Chapter 37
Dengue Viral Pathogenesis and Immune Responses in Humanized Mice

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37.1 Introduction

Dengue virus, the causative agent of dengue fever, is transmitted to humans by the bite of an infected *Aedes* mosquito. Over 3 billion people live in endemic areas and therefore are at increased risk to contract the disease with an estimated 50–100 million new infections a year [1, 2]. Dengue virus (DENV) belongs to the family Flaviviridae and is comprised of four closely related serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. In the continental USA, while cases of dengue were nonexistent until 1980, there have been outbreaks of laboratory-confirmed, locally acquired dengue cases along the Texas-Mexico border. More recently, between 2009 and 2011, autochthonous dengue fever was discovered in several Florida counties with a number of cases emerging in Key West, Florida [3]. The viral ribonucleic acid (RNA) genome encodes a single polyprotein which is processed by viral and host proteases to produce three structural proteins (core [C], membrane [M], and envelope [E]) and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins. The study of the molecular characteristics of the virus has provided new insights into its biology [4, 5]. A complex interplay between the virus and the host's immune system is widely hypothesized to precipitate the serious and fatal form of the disease [6].

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37.2 Dengue Disease, Immune Correlates of Protection and Pathogenesis

Most DENV infections are asymptomatic; the majority of subjects with symptomatic infections experience an uncomplicated acute illness, dengue fever (DF) that lasts for 3–7 days. This may be accompanied by headache, myalgia, and fatigue after resolution of the illness [7, 8]. Laboratory findings include leucopenia, thrombocytopenia, and mild elevations in serum hepatic transaminases. Less than 3% of infected individuals present with a more severe form of the disease, dengue hemorrhagic fever (DHF), which is distinguished from DF primarily by the occurrence of vascular leakage. When severe, the leakage can lead to hypotension and circulatory shock. Thrombocytopenia is another hallmark of DHF with a platelet count of $\delta 100{,}000/mm^3$ required to fulfill the case definition of DHF [9, 10]. Volume repletion is a highly successful strategy to treat DHF and the case fatality rate is less than 1% in endemic areas when experienced clinicians and nursing staff are available to provide care to hospitalized patients [10–12].

Individuals who have been infected for the first time with one dengue virus serotype (primary infection) have long-term protective immunity against reinfection with the same serotype [13]. There is transient resistance to infection with other dengue virus serotypes after which individuals are susceptible to infection with other serotypes (secondary infection). Epidemiological observations indicate that 90% of the cases of DHF occur during secondary heterologous DENV infections and that the risk of DHF is increased 15–80 times in secondary DENV infections. Memory cellular immune responses and/or antibody-dependent enhancement (ADE) of infection—wherein subneutralizing levels of anti-DENV antibodies that are present from a previous heterologous infection or passively acquired by an infant from its mother—are widely hypothesized to trigger the massive immunological cascade responsible for DHF [6].

Significant progress in our understanding of the immunity to dengue viral infection and the pathogenesis of DHF has come from a wide range of clinical observations. High titers of dengue virus-specific neutralizing antibodies have been associated with a lower likelihood of severe disease during secondary infection [14]. In patients with severe disease, T cell associated cytokines and markers of activation are found to be elevated [6]. Host factors including human leukocyte antigen (HLA) alleles, age, nutrition status, and prior T and B cell immunity are key determinants of susceptibility to DHF. Individuals carrying the HLA-A*0203 and HLA-A*33 alleles have been associated with a more resistant phenotype, whereas in contrast, patients carrying HLA-A*0207 and HLA-A*24 were found to have increased susceptibility as determined in ethnic Thai and Vietnamese populations, respectively [15–17].

Since antibodies and T cells are critical contributors to DHF pathogenesis, characterizing the nature and fine specificity of adaptive immune responses during a second infection with any of the four viral serotypes is critical to understand how these components can exacerbate severe illness. However, there are signifi-

cant challenges with clinical samples which include identifying the serotype of the previous DENV infection, varying levels of preexisting immunity and transporting patient samples in a timely manner to the laboratory from the endemic areas of the world.

