Programming the Immunogenicity of Dengue Virus Envelope Protein Domain III (EDIII): An Approach to Develop a Tetravalent Vaccine Formulation against Dengue Virus

A thesis submitted to the University of Hyderabad for the degree of DOCTOR OF PHILOSOPHY

by

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CERTIFICATE

This is to certify that the thesis entitled ***"Programming the Immunogenicity of Dengue Virus Envelope Protein Domain III (EDIII): An Approach to Develop a Tetravalent Vaccine Formulation against Dengue Virus"*** submitted by **Mr. Rafiq Ahmad Khan** bearing Registration Number **17LTPH02** in partial fulfilment of the requirements for award of Doctor of Philosophy in the Department of Biotechnology and Bioinformatics, School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from Plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Further, the student has the following publications:

1. Biomaterials Science 2022 Apr; 10.1039/d2bm00167e *(doi: 10.1039/d2bm00167e)*
2. Cytokine. 2019 Nov; 123:154780. *(doi: 10.1016/j.cyto.2019.154780)*
3. Cellular Immunology. 2018 Dec 1; 334:42-8. *(10.1016/j.cellimm.2018.09.003)*
4. Journal of Nanobiotechnology 2022 20:317. *(doi. 10.1186/s12951-022-01496-5)*
5. PLoS biology. 2018 Apr 5; 16(4):e2005317. doi: *(10.1371/journal.pbio.2005317)*
6. European Journal of Immunology 2019 Feb; 49(2):277-289. *(doi: 10.1002/eji.201847513)*
7. Clin Immunol. 2022 Mar 17; 237:10898. *(doi: 10.1016/j.clim.2022.108981)*
8. Immunol Lett. 2022 Feb; 242:27-36. *(doi: 10.1016/j.imlet.2022.01.001)*
9. Nanoscale 2020, 12, 2028-2039. *(doi: 10.1039/c9nr05211a)*

and has made poster presentations in the following conferences:

1. Indian Immunology Society conference IMMUNOCON 2019, DAE convention centre Mumbai, India.
2. Combat HIV-International Conference on Biology and Therapeutics of HIV and Associated Viral Infections, 2019, University of Hyderabad, India.
3. Indian Immunology Society conference IMMUNOCON 2018, THSTI, Faridabad, India.
4. International Vaccine Conference held on 25th to 29th Nov 2017 at ICGEB, New Delhi.

Further, the student has passed the following courses towards fulfilment of coursework requirement for Ph.D.

Course Code	Name	Credits	Pass/Fail
1. BT801	Research Methodology/Analytical techniques	4	PASS
2. BT802	Research Ethics, biosafety, Data analysis and Biostatistics	4	PASS
3. BT803	Scientific Writing and Research proposal	4	PASS

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DECLARATION

I, Rafiq Ahmad Khan hereby declare that this thesis entitled *"Programming the Immunogenicity of Dengue Virus Envelope Protein Domain III (EDIII): An Approach to Develop a Tetravalent Vaccine Formulation against Dengue Virus"* submitted by me under the guidance and supervision of **Dr. Nooruddin Khan** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Date: 11-07-2022

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Rafiq Ahmad Khan

Table of Contents

List of Figures and Tables

Figures

Tables

Acronyms

- (PrM) Precursor membrane protein, 2
- (qRT-PCR) Quantitative real time PCR, 41
- (rOMVs) Recombinant OMVs, 32
- (ROS) Reactive Oxygen Species, 29
- (RT) Room temperature, 41
- (RT-PCR) Reverse transcriptase-polymerase chain reaction, 9
- (TEM) Transmission electron microscopy, 46
- (Tfh) T follicular helper cells, 74
- (THP1) Human monocyte leukaemia cell line, 38
- (TLRs) Toll like receptors, 17
- (TNF-α) Tumor necrosis factor-α, 16
- (VLPs) Virus like particles, 29

INTRODUCTION AND REVIEW OF LITERATURE

1 Introduction and Review of Literature

1.1 Dengue Infection

Dengue has been ranked as one of the most serious vector-borne disease, posing a global threat to public health and the economy. Dengue infects more than 390 million people annually, of which 96 million develop clinical symptoms (Bhatt et al., 2013). Dengue virus spreads through the bite of an infected mosquito *Aedes aegypti* or *Aedes albopictus*. There are four distinct yet antigenically related serotypes of the dengue virus named DENV 1-4. The virus belongs to the genus Flavivirus of the family Flaviviridae. These are positive-sense, single-stranded RNA viruses. To the best of our knowledge, there are no effective antiviral therapies, leaving vaccines as an alternative strategy to stop the spread of the dengue virus. Dengvaxia is a live-attenuated chimeric tetravalent vaccine licensed for dengue. However, it demonstrated protection only toward seropositive individuals and enhanced the risk of developing severe dengue infection in dengue naïve individuals. Hence, there is an urgent need for a vaccine that can stop the spread of the dengue virus and decrease the disease burden on society.

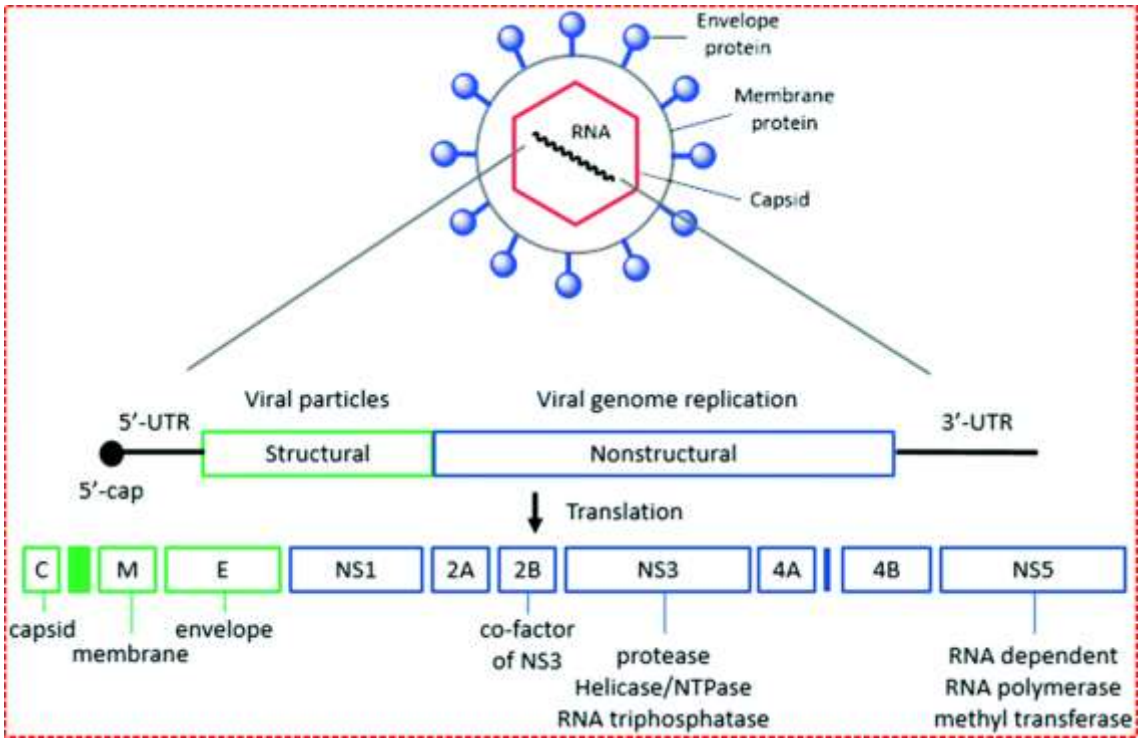
1.2 Dengue Virus: Causative Agent

Dengue is caused by four serotypes of antigenically related dengue virus (DENV) named DENV 1, DENV 2, DENV 3, and DENV 4 (Afroz et al., 2016; Tuiskunen Bäck and Lundkvist, 2013). Dengue virus belongs to *Flavivirus* genus of Flaviviridae family. Examples of viruses that belong to this family are the Zika virus, yellow fever virus etc. (Murugesan and Manoharan, 2020).

1.2.1 Structure and Genome Organization

Dengue virus is surrounded by an envelope with a diameter of ~50 nm (Mebus-Antunes Id et al., 2022). At the core of the virus, there is a structure called nucleocapsid which consists of the viral genome and capsid protein. A lipid bilayer membrane called viral envelope surrounds the nucleocapsid. The viral envelope comprises of equal number of copies of envelope and membrane proteins that span through the lipid bilayer. It forms the outer layer of the virus, which is needed for the viral entry into the host cells (Kuhn et al., 2002). DENV has positive-sense and single-stranded RNA genome of 11 kb. The RNA genome

produces a polyprotein, which is processed by host and virus-encoded proteases. This leads to the production of three structural proteins; capsid protein (C), precursor membrane protein (prM), envelope protein (E) and seven non-structural proteins namely NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Figure 1) (Lopez-Denman and Mackenzie, 2017).



***Yokokawa, Fumiaki RSC Medicinal Chemistry* 11.5 (2020): 541-551.**

Figure 1. Structure and Genome of Dengue virus.

Structure of DENV and schematic of genome organization consisting of precursor membrane protein, capsid, E protein, and non-structural proteins NS1-5 proteins in the open reading frame between the 5′ to 3′ untranslated regions.

1.2.2 **Viral Proteins**

Non-structural proteins

The non-structural proteins produced by the virus play a crucial role in viral RNA replication, assembly, and regulation of cellular and immunological responses of the host (Guabiraba and Ryffel, 2014; Navarro-Sanchez et al., 2005). During the severe phase of infection, the NS1 protein acts as a crucial immunogen that can elicit a potent anti-NS1 response in the host (Wahala and de Silva, 2011). Emerging evidence sheds light on the

crucial role that NS1 plays in the activation of the complement system during infection (Avirutnan et al., 2011). Another role of NS1 is to interact with NS4A/B and helps in viral replication. Type I interferon (IFN) signaling is modulated by non-structural proteins like NS2A, NS2B, NS4B and NS5 (Muñoz-jordán et al., 2005; Srikiatkhachorn et al., 2017) while simultaneously NS5 and NS4B proteins induce the production of chemokines and pro-inflammatory mediators (Kelley et al., 2012; Medin et al., 2005). NS3 also functions as a helicase and protease (Apte-Sengupta et al., 2014; Erbel et al., 2006) whereas NS4B helps in the dissociation of NS3 helicase from the viral RNA. Of all the non-structural proteins, NS5 is the largest and highly conserved protein (Sahili and Lescar, 2017). The two main functions of NS5 include acting as RNA-dependent RNA polymerase, which is involved in the replication of virus, and acting as RNA methyltransferase used for capping during translation (Liu et al., 2010).

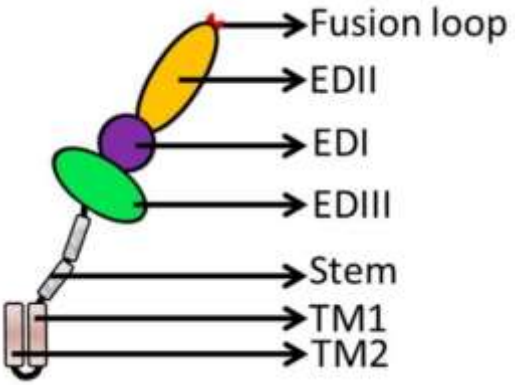
Structural proteins

Nucleocapsid protein: The nucleocapsid protein plays a crucial role in protecting the genetic material by encapsidation. It is the first protein encoded by the viral genome followed by precursor membrane protein (prM). The capsid is a 12 KDa basic protein (Wang et al., 2004). It is known to assist in the nucleic acid arrangement by acting as RNA chaperon (Byk and Gamarnik, 2016; Pong et al., 2011).

Precursor membrane protein: prM is glycosylated, and its formation begins by protease hydrolyzation at the time of late viral infection. It is crucial for forming E protein, and maintenance of its spatial structure (Roby et al., 2015). prM, along with E protein forms the surface structure of virions.

Envelope protein: E protein plays a vital role in the infection of host cells. This happens when the E protein helps in fusion of membranes between the virus and the host cell (Tang et al., 2015). This property of E proteins makes it the primary target protein to produce neutralizing antibodies. The structure of E protein looks like a raft with 90 anti-parallel homodimers present on the viral membrane (Zhang et al., 2017). The envelope protein consists of three distinct domains named as EDI (purple), EDII (orange), EDIII (green) and the TM domains (brown) linked by the stem-like structure (Figure 2). EDI is located in the middle of the E protein toward the N-terminus. EDI helps in the stabilization of the complete orientation of E protein (Wu et al., 2003; Zhang et al., 2017). EDI mostly

contributes toward non-neutralizing antibodies (Chiou et al., 2012; Williams et al., 2012). There are a few conserved Asn amino acid sites at Asn 67 and Asn 153 at the N-linked glycosylation site of E protein (Adachi et al., 2017). When amino acids at these sites are substituted with other non-glycosylated amino acids, it leads to lower cellular attachment (Kariwa et al., 2013), suggesting that these sites are involved in neuroinvasiveness in mice, pH sensitivity and virus production (Hanna et al., 2005). Between the three EDI segments, two elongated loops form a dimerization domain called EDII. EDI and EDII peptides when connected by four linkers form EDI/EDII hinge region (Messer et al., 2014). At its distal end, EDII contains a loop which is highly conserved and functions as a fusion peptide in amino acids 98 to 110 (Allison et al., 2001; Fritz et al., 2011; Huerta et al., 2014). The fusion peptide region is involved in interactions of a virus with the host cell receptor and helps in virus-mediated membrane fusion. EDII also causes mutations and homodimerizations which reduce virulence and impact viral replication (Yu et al., 2013). EDIII is a globular structure located at the C-terminal end of the E protein. EDIII is connected to the two stem helices and two transmembrane helices. It exists in a β-barrel shape which further consists of six anti-parallel β-strands (β1 to β6) (Hanna et al., 2005). EDIII interacts with the potent neutralizing antibodies with the linear antigenic epitopes present on it (Matsui et al., 2010). These epitopes act as the target cell receptor-binding sites which are used for the entry of the virus into the host cells (Roehrig et al., 2013). Most of the receptors present on the target cells are made of heparan sulfates, carbohydrate receptors and ribosomal protein (Huerta et al., 2014). Among all the four serotypes, EDIII is the domain that has the highest variability and this leads to the production of antibodies that are highly specific and have high neutralizing ability (Crill and Roehrig, 2001; Pierson et al., 2009; Wahala et al., 2009).



Zhang, Xingcui, et al. Viruses 9.11 (2017): 338.

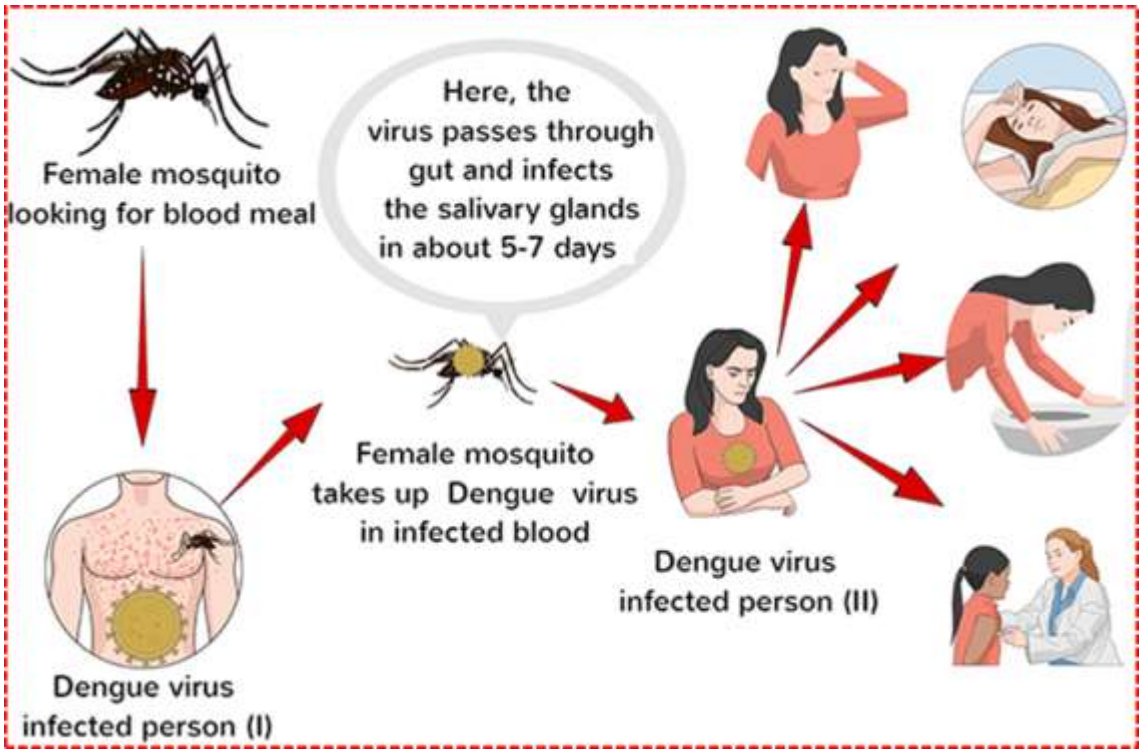
Figure 2. Structure of E protein.

Figure showing three distinct domains include EDI, EDII and EDIII and helix transmembrane domains.

1.3 Mode of Transmission

Transmission of dengue infection from mosquito to human occurs when a mosquito feeds on a dengue-infected person. The infected person can either be asymptomatic or symptomatic. The transmission can occur as early as two days before the infected person starts showing any symptoms and can be transmitted even two days after the symptoms have been resolved. With high viremia and fever in patient, the risk of mosquito infection also increases. Contrary to this, with a high-level of antibodies specific to DENV, the risk of mosquito infection decreases (Nguyen et al., 2013). While most people are viremic only for 4 to 5 days, viremia can last for 12 days (Gubler et al., 1981). Once the mosquito has ingested the virus along with the blood, the virus replicates in the midgut first and spreads to the salivary glands (Figure 3). The extrinsic incubation period (EIP) is the time starting from the virus ingestion to the transmission of the virus to a new host and it lasts from 8 to 12 days at temperatures between 25 to 28°C (Cassetti and Thomas, 2014; Tjaden et al., 2013). Factors such as temperature, viral genotype (Anderson and Rico-Hesse, 2006) and initial genome concentration (Duong et al., 2015) influence EIP. Once a mosquito has been infected it can transmit the virus for the rest of its life. Although most evidence suggests that mosquito vectors are the key mode for transmission of DENV between humans, some

studies suggest that DENV can be transmitted from a pregnant mother to her child leading to fetal distress, pre-term birth and low birthweight (Bingham et al., 2010).



Irfan A. Rather et al; Front. Cell. Infect. Microbiol. 7 (2017): 336

Figure 3. Mode of transmission of dengue virus.

Figure showing transmission of dengue virus by *Aedes mosquito*.

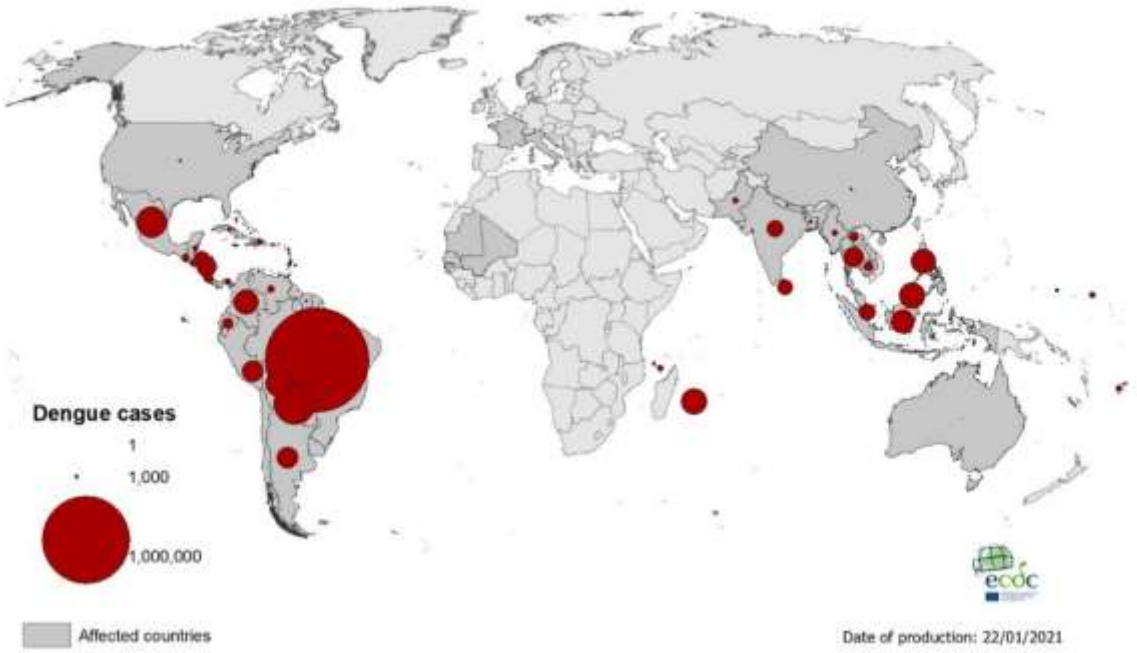
1.4 Disease Burden and Epidemiology

Dengue is a common viral infection prevalent worldwide, which has shown an increase in the spread as well as epidemic incidences over the past few decades (Bhatt et al., 2013; Guzman and Harris, 2015). The incidence of dengue is expanding at an alarming rate and has become a global burden with an estimated 390 million infections occurring annually out of which 96 million develop pathologies (Bhatt et al., 2013) and nearly 2 million are severe dengue (Woon et al., 2018). Around 21,000 deaths are caused annually due to dengue (WHO, 2012). Before World War II, DENV was distributed globally where most regions had only one or two serotypes and epidemics were reported infrequently. During World War II, the transport of troops and war materials led to the prominent spread of DENV. In many

Asian countries, it was seen that all four serotypes were co-circulating, thus causing a hyperendemic (Lambrechts et al., 2010).

1.4.1 **Global burden of Dengue**

Dengue is an epidemic in 128 countries including parts of the globe like Central America, South America and Southeast Asia, Africa, and other developing countries (Figure 4). Around 87% of the population in Southeast Asian countries is at risk of dengue infection (Mutheneni et al., 2017). In Asian countries, children between the age of 5 to 15 years are primarily affected, and in the tropical parts of America, people of age 19 to 40 are affected (Gubler, 2011). Children living in endemic areas have the highest incidence rate and in non-endemic areas incidence rate is high in young adults. While most people recover from dengue infection, the main reason for hospitalization and death is dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) (Sharp et al., 2017).



https://www.ecdc.europa.eu/

Figure 4. Global distribution of Dengue cases reported in 2020.

1.4.2 **Dengue Prevalence in India**

India witnessed the first ever case of dengue in the year 1946 (P.V. Karamchandani, 1946). In the last few decades, dengue has spread to peri-urban and rural areas as well and is not just limited to urban areas. This led to an endemic in all states of the country and is one of the leading causes of hospitalization in many cities (Chakravarti et al., 2012). The first dengue epidemic was observed in the eastern coast of India in 1963 (Carey et al., 1966), followed by Delhi in 1967 (Gupta et al., 2012) Kanpur in 1968 (Chaturvedi et al., 1970) and then it reached the southern part of the country. Each time a dengue epidemic hit the country it was by a different serotype. Kanpur was first hit by DENV 4 in 1968, and then by DENV 2 and DENV 4 in 1969. Vellore was hit by DENV 3 during an epidemic in 1966. In India, the first epidemic was reported in 1963 in the eastern coastal part of the country after which it was reported in Delhi in 1967 and Kanpur in 1968 which then further spread to the southern part of the country. During each epidemic, India was exposed to a different serotype leading to the spread of all the four serotypes of dengue in the country (Gupta et al., 2012). From 1998 to 2009, the number of dengue cases increased by 2.6-fold. One of the reasons for the rapid spread of dengue in India is the reduction in EIP. EIP determines the transmission of dengue virus and is mainly affected by temperature. When mosquitoes are in high yet tolerable temperatures, the dengue virus replicates rapidly, which leads to shortening of EIP (McLean et al., 1974). Heavy rainfall and humidity are common in India which also contributes to the rapid spread of dengue as these conditions are suitable for *Aedes aegypti* breeding (Mutheneni et al., 2017). Eventually, this leads to an increase in vector population.

1.5 Diagnosis

Detection of DENV is key towards implementation of appropriate treatment strategy for the patients. The viral antigens, genomic RNA, or the antibodies induced are the crucial macromloeclues used for the diagnosis of DENV in the infected patients. Antigens such as non-structural protein 1 (NS1), which is released from the cells that are infected can be detected early in the bloodstream through enzyme-linked immunosorbent assay (ELISA). There are several commercial diagnostic tests available based on detection of viral antigens. For simultaneous detection of NS1, immunoglobulin M (IgM), and IgG, a 3-in-1 test is

available. Serological tests like ELISA are not only easy to perform but also are cost-effective for dengue detection. Unprecedented efforts are being made to develop new diagnostic procedures which have increased the efficacy, affordability, scalability that might help in rapid and early detection of dengue and improve disease surveillance and vector control.

1.5.1 **Detection of viral RNA**

The isolation of virus had been the traditional method of detecting dengue infection, however it has been replaced by reverse transcription PCR (RT-PCR) and ELISA for rapid diagnosis. RT-PCR has been used to detect viral RNA and is being used for fast and inexpensive diagnosis of dengue. The virus is isolated from the serum or plasma of patients followed by cDNA synthesis by reverse transcription reaction and amplification by polymerase chain reaction (PCR). Several protocols for PCR have been developed depending upon the genomic sequence targeted. Some protocols use primers directed to the serotype-specific regions of the genome in single reaction or use primers for consensus region of virus followed by another amplification termed as nested PCR with serotype specific primers. The Nested PCR helps improve the sensitivity and specificity of the detection procedure (Guzmán and Kourí, 2004; Johnson et al., 2005; Wu et al., 2001).

1.5.2 **Antigen detection**

Recently there have been new developments in a technique called ELISA that targets antigens of the virus. NS1 antigen of DENV is considered as the ideal target for diagnosis. It has been seen that NS1 protein circulates in high concentrations in patients with primary and secondary infections up to 9 days after the onset of illness (Muller et al., 2017). NS1 antigen can be detected before antibody response is generated, thus helps in early detection. The commercial development of NS1 capture ELISA and immunochromatographic rapid strip tests have revolutionized dengue diagnosis. Another way to detect the presence of dengue infections is by using tissue sections to visualize dengue NS1 antigen. This is done by using labelled mAbs specific to DENV NS1 that is visualized with markers such as fluorescent dyes, enzymes. This technology is not extensively used in dengue endemic countries (Guzmán and Kourí, 2004; Jessie et al., 2004).

1.5.3 Serological tests

When a person is infected with dengue, the acquired immune response involves the production of immunoglobulins like IgM, IgG and IgA that are specific for the virus E protein. There are various approaches to serological diagnosis like hemagglutination inhibition (HI) assay, western blotting, and IgM, IgG capture ELISAs. HI assay along with capture ELISA for IgM and IgG is routinely used serological test for the detection of DENV (Muller et al., 2017). The IgM antibody is first isotype to appear in primary infection and IgG is not detectable in acute phase and peaks slowly after one week. However, IgG responses increase rapidly in secondary infection and are detectable in acute phase of disease and on the other hand IgM responses appear late. Thus the serological tests done parallelley for IgM and IgG provide an indication for a primary or secondary infection by comparing levels of both immunoglobulins during acute phase of disease (Hunsperger et al., 2009; Muller et al., 2017).

1.6 Clinical Manifestations of Dengue Virus

Dengue infection can vary from mild illness lasting for about one week to severe symptomatic conditions that requires medical attention and may lead to death if neglected. According to WHO the clinical manifestations of dengue are classified as mild febrile illness dengue fever (DF), and severe conditions like DSS and DHF. DHF/DSS is clinical condition that requires hospitalization, and it is characterized by features such as plasma leakage, thrombocytopenia, etc. which together makes it life threatening (Narvaez et al., 2011).

1.6.1 Dengue Fever

Dengue fever is mostly encountered in older children and adults. The onset of symptoms begins with a high-grade fever which lasts from three to seven days. Headache (mainly retrobulbar, behind the globe of the eye), nausea, vomiting, muscle, joint pain, rash, generalized myalgias, and arthralgias. During the initial febrile period, 50 to 80% of patients report the first cutaneous rash which is a result of capillary dilation. During the acute febrile infection period facial, neck and chest flushing is seen followed by maculopapular

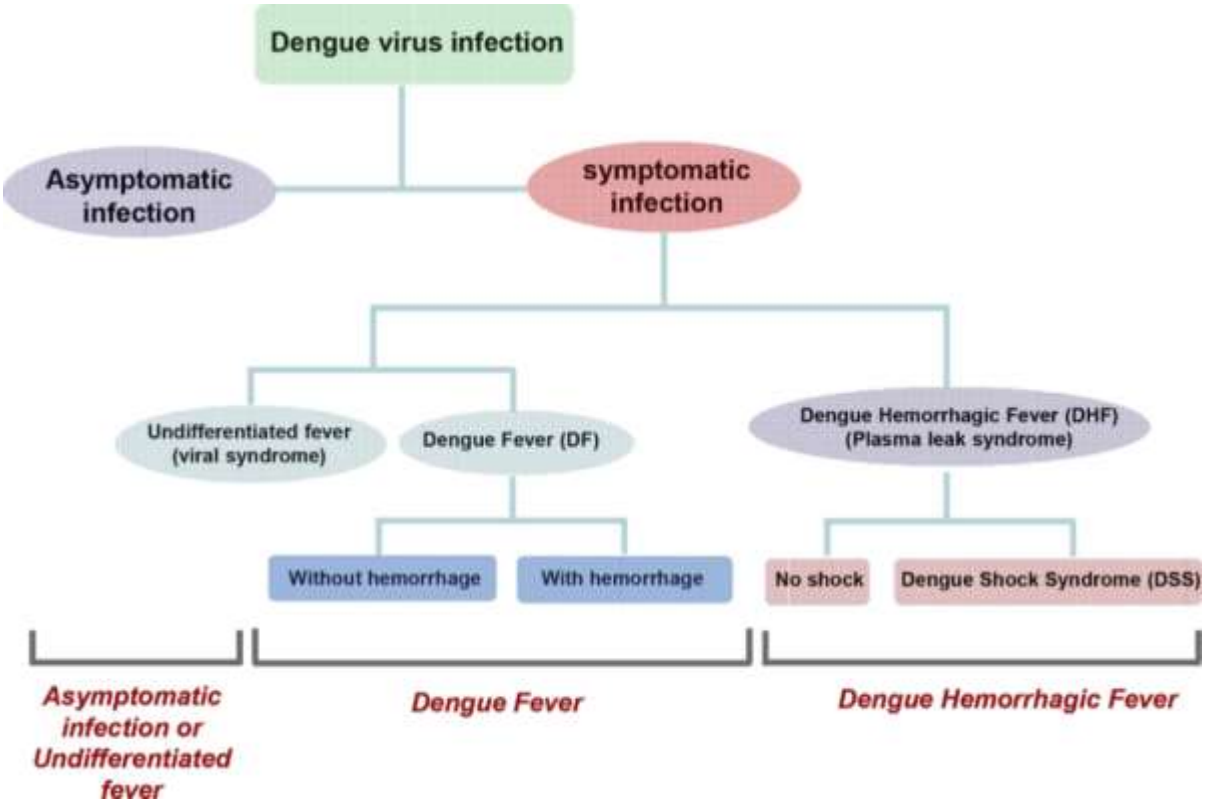
eruptions. These individual lesions merge as erythematous areas with bleeding spots and sparing (Rigau-Pérez et al., 1998).

1.6.2 **Dengue Hemorrhagic Fever**

DF most commonly manifests into DHF and primarily occurs in children under the age of 15 years. It is associated with multifunctional pathogenesis that includes hemorrhagic episodes, deficiency and dysfunction of platelets which lead to defects in blood coagulation pathways. Blood vessels become fragile due to dysfunctional platelets resulting in hemorrhage. Hemorrhagic manifestations take place during the time three to seven days of illness when the fever subsides and the viral load increases. The increase in vascular permeability and abnormal homeostasis results in the loss of plasma volume (Jayashree et al., 2011; Narvaez et al., 2011; Rigau-Pérez et al., 1998; White et al., 2007).

1.6.3 **Dengue Shock Syndrome**

DSS is characterized by DHF symptoms along with narrow and unstable pulse pressure (<20 mm Hg), restlessness, extensively worsening shock, and multiorgan damage (Patey et al., 1993). The hypovolemic shock occurs as a results of plasma volume loss (Jayashree et al., 2011). The high mortality rate associated with DSS is mostly through spread of intravascular coagulation and the patient may die within 12 to 24 hrs of going into shock or recover rapidly with volume replacement therapy. The prognosis of DSS depends on its early detection and prevention to avoid death.



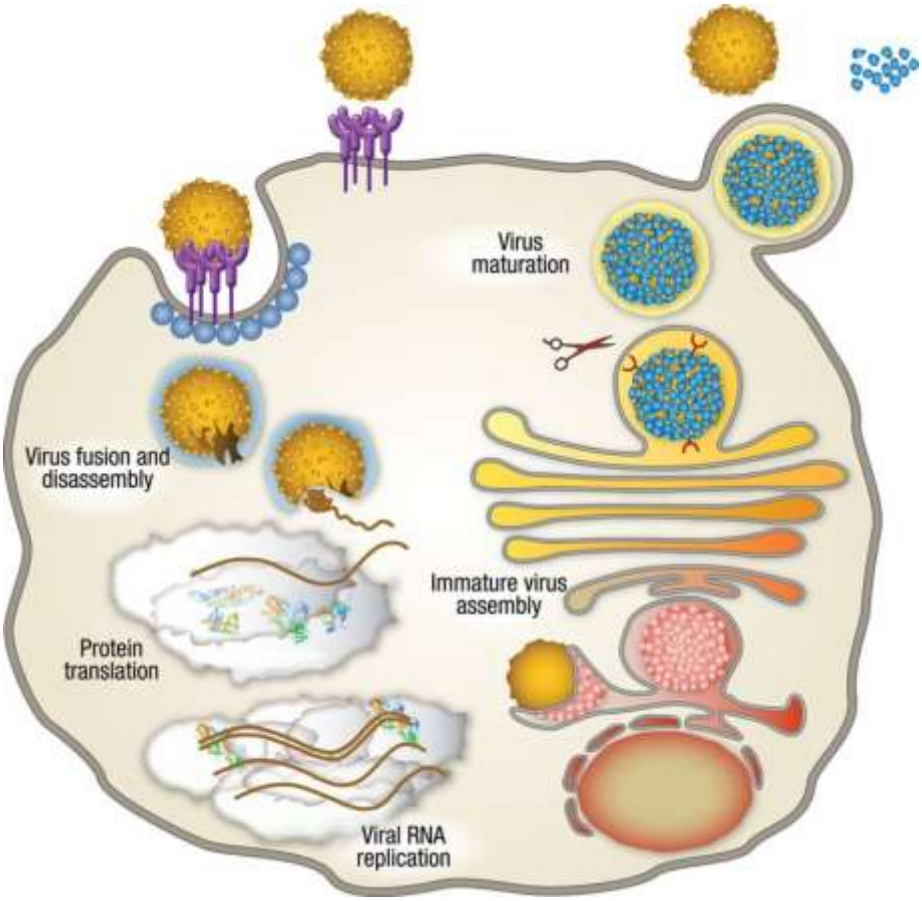
Wang, Wen-Hung, et al. Journal of Microbiology, Immunology, and Infection 53.6 (2020): 963-978.

Figure 5. Classification of dengue infection and its clinical presentation.
Flowchart showing the classification of clinical manifestations of dengue.

1.7 Dengue Virus Life Cycle and Host Immune Responses

Dengue infection begins when the vector takes a blood meal, and the virus is introduced into the host. The virus enters the host cell by clathrin receptor-mediated endocytosis (Freitas et al., 2020). The acidic environment of the late endosomes induces fusion leading to the uncoating of the viral RNA. The viral RNA is translated in association with ER (Salonen et al., 2005). After translation, the proteins are processed into three structural and seven non-structural proteins with the help of viral and cellular proteases (Lindenbach and Rice, 2003). The NS5 protein is a viral replicase that associates with NS2B3 protease and forms an antisense intermediate (-ssRNA). The -ssRNA and +ssRNA together form dsRNA which acts as a template for the replication of positive sense RNA (Clyde and Harris, 2006). After translation and replication, all the viral components get assembled and transported to the

Golgi where they form mature virions. The mature virions are released out of the cells through exocytosis (Figure 6) (Mukhopadhyay et al., 2005).



Laughlin, Catherine A., *et al. The Journal of infectious diseases* 206.7 (2012): 1121-1127.

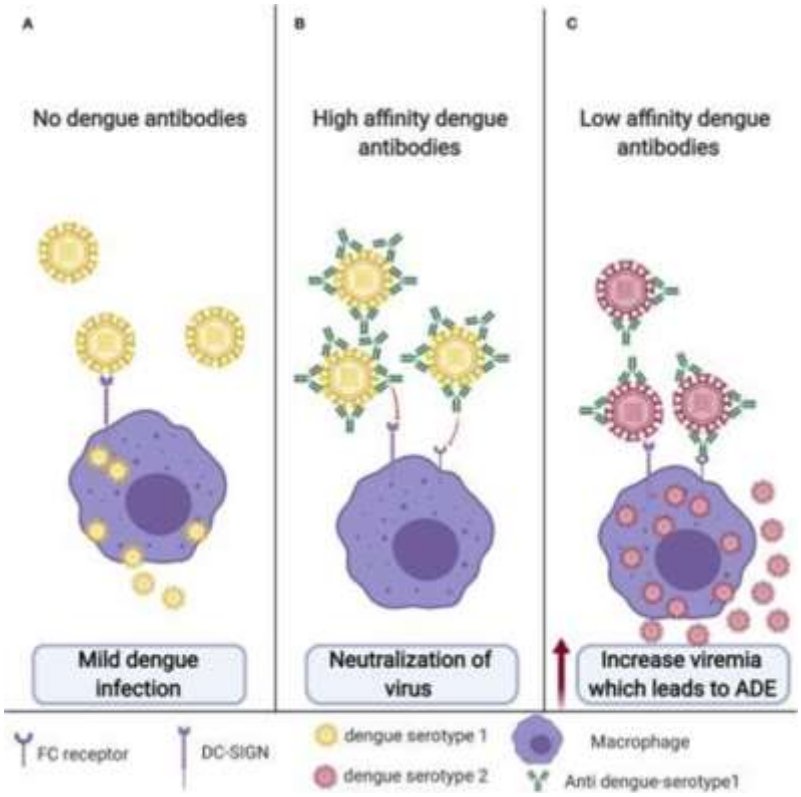
Figure 6. Dengue replication cycle.

