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Immunopathogenesis versus protection in dengue virus infections

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Immunopathogenesis versus protection in dengue virus infections

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Abstract

Dengue viruses (DENV) are mosquito-borne viruses that cause significant morbidity. The existence of four serotypes of DENV with partial immunologic cross-reactivity creates the opportunity for individuals to experience multiple acute DENV infections over the course of their lifetimes. Research over the past several years has revealed complex interactions between DENV and the human innate and adaptive immune systems that can have either beneficial or detrimental influences on the outcome of infection. Further studies that seek to distinguish protective from pathological immune responses in the context of natural DENV infection as well as clinical trials of candidate DENV vaccines have an important place in efforts to control the global impact of this re-emerging viral disease.

Keywords: dengue, immunopathogenesis, vaccine, T lymphocyte, innate immunity

Introduction

Two seminal observations have had a major and long-standing impact on progress in development of vaccines for prevention of infection with dengue virus (DENV). First, work done during World War II by Sabin and others demonstrated long-lasting resistance to reinfection with the same DENV serotype, in contrast to only short-term resistance to infection with heterologous serotypes [1]. Having established the generation of protective immunity, the path to development of an effective vaccine seemed straight-forward. Subsequently, Halstead and colleagues noted the association of dengue hemorrhagic fever (DHF) with secondary DENV infections [2], an association that was later convincingly demonstrated in prospective cohort studies [3-5]. The immune enhancement model of DHF pathogenesis, developed from these observations [6], has presented a cautionary note for vaccination against DENV. The 'holy grail' in DENV immunology has therefore been to achieve a sufficiently comprehensive base of knowledge to distinguish protective DENV-specific immunity from pathological DENV-specific immunity.

A brief summary is in order. DENV refers to a complex of four closely related flaviviruses, termed serotypes and designated DENV-1, DENV-2, DENV-3, and DENV-4 [7]. All four viruses are transmitted between humans and mosquitoes of the genus *Aedes*, demonstrate tropism for monocytes, macrophages, and dendritic cells, and cause similar clinical syndromes, including mild dengue, classical dengue fever (DF), and DHF. Although its name suggests otherwise, the principal feature of DHF is increased vascular permeability, which causes excessive plasma leakage manifesting as increased hematocrit (hemoconcentration) and effusions in pleural and peritoneal spaces and can result in life-threatening shock.

The focus of this review is recent virology and immunology research on DENV that has yielded a better, if ever more complex, picture of the contribution of the immune responses to the outcomes–

from both viral and host perspectives– of infection (Figure 1). As the field moves towards an improved understanding of the kinetics of infection and disease, and key pathways of innate and adaptive immunity and their interactions, potential implications for vaccine development and testing are also coming into sharper perspective.

Kinetics of dengue viral replication and immune responses in vivo

Prospective longitudinal studies of individuals with acute dengue illness have revealed a highly dynamic interaction between the virus and the host immune response. A consistent finding is that peak viremia titers are coincident with the onset of fever and other classical dengue symptoms [8, 9]. Levels of DENV RNA in both plasma and peripheral blood leukocytes decline thereafter [10] and are <1% of their peak values by the time plasma leakage occurs (in patients with DHF). A significant positive correlation can be demonstrated between viremia titers and illness severity, but only when looking at peak values. Field studies involving more active blood sampling in dengue-endemic areas have extended these findings, showing significantly lower viremia titers in individuals with very mild dengue illness and in asymptomatic individuals [11]. At the same time, a substantial fraction of individuals with high peak viremia titers do not develop plasma leakage. Taken as a whole, these observations suggest that high viremia is necessary but not sufficient for the development of plasma leakage and may act as a trigger for other responses that directly lead to increased vascular permeability.

