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Review

Viral toxin NS1 as pivotal target in development of efficient dengue vaccine

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Abstract: Mosquito-borne viral disease dengue is a global public health problem causing a wide spectrum of clinical manifestations ranging from mild dengue fever to severe dengue with plasma leakage and bleeding which are often associated to fatality. To date, there are no specific medications to treat dengue and prevent the risk of hemorrhage. Dengue is caused by one of the four related antigenically distinct serotypes, DENV-1 to DENV-4. The growing burden that represents the four DENV serotypes has intensified both basic and applied researches to better understand the dengue physiopathology. It has been proposed a significant role for the secreted soluble DENV nonstructural protein 1 (sNS1) glycoprotein in the pathogenesis of severe dengue. Here, we provided an overview on current knowledge about the role of sNS1 in the immunopathogenesis of dengue disease. The reasons for the consideration of sNS1 in the design of future dengue vaccine candidates will be discussed.

Keywords: arbovirus; dengue; viral hemorrhagic fever; viral immunopathogenesis; viral toxin; NS1; dengue vaccine strategies

1. Dengue disease

Dengue disease became one of the most significant mosquito-borne viral disease worldwide. The dengue global incidence has dramatically increased over the last decades causing a major public health problem in tropical and subtropical regions where endemicity relates to the profusion of *Aedes aegypti* and at lesser extent *Aedes albopictus*, as major vectors for dengue virus (DENV) transmission. The four serotypes of DENV, DENV-1 to DENV-4, sharing 60-80% homology in their genomic sequences can cause a flu-like illness but some individuals can experience severe plasma leakage associated to exacerbated inflammatory responses leading to a shock which often engages the patient vital prognosis. The mechanisms of severe dengue are poorly understood and presumably multifactorial with viral and host factors having significant roles. The immune status of patients might play a key role in the risk of severe dengue. Indeed, the antibody-dependent enhancement (A.D.E.) or original antigenic sin phenomena has been associated to the development of severe dengue which relates to secondary infection with a DENV serotype different of that responsible for the primary infection. Thus, a pre-existing immunity against DENV could be associated with the development of severe forms of dengue disease during a secondary infection. To date, no specific treatments nor therapies are available for clinical management of severe dengue disease.

DENV is a positive single-stranded RNA virus which belongs to flavivirus genus of *Flaviviridae* family sharing a great similarity with other medically-important arboviruses such as yellow fever virus, West Nile virus and Zika virus [1]. DENV is an enveloped virus particle of 50 nm made of a dense core of 30 nm with a lipid layer around it [1],

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containing a 11 kb-long genome [2,3]. DENV infection life cycle is initiated by the recognition of virus particles through attachment factors and receptors at the cell surface. Internalized virus particles are trafficked to endosomal compartment where low pH-mediated fusion between viral and intracellular membranes causes the release of nucleocapsid into the cytosol [1,3–5]. Once released from nucleocapsid, the free genomic RNA is translated into a long polyprotein which is co- and post-translationally processed to produce the three structural proteins C, prM/M and E followed by seven nonstructural proteins NS1, NS2A/B, NS3, NS4A/B and NS5. The productive infection process of DENV occurs side of the endoplasmic reticulum (ER) membranes (**Figure 1**). At the early stages of DENV replication cycle, the nonstructural proteins are mostly involved in viral RNA replication and also subversion of antiviral innate immune responses in the host-cell [6]. At the later stages, viral assembly process occurs at the ER-Golgi intermediate compartment (ERGIC). The assembled virus particles are trafficked through the secretory pathway and then released as infectious virions by exocytosis.

The NS1 glycoprotein is the only non-structural viral protein detected in the bloodstream of dengue patient during the acute phase of infection. Several reports highlight the involvement of soluble NS1 in pathogenesis of severe dengue [7–12]. In this review on the role of NS1 in immunopathogenesis of dengue, a particular attention will be paid to the reasons for the current consideration of NS1 in the design of dengue vaccine candidates.

