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Humanized mouse models to study human cell-mediated and humoral responses to dengue virus

Anuja Mathew

Institute for Immunology and Informatics, University of Rhode Island, Providence, RI, USA

Abstract

Several candidate dengue virus vaccines are in clinical trials and show promise as an effective measure to control dengue. However, it is becoming clear that additional vaccine candidates may be needed as there is concern about the durability of the immune response to all four serotypes of vaccine components and efficacy varies dependent on the immune status of the individual. The lack of an appropriate animal model to mimic human dengue has deterred the development of vaccines and anti-viral therapies to dengue virus. The focus of this review is to discuss advances in the development of humanized animal models and to highlight how they could be used for antiviral and dengue vaccine testing if limitations with cell-mediated immunity and seroconversion to IgG are overcome.

Introduction

Dengue is a systemic disease caused by a mosquito borne flavivirus dengue virus (DENV). It is estimated that 390 million people are exposed to DENV annually with approximately 96 million infections that manifest with varying levels of disease severity [1]. There are four serotypes of DENV (DENV1-4) that cause clinically indistinguishable disease. The vast majority of severe cases, dengue hemorrhagic fever (DHF) occur during a second infection with a serotype different than what caused the primary infection. While there are many risk factors for developing severe dengue, prior immunity is considered a major risk factor [2]. Since immune responses (both B and T cells) have been shown by many different groups to contribute to protection from and to pathogenesis seen during severe dengue disease it is important that any animal model mimic immune responses detected in humans. Non-human primates have traditionally been used for dengue vaccine testing since they develop viremia and neutralizing Abs. to dengue after challenge. Significant advances have been made to refine immunodeficient mouse models for dengue as immunocompetent mice do not develop clinical signs of dengue infection. Conditional knockdowns of IFN-R signaling and non mouse-adapted strains of virus that cause significant disease and uniform lethality are being tested and may provide a more cost effective rigorous model to test the protective efficacy of

*Corresponding author: Dr. Anuja Mathew, Institute for Immunology and Informatics, Department of Cell and Molecular Biology, Room 334B, 80 Washington Street, Providence, RI 02903, USA., mathewa@uri.edu, Phone: 401-277-5309, Fax: 508-277-5154.

dengue vaccines. The advantages and disadvantages of non-human primate models and immunodeficient mouse models have been described in excellent reviews [3,4].

Dengue virus vaccines

A number of DENV vaccines are now in clinical testing. Vaccine candidates are diverse and include live attenuated dengue viruses, inactivated dengue antigens and recombinant protein antigens [5]. The Sanofi Pasteur dengue vaccine is a live attenuated Yellow fever (YF)-dengue chimeric vaccine (CYD-TDV) created by inserting the DENV pre-membrane (prM) and envelope (E) genes into the cDNA backbone of the YF 17D genome. Takeda Pharmaceuticals is developing a tetravalent chimeric dengue vaccine candidate by introducing DENV-1, -3, and -4 prM and E genes into cDNA derived from a live attenuated DENV-2 component. The NIH/Butantan dengue vaccine is a tetravalent vaccine where the viruses are attenuated by constructing a 30-nucleotide deletion in the 3′ untranslated region. WRAIR/GSK are evaluating a prime-boost strategy between an attenuated live and inactivated dengue vaccine in addition to each of the components individually. Merck & Co. is developing a recombinant E protein candidate produced in a Drosophila S2 cell expression system. The pathogenesis of dengue is complex and correlates of protection are not well understood in part due to the lack of appropriate animal models available [6]. Many vaccine candidates have been evaluated in immunodeficient mice where the deficiency of IFN impacts the ability to generate both cellular and humoral immunity [4].

Humanized mice

There has been significant growth in the use of humanized mice, defined as immunocompromised mice that have been engrafted with human CD34+ hematopoietic stem cells (HSCs) and tissues to study infectious diseases in the past 10–15 years [7–9]. While many improvements have been made, there continue to be limitations in the ability to generate a fully functional human immune system [10]. NOD-SCID and NOD-SCIDIL2Rγ^{null} mice have been used for DENV infection and pathogenesis research [11]. Infected mice present with some clinical manifestation of disease as in humans such as viremia, fever, rash, thrombocytopenia, and erythema [12,13]. Human cytokines have been detected following DENV infection, and infected human cells have been detected in blood, bone marrow, and spleen cells [13–17]. Furthermore, clinical signs of dengue fever varied according to the strain of DENV-2 used and infection with low passaged clinical strains resulted in viremia and clinical disease [12]. However, symptoms of dengue disease and viremia have not been consistent in the animals tested to date.

Since prior immunity is a well-established risk factor for developing severe dengue disease [18], it is important that any humanized animal model mimic humoral and cellular responses detected following primary DENV infection in humans. An ideal model would reproduce the quality and quantity of DENV-specific antibodies (Abs.) and HLA-restricted T cells generated in humans. An effective animal model would also be able to accurately assess whether DENV vaccines can protect from a challenge with clinical or laboratory strains of DENV.

