Role of Integrated Stress Response (ISR) pathway in programming the immunogenicity of antigens: An approach to develop a novel vaccine adjuvant

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Doctor of Philosophy

By

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CERTIFICATE

This is to certify that the thesis, entitled *"Role of Integrated Stress Response (ISR) pathway in programming the immunogenicity of antigens: An approach to develop a novel vaccine adjuvant"* submitted by **Mr. Shaikh Matin Rahim** bearing registration number **15LTPH02** 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.

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1. Afroz, S., Shama, Battu, S., **Matin, S.**, Solouki, S., Elmore, J. P., Minhas, G., Huang, W., August, A. and Khan, N. "Amino Acid Starvation Enhances Vaccine Efficacy By Augmenting Neutralizing Antibody Production". *Science Signaling*, vol 12, no. 607, 2019. *American Association For The Advancement Of Science (AAAS)*, doi:10.1126/scisignal.aav4717.
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And has made presentations in the following conferences:

1. As a poster in Indian Immunology Society conference IMMUNOCON 2019, DAE convention centre Mumbai, India.
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Further, the student has passed the following courses towards fulfilment of coursework requirement for Ph.D.

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DECLARATION

I Shaikh Matin Rahim, hereby declare that the work presented in this thesis, entitled as *"Role of Integrated Stress Response (ISR) pathway in programming the immunogenicity of antigens: An approach to develop a novel vaccine adjuvant"* has been carried out by me under the supervision of **Prof. Nooruddin Khan** at Department of Biotechnology and Bioinformatics. To the best of my knowledge, this work has not been submitted for the award of any degree or diploma at any other university or institution. A report on plagiarism statistics from the University Librarian is enclosed.

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Dedicated to my parents and grandparents

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Shaikh Matin Rahim

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ACRONYMS

- (µl) Microlitre
- (µM) Micromolar
- (µm) Micron
- (ADE) Antibody-dependent enhancement
- (APCs) Antigen-presenting cells
- (ATF) Activating transcription factors
- (ATF4) Activating transcription factors
- (Ar) Arsenite /Sodium arsenite / Sodium metaarsenite
- (BSA) Bovine serum albumin
- (bZIP) Basic leucine zipper
- (CD) Cluster of differentiation
- (CDS) Coding DNA sequence
- (CFA) Complete Freund's adjuvant
- (CpG) Cytosine phosphate guanidine
- (CREB) Cyclic AMP response element binding proteins
- (CReP) Constitutive repressor of eIF2α phosphorylation
- (CTL) Cytotoxic T lymphocytes
- (DC) Dendritic cells
- (DENV-1) Dengue virus type 1
- (DENV1rEDIII) Envelope domain III protein of dengue virus
- (DENV-2) Dengue virus type 2
- (DENV2rEDIII) Envelope domain III protein of dengue virus
- (DENV-3) Dengue virus type 1
- (DENV-4) Dengue virus type 1
- (DF) Dengue Fever
- (DHF) Dengue haemorrhagic fever
- (dsRNA) Double-stranded RNA
- (DSS) Dengue shock syndrome
- (ED-I) Domain I of the envelope protein of dengue virus
- (ED-II) Domain II of the envelope protein of dengue virus
- (ED-III) Domain III of the envelope protein of dengue virus

- (eIF1) Eukaryotic initiation factor 1
- (eIF1A) Eukaryotic initiation factor A
- (eIF2) Eukaryotic initiation factor 2
- (eIF2B) Eukaryotic initiation factor 2 B
- (eIF3) Eukaryotic initiation factor 3
- (eIF4F) Eukaryotic initiation factor 4F
- (eIF5) Eukaryotic initiation factor 5
- (FBS) Fetal bovine serum
- (ER) Endoplasmic reticulum
- (GADD34) Growth arrest and DNA damage-inducible protein GADD34
- (GC) Germinal centre
- (GCN2) General control non-depressible protein 2
- (GDP) Guanosine diphosphate
- (GTP) Guanosine triphosphate
- (HA) Hemagglutinin
- (HCV) Hepatitis C virus
- (HiB) Haemophilus influenza type B
- (HIV) Human Immunodeficiency Virus
- (HRI) Heme-regulated eIF2α kinase
- (IFA) Incomplete Freund's adjuvant
- (IFN-γ) Interferon-γ
- (IL-12) Interleukin-12
- (IRAK) Interleukin (IL)-1 receptor-associated kinase
- (IRIV) The immunopotentiating reconstructed influenza virosome
- (ISR) Integrated stress response
- (kDa) Kilo Dalton
- (LPS) Lipopolysaccharides
- (MCs) Macrophages and mast cells
- (Met-tRNAi) Methionyl tRNA
- (MHC I) Major histocompatibility complex I
- (MHC II) Major histocompatibility complex II
- (MPL) Monophosphoryl lipid A

- (MyD88) Myeloid differentiation primary response 88
- (NA) Neuraminidase
- (NF-kB) Nuclear factor kappa B
- (NLRs) Nucleotide-binding domain and leucine-rich repeat-containing receptors
- (ODN) CpG Oligodeoxynucleotides
- (OVA) Ovalbumin
- (PAGE) Polyacrylamide gel electrophoresis
- (PERK) PKR-like ER kinase
- (PKR) Double-stranded RNA-dependent protein kinase
- (Poly(I:C)) (polyinosinic: polycytidylic acid) is a synthetic double-stranded RNA.
- (PP1) Protein phosphatase 1
- (PP1c) PP1 catalytic subunit
- (PRNT) Plaque reduction neutralizing tests
- (RBPs) RNA binding proteins
- (RLRs) Retinoic acid-inducible gene-I (RIG-) -like receptors
- (RT) Room Temperature
- (RT-PCR) Reverse transcription polymerase chain reaction
- (S.C.) subcutaneous
- (SD) Severe Dengue
- (SDS) Sodium dodecyl sulfate
- (SGs) Stress granules
- (tDENVrEDIII) Tetravalent combination of EDIII domain antigen from all the four serotypes of dengue virus
- (Th1) T helper 1
- (Th2) T helper 2
- (TIA-1) T cell-restricted intracellular antigen-1
- (TIAR) TIA-1 related
- (TLRs) Toll-like receptors
- (TNF-α) Tumour necrosis factor- α
- (TRAF-6) Tumour necrosis factor receptor (TNFR)-associated factor 6
- (TRIF) TIR-domain-containing adapter-inducing interferon-β
- (uORF1) Upstream open reading frame

- (UTRs) Untranslated regions
- (vRNA) Viral RNA
- (VLPs) Virus-like particles
- (YF17D Yellow Fever Vaccine

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 Vaccine

The discovery of vaccines has been one of the greatest triumphs of modern medicine. It has been used to save millions of lives globally. A vaccine is a biological product that protects against infection or disease on exposure to a pathogen. An essential part of the vaccine is one or more antigens in combination with appropriate adjuvants that enhance the immune response and provide protection. Vaccines are generally classified as live or non-live (Pollard & Bijker, 2021). Live attenuated vaccine is made up of an attenuated form of the bacteria or virus. In a live attenuated vaccine, the pathogenic organism or virus is attenuated by the serial passage and involves the selection of the mutant, which has reduced virulence or toxicity. The advantage of live attenuated vaccines it can replicate similarly to their pathogenic form without administration of the disease. It gives a long-lasting humoral and cell-mediated immune response. Examples of live attenuated vaccines are polio and Bacille Calmette-Guerin (Jiskoot et al., 2013). Subunit vaccines are made up of the only antigenic components from the pathogen which elicit an effective immune response. In subunit vaccines, proteins, nucleic acid and polysaccharides are used as antigens (Heidary et al., 2022). Subunit vaccines are safer than live attenuated ones (Wang et al., 2016). In inactivated or killed vaccines, the pathogen is inactivated using different methods such as chemical, heat or radiation. Pathogen is inactivated, so it can't be replicated in the host cell. It is safe to use in immunosuppressed patients. Recombinant/DNA vaccine consists of the gene encoding antigen inserted into the vector. It is more stable as compared to the traditional vaccines. There is good control over the recombinant vaccine design, as any gene can be inserted or deleted (Vartak & Sucheck, 2016a).

1.2 Limitations of vaccines

In the live attenuated vaccine, the reversion of the pathogen is a major limitation factor, and it is not suggested for the immunosuppressed individual. The inactivated vaccine requires the booster dose because it protects for a limited period of time (Bowick & McAuley, 2011). The recombinant vaccine has a high production cost and may cause a mutation in host DNA. The subunit vaccine is less immunogenic as compared to the live attenuated vaccine. In the case of the conjugated vaccine, it isn't easy to control the batch-wise variation (Vartak & Sucheck, 2016b). Vaccines require stringent safety requirements and the cold chain to maintain the efficacy of vaccines. Some pathogens require a more complicated vaccine, while some available licensed vaccines have low efficacy. There is a significantly lower number of available licensed adjuvants (Kennedy et al., 2020).

1.3 Challenges to develop new Vaccines

1.3.1 The immune system is complex to understand

The immune system consists of different types of tissue and organs. Many signaling pathways (Breuer et al., 2013) and hundreds of other cells have different effector molecules that recognize the foreign antigen (Kennedy et al., 2020). Everything should work effectively in proper sequence. Immunologists develop a catalogue that consists of collections of the immune system (Hurley, 2021), but it isn't easy to understand how this immune system collaboratively works together (Cady et al., 2020; Kennedy et al., 2020).

1.3.2 Variability of host

The host gene variation impacts the vaccine's immunological response (Kennedy et al., 2020). Due to the genetic diversity of the human population, vaccine-immunogenetics research has emerged. This field examines the links between genetic variables and immune response variability to identify significant genetic variants linked to variances in immune responses (Brodin & Davis, 2017).

1.3.3 Variability of pathogen

Variation in the Genetic sequence of pathogens is a significant restriction in the development of vaccines. Variability of pathogens can occur in multiple ways, such as 1) more sequence diversity among the viral strains that lead to the cross-protective immune response (Poland & Barry, 2009), 2) Antigenic shift or drift in the case of influenza strains that lead to the yearly formulation of vaccine (Kennedy et al., 2020) 3) complicated life cycle of pathogen due to its large genome, e.g. *Plasmodium falciparum* (Kennedy et al., 2020) 4) rapid mutation rate of the pathogen such as HIV and HCV (Kennedy et al., 2020) .

1.4 How do vaccines activate immune responses?

Vaccine functions through the activation of innate and adaptive arms of the immune system, which work hand-in-hand to mount an effective protective immune response (Clem, 2011).