The image depicts the DENV genome structure, replication, transcription, and translation process that occurs in the host cell. *Original Image Richard Kuhn Purdue university.*

1.7.1 **Pathogenesis**

When an individual is exposed to any of the four dengue virus serotypes for the first time, it is known as primary dengue infection. These infections can either be symptomatic or asymptomatic. During primary infection, IgM appears in three to five days and IgG appears in six to ten days after the onset of infection. While IgM disappears in two to three months

after the onset of infection while IgG persists for longer period (Maria G Guzman et al., 2010). Primary infection with a particular serotype provides immunity against that serotype and not against the other serotypes. The secondary infection with a DENV serotype which is previously encountered results in classical DF only. However, around 2 to 3% of the secondary infections result in DHF and progress to DSS and death. On the other hand, when an individual encounters DENV for the second time but with a different serotype, a low number of heterotypic antibodies promote an increase in viral load and severity of the disease. This phenomenon is known as antibody-dependent enhancement (ADE) (Figure 7). It should be noted that all the severe cases are not due to secondary infection and not all secondary infections result in DHF or DSS. Cross-reactive memory T cells also play an essential role in either protective immunity or causing immunopathology (Wan et al., 2013). After the infected mosquito bites, dengue virus enters the host body and replicates within the mononuclear phagocyte lineage cell which includes monocytes, macrophages, and B cells. In addition, the virus is known to infect mast cells, dendritic cells and endothelial cells. The incubation period lasts for 7 to 10 days and the immune responses including the antibody, cytokine, and T cell vary depending of the clinical manifestation.



Izmirly, Abdullah M.*, et al. Frontiers in Immunology* 11 (2020): 1055.

Figure 7. Mechanism of Antibody-dependent enhancement elicited during heterotypic Dengue Infection.

Figure showing primary infection in absence of neutralizing antibodies (A) in presence of same serotype neutralizing antibodies (B) and in presence of cross-reactive antibodies from previous infection (different serotype).

1.7.2 **Innate immune responses**

Innate immunity is the first line dense system of the body, which is triggered in response to pathogenic invasion. However, dysregulation in the innate immune activation has been implicated in the disease pathologies of many diseases including dengue. Growing evidences categorically manifest between a direct link between dengue induced disease severity and a phenomenon known as a "cytokine storm". Several studies have shown that the cytokine storm is triggered because of exorbitant production of pro-inflammatory cytokines. Apart from cytokine storm there are other factors such as continuous activation of immune cells, breakdown of endothelial barrier which leads to the virus spread to the

bloodstream that are associated with the manifestation of clinical severity. A major reason for clinical DHS/DSS is the rapid production of cytokines and chemokines such as IL-1β, IL-2, IL-6, IL-8, IL-10 and tumor necrosis factor-α (TNF-α). Interferons like IFN-α and IFN-γ and chemokines such as CCL2, CCL3, CCL4, CCL5, CCL8 together are implicated in disease severity. Immune cells like monocytes, macrophages, hepatocytes, endothelial cells, B cells and T cells also contribute in causing severe dengue (Costa et al., 2013a). Several studies have emphasized the significance of the activation of inflammasomes in the regulation of DENV infection. Inflammasome is a component of innate immune system, is a multimeric protein complexes that are formed in the cytosol after detecting pathogen associated molecular patterns (PAMP). Activation of inflammasomes in response to PAMP leads to an autocleavage process where pro-caspase-1 is activated to caspase-1 (Lin et al., 2002). Inflammasomes also play an important role in the regulation of proinflammatory cytokines IL-1β and IL-18 and subsequently induce programmed cell death called pyroptosis. Dengue infected patients show a variety of clinical manifestations that include sudden onset of fever, headache, muscle, and joint pain. DENV triggers high levels of endogenous pyrogens such as IL-1β and TNF-α which could be one of the meditators responsible for the onset of high fever observed in the DENV infected patients.

It has been reported that in severe dengue infections, hyperactivation of complement system plays an important role in the pathogenesis of DENV (Shresta, 2012). During DSS condition, high levels of terminal complement protein C5b-9 and anaphylatoxin C3a, and C5a have been reported in the plasma of patients with severe dengue suggesting that there is an association between complement activation and severe dengue conditions (Kraivong et al., 2021). Thus, complement activation could play an important role in the pathophysiology of severe dengue.

1.7.3 **Adaptive immune responses**

Adaptive immunity is another crucial arm of host defense system which work hand in hand with the components of innate system, which is the first line defense that shapes the quality, type and effector commitment of adaptive immune response. After a host is infected with DENV, the viral ssRNA and dsRNA act as a pathogen associated molecular patterns PAMP are recognized by innate sensor such as pattern recognition receptors including Toll-like

receptors (TLRs). It is well documented that TLR7 recognizes the single-stranded RNA, whileTLR3 recognizes dsRNA (Cumberworth et al., 2017; Tremblay et al., 2019; Uno and Ross, 2018). The involvement of these receptors with its counterpart ligand induces the type 1 interferon (IFN) response (Howell, 2013; Orr et al., 2014). Such recognition leads to further activation of innate immune transcription factors such as IRF3, IRF7, and NF-κB. These transcription factors leads to the transcription and secretion of IFN-α and IFN-β (Cumberworth et al., 2017; Morrison et al., 2003; Tremblay et al., 2019). Type 1 interferon plays an important part in a wide range of clinical symptoms such as fever, fatigue, chills, and headache (Sleijfer et al., 2005). After the primary infection, memory T cells for both serotype-specific and serotype cross-reactive cells are formed (Sierra et al., 2002). CD8+ T cells respond against different types of viral proteins including NS3 (Mathew et al., 1996). The role of CD4+ T cells when encountered by dengue viral antigens is to produce IFN-γ, TNF-α, and TNF-β, this may contribute to the pathogenesis of secondary dengue infections (Gagnon et al., 1999). Studies suggest that during dengue infections CD4+ T cell clones can destroy non-antigen presenting cells such as hepatocytes thus, causing liver injury. IFN-γ increases DENV infection of human monocytes by up-regulating the formation of Fc gamma receptors on them (Kurane et al., 1989). In dengue infections, the number of CD4+ and CD8+ T cells are lowest on the day when the fever subsides and tends to increase thereafter (Manh et al., 2020). Apart from these immunological attributes of DENV pathogenesis, adaptive memory of previous infection plays and important role for severe form of dengue pathogenesis.

The probability of an individual to be infected with dengue is multiple in the lifetime and each time individual displayed different clinical and immunological features which distinctive to the dengue virus infection. Remarkably, secondary and subsequent viral infection and pathogenesis always involves different viral serotypes than the previous infection, thanks to the homotypic immune protection against the primary strain. However, immune responses against secondary virus infection are different as it occurs in the context of pre-existing immunity against the previous strain which is cross-reactive. Memory B and T cells generated by the previous infection to a different virus serotype could respond more intensely than naïve cell during the acute subsequent infection. Nevertheless, due to the sequence dissimilarities between the virus serotypes, re-activated memory T and B cell

during secondary infection may not confers the optimal affinity and avidity to the corresponding epitopes of the secondary infecting virus. This variation in the immune response driven by the memory of previous infection is popularly known as “original antigen sin” (Rothman, 2011).

Secondary dengue virus infection alters the kinetics and specificity of immune response due the preexisting memory against the cross antigen which drive the unique pattern of antibody response during secondary infection in which titer of antibodies against the previous strains are higher as compared to currently infecting strain (Halstead et al., 1983; Midgley et al., 2011). These cross-reactive antibodies are incapable of neutralizing the currently infecting strains rather it enhances the infection because it facilitates the virus entry into the cells which is known as ADE (Mongkolsapaya et al., 2003). The increased viral burden in the immune cells due to ADE may produce excess cytokines, leading to “cytokines storm” (Rothman, 2011). After an individual encounters DENV for the first time (Primary dengue infection), antibodies are formed against both structural and non-structural viral proteins. Of all the non-structural viral proteins, antibodies against NS1 have been shown to induce caspase dependent endothelial cell apoptosis (Rivino et al., 2013). Once the IgG antibody binds the antigen, different subclasses of IgG in varying capacities help in the activation of the classical complement pathway. High levels of IgG1 and IG4 dengue virus specific antibodies are seen in patients with DHF and DSS (Koraka et al., 2001; Thein et al., 1993). Complement activation may contribute to increased vascular permeability and coagulation abnormalities. Hence DENV specific IgG subclass plays a crucial role in the pathogenesis of severe disease (Koraka et al., 2003). In patients with DHF and DSS the total IgE antibody levels and dengue specific IgE antibodies are high (Míguez-Burbano et al., 1999).

1.8 Prevention and Control of Dengue

As dengue is the most rapidly spreading vector-borne viral disease, its prevention and control has become extremely necessary. Detailed vector control strategies and treatment options currently available are discussed below.

1.8.1 Vector control

Due to lack of dengue antiviral therapy and a successful vaccine, at present mosquito control is the one of the important strategies employed to contain the spread of the virus.

Several measures are being made worldwide to control the spread of vector. Government across globe has been extensively campaigning on reducing the contaminated water sources. This effort has been proving successful, although it is labor intensive and time consuming. The use of organochloride insecticides, organophosphorus larvicides, and aerosols mostly applied outdoors as ultra-low volume concentrates are widely used in controlling vector (Newton and Reiter, 1992). Aerosols have been recommended to be used for emergency vector control during epidemics. However, the major drawback of aerosols usage is that they have a very limited impact on the adult *A. aegypti* and it is not effective in the immature stages of the mosquito (Gubler, 1998). When compared to the effects of outdoor usage, indoor usage of aerosols is effective but is very labor intensive and intrusive. Although the use of several vector control strategies is helpful, each strategy has several drawbacks, proving that these strategies for vector control have limited effectiveness.

1.8.2 **Treatment**

To date, there is no effective antiviral drug available that can eradicate dengue. The treatment strategy is mainly management of patients suffering from different stages and severity of dengue (World Health Organization (WHO), 2016). In case of DF, patients are advised to take primary care including rest, intake of oral fluids to compensate body fluid loss due to diarrhoea and vomiting. Acetaminophen which is an analgesic and antipyretic is also given to control high fever. Non-Steroidal drugs like aspirin should not be used for patients with DF because they may impair the platelet function and aggravate the bleeding tendency which is commonly seen in DENV infection (Rigau-Pérez et al., 1998). The patient's health is monitored through various blood tests from fever day 3 onwards till the condition improves. Tests include monitoring of blood pressure, hematocrit, platelet count, urinary output and hemorrhagic manifestations. Some of the clinical signs that signal progression to serious disease include low pulse, low urine output, cold limb extremities, mucosal bleeding, and abdominal pain. DHF is indicated when there is a rise in hematocrit ($\geq$20%) and a fall in platelet count (>100,000/mm^3). In any such cases, immediate hospitalization is necessary. DHF patients are supplemented with intravenous fluids like plasma expanders, and isotonic solutions (e.g., Ringer's lactate, dextran-40, plasma protein fraction etc.) to compensate huge loss of plasma. Depending on the patient's hematocrit,

urinary output data and blood pressure, intravenous fluids are adjusted to avoid excessive fluid intake as it might cause heart failure, massive effusions and respiratory disorders. In severe cases such as internal hemorrhage, whole blood transfusion may be carried out (Rigau-Pérez et al., 1998).

1.9 Current Status of Dengue Vaccine Development

As there are no effective antiviral therapies and vector control is the only method to limit the dengue infection. However, the vector control alone is not able to reduce the infection satisfactorily; therefore there is a need for a safe and efficacious vaccine that can protect against all the four serotypes of DENV. The major challenge in vaccine development is the lack of animal models, unclear understanding of immunopathogenesis, ADE effect and genomic alterations that occur over time also pose a challenge in vaccine development (Izmirly et al., 2020; Wilder-Smith, 2020). Despite these challenges, there are batteries of vaccines developed by scientists over the globe that have moved from the developmental stage to different phases of clinical trials.

1.9.1 Vaccines currently in Clinical Trials

D1ME100

This vaccine is made of a monovalent DNA plasmid that codes for prM and E proteins from DENV 1 serotype. Although the vaccine was safe, but high doses of D1ME100 vaccine were required for vaccine to be effective (Beckett et al., 2011).

TVDV VAX

TVDV VAX is a tetravalent DNA vaccine that is made by combining four monovalent DNA plasmids that encode for prM and E genes from the four serotypes. The tetravalent DNA in combination with Vaxfectin which acts as an adjuvant elicits a strong immune response. TVDV VAX showed better immune response than D1ME100 in the pre-clinical trials (Porter et al., 2012). Although TVDV VAX was well-tolerated in Phase I trials, its immunogenicity was low as tetravalent antibody response was seen in only 10% of the participants (Danko et al., 2018). During the clinical trials it was seen that TVDV VAX induced intracellular antigen processing for activating adaptive immunity, however, it showed low immunogenicity and booster doses were needed.

V180

A vaccine made by using the recombinant forms of DENV envelope glycoprotein was initially developed by Hawaii Biotech. The vaccine was named DEN-80. This vaccine product is being developed by Merck while including ISCOMATRIX as an adjuvant in the vaccine formulation (Clements et al., 2010). Although the V180 vaccine showed a good immunogenic response even with low amount of vaccine antigens, the drawback is that the immunogenicity of the vaccine depends on the adjuvants and that the vaccine needs to be given in three doses which is not very convenient (Mamo and Poland, 2012).

TV003/TV005

TV003/TV005 vaccine is also known as TetraVax-DV. This is a live-attenuated tetravalent vaccine made by National Institute of Health, US. The vaccine was made by using DENV 1 and 4 serotype wild strains and then deleting nucleotides 30/31 from the 3' untranslated region and components of DENV 2 and 3 were constructed from DENV 4 backbone (Whitehead et al., 2003). Although TetraVax-DV produced encouraging results in phase II clinical trials, its long-term protection data is not yet available (Torres-Flores et al., 2022).

1.9.2 **Licensed dengue vaccine**

After extensive studies on dengue vaccine development for decades, a vaccine CYD-TDV for dengue has been licensed. The brand name for this vaccine is Dengvaxia which has been made by Sanofi-Pasteur. Dengvaxia was first released in Mexico in December 2015 and is now released in over 20 dengue endemic countries after being licensed. This vaccine can be given to individuals of 9 to 45 years of age only. CYD-TDV is a live-attenuated tetravalent vaccine. The tetravalent nature of this vaccine is meant to provide immunity against all four serotypes. CYD-TDV vaccine is made by using the backbone of the yellow fever virus vaccine in which all four chimeric viruses are incorporated (Chambers et al., 1999; Guy et al., 2011) .The following are the limitations of Dengvaxia that draw special attention to the fact that a safer and versatile dengue vaccine is needed which are : (a) Dengvaxia cannot be given to children below the age of 9 years. (b) In dengue naïve individuals, Dengvaxia increases the risk of developing severe dengue infection. (c) Testing of serostatus before administration of Dengvaxia is difficult and too costly for many endemic countries (Bos et al., 2018). The main challenge is to obtain a balanced replication of four live vaccine

components. As these vaccines are inactivated or attenuated pathogens, there is a risk of reversion in immune-compromised people and pose as a potential health hazard (Belshe et al., 2004). It is also difficult to control the quantity and trafficking of individual vaccine antigens in a multicomponent live-attenuated vaccine. Moreover it is also difficult to control factors that have an impact on immunogenicity like the particle maturation state, cryptic epitope exposure and temperature-dependent virion dynamics. Therefore, these challenges highlight the need for finding an alternate strategy for the delivery of multicomponent DENV vaccines.

1.9.3 **New generation vaccines: Subunit Vaccines**

Over the years, a lot of effort has been taken for research on development of noninfectious, non-replicating subunit vaccines. Protein subunit vaccines are non-replicating vaccines, and they eliminate the risk of reversion. They are suitable for people with compromised immune systems and are relatively stable. Another advantage of using subunit vaccines is the low cost when compared to the cost of the inactivated vaccines. DENV EDIII has been considered as a potential vaccine candidate in case of DENV (Chen et al., 2007; Fahimi et al., 2014).

1.9.4 **Dengue EDIII as subunit vaccine**

Several DENV viral proteins have been extensively explored, particularly the immune-dominant E protein that has been identified as a potential vaccine candidate. The E protein consists of three structurally and functionally distinct, EDI, EDII and EDIII domains. EDIII is exposed and accessible on the virion surface, thus plays a crucial role in host cell recognition and attachment. This domain also plays an important role in inducing long-lasting protective immunity against DENV infections. Antibodies against EDI and EDII are weakly neutralizing and tend to cross neutralize other Flaviviruses (Oliphant et al., 2006) leading to ADE. Thus, choosing only EDIII as a vaccine candidate and not the entire E protein helps in avoiding the risk of ADE as there are no other cross-reactive and non-neutralizing epitopes present. There are vaccine studies in which EDIII has been used in various formulations (monovalent, bivalent, tetravalent) and have been tested on mice and non-human primate animal models (Fahimi et al., 2018). EDIII proteins expressed in *E. coli* have been used for developing tetravalent dengue vaccine candidates and it has been

reported that a tetravalent formulation of four EDIII fusion protein, EDIII from DENV (1-4) elicits very high titers of neutralizing antibodies against all four serotypes (Simmons et al., 2001). EDIII subunits in the tetravalent formulations have the capability to induce neutralizing antibodies that are serotype-specific (Henchal and Putnak, 1990). However, it is important to note that these antibody titers fall quickly leading to a demand for multiple booster doses in a short period of time. Although subunit vaccine offers enormous benefits including safety as they are non-replicating and non-infectious but they exhibits weak immunogenicity. To overcome the limitation of subunits vaccine a few ways have been employed to enhance their immunogenicity including the use of adjuvants and delivery vehicles.

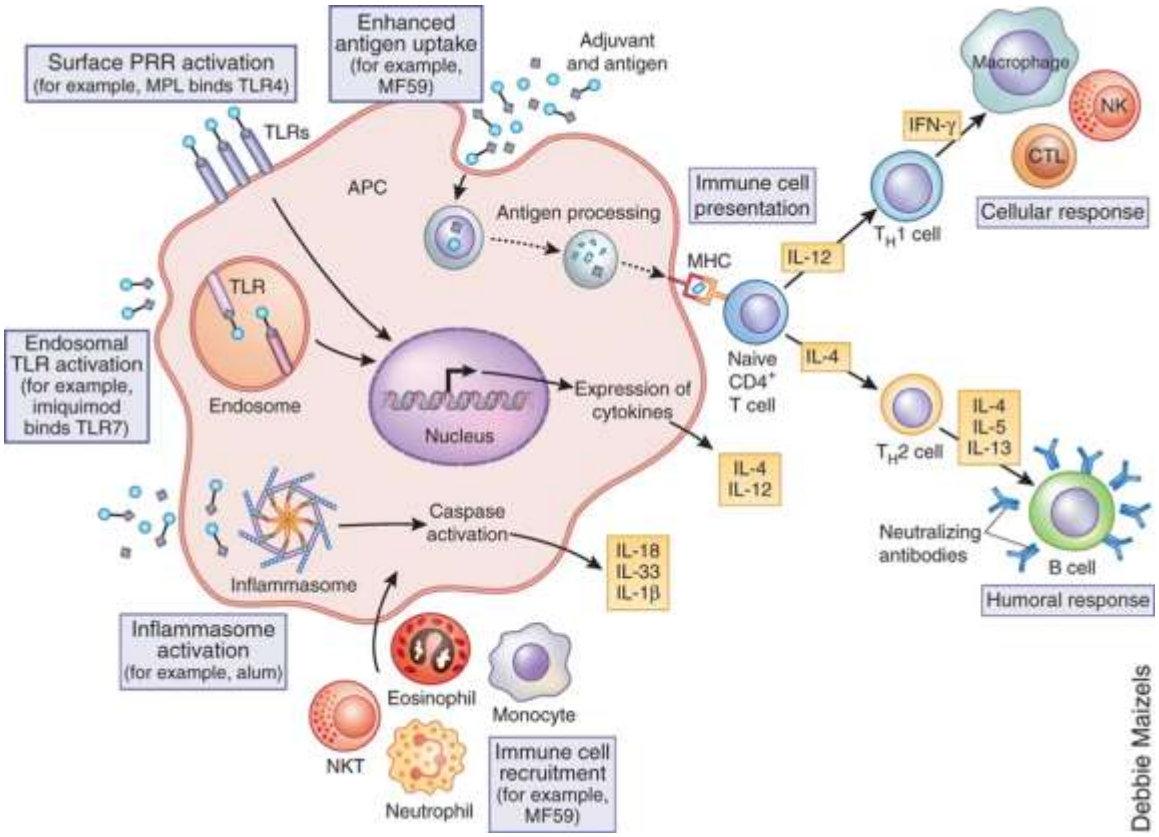
1.9.5 **Role of Adjuvants in Vaccine formulations**

Adjuvants act as immune-stimulators and are an important component in vaccine formulations. They can activate immune-recognition pathways and sustain robust immune responses to microorganisms for long durations (Steinman, 2008; Zinkernagel et al., 1997). As discussed earlier, subunit vaccines are poorly immunogenic in the absence of adjuvants. The following roles are played by adjuvants when added in vaccine formulations: (1) They allow vaccine to produce immunity for specific pathogens (Edelman, 1980). (2) They also prolong the antibody response and reduce the number of vaccine doses required (Coffman et al., 2010). (3) Adjuvants improve efficacy in neonates and geriatrics causing increased seroconversion and sero-protection rates (Tan et al., 2018). (4) They also facilitate the uptake of pathogens by the mucosal epithelia (Srivastava et al., 2015). These properties of adjuvants make them a candidate of interest in development of a wide range of vaccine formulations. Adjuvants can also control the rate at which the antigen is released into the bloodstream by a mechanism called depot effect. This effect is seen when the antigen is trapped in a polymer along with the antigen.

1.9.5.1 **Mechanism of action of Adjuvants**

There are several mechanisms through which adjuvants mediate their activity when used in vaccine formulations. Most of the adjuvants act as ligands by binding to pattern recognition receptors and activating the innate immune response. This signaling leads to the production of cytokines and chemokines that induces a Th1 and Th2 immune response as well as

influences the immune cells at the injection site. Some adjuvants mediate their mechanism of action by activation of inflammasomes leading to proinflammatory cytokine production. The other mechanism of action of some adjuvants is influencing antigen presentation by major histocompatibility complex (Figure 8).



Reed, Steven G., Mark T. Orr, and Christopher B. Nature medicine 19.12 (2013): 1597-1608.

Figure 8. Mechanisms of action of adjuvants.

Figure showing different mechanisms through which adjuvants carry out their functions of enhancing immune responses of given antigens.

1.9.5.2 **Various Vaccine adjuvants**

Aluminum-based Adjuvants:

Most widely used adjuvant in veterinary and human vaccine formulations. It has been confirmed as safe and effective Th2 immune stimulator for preventing infections such as malaria and has been approved in the market (Vogel and Powell, 1995). Despite this, the adjuvant has several limitations such as aluminium being toxic when used in high concentrations (Eldred et al., 2006) causing adjuvant arthritis, sterile abscess, neurotoxicity and allergenicity (Allison and Byars, 1991). With these limitations, aluminium adjuvants also show poor induction of mucosal immunity and Th1 immunity (Gupta et al., 1995).

Freund's adjuvant:

Freund's adjuvant is the most commonly used adjuvant. There are two types of Freund's adjuvant; Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA). FCA consists of heat-killed *Mycobacterium tuberculosis* (M. tb) in an emulsion of water and nonmetabolizable oils like paraffin and mannide monooleate. The antigenic property of heat-killed M. tb is responsible for attracting macrophages and other cells to the injection site (Coffman et al., 2010). FICA does not contain heat-killed M. tb. It is used to produce water-in-oil emulsions of antigens. It stimulates T helper type 2 cells. It is less toxic because it does not contain M. tb. Freund's adjuvants are useful for antigens that are difficult to obtain, smaller molecular weight, and are weakly immunogenic. However, the disadvantage of FCA is that it may lead to disease development in immune-compromised individuals. Whereas the disadvantage of FICA is the difficulty in preparation of vaccine formulation and poor immunomodulatory effects (Sivakumar et al., 2011).

1.9.5.3 **TLR ligands as adjuvants**

Based on the emerging evidence it is seen that TLRs play an important role in inducing a persistent antibody response by activating dendritic cells (DC) (Kawai and Akira, 2010). TLRs belong to type I membrane glycoproteins (Jin and Lee, 2008). They have an extracellular domain made of Leucine Rich Repeats for PAMP recognition and an intracellular cytoplasmic homology domain Toll/interleukin-1 receptor which is involved in downstream signaling (Kawai and Akira, 2010). TLR agonists are promising adjuvants used

in vaccine development as they are predominantly expressed on the membranes of innate immune cells. The TLR agonists used as adjuvants signal through their cognate TLRs to induce an effective immune response leading to long-lasting protection.

Poly-IC: The synthetic dsRNA molecule polyinosinic-polycytidylic acid (poly-IC) acts on TLR3 and induces both innate and adaptive response. It also increases seroconversion, increased antibody titter, and induces CD4 and CD8 T cells (Sabbatini et al., 2012).

MPL: Monophosphoryl lipid (MPL) is an adjuvant that stimulates TLR-4, it leads to the production of proinflammatory cytokines such as TNF-α and IL-6. It is also known for inducing Th1 immune response through enhanced IFN-γ production by antigen-specific CD4 T cells.

Entolimod: It is flagellin derived recombinant protein which has been pharmacologically optimized. It is used in cancer therapy in combination with radiotherapy and has been approved by Food and Drug Administration (FDA).

CpG adjuvants: CpG oligonucleotides bind to and activate TLR 9. When used as vaccine adjuvants improve function of antigen presenting cells and boosts humoral and cellular immune responses that are specific to the vaccine (Overstreet et al., 2010). However, several studies have indicated that there is increased pain, pruritus, erythema induced by vaccines that have CpG adjuvants.

Imiquimod: It is also known as R837 adjuvant, resembles nucleoside analog binds to TLR7 and induces secretion of proinflammatory cytokines IFN-α, TNF-α and IL-12. Imiquimod is known for its use as vaccine adjuvant (Patinote et al., 2020).

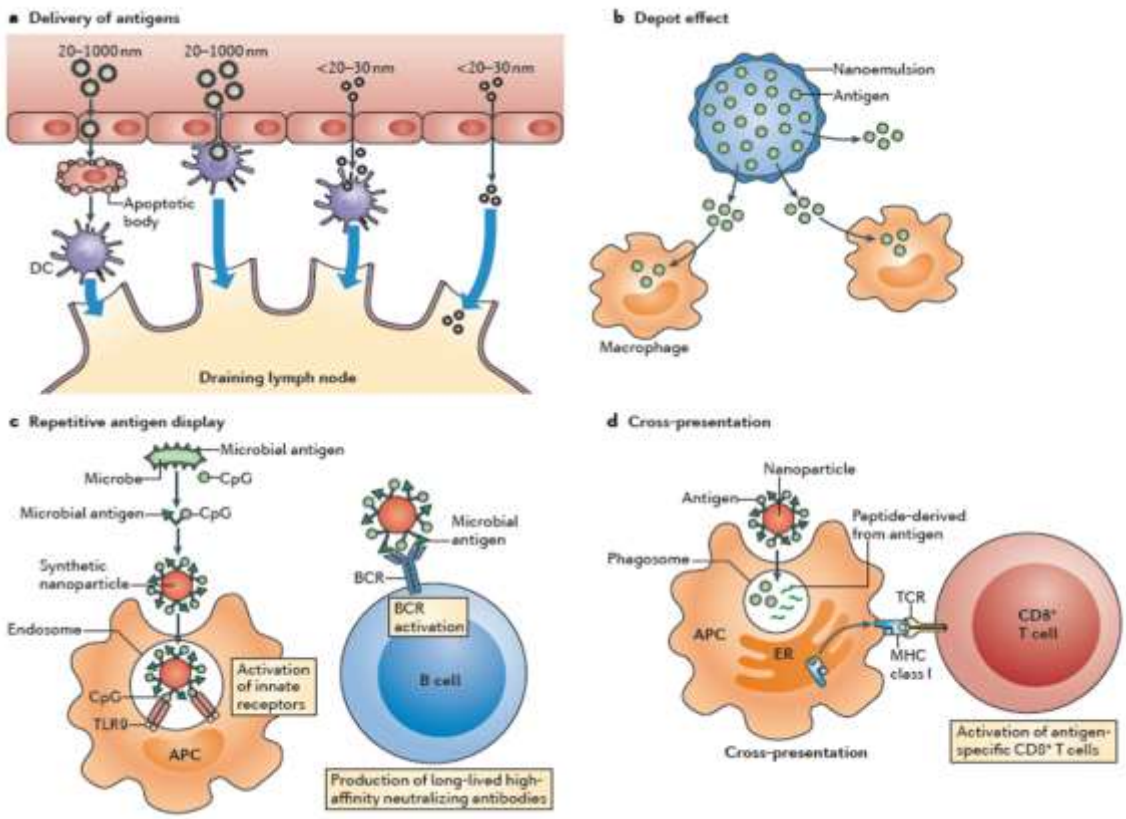
Combination of TLR ligands as adjuvants: It is of importance to note that although all TLR ligands can induce primary antibody responses, a stimulus caused by a combination of TLRs enhances the Germinal entre pathway of memory B cell formation and plasma cell responses that are long lived. Based on a study, TLR ligands MPL (TLR4) and R837 (TLR7) when loaded on poly D, L-lactide-co-glycolide (PLGA) nanoformulation containing antigen induce a synergistic response of increased antigen-specific neutralizing antibodies as compared to immunization with a single TLR ligand. PLGA (MPL+R837) showed an enhanced secretion of proinflammatory cytokines by DC *in vitro* when compared to the

response induced by a single ligand (Kasturi et al., 2011). Therefore, combination of TLR agonists can be used as vaccine adjuvants as they produce synergistic immunomodulatory effects.

1.9.6 **Role of Nano based Delivery Systems in Vaccinology**

To suffice the challenges of vaccine antigen delivery, nanotechnology holds a promising platform. Extensive application of nanotechnology in vaccinology has led to the development of "Nanovaccinology". Several candidate nanoparticles (NPs) are being employed to suffice the problem of delivery and adjuvanting. In general, nanoparticles have a size range of 1 to 1000 nm. They are as small as pathogens, making it easy for the immune cells to recognize them. This property of NPs makes them a great candidate for vaccine carriers (Zhao et al., 2014). They also have the potential to activate the immune system. NPs can also be used as adjuvants and have been included in several vaccine formulations. They have been reported to enhance the stability of antigens and help in targeted antigen delivery. They are also known for aiding in a very slow release of antigens which in turn excludes the need for booster doses. Several types of NPs have been used in designing subunit vaccines such as polysaccharides, liposomes, immune-stimulating complexes (ISCOM) and inorganic NPs.

Mechanism of action of nanoparticles: Nanoparticles alter the induction of immune response by various mechanisms. One mechanism by which nanoparticles induce immune response is through the depot effect during which nanoparticles release the antigen gradually and persistently. Another mechanism is through repetitive display of antigen to the B cell receptor, thus co-aggregation and activation the B cell. Nanoparticles also activate the innate immune response by slow leakage of antigens into the cytosol where they are taken up by phagosomes and activating MHC class I pathway through cross-presentation which leads to release of chemokines, cytokines that regulate the innate immune response (Figure 9).



Smith, Douglas M., Jakub K. Simon, and James R. Baker Jr. Nature Reviews Immunology 13.8 (2013): 592-605.

Figure 9. Mechanisms through which nanoparticles enhance immunogenicity of antigens.

Nanoparticles mediate their action through the following mechanisms: a) Delivery of antigens, b) Depot effect, c) Repetitive antigen display and, d) Cross-presentation.

1.9.6.1 Commonly used Nanodelivery systems in Vaccinology

Metallic Nanoparticles (MeNPs): Metallic nanoparticles (MeNPs) are non-biodegradable and have rigid structure. Several strategies have included MeNPs as vaccine platforms involving different materials such as gold, nickel, and iron oxide. Many metals have been studied for their immunostimulatory properties (Hofmann-Amtenbrink et al., 2015). The impact of characteristics such as material composition, size, shape, hydrophobicity and surface charge on vaccine immune response (Di Pasquale et al., 2015). The major drawback of MeNPs is the involvement of toxic chemicals, thus making it difficult to synthesize.

These lead to production of reactive oxygen species (ROS) which has the potential to cause oxidative stress, inflammation, and damage to proteins, cell membranes and DNA predominantly leads to toxicity (Fard et al., 2015; Fu et al., 2014).

Organic Nanoparticles Polysaccharides: Polysaccharides are natural polymers whose carbohydrate monomers are linked to each other by glycosidic bonds. The property of polysaccharides makes them suitable to be used in nano vaccine development as they are biocompatible and biodegradable. The chitosan present in polysaccharides is known to function as adjuvants because of their ability to induce antigen-specific immune responses (Mbow et al., 2010). Chitosan is FDA approved in the pharmaceutical and food industries but has low solubility (Singla and Chawla, 2001). To overcome this disadvantage, other forms of chitosan like trimethyl chitosan and alginate has been used in vaccine preparation.

Liposomes: Liposomes are biomolecules, and they fall under the category of synthetic nanoparticles. The formation of liposomes begins when certain amphiphilic lipids disperse and self-assemble in an aqueous solution (Bangham, 1972). Depending on the application the size and charge of liposomes can be modified to suit the purpose. In vaccine development liposomes have the ability to provide a slow and controlled release of the antigen. The versatile nature of liposomes makes it easy to cross the mucosal and skin barriers and reach the targeted site for antigen release. There has been a development in methods to produce liposomes which include lyophilization, cryoprotection and sterilization. These methods help in improving their stability and make it a reality usable for furthermore vaccine development (Mohammed et al., 2006).

Virus like particles (VLPs): Virus like particles are nanoscale structures that are made up of viral proteins but lack genetic material and are therefore non-infectious (Bai et al., 2008). VLPs are produced by plants, mammals, bacteria, and insects in dispersed form. They are exploited as carriers for the delivery of drugs, vaccines in the cavity within their structure (Chung et al., 2020; Steinmetz, 2010). VLPs can be generated in the laboratory using recombinant viral proteins that are expressed in a range of experimental systems that include yeast, prokaryotic cells, plant and mammalian cells (Mohsen et al., 2018; Shiri et al., 2020). Structural proteins of several viruses such as Hepatitis B virus, Hepatitis C virus, and Human immunodeficiency virus have been used to produce VLPs. VLPs are most widely

exploited for their application in vaccine development (Cimica and Galarza, 2017; Donaldson et al., 2018). VLPs have a size and shape similar to native viruses, these structures can efficiently elicit immune responses and because the VLPs lack viral genome there is no risk of replication within the target cells and offers improved safety (Balke and Zeltins, 2019). VLPs can stimulate both humoral and cellular immune responses (Lee et al., 2018; Wang et al., 2017). However, low protein yield, high production cost, and long expression time are some of the major disadvantages of VLPs.

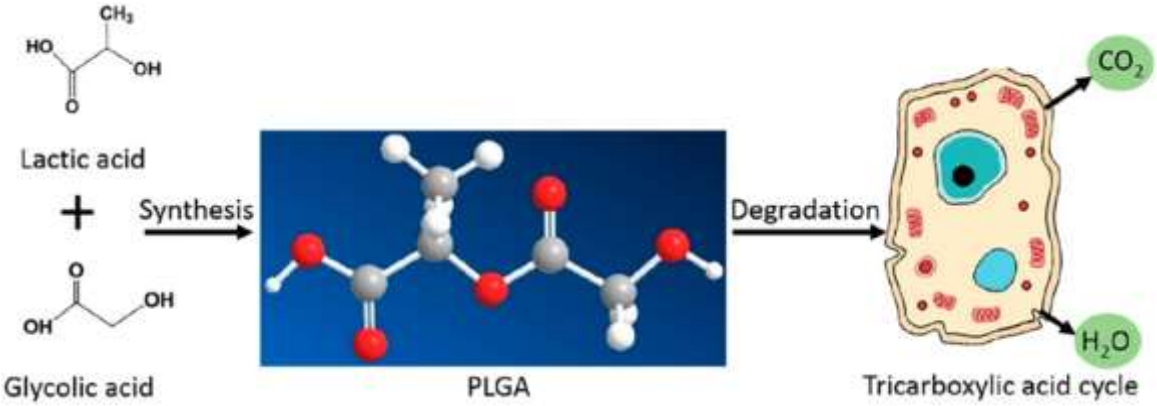
Exosomes: Exosomes are extracellular vesicles which come under a size range of 30-150 nm. They are secreted from living cells and are involved in intracellular communication using signaling molecules such as proteins, lipids, DNA, and RNA (Aheget et al., 2020; Ludwig and Giebel, 2012). Exosomes play an important role in triggering an immune response against pathogens (Schorey and Harding, 2016). However, the disadvantages of using exosomes in vaccine delivery are short half-life, sensitivity to sterilization, and low stability in blood circulation (Elsharkasy et al., 2020; Wang et al., 2021).