37.3 Animal Models for Dengue

Given the complex pathogenesis of severe dengue disease, a suitable animal model that can mimic clinical disease would be invaluable. The following criteria should be considered for all animal models. The ideal animal model should permit natural insect transmission, elicit classical disease symptoms, generate protective/enhancing antibodies and T cell responses, permit vaccine testing, and finally, aid in drug discovery. Over the years, mouse and primate models have shed light on protective and pathological responses to dengue albeit with some limitations (Table 37.1).

Immunocompetent mice such as C57BL/6 and BALB/c mice require very high doses of input virus ($>10^8$ Plaque Forming Unit (PFU)) to induce disease, far greater than the amount of virus ($\sim 10^4$ PFU) believed to be injected subcutaneously into the human host by an infected mosquito bite [18, 19]. The intracranial route of inoculation has been used in some studies, which is not the natural route of human infection. DENV replication is not typically detected in extraneural sites or in the cell types (monocytes, dendritic cells, and lymphocytes) thought to be most relevant to DENV infection in humans. To assess the contribution of memory DENV-specific CD8 T cells to the immune response in secondary DENV infection, sequential DENV infections were performed in immunocompetent BALB/c mice [20]. However, major limitations with immunocompetent mice include the lack of disease symptoms after primary or secondary infection and the cells that respond are murine in origin.

Productive replication of laboratory strains of DENV was reported after intravenous (i.v.) and subcutaneous (s.c.) infection of mice deficient for both interferon (IFN)-α, -β, and -γ receptors in a 129 background [21]. Mice developed paralysis and other neurological symptoms which are not cardinal features of dengue disease. Generation of a mouse-adapted strain of DENV, designated D2S10, and a triple-plaque-purified clone of DENV-2 D2S10, designated S221, resulted in a vascular leak syndrome with minimal neurological symptoms [22, 23]. AG129 mice develop a broadly cross-reactive and long-lasting antibody responses to DENV [24]. ADE was demonstrated in AG129 mice by passive transfer of dengue monoclonal antibodies, subneutralizing homotypic serum or cross reactive immune serum. This mouse model has also been used for antiviral testing [19, 25, 26]. An obvious limitation of immunodeficient models is the lack of a critical component of the host antiviral system. Furthermore, the infected cells, antibody, and T cell responses are murine in origin and may not truly reflect human responses to dengue infection. While recent work indicates that selective nonadapted strains of virus can induce disease [27], most studies also require the use of mouse adapted viral strains to cause disease symptoms similar to human dengue.

Table 37.1 Animal models for dengue

	Humanized mice			Nonhuman primates	Standard immunocompetent mice	Immunodeficient mice	
	Hu-HSC NSG	BLT-NSG	Hu-HSC RAG$^{-/-}$ γc$^{-/-}$	NHP	BALB/c C57BL/6	A129	AG129
Functional immune system	+++	+++	+++	+++	+++	±	±
Human cells	+++	+++	+++	n/a	n/a	n/a	n/a
Human antibody response	+++	+++	+++	n/a	n/a	n/a	n/a
Human T cell response	+++	+++	n/t	n/a	n/a	n/a	n/a
Cost	++	+++	++	++++	+	+	+
Disease symptoms	Rash fever weight loss	n/t	Fever weight loss	Only using high dose	Only using high dose or intracerebral inoculation	CNS syndrome with lab DENV strain	Vascular syndrome with mouse DENV strain
Dose required for disease	Moderate	Moderate	Moderate	High	High	Moderate	Low
References	[37–40]	[44]	[38]	[34]	[49, 50]	[18, 19, 51]	[18, 19, 22, 51]

HSC hematopoietic stem cell, *RAG* recombination-activating gene, *DENV* dengue virus, *BLT* bone marrow-liver-thymus, *NHP non human primate*, *CNS central nervous system*, *NSG NOD-scid IL2rγ^{null}*, *n/a* not applicable, *n/t* not tested

