Secondary heterologous dengue infection risk: disequilibrium between immune regulation and inflammation? Cell Immunol 2010, 262:134−140
9. Rothman AL: Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 2011, 11:532−543
10. Zompi S, Harris E: Animal models of dengue virus infection. Viruses 2012, 4:62−82
11. Srikiatkhachorn A: Plasma leakage in dengue haemorrhagic fever. Thromb Haemost 2009, 102:1042−1049
12. Clyde K, Kyle JL, Harris E: Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 2006, 80:11418−11431
13. Diaz FJ, Black WCt, Farfan-Ale JA, Lorono-Pino MA, Olson KE, Beaty BJ: Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 2006, 37:760−773
14. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM: Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 2003, 9:800−809
15. Rico-Hesse R: Dengue virus virulence and transmission determinants. Curr Top Microbiol Immunol 2010, 338:45−55
16. Halstead SB: Neutralization and antibody-dependent enhancement of dengue viruses. Adv Virus Res 2003, 60:421−467
17. Guzmán MG, Kouri G, Valdes L, Bravo J, Alvarez M, Vazques S, Delgado I, Halstead S: Epidemiologic studies on dengue in Santiago de Cuba, 1997. Am J Epidemiol 2000, 152:793−799
18. Guzmán MG, Kourí G: Dengue: na update. Lancet Infect Dis 2001, 2: 33−42
19. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, Limpitikul W, Puttikhunt C, Edwards C, Duangchinda T, Supasa S, Chawansuntati K, Malasit P, Mongkolsapaya J, Screaton G: Cross-reacting antibodies enhance dengue virus infection in humans. Science 2010, 328:745−748
20. Zellweger RM, Prestwood TR, Shresta S: Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 2010, 7:128−139
21. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E: Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 2010, 6:e1000790
22. Halstead SB: In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis 1979, 140: 527−533
23. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ: Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci U S A 2007, 104:9422−9427
24. Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S, Yenchitsomanus PT, McMichael A, Malasit P, Screaron G: Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 2003, 9:921−927
25. Midgley CM, Bajwa-Joseph M, Vasanawathana S, Limpitikul W, Wills B, Flanagan A, Waiyaiya E, Tran HB, Cowper AE, Chotiyarnwong P, Grimes JM, Yoksan S, Malasit P, Simmons CP, Mongkolsapaya J, Screaton GR: An in-depth analysis of original antigenic sin in dengue virus infection. J Virol 2011, 85:410−421
26. Nogueira RM, Schatzmayr HG, de Filippis AM, dos Santos FB, da Cunha RV, Coelho JO, de Souza LJ, Guimarães FR, de Araújo ES, De Simone TS, Baran M, Teixeira G Jr, Miagostovich MP: Dengue virus type 3, Brazil, 2002. Emerg Infect Dis 2005, 11:1376−1381
27. Rosen L: The pathogenesis of dengue haemorrhagic fever: a critical appraisal of current hypotheses. S Afr Med J 1986, (Suppl):40−42
28. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn A, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A: Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 2000, 181:2−9
29. Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, Pattanakitsakul SN, Yenchitsomanus PT, Mongkolsapaya J, Kasinrerk W, Sittisombut N, Blettner M, Vasanawathana S, Bhakdi S, Malasit P: Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 2006, 193:1078−1088
30. Libraty DH, Young PR, Pickering D, Endy TP, Kalayanarooj S, Green S, Vaughn DW, Nisalak A, Ennis FA, Rothman AL: High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 2002, 186:1165−1168
31. Murgue B, Roche C, Chungue E, Deparis X: Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996-1997 dengue-2 outbreak in French Polynesia. J Med Virol 2000, 60:432−438