ISCOM: ISCOM are composed of cholesterol, phospholipids, saponins and antigens (Lovgren and Morein, 1988). They appear like a hollow cage with a diameter up to 40 nm (Kersten et al., 1991). ISCOM are used as antigen carrier systems. ISCOMs have been found to be more immunogenic than liposomes because of the presence of an in-built immunopotentiator Quil A in them (Sanders et al., 2005). To produce excellent quality vaccines the heterogeneous mixture of ISCOM components is separated and purified using reverse phase HPLC which eliminates any toxic elements present during vaccine preparations (Daming and Wenbin, 2016). Use of ISCOMs in vaccine formulations still requires detailed study and standardized procedures to ensure vaccine preparations with consistency of quality in each batch.

Inorganic Nanoparticles: The use of inorganic NPs as adjuvants in vaccine development has been of interest for several years. A wide range of nanoparticles are included in inorganic nanoparticles such as elemental metals, metal oxides and metal salts. Gold NPs is an example of inorganic NPs. It has an antigen conjugating property which will induce carrier-specific immunity after immunization. These nanoparticles also induce cellular response and IFN-γ secreting CD4+ cell proliferation (Wang et al., 2018).

1.9.6.2 PLGA as Nanovaccine delivery Vehicle

Polymer-based nanoparticles have a small particle size enabling them to cross the biological barrier (Vela-Ramirez et al., 2015). This property of nanoparticles makes it easy to cross the biological barrier. The unique property of synthetic polymers is their biocompatibility and their versatile chemical nature (Figure 10). The versatile nature of synthetic polymers makes them easy to modify their size, composition, surface charge, surface property (Pawar et al., 2013). The versatility of these NPs also results in achieving a controlled antigen release system. One such example of a polymer NP is PLGA. PLGA is FDA approved and biodegradable, thus making it suitable for use as drug delivery systems in humans (Jain et al., 2011). The flexible nature of PLGA makes it easier to modify the outer surface by incorporating polymers such as chitosan and molecular markers such as TLRs for effective vaccine delivery. Another advantage of loading antigens into the PLGA NPs is that they prevent antigen degradation and help in controlled antigen release. It has been reported that PLGA NPs improve the immunogenicity of vaccines and induce a potent and long-lasting immune response.



Divesha Essa et al; Front. Bioeng. Biotechnol. (2020):48

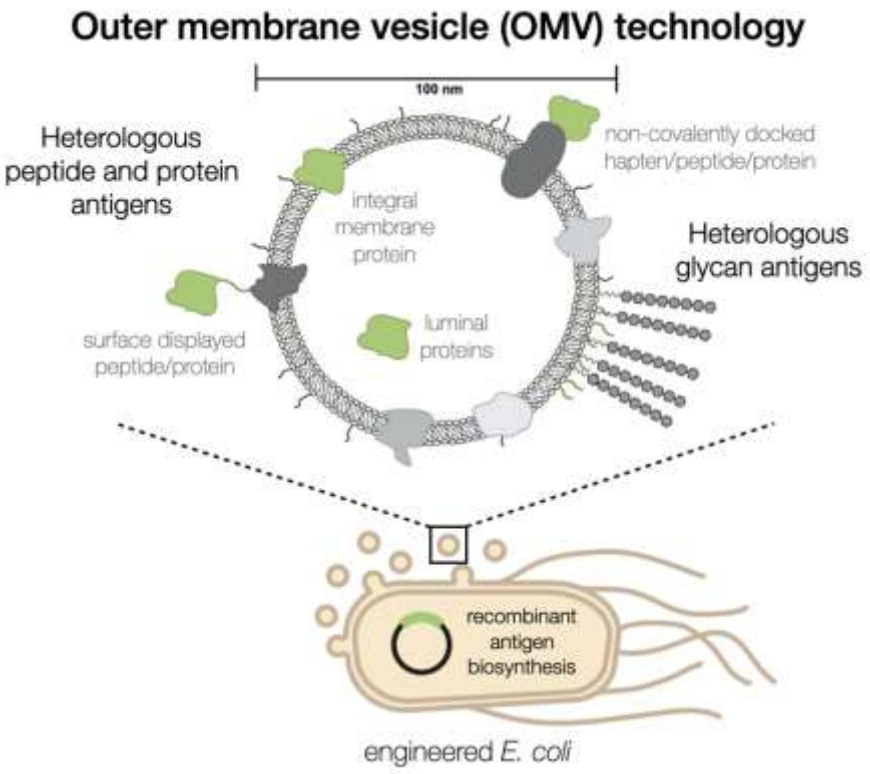
Figure 10. Synthesis and degradation of PLGA.

Figure showing synthesis and degradation of PLGA nanoparticles.

1.9.6.3 Outer Membrane Vesicles as a Vaccine Delivery and adjuvanting Platform

Outer membrane vesicles (OMVs) are released by Gram-negative bacteria during growth in vitro and in vivo (Schwechheimer and Kuehn, 2015). These bacterial nanoparticles are

bilayered and spherical nanoparticles containing many components on its external surface(Mashburn-Warren and Whiteley, 2006). OMVs are a promising candidate for vaccine delivery when compared to other delivery agents available. OMVs are known to have functions such as delivery to target locations, they also protect molecules from degradation. They also help in collecting nutrients, transfer of toxins and virulence factors (Bonnington and Kuehn, 2014; Horstman and Kuehn, 2000). OMVs have a wide variety of bacterial antigens on their surface, they have the ideal size thus making it easy to be up taken by immune cells. In addition to this OMVs have immunopotentiators such as TLR agonists which brings about the self-adjuvanticity nature of OMVs. This property of OMVs makes them strong activators of innate immune response (Dowling et al., 2016). In addition, various studies also emphasize on OMVs ability to activate the humoral and cellular immune responses (Bottero et al., 2016; Tan et al., 2018). The long-lasting immune response and ease in production of OMVs make them a promising candidate for vaccine development. Over the past few decades, scientists have highlighted the above benefits of using OMVs as a tool for vaccines as well as drug delivery. Thus, OMVs are a unique and powerful platform to display native antigens and provide simultaneous defined adjuvant activity. OMV-based vaccines are approved for use in humans, most notably the formulation approved in the European Union and by the FDA (Bexsero®, Novartis) to prevent infection of Neisseria meningitides serogroup B. Recently the ability of engineered strains of *E. coli Nissle* 1917 to generate recombinant OMVs (rOMVs) that induce a TH1-biased response (Rappazzo et al., 2016; Watkins et al., 2017) and protect in an influenza vaccine-challenge model has been demonstrated.



Dr. Matt Delisa, Locally Sourced Science. 2020

Figure 11. Outer Membrane Vesicles.

Diagram of OMVs displaying expressed antigens. *Note: Figure adapted from [Antibuddies](#) Science Communication & Immunology 2021*

To overcome the ongoing problems of antigen delivery and adjuvant supplementation using OMVs might help. OMV producing E. coli can be engineered to express a wide variety of heterologous proteins which are not inherently expressed by the host bacteria. Engineered OMVs are made based on a simple technique where the protein of interest is fused to a membrane protein anchor which then co-localizes this fused protein to the outer membrane to generate rOMVs displaying antigen of interest (Figure 11). One such example of a transmembrane protein is cytolysin A (Cly A). Cly A is a pore-forming cytotoxin found in enterobacteria such as *E. Coli.* Cly A is highly enriched in the outer membranes of OMVs. This nature of the OMVs can be exploited by generating fusion proteins between candidate antigens and Cly A (Rappazzo et al., 2016; Watkins et al., 2017). Thus, making rOMVs a

unique and powerful tool to display native antigens and simultaneously provide an adjuvant property in vaccine development.

1.10 Rationale

Dengue is expanding at an alarming rate and has become a global burden with an estimated 396 million infections annually and 96 million cases of severe infection. The enormous rise in dengue cases could be attributed to rapid urbanization, climatic changes including global warming, lack of proper animal models, and unavailability of antivirals and efficacious vaccines which could elicits a balanced tetravalent response is adding to the owes of growing infection. Availability of some of the most successful vaccines against other Flaviviruses such as Japanese encephalitis and yellow fever to which dengue belong gives a hope that vaccination might be an effective tool in the fight against DENV too. Over the last three decades, tremendous efforts have been made to develop a potent vaccine that would induces a robust and long-lasting correlates of protective immunity against all the four serotypes of DENV but until now no such vaccine has been made that can potentially mount protective immunity against all the serotypes. Although a live attenuated tetravalent vaccine "Dengvaxia" have been has been licensed for its application in a few countries against DENV, however, its efficacy is questionable. It has been reported that live attenuated Dengvaxia vaccine failed to provide balanced protection for all the four serotypes of the virus and is not recommended for seronegative individuals. The persistent challenges with live-attenuated tetravalent vaccines have led researchers to explore non-replicating, non-infectious subunit vaccine preparations. Several DENV proteins including both structural and non-structural proteins have been extensively explored and E protein particularly grabbed attention due to its immunodominance. As discussed above the E protein has three domains (EDI, EDII and EDIII) and out of all domains EDIII is known for its role in host cell infectivity and capability to produce neutralizing antibodies. However, when EDIII antigen is administered alone, exhibits weak immunogenicity and requires multiple boosters and adjuvants. To enhance the immunogenicity of EDIII domain, various strategies are being tried that include use of multiple adjuvants, antigen delivery system and a combination of both. To suffice the problem of antigen delivery and adjuvanting nanotechnology in vaccine have gained significant interest which has led to the advent of

Nanovaccinology. It has been well documented that application of nanoparticles enhance the magnitude and persistence of antigen-specific immune responses by modulating antigenic release pattern, activating the innate immunity, and targeting the antigen to antigen presenting cells (APCs), thereby suggesting that involvement of nanoparticle will result in development of novel nano-vaccine formulations. Considering this, we hypothesized that using immuno-dominant EDIII antigens from all the four serotypes of DENV, encapsulated in PLGA nanoparticles and in another setup displaying these antigens in nano OMVs would trigger a strong immune response that can neutralize the dengue virus completely and provide a long-lasting memory. Therefore, to test our hypothesis we have framed the following objectives.

2 Objectives

Objective 1

Expression, Purification, and immunological characterization of DENV EDIII of all the serotypes of DENV (1-4)

Objective 2

Developing a DENV EDIII based tetravalent nano-vaccine formulation using: (a) PLGA; (b) rOMV delivery systems followed by its biophysical characterizations and immunological screening upon immunization in mouse model system

Objective 3

Dissecting the potency of the EDIII based tetravalent nano-vaccine formulation in terms of neutralizing capability against all the serotypes of DENV (1-4)

3. MATERIALS AND METHODS

3 Materials and Methods

3.1 Cells, Chemicals and Reagents

The human monocyte leukaemia cell line THP1 (American Type Culture Collection, Rockville, Md. USA) was cultured in RPMI 1640 culture medium (Gibco, Life Technologies, USA) augmented with 10% fetal bovine serum (FBS) (Invitrogen, UK) and 1% penicillin/streptomycin (P/S) (Invitrogen, UK) at 37°C and 5% CO 2 in ahumidified incubator. Lipopolysacharides (LPS) (E.coli 0111:B4) (Sigma Aldrich, Merk Germany), Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich), Proteinase K, Isopropyl lβ-D-1-thiogalactopyranoside (IPTG) (G.Biosciences, USA), Caspase-1 inhibitor Ac-YVAD- CHO (Calbiochem- Merck Germany), N-acetyl cysteine (NAC), Polymyxin B (Sigma Aldrich, USA), Monophosphoryl Lipid A (MPL) (Avanti Polar lipids Albama), Imiquimod (R837) (Invivogen Toulouse France), PLGA (50:50) (Sigma Aldrich, Merk Germany), kanamycin, chloramphenicol (Himedia India), Lysozyme, DNase, phenylmethylsulfonyl fluoride (PMSF) (G.Biosciences Geno Technology USA).

3.2 Macrophage Differentiation

The THP-1 monocytes were differentiated by treating THP-1 cells (10^6 cells/well) for 24 hrs with 50 ng/ml PMA in a 12-well cell culture plate with 1 ml cell suspension in each well. It is well reported that the PMA-induced THP-1 cell differentiation results in the expression of certain macrophage associated surface markers, CD11b and CD36, and these differentiated cells demonstrate phagocytic activity as well (Chanput et al., 2010). Differentiated, adherent cells were washed with sterilized phosphate-buffered saline (PBS; pH 7.4) and subsequent addition of fresh RPMI 1640 medium without PMA before further treatment and stimulation.

3.3 Expression and Purification of Dengue Envelop Protein Domain III (EDIII)

Dengue EDIII from all the four serotypes of dengue (DENV 1-4) were purified in *E.Coli Rosetta* cells using IMAC as described previously (Puerta-Guardo et al., 2013). The respective clones of EDIII were transformed in *Rosetta* cells and single colonies were picked and inoculated in Luria Bertani (LB) broth with antibiotics kanamycin (50 mg/ml) and chloramphenicol (35 mg/ml) and kept at 37°C overnight with continuous agitation. The

primary culture obtained was used for making glycerol stocks and 1% of it was used for growing secondary cultures for protein purification. Secondary cultures were grown in shaking incubator at 37°C and were induced with 0.5 mM IPTG when OD reached ~0.6. The cultures were allowed to grow for 3.5 hrs after IPTG induction. Finally, cultures were centrifuged at 5000 rpm for 10 min and pellet was resuspended in lysis buffer previously mentioned (Khan et al., 2019) and were sonicated for ~15 min followed by centrifugation at 12000 rpm for 30 min. Supernatants were collected and His tagged EDIIIs were purified using Ni-NTA columns (GE Health Care, USA). Protein was eluted with 300 mM imidazole and dialyzed with appropriate buffer before storage or further use.

3.4 Immunoblotting

Immunoblotting was done to analyze the expression of different proteins. Cells were either left unstimulated or stimulated with the DENV EDIII proteins for 12 hrs, LPS was used as a positive control. Post incubation, adherent cells were washed gently with ice cold 1× PBS. The cells were scraped and lysed in cell lysis buffer (20 mM HEPES (pH 7.5),150 mM NaCl, 1% Triton-X 100, 100 mM NaF, 1 mM EDTA, 17.5 mM β-glycerophosphate, 10% glycerol, 1 mM Phenyl methyl sulphonyl fluoride, 2 mg/ml pepstatin and 4 mg/ml aprotinin), supplemented with protease cocktail inhibitor (Sigma Aldrich, St. Louis, MO, USA) and incubated on ice for 30 min with intermittent vortexing as described earlier (S. Battu et al., 2018). Lysates were later pelleted down at 12,000 rpm for 15 min at 4 °C. Equal number of proteins were loaded and run on SDS-PAGE (12% Tricine gel). Separated proteins on SDS-PAGE were transferred onto the nitrocellulose membrane (Pall Corporation, New York, USA) following electroblotting. 5% skimmed milk powder (Himedia, India) was used for blocking the nitrocellulose membrane for 1 hr, followed by overnight incubation with appropriate primary antibodies at 4 °C. The antibodies used for immunoblotting were rabbit anti-procaspase-1 and goat anti-cleaved caspase-1 (Santa Cruz, USA), rabbit anti-IL-1β (Santa Cruz, California USA), mouse anti-β-actin (Cell SignalingTechnology, USA), mouse anti-NALP3 antibody (Novus Biologicals, USA), rabbit anti-P65 (NFκB) (Cell SignalingTechnology, USA), rabbit anti-Phospho-IKBα, and Total-IKBα (Cell Signaling Technology, USA). The membranes were washed thrice with 1× TBST to remove any non-specifically bound antibodies and were then incubated with Horse

radish peroxidase (HRP) conjugated appropriate secondary antibodies for 1 hr. After the incubation with secondary antibodies, the membrane was again washed thrice to remove non-specifically bound antibodies. Protein bands were finally visualized with femto-ECL-prime chemiluminescent substrate (Geno Biosciences, USA) and chemiluminescence was captured on a Chemidoc (BioRad, California USA).

3.5 Measurement of Mature IL-1β and Cleaved Caspase-1

PMA differentiated THP-1 cells were stimulated with different concentrations of DENV EDIII protein or LPS or were left unstimulated. After the requisite treatment, culture supernatants were harvested and methanol-chloroform precipitation was employed to precipitate the proteins (Khan et al., 2019). Briefly, to 500 µl of the culture supernatant harvested, 500 µl of methanol was added. The mixture was vortexed and then 100 µl of chloroform was added and vortexed, followed by centrifugation at 14,000 rpm for two min. The top aqueous layer was pipetted off and again 500 µl methanol was added, vortexed, and centrifuged at 14,000 rpm for two min. Supernatant was again pipetted off as much as possible without disturbing the pellet. The pellet obtained was then dried by heating at 50°C for five min. Later the pellet was dissolved in 50 µl of 2× sample buffer and then boiled at 95°C for five min. Equal amount of protein was loaded and run on 12% SDS-PAGE (Tris-glycine gel). Separated proteins on SDS-PAGE were transferred onto nitrocellulose membrane (Pall Corporation, USA) following electro blotting. Expression levels of mature IL-1β and cleaved caspase-1 protein were evaluated by immunoblotting as described earlier.

3.6 Subcellular Fractionation for P65 NF-κβ Translocation

Isolation of the nuclear and cytosolic proteins was carried out by differential centrifugation as described earlier (Khan et al., 2007; Wan et al., 2011). Briefly 5×10^6 THP-1 treated cells were pelleted down and resuspended in 200 µl of cold buffer A (10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.4% (vol/vol), NonidetP-40, 0.5 mM PMSF and protease inhibitor cocktail) by pipetting gently. The homogenate was centrifuged for three min at 500 g at 4°C in a microfuge and the supernatant demonstrating the cytosolic fraction was collected. The pellet comprising the nuclei of cells was incubated at 4°C for 10 min in buffer (20 mM HEPES/KOH (pH 7.9), 420 mM NaCl, 1 mM EDTA,

1.5 mM MgCl2, 25% (vol/vol) glycerol 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) and was further incubated for 15 min on ice with brief vortexing every three min. The sample was centrifuged at 13,800 g for 10 min at 4°C; supernatant was collected and examined by Western blotting.

3.7 Confocal Microscopy

Nuclear translocation of P65 was carried out by confocal microscopy. Cells were seeded on coverslips followed by treatments of recombinant protein and LPS for 2 hrs. After the completion of experiment, media was removed, and cells were washed with 1× PBS followed by incubation with primary antibody (anti-P65) at 37°C for 1 hour. Coverslips were washed with 1× PBS and were incubated for 1 hr at 37°C with secondary antibody conjugated with Alexa Red followed by washing with 1× PBS. Finally, the cover slips were mounted on slides using mounting media containing DAPI (Vectashield Invitrogen). Images were taken at 63× in Confocal microscope (Ziess Germany) and were analyzed using LSM browser (Ziess).

3.8 Quantitative Real time PCR (qRT-PCR)

For isolation of RNA in THP-1 cells media was aspirated off after specific treatment with or without recombinant protein, and the cells were washed gently with ice-cold 1× PBS. Cells were then scraped in cold 1× PBS and centrifuged at 1200 rpm for five min at 4°C. Total RNA was isolated from the cells using the Trizol-chloroform extraction protocol as described earlier (Afroz et al., 2016). Briefly, 1 ml Trizol reagent was added to the cell pellet, resuspended, and kept at room temperature (RT) for five min. After the incubation, 200 µl of chloroform was added and the tube was shaken vigorously for 15 sec by hand and was further incubated on ice for 15 min. The samples were centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, aqueous phase of the sample was carefully removed without disturbing the interphase and was transferred into a new tube. In the aqueous phase, 500 µl of isopropanol was added, mixed gently, and was incubated on ice for at least 15 min, followed by another centrifugation at 12,000 rpm for 30 min at 4°C. The supernatant was removed to obtain the RNA pellet, which was resuspended in TE buffer. RNA from lymph nodes (Inguinal, Branchial, and Axillary) of the euthanized mice were also isolated

and a total of 4 μg of RNA was reverse-transcribed into cDNA using Verso cDNA synthesis kit (Thermoscientific, USA) according to the manufacturer's protocol. The cDNA were amplified using Syber-Green master mix (Kappa Biosystems, USA) with gene specific primers (Table 1) using the following thermal cycler parameters: initial cycle at 94°C for two min, followed by 40 cycles of 30 sec at 94°C, 30 sec annealing at 56°C and 40 sec extension at 68°C, in Quant studio five Real time PCR System (Applied Biosystems USA). The relative mRNA expression of each sample was calculated relative to the house-keeping gene GAPDH/βActin as described (Ravindran et al., 2014).

Table 1 List of primers for qRT-PCR

S. No	Gene	Primer Sequence
1	BCl-6 (Mouse)	FP 5' CCGGCACGCTAGTGATGTT 3' RP 5' TGTCTTATGGGCTCTAAACTGCT 3'
2	BCL-2 (Mouse)	FP 5' ATGCCTTTGTGGAACTATATGGC 3' RP 5' GGTATGCACCCAGAGTGATGC 3'
3	BCOR1 (Mouse)	FP 5' ATGCCTTTGTGGAACTATATGGC 3' RP 5' GGTATGCACCCAGAGTGATGC 3'
4	CXCR5 (Mouse)	FP 5' TGGCCTTCTACAGTAACAGCA 3' RP 5' GCATGAATACCGCCTTAAAGGAC 3'
5	ICOS (Mouse)	FP 5' TCCAGCAGTTAAAAATGCGATTG 3' RP 5' ATCCTCCACTAAGGTTCCTTTCT 3'
6	IL-10 (Mouse)	FP 5' AGGATGCACATCAAAAGGCTT 3' RP 5' GGCCTCGGTTAGGAAGGATAC 3'
7	β-ACTIN (Mouse)	FP 5' GTGACGTTGACATCCGTAAAGA 3' RP 5' GCCGGACTCATCGTACTCC 3'
8	IL-1β (Human)	FP: 5' ATGATGGCTTATTACAGTGGCAA 3' RP: 5' GTCGGAGATTCGTAGCTGGA 3'
9	TNF-α (Human)	FP: 5' GAGGCCAAGCCCTGGTATG 3' RP: 5' CGGGCCGATTGATCTCAGC 3'
10	NLRP3 (Human)	FP: 5' GATCTTCGCTGCGATCAACAG 3' RP: 5' CGTGCATTATCTGAACCCCAC 3'
11	GAPDH (Human)	FP: 5' ACAACTTTGGTATCGTGGAAGG 3' RP: 5' GCCATCACGCCACAGTTTC 3'

3.9 ELISA

Human monocytes differentiated with PMA were treated with or without DENV EDIII proteins, LPS was used as a positive control, and cytokines level were quantified in the culture supernatants by ELISA as described earlier (S. Battu et al., 2018), using human

TNF-α, IL-1β, IL-6 ELISA kits (BD OptEIA, BD Biosciences, USA) as per the manufacturer's instructions. Briefly, 96-well poly-vinyl chloride microtiter plates (NUNC maxisorp, Thermoscientific, USA) were coated with the respective capture antibodies diluted in suitable coating buffer and were incubated overnight at 4°C. The plates were washed and blocked with 10% FBS in PBS, followed by incubation with standard or test samples. After washing, plates were incubated with anti-cytokine detection antibodies along with HRP conjugate. Next, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (BD Biosciences, USA) was added, and the plates were incubated at RT in dark, until the development of color. 2 N Sulfuric acid (H_2SO_4) was used to terminate the reaction, and absorbance was read at 450 nm with background correction at 570 nm.

3.10 Estimation of Reactive Oxygen Species

For measurement of ROS levels, 1.5–2×10^6 THP-1 cells were seeded; PMA treated and allowed to differentiate for 24 hrs. Fresh media was added to the cells and treated with LPS or different concentrations of DENV 2 EDIII protein. Following the treatment, cells were washed in 1× PBS, harvested and incubated with 25 μM CM-H2DCFDA (Life Technologies, USA) for 30 min at 37°C in FACS buffer as described earlier (Khan et al., 2006). Later, these cells were washed extensively with Hank's balanced salt solution and resuspended finally in FACS buffer before flow cytometry analysis.

3.11 Preparation of DENV EDIII Loaded Nanoparticle

Nanoparticles were prepared using water-in-oil-in-water (W/O/W) double emulsification method as described previously with slight modification (Kasturi et al., 2011). In-brief, 1 ml of antigen solution (DENV EDIII 2 mg in 1× PBS + 0.5% poly-vinyl alcohol) was added into the 3 ml of 10% PLGA solution in dichloromethane and the resulting solution was homogenized at the speed of (12,000 rpm) for two minutes to obtain primary water-in-oil (W/O) emulsion. Subsequently, the primary emulsion was added into the 20 ml of 5% PVA solution and further homogenized at similar parameters. The obtained double emulsion (W/O/W) was left for 4 hrs at RT to evaporate the solvent. Now nanoparticle was pelleted down by centrifugation at 5000×g for 30 min and washed three times with deionized water.

Finally, the nanoparticle was snapped frozen in the liquid nitrogen and lyophilized (Figure 12).

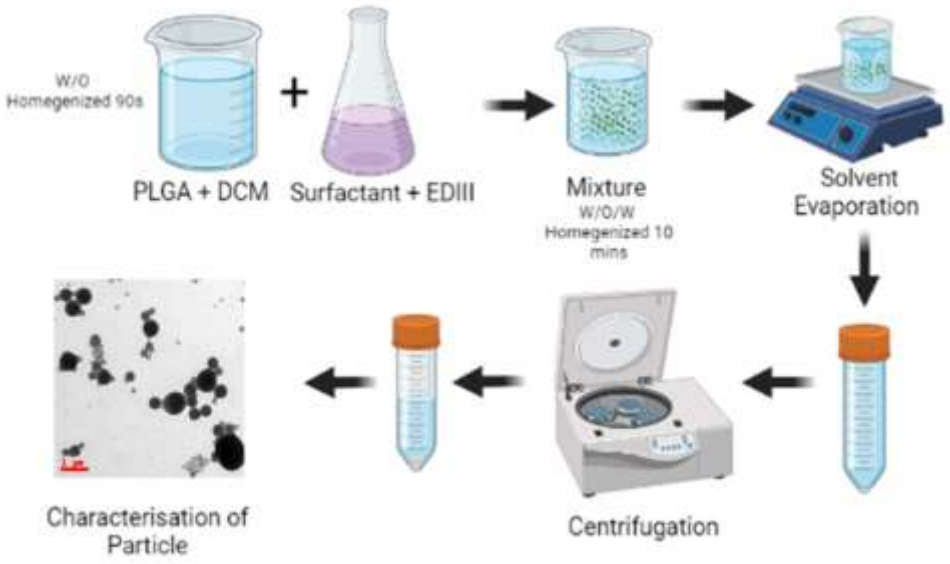


Figure 12. Encapsulation of EDIII in PLGA using w/o/w emulsion.

3.12 Cloning of EDIIIs of DENV Serotypes in PBAD-ClyA Vector for Purification of OMVs

The template for cloning of serotype-specific EDIIIs in pBAD-ClyA vector was obtained from pET28a DENV EDIII constructs. Primers were designed with restriction sites XbaI and HindIII at 5'and 3' ends of EDIII, respectively. The PCR was run for 35 cycles of denaturation (30 sec, 95°C), annealing (30 sec) and extension (25 sec, 72°C) in Thermocycler (Applied Biosystems, USA). The correctly sized amplification products were obtained for all the four serotypes. The inserts obtained from PCR were double digested using XbaI and HindIII restriction enzymes and pBAD-ClyA vector was also digested simultaneously using same pair of restriction digestion enzymes to generate sticky ends. The double digested fragments were retrieved from gel by using Nucleospin gel/PCR cleanup (Machery Nagel). Digested insert was ligated to vector using DNA Ligase (NEB) followed by transformation in *E. coli* DH5-alpha cells. Colonies were selected based on antibiotic resistance and plasmids were isolated from selected colonies. Double digestion using same pair of enzymes (XbaI and HindIII) was performed to confirm the clones (Figure 13).

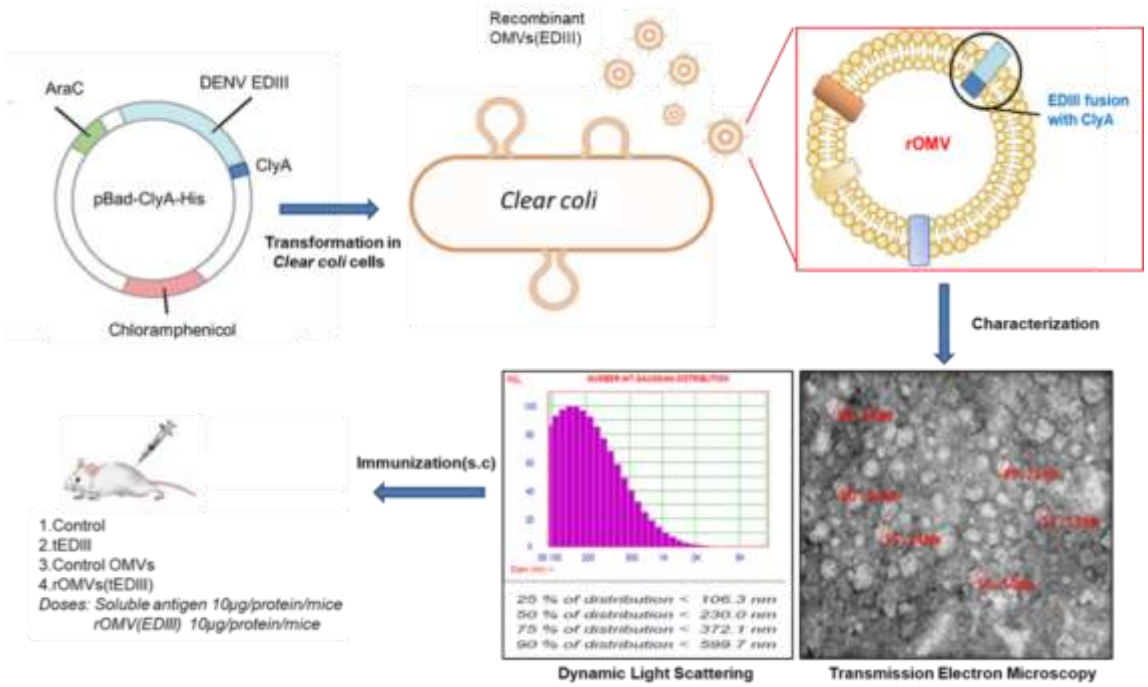


Figure 13. Strategy of expression and purification of rOMV.

3.13 Purification of rOMVs Expressing EDIII

OMVs were purified using the protocol described previously (REF). The clones obtained were transformed into hyper vesiculated *clear coli* cells by heat shock. Single colonies were picked and inoculated into liquid culture (LB broth) containing chloramphenicol (35 µg/ml) and grown at 37°C overnight in a shaking incubator. Next day the 500 ml flasks containing LB broth and chloramphenicol were inoculated with overnight culture and kept in shaking incubator till OD_{600} reached ~0.5 and the expression was induced with L-arabinose (0.2 %) and culture was allowed to grow for 16 hrs followed by centrifugation. Supernatants were collected and filtered using 0.2 µm filter and ultracentrifugation (26000 g for 3-4 hrs) was used to isolate vesicles and were resuspended in 1× PBS and stored in −20°C.

3.14 Physicochemical characterization of DENV EDIII Nanoformulations

PLGA encapsulated EDIII nanoparticles and rOMV expressing EDIII were characterized by transmission electron microscopy (TEM) (TEM-FEI Tecnai), Dynamic Light scattering (DLS) from (Malvern Pan analytical Inc.). For TEM, the samples were suspended water and contrasted with 2% uranyl acetate for two min, dried and placed on a 200-mesh type-B

carbon coated copper grid. The samples were observed under TEM and data were collected and analyzed. Further size distribution of nanoparticles and OMVs in the suspension phase was carried out by DLS. Total protein concentration of OMVs was determined by Bicinchoninic acid assay (BCA) estimation.

Release assay was also performed to check the sustained release of antigens from PLGA NPs. Small quantity of nanoparticles was resuspended/dispersed in PBS buffer and was kept on slight agitation at 37°C. Samples were obtained at the specified time points (0, 6, 12, 24, 48, and 72 hrs) followed by BCA estimation to estimate the amount of protein released from NPs. Encapsulation efficiency was calculated by the indirect method in which protein measured from the supernatant obtained after centrifugation of nanoparticle using BCA method.

Percent Encapsulation efficiency (%EE) was calculated by the following formula.

$$\%EE = Total\ amount\ of\ protein — amount\ of\ protein\ in\ the\ supernatant / Total\ amount\ of\ protein \times 100$$

3.15 Immunization of Mice

Six to eight week-old male Balb/c mice were immunized with various groups: (i) PBS control; (ii) Tetravalent formulation of soluble purified antigens (tEDIII) from all the four DENV serotypes; (iii) tEDIII in combination with TLRs agonist (tEDIII+TLRs); (iv) Tetravalent nano formulation of PLGA encapsulated antigens from all the serotypes of DENV PLGA(tEDIII); and (v) PLGA(tEDIII) in combination with the TLR agonists (adjuvants) (PLGA(tEDIII)+TLRs) via subcutaneous route at the concentration of 10 µg each antigen from all the four serotypes (Figure 14).

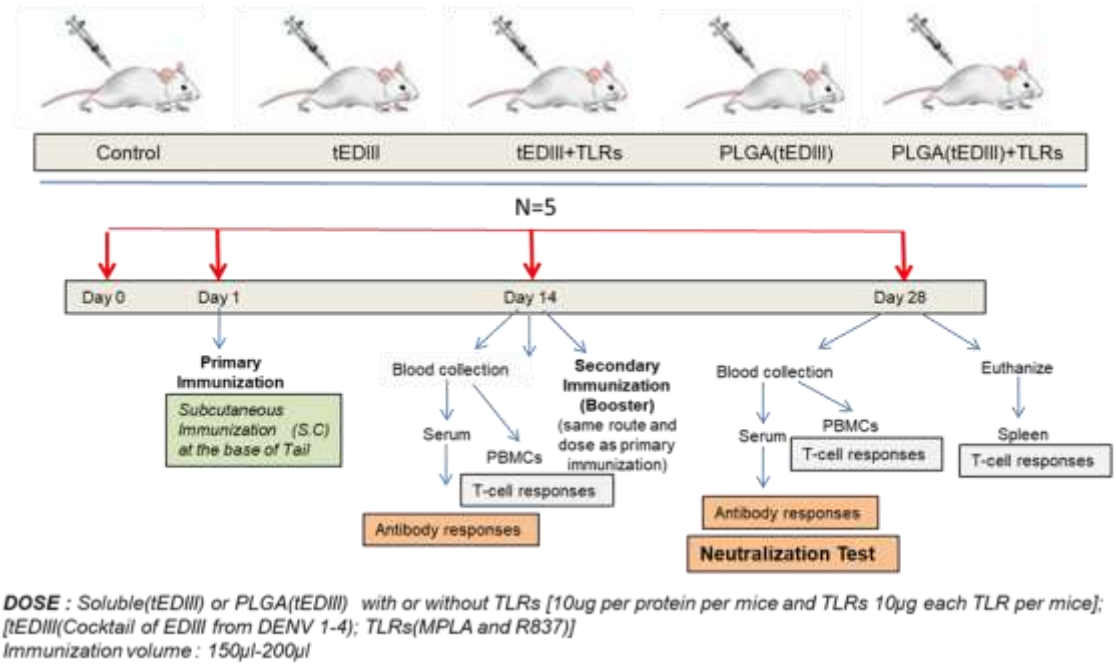
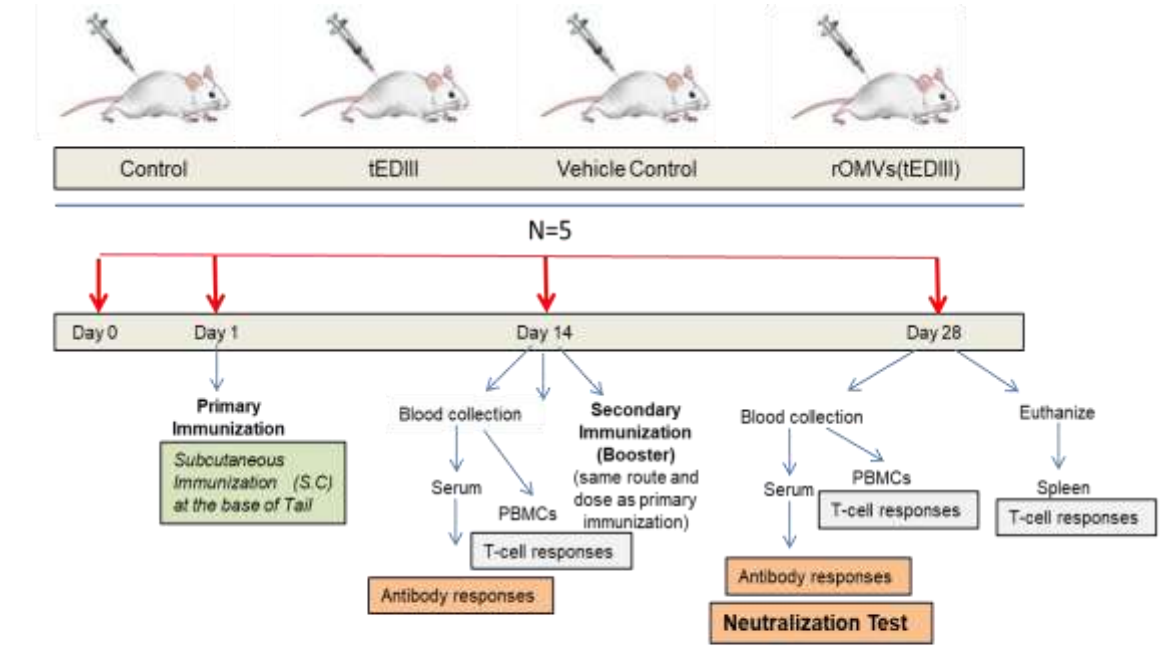


Figure 14. Immunization Schedule of mice.

(i) In separate experiment Balb/c mice of same age were immunized subcutaneously with PBS control; (ii) tetravalent formulation of soluble purified antigens (tEDIII) from all the four DENV serotypes; (iii) empty OMVs (Vehicle Control) (iv) Tetravalent formulation of OMVs expressing EDIII (rOMV expressing EDIII from four serotypes mixed physically) (rOMV(tEDIII)). After 15 days post priming, blood samples were taken from the tail of mice. PBMCs and serum samples were isolated for T and B- cell response studies. At day 15 mice from all the experimental groups were injected with a booster dose. Mice were euthanized on day 28 post priming dose, the blood, spleen, and lymph nodes were collected for various immunological assays (Figure 15). All animal experiments were performed according to the animal ethics guidelines of the University of Hyderabad.