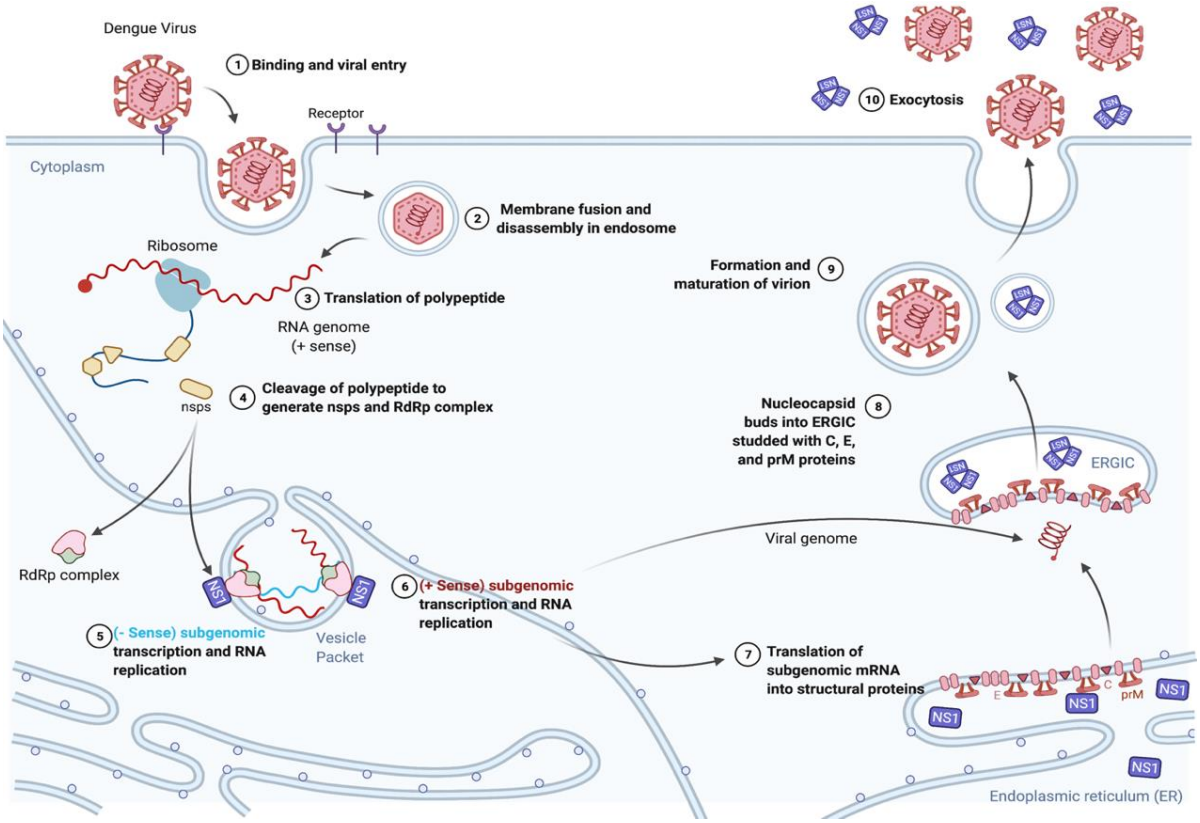

Figure 1. DENV NS1 protein involvement in viral cycle. Dimer NS1 is associated to viral RNA complexes (5, 6) and contributes to viral morphogenesis (7, 8). The release of soluble NS1 requires the protein transport into the ERGIC (10).

2. Biology and function of DENV NS1 protein

DENV NS1 protein (352 amino-acids) is divided in three functional regions designed as β-roll, Wing and β-ladder domains [13]. Upon synthesis and proteolytic processing of viral polyprotein, NS1 enters into the lumen of the ER where glycans are linked to residues N130 and N207. The post-translational maturation process is participating in the formation of homodimer NS1 which together with other NS proteins compose the viral replication complexes for copying the viral genome (**Figure 1**). In relation to its hydrophobic properties and membrane affinity [13], NS1 acts as a scaffolding protein leading to vesicle

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packets formation, a structure known to host viral replicase machinery [14,15]. During DENV infection, dimer NS1 has ability to interact with other viral proteins such as prM and E [14] as well as NS4A [16,17]. Such interactions participate to viral RNA replication [17] and virus assembly [14].

A part of mature NS1 glycoprotein follows a trafficking route through Golgi compartment to reach either the plasma membrane as a membrane-associated homodimer or the extracellular compartment as hexameric lipoprotein particles (**Figure 1**). Secreted soluble NS1 (sNS1) displays a great structural similarity with high density lipoproteins carrying triglycerides, cholesteryl esters and phospholipids (Gutsche et al. 2011). During the acute phase of DENV infection, high levels of sNS1 circulate in the bloodstream up to 50 $\mu g.mL^{-1}$ leading to the development of diagnostic kits based on the immunocapture of sNS1 [18]. A relative positive correlation between antigenemia sNS1 and severity of DENV infection has been documented [19].