T cell responses to natural dengue infection in humans and humanized mice

In humans, both CD4+ and CD8+ T cells are generated in response to natural infection with DENV [19]. A number of CD4+ and CD8+ T cell epitopes have been defined and are directed against both structural and non-structural proteins on DENV although non-structural proteins are a dominant target for CTL recognition [20]. Responses are directed to a few epitopes in each individual. The amino acid homology can vary among the four DENV serotypes and typically the strongest T cell response is to the infecting serotype during a primary infection. In many animal models that have been used to characterize cellular immunity, the T cells that respond to infection or vaccination are either of murine or primate origin. A significant advantage of a humanized mouse model is the ability to assess human CD4+ and CD8+ T cell responses directed at viral peptides presented on human HLA in contrast to murine T CD4+ and CD8+ cells that recognize viral peptides presented on human HLA evaluated commonly using HLA transgenic mice.

In cord blood engrafted humanized mice, while human T cells develop, the education of human T cells occurs on murine thymus which makes it challenging to assess authentic human T cell responses. The number of cells recovered from a spleen in cord blood engrafted mice is low which limits a comprehensive evaluation of responses to peptides covering all the structural and non-structural proteins of DENV [15]. In BLT NSG mice, since T cells are educated on autologous thymus tissue, T cells that appear in the periphery are educated on the HLA alleles of the engrafted tissues. Our group used HLA A2 transgenic BLT NSG mice engrafted with HLA A2 stem cells and assessed T cell responses in the spleen of DENV infected mice [21]. An additional advantage of BLT NSG mice is the number of cells obtained in the spleens ($50–100 \times 10^6$/spleen) compared to cord blood engrafted mice ($5–20 \times 10^6$/spleen). We used overlapping peptides encompassing the entire DENV genome and were able to detect significant IFN-γ responses to many peptide pools. Of note, IFN-γ responses were detected more frequently against non-structural pools similar to what has been detected in humans [19]. Our efforts to expand T cells in splenocytes with either specific DENV peptides or infectious DENV from immune BLT NSG mice were not successful (data not shown) as we have routinely performed using dengue immune PBMC in humans.

Frias-Staheli et al isolated T cells from HLA A2 DENV infected BLT NOD SCID mice and stimulated them in vitro with HLA A2 PBMC that were differentiated into dendritic cells [17]. Supernatants collected from immune T cells stimulated for four days had higher IFN-γ levels compared to naïve T cells stimulated similarly. Blocking Abs. to Class I and II HLA decreased cytokine secretion suggesting that the response was generated in the context of human MHC molecules.

Overall, experiments performed in BLT humanized mice indicate that IFN-γ responses can be detected following peptide stimulation of human CD8+ T cells in splenocytes from immune BLT mice. Similar to what is detected in humans, responses to non-structural proteins were strong compared to responses against structural proteins. While cytokine responses have been detected to specific viral peptides, efforts need to be made to improve

antigen-presenting cell function which should further improve human CD4+ and CD8+ T cell function.

B cell and antibody responses to natural dengue infection in humans and humanized mice

Following a primary DENV infection, humans develop Abs. that cross-react with all 4 serotypes but mainly neutralize the homologous serotype responsible for the infection. Following a secondary infection with DENV, Abs. typically have a higher titer with a broader pattern of neutralization to all four serotypes [22,23]. Due to the broad pattern of Ab reactivity, it is difficult to identify the specific serotype of the first DENV infection which is a significant challenge in humans who live in endemic areas as most are experiencing a second infection. While durable neutralizing Abs. (IgM and IgG) are likely to be critical effectors in the resolution of dengue viremia and long-term immunity, results from Sanofi Pasteur's clinical trials indicate moderate efficacy despite neutralizing titers in vaccinated children [24]. Antibody-dependent enhancement (ADE), whereby IgG DENV Abs. at sub-neutralizing concentrations enhance DENV infections in Fc-receptor bearing cells has been demonstrated in vitro. In infants born to DENV immune mothers a correlation between the decay of maternal IgG Abs. and the peak incidence of severe disease has been found in some studies while others have not found an association [25]. Multiple Ab functions in addition to neutralization are likely to contribute to the humoral response to DENV infection.

Since B cells are the central source of Abs, significant effort has been spent more recently to better define acute and memory B cell responses to DENV [18,26]. There is a massive expansion of plasmablasts during acute DENV infection with frequencies reaching up to 50–80 % of total B cell responses [27,28]. A very small frequency of memory B cells is found in immune donors however and little is known about the transition of B cells into memory [27,29]. Several groups have generated and characterized human monoclonal Abs. isolated from memory B cells in DENV-immune donors and vaccine recipients [22,30]. These studies have generated cross-reactive Abs. specific for the envelope (E), pre-membrane (prM) protein and non-structural protein −1 (NS1) with poor, moderate or potent neutralizing activity with potent neutralizing Abs. recognizing epitopes present only on intact virions.