DOSE : *Soluble (tEDIII) or rOMVs(Vehicle control) or rOMVs expressing EDIII (rOMVs(tEDIII) [10ug per protein per mice] [tEDIII(Cocktail of EDIII from DENV 1-4); Vehicle Control (OMVs) 10ug/ mice]*
Immunization volume : 150µl-200µl

Figure 15. Immunization Schedule of mice for OMV.

3.16 Antigen-Specific T Cell Assay by Flow Cytometry

Antigen-specific T cell responses were studied in the PBMCs and spleens following immunization with various immunizing group as discussed above. Spleen from mice were isolated and single cell suspension was obtained by mechanical disruption followed by filtration with 70 µm cell and pellet down at 350×g for five min. The cells were suspended in the RBC lysis buffer for 10 min to lyse the erythrocytes (Figure 16). PBMCs were also isolated by well-defined sucrose gradient density separation method as described earlier (Naz et al., 2018)**.** Single suspension of splenocytes and PBMCs were seeded with RPMI 1640 in the 96 well flat bottom plate and re-stimulated with purified DENV EDIII antigen (10 µg/ml) in the presence of GolgiPlug and GolgiStop (BD Biosciences) at 37°C for 10 hrs. The cells were stained with anti-mouse CD4 antibodies labeled-Fluorescein Isothiocyanate and Alexa Fluor 488–labeled anti-mouse CD8 (BD Biosciences) for 60 min at 4°C. The stained cells were washed thrice with FACS buffer and fixed with 4% paraformaldehyde. The cells were permeabilized using 1× Perm Wash Buffer (BioLegend), stained with phycoerythrin-conjugated anti-mouse IL-2 antibody and APC-labeled anti-mouse IFN-g

(BD Biosciences) and (BD Biosciences) for 60 min at RT. Finally, cells were washed with FACS buffer, and data were captured in BD LSRFortessa (BD Biosciences) cytometer and analyzed using FlowJo (Tree Star Inc.).

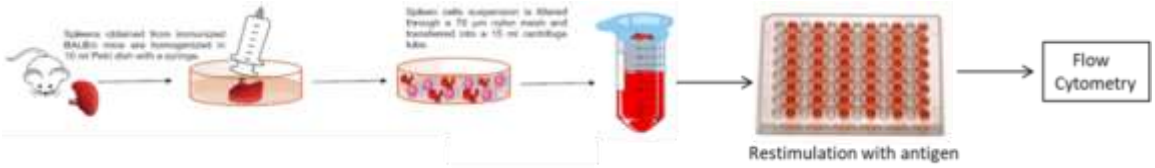


Figure 16. Isolation of spleenocytes from mice.

3.17 Serum Antibody ELISA

96 well ELISA plate (maxisorp-NUNC) was coated with 100 µl/well of DENV EDIII protein (10 µg/ml) from all the four serotypes in the bicarbonate coating buffer (pH = 9.5) and incubated at 40°C overnight. Plates were washed three time in washing buffer (1XPBS + 0.05% tween-20) and blocked with 200 µl of 4% skimmed milk for 2 hrs at RT and further washed thrice. Serum samples were diluted in 0.1% skimmed milk prepared in 1× PBST and added into the well and incubated for 2 hrs at RT. Post incubation, plates were washed four times incubated with HRP conjugated anti-mouse secondary antibody for 1 hour at RT. Plates were washed five times and TMB substrate 50 µl/well were added in the plate, after certain time-points reaction was stopped using 0.2 N H_2SO_4, finally absorbance were taken at 450 nm and plotted on Y axis. In another set of experiment to evaluate the antibody titer, antigen-coated plates were incubated with various dilutions of pooled serum from 10 mice in two groups of five each for 2 hrs at RT and rest of the steps were same as above. Antibody titers were calculated by the reciprocal value of the highest dilution at which absorbance values were more than or equal to twice the value of control in the same dilution series.

3.18 Preparation of DENV Stocks

DENV 2 (TR1751), DENV 1 (Hawaii), DENV 4 (Columbia 1982), and DENV 3 (Thailand 1973) viral strains were propagated in C636 cells as described earlier (Afroz et al., 2016). These cells were cultured in α-MEM media supplemented with 10% FBS and 1% antibiotic/antimycotic (Gibco) in T75 flasks. The cells were infected with virus (0.1 moi) for

30 min at 33°C. The inoculum was removed and fresh media with reduced FBS was added to cells and kept for five to seven days until the Cytopathic effect (CPE) was observed. The viruses were harvested by centrifugation and supernatants containing viruses were stored at −80°C. The titers of virus were quantified by FACS titration assay in vero cells as described elsewhere (Lambeth et al., 2005).

3.19 Serotyping of DENV

The RNA from culture supernatants were isolated using Viral RNA Isolation kit (Macherey Nagel Germany) according to instructions provided by manufacturer. The RNA isolated from all the four serotypes (DENV1-4) were converted into cDNA using cDNA synthesis kit (Takara Bio Inc., Japan) and using D2 reverse primer (Lanciotti et al., 1992). The cDNA obtained was amplified by PCR using D1 and D2 primers (consensus forward and reverse primers). This was followed by another amplification reaction wherein the product from first amplification was used as template and D1 forward primer and serotype-specific reverse primers (Table 2) TS1, TS2, TS3, and TS4 as described earlier (Afroz et al., 2016).

Table 2. Primer Sequences of serotyping primers

Primer	Sequence	Serotype
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	Universal
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	Universal
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	DENV 1
TS2	5' CGCCACAAGGGCCATGAACAG-3'	DENV 2
TS3	5' TAACATCATCATGAGACAGAGC-3'	DENV 3
TS4	5' CTCTGTTGTCTTAAACAAGAGA-3'	DENV 4

3.20 Quantification of DENV

The viruses were quantified by Facs titration assay as described previously (). Vero cells were cultured in DMEM with 10 % FBS and were seeded in 12 well flat bottom plate at

density of 1× 10^6 per well. Cells were incubated for 24 hrs at 37°C in incubator with 5% CO_2. Culture media was removed and cells were washed with 1× PBS and were infected neat or different dilutions of virus (10^{-2}, 10^{-4}, 10^{-6}) and incubated for 2hrs at 37 °C. The plates were rocked at an interval of 15 min to avoid drying of surface. The inoculum was removed after the incubation and cells were washed with 1× PBS and fresh media containing 2% FBS was added to the cells and incubated for 24 hrs. After the incubation cells were then washed with 1× PBS after which trypsin-EDTA was added to each well and further incubated at 37°C for two to five min and to stop the action of trypsin on the cells a mixture of 1× PBS and 10% FBS was added. Cells were harvested by centrifugation at 1000 x g for five min following which the supernatant were removed and the clump free cells were fixed with 4% Paraformaldehyde and permeabilized followed by staining with an anti-dengue antibody (Genetex Inc., USA). Samples were washed and stained with Alexa Flour 488 tagged secondary antibody (Invitrogen, USA) and incubated for 1 hr at RT. After incubation, cells were washed and resuspended in Facs buffer and analyzed on flowcytometery analyzer(BD Fortessa LSR). Atleast 20,000 events were collected and the data was analyzed using Flow Jo software (Becton Dickinson USA). The titer of virus was calculated using the following formula:

FACS Infectious units per ml (FACS IU/ml) = [(average % positive DENV infected cells-average % positive mock infected cells) X (total number of cells in well) X (dilution factor)] /volume of inoculum (in ml) added to the cells.

3.21 Flowcytometry Based Neutralization Test (FNT)

Sera from mice immunized with different combinations of antigens were assayed for neutralizing antibodies against DENV by FNT (Lambeth et al., 2005). For neutralization test, Vero cells were cultured in DMEM (Gibco Life Technologies USA) media with 10% FBS (Gibco Life Technologies USA) and 1% antibiotic/antimycotic (Gibco Life Technologies USA) and seeded in a 96-well plate at a density of 2.5×10^4 cells/well. In the preceding step these cells were incubated in a mixture of heat-inactivated serum (56°C for 30 min) pooled from 10 mice in two groups of five each and virus. The virus serum mixture was removed after 2 hrs and 200 µl of DMEM media containing 2% FBS was added to each well and plates were kept in in incubator at 37°C with 5% CO_2 for 24 hrs. Each serum

dilution was assayed in triplicates. After the incubation cells were then washed with 1× PBS followed by staining with anti DENV antibody for Facs analysis as described above. For each sample, 10,000 to 20,000 cells were collected and analyzed. The percent reduction of infection for each serum dilution was calculated and the titers were expressed as the reciprocal of the serum dilution that inhibited 50% of virus (FNT50) (de Alwis and de Silva, 2014; Lambeth et al., 2005).

3.22 Statistical Analysis

All data are represented as mean ± S.E.M of three or four independent experiments. Two sample unpaired Student's t-Test was executed using Graphpad Prism software to evaluate the significance of difference between groups. A *P*-value less than 0.05 was significant.

4. RESULTS

4 Results

4.1 Objective 1

Expression, Purification, and immunological characterization of DENV EDIII of all the serotypes of DENV (1-4)

4.1.1 Expression and Purification of EDIII from all the four serotypes of DENV

We have successfully purified the EDIII proteins from all the four serotypes of DENV. The cloned constructs DENV (1-4)-pET28a-domain III (synthesized from Gnescript USA) were transformed into *E. coli* Rosetta cells for checking the expression of the clones. Briefly, a single isolated colony from the transformed plates were used as an inoculum for the primary culture in kanamycin (50 µg/ml) and 35 µg/ml chloramphenicol containing LB media. The primary culture was incubated overnight in a shaking incubator and 1% of the primary culture was used as a starting material for secondary culture. The secondary culture was grown in a shaking incubator at 37°C until an OD of 0.5 was reached. 1 ml of the culture was removed as uninduced while the remaining culture was induced using 1 mM IPTG for the induction of expression of the domain III recombinant protein. Following incubation of 4 hrs, the cells were harvested by centrifugation and the pellet was resuspended in 1:1 ratio of 1× PBS and 1× loading dye (4× dyes, 1 M DTT, Milli Q water). A typical experiment comparing the induced and uninduced culture reveals that the IPTG induction resulted in the expression of DENV EDIIIs (Figure 17 A). Next, we investigated the solubility of the expressed proteins. Culture was induced with IPTG and the pellet obtained was resuspended in lysis buffer, with and without detergent (TritonX-100 0.2%) (Figure 17 B). The expressed protein was found in pellet 8 M urea was used in the lysis buffer to solubilize the proteins.

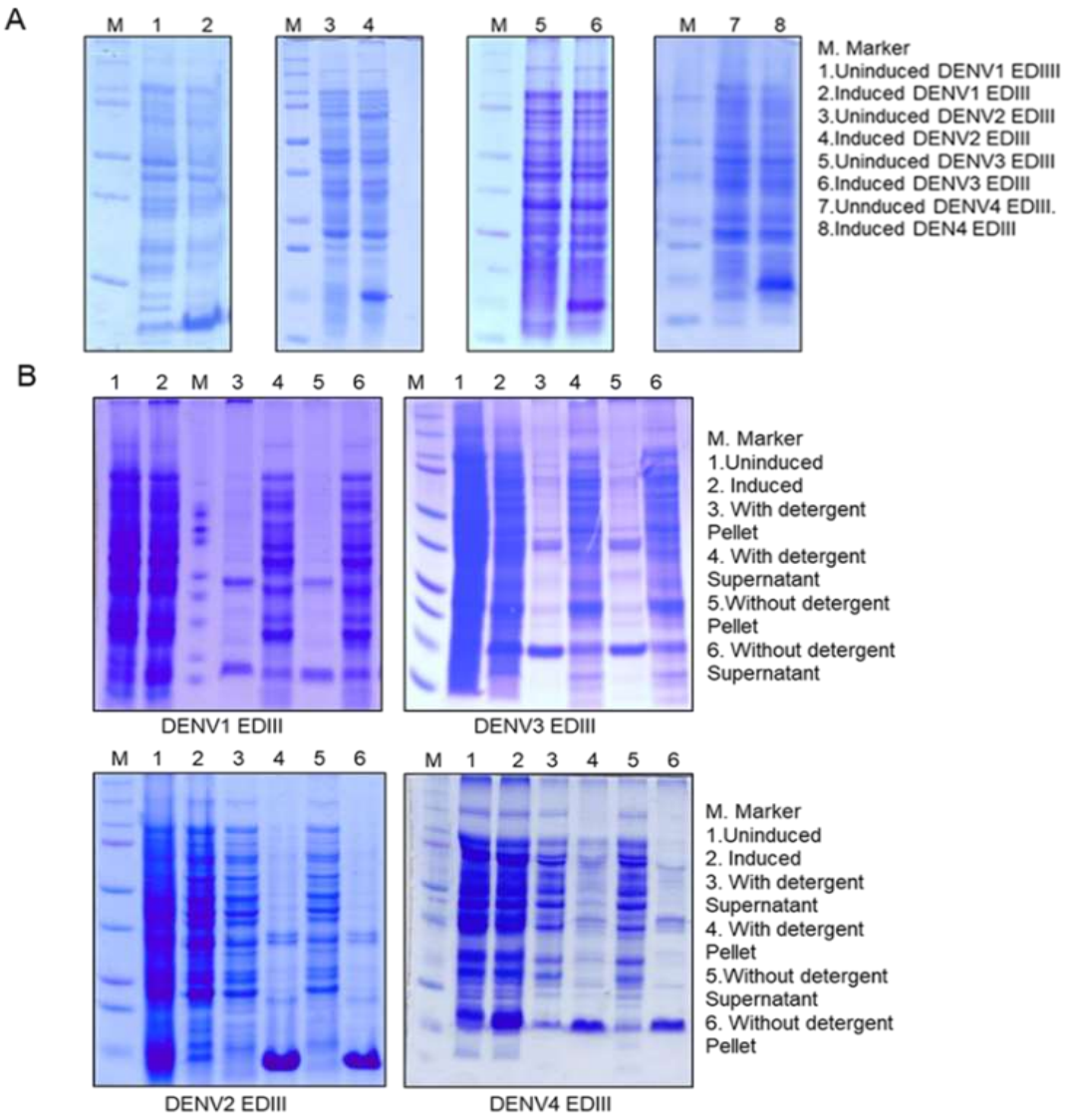


Figure 17. Expression and purification of recombinant EDIII of DENV (1-4).

(A) SDS-PAGE showing overexpression expression of EDIII in *E. coli* Rosetta cells. (B) Protein solubility: Solubility of proteins was checked in presence or absence of a detergent (0.2% Triton-X 100).

The recombinant proteins were purified using Ni – NTA column. The overnight Rosetta culture was inoculated into 1 L LB (containing 50 µg kanamycin/ml and 35 µg/ml chloramphenicol) in a flask, which was placed in the shaker at 37°C for about 2–3 hrs at 150 rpm. When the OD_{600} of the culture reached ~0.5 to 0.6 (a small aliquot of the

uninduced culture was set aside for subsequent SDS-PAGE analysis), it was induced by the addition of IPTG to a final concentration of 1 mM. Induction was allowed to proceed for 4 hrs before harvesting the cells. The induced culture was centrifuged, and the cell pellet was resuspended thoroughly in buffer containing 8 M urea and 1 mM PMSF (Tris 50 mM, NaCl 300 mM, 20 mM imidazole). The resuspended cell pellet was sonicated for 10 minutes and centrifuged at 12,000 rpm for 30 minutes. The supernatant was collected, kept for binding with Ni-NTA beads for 1 hr at 4°C, and was passed through Ni-NTA column. Flow-through, wash and different fractions (eluted with 300 mM imidazole) were collected, and run on SDS-gel. The desired band size of 13 kDa (DENV 1, 2 and 4) and 14.5 kDa (DENV 3) were observed after Coomassie staining of SDS gels (Figure 18A). The four DENV purified EDIII proteins were also confirmed through Western blot using an anti-His antibody (Figure 18 B).

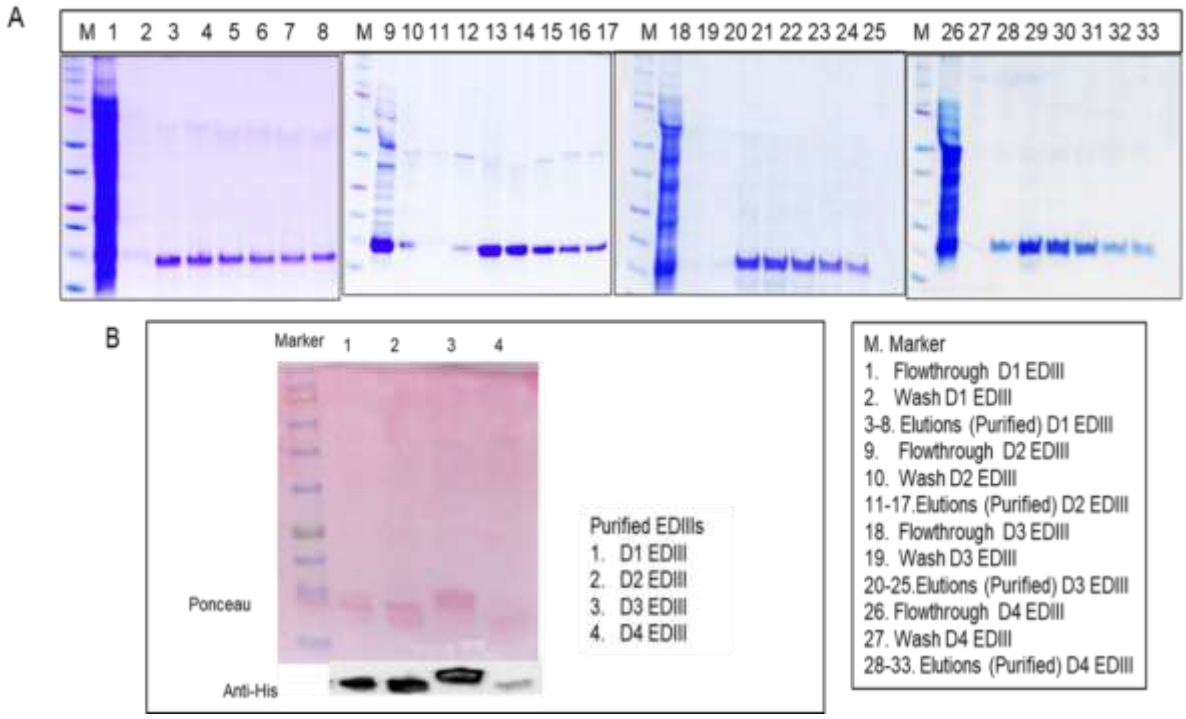


Figure 18. EDIII purification using Ni-NTA column for DENV 1, 2, 3&4.

(A) Proteins were washed with 50 mM Imidazole and eluted at concentration of 300 mM Imidazole (B) Western blot detection of the purified protein using anti-His antibody.

4.1.2 **DENV EDIII protein induces a robust proinflammatory cytokine signature in human macrophage cell line.**

The excessive production of inflammatory mediators including cytokines and chemokines from the deregulated immune cells has been mainly attributed toward the severity of dengue. However the components and the mechanisms involved in triggering such response is not well understood (Costa et al., 2013b). Although several non-structural proteins of DENV have been shown to modulate inflammatory responses, however, very less is known about the innate immune responses elicited by structural proteins like E protein. The EDIII of DENV E protein encapsulated in nanospheres has been recently shown to induce innate immune responses in neutrophils of mice (Hua et al., 2021) . However, the mechanisms through which DENV EDIII protein induces innate inflammatory responses in immune cells like monocytes/macrophages have not been extensively investigated. Therefore, we have demonstrated the effect of EDIII protein on innate inflammatory responses in the differentiated THP-1 cells. For this, we have incubated the purified recombinant EDIII of DENV 2 serotype (DENV 2 EDIII) with the differentiated THP-1 cells and the levels of proinflammatory cytokines IL-1β, TNF-α, and IL-6 were detected using ELISA and qRT-PCR in the culture supernatants and cells, respectively. The differentiated THP-1 cells were treated with different non-toxic concentrations of DENV 2 EDIII (Figure 19 A), while LPS was used as a positive control. Following stimulation, the cells and culture supernatants were harvested for the examination of cytokines levels. Interestingly, the data (Figure 19 B-C) reveals that the levels of cytokine IL-1β and TNF-α showed a remarkable increase with increasing concentrations of DENV 2 EDIII protein. However, IL-6 did not show any dose dependent change with protein treatments (Figure 19 D). To rule out the possibility of endotoxin contamination, the purified heterologous protein was subjected to proteinase K treatment (Khan et al., 2008). The EDIII protein treated with proteinase K was not able to induce proinflammatory cytokines in THP-1 cells, thereby suggesting that the observed phenomenon is the result of protein activity and not because of the LPS contamination. Concomitantly, we also examined the mRNA levels of these cytokines using qRT-PCR. For qRT-PCR the cells were treated with different concentrations of EDIII protein and LPS for 6 h. The qRT-PCR results were in line with the ELISA data and showed a similar trend (Figure 19 E-F), thereby suggesting that the DENV 2 EDIII protein influences

transcriptional machinery to program the proinflammatory environment of the cells.

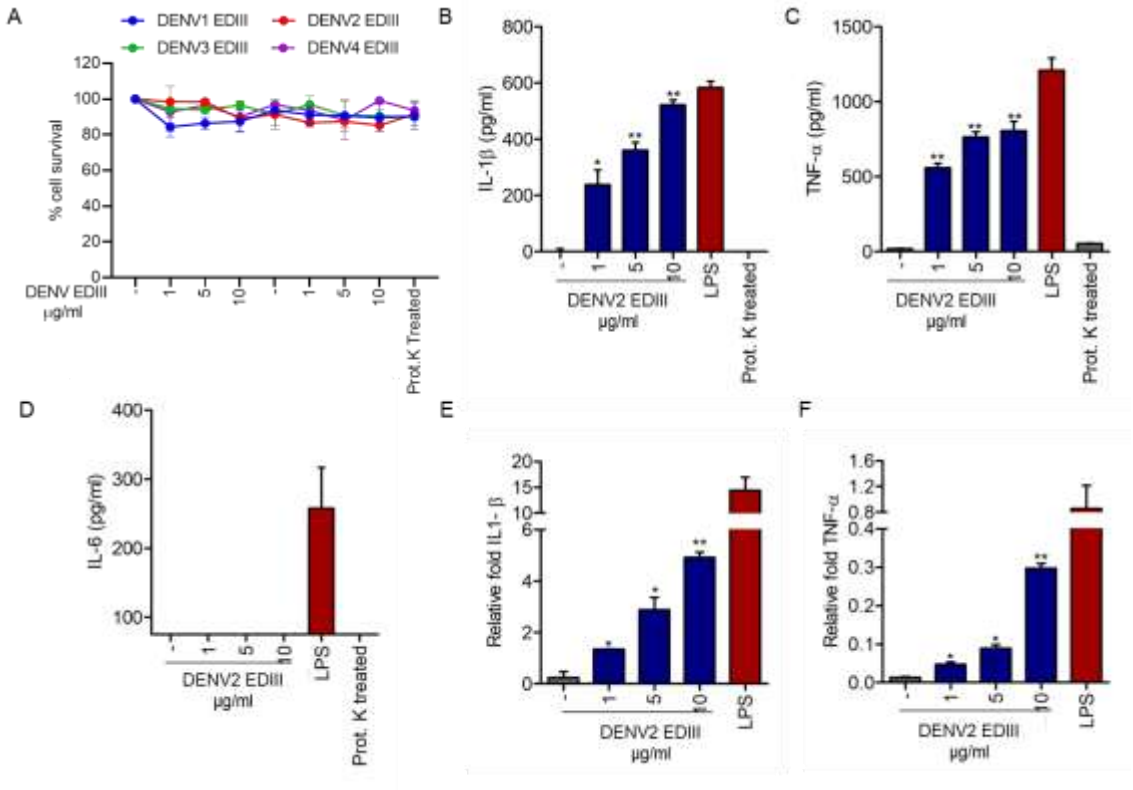


Figure 19. Cytokine profile in differentiated THP-1 cells stimulated with EDIII of DENV 2.

(A) MTT assay to evaluate cytotoxicity of DENV EDIII proteins and LPS (500 ng/ml) for 24 hours in THP-1 cells (B-D) ELISA for cytokine estimation from THP-1 cells untreated or treated with different concentrations of DENV 2 EDIII for 24 hrs. LPS (500 ng/ml) was used as positive control and Proteinase K treated protein was used as a negative control. (B) IL-1β (C) TNF-α (D) IL-6 is the ELISA data. (E) qRT-PCR analysis to check mRNA expression of (E) IL-1β, and (F) TNF-α in macrophages treated with EDIII and LPS. Statistical significance was calculated by Student t-test. $*P \leq 0.05$, $**P \leq 0.005$, were considered statistically significant. Error bars represent mean ± SEM. Data are representative of one of three independent experiments.

Having observed that DENV 2 EDIII protein influences enhanced production of IL-1β and TNF, we were interested to examine whether this phenomenon was restricted to the EDIII of DENV 2 serotype or mediated by other serotype EDIII proteins. To test this hypothesis, we used EDIII protein of DENV 1, 3 and 4 serotypes. Interestingly, we observed similar results

with other serotype EDIII proteins (Figure 20), thereby suggesting that EDIII mediated tailoring of inflammatory cytokine production is not serotype restricted. Furthermore, the above finding suggests that EDIII protein induces proinflammatory signatures by increasing the production of proinflammatory cytokines IL-1β and TNFα in THP-1 cells.

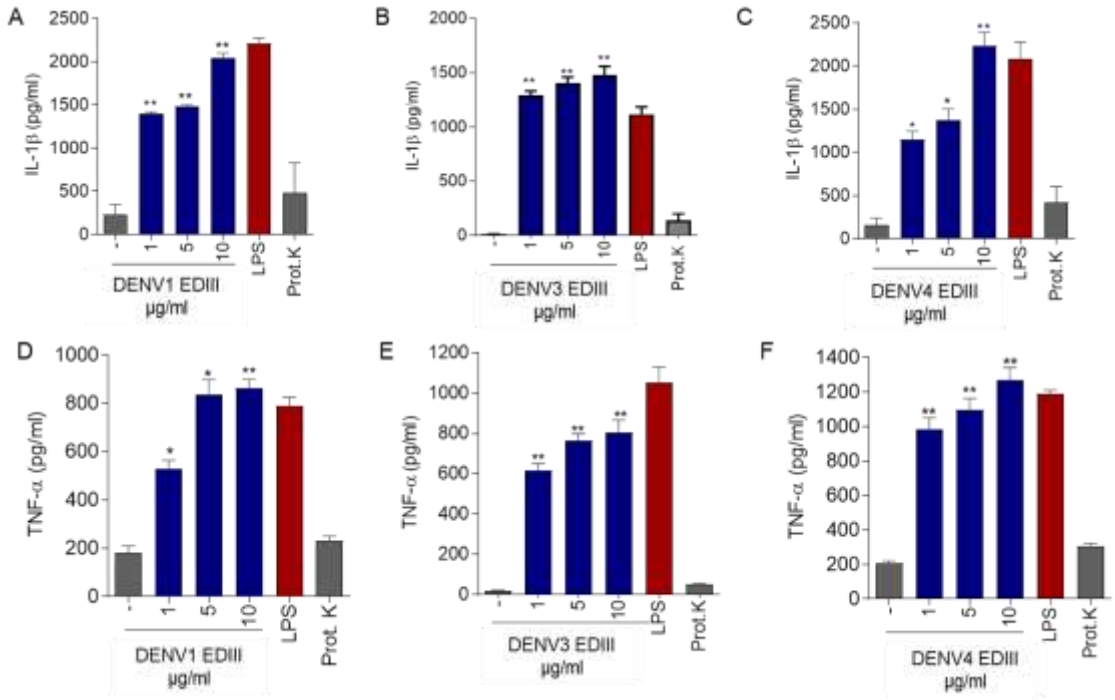


Figure 20. Cytokine profiling of THP-1 cells untreated or treated with different concentrations of EDIII of DENV serotypes 1, 3, and 4.

(A-C) ELISA to check the expression of IL-1β in THP-1 cells treated with (A) DENV 1 EDIII, (B) DENV 3 EDIII, and (C) DENV 4 EDIII. TNF-α expression by ELISA in THP 1 cells treated with (D) DENV 1 EDIII, (E) DENV 3 EDIII, and (F) DENV 4. Statistical significance was calculated by Student t-test. $*P \leq 0.05$, $**P \leq 0.005$, were considered statistically significant. Error bars represent mean ± SEM. Data are representative of one of three independent experiments.

4.1.3 **Proinflammatory response induced by recombinant DENV 2 EDIII protein is mediated by activation of NF-κB.**

Having observed that EDIII proteins trigger IL-1β and TNF-α at mRNA and protein levels, we were next interested to understand the mechanism through which these cytokines might

be regulated. It is well documented that the transcription factor NF-κB regulates wide range of genes involved in regulating inflammatory responses (Liu et al., 2017; Oeckinghaus and Ghosh, 2009). NF-κB represents family of transcription factors composed of five structurally related members including p50, p52, p65, RelB and c-Rel (Sun et al., 2013). Even though all these members are well characterized but p65 has received the most attention. The phosphorylation of NF-κB results in the nuclear translocation of p65 subunit thereby activating the transcription of downstream genes by binding to target DNA elements (Hayden and Ghosh, 2012; Hochrainer et al., 2013). Therefore, to determine whether IL-1β and TNF-α production are regulated by NF-κB activation in response to DENV 2 EDIII stimuli, we evaluated the NF-κB levels in nuclear and cytoplasmic fractions in EDIII protein stimulated THP-1 cells. A clear and enhanced nuclear translocation of p65 in DENV 2 EDIII treated cells was observed in a time dependent manner as indicated (Figure 21 A; upper panel**)**. Time-course measurements suggested an upsurge in nuclear p65 levels 2 hrs post treatment when compared to untreated control. This was in line with the declining p65 levels in the cytoplasm and its rising translocation to the nucleus. Under resting conditions NF-κB is bound by inhibitory IKB proteins including IKBα, thereby rendering it into inactive form(Mathes et al., 2008). In response to external stimuli, IKB proteins are phosphorylated which leads to their degradation by the proteasome. Thus NF-κB is released from complex and is translocated to the nucleus (Viatour et al., 2005). Therefore, we checked for IKBα phosphorylation under the same conditions as were employed for p65. We observed time dependent increase in the phosphorylation of IKBα (Figure 21 A; lower panel**)**. We also examined the NF-κB nuclear translocation with the help of confocal microscopy. THP-1 cells were seeded on coverslips and were treated with recombinant DENV 2 EDIII protein and LPS. The microscopic images clearly show NF-κB translocation in nucleus of the treated cells as compared to media control (Figure 21 B**)**.

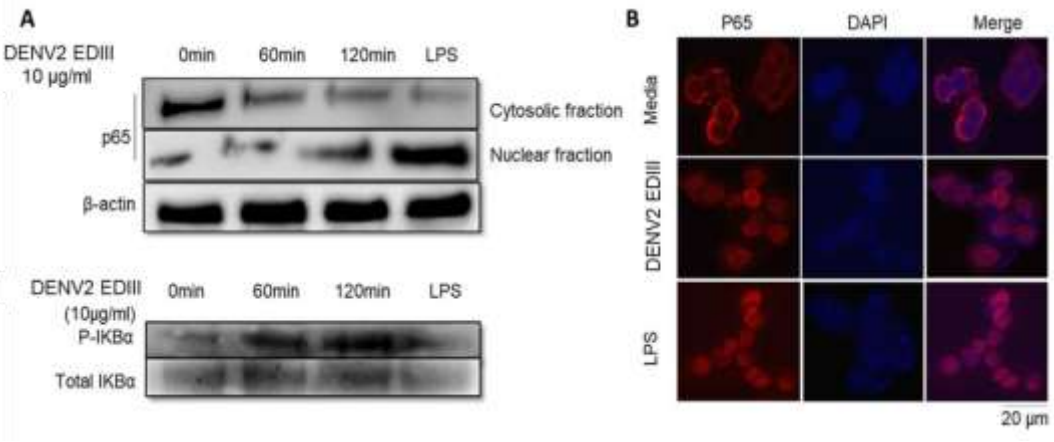


Figure 21. Activation of NF-Kappa β following stimulation with DENV2 EDIII protein in THP-1 cells.

(A) Immunoblot showing nuclear translocation of p65 and phosphorylation of IKBα in THP-1 cells after stimulation with EDIII in a time dependent manner, LPS was used as a positive control. (B) Confocal microscopy mages showing p65 (red) translocated to nucleus in EDIII and LPS stimulated macrophages, nuclei were stained with DAPI (blue). Scale bars, 20 µm. Cells were stimulated for 1 hr. Data are representative of one of three independent experiments

It is reported that TLR signaling is involved in the phosphorylation of IKB proteins and their subsequent degradation allows NF-κB to move into the nucleus thereby triggering the induction of inflammatory cytokines (Hayden et al., 2006). Since TLRs are involved in the activation of NF-κB, therefore we wanted to check which TLR has involved in the production of IL-1β mediated by DENV 2 EDIII. We incubated cells with TLR2 and TLR 4 neutralizing antibodies (20 µg/ml) to block respective receptors followed by treatments of DENV 2 EDIII. An isotype control IgG2a was also used. We observed significant inhibition in IL-1 β production in the cells where TLR4 antibodies were preincubated compared to TLR2 and isotype control (Figure 22 B). This shows that DENV 2 EDIII mediates signaling via TLR 4.

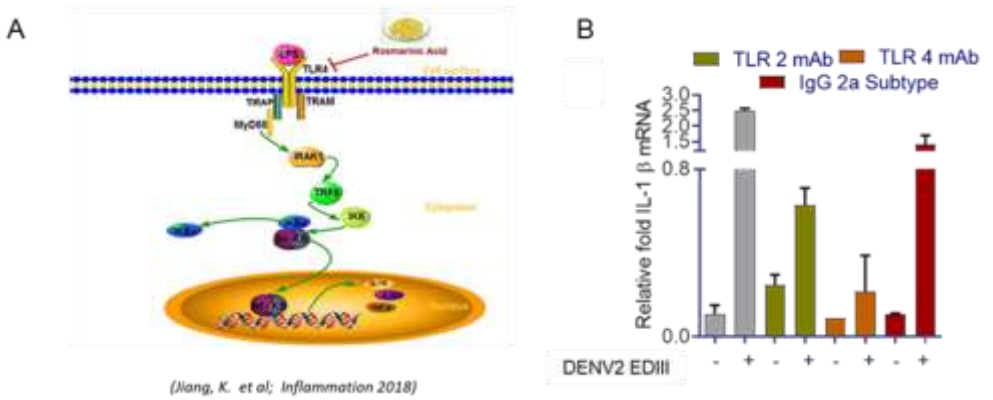


Figure 22. DENV 2 EDIII induces NF-κB activation via TLR 4

(A) NF-κB activation via TLR-4 signalling (B) THP-1 cells were preincubated with TLR2 or TLR4 or IgG2a (isotype control) antibodies (20 µg/ml) and stimulated with DEN2 EDIII (10 µg/ml). qRT-PCR was performed to check the expression of IL1β. Data are representative of one of three independent experiments.

4.1.4 **IL-1β maturation induced by DENV 2 EDIII requires caspase-1 activation and involves NLRP3 inflammasome**

IL-1β is one of the most proinflammatory cytokines which is produced by activated monocytes and macrophages in response to proinflammatory signals like LPS (Matsushima et al., 1986). IL-1β is produced as biologically inactive 31 KDa polypeptide called as pro-IL-1β and is cleaved by caspase-1 to produce 17 KDa mature IL-1β (biologically active form) (Sutterwala et al., 2006). Caspase-1 is itself activated to active enzyme by the autocatalytic reaction which is tightly controlled by molecular scaffold termed as inflammasome (Mariathasan and Monack, 2007). Since IL-1β maturation and secretion is controlled by caspase-1 upon inflammasome activation, we tested whether DENV 2 EDIII protein has any role in the maturation of pro-IL-1β and inflammasome activation. We assessed the release of both, the active caspase-1 and IL-1β, from the differentiated human monocytes by immunoblotting. Cells were seeded in Opti-MEM media containing reduced serum followed by treatment with different concentrations of protein as well as LPS. Immunoblotting results clearly show increase in pro- and mature IL-1β, as well as cleaved caspase 1, however, procaspase one levels were comparable (Figure 23 A). These data suggests that the IL-1β maturation induced by DENV 2 EDIII protein is dependent on

caspase-1 activation. In order to check whether caspase-1 is required for DENV 2 EDIII induced maturation of IL-1β we used caspase-1 inhibitor (Ac-YVAD-CHO) and examined its effect on mature IL-1β (Zhou et al., 2000). We incubated cells with DENV 2 EDIII in presence or absence of Ac-YVAD-CHO and observed that mature IL-1β was drastically reduced in presence of Ac-YVAD-CHO compared to only DENV 2 EDIII treated cells (Figure 23 B). Further, we also investigated whether NLRP3 inflammasome activation has a role to play in maturation of IL-1β; we stimulated the cells with recombinant protein and probed for the expression levels of NLRP3. Immunoblotting results show that DENV 2 EDIII treatment induces NLRP3 activation (Figure 23 D). NLRP3 expression was also evaluated using qRT-PCR, which also indicates an increased expression of NLRP3 in LPS and DENV 2 EDIII protein treated cells as compared to the untreated (Figure 23 C). These data, therefore, demonstrates that the NLRP3 inflammasome activation is involved in DENV 2 EDIII protein induced IL-1β maturation and is mediated via caspase-1.