32. Noisakran S, Chokephaibulkit K, Songprakhon P, Onlamoon N, Hsiao HM, Villinger F, Ansari A, Perng GC: A re-evaluation of the mechanisms leading to dengue hemorrhagic fever. Ann N Y Acad Sci 2009, 1171(Suppl 1):E24−E35
33. Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG: A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis 2007, 195:665−674
34. Basu A, Chaturvedi UC: Vascular endothelium: the battlefield of dengue viruses. FEMS Immunol Med Microbiol 2008, 53:287−299
35. Garcia CC, Guabiraba R, Soriani FM, Teixeira MM: The development of anti-inflammatory drugs for infectious diseases. Discov Med 2010, 10:479−488
36. Lee YR, Liu MT, Lei HY, Liu CC, Wu JM, Tung YC, Lin YS, Yeh TM, Chen SH, Liu HS: MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells. J Gen Virol 2006, 87:3623−3630
37. Nielsen DG: The relationship of interacting immunological components in dengue pathogenesis. Virol J 2009, 6:211
38. Medin CL, Fitzgerald KA, Rothman AL: Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol 2005, 79:11053−11061
39. Navarro-Sanchez E, Despres P, Cedillo-Barron L: Innate immune responses to dengue virus. Arch Med Res 2005, 36:425−435

40. Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS: Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. FEMS Immunol Med Microbiol 2000, 28:183−188
41. Chen JP, Lu HL, Lai SL, Campanella GS, Sung JM, Lu MY, Wu-Hsieh BA, Lin YL, Lane TE, Luster AD, Liao F: Dengue virus induces expression of CXC chemokine ligand 10/IFN-gamma-inducible protein 10, which competitively inhibits viral binding to cell surface heparan sulfate. J Immunol 2006, 177:3185−3192
42. Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, Assis EF, Bozza PT, Kubelka CF: Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 2008, 8:86
43. Tolfvenstam T, Lindblom A, Schreiber MJ, Ling L, Chow A, Ooi EE, Hibberd ML: Characterization of early host responses in adults with dengue disease. BMC Infect Dis 2011, 11:209
44. Brasier AR, Ju H, Garcia J, Spratt HM, Victor SS, Forshey BM, Halsey ES, Comach G, Sierra G, Blair PJ, Rocha C, Morrison AC, Scott TW, Bazan I, Kochel TJ: A three-component biomarker panel for prediction of dengue hemorrhagic fever. Am J Trop Med Hyg 2012, 86:341−348
45. Wu SJ, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovich MA, Wong HK, Blauvelt A, Murphy GS, Robb ML, Innes BL, Birx DL, Hayes CG, Frankel SS: Human skin Langerhans cells are targets of dengue virus infection. Nat Med 2000, 6:816−820
46. Jessie K, Fong MY, Devi S, Lam SK, Wong KT: Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 2004, 189:1411−1418
47. Bowie AG, Haga IR: The role of Toll-like receptors in the host response to viruses. Mol Immunol 2005, 42:859−867
48. Meylan E, Tschopp J, Karin M: Intracellular pattern recognition receptors in the host response. Nature 2006, 442:39−44
49. Severa M, Fitzgerald KA: TLR-mediated activation of type I IFN during antiviral immune responses: fighting the battle to win the war. Curr Top Microbiol Immunol 2007, 316:167−192
50. Tsai YT, Chang SY, Lee CN, Kao CL: Human TLR3 recognizes dengue virus and modulates viral replication in vitro. Cell Microbiol 2009, 11:604−615
51. Nasirudeen AM, Wong HH, Thien P, Xu S, Lam KP, Liu DX: RIG-I, MDA5 and TLR3 synergistically play an important role in restriction of dengue virus infection. PLoS Negl Trop Dis 2011, 5:e926
52. Liang Z, Wu S, Li Y, He L, Wu M, Jiang L, Feng L, Zhang P, Huang X: Activation of Toll-like receptor 3 impairs the dengue virus serotype 2 replication through induction of IFN-beta in cultured hepatoma cells. PLoS One 2011, 6:e23346
53. Sun P, Fernandez S, Marovich MA, Palmer DR, Celluzzi CM, Boonnak K, Liang Z, Subramanian H, Porter KR, Sun W, Burgess TH: Functional characterization of ex vivo blood myeloid and plasmacytoid dendritic cells after infection with dengue virus. Virology 2009, 383:207−215