Human primates are the only vertebrates known to be infected by dengue virus in nature. Infection of chimpanzees and several species of monkeys with physiologic doses of DENV (10^4–10^6 PFU) via the s.c. route resulted in viral replication. NHPs are also used to study ADE and to test the efficacy and safety of candidate vaccines [28–31]. In vaccine studies, antibody titers and T cell responses were measured and protection was indicated by reduced/absent viremia [32, 33]. Inoculation with a higher dose of DENV via an i.v. route recently has been shown to induce hemorrhage and coagulopathy [34]. However, this is not a natural route of dengue infection. Overall, while NHPs develop viremia and neutralizing antibody responses, there is only limited evidence of disease or hematologic abnormalities. In addition, for large-scale vaccine testing NHP models involve significant cost and accessibility.

37.4 Humanized Mouse Models for Dengue

Humanized mouse models with multilineage human hematopoiesis and a capacity for eliciting human immune responses are likely to overcome many of the limitations observed in mice and NHP models [35, 36]. A major advantage of humanized models is the presence of human cells in a physiological setting. Furthermore, dengue-specific humoral and/or cellular immune responses are directed at viral antigenic epitopes recognized by the human immune system in contrast to the murine or primate system. With the advent of improved humanized mouse models, new in vivo experimental strategies are being pursued by several groups (Fig. 37.1). Two leading humanized mouse models currently employed to study dengue are the hu-HSC model in which human CD34+ HSC are engrafted, and the BLT mouse model where human fetal thymus, liver, and HSC are transplanted (Table 37.2).

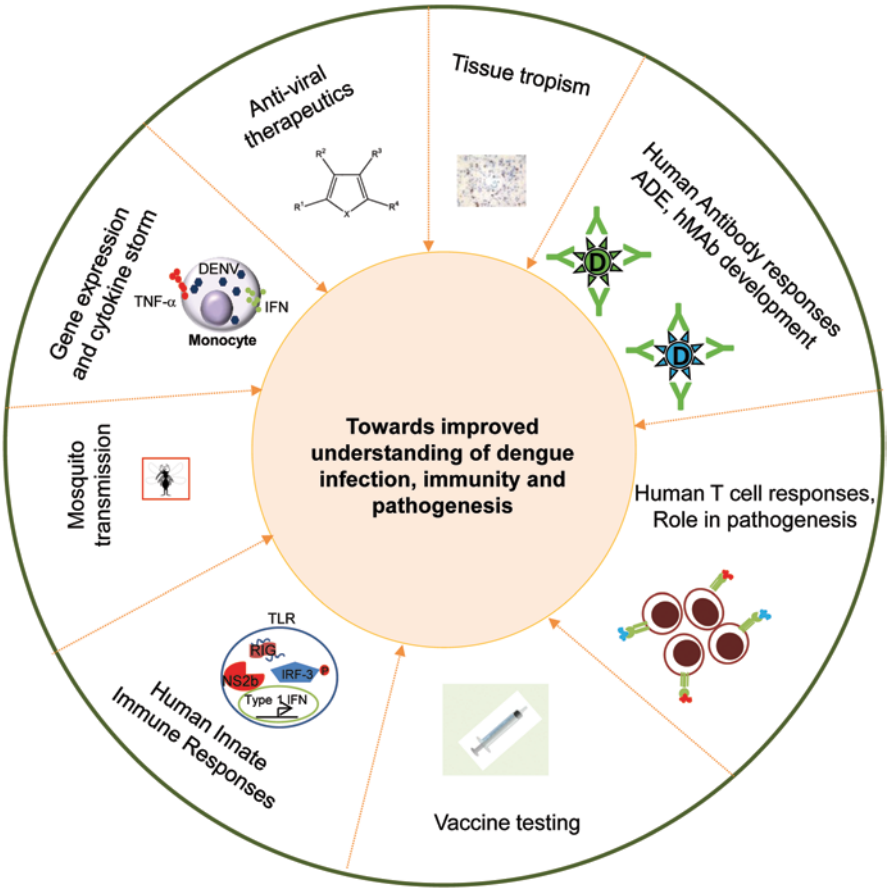

Fig. 37.1 Humanized mice: applications for dengue. *DENV* dengue virus, *IFN* interferon, *ADE* antibody-dependent enhancement, *hMAb* human monoclonal antibodies, *TLR* Toll Like Receptor