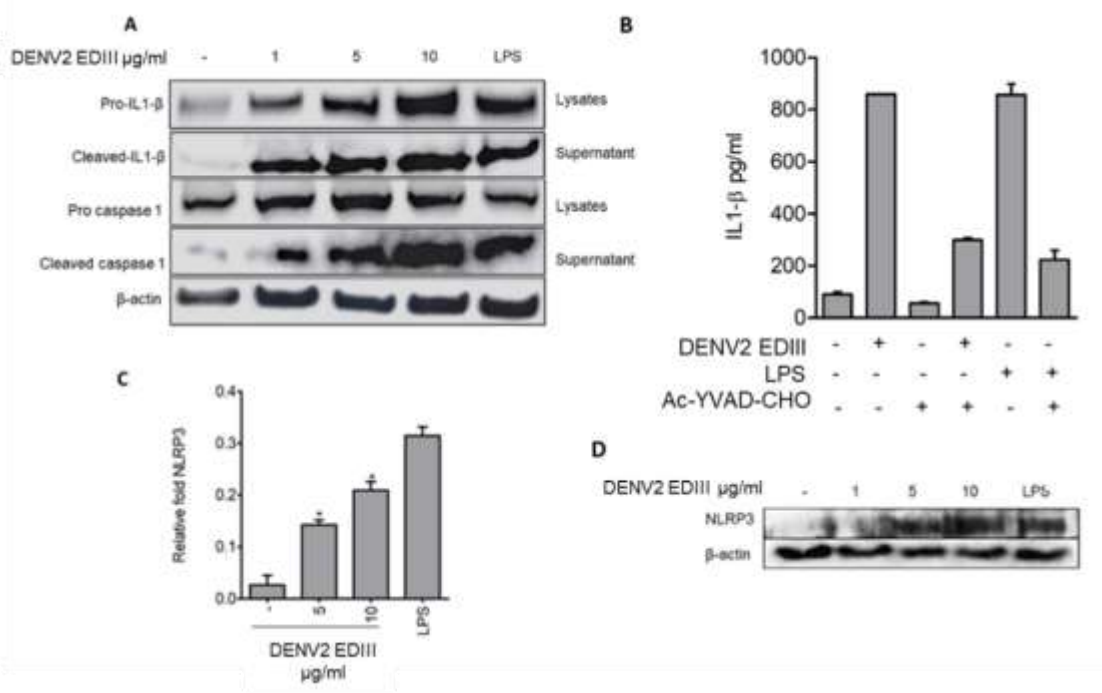


Figure 23. Maturation of IL1-β requires Caspase-1 and NLRP3 inflammasome activation.

(A) Immunoblot analysis for expression of IL-1β and caspase-1 (pro and active) from differentiated THP-1 cells untreated or treated with different concentrations of EDIII for 12 h and LPS (500 ng/ml) (Lys. cell lysates; Sup., culture supernatant;) (B) Estimation of

IL-1β in supernatants of THP-1 cells untreated or treated with DENV 2 EDIII (10μg/ml) in presence or absence of caspase-1 inhibitor Ac-YVAD-CHO (50 μM). (C) qRT-PCR to check the expression of NLRP3. (D) Production of NLRP3 from DENV 2 EDIII stimulated THP-1 cells by immunoblotting. Statistical significance was calculated by Student t-test. *$P \leq 0.05$ was considered statistically significant. Error bars represent mean ± SEM. Data are representative of one of three independent experiments.

4.1.5 **Recombinant DENV 2 EDIII induced IL-1β maturation is dependent on ROS and potassium efflux**

Since NLRP3 expression was observed in the DENV 2 EDIII treated cells, we tried to look into the mechanism of inflammasomes activation. Several mechanisms have been proposed for its activation. It is evident from previous reports that production of ROS plays a crucial role in the activation of NLRP3 inflammasomes (Zhao et al., 2020). As described above IL-1β maturation is dependent on caspase 1 which in turn is regulated by inflammasomes. Therefore, we were interested to find out whether DENV 2 EDIII triggers ROS production. To estimate intracellular ROS production, cells were stimulated with different concentrations of DENV 2 EDIII protein and LPS for 12 h, followed by incubation with CMH2DCFDA for intracellular ROS estimation. A significant increase in ROS production was observed in cells stimulated with EDIII protein as compared to the untreated cells (Figure 24 A). Next, we examined the levels of IL-1β and TNF-α in DENV 2 EDIII stimulated cells in presence and absence of ROS inhibitor, N-acetyl-L-cysteine (NAC), to find any correlation between ROS production and IL-1β maturation. Interestingly, IL-1β levels were reduced in the cells treated with (NAC) plus DENV 2 EDIII as compared to the cells stimulated with only protein (Figure 24 B). However, there was no change in the levels of TNF-α (Figure 24 C). These data suggests that the maturation of IL-1β induced by DENV 2 EDIII protein is dependent on ROS production which is in agreement with previous studies showing correlation of ROS with IL-1β maturation (Dostert et al., 2008; Franchi et al., 2009). It is well established that potassium efflux is an important signal for the activation of NLRP3 inflammasome and IL-1β maturation (He et al., 2016), we next examined the role of these signals in the DENV 2 EDIII mediated IL-1β processing and secretion. Since it is well established that IL-1β maturation is dependent on potassium efflux

(Colomar et al., 2003), therefore, to check the role of potassium efflux on the maturation of IL-1β, we treated the cells with NaCl and KCl (Kim et al., 2016) before the recombinant protein stimulation. We observed a reduction in the IL-1β levels in the DENV 2 EDIII protein treated cells with higher extracellular K+ levels as compared to the cells treated with only protein, however not much decrease was observed in the cells with higher extracellular Na+ levels (Figure 24 D). However TNF-α levels remained unchanged (Figure 24 E), which is in line with the previous findings (Groß et al., 2012), thereby suggesting that DENV 2 EDIII induced IL-1β maturation is indeed dependent on ROS and potassium efflux which supports the previous findings of the IL-1β regulations (Rivers-Auty and Brough, 2015).

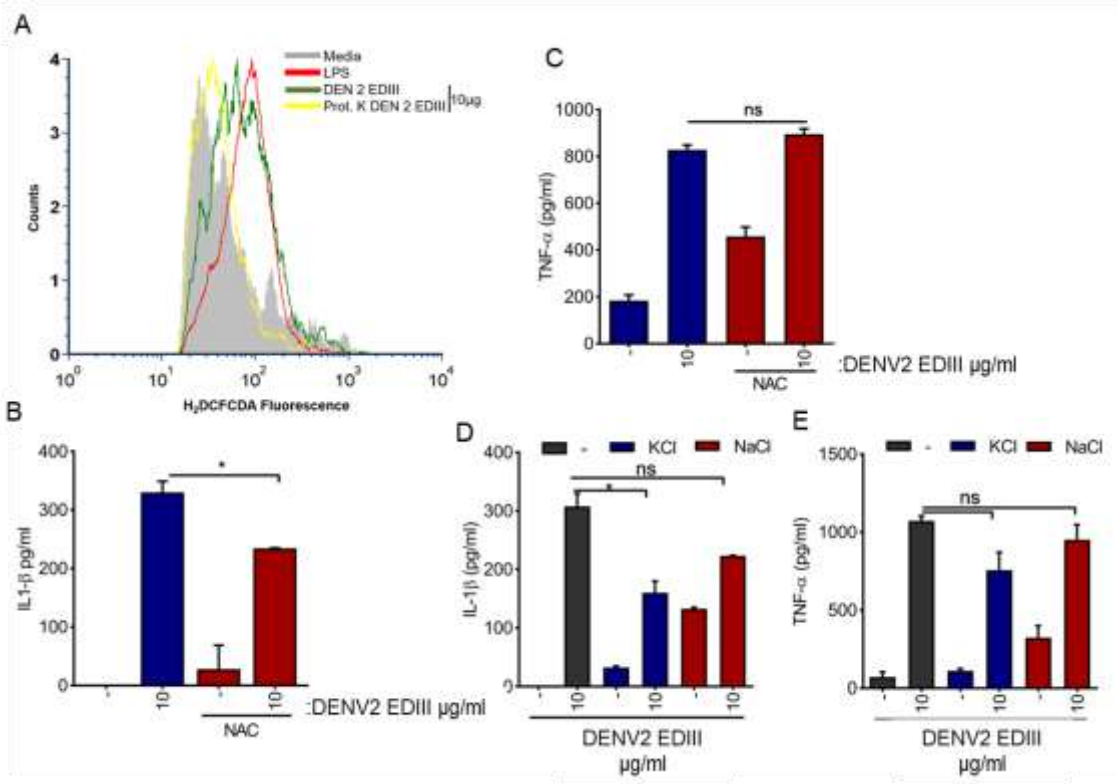


Figure 24. ROS production and Potassium efflux regulates IL1-β maturation.

(A) ROS estimation in differentiated THP-1 cells stimulated with EDIII and LPS (500 ng/ml). ELISA to check (B) IL1-β and, (C) TNF-α level in THP-1 cells stimulated with EDIII in presence and absence of NAC (ROS inhibitor). Estimation of (D) IL1-β and, (E) TNF-α level in EDIII stimulated THP-1 cells supplemented with NaCl and KCl to mimic intracellular Na+ and K+. Statistical significance was calculated by Student *t* test. $*P \leq 0.05$

was considered statistically significant, ns = not significant. Error bars represent mean ± SEM. Data are representative of one of three independent experiments.

4.2 Objective 2

Developing a DENV EDIII based tetravalent nanovaccine formulation using: (a) PLGA; (b) rOMV delivery systems followed by its biophysical characterizations and immunological screening upon immunization in mouse model system.

a) PLGA

4.2.1 Physicochemical characterization of the PLGA encapsulated DENV EDIII proteins.

The purified recombinant EDIII domain of dengue virus (1-4) envelop protein from all the dengue virus serotypes (1-4) (Figure 18) were subjected to PLGA encapsulation as described in the material and method section. The PLGA encapsulated DENV EDIII proteins were subjected to various biophysical characterizations. The shape, size and surface morphology of nanoparticle was evaluated by TEM. Our result categorically demonstrates that the PLGA nanoparticle encapsulated with DENV tEDIII is in the size ranging from 100 nm-1 μm (Figure 25 upper panel**)** with spherical morphology in the entire formulation group. The hydrodynamic radii of particles were measured by DLS method, and it shows that the majority of the particle size ranges from 250 nm to 2 μm (Figure 25 lower panel**)**.

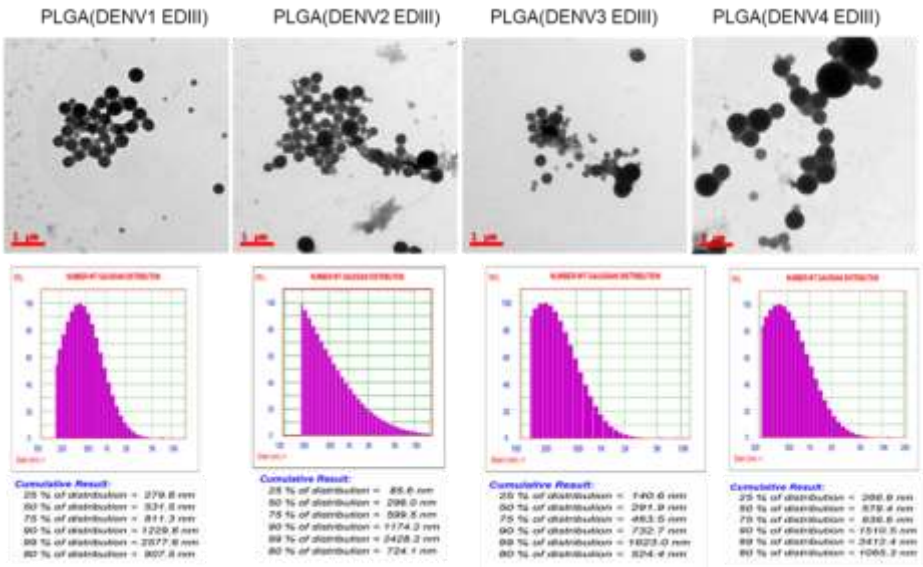


Figure 25. Physical characterization of EDIII encapsulated PLGA nanoparticles.

TEM for surface morphology and size of PLGA encapsulated EDIIIs of all four serotypes of DENV (Upper Panel). DLS to check size distribution of EDIII encapsulated nanoparticles (Lower panel)

4.2.2 **Encapsulation efficiency and release of EDIII antigens from the PLGA Nanoparticles**

After encapsulation of the DENV EDIII proteins in the PLGA, next, we evaluated the encapsulation efficiency of PLGA nanoparticle. Our results show that a significant amount of DENV EDIII (1-4) in the nanoparticles was encapsulated (Table 3) represented in the terms of per cent encapsulation efficiency as calculated by the formula described in the methodology section. Loading of protein in nanoparticles was also checked by SDS-PAGE analysis where we observed a single band at corresponding size for EDIII for all the four serotypes of DENV (Figure 26 A) which indicated that EDIII antigens were successfully loaded in the nanoparticle. Samples were lysed and mixed with SDS sample buffer contains β-mercaptoethanol and heated at 95°C for five minutes before loading in SDS-PAGE gel. In vitro release assay of antigen loaded PLGA nanoparticle shows that in the first 24 hrs of incubation nanoparticle releases ~20% of content and then sustained beyond 72 hrs which indicates a biphasic release pattern, burst followed by sustained release (Figure 26 B). In another observation, the release of antigens was significantly enhanced at acidic pH (Ph =

5), which is the pH of endolysosomes (Figure 26 C), and it is important for antigen processing by the APCs. This kind of release pattern is very important for the optimal and balanced immune response against antigens which could serve as a sustained antigen depot.

Table 3. Encapsulation efficiency of EDIII antigen from all the four serotypes of DENV

Formulation	Encapsulation Efficiency (%)
PLGA (DENV 1 EDIII)	47.77
PLGA (DENV 2 EDIII)	57.55
PLGA (DENV 3 EDIII)	52.2
PLGA (DENV 4 EDIII)	44.34

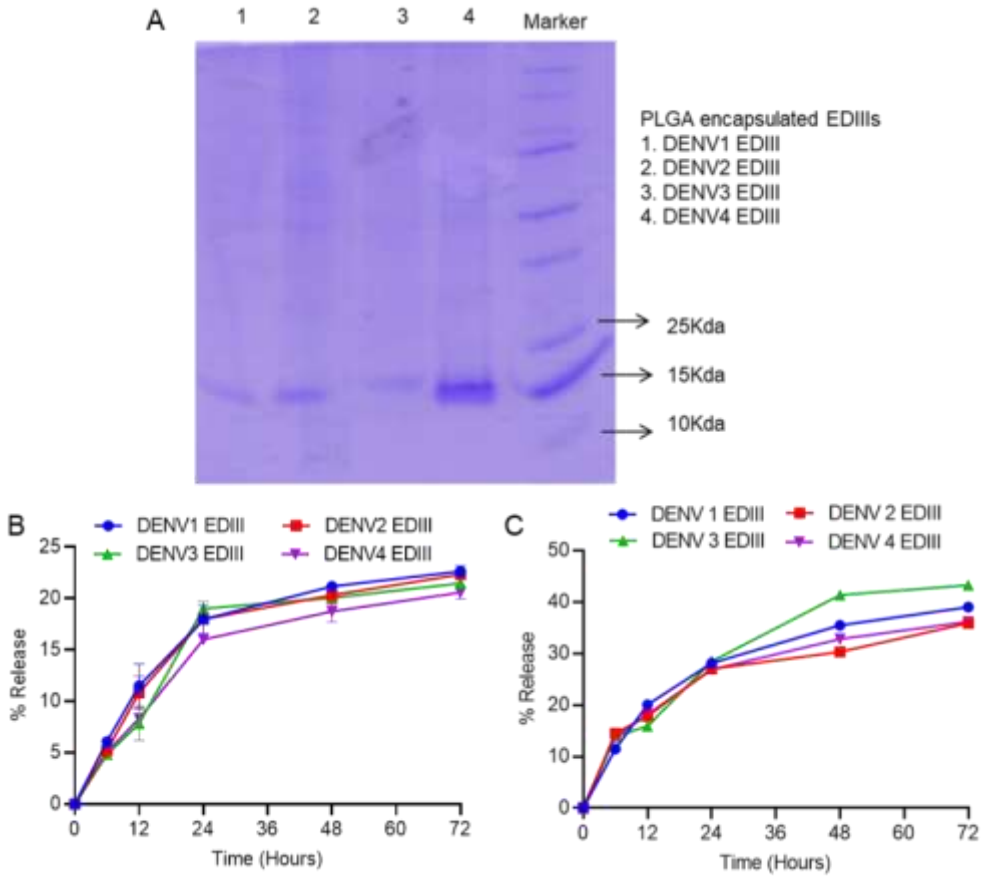


Figure 26. Loading of EDIII in PLGA nanoparticles and its release kinetics.

A) Loading of EDIII in PLGA nanoparticle analyzed by SDS-PAGE. Lane 1, 2, 3, and four

represent the encapsulated EDIII antigen from DENV1, DENV2, DENV3, AND DENV4 respectively (B) In vitro release profile of EDIII loaded PLGA nanoparticles evaluated by suspending into the encapsulated PLGA nanoparticle in 1× PBS followed by incubation at 37°C. Sampling was done at defined time points. Released antigens were measured using the BCA protein estimation method. (C) Antigen release profile under acid condition (pH = 5) which emulates the pH of endolysosomal compartment. Data are representative of one of three independent experiments.

4.2.3 **Tetravalent formulation of EDIII encapsulated PLGA nanoparticle along with TLR adjuvants elicits a robust antigen-specific effector T cell response**

Vaccinology in the modern era is challenged with several problems including antigen delivery and adjuvating (Gregory et al., 2013). Here, we have explored the efficacy of PLGA nano delivery system in enhancing the immunogenicity of vaccine antigens using EDIII antigen of DENV. PLGA is a biocompatible FDA approved material and has been shown immunomodulatory properties (Kasturi et al., 2011). We examined the potential of PLGA nanoparticles with and without TLR agonist in eliciting the potent antigen-specific effector T cell response against the EDIII domain of dengue virus envelop protein. For that we primed mice with PBS, tEDIII, tEDIII+TLRs, PLGA (tEDIII) and PLGA (tEDIII)+TLRs, in a separate group and gave a booster of the same formulation post 14 days of priming. After immunization with the prime and booster dosages of formulation, the animals were sacrificed on day 28 and an ex-vivo recall assay of splenocytes was performed, which shows that tetravalent formulation of PLGA (tEDIII) nanoparticles induces a significant amount of antigen-specific CD4+ and CD8+ T cells response as compare to tetravalent soluble antigens alone and it was further showed synergy in term of enhanced T cells response in the groups administered with TLRs adjuvant. Notably, the frequency of antigen-specific IFNγ and IL-2 secreting polyfunctional CD4+ (Figure 27) and CD8+ (Figure 28) cells enhance in the animals immunized with a tetravalent formulation of nanoparticle with TLRs as compared to antigen alone or control group.

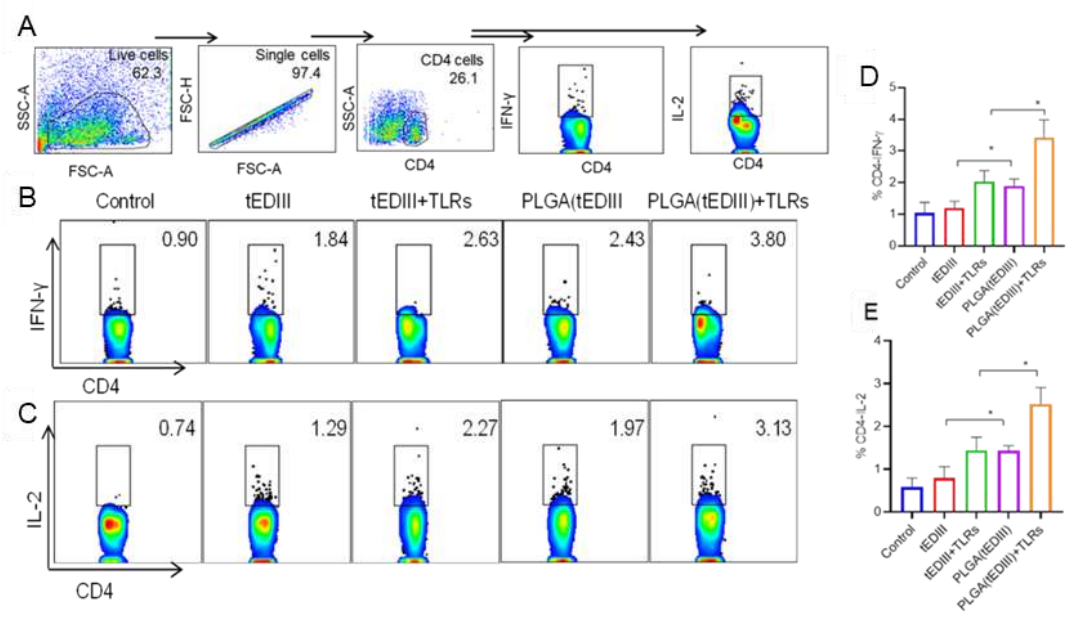


Figure 27. Tetravalent formulation of EDIII encapsulated PLGA nanoparticles along with TLR adjuvants elicits robust antigen-specific T cell responses in mice.

(A) Gating strategy for selecting CD4+IFN-γ and IL2 cells. Antigen-specific T cell responses following immunization in mice were measured by the antigenic restimulation of splenocytes obtained from day 28 of immunized mice. Flow cytometry analysis of DENV EDIII specific T cell response. (B, D) antigen-specific IFN-γ producing CD4+ and (C, E) IL2 producing CD4+ cells. (B) and (C) is a representative FACS plot, box plot (D) and (E) represents the average percentage of IFN-γ and IL2 producing CD4+ cells respectively from the two independent experiments each n = 5 per treatment group per experiment. Data are represented as the mean of multiple biological and experimental replicates with standard error of mean and value of significance $p < 0.05$ was considered as significant.

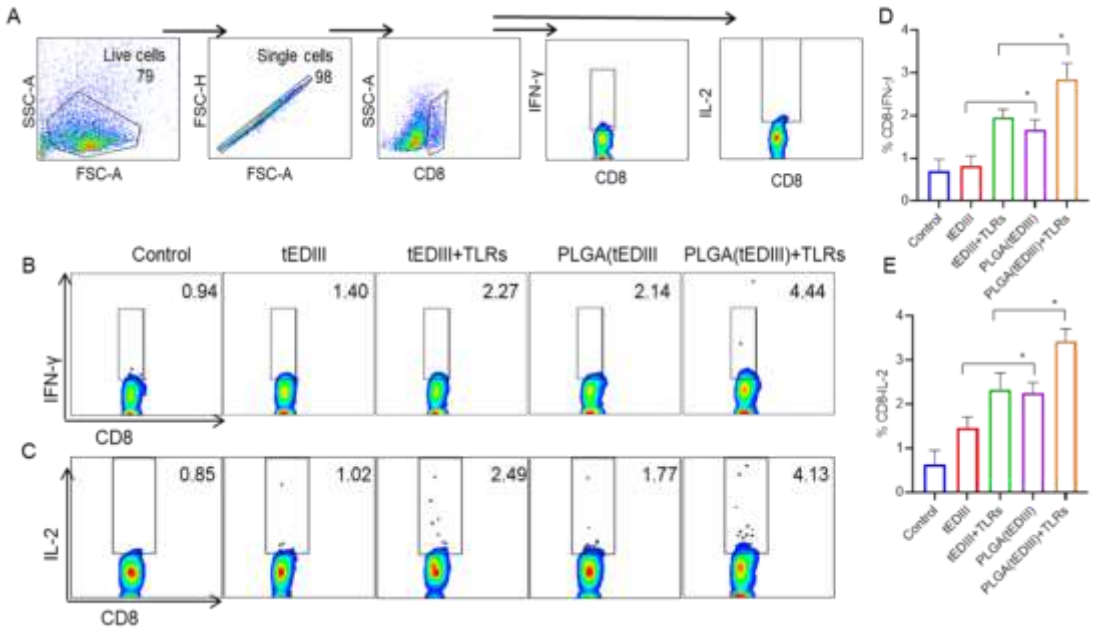


Figure 28. Antigen-specific CD8+ responses.

(A) Gating strategy for selecting CD8+IFN-γ and IL2 cells. Antigen-specific IFN-γ (B, D) and IL2 producing CD8+ (C, E) T cell response were analyzed by flow cytometer in the splenocytes of the immunized mice stimulated with the antigens. (B) and (C) is showing representative FACS plot, box plot (D) and (E) represents the average percentage of IFN-γ and IL2 producing CD8+ T cell respectively from the two independent experiments each n = 5 per treatment group per experiment. Data are represented as the mean of multiple biological and experimental replicates with standard error of mean and value of significance $P < 0.05$ was considered as significant.

A similar pattern of T cell response was also observed in the PBMCs (Figure 29) thereby suggesting that encapsulation of the vaccine antigens in the PLGA nano delivery system enhances the immunogenicity of the soluble purified antigens which alone if administer manifests weaker response. Further supplementary with nano delivery system with TLR adjuvant showed synergy in terms of enhancing the antigen-specific responses.

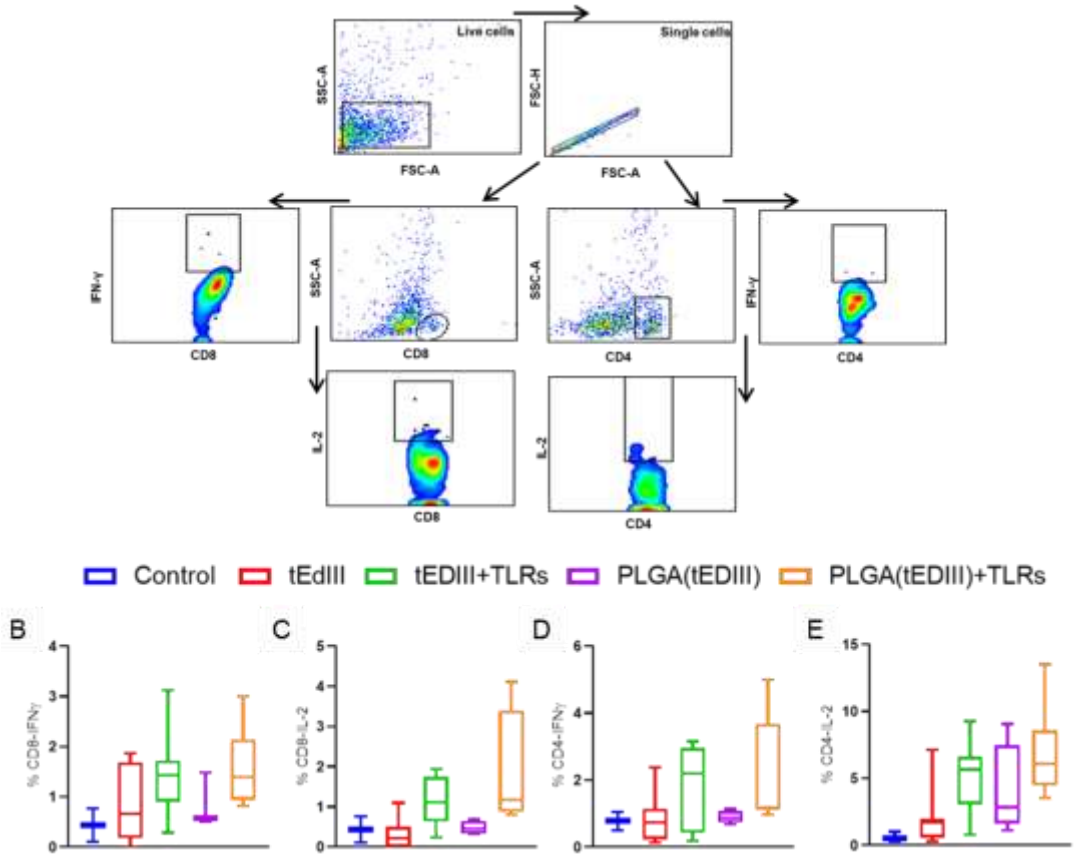


Figure 29. PLGA nanoparticle loaded with tetravalent EDIII along with TLRs elicits potent antigen-specific T cell response in PBMCs of the mice.

(A) Gating strategy of IL-2 and IFN-γ expressing CD4+ and CD8+ T cell from peripheral blood mononuclear cells (PBMCs). Flow cytometry analysis of antigen-specific IFN-γ producing CD8+ (B), CD4+ (D) T cell and IL2 producing CD8+ (C), CD4+ (E) T cell in PBMCs. The box plot represents the average percentage of double-positive cells and represents the data from two independent experiments, each with n = 5 mice per treatment group per experiment.

4.2.4 **Tetravalent nano formulation of dengue (1-4) EDIII antigen in combination with TLR agonists significantly enhances the antigen-specific antibody responses in the immunized mice**

The quality and quantity of antibody responses are mediated by T cells help (Pasare and Medzhitov, 2005). T cell microenvironment modulates the humoral arm of adaptive immunity by modulating the effector and memory B cell response. To modulate and sustain

this process, activated naïve CD4+ T helper cells differentiate into T follicular helper (Tfh) cells that ultimately help in B cell differentiation into high affinity antibody producing plasma cells and memory B cells (Fillatreau, 2014). Promising results of robust antigen-specific CD4+ T cell response in the tetravalent formulation of PLGA nanoparticles along with TLRs coadjutants prompted us to evaluate the antigen-specific antibody response. So, antigen-specific total serum IgG level against all the serotypes (DENV1, DENV2, DENV3 and DENV4) was measured from serum after 14 days of antigen priming and it demonstrates that antigen loaded nanoparticles PLGA (tEDIII) induce significantly higher antibody response as compared to antigen alone (tEDIII) and additionally enhanced in the PLGA (tEDIII)+TLRs group (Figure 30 A-D). We observed further enhancement in total serum IgG level after secondary immunization which shows almost two-time increment in the PLGA (tEDIII), (tEDIII) +TLRs and PLGA (tEDIII)+TLRs immunized group as compared to antigens alone (tEDIII) (Figure 30 A-D).

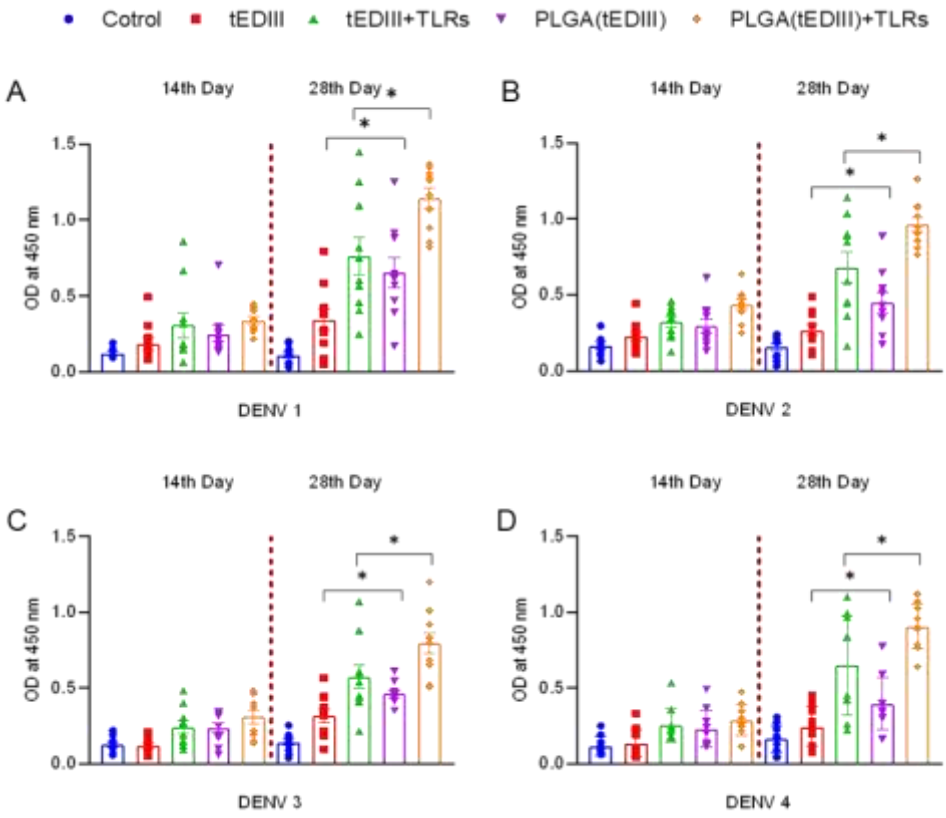


Figure 30 Tetravalent nano formulation of dengue (1–4) EDIII antigen in combination with TLR agonists significantly enhances the antigen-specific antibody responses in the immunized mice.

(A-D) Antigen-specific total serum IgG was measured in mice after 14 days and 28 days of immunization from all the treatment groups (PBS, tEDIII, tEDIII + TLRs, PLGA (tEDIII) and PLGA (tEDIII) + TLRs) using ELISA in which plates were coated with the DENV 1 EDIII (A), DENV 2 EDIII (B), DENV 3 EDIII (C), and DENV 4 EDIII (D). Data are represented as the mean of multiple biological and experimental replicates with a standard error of mean and value of significance $P < 0.05$ was considered as significant.

In another set of experiments, we measured the titer of the antibody from the serum of immunized mice. The antibody titer was found to be significantly high in PLGA (tEDIII)+TLRs group as compared to other immunization groups (Figure 31 E). The high titer value of the antibody also indicates the quantity and affinity of produced antibody which might help in neutralizing the virus. Furthermore, we analyzed the level of IgG subclasses IgG1, IgG2a and IgG2b from the serum of immunized mice. We observed that level of IgG subclasses was significantly enhanced in the PLGA (tEDIII)+TLRs as compared to other groups (Figure 32).

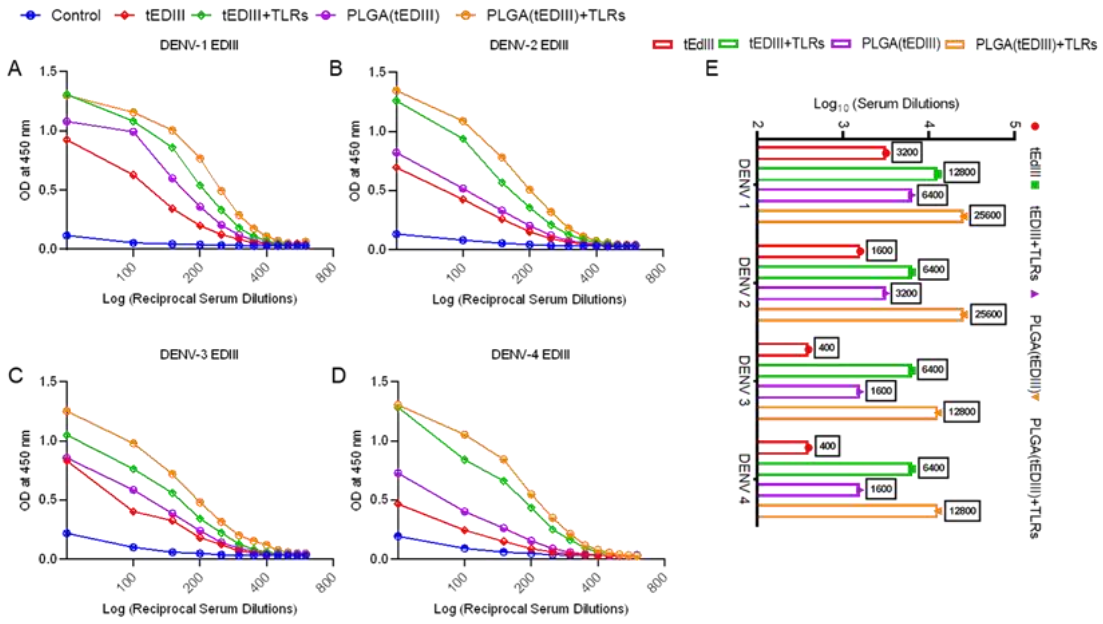


Figure 31. Antigen-specific antibody titer against all the four EDIII antigens.

(A-E) Antibody titer was measured by the indirect ELISA in which antigen-coated DENV 1 EDIII (A), DENV 2 EDIII (B), DENV 3 EDIII (C), and DENV 4 EDIII (D) plates were incubated with serially diluted serum (1:100 to 1:20480). (E) Antibody titers were calculated by the reciprocal value of the highest dilution at which absorbance values were ≥twice the value of control in the same dilution series.

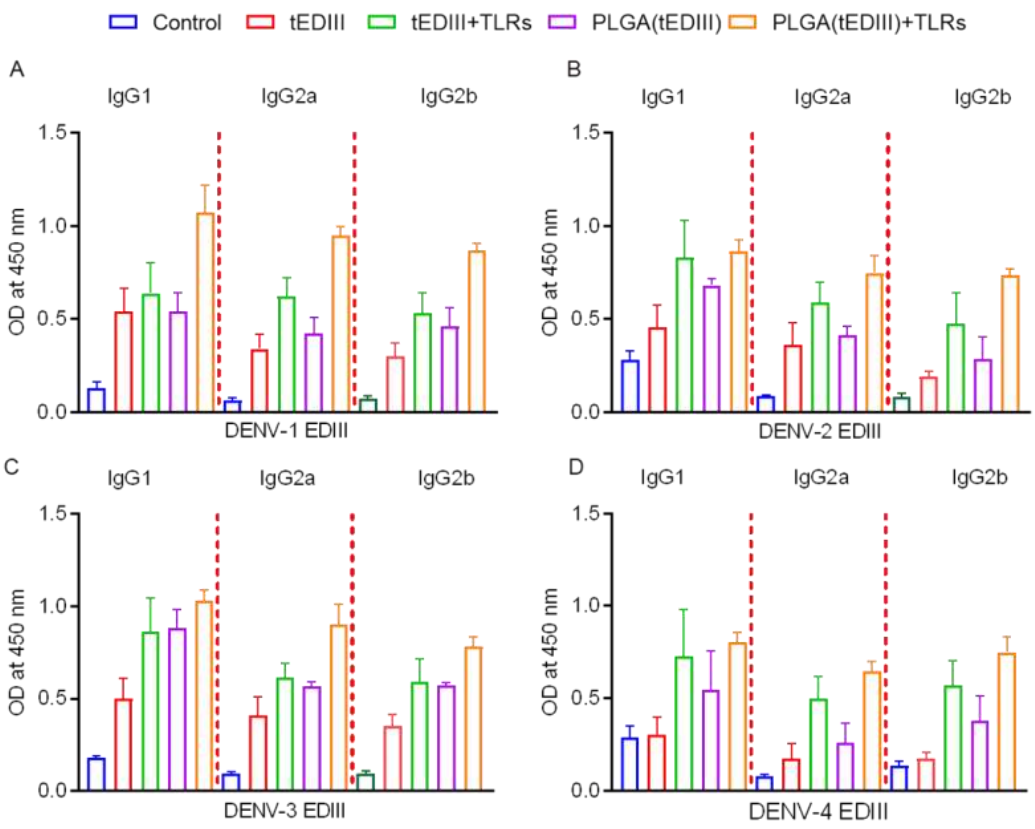


Figure 32. PLGA (tEDIII)+TLRs induce enhanced IgG subclasses as compared to other groups.