1.4.1 Innate arm of immunity

The first line of protection for the immune system is provided by innate immunity. This response is not specific to the particular pathogen. All microbes share the same molecular pattern, which is conserved in innate immune cells. The innate immune system lacks memory. The anatomic barrier, which stops many microbes from entering through the skin and mucous

membrane, is where the innate immune system's defensive mechanism starts. The innate immune system has physiological defences, including the body's average temperature, lysozyme, interferon, collectins, and gastric acidity. Body temperature variations inhibit many pathogen's growth. Hydrolytic enzyme lysozyme is found in the mucous membrane and cleaves the bacterial cell wall made up of the peptidoglycan layer (Clem, 2011). Collectins are surfactant proteins that break the lipid membrane of bacteria to kill them, or they might clump the bacteria to promote phagocytosis (Hartshorn et al., 2002). Pattern recognition receptors are also part of the innate immune response. They offer a quick response against the respective pathogen despite not being specific to any one pathogen. All cells of the innate immune system have PRRs as membrane proteins. PAMPs (pathogen-associated molecular patterns) that cause the production of cytokines are recognised by PRRs. PRRs recognising PAMPs cause the activation of phagocytes and complement (Pashine et al., 2005). Monocytes are essential in connecting the innate to the adaptive immune response. Monocytes are also crucial in antigen presentation and phagocytosis (Clem, 2011). Blood-circulating monocytes undergo differentiation to become tissue-specific macrophages or dendritic cells. The diverse immune system tasks are carried out by various types of dendritic cells. Dendritic cells deliver antigens to T lymphocytes (Pashine et al., 2005). Granulocytes consist of neutrophils, eosinophils, and basophils/mast cells. The first phagocytic cells to reach the site of inflammation are neutrophils. Eosinophils are also phagocytic cells that play an essential role in parasite resistance. Basophils and mast cells affect allergies. To fight the pathogenic agent, innate immunity activates the adaptive immune response (Clem, 2011).

1.4.2 Adaptive arm of immunity

The innate immune system responds quickly and without memory. The B and T cells are the adaptive immune response components. B cell consists of antibody-mediated immunity as the humoral arm of the immunity, while T cell immunity refers to cell-mediated immunity (Clem, 2011). The lymph node is where B lymphocytes contact a specific pathogen after they are generated in the bone marrow. B cells recognise the antigen in its native form, meaning it does not require antigen processing and presentation by the T cells. These antigens are called T-independent antigens because T cells do not require B cell activation. Dextran, bacterial polymeric flagellin, and lipopolysaccharide are some such antigens. T-cell activation of B cells improves immune response and memory. The somatic hypermutation in the B cells that results in the fit between the Fab region and antigen is initiated by the antigen binding to the Fab Region of the antibody and the secondary signals from cytokines by the T cell. This procedure

encourages B cells to develop into plasma cells, which triggers the production of antibodies. From the stimulated B cells, the clones of the B cells are produced. These cells become the plasma cells to secrete antibodies and memory B cells to respond against exposure to the same pathogen. It will take many days for the clonal selection and growth procedure to take place. The clonal selection primarily involves the production of IgM, then the IgG, and later IgD, IgA, and IgE antibodies. IgG is better known for its neutralization and is best for the vaccine, while IgA is present in the saliva and mucous secretions. IgE is involved in allergic and parasitic infections. Cell-mediated immunity is a component of adaptive immunity that primarily protects against intracellular pathogens. T cell maturation occurs in the thymus before being discharged into the bloodstream. Two categories of T cells exist: CD4 and CD8 cell types. T helper cells with the CD4 coreceptor recognise the MHC II receptor. For humoral immunity mediated by antibodies to regulate extracellular pathogens, CD4 cells are necessary. CD4 cells have two subsets, Th1 and Th2. While Th2 is linked to antibody-mediated immunity, Th1 is connected to cell-mediated immunity. T cytotoxic cells with the CD8 coreceptor recognize the major histocompatibility complex (MHC) I. CD8 cells are required for the cell-mediated immunity that controls Intracellular pathogens. T cells recognize the antigen, which is processed and presented by the antigen-presenting cells (Clem, 2011).

1.5 Adjuvants

The Latin verb adjuvare, which means "to help," is where "adjuvant" comes from. Adjuvant increasing immunogenicity of vaccine antigen. The adjuvant enhances humoral and T-cell-mediated immunity. There are many different adjuvant systems, such as the delivery system, which includes mineral salts, aluminium salt (Alum), calcium phosphate and Freund's adjuvant. Microparticle delivery systems, another class of adjuvants, are immune potentiators and mucosal adjuvants (Apostólico et al., 2016). Adjuvants interact with the innate immune system's many receptors and signalling molecules, and these innate reactions powerfully promote the adaptive immunological response. Adjuvants also guide the adaptive immune response to produce an effective form of immunity for each specific pathogen. Two main reasons to incorporate the adjuvant in the vaccine. The first is that adjuvants are used in medicine to improve vaccine response in the general public by raising antibody titers. The other reason is to improve the qualitative alteration of an immune response. Adjuvants used in the clinical and pre-clinical studies to 1) provide the functionally appropriate types of immune response TH1 (T helper 1) versus TH2 (T helper 2) response, $CD4^+$ T cells versus $CD8^+$ T cells response (Coffman et al., 2010). 2) increase in the generation of T cell memory (Galli et al.,

2009). 3) Accelerating early response time could be crucial in the pandemic infection breakout (Khurana et al., 2010).

1.5.1 Classification of adjuvants

1.5.1.1 Delivery system

1.5.1.1.1 Mineral Salts

The delivery system has a variety of components, including microparticles, lipid particles, and aluminium salts (alum). Since 1920, alum has been the most frequently used adjuvant (Lindblad, 2004). Pneumococcus and Haemophilus influenza type B (HiB) vaccines contain alum in their formulation (Apostólico et al., 2016). Recently, it was thought that alum caused the slow release of a related antigen (Gupta et al., 1995). Recent reports show that if the antigen's depot effect were removed, the immunogenicity of the antigen would be unaltered; it proves that alum is working through the other mechanism rather than the depot effect (Hutchison et al., 2012). Without the depot effect, alum activates the innate immune response. Adjuvants that contain aluminium do not utilise the conventional MyD88 and TLRs pathway; instead, they use the inflammasome complex pathway (Lambrecht et al., 2009).

1.5.1.1.2 Emulsion Adjuvants

1.5.1.1.2.1 Freund's Adjuvants.

Mycobacteria that have been heat-killed and an oil-in-water emulsion make up Complete Freund's adjuvant (CFA). CFA represents this group of adjuvants (Billiau & Matthys, 2001). Only the oil-in-water emulsion made with incomplete Freund's adjuvant (IFA) is free of mycobacteria. IFA was widely used in the 50s to prepare the influenza vaccine. This vaccine gives a long-lived antibody response compared to one without an adjuvant (Salk & Laurent, 1952). The antigen-loaded oily deposit helps the continuous release of antigens, extending the antigen's half-life and boosting local immunity via phagocytosis and the generation of cytokines (MUSSENER et al., 1995).

1.5.1.1.2.2 MF59

It is a water-in-oil emulsion compound that belongs to the squalene family and is approved for use in flu vaccines. It is also permitted in the H1N1 pandemic in young children and pregnant women (O'Hagan et al., 2013). Similar to other adjuvant mechanisms through which MF59

works, it is not completely understood. Like an alum, it does not depend on the depot formation (Awate et al., 2013). MF59 activates both the humoral and cell-mediated immune response, in which the humoral immune response gives a high titer of functional antibodies (Stephenson et al., 2005). The macrophages, local monocytes, and DCs are stimulated by MF59 to release cytokines that cause leukocyte recruitment and antigen absorption, which migrate to the lymph node and help in activation of adaptive immunity (O'Hagan et al., 2013).

1.5.1.1.2.3 AS03

ASO3 is a water-in-oil emulsion consisting of polysorbate 80, squalene, and α-tocopherol. It is first used in the human malaria vaccine (Morel et al., 2011). By triggering the immune cell recruitment, NF-kB activation, and development of high antibody titers, AS03 enhances the immunological response (Garçon et al., 2012).

1.5.1.1.3 Microparticles

1.5.1.1.3.1 Virus-Like Particles

The structural proteins of viruses, such as the capsid or envelope, which resemble the size, shape, and molecular organisation of viruses and have self-assembling properties, create virus-like particles (VLPs) (Muratori et al., 2010). VLPs have a high level of immunogenicity due to their lack of replication and infectious potential (Huret et al., 2013). Depending on the parental virus VLPs can be enveloped or nonenveloped virus-like particles. Enveloped virus particles comprise the virus-cell membrane (envelope) and the antigen of interest. Nonenveloped virus-like particles comprise the only pathogen component that can self-assemble (Kushnir et al., 2012). VLPs are involved in T cell activation through the dendritic cell's activation, so they activate both branches of immunity, humoral and cell-mediated (Smith et al., 2013).

1.5.1.1.3.2 Virosomes

Virosomes include in the type of VLP. The native virus is made up of lipids and envelope proteins, which function as a transport system for both antigens and adjuvant. It lacks genetic material, and doesn't replicate (Zurbriggen, 2003). By employing detergent to dissolve the virus's envelope proteins and extracting the virus' genetic material and non-membranous proteins, virosomes can be created (Copland et al., 2005). The immunopotentiating reconstructed influenza virosome (IRIV), which has the proteins neuraminidase (NA) and hemagglutinin (HA) intercalated within a lipid membrane, is an illustration of the virosomes

system (Morse et al., 2011). IRIV functions by endocytosis, in which sialic acid and virosomal HA bind with APCs. The HA modifies the confirmation and fused antigen released in the cytoplasm or stays in the endosomes to follow either the MHC I or MHC II route after endosome acidification. Virosomes boost the costimulatory molecules expression on the APC, such as CD40, CD80, and CD86. Additionally, this mechanism results in the activation of $CD8^+$ and $CD4^+$ T cells and also the generation of cytokines (Felnerova et al., 2004).

1.5.1.2 Immune Potentiators

Immune potentiators influence innate immunity via PRRs such as RLRs, TLRs, and NLRs. The agonists' activation of the PRRs results in the activation of the APC and the generation of cytokines, which in turn triggers an adaptive immune response. MPL, flagellin, Imiquimod, resquimod and ODN are PRRs agonists (Apostólico et al., 2016).

1.5.1.2.1 Agonists of TLR3

Poly (I:C) is an agonists of TLR3, which is extensively studied adjuvant in conditions like HIV, malaria, dengue, and cancer. Antigen processing, APC maturation, and ultimately, B and T cell immunity are all improved by poly (I:C) (Apostólico et al., 2016).