Table 37.2 Dengue viral infection in human immune system models

Mouse strain/ model	Human HSC source	Human lymphoid tissues	Dengue viremia/ symptoms	Human cytokine response	Human T cell responses	Human Ab responses	MAbs isolated
Hu-HSC NSG	Cord blood	n/a	+	+	±	+ (weak IgM)	No
Hu-HSC NSG-HLA Tg	A2+ cord blood	n/a	n/t	n/t	DENV-A2 peptides	++ (moderate IgM)	No
Hu-HSC RAG$^{-/-}$ γc$^{-/-}$	Human fetal liver	n/a	+	n/t	n/t	++ (moderate IgM), weak IgG	No
BLT-NSG	Autologous fetal liver	Autologous fetal thymus and liver	±	n/t	Viral non-structural overlapping peptides	++ (moderate IgM)	Yes
BLT-NSG-HLA Tg	Autologous A2+ fetal liver	Autologous fetal thymus and liver	n/t	n/t	DENV-A2 peptides	++ (moderate IgM)	No
References			[37–27, 39]	[40]	[39, 44]	[41, 39, 44, 38]	Unpublished data

HSC hematopoietic stem cell, *RAG* recombination-activating gene, *DENV* dengue virus, *HLA* human leukocyte antigen, *IgM* immunoglobulin M, *IgG* immunoglobulin M, *BLT* bone marrow-liver-thymus, *NSG*, *MAbs* monoclonal antibodies, *n/t* not tested, *n/a* not applicable

37.5 hu-HSC NOD-SCID Mice

Bente et al. used nonobese diabetic–severe combined immune deficient (NOD-SCID) mice transplanted with human CD34+ HSC in their early studies [37]. Mice were inoculated with DENV-2 by the s.c. route. In addition to viremia, clinical signs of DF characterized by fever, rash, and thrombocytopenia were seen. However, due to the lack of sustained multilineage hematopoiesis and paucity of a full complement of human immune cells, these mice were incapable of human immune responses. Therefore, the utility of this model for immunopathogenesis studies has been limited.

37.6 hu-HSC Rag2$^{-/-}$cγ$^{-/-}$and NSG Mice

Studies of Kuruvilla et al. employed Balb/c recombination-activating gene (Rag)2$^{-/-}$cγ$^{-/-}$ mice which due to more severe immunodeficiency permitted higher human cell reconstitution levels and sustained multilineage human hematopoiesis