(A-D) Antigen-specific IgG subclasses in the serum were measured by indirect ELISA for all the serotypes (A) DENV 1, (B) DENV 2, (C) DENV 3 and (D) DENV 4. Data are represented as the mean of multiple biological and experimental replicates with a standard error of the mean.

4.2.5 **Tetravalent EDIII formulation of PLGA nanoparticles along with TLR agonists elicits the Tfh and GC-B cell response.**

Germinal center (GC) is a temporary and important structure in the lymph node and spleen where B cell affinity maturation and thereafter differentiation into antibody secreting plasma cell and memory B cell happens, which is an important phenomenon for potent humoral immunity (Pasare and Medzhitov, 2005). This process is constantly supported and sustained by ZX cell which provides an important signal for the differentiation of the naïve B cell into the antibody producing plasma cell and memory B cell (Leo et al., 2011). Therefore, in this

study we isolated the cell from draining lymph node and stained for the Tfh marker (CXCR5+, PD-1+ and CD4+) (Jia et al., 2018) and GC-B markers (GL7+ and Ig+) (Kasturi et al., 2011) and analyzed under flow cytometry. Interestingly, we found that the frequency of Tfh cells (CXCR5+, PD-1+ and CD4+) in the PLGA (tEDIII) group are significantly high as compared to the (tEDIII) alone and even slightly better than (tEDIII)+TLRs. However, we found a profound enhancement in the PLGA (tEDIII)+TLRs (Figure 33 B, E) group suggesting that the encapsulation of antigens in PLGA nanoparticles in combination with TLR adjuvant trigger better programming of Tfh cells. Activated Tfh cells are destined to modulate the initiation process of the B cell affinity maturation and differentiation within the GC (Crotty, 2014) so the frequency of GC-B cell (GL7+ and Ig+) in lymph nodes was found to be significantly high in the PLGA (tEDIII)+TLRs immunized group (Figure 33 D, F).

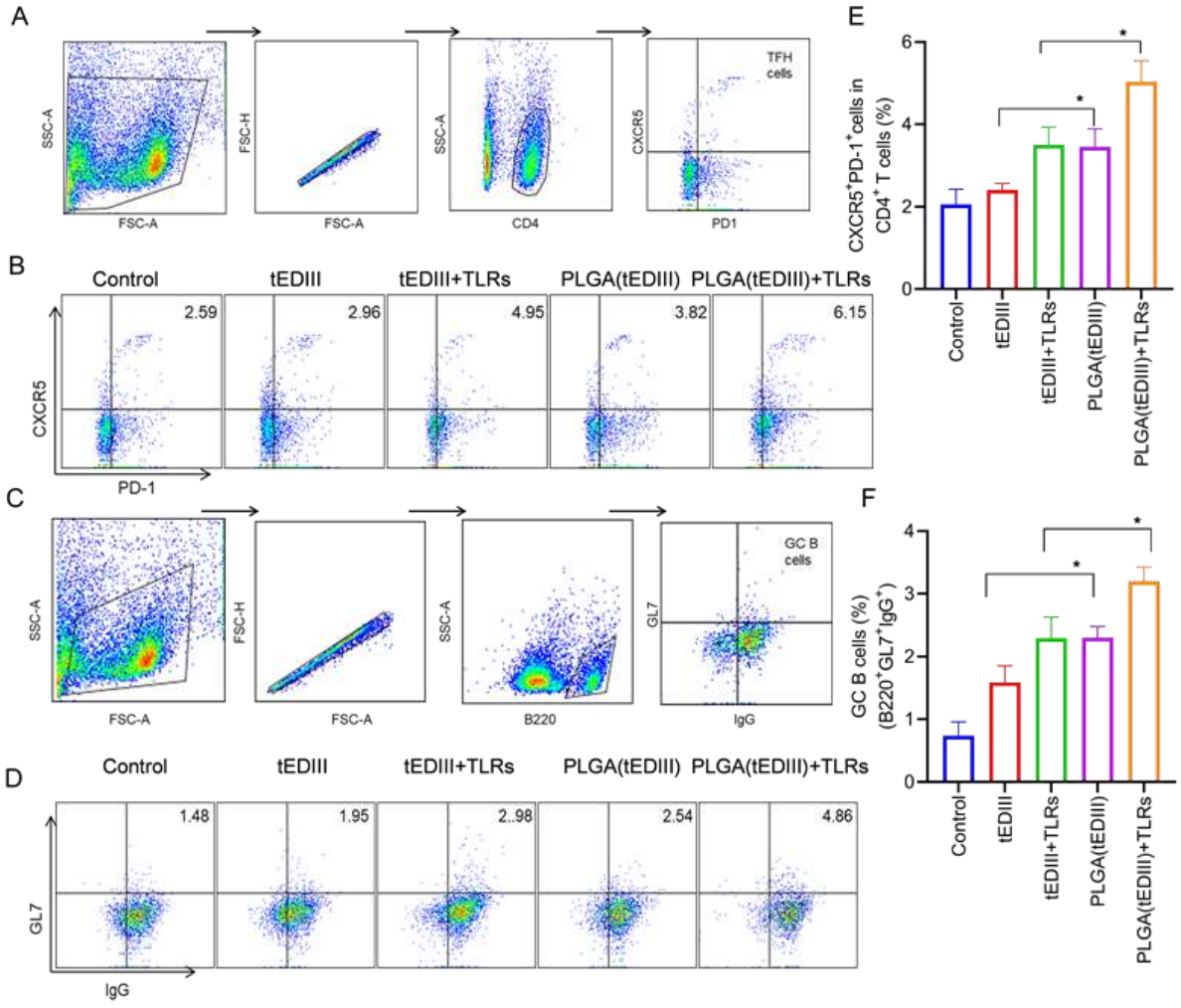


Figure 33. Tetravalent (DENV 1–4) EDIII formulation of PLGA nanoparticle along with TLRs agonist enhanced Tfh and GC-B cell response in the lymph nodes of immunized mice.

(A) Gating strategy for selecting Tfh cells and (C) GC-B cells. (B) Analysis of Tfh cells (B and E), and GC B cell (D and F) was carried out by measuring the frequency of these cells in the lymph nodes of the mice immunized with EDIII and PLGA encapsulated EDIII antigens from DENV (1–4) using flow cytometer. Double-positive CD4+ cells (CD4+, CXCR5+ and PD-1+) were considered as Tfh cells, whereas triple positive cells (B220+, GL7+ and IgG+) were considered as GC-B cells. Data are represented as the mean of multiple biological and experimental replicates with standard error of mean and value of significance $P < 0.05$ was considered as significant.

All the genes associated with initiation and sustenance of Tfh cell (Bcl-6, CXCR5, BCOR 1) and GC-B Cell (Bcl2, and IL-10) were upregulated in the immunization group which has induced Tfh and GC-B cell differentiation (Figure 34). This observation indicates that PLGA (tEDIII)+TLRs induces the reaction of GC efficiently which ultimately mounts strong humoral immune response by producing significant amount of IgG with relatively higher titer that is prerequisite for potent antiviral immunity.

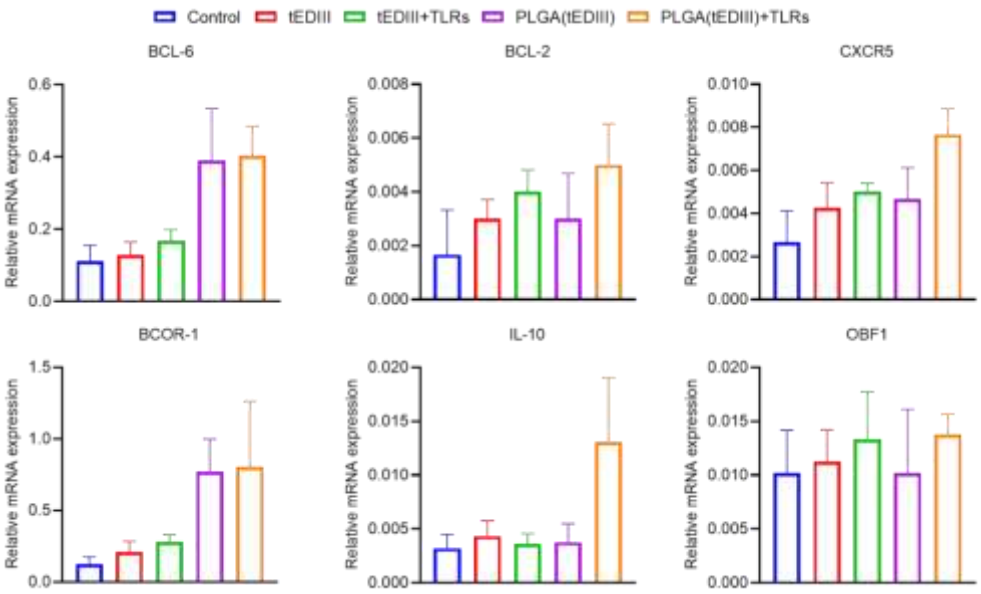


Figure 34. Nano formulation of DENV tEDIII induces upregulation of genes regulating Tfh cells and GC B cell maintenance.

qRT-PCR analysis of gene expression in the lymph node cells of immunized mice. The data were presented as relative mRNA expression. Data are represented as the mean of multiple biological and experimental replicates with a standard error of the mean.

b) rOMV

4.2.6 Cloning, expression, and purification of rOMVs expressing EDIII from all the four serotypes of DENV

The cloned constructs for DENV EDIIIs in pET28a vector were used as a template for cloning serotype-specific EDIIIs in pBAD-ClyA vector to produce rOMVs. Restriction sites XbaI and HindIII were introduced at 5' and 3' ends of EDIII through forward and reverse primers. The PCR was run for 35 cycles of denaturation (30 sec, 95°C), annealing (30 sec) and extension (25 sec, 72°C) in a Thermocycler (Applied Biosystems). The correctly sized amplification products were obtained for all four serotypes. The inserts obtained from PCR were double digested using XbaI and HindIII restriction enzymes. pBAD-ClyA vector was also digested simultaneously using the same pair of restriction digestion enzymes to generate sticky ends compatible with the insert and double digested fragments were

retrieved from the gel by using Nucleospin gel/PCR cleanup (Machery Nagel). The digested insert was ligated to vector using DNA Ligase (NEB) followed by the transformation in *E. coli* DH5-alpha cells. Colonies were selected based on antibiotic resistance and plasmids were isolated from selected colonies. Double digestion using the same pair of enzymes (XbaI and HindIII) was performed to confirm the clones. The digested plasmids were subjected to agarose gel electrophoresis and bands corresponding to the size of EDIII were observed for all four serotypes of DENV which confirms the successful cloning of EDIIIs in pBAD-ClyA vector (Figure 35).

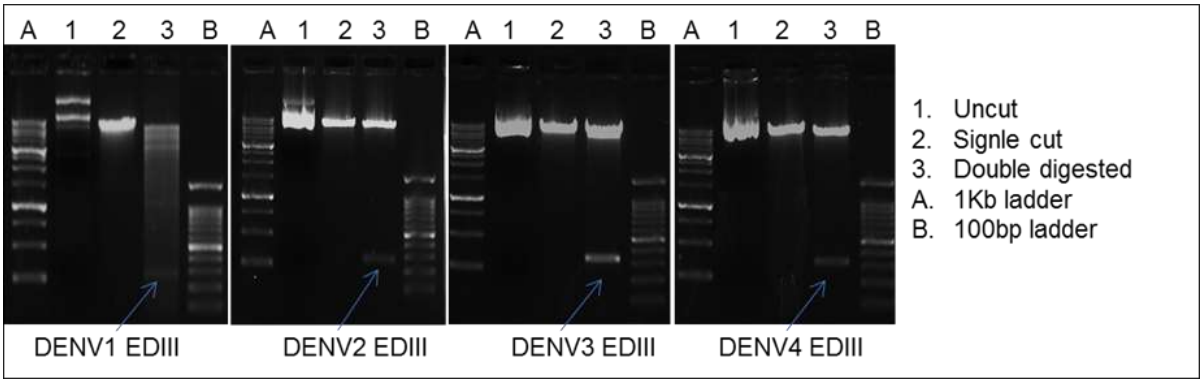


Figure 35. Cloning of DENV EDIII from all the four serotypes in pBAD-ClyA for expression of OMVs.

Double digestion using XbaI and HindIII to confirm clones in pBAD-clyA vector for expression and purification of rOMVs expressing EDIIIs from all the four serotypes of DENV (Insert size: D1, D2, and D4 273bp; D3 366bp).

After cloning we proceeded for expression and purification of rOMVs expressing EDIIIs according to the methods described earlier with modifications (Watkins et al., 2017) as well as the methods we have adopted to purify rOMVs expressing GFP proteins. Vesicles were isolated by ultracentrifugation and resuspended in sterile PBS. Total protein from rOMVs was determined by BCA estimation and an equal amount of protein (vesicles) was loaded in SDS-gel. rOMVs expressing EDIIIs were confirmed using anti-sera from EDIII immunized mice and anti-His was used for confirmation of empty OMVs (Figure 36).

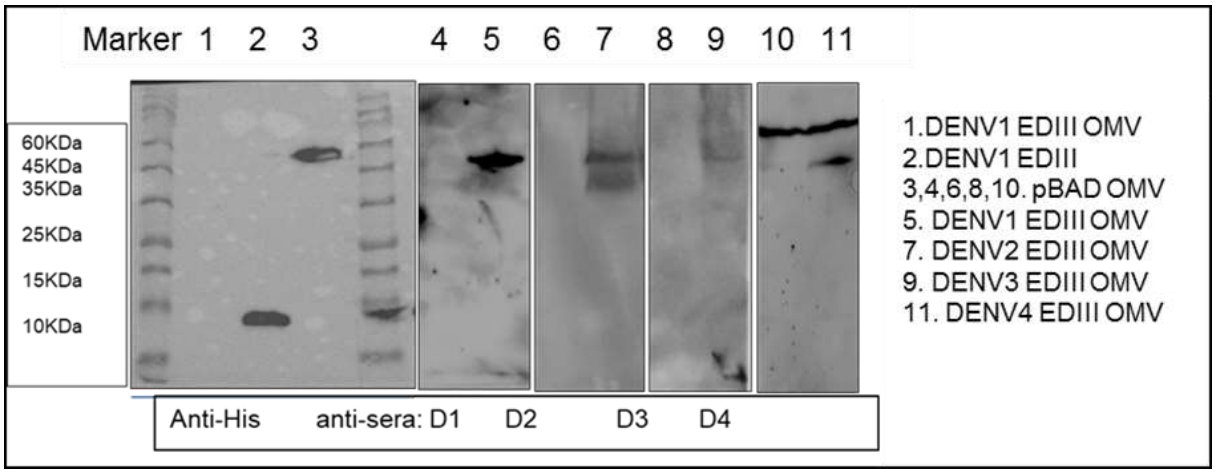


Figure 36. Expression and purification of OMV expressing EDIII from DENV serotypes (1-4).

Western blotting using anti-His and anti-sera from EDIII immunized mice (D1, D2, D3, D4) to confirm purified empty OMVs and rOMVs expressing EDIIIs respectively.

4.2.7 **Physical characterization of rOMVs**

Isolated rOMVs displayed with DENV EDIII of all the four serotypes were characterized for their shape and size by TEM and the result shows that vesicles are spherical in shape and less than 100 nm in size (Figure 37 upper panel**)**. Size distribution of vesicles was analyzed by DLS, and data has shown that size of majority of rOMVs is less than 100 nm except for DENV1 EDIII rOMVs (Figure 37 lower panel**).** The size obtained in the DLS is bigger than the TEM is most likely because DLS measured hydrodynamic radii that includings size of the water shell around the particle whereas TEM measured the size of dry particles.

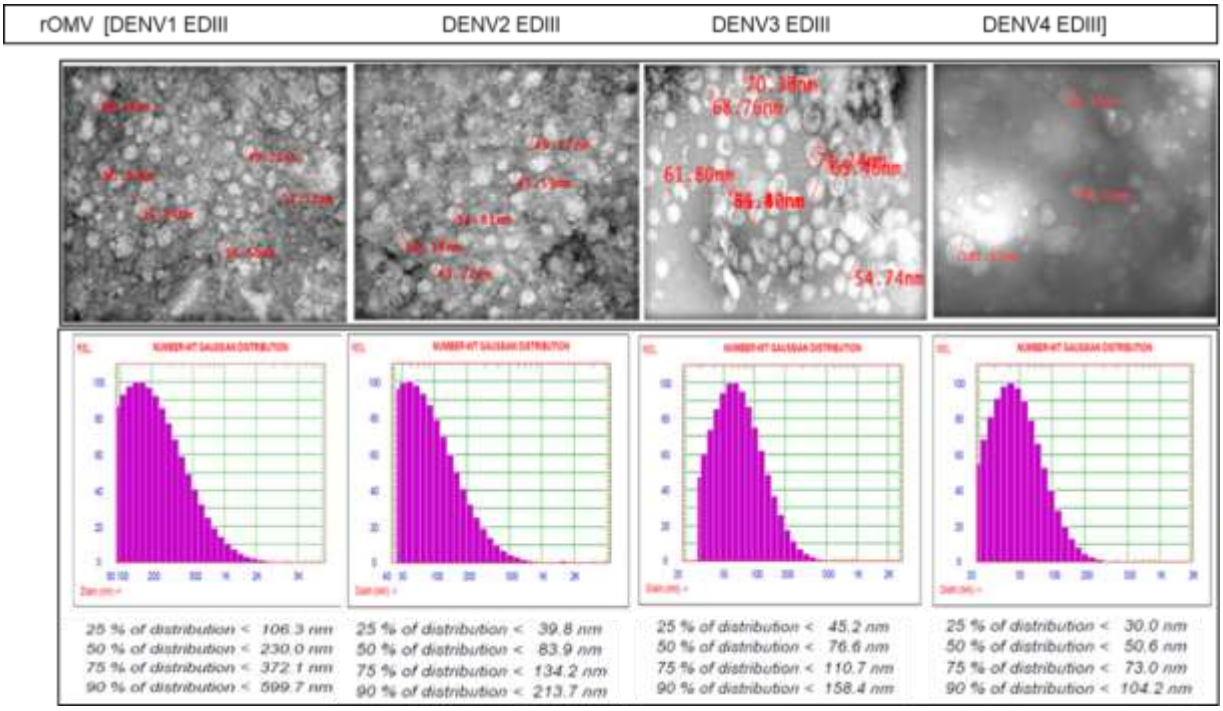


Figure 37. Physical characterization of rOMV expressing EDIII.

(A) TEM analysis of rOMVs samples stained with uranyl acetate and layered over the carbon coated copper grids. B) Size distribution of various rOMVs analyzed by DLS.

4.2.8 **rOMVs expressing DENV EDIII induce strong antigen-specific T cell response in the mice.**

After successfully designing and characterization of rOMVs displayed with DENV EDIII antigen, we further explored the ability of rOMVs in stimulating the antigen-specific effector T cell response against the dengue antigen. To investigate that, the immunized mice with different formulations were sacrificed after 28 days, splenocytes were collected from the spleen and cells were reactivated with the same antigen in the culture medium. Recall assay data has shown that rOMV(tEDIII) formulation induces considerable degree of antigen-specific CD4+ and CD8+ T cells responses as compared to EDIII antigen alone and other control groups. Specifically, the frequency of polyfunctional T cells which is known as IL-2 and IFN-γ secreting CD4+ (Figure 38 A-E) and CD8+ T cells (Figure 39 A-E) has enhanced in the animals those were immunized with rOMV(tEDIII) formulation compared to free antigens or control groups polyfunctional T cells played an important role in inducing the effective and durable immunity against viral antigens.

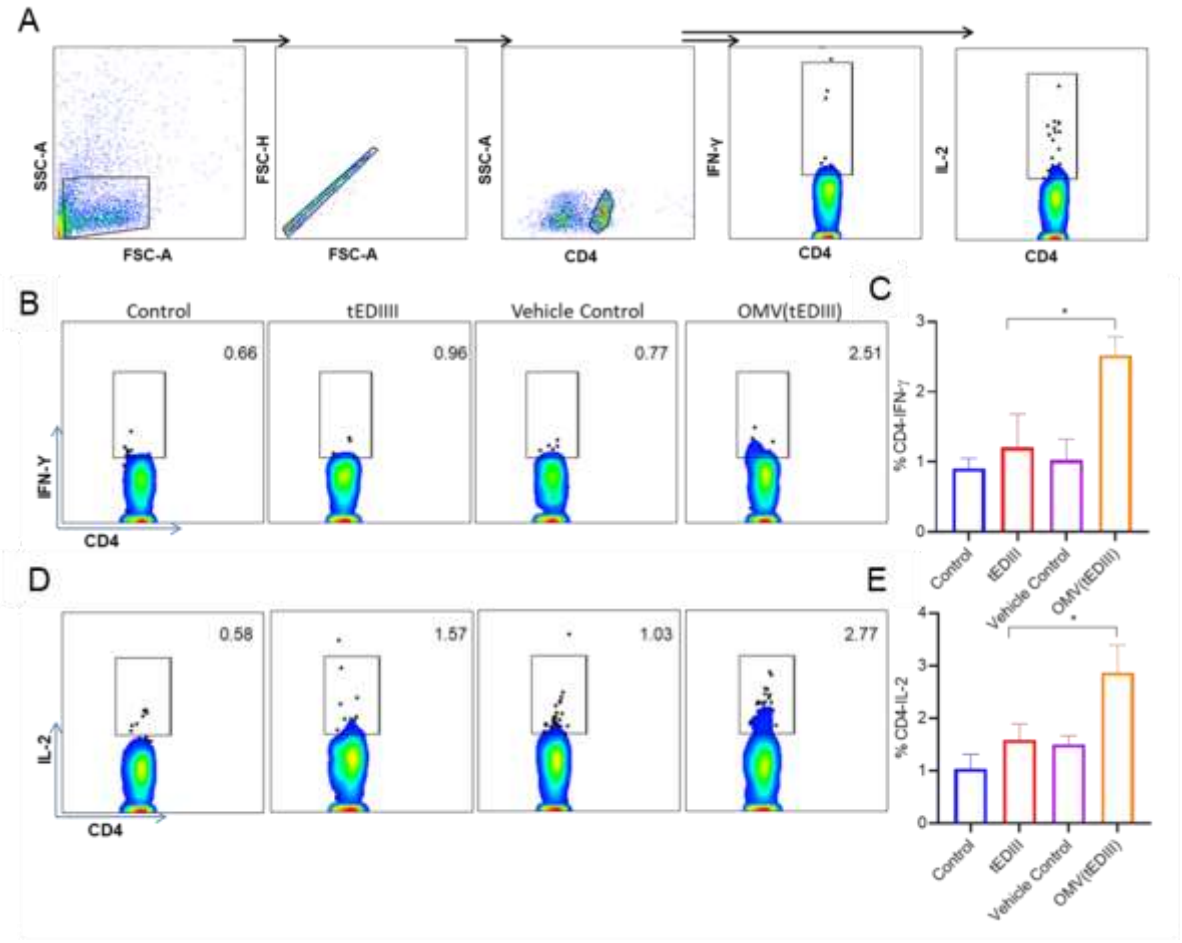


Figure 38. rOMVs engineered with DENV EDIII induce strong antigen-specific CD4+ T cell response in the mice.

(A) Gating strategy to distinguish cells populations, (B and C) Frequency of IFN-γ secreting CD4+ cells, D and E) frequency of IL-2 secreting CD4+ in various immunization groups; Control(PBS), tEDIII (soluble EDIII), Vehicle Control (empty OMVs), OMV(tEDIII) OMV expressing EDIII.

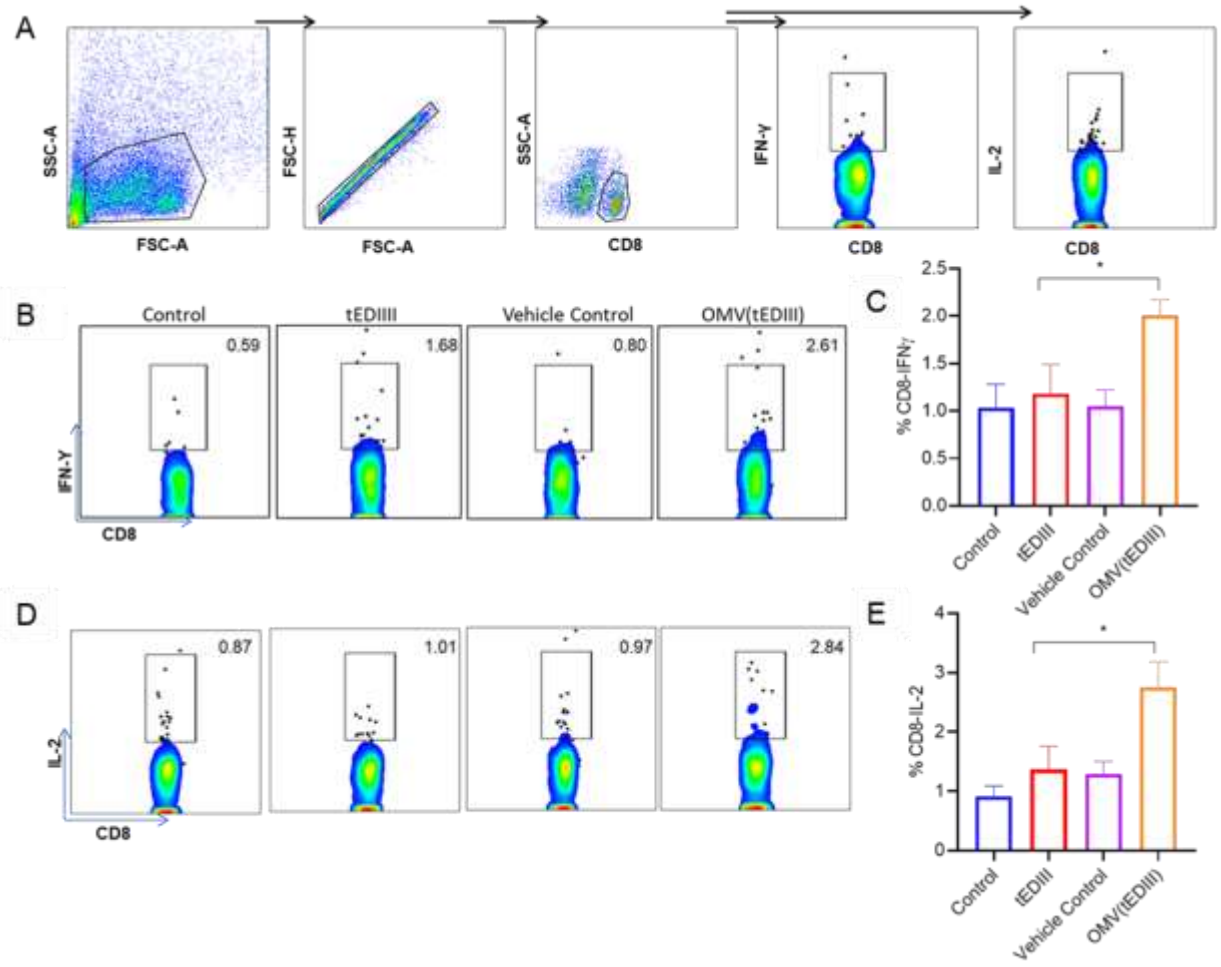


Figure 39. rOMVs engineered with DENV EDIII induce strong antigen-specific CD8+ T cell response in the mice.

(A) Gating strategy to identify defined cell population, B, E) Best representative image of FACS plot C, E) and mean % frequency distribution of IFN-γ secreting CD4+ cells and IL-2 secreting CD4+ cells, respectively. Each data set is a representative of 10 animals (5×2) and data is presented as a mean with SEM. Value of significance $*P < 0.05$ is considered as significant data.

4.2.9 rOMV expressing DENV EDIII in tetravalent formulation induces a significant antigen-specific antibody response

The effectiveness of a successful viral vaccine is measured by the propensity of B cell response in producing the antigen-specific high affinity antibody that ultimately helps in neutralizing the viruses. Therefore, DENV EDIII specific total IgG from serum was

measured by indirect ELISA. Result has shown that antigen expressed rOMVs induces significantly higher antibody level against all the four distinct serotype antigens as compared to antigens alone or control groups after 14 days of priming. After the booster dose, the level of antibody was further enhanced on day 28 (almost twice the value of the fourteenth day) in the groups of animals that were immunized with rOMV (tEDIII) as compared to (tEDIII) alone or in controls groups (Figure 40).

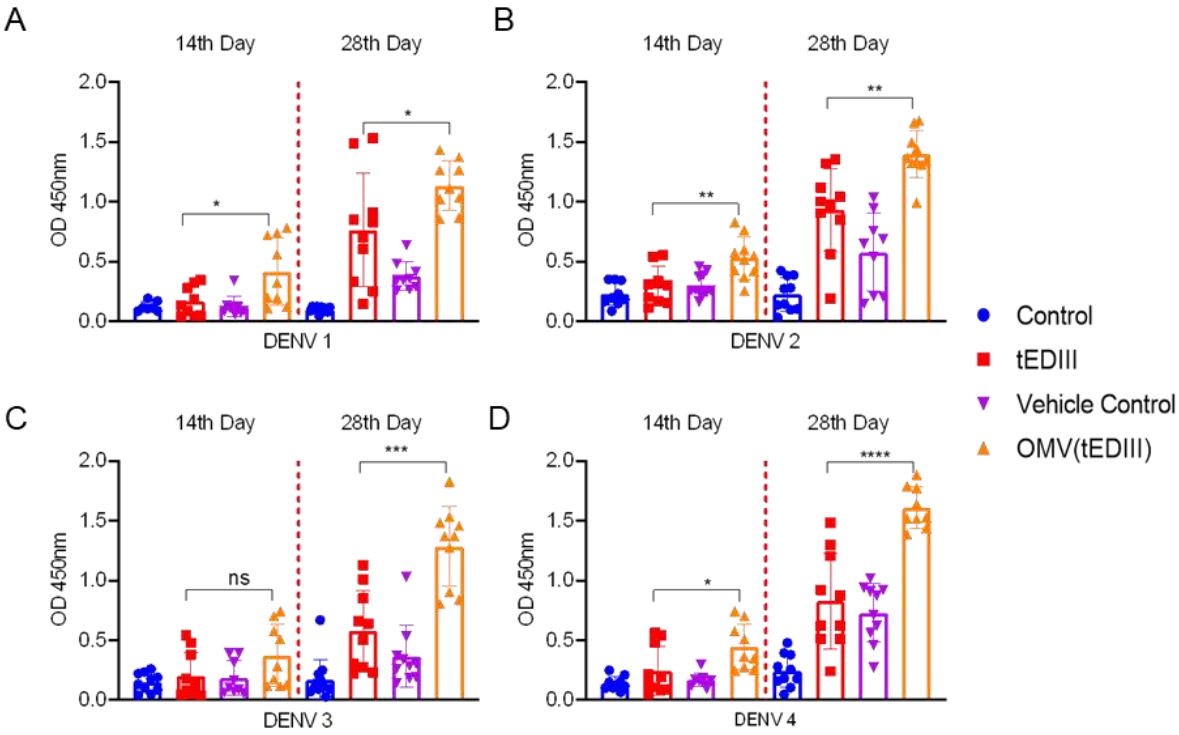


Figure 40. rOMV expressing DENV EDIII in tetravalent formulation induces significant antigen-specific antibody response.

Antigen-specific total IgG from serum of mice was estimated by indirect ELISA at day 14 and 28 post immunization A) DENV1, B) DENV2, C) DENV3 and D) DENV4. Data of each groups contains 10 animal (5×2) and data represented as mean with SEM. Value of significance *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ and ****$P < 0.00005$).

4.2.10 **Tetravalent (DENV 1-4) EDIII expressed in OMVs enhanced Tfh and GC-B cell response in the lymph nodes of immunized mice**

Strong humoral immunity plays a vital role in tackling viral infections by producing abundantly high affinity antibodies. GC is an important temporary structure in the lymph

nodes where specialized B cell differentiates into memory B cell and longlived antibody-secreting plasma cells with the help of $CD4^+$ T cells which is known as Tfh cells. So, in the current study, cells were isolated from the draining lymph node of immunized mice and stained with fluorophore-conjugated Tfh marker antibodies ($PD\text{-}1^+$, $CXCR5^+$ and $CD4^+$) and GC B cell (GC-B cell) marker antibodies ($B220^+$, $GL7^+$ and Ig^+) which further analyzed by flow cytometry. Remarkably, result has shown that frequency of Tfh cells ($PD\text{-}1^+$, $CXCR5^+$, and $CD4^+$) in the animals immunized with rOMV(tEDIII) formulation are significantly enhanced as compared to the (tEDIII) or control groups (Figure 41 A-B). Activated Tfh cells are known to assist the process of B cell affinity maturation and differentiation in the GC. Therefore, we measured the frequency GC-B cell in the lymph node and result suggest that the frequency of GC-B cell ($GL7^+$ and Ig^+) are remarkably high in the rOMV(tEDIII) immunized group as compared to other groups (Figure 41 C-D).

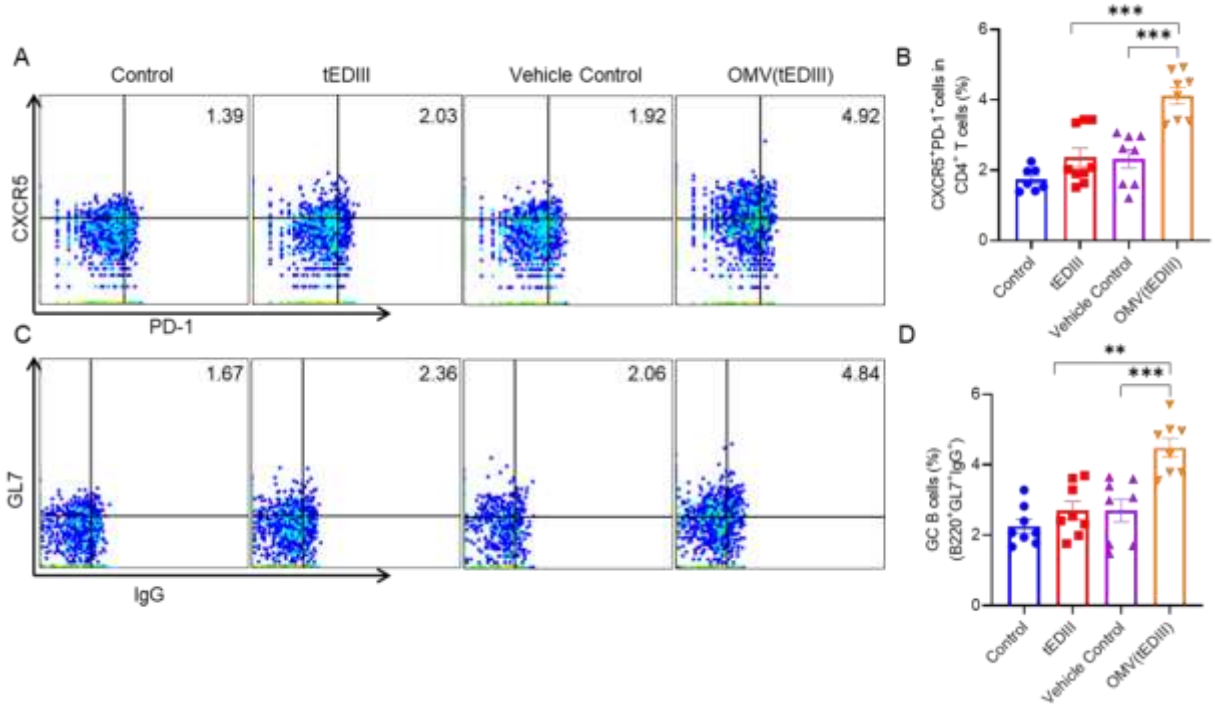


Figure 41. Tetravalent (DENV 1-4) EDIII expressed in OMVs enhanced Tfh and GC-B cell response in the lymph nodes of immunized mice.

(A) FACS plot of best representative data of Tfh cells (CXCR5+ and PD-1+ cells) B) % frequency distribution of triple-positive (CXCR5+, CD4+, PD-1+ cells) Tfh cells C) FACS plot of representative data of GC-B cells (B220+, GL7+ and IgG+) D) % frequency

distribution of GC-B cells in various immunization groups. Each data sets contains 10 animals (5×2) and data represented as mean with SEM.

4.3 Objective 3

Dissecting the potency of the EDIII-based tetravalent nano-vaccine formulation in terms of neutralizing capability against all the serotypes of DENV (1-4)

4.3.1 Propagation and Serotyping of DENV serotypes

All the four serotypes of DENV were propagated in C636 cells in in vitro conditions and the propagated viruses were further identified through serotyping. Cells infected with viruses showed CPE on the 5^{th} or 6^{th} day of infection (Figure 42 A). Viruses were harvested after observation of the CPE effect and RNA was isolated from all the serotypes and analyzed by semiquantitative RT-PCR. The amplicon size of 511 bp was obtained upon amplification with primers D1 and D2 (Lanciotti et al., 1992) which indicates the presence of DENV. Further to identify serotypes of DENV, the nested PCR was performed using forward primers D1 and reverse erotype-specific primers (TS1, TS2, TS3 and TS4) for DENV 1, DENV 2, DENV 3 and DENV 4, respectively. Correctly sized products were obtained for all the four serotypes as indicated (Figure 42 B-C).