54. Sariol CA, Martinez MI, Rivera F, Rodriguez IV, Pantoja P, Abel K, Arana T, Giavendoni L, Hodara V, White LJ, Anglero YI, Montaner LJ, Kraiselburd EN: Decreased dengue replication and an increased anti-viral humoral response with the use of combined Toll-like receptor 3 and 7/8 agonists in macaques. PLoS One 2011, 6: e19323
55. Ramirez-Ortiz ZG, Warke RV, Pacheco L, Xhaja K, Sarkar D, Fisher PB, Shaw SK, Martin KJ, Bosch I: Discovering innate immunity genes using differential display: a story of RNA helicases. J Cell Physiol 2006, 209:636−644
56. Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M Jr: Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol 2008, 82:335−345
57. Brown MG, McAlpine SM, Huang YY, Haidl ID, Al-Afif A, Marshall JS, Anderson R: RNA sensors enable human mast cell anti-viral chemokine production and IFN-mediated protection in response to antibody-enhanced dengue virus infection. PLoS One 2012, 7: e34055
58. da Conceição TM, Rust NM, Berbel AC, Martins NB, do Nascimento Santos CA, Da Poian AT, de Arruda LB: Essential role of RIG-I in the activation of endothelial cells by dengue virus. Virology 2013, 435:281−292
59. Perry ST, Prestwood TR, Lada SM, Benedict CA, Shresta S: Cardif-mediated signaling controls the initial innate response to dengue virus in vivo. J Virol 2009, 83:8276−8281
60. Kurane I, Innis BL, Nimmannitya S, Nisalak A, Meager A, Ennis FA: High levels of interferon alpha in the sera of children with dengue virus infection. Am J Trop Med Hyg 1993, 48:222−229
61. Shresta S, Sharar KL, Prigozhin DM, Snider HM, Beatty PR, Harris E: Critical roles for both STAT1-dependent and STAT1-independent pathways in the control of primary dengue virus infection in mice. J Immunol 2005, 175:3946−3954
62. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A: Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A 2003, 100:14333−14338
63. Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL: CLEC5A is critical for dengue-virus-induced lethal disease. Nature 2008, 453:672−676
64. Tsai JJ, Liu L, Chang K, Wang S, Hsiao H, Clark KB, Perng GC: The importance of hematopoietic progenitor cells in dengue. Ther Adv Hematol 2012, 3:59−71
65. Gomes AL, Wee LJ, Khan AM, Gil LH, Marques ET Jr, Calzavara CE, Tan TW: Classification of dengue fever patients based on gene expression data using support vector machines. PLoS One 2010, 5:e11267
66. Modhiran N, Kalayanarooj S, Ubol S: Subversion of innate defenses by the interplay between DENV and pre-existing enhancing antibodies: TLRs signaling collapse. PLoS Negl Trop Dis 2010, 4:e924
67. Souza DG, Fagundes CT, Sousa LP, Amaral FA, Souza RS, Souza AL, Kroon EG, Sachs D, Cunha FQ, Bukin E, Atrasheuskaya A, Ignatyev G, Teixeira MM: Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection. Proc Natl Acad Sci U S A 2009, 106:14138−14143
68. Fagundes CT, Costa VV, Cisalpino D, Amaral FA, Souza PR, Souza RS, Ryffel B, Vieira LQ, Silva TA, Atrasheuskaya A, Ignatyev G, Sousa LP, Souza DG, Teixeira MM: IFN-gamma production depends on IL-12 and IL-18 combined action and mediates host resistance to dengue virus infection in a nitric oxide-dependent manner. PLoS Negl Trop Dis 2011, 5:e1449
69. Costa VV, Fagundes CT, Valadao DF, Cisalpino D, Dias AC, Silveira KD, Kangussu LM, Avila TV, Bonfim MR, Bonaventura D, Silva TA, Sousa LP, Rachid MA, Vieira LQ, Menezes GB, de Paula AM, Atrasheuskaya A, Ignatyev G, Teixeira MM, Souza DG: A model of DENV-3 infection that recapitulates severe disease and highlights the importance of IFN-gamma in host resistance to infection. PLoS Negl Trop Dis 2012, 6:e1663