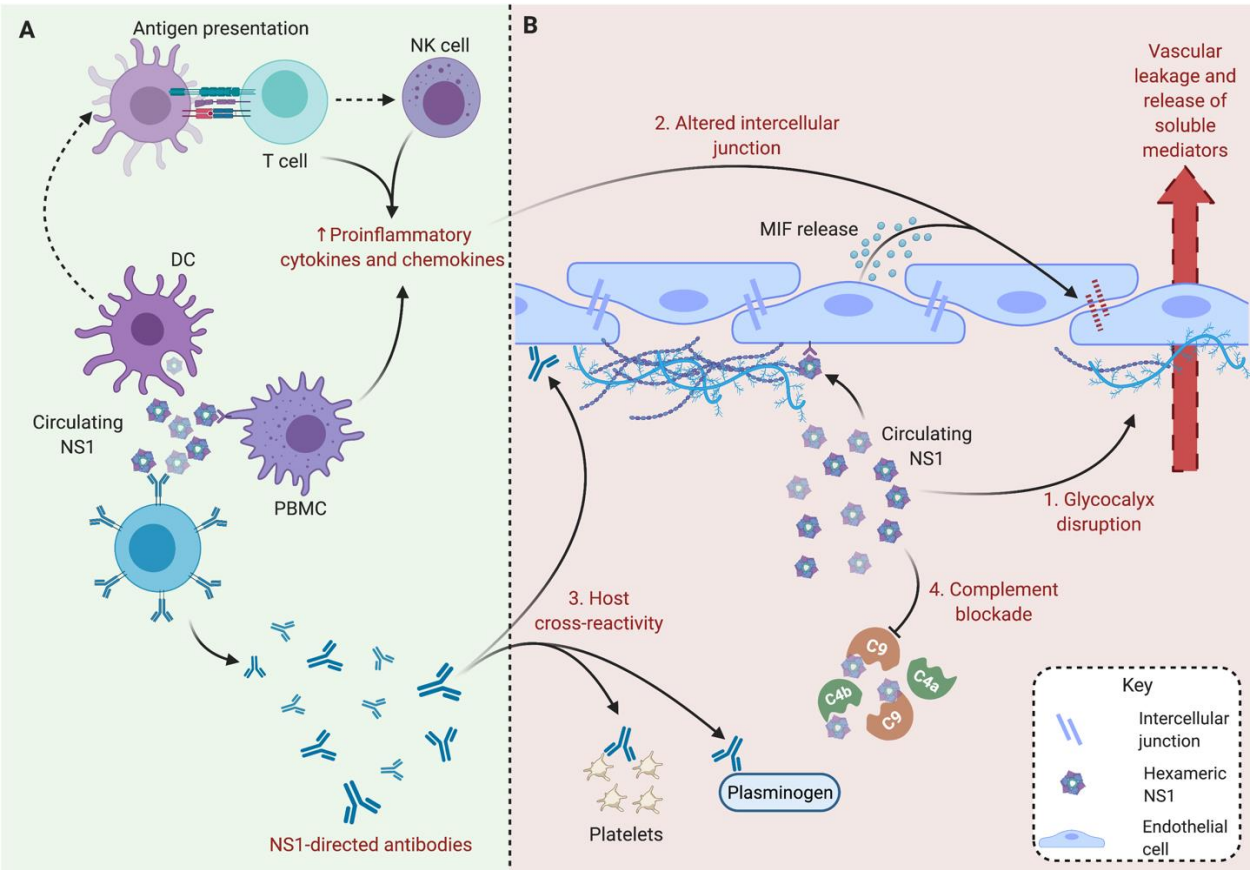

Figure 2. Secreted NS1 implications during DENV infection. (A) NS1-directed immune response. Release of hexameric form of NS1 by infected cells triggers humoral and cell-mediated immune response. B-cell will produce NS1-directed antibodies which participate to NS1 clearance from blood. Whereas, peripheral blood mononuclear cells (PBMC), like T-cell and NK-cell, produce proinflammatory cytokines and chemokines. (B) NS1-mediated pathogenesis. Circulating NS1 is directly linked to glycocalyx disruption (1), as well as, Macrophage Migration Inhibitory Factor (MIF) release by endothelial cells, through Toll-Like Receptor 4 (TLR4) signaling. MIF, as other proinflammatory mediators released by immune cells in response to NS1, lead altered intercellular junctions between endothelial cells (2). On the course of severe dengue, NS1 has ability to contribute to vascular leakage and cytokine storm. Also, soluble NS1 is associated to production of cross-reactive antibodies that will target host endothelial cells, platelets and plasminogen (3). Finally, NS1 may participate to immune evasion through complement blockade.

