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REVIEW ARTICLE

Autoimmunity in dengue pathogenesis

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Dengue is one of the most important vector-borne viral diseases. With climate change and the convenience of travel, dengue is spreading beyond its usual tropical and subtropical boundaries. Infection with dengue virus (DENV) causes diseases ranging widely in severity, from self-limited dengue fever to life-threatening dengue hemorrhagic fever and dengue shock syndrome. Vascular leakage, thrombocytopenia, and hemorrhage are the major clinical manifestations associated with severe DENV infection, yet the mechanisms remain unclear. Besides the direct effects of the virus, immunopathogenesis is also involved in the development of dengue disease. Antibody-dependent enhancement increases the efficiency of virus infection and may suppress type I interferon-mediated antiviral responses. Aberrant activation of T cells and overproduction of soluble factors cause an increase in vascular permeability. DENV-induced autoantibodies against endothelial cells, platelets, and coagulatory molecules lead to their abnormal activation or dysfunction. Molecular mimicry between DENV proteins and host proteins may explain the cross-reactivity of DENV-induced autoantibodies. Although no

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[j] Dr Huan-Yao Lei passed away during the preparation of this manuscript. This review article is dedicated to Dr Lei.

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licensed dengue vaccine is yet available, several vaccine candidates are under development. For the development of a safe and effective dengue vaccine, the immunopathogenic complications of dengue disease need to be considered.

Introduction

Dengue virus (DENV) belongs to the genus *Flavivirus* of the family Flaviviriade. Based on neutralization assay data, four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) can be distinguished. DENV is transmitted to humans mainly by *Aedes aegypti* and *Aedes albopictus*.[1] About 50 million dengue infection cases, with around 500,000 cases per year of severe dengue, have mainly been reported in the Asia-Pacific region, the Americas, and Africa. All four DENV serotypes are now circulating in these areas.[2] The transmission efficiency and disease expression between the serotypes are still uncertain, but DENV-2 and DENV-3 might contribute the most to disease severity and mortality.[3] There have been several major outbreaks of dengue in Taiwan, particularly in 1981, 1987—1988, 2001—2002, and 2007. Dengue outbreaks involve various combinations of dengue serotypes, with certain serotypes predominating, such as DENV-2 in the year 2002.[4,5] Recent reports have clarified the usual pattern in Taiwan outbreaks: starting by import from abroad in early summer, spreading out locally, and ending in the winter. Dengue is primarily an adult disease in Taiwan. Most cases of dengue fever (DF) have been reported in individuals in the 50—54-year age range and most cases of dengue hemorrhagic fever (DHF) in the 60—64-year range.[4] However, dengue usually occurs in children in hyperendemic Southeast Asia. Secondary infection of DENV-2 was prevalent in the year 2002, but primary infection of DENV-1 or DENV-3 in 2004—2007. In addition, adults or the elderly have a greater risk of developing the severe dengue disease.[4]

DENV is a lipid-enveloped, single-positive-RNA virus, with a genome of about 10.7 kb. RNA of the virus is translated to three structural proteins, namely capsid protein (C), precursor membrane protein (prM), and envelope protein (E). Besides the structural proteins, there are seven nonstructural proteins (NS), which are involved in various functions affecting viral replication and disease pathogenesis.[6,7] The replication cycle of DENV begins when the virions attach to the surface of host cells and subsequently enter the cells by receptor-mediated endocytosis. Acidification of the endosomal vesicle triggers conformational changes in the virion, which results in the fusion of the viral and cell membranes. After the fusion has occurred, the nucleocapsid is released into the cytoplasm. The positive-sense RNA is translated into a single polyprotein that is processed cotranslationally and post-translationally by viral and host proteases. Genome replication occurs on intracellular membranes. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and the newly synthesized RNA bud into the lumen of ER. The virion is maturated in the Golgi compartment and exits by the secretory pathway. Two processes are involved in virus maturation. First, the prM protein is cleaved by host furin and forms the M protein in the trans-Golgi network. Second, the E protein undergoes a major conformational rearrangement during the maturation of virus particles during exocytosis.[7,8]