Activation of innate and adaptive immune mechanisms is also a consistent finding in patients with acute dengue illness. Based on the plasma levels of cytokines and soluble cell activation markers [12] as well as by gene expression analysis [13, 14] and flow cytometry [15] of circulating leukocytes, multiple pathways are activated. A somewhat counterintuitive finding is that immune responses that are associated with both the promotion of inflammation (e.g., IFN-γ and TNFα) and the inhibition of inflammation (e.g., IL-10) are activated in dengue. Studies have shown significant correlations among

these different measures and between immune activation and viremia, however, which complicate efforts to infer causality with regard to disease. In this regard, the kinetics of immune activation appears to be a critical consideration. Whereas peak viremia uniformly occurs early in illness, each of the different immune responses follows a distinct temporal pattern. Type I IFN levels, for example, peak early during illness, in parallel with viremia levels, whereas levels of IL-10 and VEGF peak later, closer in time to plasma leakage. In one study, the relative timing of peak viremia, IFN-γ, and IL-10 was significantly different between patients with DF and those with DHF [16]. We have favored the interpretation that elevated levels of IL-10 represent a counter-regulatory response to the activation of pro-inflammatory pathways.

Effects of dengue virus infection on host cells- intersection of innate and adaptive responses

While the association of severe disease with secondary DENV infection has focused attention on the role of adaptive immune responses, substantial progress has been made in understanding innate immunity in dengue and its interactions with adaptive immunity. In vitro studies have elucidated a variety of structural changes within the cell that are induced by DENV infection and affect (or are predicted to affect) the immune response to acute infection. Some of the most striking changes are an increase in ER-derived membranes, structural changes to mitochondria, and an abundance of vesicles containing viral particles [17, 18]. Several studies have shown that autophagosomes are induced by DENV infection and contribute to viral replication [19-23]. Many of the organelles affected by DENV infection also play a role in the cellular antiviral response. DENV triggers type I IFN production through the RIG-I signaling pathway [24, 25]. RIG-I and a recently identified molecule, STING (also known as MITA, ERIS, and TMEM173), interact with MAVS at the mitochondrial membrane to activate IRF3 and induce type I IFN production [26]. Autophagosomes deliver viral proteins to the antigen presentation pathway and also regulate Toll-like receptor activation [27].

The regulation of these innate immunity pathways by viruses such as DENV has also been an active area of research. The DENV replication complex is located in close association with cellular membranes, where the viral proteins and RNA appear to interact with essential host cell factors [28]. DENV has been shown to inhibit type I IFN signaling through the action of viral proteins (NS2A, NS4A, NS4B, and NS5) targeting cellular STAT signaling proteins [29, 30]. Various other viruses have also been shown to modify mitochondrial function either through direct interaction of viral proteins with mitochondrial components or indirectly via association with structures that interact with the mitochondria such as the mitochondria-associated membrane (MAM) fraction of the ER [31]. Whether DENV similarly directly modifies mitochondrial function has yet to be determined, but the DENV NS2B-3 protease has been shown to cleave the MAM-associated STING protein, thereby inhibiting the production of type I IFN [32]. DENV NS4A was shown to induce autophagy and enhance viral replication [23]. Inhibition of autophagy reduced DENV-2 infection, whereas maturation of autophagosomes to autolysosomes was important for DENV-3 replication [20, 22, 33]. These results suggest serotype-specific regulation of autophagosome maturation.

As noted above, studies have shown T cell activation in acute DENV infections, with higher levels of T cell activation markers such as CD69 and soluble CD4 and CD8 in patients with DHF [34]. The main targets of DENV infection, dendritic cells and monocytes, are important antigen-presenting cells. Autophagosomes can facilitate loading of antigen onto MHC class II molecules for presentation to CD4+ T cells [35-37] and can also cross-present antigen in the context of MHC class I molecules [38]. The regulation of autophagy by DENV may therefore influence the repertoire of epitopes presented by antigen-presenting cells and alter the type and intensity of the T cell response.