3. Secreted soluble NS1 contributes to the pathogenesis of severe dengue

During the last decade, a great effort has been made to better understand the role of sNS1 in the pathogenesis of severe dengue. It is now admitted that sNS1 contributes to vascular leakage that is indicative of dengue severity. Indeed, sNS1 has ability to trigger hyper-permeability of human endothelial cells in relation to disruption of endothelial glycocalyx layer and rearrangement of VE-cadherin in adherent junctions [12,20,21]. Also,

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sNS1 has an effect on vascular endothelium through overexpression of sialidases (Neu 1, Neu 2, Neu 3) and heparanase resulting to a barrier dysfunction which participates to plasma leakage [12,20].

DENV sNS1 has also ability to induce expression of vasoactive cytokines notably IL-6 and TNF-α which may be associated with development of vascular disorders in severe dengue (**Figure 2B**) [9]. Expression of cytokine Macrophage Migration Inhibitory Factor (MIF) as part of the response of endothelial cells to sNS1 might play a key role in intercellular junctions' impairment and subsequent vascular leakage (**Figure 2B**). Indeed, MIF participates to cell-to-cell contact disruption through internalization and autophagic degradation of intercellular junction proteins, including Zonula Occludens-1 and Vascular Endothelial Cadherin factors [21,22]. Lastly, sNS1 might play a role in the viral immune evasion strategies mostly through complement blockade (**Figure 2B**). Indeed, DENV sNS1 functions as a complement-fixing protein for several complement actors (C4, C4b, C9 and mannose-binding lectin) [7,8,23,24] and regulators (vitronectin) [24]. Consequently, these interactions have an impact on complement activity and complement-mediated neutralization [15]. In conclusion, the fact that sNS1 has been identified as a viral factor of virulence comparable to a toxin making it a target of great interest for dengue vaccine strategy.

4. Soluble NS1 as significant focus for dengue vaccine strategies

4.1. Immunity to DENV NS1 protein

At the onset of DENV infection, sNS1 elicits a potent humoral and cell-mediated immune response (**Figure 2A**). The NS1-directed antibody response is essentially based on the recognition of B-cell epitopes located in the Wing and C-terminal regions as immunodominant domains of hexameric NS1 lipoparticles. The NS1-directed antibodies participate in sNS1 clearance in the bloodstream during DENV infection and are beneficial to protect against severe dengue, limiting sNS1-associated dengue immunopathogenesis. However, these antibodies could also be involved in pathology of severe dengue. As part of dengue immunopathogenesis, sNS1-directed antibodies have ability to interact with host proteins presumably in relation to a molecular mimicry as a mechanism of autoimmune disease associated to DENV infection (**Figure 3**) [25–27].The self-antigen recognition by sNS1-directed antibodies might play a role in the triggering of apoptosis and complement-dependent cell cytotoxicity in endothelial cells [25]. Consequently, humoral immunity to DENV sNS1 could be a key effector of coagulopathy observed in dengue patient through activation of plasminogen and platelet activation which both participate to thrombocytopenia.

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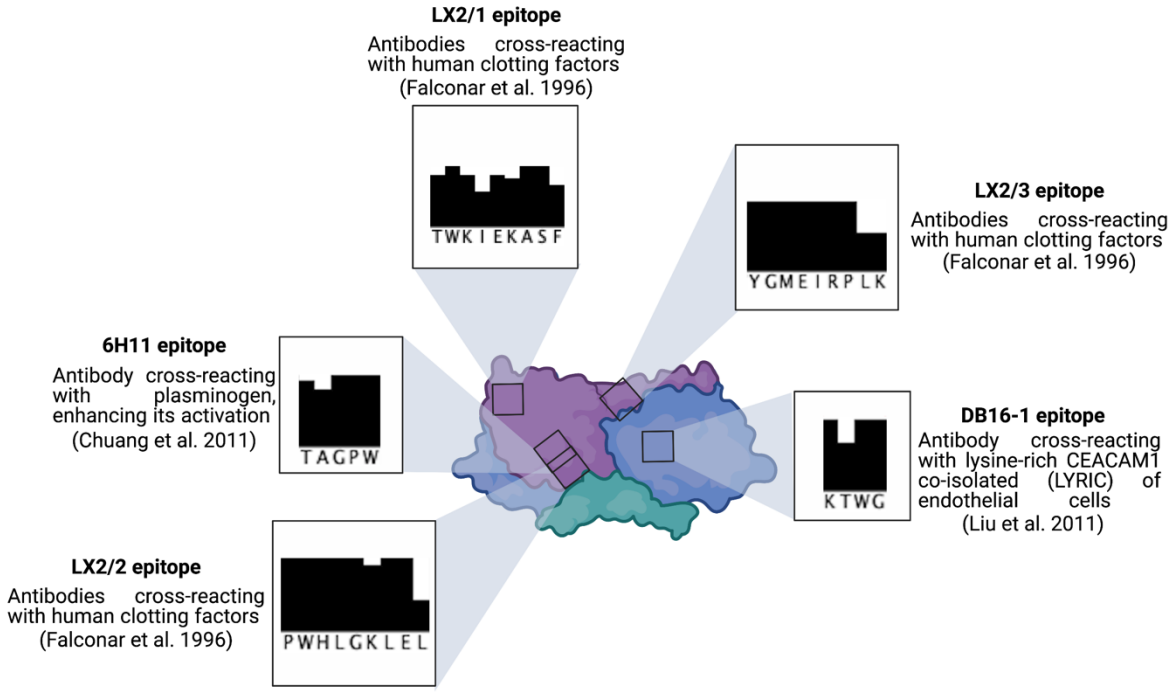