1.5.1.2.2 Agonists of TLR4

Monophosphoryl lipid A (MPL) is an example of the TLR4 agonist. Similar to LPS, MPL interacts with TLR4 to activate the immune system. (Duthie et al., 2011). MPL function involves activating the TRIF signalling pathway, which causes the production of several cytokines, including the TNF-α (Mata-Haro et al., 2007). MPL contributes to the synthesis of IL-12 and IFN-γ, which encourages Th1 responses (Apostólico et al., 2016).

1.5.1.2.3 Agonists of TLR5

Both gram-positive and Gram-negative bacteria consist of flagellin as a component of the flagella. The TLR5 recognises flagellin. TNF-α production is induced by flagellin, and mixed TH1/TH2 responses and high antibody production are also induced (Didierlaurent et al., 2009)

1.5.1.2.4 Agonists of TLR7/8

Resiquimod and Imiquimod imidazoquinolines with antiviral properties (Weeratna et al., 2005) (Harrison et al., 1994). Similar to single-stranded RNA, imidazoquinolines are recognised by

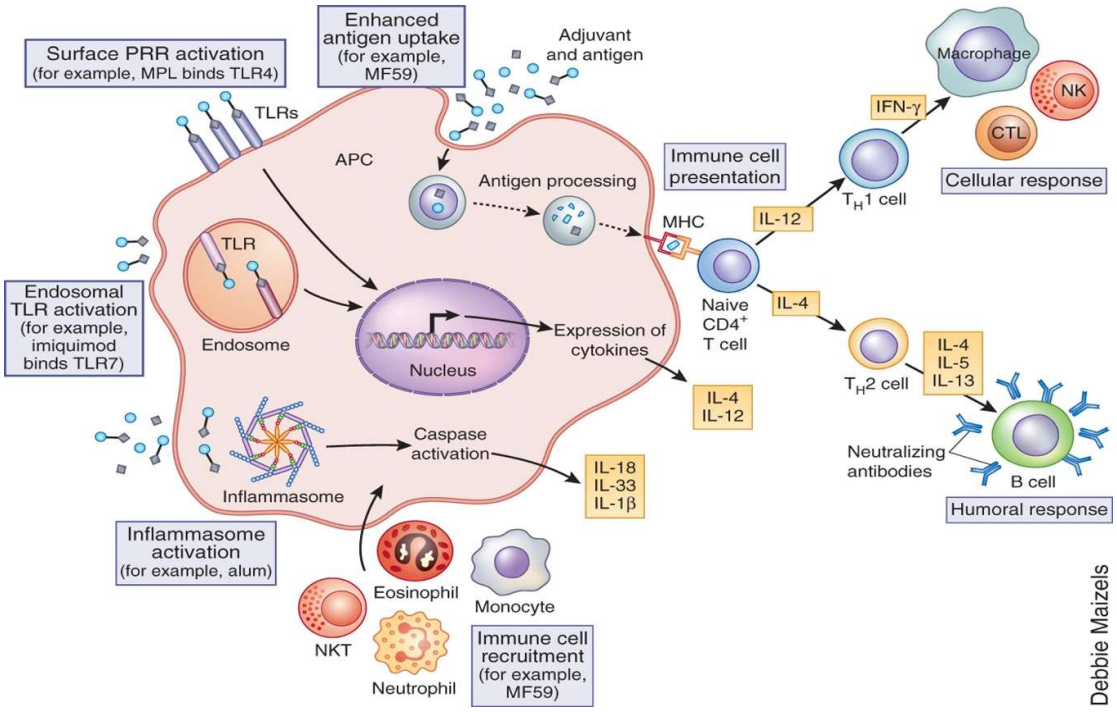
the TLR7/8 on endosomes and cause signals to be sent via the MyD88 signaling (Hemmi et al., 2003). Resiquimod stimulates TLR7 and TLR8, whereas imiquimod activates TLR7 (Ma et al., 2010).

1.5.1.2.5 Agonists of TLR9

CpG (cytosine phosphate guanidine) and ODNs (oligodeoxynucleotides) are unmethylated CG motifs having a length of 18-25 oligonucleotides. The TLR9 recognizes CpG ODNs (Dasari et al., 2005). TLR9 signal works through the TRAF-6, IRAK and MyD88 that, finally, leads to the activation of proinflammatory cytokines and costimulatory molecules (CD80, CD86, and CD40) (Krieg, 2003) (Klinman, 2004).

1.5.2 Adjuvant's mechanisms of action.

Adjuvants act by various mechanisms such as depot effect formation, cytokine and chemokine induction, antigen presentation, immune cell recruitment and the transport of antigens to draining lymph node. Adjuvants that trigger innate immune responses assist in changing the type and extent of the adaptive immunological response (Awate et al., 2013). Depot development at the injection site is the oldest and most well-known mode of action for adjuvants. The antigen is trapped at the injection site and released slowly, which causes the immune system to be constantly stimulated and produce antibodies (Siskind & Benacerraf, 1969). The electrostatic interaction of the antigen absorbed on the alum enhances antigen uptake and presentation (Mannhalter et al., 1985). A key component of triggering the adaptive immune response is dendritic cell activation. *In vitro*, human cell experiments found that alum and MF59 did not enhance direct dendritic cell activation. Still, they did enhance the expression of co-stimulatory molecules (CD86 & CD83) and MHC class II on granulocytes, macrophages and monocytes, which promoted the proliferation of T cells (Sun et al., 2003).



Reed, S. et al Nature Medicine, 19(12) (2013), 1597-1608

Figure 1: Adjuvant mechanisms of action

There are several methods by which adjuvants mediate their function.

1.5.3 Limitations of Current adjuvants

Some adjuvants are limited to certain antigens, e.g., the aluminum compound does not give an adjuvant effect when mixed with the typhoid vaccine, Hemagglutinin antigen (Gupta & Siber, 1995). The dose of adjuvant also affects the immunogenicity of antigen, e.g., aluminum compound, if a small amount of it is required to complete antigen absorption but may not be sufficient to enhance the immunogenicity of the antigen. So, an excess adjuvant is needed to obtain the maximum immune response. As the amount of the aluminum adjuvant increases, its effect increases to a specific limit, after which it decreases even though the aluminum increases. It has been speculated that a certain minimum amount of the antigen is necessary at that injection site for the depot effect or to stimulate the macrophages. A higher aluminium concentration may be toxic to macrophages or suppress the immunogenicity by covering the antigen completely with mineral salts. There are no authentic animal models for most diseases for which vaccine is being developed. So, it isn't easy to assess adjuvants for these vaccine antigens. Immune response was first evaluated in the animal models. Biological difference

between animal model and human varies in the clinical trials of adjuvants. Different animal species respond differently to other adjuvants (R. K. Gupta & Siber, 1995).

1.5.4 New Adjuvants are needed, but what is the way forward?

A better understanding of the vaccine immunobiology can provide insights into the new adjuvant discovery; one example is the Yellow Fever Vaccine (YF17D). Yellow fever vaccine is an attenuated virus that can be easily passaged in monkeys, mouse embryo tissue culture, and chicken embryo culture**.** It Induces multi-pronged immunity such as Th1, Th2, CTL and antibodies. Antibody titers persist for more than 30 years after vaccination. To date, more than 600 million people have been vaccinated. YF-17D activates multiple DC subsets via multiple TLRs to induce polyvalent immunity (T. Querec et al., 2006). Recent system vaccinology approaches have dissected the mechanism of YF17D tailors vaccine-induced protection. It was found that YF17D triggers an integrated stress response (ISR) pathway to program the magnitude of antigen-specific vaccine-induced protective immunity. YF17D induced enhanced T cell response by activating the Integrated stress response (ISR) pathway, which regulates autophagy and enhanced antigen presentation. It has been observed that the deletion of ISR components impairs CD8 T cell response in YF17D immunized mice (Ravindran et al., 2014a) (Nakaya et al., 2011).

1.6 Integrated Stress Response Pathway and its immunomodulatory functions

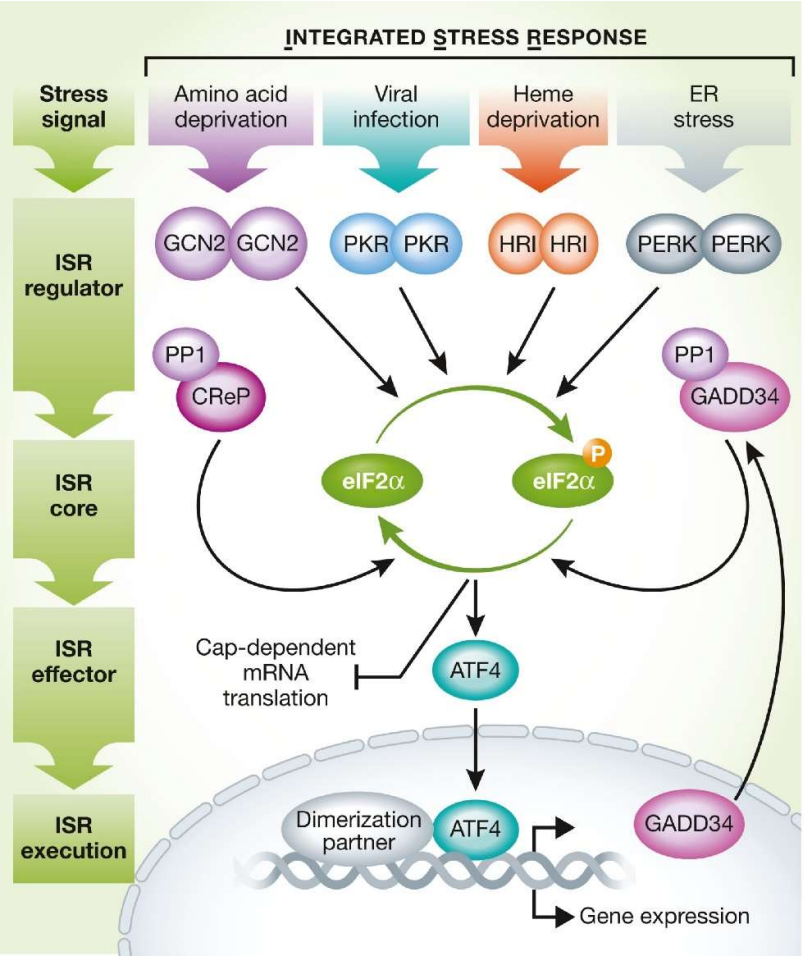
Arsenite acts as the inducer for the ISR pathway. It has been demonstrated that arsenic compounds trigger ISR signaling by phosphorylating eIF2 and forming SGs (Stress granules) (McEwen et al., 2005). Numerous biological processes, including apoptosis, proliferation, inflammation, angiogenesis, and immunological responses, have been demonstrated to be affected by arsenic compounds (GHOSH et al., 2008). Western medicine and traditional Chinese medicine have long employed arsenic compounds therapeutically to treat a range of ailments, including dental problems, rheumatism, psoriasis, syphilis, and trypanosomiasis (Kedersha et al., 1999).