Kinetics and specificity of T lymphocyte responses during acute dengue virus infection

The current understanding of immune responses to DENV infection has been driven by paradigms related to kinetics, frequency, and serotype-cross-reactivity. Due to the amino acid sequence homology among the four DENV serotypes, the kinetics of the adaptive immune response to secondary heterologous DENV infection have been presumed to reflect the preferential activation of cross-reactive memory T and B cells, i.e., more rapid and greater expansion and activation in secondary infection than in primary infection. In the absence of prior laboratory data, the pattern of serotype-cross-reactivity observed in secondary infection was therefore used to infer the serotype of previous DENV infections [39]. These assumptions have recently been challenged, however. Daily sampling and analysis of PBMC from patients with acute DENV infection revealed earlier peak frequencies of DENV-specific T cells in primary infection than in secondary infection [40]. Given the more rapid proliferation of memory T cells, and the more rapid clearance of viremia in secondary infection [8], this result was unexpected and requires revision of the model. As the kinetics of expansion and activation are critical to an effective immune response, this finding may reflect an effect of secondary heterologous DENV infection independent of viremia that delays T cell expansion, leading to an increased risk of severe disease. Seemingly contrary to a delay in T cell responses, however, is the finding that an earlier peak IFN-γ response was seen in DHF versus DF cases [9]. This implies that the functional profile of DENV-reactive T cells is as important as their expansion to their influence on disease outcome, as has been suggested previously [41-43].

The relationship between the frequency of DENV-specific T lymphocytes and clinical outcome appears to be dependent on the epitope studied, among other factors. For example, T cell responses to HLA-A24-restricted [44] and HLA-B7-restricted [45] epitopes appeared to correlate with disease severity, whereas T cell responses to an HLA-B57-restricted epitope [46] did not. Perhaps the most studied DENV epitope, HLA-A11/$NS3_{133}$, induced a tetramer-positive population that appeared to correlate with

disease outcome in one population of patients [47], but not others [40, 48], and cytokine responses, but not degranulation, to this epitope were associated with disease outcome [41]. Perhaps most intriguing was the finding that the frequency of HLA-B7-restricted tetramer-positive T cells appeared to correlate with disease severity only in HLA-A11-negative subjects and not in HLA-A11-positive individuals [40]. This supports an influential role for specific HLA haplotypes in crafting DENV-specific T cell responses that inform disease outcome [49].

Another recent finding challenging previous models was that the extent of DENV serotype-cross-reactivity among T cells was comparable in primary and secondary DENV infections [40]. While counterintuitive, this finding indicates that the T cell response is poised to respond to the variant epitopes encountered in secondary heterologous DENV infections. In the case of the HLA-A11-restricted CD8+ T cell epitope $NS3_{133}$ [39], some variants induced comparable responses while others induced rather poor responses [50]. This fits with the epidemiological data, in that many cases of DENV infection result in mild disease and others manifest with more severe disease, and supports the model that T cell responses can contribute to either protection or immunopathogenesis.

Some of the above findings have been replicated in mouse models of DENV infection. Sequential infection of immunocompetent mice with different DENV serotypes has recapitulated skewing of the DENV-specific T cell response [51, 52]. Additional studies have shown differences in the ability of epitope-specific T cells to clear antigen-presenting cells in vivo [53]. HLA-transgenic mice and immunodeficient mice reconstituted with human hematopoietic stem cells have been shown to recognize some of the same epitopes as humans with the corresponding HLA allotypes [54-56]. Given the challenges presented by heterogeneity of both HLA alleles and infection sequences in the setting of natural DENV infection in humans, these results raise the possibility that such mouse models can be used to further explore the immune response to secondary DENV infection, although the lack of a reliable mouse model of human dengue disease will necessitate cautious interpretation of the results.

Immunological memory in natural infection and vaccination- relation to outcomes

In 2008, the World Health Organization Initiative for Vaccine Research convened an expert scientific panel to consult on cell-mediated immunity in dengue and DENV vaccine development [57]. A key recommendation of this committee was that there would be great interest in documenting the cellular response to DENV vaccination during clinical phase I-III vaccine testing to better understand the short- and long-term safety and immunogenicity of the vaccine candidates and potentially to define an immunological correlate of protection. Most focus has been placed on the role of neutralizing antibodies as a correlate of protection, without success (to date). A role for T cell responses in vaccine-mediated protection against flavivirus infection has been suggested by systems biology studies of recipients of the live attenuated yellow fever vaccine [58].