Figure 3. NS1 epitopes in relation to autoantibodies. The recognition of NS1 epitopes LX2/1, LX2/2 and LX2/3 can engender antibodies cross-reacting with human clotting factors [27]. Whereas, NS1 epitope KXWG epitope leads to production of DB16-1 antibody cross-reacting with Lysine-rich CEACAM1 (LYRIC) of endothelial cells, promoting cell death by apoptosis [25]. Finally, 6H11 antibody, which recognize NS1 TAGPW epitope, also interacts with plasminogen and enhance its activation [28]. *β-roll domain (green), Wing domain (blue), β-ladder domain (purple). Black bars illustrate conservation degree of epitope sequence across Dengue Virus serotypes and genotypes.*

On the other hand, anti-NS1 cell-mediated response is based on DENV sNS1 antigen recognition by peripheral blood mononuclear cells via pattern recognition receptors (PRRs). Activated PRRs from the Toll-like receptors family have ability to trigger major pro-inflammatory cytokines and chemokines release involved in DENV immunopathogenesis [9,11]. sNS1 was able to activate TLR4 and the downstream signaling pathway leading to IL-6 and IL-8 production, as well as IL-1β and TNF-α expression [11]. Furthermore, TLR6-/- mice exhibited a higher survivability in presence of sNS1 [29]. However, these data should be interpreted with caution because NS1-mediated TLR2/6 activation was observed with recombinant NS1 proteins produced in bacteria but not in invertebrate cells [30].

4.2. The current challenges for dengue vaccine development

The safe and effective vaccine development against all of the four serotypes of DENV is challenging because of a limited understanding of mechanisms of sever dengue in relation to immunopathogenesis of disease. To date, the only licensed dengue vaccine currently available on the market is Dengvaxia developed by Sanofi Pasteur and registered in 20 dengue endemic countries, European Union and United States regulatory authorities. This is a live-attenuated tetravalent dengue vaccine which contains DENV E and prM genes from the four serotypes inserted in the yellow fever 17D backbone. The objective of this vaccine is to induce preventive humoral and cell-mediated immune responses [31,32]. However, it has recently revealed some major weaknesses. Firstly, it has unequal efficacy against all four DENV serotypes and elicits only a limited protection against serotype 2 DENV infection. Secondly, efficacy of Dengvaxia is effective only in individuals who have been previously infected with DENV. Lastly, the vaccination on children younger than nine years old was associated with an increased incidence of hospitalization for severe dengue [33]. Although neutralizing E-directed antibodies are assumed to be the main correlate of protection against DENV infection, importance of NS proteins for developing an effective dengue vaccine merits a greater consideration. Indeed, T-cell-based immunity is

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necessary in controlling DENV infection and most of the key targets of these responses are located in the NS proteins [34,35]. Indeed, non-structural proteins should be part of the vaccine approach, especially sNS1 which exists as a circulating hexameric lipoparticles. Given the significant role of sNS1 in immunopathogenesis of severe dengue, a lack of NS1-associated immunity could be a possible explanation for the limited performance of vaccine candidates essentially based on expression of DENV structural proteins prM and E [12].