1.7 Integrated stress response pathway (ISR)

To re-establish cellular equilibrium, eukaryotic cells start the integrated stress response system. This pathway's key event is the eukaryotic translation initiation factor eIF2-α phosphorylation, which stops all protein synthesis globally but activates a few particular genes, including ATF4. Cell-extrinsic factors, including hypoxia, glucose deprivation, viral infection, and amino acid deprivation, are the leading causes of stress (Pakos-Zebrucka et al., 2016a). The phosphorylation of eIF2-α on serine 51 is the point at which all stress responses converge (Ron, 2002). dephosphorylation of the eIF2-α leads to the ISR pathway termination and the start of normal protein synthesis (Novoa et al., 2001).

1.7.1 Elements of the ISR pathway

eIF2 complex consists of 3 subunits, α, β, and γ. The primary regulatory component, eIF2-α, has phosphorylation and RNA binding sites. eIF2 is essential in initiating mRNA translation, and AUG starts codon recognition (Jackson et al., 2010). The 43s pre-initiation complex (PIC), which is made up of eIF2 and the initiation factors eIF1 and eIF1A, GTP, and Met-tRNA, binds the 40S ribosomal subunit (Lomakin & Steitz, 2013). The 43S PIC complex binds to the 5'methylgunaine cap of mRNA, mediated by the eIF4F complex and other protein molecules. The cap-binding protein eIF4E is a component of the eIF4F complex, which also contains the scaffold protein eIF4G and the RNA helicase eIF4A. PIC is stabilised by interactions between eIF4G and eIF3, which causes it to move to the AUG start codon (Aitken & Lorsch, 2012). With the aid of eIF5, GTP on eIF2 is hydrolyzed, and eIF1 dissociates from the complex after binding the Met-tRNAi anticodon and the AUG start codon. As a result, the 40S ribosomal complex and the eIF2-GDP complex separate, making way for another round of mRNA translation initiation (Pakos-Zebrucka et al., 2016). To restore the eIF2 to its active state, the guanine nucleotide exchange factor converts the exchange of GDP for GTP (Jackson et al., 2010). Phosphorylated eIF2 restricts the eIF2B-mediated conversion of GDP to GTP during ISR activation, blocking the creation of the 43S PIC (Pakos-Zebrucka et al., 2016b). This results in the translation of specific mRNAs like ATF5, ATF4, and GADD34 and the induction of the 5′ Cap-dependent protein synthesis (Hinnebusch, 2011). eIF2α phosphorylation occurs at S51. This site's homozygous mutation precludes the phosphorylation of eIF2 (Scheuner et al., 2001). ER stress, starvation, and viral infection do not cause autophagy to be induced in cells with homozygous eIF2 mutation (Kouroku et al., 2007).



Pakos-Zebrucka et al (2016), EMBO Reports 17(10), 1374-1395

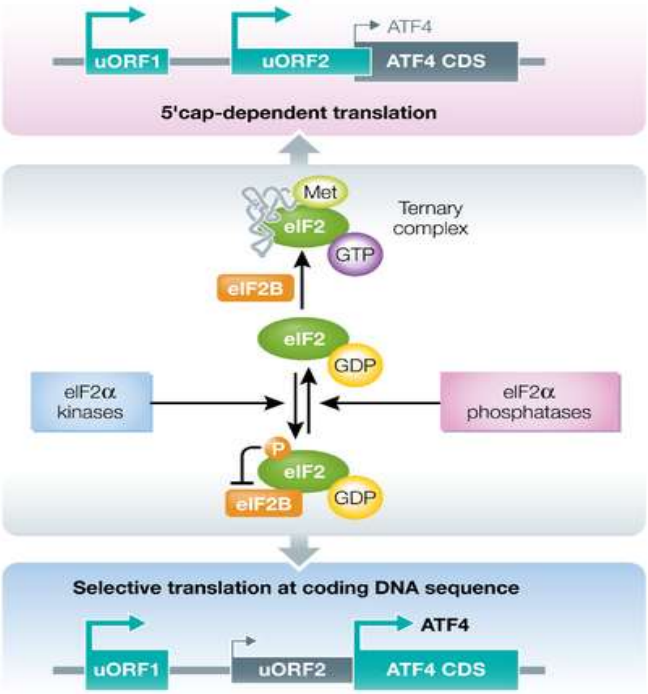
Figure 2: Integrated stress response pathway

The ISR's main protein, eIF2, is phosphorylated by the PERK, PKR, HRI, and GCN2 in response to stress signals. As a result, mRNAs that are unique to ISRs, including ATF4, are translated preferentially while concurrently attenuating Cap-dependent translation globally. The ISR's primary effector is that ATF4 can bind to the targeted DNA sites in the homo or heterodimerized form. ISR pathway terminated by the GADD34, which helps dephosphorylation of eIF2- α.

1.7.2 Activation of Integrated Stress Response Pathway

When there is a disruption in cellular homeostasis, the eIF2 kinase is activated (Pakos-Zebrucka et al., 2016b). There are four members of eIF2 kinase: double-stranded RNA-dependent protein kinase (PKR) (Donnelly et al., 2013), PKR-like ER kinase (PERK) (Perkins & Barber, 2004), general control non-derepressible 2 (GCN2) (Wek et al., 2006), and heme-regulated eIF2α kinase (HRI) (Donnelly et al., 2013). The catalytic domains of all four eIF2

kinases are very homologous, but their regulatory domains differ (Harding et al., 1999) (Berlanga et al., 1998) (J. J. Chen et al., 1991) (Shi et al., 1998). Kinase dimerizes and autophosphorylates for the complete activation of eIF2α (Lavoie et al., 2014). Each kinase shows a different regulatory mechanism. PERK is found in the metazoans in the endoplasmic reticulum (ER). Stress in the endoplasmic reticulum causes the activation of PERK (Pakos-Zebrucka et al., 2016). There are two pathways of PERK activation (Korennykh & Walter, 2012). According to the conventional theory, the build-up of a misfolded protein in the ER lumen causes GRP78 to separate from PERK, which then undergoes autophosphorylation and activation (Harding et al., 1999). According to recent research, PERK gets activated by binding unfolded or misfolded proteins to its luminal domain (Korennykh & Walter, 2012). GCN2 binds to deacylated transfer RNAs (tRNAs) in amino acid deprivation by the yeast's histidyl-tRNA synthetase-related domain (Vazquez de Aldana et al., 1994). Prolonged glucose deprivation in tumour cells leads to GCN2 activation. This is due to consuming amino acids as an alternative energy source without glucose (Ye et al., 2010). During viral infection, double-stranded RNA (dsRNA) activates the mammalian PKR (CLEMENS & ELIA, 1997). The autophosphorylation at T446 caused by PKR dimerization via its c terminal kinase domain culminates in the kinase's functional activation (Dey, Cao, et al., 2005). The presence of dsRNA during viral infection prevents the synthesis of viral and host proteins by phosphorylating eIF2-α (CLEMENS & ELIA, 1997). During erythropoiesis, when erythroid cells are involved in erythrocyte differentiation, HRI is primarily expressed in these cells (Han, 2001). For the activation of the eIF2-α kinase, HRI requires the autophosphorylation and dimerization of its kinase domain (Rafie-Kolpin et al., 2000). Heme inhibits the HRI kinase activity by forming the disulfide bond between HRI monomers, leading to inactive dimer conformation. The HRI molecule interacts non-covalently in the absence of heme, creating an active dimer. HRI is activated by heat shock, arsenite-induced oxidative stress, 26s proteasome inhibition, and osmotic stress. These HRI stresses can be activated without the heme (Han, 2001).



Pakos-Zebrucka et al (2016), EMBO Reports 17(10), 1374-1395

Figure 3: ATF4 translational control is mediated by eIF2.

The eIF2 component of the eIF2 complex is phosphorylated during the ISR, preventing eIF2B from mediating the exchange of eIF2-GDP to eIF2-GTP. This inhibits the formation of the ternary complex composed of eIF2-GTP-methionyl-initiator tRNA. Ribosomes often begin scanning at the upstream open reading frame (uORF1) of the ATF4 transcript and quickly resume at the uORF2 due to the abundance of the ternary complex. Because uORF2 overlaps the ATF4 coding DNA sequence (CDS) out of frame, ATF4 cannot be translated. ATF4 is a member of the family of activating transcription factors and cyclic AMP response element binding proteins (ATF/CREB). It is a transcription factor, a basic leucine zipper (bZIP) (Ameri & Harris, 2008). ATF4 consists of the many dimerization partners regulating the gene transcription, which guides the cellular outcome (Pakos-Zebrucka et al., 2016b). When the ISR is activated, ATF4 plays a crucial role in cellular fate determination. ATF4 is regulated at the three levels of transcription, translation, and post-translation levels.

1.7.3 Termination of the ISR pathway

eIF2 dephosphorylation is necessary for eIF2 to function normally and to resume protein synthesis (Novoa et al., 2001). The protein phosphatase 1 (PP1) complex, which recruits one of two regulatory subunits and the PP1 catalytic subunit (PP1c), accomplishes this (Pakos-

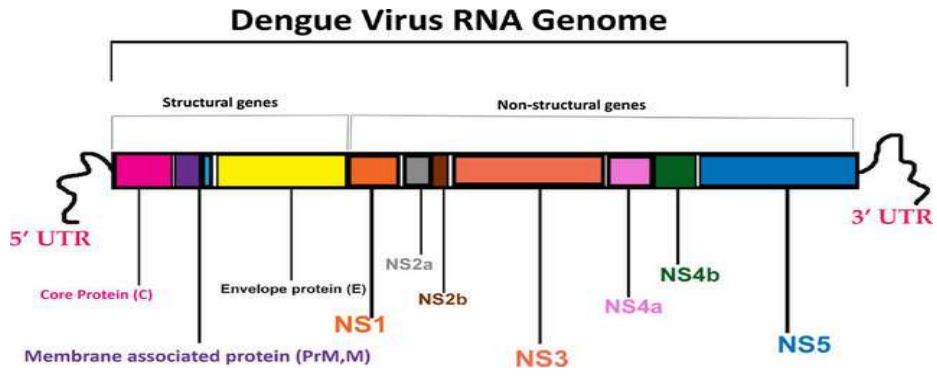
Zebrucka et al., 2016b). In mammalian cells, PPP1R15A is involved in the regulation of phosphatase activity (also known as growth arrest and DNA damage-inducible protein, GADD34), which induces as part of ISR or by constitutively expressed paralogue PPP1R15B (also known as a constitutive repressor of eIF2α Phosphorylation, CReP) which is responsible for the targeting enzyme eIF2α (Jousse et al., 2003). GADD34 expression leads to dephosphorylation of eIF2α, which helps in the expression of ATF4 (Kojima et al., 2003). Dephosphorylation of eIF2 caused by the production of GADD34-PPI aids in the expression of ATF4 (Y. Ma & Hendershot, 2003). Accumulated mRNA translation for stress genes in ER and oxidative stress requires eIF2-α dephosphorylation (Novoa et al., 2003).