Evidence from a prospective dengue cohort study points to the potential for pre-existing DENV-specific memory T lymphocytes to either reduce or increase the severity of illness during a subsequent DENV infection. In this study, PBMC samples were obtained from a study cohort in an endemic region, and subjects were then monitored for the development of acute febrile illnesses [59, 60]. Serologic testing in the entire study cohort after each DENV transmission season also identified subjects who had serologic evidence of an intervening DENV infection despite having no recognized dengue illness (subclinical infections). Two immunology studies in this cohort have suggested that Th1-biased memory T cell responses dominated by IFN-γ are associated with less severe secondary DENV infection. In the first study, IFN-γ production by PBMC stimulated for 7 days with inactivated DENV antigens showed broader serotype-cross-reactivity in a subset of subjects with non-hospitalized symptomatic DENV infections than in subjects who were hospitalized by the treating clinician [61]. In the second study, the mean frequency of CD4+ T cells producing IFN-γ in response to short-term (16-18 hrs) stimulation with inactivated DENV antigens was significantly higher in subjects who had subclinical DENV infection than in subjects who had a symptomatic DENV infection [62]. On the other hand, the same studies also

suggest that T cell production of TNFα, which seems to be a preferential feature of serotype-cross-reactive responses [63], is associated with more severe infection. In the first study mentioned above, TNFα was detected in culture supernatants of antigen-stimulated PBMC only from a subset of subjects who were hospitalized during the acute DENV infection [61]. This implies that this specific pattern of memory T cell responses represents a higher risk for more severe disease, but does not explain all severe dengue cases.

Several additional lines of evidence support the potential for memory DENV-specific T lymphocytes to contribute to protective immunity against severe dengue disease. While some HLA alleles are more frequently found in patients with severe dengue disease than in those with milder illness (or in the general population), other alleles are significantly less common in subjects with severe illness [49]. Furthermore, these positive and negative associations appear to be specific for secondary DENV infections and depend on the DENV serotype causing the secondary infection [64]. In a recent study of healthy adults in Sri Lanka, two-thirds of whom had serologic evidence of multiple prior DENV infections, the frequency of IFN-γ-secreting T cells measured in ELISPOT assays in response to DENV peptide stimulation showed a weak but statistically significant correlation with the ranking of that particular HLA allele in terms of susceptibility or resistance to severe disease [65]. This finding also supports a protective role for IFN-γ responses, as suggested by the cohort study described above.

Studies of T cell responses induced by DENV vaccines have mainly been confined to small phase I or phase II studies of live attenuated vaccines [66-70]. Similar to the findings in natural DENV infection, these vaccines induce predominantly Th1-type responses to multiple serotypes. In at least one study, these responses included polyfunctional T cells producing IFN-γ, TNFα, and IL-2, which have been associated with protective immunity for other infections [71-73]. For most of these studies, no association with protection could be evaluated because subjects were not known to have subsequent exposure to DENV. In one study, 10 subjects who had previously received a tetravalent live attenuated

vaccine were experimentally infected with either DENV-1 or DENV-3 [74]. All five subjects challenged with DENV-1 were protected, whereas only 2 of 5 subjects challenged with DENV-3 showed evidence of protective immunity. PBMC were collected from subjects at serial time points during the 14-day challenge period and tested for cytokine production in response to in vitro stimulation with DENV. PBMC from subjects who were protected from the challenge infection showed sustained production of IFN-γ, whereas PBMC from subjects who were not protected lost this response by day 5 post-challenge.