Infection with DENV causes diseases ranging from mild DF to severe DHF and dengue shock syndrome (DSS). DHF/DSS usually occurs in patients who are secondarily infected with heterotypic DENV, but it also occurs in case of primary infection.[9] DF presents with an onset of fever accompanied by severe headache, retro-orbital pain, myalgia, arthralgia, abdominal pain, rash, and minor hemorrhage in the form of petechiae, epistaxis, or gingival bleeding. Leukopenia is a common finding in laboratory tests, whereas thrombocytopenia may occasionally be observed in DF patients.[10] In addition to all the symptoms of DF, DHF is characterized by severe hemorrhage (positive tourniquet test or spontaneous bleeding), thrombocytopenia (platelet counts $<$100,000/mm^3), plasma leakage (increased hemoconcentration or fluid effusion in chest or abdominal cavities), and hepatomegaly (elevation of serum transaminases). The World Health Organization (WHO) classifies DHF into four grades (I—IV). DHF grades I and II represent relatively mild cases without shock, whereas grades III and IV cases are more severe and may lead to disseminated intravascular coagulation.[11—13] There has been a systematic literature review summarizing the difficulties in applying the criteria for DHF in the clinical situation. For example, the positive tourniquet test indicative of hemorrhagic manifestation does not significantly distinguish between DHF and DF. In addition, the incidences of major manifestations (hemorrhage, thrombocytopenia, and plasma leakage) observed in DHF patients span a large range.[13] Accordingly, the WHO classification system is currently being reconsidered to be more suitable for clinical practice. The new guidelines include dengue without warning signs, dengue with warning signs, and severe dengue. From recent studies, 13.7% of dengue cases could not be classified using the DF/DHF/DSS classification, whereas only 1.6% could not be classified using the revised classification.[14] Hence, assessments of the new classification are still continuing, and the potential implementation of the revised classification has been proposed.

The pathogenic mechanisms in DHF/DSS are complicated and not fully resolved. Several mechanisms are involved in the pathogenesis of DHF/DSS progression, including viral pathogenesis and immunopathogenesis. Viral pathogenesis reflects the pathology directly caused by the virus, and is subject to serotypic or genotypic differences. In contrast, immunopathogenesis encompasses other factors involving the host immune response, which may be involved in the pathogenesis.[15] For example, during secondary infection, the critical phase of disease occurs when the viral burden declines. This has led to the suggestion that

immunopathogenic mechanisms, such as the adaptive immune response, inflammatory mediators, and autoimmunity, are important in the pathogenesis of dengue disease. Such mechanisms play significant roles in major manifestations of DHF, including hemorrhage, thrombocytopenia, plasma leakage, and hepatomegaly (summarized in Fig. 1).[15–17]

Viral pathogenesis

Virus variation

Virus variation indicates the capacity of a virus to produce disease in a host. In the case of dengue, genetic differences among DENV isolates contribute to the severity of dengue disease. There are four antigenically distinct serotypes of DENV, each of which can cause an outbreak of dengue disease.[2] However, DENV-2 and DENV-3 may contribute the most to disease severity and mortality.[3] Viral genetic[18–20] and structural[21] differences have been shown to influence human disease severity. Recently, viral genetic differences were demonstrated to be a contributing factor to virulence in a mouse model.[22] However, it remains to be determined whether these serotypic or genotypic differences observed *in vitro* or in mouse models, respectively, contribute to virulence differences in humans.

Cell and tissue tropism

Cell and tissue tropism of DENV likely have a major impact on the outcome of DENV infection. Langerhans cells (dermal dendritic cells) are generally proposed to be the initial target for DENV infection at the site of the mosquito bite,[23] followed by the systemic infection of macrophages/monocytes[24] and viral entry into the blood. From autopsies of fatal cases, DENV has been found in the skin, liver, spleen, lymph node, kidney, bone marrow, lung, thymus, and brain.[11] Besides the primary targets (dendritic cells and macrophages) of DENV, other potential target cells including hepatocytes, endothelial cells, and neuronal cells have been detected in mouse models. DENV can not only replicate in these cells but also contribute to their damage and/or dysfunction. For example, mice inoculated with DENV by intraperitoneal,[25] intradermal,[26,27] or intracerebal[28] routes have been shown to display liver pathology, hemorrhagic or neurological symptoms. Elevation of serum transaminases, hemorrhage, and fatal encephalitis have been observed in these mouse models, and provide mechanistic insights for the manifestations of dengue disease.[25–28] The range of these cell or tissue types infected with DENV suggest that the receptors of DENV are diverse or broadly distributed. The affinity of DENV with those receptors might influence virus infectivity as well as virulence. A single amino acid mutation on E protein of the flavivirus (Murray Valley encephalitis virus) have been demonstrated to cause altered cell tropism, including differences in entry kinetics, attachment to mammalian cells, and virulence in mice.[29] In summary, the factors that determine the numbers and fates of infected cells at specific sites likely contribute to the pathology of dengue disease.