4.3. DENV vaccine candidates expressing sNS1

The targeting of NS1 for dengue vaccine development may have many advantages. The benefits of a sNS1-based dengue vaccine relate to a high degree of NS1 conservation amongst DENV serotypes (about 70% of amino-acid identity), a strong immunogenic potential of sNS1 and the evidence of an efficient anti-DENV immune response based on stimulation of B- and T-cell dependent immunity. According to the immunopathogenesis of dengue, a major advantage of use NS1 is to by-pass a risk of A.D.E. which mainly relates to the production of antibodies enable to raise viral growth and enhance the severity of disease

The second generation of dengue vaccine candidates based on live-attenuated viruses (LAV) include all proteins of DENV whereas licensed Dengvaxia elicits immunity only against prM and E proteins as dengue antigens. The more advanced LAV dengue vaccines is the tetravalent TAK-003 developed by Takeda [36]. TAK-003 consists of an attenuated DENV-2 strain together with chimeric DENV-2 in which the prM and E genes were substituted by the counterparts from DENV-1, DENV-3 and DENV-4 [36]. A single dose of TAK-003 vaccine can elicit a durable T-cell mediated immunity against both structural and non-structural proteins of all four DENV serotypes for at least 4 months post-immunization. Notably, TAK-003 elicits a broad response directed across the DENV-2 proteome, with focused reactivity against NS1 and NS3 [37]. The DENV-2 NS1-directed IgGs are reactive with NS1 of the three other serotypes [38]. In TAK-003 vaccines, hyperpermeability of capillary vessels and degradation of endothelial glycocalyx components were not observed regardless the DENV serotypes [38]. Consequently, a such LAV vaccine provides functional NS1-specific IgG responses which confer protection against the effects of the viral toxin NS1.

Several technologies are currently in use to elicit immune response against DENV NS1 protein (**Figure 4)**. There are LAV, recombinant antigen, and encapsulated protein-encoding gene driving B- and T-cell immune responses targeting DENV NS1.

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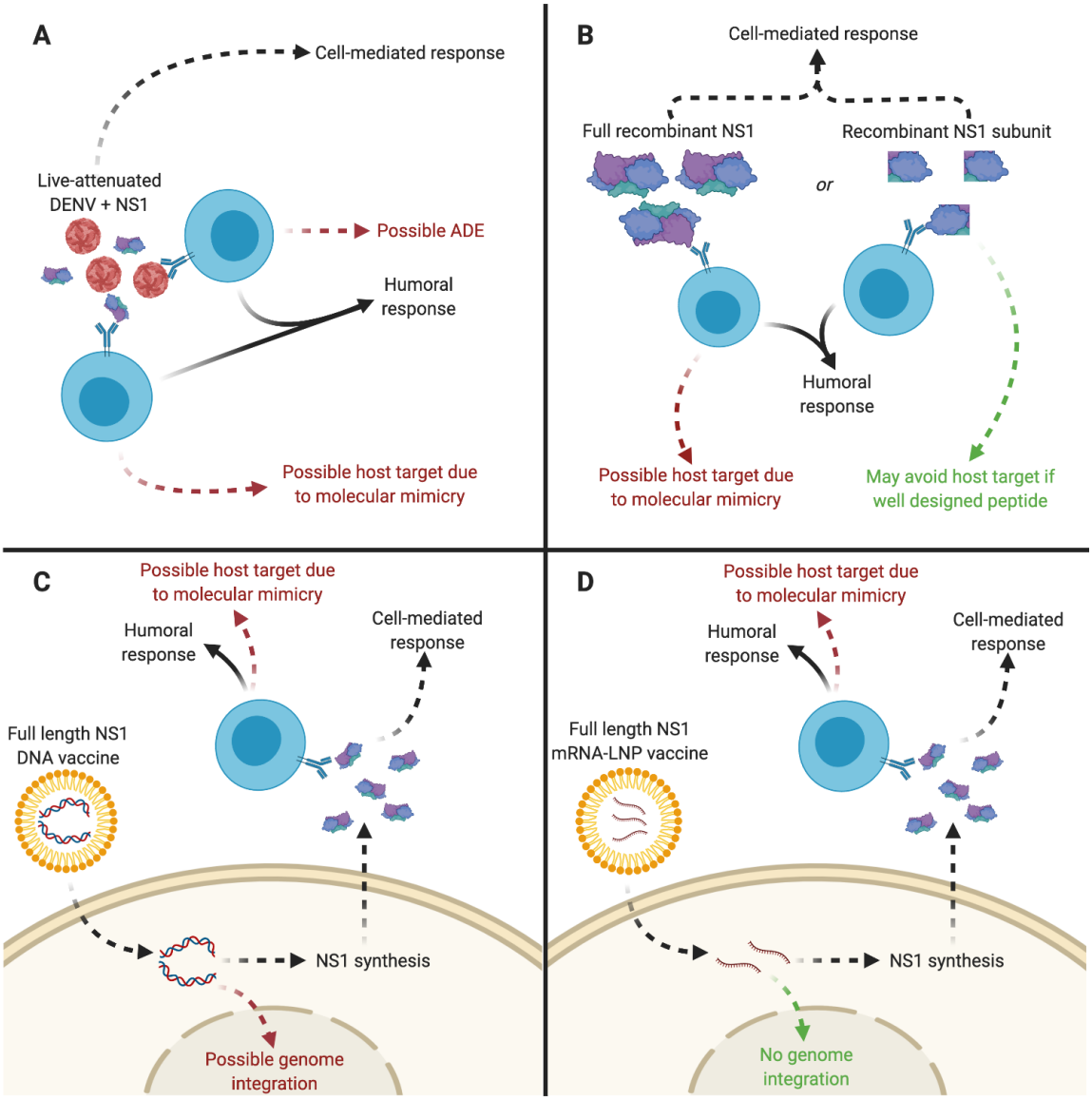