Improving B cell and Antibody responses in humanized BLT NSG mice

In the last several years it has been recognized that antigen-specific antibody responses to pathogens or protein antigens generated in humanized mice have been predominantly of the IgM isotype with minimal class switching to IgG although considerable progress has been made in humoral responses to HIV [31,32]. A number of factors have been proposed to contribute to poor class switching to IgG including the lack of T cell help for Ab class switching, inadequate amounts of human cytokines available to activate B cells, the lack of formation of proper germinal centers in peripheral lymphoid organs and significant numbers of immature B cells in the periphery of humanized mice. This blunted B cell and subsequent Ab response generated in many models of humanized mice continues to be a major limitation as seroconversion is used typically to validate the efficacy of vaccines.

Our group and others have shown that humanized mice develop predominantly IgM Abs to DENV during acute infection and in convalescence [14,15]. Therefore humanized mice cannot be used to study aspects of ADE currently as IgG Abs. bound to pathogen complexes trigger signals in multiple FcRs for effective ADE [25]. We demonstrated heightened DENV-specific IgM antibody responses in BLT-NSG mice compared to cord blood HSC engrafted mice [21,33]. Human IgM Abs isolated from recognized the dengue envelope protein produced as a soluble recombinant while a number of human Abs only recognized epitopes on intact virions. The majority of the human Abs isolated during acute infection and in convalescence in BLT NSG mice was serotype-cross-reactive and poorly neutralizing. These findings are similar to what has been detected in humans.

B cells maintained long-term in immunized BLT-NSG mice were able to secrete DENV-specific neutralizing IgM Abs.[33]. Our findings that hAbs isolated in immune BLT-NSG mice had a higher avidity and better neutralization capacity compared to Abs isolated during acute dengue infection suggested that B cells present after resolution of DENV infection in BLT-NSG mice may be functionally more mature and perhaps have undergone some form of affinity maturation. However, the sequences of Abs. characterized at day 45 were not mutated and were similar to germline Abs.

Given the blunted DENV-specific Ab responses we detected in BLT NSG mice, we infected novel strains of transgenic BLT NSG mice where human cytokines known to be important in B cell function and activation were expressed. The sera from SGM3 (Stem cell growth factor, GM-CSF, and IL3) transgenic BLT NSG mice infected with DENV elicited modest IgG Abs. which was a significant improvement compared to standard BLT NSG mice [34]. Studies using sera from DENV immunized IL6 transgenic BLT NSG mice also showed improved IgG responses compared to BLT NSG mice (unpublished data). A recent report suggests that IL6 knock-in mice had improved class-switched memory B cells and ovalbumin specific IgG in response to immunization with OVA [35].

The data suggest that further refinements are required to improve Ab responses in humanized mice and support further testing of humanized mice where human cytokines known to be important for B cell function are injected or expressed constitutively to improve Ab responses. Models such as NOD.Rag1KO.IL2Rγc KO mice that express HLA-DR0401 molecules (DRAG mice) with improved Class II expression may prove to have enhanced Ab responses to DENV as they are known to have elevated Tfh cells critical for class switching [6,36]. DRAG mice are infused with HLA-DR matched human hematopoietic stem cells and unlike the BLT mice do not require human fetal liver and thymus transplants to generate human immune cells. Whether DRAG mice infected with DENV generate E-specific IgG Abs. following infection is not yet known.

Humanized mice for anti-viral drug testing

DENV is extremely sensitive to IFN and inhibits IFN signaling in humans but not in mice which explains why clinical and lab isolates of DENV do not easily replicate in WT mice [37]. DENV replicates to high levels in immunocompromised mice (mice deficient in IFN-α/β and -γ receptors on the129/Sv genetic background (AG129). In recent years, AG129

mice have increasingly become the standard mouse model for *in vivo* testing of anti-viral candidates that impact viral replication through type I and II IFN-independent pathways [4]. The antivirals that have been tested include iminosugars, ribavirin, adenosine nucleotides, inhibitors of NS3 helicase and capsid proteins [3].

An adenosine nucleoside inhibitor, NITD008, was previously shown to inhibit the RNA-dependent RNA polymerase of DENV. NITD was shown to reduce viremia in AG129 mice infected with DENV-2 strain TSV01 [38]. Frias-Staheli et al evaluated NITD008 in BLT NOD scid mice and demonstrated reduced viremia in mice infected with a DENV-2 patient isolate [17]. This provides proof of concept for the use of humanized mice for preclinical testing of antivirals. An added advantage of using humanized mice is the evaluation of immune-mediated drug toxicities detected only in humans.

Although significant advances have been made and are continuing to be made in humanized mouse models, the blunted immune functions and the absence of severe dengue symptoms such as vascular leakage and hemorrhage still limit their utility. Improving both cellular and humoral responses in humanized mice will contribute greatly to the ultimate goal of creating a small animal system that faithfully models human immunity to allow accurate assessment of candidate human vaccines.

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Highlights

1. Human CD8+ T cells respond to dengue viral peptides in immune humanized mice.
2. Dengue virus-specific human IgM antibodies are generated during acute infection and convalescence.
3. B-cells at all major stages of the developmental pathway are detected in the periphery of humanized mice.
4. There is a continued need to identify factors that improve human T cell, B cell and antigen-presenting cell function.