1.8 Dengue virus

Dengue is a *Flavivirus* that belongs to the Flaviviridae family. There are four serotypes of dengue virus, i.e., DENV-1, DENV-2, DENV-3, and DENV-4. The World Health Organization (WHO) declares dengue a serious global public health issue in tropical and subtropical nations. There are 2.5 billion individuals who live in dengue-endemic areas, and 400 million infections happen every year. Dengue increased between 1960 to 2010 due to urbanization, failure in mosquito control, air travel, increased population growth rate, increase in global warming, and lack of health care facilities. Dengue infection involves more than 100 countries (Hasan et al., 2016a).

1.8.1 The genome of the dengue virus

Each serotype of the dengue virus is made up of a spherical viral particle with a diameter of approximately 500A˚. Each virus particle has an 11 kb positive sense. These are single-stranded RNA genome that codes for ten proteins. Three of these are structural proteins capsid (C) protein, envelope (E) protein, membrane (M) protein, and non-structural (NS) proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Guzman et al., 2016).



Abdul Wadood et al., Computational Biology and Chemistry, 2017.

Figure 4: RNA genome of dengue virus

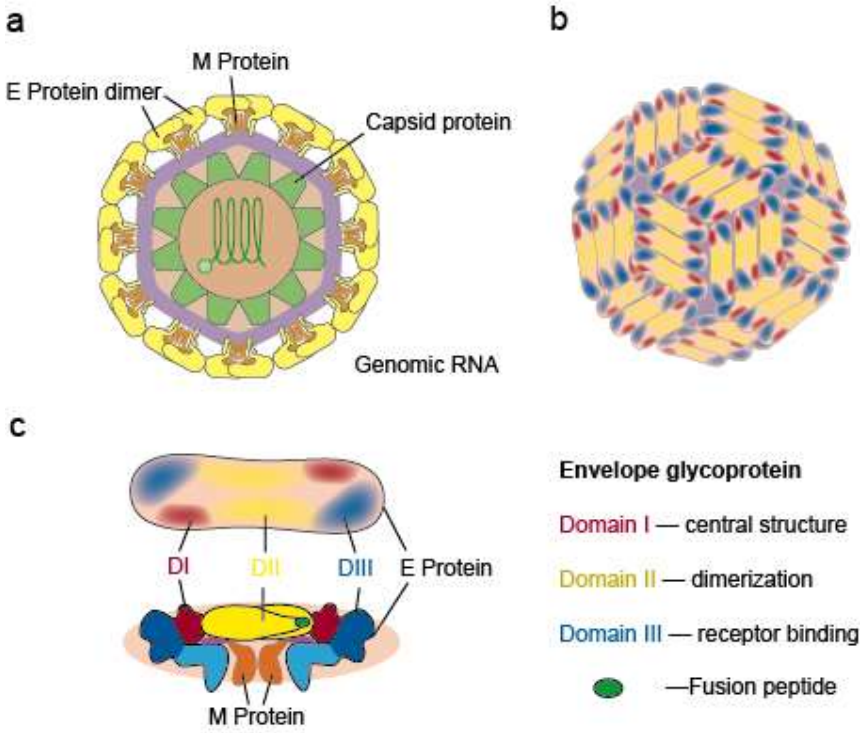
Different colours denote structural and non-structural genes. Three structural proteins are represented at 5′ UTR, while the seven non-structural proteins are at 3′ UTR.

1.8.2 The function of structural proteins

The genome of the dengue virus, i.e., RNA, is locked in the capsid protein, which is covered in a lipid bilayer membrane. E and M proteins are present on the surface of the mature dengue virus. The major surface protein of the dengue virus is the E protein. It has an essential role in receptor binding and fusion. Envelope protein (E) has three domains: ED-I, ED-II and ED-III. ED-II has a vital role in the fusion, while Domain III has an essential role in receptor binding and fusion (Q. Zhang et al., 2012).

1.8.3 The function of non-structural proteins

Dengue virus non-structural proteins are essential in viral replication and packaging (Guzman et al., 2016). NS1 protein is 46 kDa. It can be membrane-associated, endoplasmic reticulum anchored or secreted (sNS1) and is involved in early viral replication, whereas NS1 activates the innate immune system (Muller & Young, 2013). NS2A is 22 kDa in size and is a hydrophobic integral membrane protein involved in RNA replication and virion assembly**.** NS2B has a length of 14 kDa, a small hydrophobic protein that acts as a cofactor for NS3 (Xie et al., 2015). NS3 is a 69 kDa protein, and it is a multifunctional protein with several catalytic domains; these domains are involved in helicase functions and nucleoside triphosphatase and during RNA synthesis. NS4A is a 16 kDa hydrophobic integral membrane protein required to form replication vesicles. NS4B, having a size of 30 kDa Small hydrophobic domain, has a role in the suppression of IFNβ and IFNγ signaling (Muñoz-Jordán et al., 2005). NS5 protein is involved in RNA synthesis and has a size of 105 kDa (Muñoz-Jordán & Santiago, 2014).



https://www.creative-diagnostics.com/Dengue-Virus.htm

Figure 5: Assembly of a dengue Virus particle

The surface protein (a) and Envelope glycoproteins are shown (b,c).

1.8.4 Structure and Function of envelope glycoprotein Domain III

Domain III has a structure similar to the immunoglobulin-like module and has an essential role in the fusion transition. Domain III is involved in receptor binding. Antigenic and structural integrity domain III depends on the single disulfide bond between Cys302 and Cys333. Receptor binding to host cells localised to this domain. Envelope domain III is accessible to the virion surface and exposed. Domain III contains dominant epitopes that are neutralizing in nature. Studies on monoclonal antibodies reveal that neutralizing antibodies largely interact with domain III's surface-exposed loops. It has been proposed that if mutations occur in the specific residues of domain III, it loses its bonding to neutralizing mAbs. Domain III dengue viruses have an identical fold among the four viruses. Due to variations in the amino acid residues, domain III is unique among the four viruses. Anti-E domain III antibodies have been demonstrated to be virus infectivity blockers. Domain III shows low cross-reactive antibodies to heterologous dengue serotypes (Guzman et al., 2010).

1.8.5 Infection with Dengue virus

While other viruses enter the bloodstream directly, DENV enters the dermis and epidermis through a mosquito bite. Dendritic cells, Langerhans cells, and macrophages are infected in the skin. Such infected cells go from the primary infection site to the lymph node, where they enable the recruitment of monocytes and macrophages that serve as the target of the dengue infection.

1.8.5.1 Dengue Fever (DF)

Dengue fever lasts for 5-7 days. It is a self-limiting fever. Depending on the patient's age, dengue fever has different symptoms. It can be associated with any serotype. It produces fever, rash, retro-orbital pain, flu-like symptoms, painful joints and muscles, extreme nausea and headache (Khetarpal & Khanna, 2016). Up to 50% of patients may experience an early or late rash eruption. Facial flushing may appear one to two days after the start of the symptoms, along with or just before the onset of the fever. A second rash that can appear from scarlatina type to maculopapular may arise between days 2 and 6 of the illness. Usually, a rash develops on the trunk before spreading to the face and limbs. On rare occasions, a severe erythematous pattern with islands of normal skin might be noticed. The second rash often lasts two to three days. When the disease's feverish phase is over or the temperature reaches or drops below the average, petechiae, which can be scattered or confluent, may begin to emerge (Gubler, 1998).

1.8.5.2 Dengue haemorrhagic fever (DHF)

DHF is primarily a disorder affecting children under 15 years old, but it can also affect adults. It is distinguished by an abrupt onset of fever, typically lasting 2 to 7 days and various vague symptoms. It may be challenging to distinguish DHF from dengue fever and other tropical illnesses when the condition is acute. The acute phase of sickness should be treated as a differential diagnosis for viral hemorrhagic fevers, malaria, leptospirosis, typhoid, influenza, rubella, measles, and any other disease that shows symptoms as a nonspecific viral syndrome. Children typically experience upper respiratory symptoms from multiple viruses and bacteria infected simultaneously (Gubler, 1998).

1.8.5.3 Dengue shock syndrome (DSS)

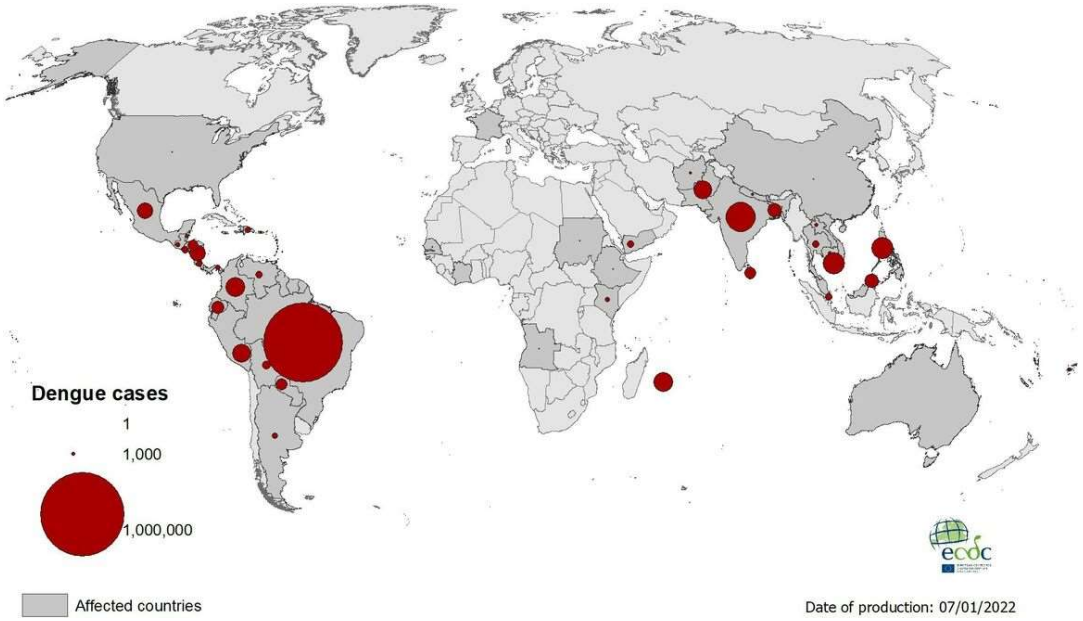
starts after 3 to 5 days of fever. In DSS, critical bleeding, fluid buildup with respiratory difficulty, and a drop in platelet counts, which results in plasma leakage, cardiorespiratory failure, cardiac arrest, and hypotension are all side effects.