Figure 4. DENV NS1-based vaccine platforms. In A), immunization of live-attenuated DENV (LAV) expressing NS1 induces both humoral and cell-mediated responses. However, vaccination with LAV DENV has a risk of exacerbating dengue severity via antibody-dependent enhancement phenomenon whereas NS1 has ability to generate autoantibodies in relation to molecular mimicry. In (B), a safe immunization with recombinant DENV NS1 or antigenic parts of the protein requires a lack of autoimmune antibodies. In (C), immunization with an encapsulated DNA vaccine expressing DENV NS1 induces both humoral and cell-mediated responses. It cannot rule out a risk of DNA integration into the host-cell genome. In (D), encapsulated NS1 mRNA is a promising approach with lipid nanoparticle (LNP) playing the role of RNA cargo transporter. Released NS1 mRNA is directly translated by the cellular machinery without risk of integration. However, a such NS1-based vaccine should ideally avoid a risk of induction of autoantibody production.

A number of DENV NS1-based vaccines have been and still in development (Table 1).

Table 1. Different NS1-based vaccine strategies.

Platform	Methods	Preclinical	Outcome	Reference
LAV	Tetravalent dengue vaccine (TDV)	NHP	Neutralizing anti-DENV antibodies	[39]
VLPs	$NS1_{1-279}$TMC NPs	BALB/c mice	Protection efficacy 97.47%	[40]
DNA	pcTPANS1*	BALB/c mice	Protection efficacy 100%	[41,42]
	pcENS1**	BALB/c mice	Protection efficacy <90%	[43]
	pE11D2*** and pcTRANS1****	BALB/c mice	Protection efficacy <90%	[44]

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	pD2NS1/pD2NS1 + pIL-2	C3H mice	Protection efficacy 50-80%	[45]
mRNA	DENV-2 E80-mRNA, NS1-mRNA	BALB/c mice	n.a.	[46]
Viral antigen	rNS1 + LTG33D adjuvant	BALB/c mice	Protection efficacy 50%	[47]
	ΔC NS1# + CFA adjuvant	C3H/HeN mice	Protection efficacy 65%	[48]
	Chimeric DJ NS1## + CFA adjuvant	C3H/HeN mice	Protection efficacy 65%	[48]
	Full DENV 1-4 NS1+ MPLA/AddaVax adjuvant	Ifnar -/- C57BL6 mice	Protection efficacy 60-100%	[9]
	Recombinant DENV NS1 + cyclic dinucleotides (CDNs) adjuvant	Ifnar -/- C57BL6 mice	Protection efficacy 60-70%	[49]
Synthetic peptide	Modified NS1-WD* + CFA adjuvant	C3H/HeN: STAT1 -/- C57BL6 mice	Protection efficacy 100%	[50]

*TPA: human tissue plasminogen activator; a secretory signal sequence; **pcENS1: encoding the C-terminal E protein plus the full NS1 region; ***pE11D2: encoding the envelope (E) ectodomain (domains I, II, and III); ****pcTRANS1: encoding the non-structural 1 (NS1) protein of DENV2; NS1_WD: NS1 wing domain.