Immunopathogenesis

Antibody-dependent enhancement

Antibody-dependent enhancement (ADE) is a well-known hypothesis of dengue disease pathogenesis. Epidemiological evidence suggests that the presence of pre-existing subneutralizing antibodies (Abs) is a major factor for developing DHF/DSS in both infants and adults.[30] Enhancing Abs increase the efficiency of virus attachment and

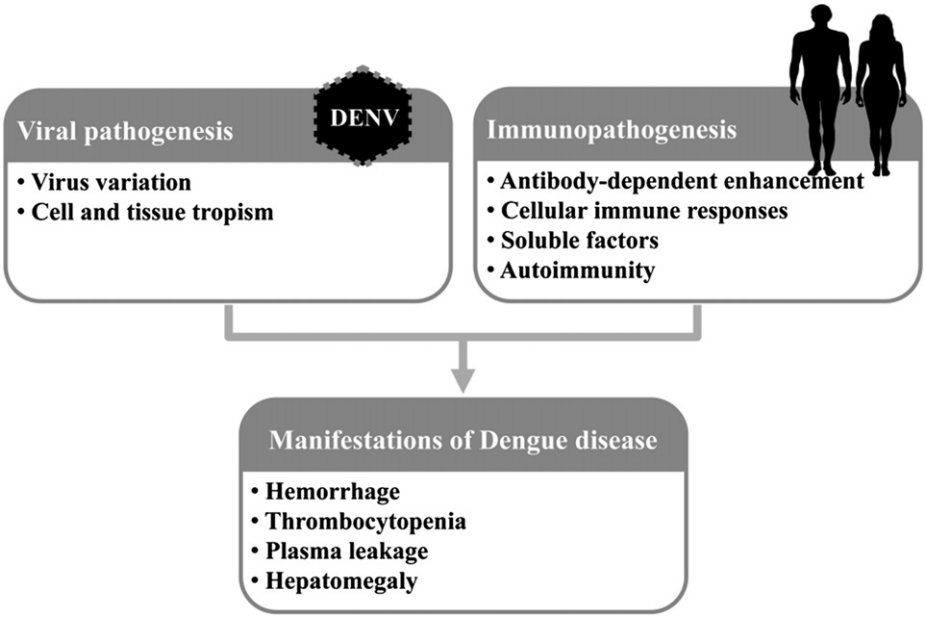

Figure 1 A hypothetical model of dengue pathogenesis. Viral and immunological factors contribute to clinical manifestations, including severe hemorrhage, thrombocytopenia, plasma leakage, and hepatomegaly. DENV = dengue virus.

internalization through Fcγ receptor (FcγR)-dependent[30] or FcγR-independent mechanisms.[31] Enhancing Abs also contribute to the binding of DENV to platelets.[32]

Recently, a new hypothesis (termed intrinsic ADE) postulates that FcγR-mediated DENV internalization suppresses the type I interferon (IFN)-mediated antiviral responses by inhibiting antiviral genes and enhancing interleukin-10 (IL-10) production, which suppresses the IFN-γ signaling pathway and promotes T-helper-2 responses.[33–35] T-helper-1 responses are required for virus clearance; however, T-helper-2 responses have limited antiviral effect and enhance the production of Abs. This may lead to high levels of both viral loads and Abs in dengue patients. Besides the amplification of viral output, ADE enhances cytokine and chemokine production,[36–39] cell apoptosis,[40] and tumor necrosis factor-α (TNF-α)-mediated endothelial cell activation.[36,41]