1.8.6 The life cycle of the dengue virus

When a female mosquito takes blood during the acute febrile stage of the illness, she becomes infected. The virus enters the midgut and moves to the salivary gland during the extrinsic incubation phase; this typically takes 8 to 10 days. This process is generally affected by the ambient temperature, mosquito competence and viral strain. When the salivary gland gets infected, the mosquito develops an infection and can spread it to the next person while feeding on blood. The mosquito can spread the virus to multiple people as it feeds on blood, staying infectious for life (Guzman et al., 2016).

1.8.7 Global burden of dengue infection

DENV achieved its distribution in tropical areas in the eighteenth and nineteenth centuries. Globalization helps more spread the virus during the twentieth and twenty-first centuries. Additionally, the tropical region is becoming more hyperendemic due to globalisation. The increased epidemic activity during the 1970s and 1980s resulted in the spread of viruses and mosquito-borne diseases (Gubler, 1998). a lack of effective mosquito control in endemic nations, global trade, urbanisation, modern transportation and human population expansion are the leading causes of the virus spread in the 20th century (Messina et al., 2014) (Gubler, 1998). The recent estimate of the dengue burden shows that nearly the world's half population, i.e., 3.6 billion people, live in the highly-dense infectious region. There is the occurrence of 390 million overall DENV infections per year (Guzman et al., 2016) and 21000 death per year globally (Bhatt et al., 2013). The highest dengue infection occurred in Asia, followed by the American tropics (Gubler, 2011).



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

Figure 6: Reports of dengue cases around the world in 2020

1.8.8 Dengue prevalence in India

The first report of dengue's prevalence occurred in India in 1946 (Karamchandani, 1946). Later, the first dengue fever epidemic was discovered in India in 1963–1964. (Rodrigues et al., 1966). It was first noted on India's eastern coast; after spreading to the country's southern region and eventually covering the entire nation with the spread of all four dengue virus serotypes, it then migrated to Delhi in 1967 and Kanpur in 1968 (N. Gupta et al., 2012). The dengue virus's epidemiology and serotypes are constantly evolving. DENV 4 caused the outbreak in Kanpur in 1968, and DENV 2 and DENV 4 caused it in 1969. The DENV2 completely replaced this scenario in the adjoining city in 1970 (Chaturvedi et al., 1972).

1.8.9 Dengue infection and host immune response

1.8.9.1 Innate immune response

People can acquire DENV via a mosquito bite. After infection, skin cells, including keratinocytes and Langerhans cells, serve as the virus' primary replication sites (Garcia et al., 2017). This will set off several innate immunological reactions in the host and trigger numerous innate immunological responses in the host. Pattern recognition receptor (PRR) helps

the innate immune cells respond to an infection by identifying pathogen-associated molecular patterns (Loo & Gale, 2011) (Akira & Takeda, 2004). These immune cells consist of monocytes, macrophages, and DCs. When the PRR is recognised, cytokines and chemokines are produced, which create an antiviral state. For instance, DENV causes the activation and maturation of both infected and uninfected bystander DCs, resulting in the production of TNF and IFNα when immature human myeloid DCs are exposed to it (Libraty et al., 2001). Abortive DENV replication in (Macrophages and mast cells) MCs also causes IFN and chemokine responses. MCs are granulated immune cells packed with immune mediators and can degranulate in response to some stimuli, such as DENV, in minutes (St. John et al., 2011). As a result, mediators like TNF are released, promoting the retention of lymphocytes in draining lymph nodes (LNs), which causes LN hypertrophy (McLachlan et al., 2003). The recruitment of monocytes into the skin, where they undergo differentiation to become DCs (dendritic cells) (Shelburne et al., 2009).

1.8.9.2 Adaptive immune response

In addition to the innate immune system, the dengue virus (DENV) employs various strategies to evade the adaptive immune system. Delivered to the draining lymphoid tissues, antigen and activated APCs initiate adaptive immunity when the innate immune response fails to eradicate the infection (Lee et al., 2022). The adaptive immune system is made up of the humoral immune response, which is mediated by B cells and neutralizing antibodies, and the cell-mediated immunological response, which is mediated by $CD4^+$ and $CD8^+$ T cells (Ye et al., 2013).

1.8.9.3 Antibody-dependent enhancement

DENV may potentially actively undermine the humoral immune system to promote infections. DENV may use immune complexes that include infectious viruses to increase the transmission of infections via the lymphatic system through a method known as an antibody-dependent enhancement (ADE) (Diamond, 2003). The pathophysiology of secondary DENV infections depends on ADE (Halstead, 2003). According to studies, neutralizing antibodies produced after the initial infection attach to DENV and facilitate the virus' entrance into cells with Fc receptors, leading to ADE and more severe dengue (Chareonsirisuthigul et al., 2007).

1.8.10 Diagnosis of dengue

Dengue can be diagnosed clinically or by different methods such as the anti-DENV specific antibodies, DENV-specific nucleic acid detection or non-structural protein 1 (NS1) antigen. Clinical diagnosis includes the clinical examination and tourniquet test (Raafat et al., 2019). For 4 to 5 days following the sickness, the virus can be found in the serum, plasma, and circulating blood cells. Antigen detection and virus isolation can be employed to identify the infection in the early stages of the disease's nucleic acid. Serological methods are used for the diagnosis at the end of the infection. (https://www.ncbi.nlm.nih.gov/books/NBK143156/)

1.8.10.1 Clinical examination

Dengue fever includes symptoms such as arthralgia, retro-orbital pain, myalgia, headache, rash and leukopenia. Due to thrombocytopenia, evidence of plasma leakage, and haemorrhagic symptoms, DHF differs from DF. In the case of DSS, it includes all the signs of the DHF (Raafat et al., 2019). According to 2009 regulations, the cases are mainly in two categories: Dengue and Severe Dengue (SD). Fever with one of the following symptoms is known as dengue, a positive tourniquet test, rash, aches and pains, leukopenia, or nausea/vomiting. Severe dengue includes bleeding and severe plasma leakage (Raafat et al., 2019)

1.8.10.2 Antibody serology

The most popular technique for diagnosing dengue is the ELISA (Enzyme-Linked Immunosorbent Assay), which measures anti-dengue IgG antibodies and immunoglobulin M (IgM) (Raafat et al., 2019) (Tang & Ooi, 2012). IgM ELISA has a role in primary infection detection, while IgG ELISA is essential in secondary infection detection because IgG titer is higher in secondary infection. Therefore, in the acute phase of the disease ratio, the IgM: IgG is used to differentiate the primary infection from the secondary infection (Raafat et al., 2019)

1.8.10.3 PRNT

Neutralizing antibodies are crucial for preventing the spread of the dengue virus (Russell et al). First developed plaque reduction neutralizing tests (PRNT) (Tang & Ooi, 2012). The best technique for establishing prior exposure to dengue is PRNT. This measures the serum titer necessary to prevent a cultivated monolayer of cells from becoming 50% to 70% virus-infective. Thus, by culturing with different serotypes, neutralizing antibody titers specific to a certain serotype can be estimated (Roehrig et al., 2008).

1.8.10.4 NS1

As hexamer can identify dengue infection, non-structural protein 1 is a conserved glycoprotein released from infected cells (Tang & Ooi, 2012). Due to its early occurrence in sera, it can be used for disease early detection. For the detection of NS1, only a single sample is required. The NS1 is a perfect candidate for the RDT (rapid diagnostic tests) due to these characteristics (rapid diagnostic tests) (Peeling et al., 2010).

1.8.10.5 Nucleic acid testing

High specificity and speedy results are provided by reverse transcription polymerase chain reaction (RT-PCR), which is used to detect viral RNA. It also uses a single sample (Tang & Ooi, 2012). RT-PCR methods differ in primers, enzymes, buffers, and cycling conditions (Tang & Ooi, 2012)

1.8.11 Prevention and treatment of dengue

Dengue prevention involves various methods, such as biological control and chemical control, as follows.

1.8.11.1 Biological control

It includes the paratransgenesis and use of wolbachia. Paratransgenesis reduces the spread of disease by recolonizing the vector population with genetically altered symbiotic bacteria (Araújo et al., 2015). According to studies, wolbachia is the most effective bacterial agent (Jeffery et al., 2009). The reproductive parasite wolbachia disrupts the cellular and reproductive processes of the vector species (Araújo et al., 2015). *Aedes aegypti* population can be controlled through vector-specific genetic alteration. It contains the effector gene for stopping and decreasing the spread of illness (Reis-Castro, 2012). After the genetically engineered mosquitoes were released in Brazil, the population of *Aedes aegypti* decreased by 85% (Rather et al., 2017). The sterile insect technique (SIT) entails dispersing male vectors sterilised in a lab among the target population. These insects aid in reducing female mosquito reproduction rates and managing the population's vector density (“Vector Control for the Chikungunya Disease,” 2010).

1.8.11.2 Chemical control

Insecticides and plant derivatives are used in it. Due to the target vector population developing resistance, an artificial substance known as an insecticide has been employed for many years, having a detrimental effect on the ecosystem. (Araújo et al., 2015). To overcome these effects, the researchers developed plant-based insecticides that are less toxic to the environment (Rather et al., 2017). These insecticides are created from the different parts of the plants, such as leaves, stems, roots and herbal extracts (Rather et al., 2017).