Next-generation of dengue vaccines in development, including DNA subunit, virus-like particles (VLP) and viral vector vaccines, are reviewed by Redoni *et al.* [51]. VLPs display viral antigens with high density on their surface, giving a potential for high antigenicity and potent immunogenicity [52]. This makes VLPs a promising approach for developing safe and effective DENV vaccines. VLPs based DENV vaccines are described by Zhang *et al.*, 2020, but none of them were developed with NS1 proteins [53]. One example is given with C-terminal truncated DENV-2 NS1 loaded in N,N,N, trimethyl chitosan nanoparticles ($NS1_{1-279}$TMC NPs) investigated in murine model and in human *ex vivo* [40]. In human *ex vivo* model, it has been demonstrated that TMC particles deliver $NS1_{1-279}$ protein in monocyte-derived dendritic cells (MODCs) and also stimulate those cells resulting in an increase expression of maturation marker (CD83), costimulatory molecules (CD80, CD86 and HLA-DR) and secreting diverse immune cytokines/chemokines [40]. Immunization with $NS1_{1-279}$TMC NPs resulted in both B cell and T cell responses leading to IgG production and CD8+ T cells activation. One important finding is that DENV2 $NS1_{1-279}$-directed antibodies have ability to kill DENV-infected cells through antibody dependent complement-mediated cytotoxicity [40]. Consequently, a such NS1-based vaccine candidate is of great interest in relation to the properties of TMC as a suitable adjuvant which enhances delivery and promote immunogenicity of viral antigen. The NLP-associated delivering of NS1 mRNA has been assessed in preclinical studies. Injection of LNP as carrier vehicle of mRNA expressing both N-terminal part of E and NS1 has resulted in high levels of neutralizing DENV antibodies and viral antigen-specific T cell responses leading to a complete protection against DENV challenge.

It is well admitted that interactions of DENV NS1 protein with clotting factors and endothelial cells contribute to the immunopathogenesis of severe dengue. Several studies have identified NS1 peptide sequences, that can generate anti-DENV antibodies displaying a cross reactivity to self-antigens [54]. Such NS1 motifs are mostly conserved across DENV serotypes and genotypes (**Figure 3**). For instance, it has been shown that antibodies directed against the C-terminal region of NS1 can cross-react with the LYRIC protein which localizes at the tight junctions of endothelial cells [55–57]. Given that immunization with a DENV NS1 peptide representing a modified LYRIC-like sequence can provide an efficient protection against DENV [50], the NS1 peptides lacking of mimicry sequences may represent an attractive platform in the development of NS1-based dengue vaccines.

5. Concluding remarks

It is widely admitted that the threat against dengue disease requires a successful vaccine against the risk of severe dengue. An efficient dengue vaccine must be able to elicit a long-term immunity to DENV regardless the serotype and genotype of infecting viral strain. At our stage of knowledge on dengue disease, key elements of the most popular dengue vaccine strategies include the notion of designing viral antigens that are target for

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effective antibody-mediated neutralization of the four DENV serotypes. Dengvaxia efficacy trials conducted to date have demonstrated that a dengue vaccine is possible and have made important contributions to our understanding of the path towards the development of such as vaccine. Since the licensed dengue vaccine Dengvaxia has shown debated efficacy, the mechanisms by which a dengue vaccine might confer efficient protection against DENV infection need to be better defined. Consequently, new insights that will help to guide rational vaccine design against DENV are necessary. The NS1 glycoprotein has recently emerged as a potential viral antigen target for development of dengue vaccine candidates. As a viral toxin, released soluble NS1 has been demonstrated playing a key role in the immunopathogenesis of severe dengue. One can estimate that LAV of DENV and NS1-based vaccines represent promising strategies that have the potential to significantly advance dengue vaccine development. Given that their efficacy could be greatly improved by reducing off-target antibody responses to DENV NS1 protein, it will be of great interest to modify by mutational approach the NS1-associated irrelevant epitopes representing a risk of mimicry with host factors involved in coagulation and integrity of vascular endothelium. Such modified NS1 proteins will have to retain their ability to elicit an effective anti-NS1 immune response.

Abbreviations

ADE	Antibody Dependent Enhancement
DENV	Dengue Virus
DHF	Dengue Hemmorhagic Fever
DSS	Dengue Shock Syndrom
E	Envelope protein
ERGIC	ER-Golgi intermediate compartment
FcγR	FC gamma receptor
GMT	Geometric mean titer
IL	Interleukine
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MIF	Macrophage Migration Inhibitory Factor
MODCs	Monocyte-derived dendritic cells
NS	Nonstructural protein
PBMC	Peripheral blood mononuclear cells
prM	Precursor of M protein
PRR	pattern recognition receptors
TLR	Toll-Like Receptor
TMC	Trimethyl Chitosan
TNF-α	Tumor necrosis factor α
VLP	Virus-Like Particle
WD	Wing Domain
WNV	West Nile Virus
ZIKV	Zika Virus

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