[38]. Hu-mice (sometimes referred to as RAG-hu) were prepared by intrahepatic injection of human fetal liver derived CD34 HSC into newborn mice. These mice generated human T cells, B cells, macrophages, natural killer (NK) cells, and dendritic cells which are chief components of innate and adaptive immune responses. Mice were injected with DENV-2 viral strains by the s.c. route. Sustained viremia was detected reaching 10^6 PFU/ml lasting up to three weeks accompanied by fever. Dengue specific antibody responses were detected with IgM appearing in 2 weeks followed by IgG responses in 6 weeks in a minority of infected mice. Most importantly, viral neutralizing antibody responses were seen with reactivity to the principal protective immunity inducing viral surface antigen E protein. However, cell mediated T cell responses were not evaluated in these studies due to lack of human HLA class restriction in this model. To overcome this deficiency, the studies of Jaiswal et al., used NSG (NOD-*scid IL2rγ*^{null}) transgenic for HLA-A2 which were humanized by transplanting with cord blood human HSC CD34 cells of the corresponding human HLA type [39]. Mice were infected via s.c. or i.p. routes. Productive viral infection was demonstrated by the presence of viral antigens and RNA in plasma and different tissue compartments. Virus-specific IgM Abs was detected 1 week post infection. Of major importance, virus-specific T cell responses were elicited with the secretion of cytokines IFN-γ, interleukin (IL)-2 and Tumor Necrosis Factor (TNF)-α in response to stimulation with A2 restricted dengue viral peptides. Thus, both antibody and cellular responses to DENV are detected in hu-HSC mice. In an extension of studies in hu-HSC NSG mice, Mota et al., used a highly virulent low passage DENV-2 viral strain [40]. Viremia, clinical signs of fever and thrombocytopenia were detected. In addition to monocytes and macrophages, B and T cells were found to be infected. Cytokine detection assays revealed increased levels of IL-6 and TNF-α in infected mice. However, dengue specific antibody and T cell responses were not assessed in this study. Since dengue is an insect transmitted disease, studies incorporating this natural transmission route are likely to increase our understanding of the dynamics of vector–host interactions and to develop ways to interfere with this process. Cox et al., used hu-HSC NSG mice to allow insect-mediated viral transmission by dengue infected *Aedes aegypti* mosquitoes during feeding [41]. More severe signs of disease characterized by higher and more sustained viremia, erythema, and thrombocytopenia were seen in mice bitten by dengue infected mosquitoes versus those infected by the s.c. route. Interestingly, only mice with insect-mediated viral infection produced IgM antibodies compared to mice infected by injection. This is in contrast to a number of other studies wherein antibody production was demonstrated in hu-mice productively infected by either s.c. or i.p. routes [39, 38].

37.7 BLT-NSG Mice

With the exception of the HLA-A2 transgenic NSG mice, standard hu-HSC mice do not permit evaluation of human HLA restricted dengue T cell responses [36]. The use of BLT-NSG mice, where developing human T cells are educated in an

autologous human thymic graft, is an important advance to generate authentic human T cell responses during viral infections [42, 43]. Thus, the hypothesis that T cells restricted by multiple HLA alleles expressed by the donor should be able to respond to DENV infection can be tested using this model. Accordingly, in a recent study, overlapping peptide pools that encompass the entire DENV genome were used to assess the breadth, magnitude, and quality of DENV-specific T cell responses [44]. The results demonstrated that nonstructural proteins are the predominant targets of CD8 T cells, which is similar to the findings seen in humans [44, 45]. CD8+ T cells in splenocytes from BLT-NSG A2+ mice engrafted with HLA A2 tissue secreted IFN-γ when stimulated with previously identified HLA-A2-restricted DENV epitopes [46]. These findings set the stage for the exploitation of BLT mice to measure human T cell responses to DENV during controlled primary and secondary homologous and heterologous DENV infections.

37.8 Summary and Future Prospects of Dengue Humanized Mouse Models

Studies from several labs have demonstrated productive DENV infection in various humanized mouse models [37, 39, 44, 38]. The induction of human DENV-specific immune responses, both humoral and cellular, represents a promising first step towards developing an ideal small animal model with a functional human adaptive immune system to study complexities of human DENV infection. Further improvement of these models will likely enable the testing of multiple aspects of the interplay between the virus, host immunity, and pathogenesis of disease (Fig. 37.1). However, there remain several important limitations and challenges in advancing these humanized mouse models to study human dengue disease. Studies performed to date have differed in the immunodeficient mouse strains used, the types of human cells transplanted, and the routes used for DENV challenge. Each of these parameters could influence the differing outcomes of infection. The variable and low IgG responses observed in NSG and BLT-NSG mice have been primarily attributed to a lack of species-cross-reactive cytokines in the xenogenic environment. Recent studies of Lang et al., attributed the inefficient Ig class switch in hu-mouse models to insufficient time allowed for the generation of adequate levels of helper T cells resulting in suboptimal T–B cell interactions [47]. Nevertheless, current ongoing efforts to improve the levels of human-cell engraftment, HLA restricted T cell help, and germinal center formation are likely to lead to more robust humanized mice for dengue studies [36, 48].

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