Cellular immune response

Although memory T cells, which cross-react with heterologous viruses, can provide partial protective immunity, they may cause immunopathology.[42] According to the "original antigenic sin" model, low-affinity memory T cells generated during primary DENV infection expand selectively during the secondary infection of another virus serotype, prior to the activation of naïve T cells of higher avidity for the second DENV serotype. The cross-reactive T cells produce high concentrations of inflammatory cytokines and may contribute to the pathogenesis of plasma leakage in dengue disease.[11,43–45] DENV-specific human $CD4^+$ cytotoxic T cells have been demonstrated to lyse bystander target cells *in vitro*.[46] This mechanism may provide an explanation for lymphocyte activation and hepatocyte damage in a DENV-infected mouse model.[47] A recent study demonstrated that regulatory T-cell frequencies and regulatory T-cell/effector T-cell ratios are increased in acute dengue infection.[48]

Soluble factors

Several studies have indicated that the concentrations of cytokines, chemokines, or other mediators might be significantly increased during DENV infection. Higher levels of IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-18, monocyte chemoattractant protein-1 (MCP-1), macrophage migration inhibitory factor (MIF), transforming growth factor-β, TNF-α, and IFN-γ have been found in the plasma of severe dengue patients.[43,49–58] These mediators play central roles in regulating the immune response to dengue. In particular, TNF-α produced by dengue-infected monocytes[36] as well as by mast cells[41] triggers the activation of vascular endothelial cells. Also, some studies demonstrated that TNF-α contributes to endothelial permeability and hemorrhage during DENV infection in animal models.[26,59] In addition to TNF-α,[60] several studies demonstrated that IL-8,[61] MCP-1,[57] MIF,[62] and metalloproteinase 9[63,64] promoted increased endothelial permeability *in vitro*. Furthermore, IL-6 and IL-8 have been found to be associated with the activation of coagulation and fibrinolysis.[65–67] IL-8 levels have been reported to be increased in most dengue patients and correlated with degranulation of neutrophils.[68] In addition, levels of IL-10 have been shown to correlate with the loss of platelets and failure of platelet function.[69]

High levels of C3a and C5a have been detected in the sera from severely affected dengue patients.[70,71] C3a and C5a, the products of C3 and C5 cleavage, are anaphylotoxins, which promote chemotoxis of immune cells and contribute to inflammatory responses. Soluble NS1 and anti-DENV Abs have also been reported to activate complement, by binding on the surface of infected endothelial cells.[70,72] High plasma levels of NS1 and terminal complement complex have been detected in DENV-infected patients, and these were correlated with vascular leakage as well as disease severity.[70] High levels of regulatory factors D and H have also been reported in DHF patients compared to those in DF patients. The imbalance of factors D and H caused alternative complement pathway deregulation and might correlate with disease severity.[72]

Autoimmunity

Autoimmunity and molecular mimicry have been demonstrated in various viral infections, such as Coxsackievirus and Epstein—Barr virus, and have been implicated in human autoimmune diseases.[73] Autoantibodies represent another important factor involved in dengue disease pathogenesis. Several studies showed that the generation of autoantibodies against platelets,[74–76] endothelial cells,[77,78] and coagulatory molecules[77–81] was associated with dengue disease. Molecular mimicry between platelets, endothelial cells, and coagulatory molecules with NS1, prM, and E proteins may explain the cross-reactivity of anti-NS1, anti-prM, and anti-E Abs, respectively, to host proteins. The consequences of these cross-reactive Abs are platelet dysfunction, endothelial cell apoptosis, coagulation defect, and macrophage activation.[73,82] A schematic model of important dengue manifestations induced by cross-reactive autoantibodies is illustrated in Fig. 2.