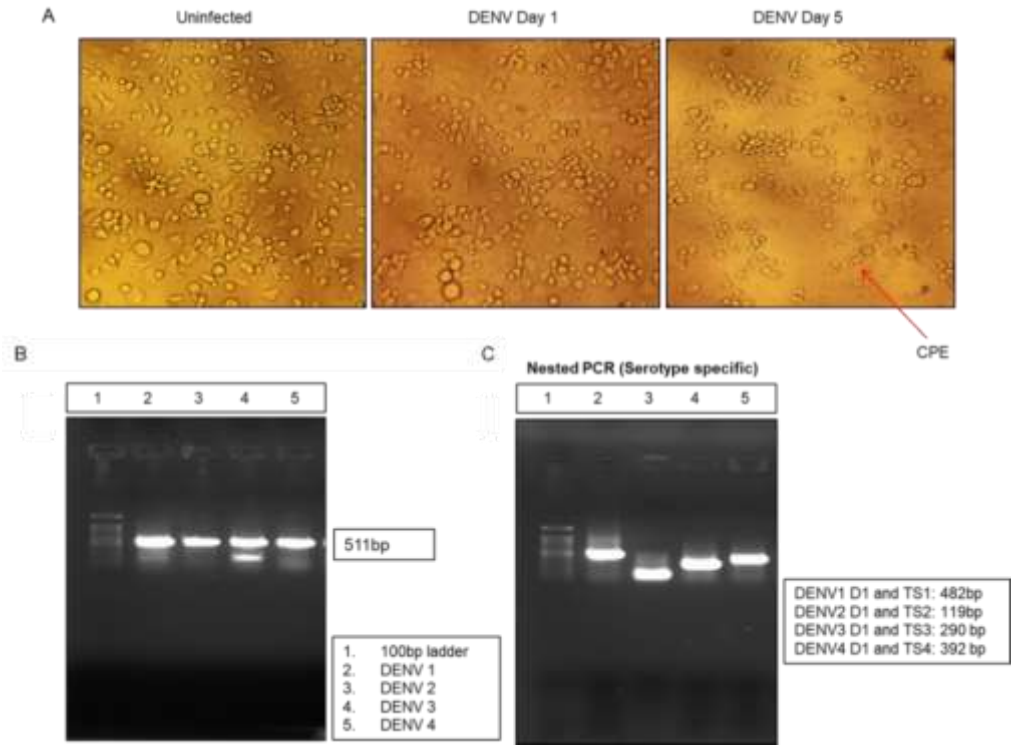


Figure 42. DENV Propagation and Serotyping.

(A) Representative image of CPE effect observed on day five in DENV 1 infected in C636 cells. (B) Gel image showing amplicon of 511 bp size obtained by Semiquantitative RT-PCR. (C) Agarose gel represents the amplified products from nested PCR using serotype-specific primers (TS1, TS2, TS3, and TS4) for DENV 1, DENV 2, DENV 3 and DENV 4, respectively.

4.3.2 **Quantification of propagated viruses**

After identification and serotyping of propagated viruses, the viruses were quantified by FACS based titration assay as described previously (Lambeth et al., 2005). The Vero cells were infected with different dilutions of virus and titers were calculated according to the formula given in methodology section (Figure 43, Table 4).

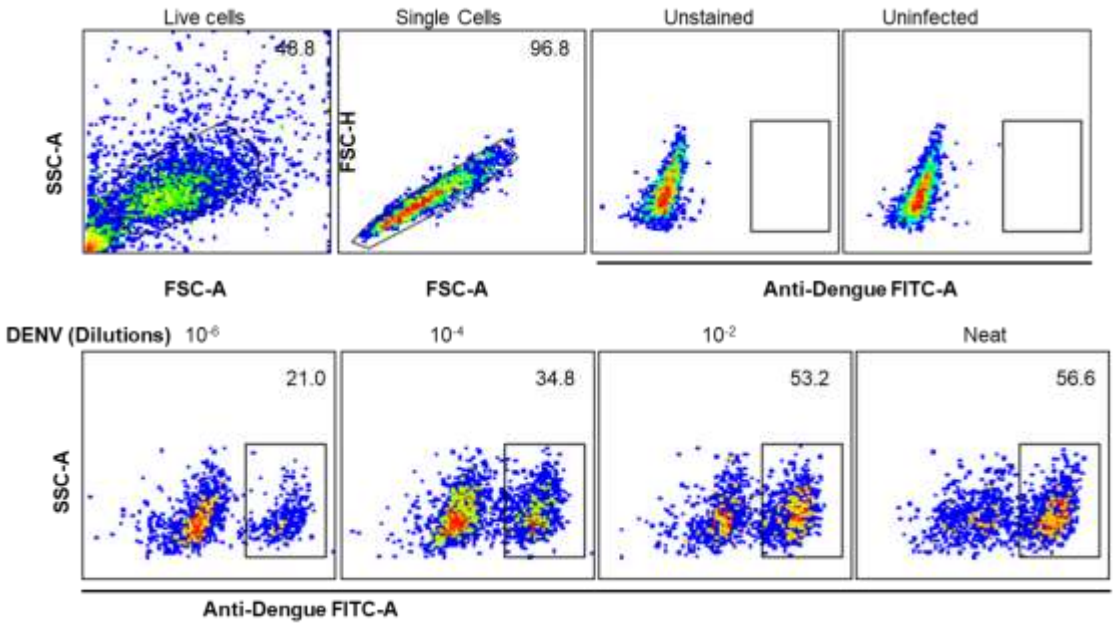


Figure 43. Quantification of DENV by FNT assay.

FACS representative plots showing Vero cells infected with different dilutions of DENV 1. Titers were calculated by FNT assay. This is a representative image for DENV 1, and all other serotypes were quantified by the same methodology.

Table 4. Titers of viruses calculated by FNT assay

Serotype	Viral Titer IU/ml
DENV 1	7.8×10^{7}
DENV 2	5.5×10^{7}
DENV 3	3.3×10^{7}
DENV 4	2.5×10^{7}

4.3.3 **PLGA encapsulated EDIII antigen of DENV (1-4) along with TLR agonists augments neutralizing antibodies capable of effectively neutralizing all the serotypes of Dengue virus (1-4)**

Virus neutralizing antibody is an important parameter to evaluate the efficacy of vaccine candidates and their formulation as the quantity and quality of neutralizing antibody are considered as surrogates of protective immunity against the various serotypes of Flavivirus. Keeping this in view, we have evaluated the virus neutralization efficacy of serum antibodies in the in vitro infection model. In this experiment, sera obtained after 28 days of full immunization from all the experimental groups were incubated with live virus separately and then subjected to infection on the Vero cells, and after defined time points, several infected cells were analyzed. Results show that serum from the PLGA (tEDIII)+TLRs significantly inhibits the virus infectivity of all the four serotypes to Vero cells at different dilutions expressed in terms of per cent normalized infection (Figure 44 A, C, E, and G) and FNT50 (Figure 44 B, D, F, and H) as compared to tEDIII. This finding corroborates the fact that nanoparticle in combination with TLR agonist co-adjuvants induces strong T cell-assisted B cell response, which produces high affinity antibody, therefore, efficiently neutralizing the dengue virus.

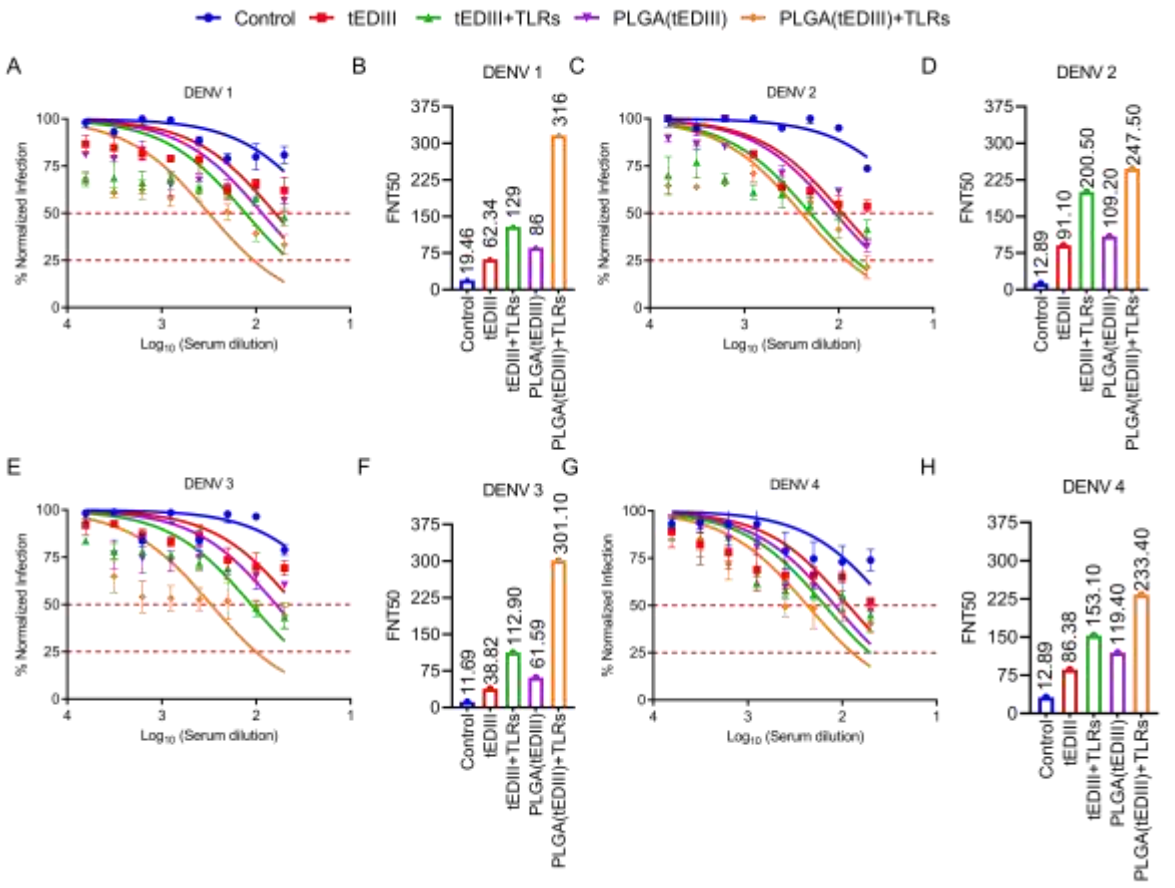


Figure 44. PLGA encapsulated EDIII antigen of DENV (1-4) along with TLR agonists augments neutralizing antibodies capable of effectively neutralizing all the serotypes of dengue virus (1-4).

In vitro virus neutralization potential of immunized serum was assessed at different serum dilutions by the FNT against all the four serotypes of dengue virus DENV 1 (A), DENV 2 (C), DENV 3 (E), and DENV 4 (G). FNT50 value of all the immunized serum was calculated by the dilution of serum at which 50% of virus get neutralized which was again estimated by FACS neutralization test for DENV 1 (B), DENV 2 (D), DENV 3 (F), and DENV 4 (H). Data are represented as the mean of multiple biological and experimental replicates with a standard deviation of mean and value of significance $p < 0.05$ was considered as significant.

4.3.4 **Serum from mice immunized with EDIII antigens expressing OMV (tEDIII) effectively neutralizes all the serotypes of Dengue**

Neutralizing antibodies (nAbs) are considered as a surrogate of protective immunity against the various Flaviviruses; therefore, it is an important functional parameter to evaluate the potency of vaccine candidates and their formulations. To evaluate virus neutralizing capability of serum antibodies, we chose in vitro infection model. In this setup, live viruses of all the four serotypes was mixed with the serum of fully immunized animal from all the immunization group separately and then subjected to infection on the Vero cells monolayer, after the defined time-points number of infected Vero cells were analyzed by flow cytometry. Obtained results demonstrate that serum from the OMVs (tEDIII) significantly inhibits the infectivity or provides protection to the Vero cells which is expressed in the terms of per cent normalized infection (Figure 45 A-D) and Flowcytometry based neutralization tests-50 (FNT50) (Figure 45 E-H) as compared to tEDIII alone or control groups. Significant potency of nAbs from the OMVs (tEDIII) substantiates the fact that antigen displayed over rOMVs induces the polyfunctional T cells which eventually modulate the potent B cell response, therefore, neutralizing the dengue virus.

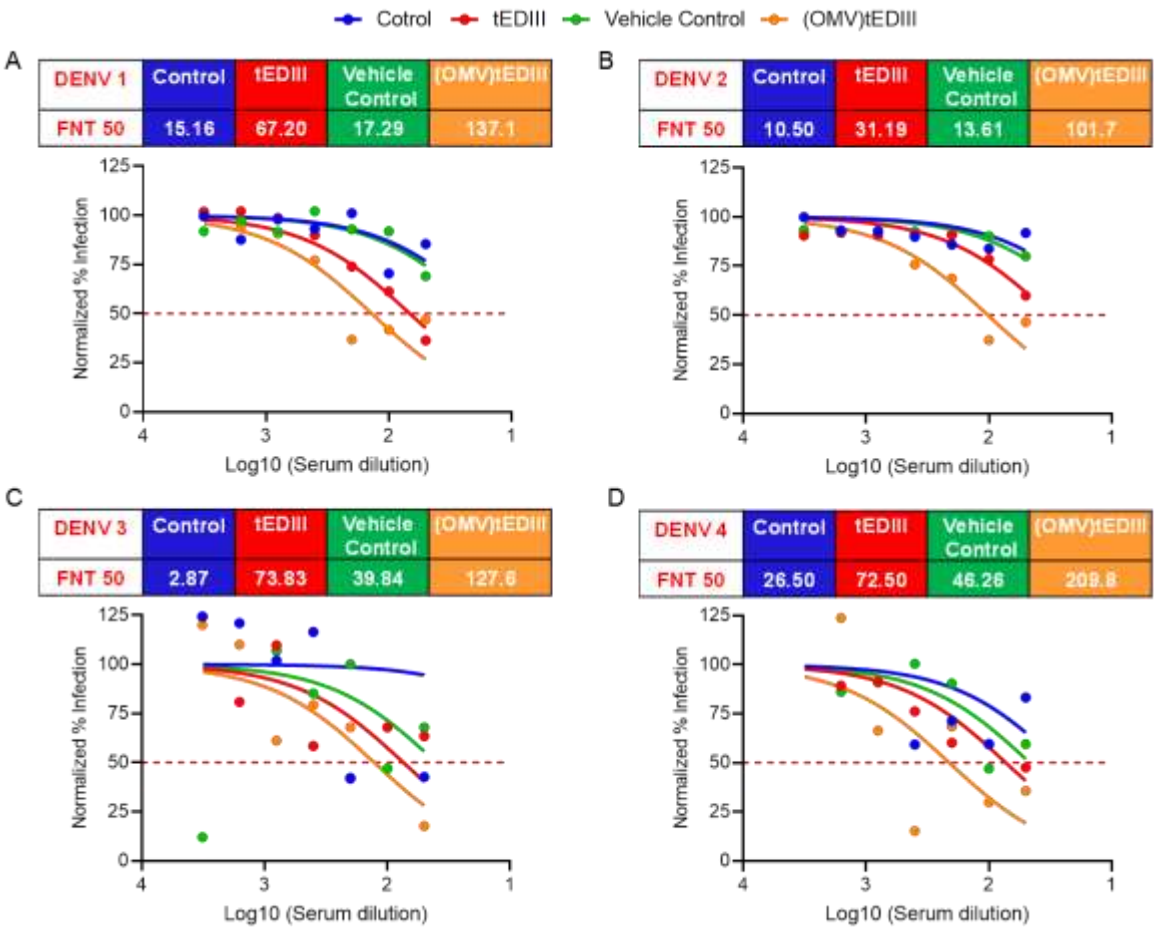


Figure 45. Serum from mice immunized with EDIII antigens expressing OMVs(tEDIII) effectively neutralizes all the serotypes of Dengue.

(A-D) Virus neutralizing capability of mice serum against all the four serotypes of DENV obtained from various immunization groups expressed as percent normalized infection at various serum dilutions. FNT-50 value represented as the dilution of serum at which 50% Vero cells protected from DENV infection of all the four serotypes.

5. DISCUSSION

5 Discussion

Dengue infection is considered as one of the most pervasive viral disease in humans transmitted by a mosquito. A spectrum of ailments from symptomless flu-like conditions to fatal Dengue Shock Syndrome/Dengue Hemorrhagic Fever (DSS/DHF) is caused by the four closely related serotypes of DENV (DENV-1 to DENV-4)(Afroz et al., 2016; Simmons et al., 2007). Dengue infection can be mild illness lasting for about one week, whereas severe dengue (DSS/DHF), is characterized by plasma leakage and considered to be life threatening (Khan et al., 2013). The upsurge in the incidence of dengue epidemics globally has led to an increase in the investigations on dengue virus biology and its pathology to design therapeutics for the prevention and containment of the infection. However, designing of adequate therapies and vaccines needs in-depth knowledge about the disease pathogenesis. Even though many efforts have been made to understand DENV pathogenesis, the underlying mechanism of this disease remains elusive, and need to be explored further. The inflammatory responses against DENV have been reported to play a crucial role in DENV pathogenesis (Ming-Fang. Wu et al., 2013). The diverse clinical manifestations between mild and severe dengue patients suggest that inflammatory responses might differ significantly between the disease conditions (Ming-Fang. Wu et al., 2013). It has been reported that DENV-infected cells produce substantially elevated levels of the proinflammatory cytokines TNF-α, IL-6, IL-12p70, as well as PGE2 (Puerta-Guardo et al., 2013). The role of non-structural proteins has been studied in pathogenesis and host immunomodulation of dengue, while structural proteins like E protein has been studied mostly from the perspective of vaccine development (Babu et al., 2008; Chiang et al., 2016). E protein is a multi-domain protein (EDI, EDII, and EDIII domains) (Modis et al., 2003; Poggianella et al., 2015), has been shown to be a major target for the antibody based immune response during dengue infection (Block et al., 2010).The EDIII domain is the region which has highest variability amongst the four serotypes, congruently the highly specific antibodies generated against this particular region are the ones with high neutralizing ability (Crill and Roehrig, 2001; Wahala et al., 2009). EDIII has also been shown to have immune-modulatory property as it tailors NLRP3 dependent innate inflammatory responses (Hua et al., 2021) . Herein, through this study, we demonstrate that the EDIII protein induces a robust proinflammatory cytokine signature in human

macrophages both at RNA as well as protein level. The levels of proinflammatory cytokines like IL-1β and TNF-α showed a dose-dependent increase in the EDIII treated cells, which is consistent with the earlier reports that these cytokines play a crucial role in DENV pathogenesis. However, IL-6 did not show an increase upon EDIII stimulation. In addition, the findings of this study reveal that EDIII induced increase in the levels of IL-1β and TNF-α is not serotype specific. Furthermore, it is well-established that the NF-κβ family of transcription factors plays a vital role in inflammation and innate immunity (Caamaño and Hunter, 2002; Tak et al., 2001). It controls the expression of cytokines, which is in agreement with our study, as the increase in cytokine levels following stimulation with EDIII protein was found to be mediated via NF-κβ activation. Activation of the NF-κβ transcription factor is firmly controlled by group of proteins called inhibitory complex (Verma et al., 1995; Viatour et al., 2005; Zhang et al., 2016). These proteins block the nuclear localization signal of NF-κβ, hence prevents its nuclear translocation. Phosphorylation of IKB proteins like IKB-α, responsible for masking p65 component of NF-κβ resulting in ubiqutination and proteosomal degradation and subsequent p65 translocation to the nucleus (Christian et al., 2016; Mathes et al., 2008). In our study, we observed a decrease in the levels of NF-κβ p65 component in the cytosolic fraction while a simultaneous increase in nuclear fraction was observed following the EDIII treatment in a time-dependent fashion, that correlated with the phosphorylation of IKBα. This suggests that EDIII induces cytokine expression via NF-κB activation and nuclear translocation of its NF-κβ p65 component, thereby suggesting that NF-κβ activation might be responsible for regulating these cytokines transcriptionally. It is well documented that production of cytokines such as IL-1β is tightly controlled not only at transcriptional levels but also at the posttranscriptional level (Srikanth Battu et al., 2018; Ganguly et al., 2016). IL-1β is mainly produced as an inactive pro-protein and a cysteine protease caspase-1 mediates its activation and release, cleaves it to biologically active form (Franchi et al., 2009). Caspase- 1 is itself cleaved to its active form by inflammasome activation. Our results indicate an increase in mature IL-1β and cleaved caspase-1 in THP-1 cells following EDIII treatment, which confirms that IL-1β activation is dependent on caspase-1. DENV EDIII also induces NLRP3 inflammasome activation, which is important in DENV pathogenesis and host immune modulation during dengue infection. ROS generation and potassium (K+) efflux have been

suggested for NLRP3 activation to trigger caspase-1 activation and IL-1β maturation (Jo et al., 2016; Pétrilli et al., 2007). The ROS production acts as both a signaling molecule as well as a mediator of inflammasome activation and inflammation (Srikanth Battu et al., 2018). DENV EDIII treatments also induced the production of ROS and the increase in IL-1β levels, was found to be associated with the increase in ROS production. Mature IL-1β levels decreased in the presence of both ROS inhibitor NAC (Gov et al., 2017; Rivers-Auty and Brough, 2015) as well as K+ ions, which shows that release of mature IL-1β is dependent on both ROS production as well as K+ efflux. The above findings categorically demonstrate the role of EDIII protein of DENV in the activation of innate immunity. Having observed the immunomodulatory function of EDIII combined with other studies showing that the EDIII specific antibodies display enhanced neutralizing capability suggests EDIII antigen from DENV as a potential vaccine candidate. However, it has been observed that purified recombinant antigen when administered alone elicits week correlates of protective immunity and thereby requires multiple boosters, multiple adjuvants and a suitable delivery system for tailoring strong immune responses.

As there are no specific antiviral therapies against DENV, therefore a potent and efficacious dengue vaccine that provides protection against all the serotypes is immediately needed. Efforts have been made over the last three decades, but no effective vaccine has been developed which could potentially mount protective immunity against all the serotypes of DENV. However, several vaccine candidates are in the preclinical and clinical trial stages, such as subunit vaccines (Chiang et al., 2016), inactivated vaccines (Redoni et al., 2020) VLP based vaccine (Boigard et al., 2018) and live attenuated vaccines (LAVs) (Biswal et al., 2019; Kirkpatrick et al., 2016). A tetravalent chimeric live attenuated vaccine has been licensed for clinical application in various endemic countries under the brand name of "Dengvaxia" developed by Sanofi Pasteur; however, its efficacy in mounting adequate protection against all the DENV serotypes is variable (Flasche et al., 2016; Halstead, 2017). The significant advantage of LAV is that it imitates the natural virus infection and therefore elicits a potent cell-mediated and humoral response. Nonetheless, LAVs carry a risk of reversion, which renders them unsuitable for immunocompromised individuals (Minor, 2015). To overcome the above shortcomings, subunit and synthetic peptide vaccines provides an alternative to LAVs in vaccine filed. Although subunit vaccine offers enormous

benefits, including safety, as they are non-replicating and non-infectious. Further they can be tailored for developing a multivalent vaccine containing multiple immunodominant antigens, which is important for dengue vaccine as it has to be tetravalent which could confer balanced response against all the serotypes of DENV. Although subunit vaccine offers several benefits, they have been shown to exhibit weak immunogenicity as compared to conventional vaccines when administered (Vartak and Sucheck, 2016). However, the problem of immunogenicity could be solved through a proper usage of adjuvants and antigen delivery systems.

Therefore, in the current study, we formulated a PLGA based particulate system loaded with immunodominant EDIII domain of dengue envelop protein from all the four serotypes. The PLGA NPs were polydisperse in nature with sizes ranging from 100 nm–200 nm, with a sustained antigenic release pattern, which is known to be an important feature of the particle system to induce strong effector and memory immune responses at a relatively lesser dose (Kasturi et al., 2011). Furthermore, smaller sized nanoparticles could be effectively taken up by APCs, therefore enhancing the antigen presentation and subsequent T cell activation (Oyewumi et al., 2010). To evaluate the potency of nano-vaccine formulation, the EDIII domain of four serotypes were separately encapsulated in PLGA nanoparticle and administered to the mice in tetravalent combination with and without TLR agonists (MPLA + R837) as coadjuvants. *In vivo* immunization study divulges that tetravalent formulation of PLGA nanoparticle loaded with EDIII domain elicits a significant antigen specific expansion of IL-2 and IFN-γ producing CD4+ and CD8+ T-cells as compared to free EDIII domain antigens, which is a prerequisite to effective T-cell based adaptive immunity that play a pivotal role in neutralizing and clearing the DENV (Waickman et al., 2019). This phenomenon was further enhanced by adjuvanting the tetravalent formulation of PLGA nanoparticles with TLR agonists (MPLA + R387), which is known to activate the innate immunity pathway to augment adaptive immunity (Kasturi et al., 2011). Expansion of balanced serotype specific polyfunctional T-cell would drive a strong antigen specific B-cell effector and memory response, which provides protective immunity against the pathogens (Harty and Badovinac, 2008; Kasturi et al., 2011). This phenomenon is mostly attributed to the particle character of PLGA nano formulation, which slowly releases the antigens, therefore giving continuous exposure, enhanced uptake, and presentation by APCs (Sahdev

et al., 2014). Apart from that, TLR agonists are also known to activate the dendritic cell, which in turn activates the CD4+ helper cell, which is essential for antibody response (Essen et al., 2000; Mitchison, 2004). Having observed the potent poly-functional T cell response, we analyzed the antigen specific antibody response, and found that tetravalent particulate formulation induces a significant level of serotype specific total IgG after priming and boosting, which was further enhanced in the group of TLR agonist-based coadjuvants. Antibody responses are established along two functionally and anatomically different pathways, extrafollicular pathways that generate rapid and short-lived antibody producing plasma cells, and another is GC pathways, which produce memory B-cell and long-lived plasma cells that generate high affinity antibody (Afroz et al., 2019; McHeyzer-Williams and McHeyzer-Williams, 2005). We have observed that tetravalent EDIII loaded PLGA nanoformulation increases the frequency of Tfh cells in the draining lymph node and it is further enhanced with TLR agonists. Differentiated Tfh cell into the GC provides a helper signal to B cell in the GC for activation and differentiation into long lasting memory B cell and antibody producing plasma cell (Crotty, 2014). Next, we examined the frequency of GC-B cell Gl7+ and Ig+ from the lymph node of immunized mice, and it has been observed that the frequency of GC-B cells is significantly enhanced in the tetravalent EDIII loaded PLGA nanoformulation, which was further improved with the TLR agonists. We also evaluated the effects of nanoformulation along with TLR agonists in the early molecular programming of Tfh and antigen specific B cell into the GC. It has been noticed that Bcl-6, Cxcr5, and BcoR1 genes were upregulated in the PLGA(tEDIII) nanoparticle and PLGA(tEDIII) nanoparticle + TLRs (MPLA + R387) animal groups, and these genes play an important role in the differentiation and maintenance of Tfh cell (Wu et al., 2018). Genes associated with memory B cell formation, i.e., Bcl2 and IL-10, were also upregulated (Kasturi et al., 2011). Tetravalent formulation of EDIII encapsulated PLGA nanoparticle in a combination of TLR agonists induces strong polyfunctional CD4+ and CD8+ effector and memory cell responses, which lead to an efficient antibody response via the GC pathway that eventually produces the high titer antibody against all the four serotypes of dengue virus. In vitro virus neutralization assay from the serum of immunized mice revealed that nanoformulation along with co adjuvants demonstrates better virus neutralizing capability as compared to soluble antigen, or only nanoparticle or only antigen plus TLR agonists.

Notably, a single tetravalent nanoformulation efficiently and equally neutralizes all four serotypes. ADE of infection happens due to the production of non-neutralizing antibodies against cross antigens (Rothman, 2011). Specifically, dengue envelop protein antigen consist of three distinct domains, a central domain (EDI), dimerization domain (EDII), and C terminal domain (EDIII) (Modis et al., 2004). While antibodies generated against EDI and EDII are weakly neutralizing and cross neutralizing against various flaviviruses, leading to immune responses causing ADE (Fahimi et al., 2018), on other hand antibodies against EDIII manifest strong neutralizing capability. Hence application of EDIII antigens in vaccine formulation might reduce the risk of ADE. Our formulation comprising of EDIII domain antigens from all the serotypes of DENV encapsulated in PLGA nanoparticle in tetravalent combination along with TLR agonists induces balanced immune correlates against all the four serotype of DENV which culminated into the efficient virus neutralization and also mitigate the phenomenon of ADE.

Having observed the promising antigen specific T and B cell responses of nanoformulation of PLGA tEDIII in combination of TLR coadjuvants along with virus neutralizing capability. Furthermore, we explored an alternate vaccine delivery platform that has stand-alone adjuvanting properties which can modulate immune response without the use of additional adjuvants or coadjuvants. In this regard, outer membrane vesicle (OMVs) of gram-negative bacteria is engineered so that it can display EDIII antigen over the surface of lipid bilayer. OMVs are naturally released by gram negative bacteria to transport various cargos to other cells and possess ability to modulate the range of various host biological processes including immune responses (Kaparakis-Liaskos and Ferrero, 2015). Since past decade, OMVs have been proved as an attractive vaccine platform due to optimal size which can be efficiently taken up by APCs, presence of various immune activating molecules such as TLR agonists which activates both the arms of immunity, and ability to display or encapsulate a range of antigens in its native conformation (Mancini et al., 2020). Therefore, we developed a recombinant engineered rOMVs on which EDIII antigen is fused with cytolysin A (ClyA) protein of bacteria that helps to target the antigen over outter membrane of OMVs. The ClyA protein is commonly used to target various antigens over OMVs membrane (Chen et al., 2010). Recombinant OMVs are released from a special kind *E. coli* strain known as Clear coli in which LPS gene is genetically modified and is less endotoxic

than wild type of LPS gene (Cardoso et al., 2022). OMVs with modified LPS can strike a fine balance between reactogenicity of endotoxin and potent immune response against carrying antigens which makes it attractive vaccine platform with stand-alone adjuvanticity. Size of rOMVs were 100 nm and smaller size of OMVs is instrumental in inducing effective T cell response due to enhanced uptake by APCs (Manolova et al., 2008; Tomić et al., 2014). To study the potency of rOMVs formulation, OMVs expressed EDIII antigens of all the four serotypes were physically mixed to form tetravalent formulation (OMV (tEDIII)) and administered in the mice. Data of immune response study showed that tetravalent formulation of OMV (tEDIII) induces a potent antigen specific enhancement of IFN-γ and IL-2 producing $CD8^+$ and $CD4^+$ T-cells as compared to EDIII antigen alone which indicates Th1 biased immunity and it has been reported earlier that OMVs drives Th1 immunity (Iwasaki and Medzhitov, 2015; Lötvall et al., 2013). Balanced serotype specific expansion of polyfunctional T-cells would drive an effector and memory B-cell response that will eventually mount a protective immunity against pathogens (Harty and Badovinac, 2008; Kasturi et al., 2011). Activation of robust polyfunctional T-cell is majorly contributed by the size of OMVs and inherent property of innate immune activations (Manolova et al., 2008) (Iwasaki and Medzhitov, 2015). It has been established that activation of innate immunity modulate adaptive immunity as OMVs possesses various PAMP which are known to modulates innate immunity (Iwasaki and Medzhitov, 2015; Tan et al., 2018) and it is well proven that OMVs induces DC activation and maturation (Alaniz et al., 2007). Poly functional T cell responses are known to activate strong B cell response; therefore, we analyzed the antigen specific humoral response and result demonstrated that OMVs (tEDIII) formulation induces significant degree of serotype specific total IgG response after priming and boosting as compared to tEDIII alone. Humoral responses are driven by two different pathways, extrafollicular pathways which induces short-lived antibody producing plasma cells that produce low affinity antibody, and another pathway is GC which occurs in lymph node, produces effective memory B-cells and long-lived plasma cells which generates high affinity antibody (Afroz et al., 2019; McHeyzer-Williams and McHeyzer-Williams, 2005). In this study we observed that OMVs (tEDIII) formulation induces the expansion of Tfh cell in the draining lymph node as compared to EDIII antigen alone. Activated Tfh cell send a helper signal to B-cell in the GC for programming into long lasting memory B cells and

antibody producing plasma cell (Crotty, 2014). Furthermore, we observed the frequency GC-B cell in lymph node has been significantly enhanced in the groups of OMVs (tEDIII) immunized mice. Tetravalent formulation of OMVs(tEDIII) generates a potent polyfunctional CD4+ and CD8+ effector T-cell response that eventually leads to a potent humoral response through GC pathway which produces a high titer antibody against all the serotypes of DENV. *In vitro* virus neutralization study from the serum of immunized animals demonstrated that OMVs(tEDIII) group exhibited significant virus neutralization potential as compared to soluble antigen alone. Remarkably, a single tetravalent rOMVs(tEDIII) formulation equally neutralizes all the four serotypes of DENV.

Overall the study demonstrate that the purified DENV EDIII antigen from all the four serotypes encapsulated in a PLGA nanoparticle as a tetravalent combination along with TLR agonists as an adjuvant triggers a robust and balanced immune correlates, which is capable of neutralizing all the dengue virus serotypes (1-4). In line with the above study, we have developed OMVs based tetravalent vaccine formulations which suffice the problem of adding additional adjuvants that will cut down the cost to benefit ratio. rOMV based tetravalent vaccine formulation induced a strong B and T cell responses with enhanced neutralization profile against the four serotypes of Dengue Virus. Given the benefits of rOMV technology that includes stable antigen delivery, intrinsic adjuvantig property combined with its economical and scalable attributes make rOMVs a lucrative vaccine platform technology. The current study provides a new strategy for enhancing the immunogenicity of dengue vaccine antigen using a suitable antigen delivery, and adjuvating system, which further could be extrapolated for the design and development of novel vaccine formulations against other infectious diseases.

6. SUMMARY

6 Summary

Dengue is a mosquito-borne viral disease caused by the four related serotypes of dengue virus (DENV 1-4), growing at an astonishing rate globally. It is primarily dominated in the tropical and subtropical regions and is endemic to almost 130 countries (Stanaway et al., 2016). There are estimates that 390 million people are infected annually with 96 million cases of severe infection (Bhatt et al., 2013) and 3 billion are at the risk of infection (Brady et al., 2012). This stupendous rise of DENV cases could be geographical expansion of the vector due to increasing urbanization, global warming, lack of proper diagnosis, unavailability of specific antiviral therapies and licensed dengue vaccine (Brady et al., 2012; Ebi and Nealon, 2016).Therefore to contain the spread of dengue cases a potent prophylactic vaccine effective against all the four serotypes of DENV is of utmost importance. Primary infections with one serotype induce strong and durable protective immunity against the same serotype, but individuals remain susceptible to other serotypes (Rothman, 2011). Subsequent infection of the same individual by another serotype causes severe clinical symptoms, i.e., DHF and DSS. The enhancement of disease symptoms during heterotypic secondary infection is possibly explained by two phenomena: (a) ADE of infection and (b) original antigen sin due to poor immunogenic response against cross antigen during the previous infection (Rothman, 2011). More specifically, ADE happens during heterotypic secondary infection because primary infection of DENV induces weak antibodies against cross antigens of other serotypes, which ultimately enhances the infection rather than neutralization of the virus. The above phenomenon during secondary infection is proved to be a major bottleneck towards the development of a potent universal vaccine against all the serotypes of DENV.

It has been well documented that the innate combined with adaptive immunity triggered by the host play a primary role in controlling dengue infection, however, the immunological mechanisms adapted by the virus to evade the host immune response still need to be investigated. DENV has been known to employ multiple routes to escape from host immune responses. Exaggerated inflammatory responses induced by the host, which account for the significant tissue damages during infection could be one of such approaches employed by the virus (Ye et al., 2013). It is also evident from the studies which report that the DENV infected cells produce significantly enhanced levels of the proinflammatory cytokines such

as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 12p70, as well as prostaglandin E2 (PGE2) (Puerta-Guardo et al., 2013). Moreover, the macrophages infected with DENV has shown the elevated levels of proinflammatory cytokines and NLRP3 in association with caspase-1 activation (Ming-Fang. Wu et al., 2013). Hence, identifying the DENV component, which elicits such response, would help in the design of therapeutic interventions. The EDIII domain has been investigated as a potential candidate for vaccine development as it has ability to elicit strong antibody responses (Fahimi et al., 2014; Maria G. Guzman et al., 2010). Nevertheless host immunomodulatory role of EDIII has not been investigated completely. Therefore, in this study, we have examined the role of EDIII in modulating innate immune responses in the differentiated human monocyte cell line (THP-1). Also we used nanoparticle delivery systems to enhance the immunogenicity of EDIII and evaluated the immune responses in mice.