70. Pacsa AS, Agarwal R, Elbishbishi EA, Chaturvedi UC, Nagar R, Mustafa AS: Role of interleukin-12 in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 2000, 28:151−155
71. Charnsilpa W, Takhampunya R, Endy TP, Mammen MP Jr, Libraty DH, Ubol S: Nitric oxide radical suppresses replication of wild-type dengue 2 viruses in vitro. J Med Virol 2005, 77:89−95
72. Gunther VJ, Putnak R, Eckels KH, Mammen MP, Scherer JM, Lyons A, Sztein MB, Sun W: A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. Vaccine 2011, 29:3895−3904
73. Mangada MM, Endy TP, Nisalak A, Chunsuttiwat S, Vaughn DW, Libraty DH, Green S, Ennis FA, Rothman AL: Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai school children. J Infect Dis 2002, 185:1697−1703
74. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G: Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol 2003, 35:33−42

75. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E: Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 2006, 80:10208−10217
76. Assuncão-Miranda I, Amaral FA, Bozza FA, Fagundes CT, Sousa LP, Souza DG, Pacheco P, Barbosa-Lima G, Gomes RN, Bozza PT, Da poian AT, Teixeira MM, Bozza MT: Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection. FASEB J 2010, 24:218−228
77. Chuang YC, Lei HY, Liu HS, Lin YS, Fu TF, Yeh TM: Macrophage migration inhibitory factor induced by dengue virus infection increases vascular permeability. Cytokine 2011, 54:222−231
78. Hsieh MF, Lai SL, Chen JP, Sung JM, Lin YL, Wu-Hsieh BA, Gerard C, Luster A, Liao F: Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. J Immunol 2006, 177:1855−1863
79. Conceicão TM, El-Bacha T, Villas-Boas CS, Coello G, Ramirez J, Montero-Lomeli M, Da Poian AT: Gene expression analysis during dengue virus infection in HepG2 cells reveals virus control of innate immune response. J Infect 2010, 60:65−75
80. Guabiraba R, Marques RE, Besnard AG, Fagundes CT, Souza DG, Ryffel B, Teixeira MM: Role of the chemokine receptors CCR1. CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice. PLoS One 2010, 5:e15680
81. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, Peters B, Prestwood TR, Sette A, Shresta S: A protective role for dengue virus-specific CD8+ T cells. J Immunol 2009, 182:4865−4873
82. Renneson J, Guabiraba R, Maillet I, Marques RE, Ivanov S, Fontaine J, Paget C, Quesniaux V, Faveeuw C, Ryffel B, Teixeira MM, Trottein F: A detrimental role for invariant natural killer T cells in the pathogenesis of experimental dengue virus infection. Am J Pathol 2011, 179:1872−1883
83. Trung DT, Wills B: Systemic vascular leakage associated with dengue infections: the clinical perspective. Curr Top Microbiol Immunol 2010, 338:57−66
84. Setiawan MW, Samsi TK, Pool TN, Sugianto D, Wulur H: Gallbladder wall thickening in dengue hemorrhagic fever: an ultrasonographic study. J Clin Ultrasound 1995, 23:357−362
85. Thulkar S, Sharma S, Srivastava DN, Sharma SK, Berry M, Pandey RM: Sonographic findings in grade III dengue hemorrhagic fever in adults. J Clin Ultrasound 2000, 28:34−37
86. Venkata Sai PM, Dev B, Krishnan R: Role of ultrasound in dengue fever. Br J Radiol 2005, 78:416−418
87. Colbert JA, Gordon A, Roxelin R, Silva S, Silva J, Rocha C, Harris E: Ultrasound measurement of gallbladder wall thickening as a diagnostic test and prognostic indicator for severe dengue in pediatric patients. Pediatr Infect Dis J 2007, 26:850−852
88. Srikiatkhachorn A, Krautrachue A, Ratanaprakarn W, Wongtapradit L, Nithipanya N, Kalayanarooj S, Nisalak A, Thomas SJ, Gibbons MP Jr, Mammen MP Jr, Libraty DH, Ennis FA, Rothman AL, Green S: Natural history of plasma leakage in dengue hemorrhagic fever: a serial ultrasonographic study. Pediatr Infect Dis J 2007, 26:283−290, discussion 291-282