Areas of uncertainty

Current knowledge regarding the relationship between pre-existing DENV-specific immune responses and clinical outcome is based on a very limited number of subjects. Given the important role of host genetics in controlling immune responses, it will be important to substantially expand these analyses to additional populations in order to evaluate the generalizability of these findings and establish robust correlates of protective or pathological immunity. The immunological characterizations of acute dengue illness are skewed by the threshold of illness severity necessary for diagnosis; that is, the individuals who enrolled in those studies felt ill enough to seek medical attention. Comparatively little is known about the immune response in very mild (or asymptomatic) infections, although these might be most informative for an effective vaccine. Subjects with acute DENV infection demonstrate potent immune activation even at the earliest stages of clinical illness, meaning that the earliest immunological events, potentially of greatest impact on viral replication and disease, were missing from analysis. None of the studies published to date has evaluated the relationships between T cell responses present prior to secondary DENV infection and those detected during acute illness.

Additional uncertainties arise in relating any immunological correlates defined in the context of natural infection to those induced by vaccination. The DENV vaccine candidates currently in clinical trials reflect a variety of vaccine modalities, including recombinant proteins, virus-like particles (VLP), killed

inactivated viruses, DNA vaccines, live-attenuated dengue viruses, and live chimeric flaviviruses [75]. These vaccines differ in the DENV components included, the valency for each component (number of DENV serotypes included), and the formulation in which the components are presented to the immune system [34]. Protective mechanisms may not be the same for each of these vaccines. Killed inactivated or recombinant protein/VLP vaccines will likely generate a very different immune response from those generated by live-attenuated vaccines. Even subtle differences in the quality of the T cell response could potentially shift the balance of immunity from protection to disease enhancement. The observation of immune enhancement of disease with other virus vaccines provides a precedent for undesirable vaccine-induced immune responses [76]. Additionally, whereas natural infection almost always involves a single DENV serotype, all of the current vaccine candidates incorporate antigens from multiple serotypes. This aspect of vaccine design is likely to alter which T cell populations are preferentially activated and expanded. While potentially circumventing the phenomenon of "original antigenic sin," at least in vaccinees who have not previously been exposed to DENV, there is insufficient evidence to predict the functional phenotypes of the responding T cell populations. Clinical studies seeking to define correlates of protective or pathological immunity will need to include subjects with a wide range of prior DENV exposure histories.

Conclusions

The paradigm of severe dengue disease is that dengue-associated plasma leakage reflects an immune response to systemic DENV infection and is enhanced in patients with prior exposure to DENV. In this context, recent findings on innate and adaptive immune responses to DENV and their interactions have added several layers of complexity. Efforts to identify a single mechanism responsible for the plasma leakage phenomenon have not borne fruit. Instead, a number of immune responses have shown correlations with either less severe disease (protective immunity) or more severe disease (pathologic

immunity), and we are left with the possibility that multiple mechanisms might converge on the same clinical outcome. Further clinical studies of well-characterized patients with natural DENV infection continue to be highly relevant to resolving these areas of uncertainty. DENV vaccines currently in advanced clinical development differ sufficiently from natural DENV infection such that detailed immunologic characterization of vaccinees will be important to support the identification of immune markers or correlates of vaccine efficacy.

Figure legend

Figure 1- Innate and adaptive immune responses to dengue virus and their relationships to protection or disease. Text boxes in the figure note immune responses activated in the infected cell (DENV-infected monocyte), in DENV-specific T lymphocytes that recognize DENV epitopes expressed by the infected cell, or in vascular endothelial cells. Green arrows denote those pathways that are primarily protective against infection or disease, red arrows denote those pathways that are primarily associated with more severe disease (pathological immune responses), and yellow arrows denote pathways that can be associated with either protection or pathogenesis, as described in the text. Monocytes (shown here), macrophages, and dendritic cells are the primary targets for dengue virus (DENV) infection; DENV infection of cells is enhanced by DENV-antibody complexes formed in the presence of pre-existing DENV-specific antibodies (not shown) from a previous DENV infection or passive transfer, e.g., in newborn infants. DENV-specific T lymphocytes responding to the current infection include both naïve T cells and serotype-cross-reactive memory T cells from a prior DENV infection. As illustrated in the figure, plasma leakage results from the action of circulating mediators on vascular endothelial cells, whereas direct infection of endothelial cells by DENV is not considered a major contributing factor in vivo.

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