1.8.11.3 Vaccines against the dengue

1.8.11.3.1 TV003/TV005

TV003/TV005 is a live attenuated DENV vaccine. The technique entails removing 30 nucleotides from positions 172-173 in the TL2 stem-loop. This loop is from the 3'-UTR of DENV 4 to utilise the DENV's untranslated regions (UTRs), which are crucial in the replication of the DENV genome (rDEN4Δ30) (Men et al., 1996). The same mutant created for the DENV 1 is called DENV 1 (rDEN1Δ30). When DENV 1 and DENV 4 wild types were challenged in rhesus macaques, both mutants had the attenuated phenotype and demonstrated lower infectivity and potential for neutralizing antibody responses (Whitehead et al., 2003). A slightly similar method to that utilised to make the DENV 3-DENV 4-chimeric virus was used in subsequent attempts to produce the attenuated DENV-2 component (Blaney et al., 2008).

1.8.11.3.2 TAK-003 (DENVax)

The DENVax was created at Mahidol University in Bangkok in the late 1980s. The attenuation of the DENV 2 strain was performed by serial passaging the dog kidney cells 53 times. This leads to the making of the PDK-53-V strain. This strain possesses a non-synonymous mutation in the NS3 gene in addition to the 5'UTR and NS1 gene mutations. This strain is the backbone for generating the DENVax vaccine (Butrapet et al., 2000).

1.8.11.3.3 Inactivated virus vaccines

The antigenic components in inactivated virus vaccines are generated from the pathogen's inactive component, providing protectivity against live infection (Putnak et al., 1996). Rhesus monkeys respond well to the formalin inactivation and sucrose centrifugal purification (Sparkuhl et al., 1996). A dengue vaccine made from four inactivated dengue serotypes is called tetravalent purified formalin-inactivated virus (TPIV) (Simmons et al., 2010).

1.8.11.3.4 Recombinant subunit vaccine

Antigenic proteins expressed in prokaryotic or eukaryotic cells make up recombinant subunit vaccines. These are designed to elicit a long-lasting, therapeutic or protective immune response (Tripathi & Shrivastava, 2018). Recombinant dengue proteins are generally straightforward to generate in *E. coli.* There are some problems with the incorrect folding of protein and endotoxin contamination (Berlec & Štrukelj, 2013). In mice, it has been successfully demonstrated that the recombinant EDIII was generated in *E. coli*. This was then purified by metal affinity membrane chromatography and induced with antibodies generated against the four serotypes of dengue (Babu et al., 2008).

1.8.12 Rationale

The vaccine is a suspension containing attenuated, dead, or fragmented microbes or toxins or another biological preparation used largely to prevent disease. The vaccine comprises different constituents such as water, preservatives and stabilisers, residual traces and two important materials, active ingredients and adjuvant. Active ingredients consist of a small part of the harmless form of bacteria or virus which is immunizing against. The adjuvant is the small ingredient in the vaccine that gives a stronger immune response. Adjuvant also helps to give more effector and memory cells. Even though currently available adjuvants have advantages, they also have certain limitations, such as the adjuvants are not universal and their inherent side effects. So, there is always a requirement for new adjuvants, which can be achieved by studying the immunobiology of the most successful vaccine. One such vaccine is the yellow fever vaccine 17D (YF17D). YF17D induces multipronged immunity (Th1, Th2, CTL, antibody) and profound antibody titers which last for more than 30 years after the vaccination, but the mechanism through which the YF17D works and induce long term vaccine induced immunity is not properly understood. Recent system vaccinology approaches have been implemenetd to dissect the mechanism through which the YF17D works. It has been discovered that it functions by triggering the Integrated Stress Response Pathway, which controls the quality and magnitude of antigen-specific immunity induced by vaccines. Additionally, it was shown that YF17D increased T cell response by activating the ISR pathway, which in turn controls autophagy and improves antigen presentation thereby enahncing T cell responses. These findings manifest that the integrated stress response pathway has immunomodulatory functions and YF17 enances the vaccine induced protecive immunity by manipulating ISR Pathway. These observations tempted us to questions that whether activation of ISR pathway using small molecule triggers an effective immune response aginst vaccine antigens, thereby

acting as an adjuvant. To address this question, we planned to use Ovalbumin and EDIII Domain of the dengue virus as a model antigens for studying the efficacy of ISR adjuvant**.** Dengue is one of the most dangerous neglected tropical diseases. Paralleling the global spread of vector mosquitoes, its prevalence has grown in recent years. There is an occurrence of 390 million infections per year and 2.5% cases of fatality annually. The prevalence of dengue infection is increasing at an alarming rate globally, including in India. There are many factors attributed to the constant upsurge in global DENV cases. One important factor is the unavailability of an effective vaccine, which might confer a balanced response against all the serotypes. No potent vaccine has been developed for dengue to date. Dengvaxia® (CYD-TDV), a licenced dengue vaccine created by Sanofi Pasteur, has restricted usage. Dengvaxia® has a lesser efficacy for dengue 1 and 2 than for dengue 3 and 4, and it only works in a limited age group. A significant medical need is the development of new vaccine formulations that can offer balanced protection against all DENV serotypes. It could be achieved by identifying potential vaccine candidates and proper adjuvanting systems. We selected EDIII domain of dengue virus enveleop protein as a potential vaccine candidate. Anti-EDIII antibodies are often thought to be the most effective inhibitors of viral infectivity. It has been suggested that changes to certain domain III residues cause neutralizing mAbs to no longer attach to them. These observation suggests the potential of domain III as an effective vaccine candidate; however, when its adminstred alone it demonstrate weeker antigen specific responses. Given the fact that activation of ISR pathway programs enhanced protective immunity, henece based on this finding, we hypothesised that using arsenite an activator of the ISR pathway might improve the immunogenicity of Vaccine antigens and might serve as an adjuvant in developing a vaccine against the dengue virus. To put our hypothesis to the test, we devised the below objectives.

2. OBJECTIVES

Objective 1

To elucidate the role of ISR pathway activation in the modulation of innate effector function using an in-vitro macrophage stimulation system.

Objective 2

To examine the adjuvanting function of ISR activator, arsenite (Ar) in programming the magnitude of antigen-specific immune responses against various antigens upon immunization in a mouse model

Objective 3

To dissect the potency of ISR activator arsenite as a Vaccine adjuvant through virus neutralization assay using a Dengue Virus infectious model system.

3. MATERIALS AND METHODS

3.1 Maintenance of cells

The mice monocyte cell line J774A.1 (National Centre for Cell Science Pune, India) was cultured in the DMEM culture medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, UK) and 1% penicillin/streptomycin (P/S) (HyClone, USA) at 37°C and 5% CO_2 in a humidified incubator.

3.2 Chemicals and Reagents

Lipopolysacharides (LPS) (*E. coli* 0111: B4) (Sigma Aldrich, Merk Germany), Isopropyl lβ-D-1- thiogalactopyranoside (IPTG) (G.Biosciences, USA), Chloramphenicol, kanamycin (Himedia India), phenylmethylsulfonyl fluoride (PMSF), Lysozyme, DNase (G.Biosciences, USA), Sodium (meta)arsenite (Sigma-Aldrich, Merk Germany).

3.3 MTT assay

J774A.1 cells from the T75 flask were resuspended in the 15 ml centrifuge tube and centrifuged for 5 min @ 1200 rpm. Media was removed, and cells were resuspended in 1 ml of complete media, after which cells were counted and recorded per ml diluted cells up to 3,00,000 per ml. Added 200 µl of cells (60,000) into each well and left overnight. The next day, cells were treated with different concentrations of arsenite such as 0.1µM, 1µM, 10µM, 100 µM and 1000µM. Cells were kept for incubation for 24 hrs, and 20 µl of MTT (5 mg/ml) was added to each well and left for another 4 hrs @37˚C in the incubator. Media was removed, formazan crystals were solubilised by adding 100µl of DMSO, and absorbance was recorded at 540 nm.

3.4 ROS Assay.

J774A.1 cells were seeded at 50,000/well in 96 well plate and treated with different TBHP, LPS and arsenite concentrations to measure the reactive oxygen species. After treatment, cells were washed with 1XPBS. After being washed, cells were extracted and incubated in FACS buffer for 30 minutes @ 37°C with 25 µM CM-H2DCFDA (Life Technologies, USA). After incubation, cells were harvested and washed with Hank's balanced salt solution (HBSS) and subjected to flow cytometry analysis.

3.5 Nitric oxide assay

J774A.1 cells were seeded in the 96 well plates in triplicate with Complete DMEM medium (including the 10% FBS) and treated with the varied concentrations of arenite, such as 0.1 µM,

1 μM and 10 μM and LPS (500 ng/ml). Cells were treated with three different time points, i.e., 24 hrs,18 hrs, and 12 hrs. After treatment, cells were kept in a CO_2 incubator. Post-treatment, 100 μl of Griess reagent was taken and mixed with 100 μl of supernatant and incubated for 10 minutes, and endpoint absorbance was recorded at 570nm. The graph was plotted as concentration vs absorbance to calculate the unknown nitrite concentration in the reaction.

3.6 OVA uptake assay

J774A.1 macrophages were seeded at 1 million cells/well in the six-well plates and treated with various concentrations of arsenite such as (0.1 μM, 1 μM and 10 μM) for 3 hr. After treatment, macrophages were incubated with OVA labelled with FITC (Invitrogen, UK) for 2 hrs. After washing the cells, they were resuspended in FACS buffer and data was acquired by the Flow cytometer (BD Accuri C6 plus).

3.7 MHC I expression analysis

J774A.1 macrophages were seeded at 1 million cells/well in the six-well plates and treated with various concentrations of arsenite such as (0.1 μM, 1 μM and 10 μM) for 3 hrs. After treatment, macrophages were incubated with MHC I antibody tagged with FITC (Invitrogen, UK) for 1 hr. After washing the cells, they were resuspended in FACS buffer and data was acquired by the Flow cytometer (BD Accuri C6 plus).

3.8 Analysis of costimulatory molecule

J774A.1 macrophages were seeded at 1 million cells/well in the six-well plates and treated with various concentrations of arsenite such as (0.1 μM, 1 μM and 10 μM) for 3 hr. After treatment, macrophages were incubated with CD86 antibody tagged with PerCP (BioLegend, Catalogue no. 105060) for 1 hr. After washing the cells, they were resuspended in FACS buffer and data was acquired by the Flow cytometer (BD Accuri C6 plus).