89. Groger M, Pasteiner W, Ignatyev G, Matt U, Knapp S, Atrasheuskaya A, Bukin E, Friedl P, Zinkl D, Hofer-Warbier R, Zacharowski K, Petzelbauer P, Reingruber S: Peptide Bbeta(15-42) preserves endothelial barrier function in shock. PLoS One 2009, 4:e5391
90. Green S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Suntayakorn S, Nisalak A, Lew R, Innis BL, Kurane I, Rothman AL, Ennis FA: Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis 1999, 179:755−762
91. Dalrymple NA, Mackow ER: Endothelial cells elicit immune-enhancing responses to dengue virus infection. J Virol 2012, 86:6408−6415
92. Cardier JE, Marino E, Romano E, Taylor P, Liprandi F, Bosch N, Rothman AL: Proinflammatory factors present in sera from patients with acute dengue infection induce activation and apoptosis of human microvascular endothelial cells: possible role of TNF-alpha in endothelial cell damage in dengue. Cytokine 2005, 30:359−365
93. Luplertlop N, Misse D, Bray D, Deleuze V, Gonzalez JP, Leardkamolkarn V, Yssel H, Veas F: Dengue-virus-infected dendritic cells trigger vascular leakage through metalloproteinase overproduction. EMBO Rep 2006, 7:1176−1181
94. Kubelka CF, Azeredo EL, Gandini M, Oliveira-Pinto LM, Barbosa LS, Damasco PV, Avila CA, Motta-Castro AR, Cunha RV, Cruz OG: Metalloproteinases are produced during dengue fever and MMP9 is associated with severity. J Infect 2010, 61:501−505
95. Ong SP, Lee LM, Leong YF, Ng ML, Chu JJ: Dengue virus infection mediates HMGB1 release from monocytes involving PCAF acetylase complex and induces vascular leakage in endothelial cells. PLoS One 2012, 7:e41932
96. Allonso D, Belgrano FS, Calzada N, Guzman MG, Vazquez S, Mohana-Borges R: Elevated serum levels of high mobility group box 1 (HMGB1) protein in dengue-infected patients are associated with disease symptoms and secondary infection. J Clin Virol 2012, 55:214−219
97. Barth OM, Barreto DF, Paes MV, Takiya CM, Pinhao AT, Schatzmayr HG: Morphological studies in a model for dengue-2 virus infection in mice. Mem Inst Oswaldo Cruz 2006, 101:905−915
98. Srikiatkhachorn A, Ajariyakhajorn C, Endy TP, Kalayanarooj S, Libraty DH, Green S, Ennis FA, Rothman AL: Virus-induced decline in soluble vascular endothelial growth receptor 2 is associated with plasma leakage in dengue hemorrhagic Fever. J Virol 2007, 81:1592−1600
99. Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, Beatty PR, Harris E: Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. Am J Trop Med Hyg 2009, 80:416−424
100. Liu P, Woda M, Ennis FA, Libraty DH: Dengue virus infection differentially regulates endothelial barrier function over time through type I interferon effects. J Infect Dis 2009, 200:191−201
101. Kiss J, Yegutkin GG, Koskinen K, Savunen T, Jalkanen S, Salmi M: IFN-beta protects from vascular leakage via up-regulation of CD73. Eur J Immunol 2007, 37:3334−3338
102. Kraus J, Voigt K, Schuller AM, Scholz M, Kim KS, Schilling M, Schabitz WR, Oschmann P, Engelhardt B: Interferon-beta stabilizes barrier characteristics of the blood-brain barrier in four different species in vitro. Mult Scler 2008, 14:843−852
103. Espada-Murao LA, Morita K: Dengue and soluble mediators of the innate immune system. Trop Med Health 2011, 39:53−62
104. Gomez D, Reich NC: Stimulation of primary human endothelial cell proliferation by IFN. J Immunol 2003, 170:5373−5381
105. Yang KD, Wang CL, Shaio MF: Production of cytokines and platelet activating factor in secondary dengue virus infections. J Infect Dis 1995, 172:604−605
106. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM: Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Rev Clin Lab Sci 2003, 40:643−672
107. Ishii S, Shimizu T: Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res 2000, 39:41−82