Our studies showed that the levels of antiplatelet and antiendothelial cell autoantibodies are higher in the sera of DHF/DSS patients than in that of DF patients. Immunoglobulin M (IgM) present in the sera of DHF patients played a more dominant role than IgG in the cross-reactivity with platelets and endothelial cells. Absorption experiments revealed that anti-DENV NS1 Abs in patients' sera are responsible for the cross-reactivity, resulting in platelet dysfunction and endothelial cell apoptosis.[74,78,83] These findings suggest that DENV-induced antoantibodies might be associated with thrombocytopenia and plasma leakage. Anti-DENV NS1 Abs, which were generated from mice, cross-reacted with endothelial cells and triggered apoptosis by nitric oxide production.[84] In addition, anti-DENV NS1 Abs induced endothelial cells to express IL-6, IL-8, MCP-1, and intercellular adhesion molecule-1. The activation of endothelial cells by anti-DENV NS1 Abs demonstrated the involvement of anti-DENV NS1 Abs in the vasculopathy of DENV infection.[85] Furthermore, mice actively immunized with NS1 proteins or passively administrated with anti-DENV NS1 Abs showed a hepatitis-like pathologic effect. These results revealed that anti-DENV NS1 Abs might play a role in liver damage, which is an important manifestation of

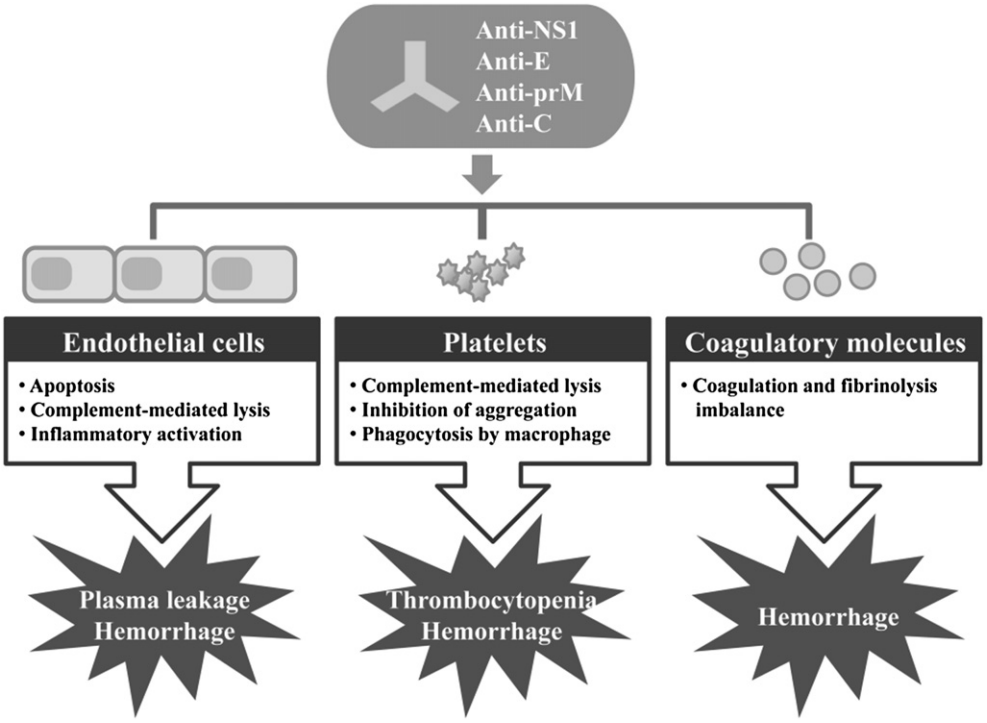

Figure 2 A schematic model of autoantibody-mediated immunopathogenesis in DENV infection. Molecular mimicry between platelets, endothelial cells, and coagulatory molecules with NS1, prM, E, and C proteins underlies the cross-reactivity of anti-NS1, anti-prM, anti-E, and anti-C Abs, respectively, to host proteins. Abs = antibodies; C = capsid protein; DENV = dengue virus; E = envelope protein; NS = nonstructural protein; prM = precursor membrane protein.

dengue disease.[86] From proteomic analysis, the potential candidate proteins on endothelial cells, recognized by anti-DENV NS1 Abs, include ATP synthase β-chain, vimentin, heat shock protein 60, and protein disulfide isomerase. The C-terminal amino acid (a.a.) 311—352 region of DENV NS1 shows certain degrees of homology with the candidate proteins.[87] Protein disulfide isomerase was recognized by anti-DENV NS1 Abs both on endothelial cells and on platelets.[87,88] We also found that the C-terminal region of NS1 was responsible for cross-reactivity with platelets. The deletion of C-terminal region (a.a. 277—352) of NS1 abolished anti-NS1-mediated platelet aggregation and bleeding tendency.[89] These results suggest a mechanism of molecular mimicry in which Abs against DENV NS1 cross-react with endothelial cells and platelets.