We have purified EDIII of all the four serotypes of DENV (1-4). To check the proinflammatory responses, THP-1 cells were treated with purified proteins (non-toxic concentrations) and the cytokines like L-1β, TNF-α and IL-6 were evaluated by ELISA and qRT-PCR. It was observed that EDIII from DENV 2 induced robust proinflammatory signatures in human macrophages. Next, we evaluated the proinflammatory responses of EDIII from other serotypes (DENV1, 3 and 4) and observed that the proinflammatory responses are general for DENV and not serotype-specific. Having observed that EDIII proteins trigger IL-1β and TNF-α at mRNA and protein level, we were next interested to understand the mechanism through which these cytokines might be regulated. It is well documented that the transcription factor NF-κB regulates wide range of genes involved in regulating inflammatory responses (Liu et al., 2017; Oeckinghaus and Ghosh, 2009). Therefore, we evaluated activation of NF-κB pathway in response to EDIII stimulation. It was observed that EDIII activates NF-κB by increasing nuclear translocation of P65 and phosphorylation of IKBα. Since TLRs are involved in the activation of NF-κB, we wanted to check which TLR is involved the production of IL-1β mediated by EDIII. Results showed that EDIII mediated NF-κB activation involve TLR4. It is known that IL-1β is produced as biologically inactive 31 KDa polypeptide called as pro- IL-1β and is cleaved by caspase-1 to produce 17 KDa mature IL-1β (biologically active form) (Sutterwala et al., 2006). Therefore, we assessed the release of both, the active caspase 1 and IL-1β, from the

differentiated human monocytes by immunoblotting. Data suggests that the IL-1β maturation induced by DENV EDIII protein is dependent on caspase-1 activation. To check whether caspase-1 is required for EDIII induced maturation of IL-1β we used caspase-1 inhibitor (Ac-YVAD-CHO) and observed that mature IL-1β was drastically reduced in presence of inhibitor compared to only EDIII treated cells. Furthermore, we checked the expression of NLRP3 in EDIII stimulated cells and data demonstrates that the NLRP3 inflammasome activation is involved in EDIII induced IL-1β maturation. Since NLRP3 expression was observed in the EDIII treated cells, we tried to look into the mechanism of inflammasomes activation. It is evident from previous reports that production of ROS plays a crucial role in the activation of NLRP3 inflammasomes (Zhao et al., 2020). As described above IL-1β maturation is dependent on caspase 1 which in turn is regulated by inflammasomes. Therefore, we were interested to find out whether EDIII triggers ROS production. To estimate intracellular ROS production, cells were stimulated with different concentrations of EDIII protein and LPS for 12 h, followed by incubation with CM-H2DCFDA for intracellular ROS estimation. A significant increase in ROS production was observed in cells stimulated with EDIII protein as compared to the untreated cells. Also, it is well established that IL-1β maturation is dependent on potassium efflux (Colomar et al., 2003), therefore, in order to check the role of potassium efflux on maturation of IL-1β we treated the cells with NaCl and KCl (Kim et al., 2016) prior to the recombinant protein stimulation. We observed reduction in the IL-1β levels in the EDIII protein treated cells with higher extracellular K+ levels as compared to the cells treated with only protein. In summary, this study highlights the role of EDIII protein of DENV in the modulation of host innate inflammatory response.

Having observed the innate immune responses induced by DENV EDIII in human macrophages, we next wanted to evaluate the antigen-specific adaptive immune responses of this protein in an in-vivo system. It is well known that EDIII, an immunodominant antigen of dengue is considered a potential subunit vaccine candidate owing to its ability to produce neutralizing antibodies (Fahimi et al., 2018). This domain also plays an important role in inducing long-lasting protective immunity against DENV infections. Antibodies against EDI and EDII are weakly neutralizing and tend to cross neutralize other Flaviviruses (Oliphant et al., 2006) leading to ADE. Therefore, selection of EDIII as a vaccine candidate

and not the entire envelope protein might help in avoiding the risk of ADE. It has been shown that EDIII protein when administered alone exhibits weak immunogenicity. Although EDIII protein induces neutralizing antibodies that are serotype-specific (Henchal and Putnak, 1990), however, the antibody titers fall quickly leading to a demand for multiple booster doses in a short period of time. To overcome these limitations of candidate recombinant subunit vaccines, a number of ways have been employed, including nanotechnology (Vartak and Sucheck, 2016). In the era of modern vaccine formulation, biocompatible nanomaterials have attained an important place, leading to a field of "Nanovaccinology." Nanoparticles fabricated from various synthetic as well as natural materials have been reported to enhance the immunogenicity of various antigens (Gregory et al., 2013) particularly related to infectious diseases (Fries et al., 2020). Specifically for the DENV, several nanoparticle formulations, including PLGA nanoparticle (Metz et al., 2018), calcium phosphate nanoparticle loaded with synthetic peptide epitope (Huang et al., 2017) multi-epitopes peptide conjugated with polystyrene nanoparticle (Chan et al., 2020) and liposomes (Swaminathan et al., 2016), have been utilized to potentiate the immunogenicity of various DENV antigens.

Based on the above background, we proposed a new strategy to enhance the immunogenicity of EDIII protein, a potential vaccine candidate (Maria G. Guzman et al., 2010) from all the DENV serotypes by encapsulating them in a polymer-based nano delivery system separately. To obtain a tetravalent nanoformulation, encapsulated NPs from all the four serotypes were physically mixed along with a combination of TLR agonists. We further evaluated the potency of the said formulation in inducing balanced immune correlates against all the serotypes. These purified antigens from DENV (1-4) were subjected to PLGA encapsulation as described in the material and method section. The PLGA encapsulated DENV EDIII proteins were characterized by TEM for size and surface morphology. Our result demonstrates that the PLGA nanoparticle encapsulated with DENV EDIII is in the size ranging from 100 nm ≤1 μm with spherical morphology in the entire formulation group. The hydrodynamic radii of particles were measured by the DLS method, and it shows that most of the particle size ranges from 250 nm to 2 μm. Next, we evaluated encapsulation efficiency of EDIII encapsulated NPs and the results show that a significant amount of DENV EDIII (1-4) in the NPs was encapsulated. Loading of protein in NPs was

also checked by SDS-PAGE analysis, where we observed a single band at the corresponding size for EDIII for all the four serotypes of DENV which indicated that EDIII antigens were successfully loaded in the nanoparticle. Further i*n vitro* release assay of antigen loaded PLGA nanoparticle shows that in the first 24 h of incubation, nanoparticle releases 20% of the content and then sustained beyond 72 h, which indicates a biphasic release pattern, burst followed by sustained release. In another observation, the release of antigens was significantly enhanced at acidic pH (pH = 5), which is the pH of endolysosomes, and it is important for antigen processing by the APCs. This kind of release pattern is very important for the optimal and balanced immune response against antigens, which could serve as a sustained antigen depot. After characterization of EDIII encapsulated PLGA nanoparticles, we examined the potential of PLGA nanoparticles with and without TLR agonists in eliciting the potent antigen-specific effector T cell response against the EDIII domain of DENV. For that, we primed mice with different formulations of soluble and encapsulated EDIII and gave a booster of the same formulation post 14 days of priming. After immunization with the prime and booster dosages of formulation, the animals were sacrificed on day 28. We observed that PLGA (tEDIII) nanoparticles induces a significant amount of antigen-specific CD4+ and CD8+ T cells response as compared to tetravalent soluble antigens alone and it further showed synergy in terms of enhanced T cell responses in spleen and PBMCs of groups administered with TLR adjuvants. Data suggests that PLGA nano delivery system enhances the immunogenicity of the soluble purified antigens, which alone if administered, manifests weaker response. Further supplementation with nano delivery systemTLR adjuvant showed synergy in terms of enhancing antigen-specific responses. Promising results of robust antigen-specific CD4+ T cell response in the tetravalent formulation of PLGA nanoparticles along with TLR co-adjuvants prompted us to evaluate the antigen-specific antibody response. Antigen-specific total serum IgG level against all the serotypes (DENV 1, DENV 2, DENV 3, and DENV 4) was measured from the serum after 14 days of antigen priming and it demonstrates that antigen loaded nanoparticles (PLGA (tEDIII)) induce significantly higher antibody response as compared to antigen alone (tEDIII) and additionally enhanced in the (PLGA (tEDIII) + TLRs group). We observed further enhancement in total serum IgG level after secondary immunization. We also measured the titer of the antibody from the serum of immunized mice. The

antibody titer was found to be significantly high in PLGA (tEDIII) + TLRs group. The high titer value of the antibody also indicates the quantity of produced antibodies, which might help in neutralizing the virus. Furthermore, we analyzed the level of IgG subclasses IgG1, IgG2a, and IgG2b from the serum of immunized mice. We observed that level of IgG subclasses was significantly enhanced in the PLGA (tEDIII) + TLRs compared to other groups. GC is a temporary and important structure in the lymph node and spleen where B cell affinity maturation and thereafter differentiation into antibody secreting plasma cell and memory B cell happens, which is an important phenomenon for potent humoral immunity (Pasare and Medzhitov, 2005). This process is constantly supported and sustained by Tfh cells, which provide an important signal for the differentiation of the naïve B cell into the antibody producing plasma cell and memory B cell (Leo et al., 2011). Since we observed high titer antibodies in serum of immunized mice, therefore we isolated the cells from draining lymph nodes to check Tfh and GC activation. The cells were stained for the Tfh marker (CXCR5+, PD-1+, and CD4+) (Jia et al., 2018) and GC-B markers (GL7+ and Ig+) (Kasturi et al., 2011) and analyzed under flow cytometry. Interestingly, we found that the frequency of Tfh cells (CXCR5+, PD-1+, and CD4+) in the PLGA (tEDIII) group is significantly high as compared to the (tEDIII) alone. However, we found a profound enhancement in the PLGA (tEDIII) + TLRs group, suggesting that the encapsulation of antigens in PLGA nanoparticles in combination with TLR adjuvant trigger better programming of Tfh cells. Activated Tfh cells are destined to modulate the initiation process of the B cell affinity maturation and differentiation within the GC (Crotty, 2014) so the frequency of GC-B cells (GL7+ and Ig+) in the lymph node was found to be significantly high in the PLGA (tEDIII) + TLRs immunized group. The genes associated with initiation and sustenance of Tfh cell (Bcl-6, CXCR5, and BCOR 1) and GC-B cell (Bcl2 and IL-10) were unregulated in the immunization group, which has induced Tfh and GC-B cell differentiation. This observation indicates that PLGA (tEDIII) + TLRs induce the reaction of the GC efficiently, which ultimately mount a strong humoral immune response by producing a significant amount of IgG with a relatively higher titer, that is a prerequisite for potent antiviral immunity. Virus neutralizing antibody is an important parameter to evaluate the efficacy of vaccine candidates and their formulation as the quantity and quality of neutralizing antibody are considered as surrogates of protective immunity against the

various serotypes of Flavivirus. Keeping this in view, we have evaluated the virus neutralization efficacy of serum antibodies in the *in vitro* infection model. The virus was incubated with different dilutions of sera obtained from all the experimental groups and subjected to infection on the Vero cells. Results show that serum from the PLGA (tEDIII) + TLRs significantly inhibits the virus infectivity of all the four serotypes to Vero cells at different dilutions expressed in terms of percent normalized infection and FNT50 as compared to tEDIII. This finding corroborates the fact that nanoparticle in combination with TLR agonist co-adjuvants induces strong T cell assisted B cell response, which produces high affinity antibody, therefore, efficiently neutralizing the dengue virus.

Having observed promising antigen-specific T cell responses with strong antibody responses in EDIII encapsulated PLGA with TLR formulation, we wanted to explore a delivery vehicle that has intrinsic adjuvanting properties and can augment the immunogenicity of antigens without the use of additional adjuvants or coadjuvants. It is well documented that OMV are naturally released by Gram-negative bacteria during growth (Schwechheimer and Kuehn, 2015). OMV have immunopotentiators such as TLR agonists which brings about the self-adjuvanticity nature of OMVs. This property of OMVs makes them strong activators of innate immune response (Dowling et al., 2016). In addition, various studies also emphasize on the ability of OMVs to activate the humoral and cellular immune responses (Bottero et al., 2016; Tan et al., 2018). The long-lasting immune response and ease in production of OMV make them a promising candidate for vaccine development. The recombinant OMV are made based on a simple technique where the protein of interest is fused to a membrane protein anchor which then co-localizes this fused protein to the outer membrane of vesicles. One such example of a transmembrane protein is Cly A. Cly A is a pore-forming cytotoxin found in the outer membrane of OMVs of enterobacteria such as *E. Coli*. This nature of the OMVs can be exploited by generating fusion proteins between candidate antigens and Cly A (Rappazzo et al., 2016; Watkins et al., 2017). This makes rOMVs a unique and powerful tool to display native antigens and simultaneously provide an adjuvant property in vaccine development. Therefore, we have used OMV and expressed EDIII antigens (fused with Cly A) from DENV serotypes individually and physically mixed to form tetravalent formulations. The cloned constructs for DENV EDIIIs in pET28a vector was used as a template for cloning serotype-specific EDIIIs in pBAD-ClyA vector to produce rOMVs.

After cloning we proceeded for expression and purification of rOMVs expressing EDIIIs according the methods described earlier with modifications (Watkins et al., 2017) and we successfully purified the OMVs expressing EDIII. The purified rOMVs were characterized with TEM and DLS and results show that vesicles are spherical in shape and less than 100 nm in size. Size distribution of vesicles was analyzed by DLS, and data has shown that size of majority of rOMVs is less than 100 nm except DENV1 EDIII rOMVs. After successful designing and characterization of rOMVs displayed with DENV EDIII antigen, we further explored the ability of rOMVs in stimulating the antigen-specific effector T cell response against the dengue antigen (EDIII) by immunizing these formulations in mice as described in methodology section. Spleenocytes were collected from euthanized mice, and we observed that frequency of polyfunctional T cells which is known as IL-2 and IFN-γ secreting CD4+ and CD8+ T cells has enhanced in the animals those were immunized with rOMV (tEDIII) formulation compared to soluble antigens or control groups. Effectiveness of a successful viral vaccine is measured by the propensity of B cell response in producing the antigen-specific high affinity antibody that ultimately helps in neutralizing the viruses. Therefore, DENV EDIII specific total IgG from serum was measured by indirect ELISA. We observed that antigen expressed rOMVs induces significantly higher antibody level against all the four distinct serotype antigens as compared to antigens alone or control groups after 14 days of priming and it has further enhanced after booster dose. Next, we carried out Tfh and GC responses in lymph nodes of immunized mice and observed that the formulation rOMV (tEDIII) induces strong Tfh and GC responses compared to soluble antigen and control groups. nAbs are considered as surrogate of protective immunity against the various Flaviviruses; therefore, it is an important functional parameter to evaluate the potency of vaccine candidates and their formulations. The results demonstrate that serum from the OMVs (tEDIII) significantly inhibits the infectivity or provide protection to the Vero cells as compared to tEDIII alone or control groups. Significant potency of nAbs from the OMVs (tEDIII) substantiating the fact that antigen displayed over rOMVs induces the polyfunctional T cells which eventually modulate the potent B cell response, therefore, neutralizes the dengue virus.

In conclusion, this study provides a new strategy for enhancing the immunogenicity of Dengue vaccine antigen using a suitable antigen delivery, and adjuvating system. Using

such approaches, herein, we have developed a novel tetravalent nano-vaccine formulation comprising of immune-dominant EDIII antigen from all the serotypes of DENV, which is able to confer strong vaccine induced protective immunity capable of neutralizing DENV (1-4) effectively. The findings of this study could be further extrapolated for the development of efficacious, safe and economical vaccine formulations against other emerging and re-emerging diseases.

6. REFERENCES

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8 PUBLICATIONS

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Tetravalent formulation of polymeric nanoparticle-based vaccine induces a potent immune response against dengue virus†

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Dengue is a mosquito-borne disease caused by the four serotypes of the dengue virus (DENV 1–4). It is growing at an alarming rate globally, which could be partly attributed to the lack of an effective therapeutic regimen. Therefore, strategies for developing an effective vaccine have gained more significance in the given scenario. Failure of the existing live attenuated vaccine candidates to mount effective and broader protection against all the four serotypes of DENV has generated a new interest in exploring novel strategies for augmenting the efficacy of non-infectious, non-replicating subunit vaccines. In the current study, we employed a new strategy of encapsulating the immunodominant EDIII domain of Envelop protein of all the serotypes of DENV (1–4) into PLGA nanoparticles separately. All four nano formulations were physically mixed to develop a tetravalent nano formulation in combination with TLR agonists. Further, we examined its immunological efficacy using a mouse and *in vitro* infection model system. Interestingly, our results demonstrate that majority of EDIII protein loaded PLGA nanoparticles were polydispersed and less than 1 μm in size with optimal encapsulation efficacy. Tetravalent nanoformulation along with TLR agonists (MPLA + R837) enhanced the magnitude of antigen-specific polyfunctional T cell response. It triggered robust antibody responses in mice concurrent with the increased level of genes involved in the programming of memory B-cell formation and the maintenance and maturation of GCs, leading to the formation of long-lived plasma cells secreting antigen-specific antibodies. Further assessment revealed that tetravalent nanoformulation in combination with TLR ligands upon immunization in mice aids in the enhanced production of serotype-specific neutralizing antibodies, which can effectively neutralize all the four serotypes of DENV (DENV 1–4). The findings of this study reveal a new strategy for enhancing the immunogenicity of vaccine candidates and might pave the way for the development of a tetravalent vaccine against all the serotypes of Dengue Virus.

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Introduction

Dengue is a mosquito-borne viral disease caused by the four serotypes of dengue virus (DENV 1–4), growing at an astonishing rate globally. It is primarily dominated in the tropical and subtropical regions and is endemic to almost 130 countries.[1] There are estimates that 390 million people are infected annually with 1 million cases of severe infection with 3–5% of case fatality rate,[2] and 3 billion are at the risk of infection.[3] As the climate becomes warmer, the rate and risk of infection will continue to grow;[3,4] therefore, a potent prophylactic vaccine or antiviral therapy effective against all the four serotypes of DENV is of utmost importance. Primary infections with one serotype induce strong and durable protective immunity against the same serotype, but individuals remain susceptible to other serotypes.[5] Subsequent infection of the same individual by another serotype causes severe clinical symptoms, *i.e.*, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[5] The enhancement of disease symptoms during heterotypic secondary infection is possibly explained by two phenomena: (1) antibody-dependent enhancement of infection (ADE) and (2) original antigen sin due to poor immunogenic response against cross antigen during the previous infection.[5] More specifically, ADE happens during heterotypic secondary infection because primary infection of DENV induces weak antibodies against cross antigens of other serotypes, which ultimately enhances the infection rather than neutralizing the viruses. The above phenomenon during secondary infection is proved to be a major bottleneck in developing a potent universal vaccine against all the serotypes of DENV.

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Dengue virus envelope protein domain III induces pro-inflammatory signature and triggers activation of inflammasome

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ABSTRACT

Dengue virus poses a considerable clinical problem, with the four closely related serotypes of dengue virus (DENV) infecting around 50–100 million people per year world-wide. The drastic increase in the dengue infection could be partly attributed to geographic expansion of the vector due to increasing urbanization, unavailability of specific antiviral therapies, licensed dengue vaccine, and poor understanding of the host immune responses. It has been reported that the immune-dominant envelope protein (E protein) domain III region (EDIII) of DENV is one of the most potent vaccine candidates because of its ability to trigger host immunity by inducing production of protective neutralizing antibodies. However, its role in the modulation of innate inflammatory responses hitherto remains unexplored. Herein, we demonstrate that EDIII protein of DENV induces pro-inflammatory signature by inducing production of inflammatory cytokines such as IL-1β and TNF-α in THP-1 cells through NF-κB pathway. Also, we observed increase in the maturation of IL-1β, which was found to be associated with increased ROS production and potassium efflux. Further, our findings reveal that the IL-1β production by EDIII protein is mediated through caspase-1 and NLRP3 inflammasome activation. In conclusion this study unearths the role of DENV EDIII protein in modulating innate inflammatory responses, which might provide possible mechanism of pathogenesis and open-up new avenues for the development of therapeutics against DENV.

1. Introduction

Dengue is a common viral infection prevalent worldwide, which has shown an increase in the spread as well as epidemic incidences over the past few decades [1–3]. This is evident from the numbers reported in the WHO report, which clearly indicates that there has been a gradual upsurge in the Dengue cases globally from less than a thousand cases in 1950 to around three million cases annually in 2015 [4].

Dengue is a mosquito-borne viral disease, transmitted by the bites of female mosquitoes, mainly of the species *Aedes aegypti,* and *Aedes albopictus* in a few cases [5,6]. It is primarily caused by four different but quite related Dengue virus (DENV) serotypes, namely, DENV-1,DENV-2, DENV-3,and DENV-4 [5]. The presence of four different causative serotypes of the virus lead to cross-reactivity and hence, additional risk on subsequent dengue infections [2]. Clinically, dengue infection is characterised by a plethora of symptoms ranging from asymptomatic mild flu called as Dengue fever (DF)to the more severe condition, which includes coagulopathy, enhanced vascular fragility and thrombocytopenia known as Dengue Haemorrhagic fever (DHF), which may lead to hypovolemic shock called Dengue Shock Syndrome (DSS) [7].

Dengue virus (DENV) is an enveloped virus with positive-sense single stranded RNA genome that codes for seven non-structural and three structural proteins [8]. The non-structural proteins (NSPs) (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) play a role in replication of viral RNA and assembly as well as regulation of the host cellular and immunological responses [9,10]. DENV NS1 protein acts as immunogen during severe phase of infection and elicits a potent anti-NS1 response [11]. Emerging evidences highlight a vital role of NS1 in the activation of immune complement system during infection [12]. NSPs like NS2A, NS2B, NS4B and NS5 have been reported to modulate type I interferon (IFN) signaling [13,14] while simultaneously NS5 and NS4B proteins induce production of chemokines and proinflammatory mediators [13,15,16]. DENV structural proteins such as envelope glycoprotein (E) is a major component of the envelope of viral membrane, which helps the virus attachment to the host cell and subsequent fusion to the cellular membrane during the endosomal-mediated virus internalization [17]. The E protein is constituted of three structural domains, namely, EDI, EDII and EDIII [18], held by a helical stem region hitched to virus membrane by *trans*-membrane anchor [17,19]. EDIII is an Ig-like domain involved in binding of the virus, which makes it to be an

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Research paper

Transcriptome meta-analysis identifies immune signature comprising of RNA binding proteins in ulcerative colitis patients

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ABSTRACT

Ulcerative colitis (UC) is a persistent inflammatory illness, which is clinically categorised as Inflammatory bowel disease (IBD), affecting millions of people worldwide. The precise cause behind the pathology of the disease remains unknown. However, the involvement of multiple factors including genetic predisposition, immunological deregulations, microbiota imbalance, and environmental triggers has been suggested. Amongst all these factors, the over-active immunological response reported in UC patients seems to be a promising target for therapy. Moreover, identification of gene signatures associated with disease onset and progression would help in better understanding of the molecular mechanisms involved in the disease pathogenesis. Here, we have conducted meta-analysis of gene expression profiles of UC patient microarray datasets accessible in public databases and further validated the *in-silico* findings in UC patients' blood samples. Our study reveals that UC pathogenesis perturbs expression of several inflammatory genes. In addition, we report a novel gene signature comprising of TIA1 (T cell restricted intracellular antigen) and TIAR (TIA1 related protein; also known as TIAL1), which were found to be significantly downregulated in UC patients. TIA1 and TIAR are RNA-binding proteins (RBPs), which function as a translational represser by binding to ARE sequences in the 3′ UTR of mRNAs encoding inflammatory mediators including cytokines. Our findings demonstrate that deletion of TIAR using gene specific siRNAs in-vitro results in enhanced production of inflammatory cytokine IL-1β. In conclusion, the findings of this study reveal that down regulation of TIA1/TIAR genes could be responsible for UC associated inflammation. This study highlights the usefulness of the meta-analysis approach in the identification of unique gene signatures that might deliver mechanistic insights into UC pathogenesis and possibly assist in discovery of prognostic markers and therapeutic interventions.

1. Introduction

Ulcerative colitis (UC) is a chronic illness of the gastro-intestinal tract, affecting millions of people world-wide every year [1]. In recent times, the prevalence rate of this disease has increased explicitly. It has been reported that the disease is prevailing with incidence from 5.3 to 63.3 per 100,000 people, whereas in North America itself, it ranges from 37.5 to 238 per 100,000 individuals [2–5]. UC is a clinical condition that belongs to a broad category of disease called as Inflammatory bowel disease (IBD), and is mainly characterized by inflammation in the colonic mucosa [6]. UC patients frequently present with a combination of symptoms that vary from abdominal pain, fever, and a clinical sign of bowel obstruction or diarrhoea in mild cases to bloody stools in severe cases [6]. However, the exact etiology of the disease still remains unknown. The reason could be multi-factorial nature of the disease, which has demonstrated a complex interplay of immunological, genetic, and environmental or extrinsic factors that have shown to significantly influence the disease susceptibility [7,8]. The pathological response commonly linked with UC is an upsurge in pro-inflammatory mediators, oxidative stress, disturbed colonic

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RESEARCH

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Milk exosomes elicit a potent anti-viral activity against dengue virus

Vengala Rao Yenuganti[1*†], Sumbul Afroz[1,2†], Rafiq Ahmad Khan[2], Chandrima Bharadwaj[2], Deepti Kailash Nabariya[1], Nagaraj Nayak[3], Madhuri Subbiah[3], Kumaraswami Chintala[4], Sharmistha Banerjee[4], Pallu Reddanna[1] and Nooruddin Khan[1,2*†]

Abstract

Background: Exosomes are nano-sized vesicles secreted by various cells into the intra and extracellular space and hence is an integral part of biological fluids including milk. In the last few decades, many research groups have proved the potential of milk exosomes as a sustainable, economical and non-immunogenic drug delivery and therapeutic agent against different pathological conditions. However, its anti-viral properties still remain to be unearthed.

Methods: Here, we have been able to isolate, purify and characterize the milk derived exosomes from Cow (CME) and Goat (GME) and further studied its antiviral properties against Dengue virus (DENV), Newcastle Disease Virus strain Komarov **(**NDV-K) and Human Immunodeficiency Virus (HIV-1) using an in-vitro infection system.

Results: TEM, NTA and DLS analysis validated the appropriate size of the isolated cow and goat milk exosomes (30–150 nm). Real-time PCR and immunoblotting results confirmed the presence of several milk exosomal miRNAs and protein markers. Our findings suggest that GME significantly decreased the infectivity of DENV. In addition, we confirmed that GME significantly reduces DENV replication and reduced the secretion of mature virions. Furthermore, heat inactivation of GME did not show any inhibition on DENV infection, replication, and secretion of mature virions. RNase treatment of GME abrogates the anti-viral properties indicating direct role of exosomes in DENV inhibition. In addition GME inhibited the infectivity of NDV-K, but not HIV-1, suggesting that the GME mediated antiviral activity might be virus specific.

Conclusion: This study demonstrates the anti-viral properties of milk exosomes and opens new avenues for the development of exosome-based therapies to treat viral diseases.

Keywords: Goat milk exosomes, Dengue virus, Anti-viral, And NDV-K

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Basic

Research Article

Activation of integrated stress response pathway regulates IL-1β production through posttranscriptional and translational reprogramming in macrophages

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Immune cells sense and programme its cellular machinery appropriately to the environmental changes through the activation of cytoprotective adaptive pathway so-called the "integrated stress response (ISR)". However, the mechanisms implicated in ISR-induced protective responses are poorly understood. Here, we show that ISR activation by arsenite (Ar) results in suppression of IL-1β production in macrophages and inhibition of DSS-induced colitis in a murine model through a novel posttranscriptional and translation regulatory (PTR) mechanism. Ar triggers PTR events through eIF2α-phosphorylation, which results in the attenuation of active polysome formation leading to the accumulation of translationally stalled IL-1β mRNAs. Translationally stalled IL-1β mRNAs recruit RNA-binding proteins (TIA-1/TIAR), resulting in the formation of RBP-RNA complexes known as stress granules (SGs). The SGs bound IL-1β mRNAs might undergo degradation through induction of autophagy. Also, we show that Ar posttranslationally impairs processing and secretion of IL-1β by diminishing inflammasome activation. Altogether, this study unveils a novel mechanism of IL-1β regulation and further suggests that pharmacological activation of cytoprotective ISR pathway might provide an effective therapeutic intervention against inflammatory diseases.

Keywords: arsenite · autophagy · integrated stress response (ISR) pathway · stress granules · TIA-1/TIAR

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Adaptation of the immune cells to the changing environmental stress cues is crucial for the maintenance of immune homeostasis, which is majorly mediated through activation of the cytoprotective adaptive pathway, so-called "integrated stress response (ISR)" [1, 2]. The ISR pathway can be activated through eukaryotic translation initiation factor-2 (eIF2α) kinases such as PKR

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Light-triggered selective ROS-dependent autophagy by bioactive nanoliposomes for efficient cancer theranostics†

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Light-responsive nanoliposomes are being reported to induce cancer cell death through heat and reactive oxygen species (ROS). Nanoliposomes (CIR NLPs) encapsulating a near-infrared (NIR) light-sensitive dye, IR780, and a bioactive chlorophyll-rich fraction of *Anthocephalus cadamba* (CfAc) were synthesized and characterized. These CIR NLPs, when activated by NIR light, displayed localized synergistic cancer cell death under *in vitro* and *in vivo* conditions. We demonstrated a NIR light-mediated release of CfAc in cancer cells. The bioactive CfAc was selective in causing ROS generation (leading to autophagic cell death) in cancer cells, while normal healthy cells were unaffected. An increase in the intracellular ROS leading to enhanced lipidation of microtubule-associated protein light chain 3 (LC3-II) was observed only in cancer cells, while normal cells showed no increase in either ROS or LC3-II. *In vivo* analysis of CIR NLPs in an orthotopic mouse model showed better anti-tumorigenic potential through a combined effect (*i.e. via* heat and CfAc). We reported for the first time induction of selective and localized, bioactive phyto fraction-mediated autophagic cancer cell death through an NIR light trigger. The synergistic activation of ROS-mediated autophagy by light-triggered nanoliposomes can be a useful strategy for enhancing the anticancer potential of combinational therapies.

1. Introduction

Light has been used as a diagnostic as well as therapeutic modality (theranostics) for several decades. It serves as a non-invasive, direct and accurate mode of treatment. It can be easily focused and tuned to localized treatment, causing relatively less damage to healthy tissues. Photo-based therapeutic modalities enable flexible dosages (*i.e.* adjustable regimen in terms of the therapeutic area, time and dosage) based on patients' physiological response and clinical needs.[1] The current light-based theranostic systems are responsive to ultraviolet (UV), visible (Vis) and near-infrared (NIR) wavelengths. UV irradiation is used for the treatment of various skin diseases.[2] As a new and preferred light source for phototherapy, blue light-emitting diodes (400–500 nm) are used for treating hyperbilirubinemia in infants.[3] Red/NIR lasers (700–1100 nm) are used for direct thermal ablation of tumors.[4]

UV/Vis light has a tissue penetration depth of a few microns. Higher scattering and absorption of UV/visible light by biological tissues prevent their penetration to deeper tissues. NIR light has relatively better tissue penetration ability compared with UV/Vis light.[5] Biological tissues are transparent to NIR light, exhibiting minimal or negligible absorbance, thereby enabling better penetration depth.[6]

The United States Food and Drug Administration (US FDA) has recently approved near-infrared autofluorescence (NIR-AF) as a high accuracy label-free modality for intraoperative parathyroid identification.[7] NIR-mediated phototherapies have emerged as a new technology for the treatment of various types of cancers. The significant advantages of these therapies are (i) minimal invasiveness, (ii) greater tissue penetration with an ability to focus on the tumor area, (iii) reduced systemic toxicity and (iv) spatio-temporal control.[4,8] NIR light-activated nanomaterials have been reported to generate heat/reactive oxygen species (ROS) resulting in photothermal therapy (PTT)/photodynamic therapy (PDT), respectively.

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PLOS BIOLOGY

RESEARCH ARTICLE

Amino acid starvation sensing dampens IL-1β production by activating riboclustering and autophagy

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Abstract

Activation of the amino acid starvation response (AAR) increases lifespan and acute stress resistance as well as regulates inflammation. However, the underlying mechanisms remain unclear. Here, we show that activation of AAR pharmacologically by Halofuginone (HF) significantly inhibits production of the proinflammatory cytokine interleukin 1β (IL-1β) and provides protection from intestinal inflammation in mice. HF inhibits IL-1β through general control nonderepressible 2 kinase (GCN2)–dependent activation of the cytoprotective integrated stress response (ISR) pathway, resulting in rerouting of IL-1β mRNA from translationally active polysomes to inactive ribocluster complexes—such as stress granules (SGs)—via recruitment of RNA-binding proteins (RBPs) T cell–restricted intracellular antigen-1 (TIA-1)/TIA-1–related (TIAR), which are further cleared through induction of autophagy. GCN2 ablation resulted in reduced autophagy and SG formation, which is inversely correlated with IL-1β production. Furthermore, HF diminishes inflammasome activation through suppression of reactive oxygen species (ROS) production. Our study unveils a novel mechanism by which IL-1β is regulated by AAR and further suggests that administration of HF might offer an effective therapeutic intervention against inflammatory diseases.

Author summary

Reduced intake of food (also known as dietary restriction) without malnutrition has been shown to benefit health in humans and animals, including an increase in life expectancy, metabolic fitness, and resistance to acute stress. Recent studies have attributed the benefits

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Equine immunoglobulin fragment F(ab')$_2$ displays high neutralizing capability against multiple SARS-CoV-2 variants

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ABSTRACT

Neutralizing antibody-based passive immunotherapy could be an important therapeutic option against COVID-19. Herein, we demonstrate that equines hyper-immunized with chemically inactivated SARS-CoV-2 elicited high antibody titers with a strong virus-neutralizing potential, and F(ab')$_2$ fragments purified from them displayed strong neutralization potential against five different SARS-CoV-2 variants. F(ab')$_2$ fragments purified from the plasma of hyperimmunized horses showed high antigen-specific affinity. Experiments in rabbits suggested that the F(ab')$_2$ displays a linear pharmacokinetics with approximate plasma half-life of 47 h. *In vitro* microneutralization assays using the purified F(ab')$_2$ displayed high neutralization titers against five different variants of SARS-CoV-2 including the Delta variant, demonstrating its potential efficacy against the emerging viral variants. In conclusion, this study demonstrates that F(ab')$_2$ generated against SARS-CoV-2 in equines have high neutralization titers and have broad target-range against the evolving variants, making passive immunotherapy a potential regimen against the existing and evolving SARS-CoV-2 variants in combating COVID-19.

1. Introduction

COVID-19 caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has resulted in over 377 million infections with more than 5.6 million deaths globally as of February 2022 [1]. Though several vaccines have been approved for immunization, it would require years of continuous vaccination drive before we defeat the disease [2,3]. Remdesivir is an antiviral drug used for treating COVID-19, though with limited efficacy [4]. The long delay in vaccination programs coupled with the unavailability of effective drugs and emergence of new variants indicates that COVID-19 is far from being over [5–7]. The situation calls for multiple approaches in countering the viral spread.

Neutralizing antibody (nAb)-based passive immunotherapy has been used as an antiviral therapy module against various intractable viral diseases [8] by blocking the viral attachment and entry into the host cell. Convalescent plasma from the recovered patient has been used as an emergency treatment plan for COVID-19 [9]. However, its scope is limited due to the lack of abundant and reliable blood source, heterogeneous antibody titer, and possible risks of transmission of blood-borne infections [7,10]. Antisera with improved efficacy purified from hyper-immunized equines are a good alternative to convalescent plasma [11,12], but they carry the risk of antibody-dependent enhancement of infection (ADE) and serum sickness [13–16] mostly due to the presence of a constant region (Fc) that allows non-neutralizing antibody attached to the virus to enter cells expressing FcγR [17]. To overcome these limitations, next-generation passive immunotherapy uses the F(ab')$_2$ fragment of antigen-specific immunoglobulins by removing the Fc region of the antibody [18–24].

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Immunogenic profiling of Mycobacterium tuberculosis DosR protein Rv0569 reveals its ability to switch on Th1 based immunity

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ABSTRACT

Mycobacterium tuberculosis (M.tb) is a multifaceted bacterial pathogen known to infect more than 2 billion people globally. However, a majority of the individuals (>90%) show no overt clinical symptoms of active Tuberculosis (TB) and, it is reported that *M.tb* in these individuals resides in the latent form. Therefore, a huge burden of latently infected population poses serious threat to the human health. Inconsistent efficacy of BCG vaccine and poor understanding of latency-associated determinants contribute to the failure of combating *M.tb*. The discovery of DosR as the master regulator of dormancy, opened new avenues to understand the pathophysiology of the bacterium. Though the specific functions of various DosR genes are yet to be discovered, they have been reported as potent T-cell activators and could elicit strong protective immune responses. Rv0569 is a DosR-encoded conserved hypothetical protein overexpressed during dormancy. However, it is not clearly understood how this protein modulates the host immune response. In the present study, we have demonstrated that Rv0569 has a high antigenic index and induces enhanced secretion of Th1 cytokines IL-12p40 and TNF-α as compared to Th2 cytokine IL-10 in macrophages. Mechanistically, Rv0569 induced the transcription of these pro-inflammatory signatures through the activation of NF-κB pathway. Further, immunization of mice with DosR protein Rv0569 switched the immune response towards Th1-biased cytokine pattern, characterized by the enhanced production of IFN-γ, IL-12p40, and TNF-α. Rv0569 augmented the expansion of antigen-specific IFN-γ and IL-2 producing effector $CD4^+$and $CD8^+$ T-cells which are hallmarks of Th1 biased protective immunity. Additionally, IgG2a/IgG1 and IgG2b/IgG1 ratio in the serum of immunized mice further confirmed the ability of Rv0569 to skew Th1 biased immune response. In conclusion, we emphasize that Rv0569 has the ability to generate signals to switch on Th1-dominated responses and further suggest that it could be a potential vaccine candidate against latent *M.tb* infection.

1. Introduction

Tuberculosis (TB) is caused by the slow-growing, intracellular pathogen *Mycobacterium tuberculosis* (*M.tb*) which is known to infect more than one-third of the world's population [1]. However, it has been shown that in majority (90%) of the infected individuals, *M.tb* resides in a metabolically reversible state known as latent/dormant form and doesn't show any overt clinical symptoms of active TB. However, the huge burden of latently infected individuals poses severe global threat to human health. These latently infected individuals are a great reservoir of the pathogen and also carry a serious risk of developing active infection any time during their lives. Although *Mycobacterium bovis* Bacille Calmette Guerin (BCG), the only licensed vaccine against *M.tb,* successfully protects infants from disseminated TB, it is highly variable in adults against pulmonary infection [2]. This failure is indicated by a huge population of vaccinated individuals carrying the pathogen in an asymptomatic form defined as dormancy/latency [2].

The outcome of a *Mycobacterial* infection is dictated by both innate

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Programming the Immunogenicity of Dengue Virus Envelope Protein Domain III (EDIII): An Approach to Develop a Tetravalent Vaccine Formulation against Dengue Virus

by Rafiq Khan

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ORIGINALITY REPORT

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R Khan

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11/07/2022

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