Previous studies in our laboratory identified important cross-reactive epitopes on the C terminus (a.a. 271—352) of DENV NS1 proteins.[87—90] Recent studies also indicated that a.a. 116—119 of DENV NS1 shared sequence similarity with human LYRIC protein (lysine-rich CEACAM1 co-isolated) a.a. 334—337.[82] Furthermore, despite the absence of an arginine—glycine—aspartic acid (RGD) motif in the DENV NS1 protein sequence, RGD structural mimicry exists within the NS1 protein. Since RGD is an important motif for matrix-integrin-mediated cell adhesion, anti-NS1 Abs could block RGD-mediated cell adhesion.[91] These findings suggest the existence of still other cross-reactive epitopes, which should be investigated in the future.

Besides thrombocytopenia and plasma leakage, abnormal coagulopathy can also be observed in severe dengue patients. Hemostatic parameters altered in DHF/DSS include prolonged thrombin time and activated partial thromboplastic time, decreased levels of fibrinogen, and increased levels of fibrinogen degradation products.[92] Several studies suggested that autoantibodies may participate in abnormal hemostasis during DENV infection. Abs against NS1 and E proteins have been shown to cross-react with human blood coagulation factors, fibrinogen, and plasminogen.[77,79,80] By sequence alignment, DENV proteins, including core, E, prM, and NS1, have shown different levels of sequence similarity with different coagulatory-associated molecules such as factor X, factor XI, and plasminogen.[73] Although the effects of these autoantibodies on coagulatory factors are still unclear, some reports demonstrated that DENV-induced autoantibodies might interfere with human fibrinolysis.[93,94]

In our previous studies, the titers of DENV-induced autoantibodies reached peak levels in the acute phase, declined during the convalescent stage, and lasted for several months.[74,78] This time course is different from chronic virus infection-associated autoimmune disease.[73] A recent case report showed a dengue patient with numerous autoimmune features,[95] and another report showed a dengue patient in whom dengue evolved into systemic lupus erythematous and lupus nephritis after a month.[96] A follow-up study reported that dengue-infected individuals have long-term persistence of clinical symptoms with complement factors, rheumatoid factor, C-reactive protein, antinuclear Abs, and immune complexes.[97] From these studies, it appears that DENV infection may trigger abnormal immune responses causing autoimmune reactions. Therefore, autoimmune

complications should be considered when developing a safe dengue vaccine.

Dengue vaccine strategy

Although no licensed dengue vaccine is yet available, several vaccine candidates are under development. Although live viral vaccines have advanced to clinical trials, they encountered new difficulties, such as viral interference among the four serotypes in tetravalent formulations. For safety concerns, nonviral vaccines have also been developed, particularly subunit vaccines mostly focused on the E protein or its derivatives. However, the challenge of eliciting balanced levels of neutralizing Abs to each of the four viral serotypes remains a major concern.[12,98]

NS1 is not a virion-associated protein, and anti-NS1 Abs do not enhance DENV infection. Anti-DENV NS1 Abs fix complement and trigger complement-mediated lysis of DENV-infected cells.[99] Previous studies showed that active immunization with NS1 proteins and passive immunization with anti-NS1 Abs could provide protection to mice against DENV challenge.[99,100] However, anti-NS1 Abs still show some pathogenic effects both *in vitro* and *in vivo*.[15,73] Further mapping and/or genetic manipulation of the relevant pathogenic epitopes will be important for the development of a safe dengue NS1 vaccine.

Conclusions

Dengue is one of the most important vector-borne viral diseases in the world. The complexity of dengue immunopathogenesis increases the difficulties associated with the development of a dengue vaccine. A successful dengue vaccine must be effective against all four serotypes, avoid potential ADE-associated pathogenic effects, as well as be free of potential autoimmune complications.

Acknowledgments

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