3.9 Western blotting

To assess the expression of various proteins, Western blotting was used. Macrophage cells were plated at a density of 1 million cells/well in a 6-well plate and treated with different concentrations of arsenite (1 μM, 5 μM and 10 μM). Post incubation, cells were washed thrice with the 1X PBS (ice cold) followed by lysis using the lysis buffer (150 mM NaCl, 1% Triton-X 100, 20 mM HEPES-pH 7.5, 10% Glycerol, 100 mM NaCl, 1 mM EDTA, 17.5 mM β-Glycerophosphate, 1 mM phenyl methyl sulphonyl fluoride) added with protease and

phosphatase inhibitor cocktail and incubated on ice for 30 min with intermittent vortexing. Furthermore, it is centrifuged @ 12000 rpm for 15 min @ 4^0C. The BCA method was used to estimate the protein, and the equivalent amount of protein was loaded on the SDS-PAGE (12% of SDS gel). The separated protein was transferred to the nitrocellulose membrane by electroblotting. After that, 5% BSA in 1XPBS was used to block the membrane. Afterwards, the membrane was incubated overnight with a primary antibody anti-eIF2α-P (Cell Signaling Technology, USA) at 4°C. After primary antibody incubation blot incubated with appropriate secondary antibody on the gel rocker for 1 hr. Blots were developed with FemtoLUCENT™ PLUS HRP Chemiluminescent reagents (G BIOSCIENCES, Cat # 786-003), and chemiluminescence was captured by Chemidoc System (BioRad) to visualize the band of interest.

3.10 Confocal Microscopy

50,0000 macrophages per well seeded in the 24 well plate in DMEM media supplemented with fetal bovine serum and cells allowed to adhere. Next day, cells were treated with 5µM concentration of the arsenite for 3 hrs. post completion of the experiment; the cells were washed three times in 1xPBS and fixed in the 4% paraformaldehyde for 20 minutes @ RT. Remove the paraformaldehyde and wash with 1xPBS. Permeabilize the cells with 0.2% Triton X-100 for 20 minutes @ RT. After permeabilization, cells were washed three times with 1X PBS. Cells were blocked with the 5% BSA @ RT for 1 hr, followed by a wash with 1X PBS. After washing, add anti-TIA1 (Santa Cruz-1751) and anti-TIAR (Santa Cruz-1749) antibodies @ RT for 2 hrs. After incubation, wash the cells with 1XPBS for three times. Add the Alexa flour tagged secondary antibodies (Alexa flour 488 and Alexa flour 555) and incubated for 2 hrs @ RT. For the removal of the nonspecific antibodies, wash the cells with 1XPBS three times. At last, mount the coverslips on the slide using DAPI (Invitrogen) (DAPI used as mounting media). LSM510 confocal microscope (Zeiss) was used to record the Immunofluorescence signal, and the Zeiss LSM Image Browser was used to analyze the images.

3.11 Sandwich ELISA

J774A.1 cells were treated in two groups: one with LPS and one without LPS (500 ng/ml) for 3 hrs and one without arsenite for another 3 hrs. After this, ATP (5 mM) was added to the culture for 30 min. Arsenite treatment is given as 0.1 µM, 1 µM, and 10 µM. After treatment, culture supernatant was collected, and sandwich ELISA was performed. IL-6, Il-1β and TNF-α levels were measured by ELISA using the kits (BD OptEIA™) by BD Biosciences, as per

the manufacturer's protocols. ELISA plates (Maxisorp-Nunc-96 well plate) were coated with TNF-α, IL-6, and Il-1β capture antibodies diluted in the carbonate/phosphate coating buffer, and the plates were left overnight @ 4°C. After incubation, plates were washed with 1X PBST thrice and proceeded for blocked with BSA or FBS. Now, plates were washed again thrice and incubated with standard or test samples for a period of 1 hr @ RT. After incubation, plates were washed with PBST four to five times, after which the plate was incubated with the respective streptavidin conjugated detection antibodies mixed with Avidin-HRP for 1 hr @ RT. Post incubation, plates were washed six times, and ELISA was developed by adding TMB substrate (G Biosciences) @ RT in the dark. To stop the reaction, 2 N H_2SO_4 was added in each well and absorbance was recorded at 450 nm with 570 nm correction.

3.12 Expression and Purification of DENV1rEDIII, DENV2rEDIII, DENV3rEDIII, and DENV4rEDIII protein

To examine the expression, E. coli Rosetta cells were transformed with the cloned constructs DENV1 pET28a-Domain III, DENV2 pET28a-Domain III, DENV3 pET28a-Domain III, and DENV4 pET28a-Domain III. A separate colony was hand-picked from the transformed plate, and it was used as inoculum for the primary culture in LB media containing chloramphenicol (35 µg/ml) and kanamycin (50 µg/ml) and left overnight @ 37^0C under shaking. Obtained primary culture was re-inoculated into 1 litre Luria Bertani media with chloramphenicol (35µg/ml), and kanamycin (50µg/ml) was allowed to grow in the incubator @ 37°C under constant shaking at 150 rpm for about 2–3 hrs or until the culture attained O.D. of ~0.5–0.6 at 600nm. A small volume (1 ml) of the un-induced culture was kept separately for SDS-PAGE analysis. IPTG was added to the remaining bulk culture to induce it at a final concentration of 1 mM. Induction was given for 4 hrs. After induction, the culture was spin down, and the cell pellet was resuspended in the buffer containing DNase, NaCl 300 mM, lysozyme, Tris 50 mM, 20mM imidazole, 1mM PMSF, and 8M urea. The suspended cell pellet was allowed to sonicate for 10 minutes and spin down at 12000 rpm for 30 minutes. Eventually, the supernatant was collected and allowed for binding with Ni-NTA beads for 1hr @ 4°C and was passed through a Ni-NTA packed column (GE Healthcare) and discarded the flow through. Then, washing buffer (20 mM imidazole) was added to the column (i.e., 25 ml in the two go) and allowed to pass away unbound protein from the column and collect wash in one Eppendorf. After washing, the elution buffer (300 mM imidazole) was added to the column and collected elution fractions. Eluted fractions were resolved on the SDS-PAGE followed by staining with Coomassie blue, and a band size of 14.5 kDa was observed for DENV1rEDIII, DENV2rEDIII, DENV4rEDIII

protein and 15 kDa for DENV3rEDIII protein. The obtained pure protein was further analysed through a western blot using the anti-His tagged antibody (Invitrogen).

3.13 Mice Immunization schedule

3.13.1 Ova (Ovalbumin) antigen

The mice (Balb/c), aged 6-8 weeks, were primed in various groups: 1) PBS, 2) arsenite (Ar), 3) Ova 4) Ova+Ar by the subcutaneous route. Mice were primed with the arsenite 5µg/ mice for three days. On the 4th day, immunization was performed with the Ova 10µg/ mice. On the 14th day, the mice blood and serum were collected. From day 15th to 17^{th,} mice were primed with arsenite with 5 µg/mice. On the 18th day, mice were injected with the booster dose of Ova 10 µg/mice. 28th day after immunization, mice blood was collected, and serum was isolated for the evaluation of antibody response. From the 29th to the 31st day, mice were again primed with the arsenite and immunized on the 32nd day with a booster dose of Ova of 10 µg/mice. On the 42nd day, mice were euthanized, serum was collected for the antibody response, and splenocytes were collected for the T-cell response.

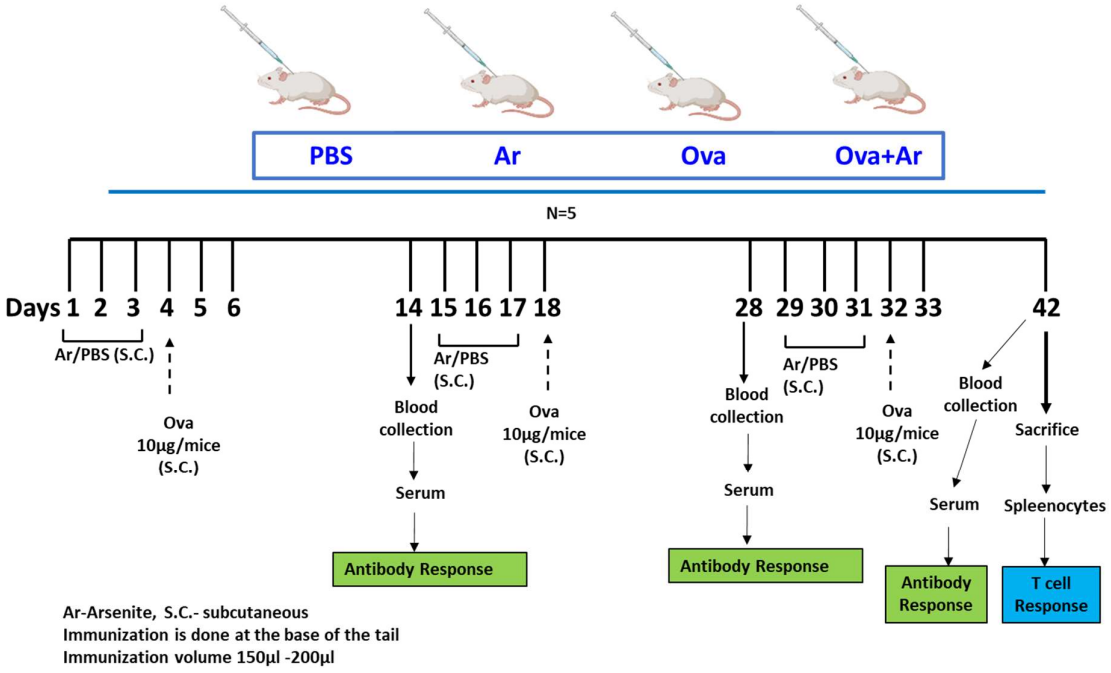


Figure 7: Immunization Schedule of mice for Ovalbumin

3.13.2 DENV1rEDIII (Envelope domain III protein of dengue virus 1) antigen

The mice (Balb/c), aged 6-8 weeks, were primed in various groups: 1) PBS, 2) DENV1rEDIII 3) DENV1rEDIII+Ar by the subcutaneous route. Mice were primed with the arsenite 5 µg/ mice for three days. On the 4th day, mice were immunized with the DENV1rEDIII 20 µg/mice. On day 14, mice blood was collected and isolated serum for the antibody response. From day 15th to 17th, mice were primed with arsenite 5 µg/mice. On the 18th day, mice were immunized with the booster dose of DENV1rEDIII 20 µg/mice. 28th day after immunization, mice blood was collected and serum isolated for the antibody response. From the 29th to the 31st day, mice were again primed with the arsenite and immunized on the 32nd day with a booster dose of DENV1rEDIII 20 µg/mice. On the 42nd day, mice were euthanized, serum was collected for the antibody response, and splenocytes were collected for the T-cell response.

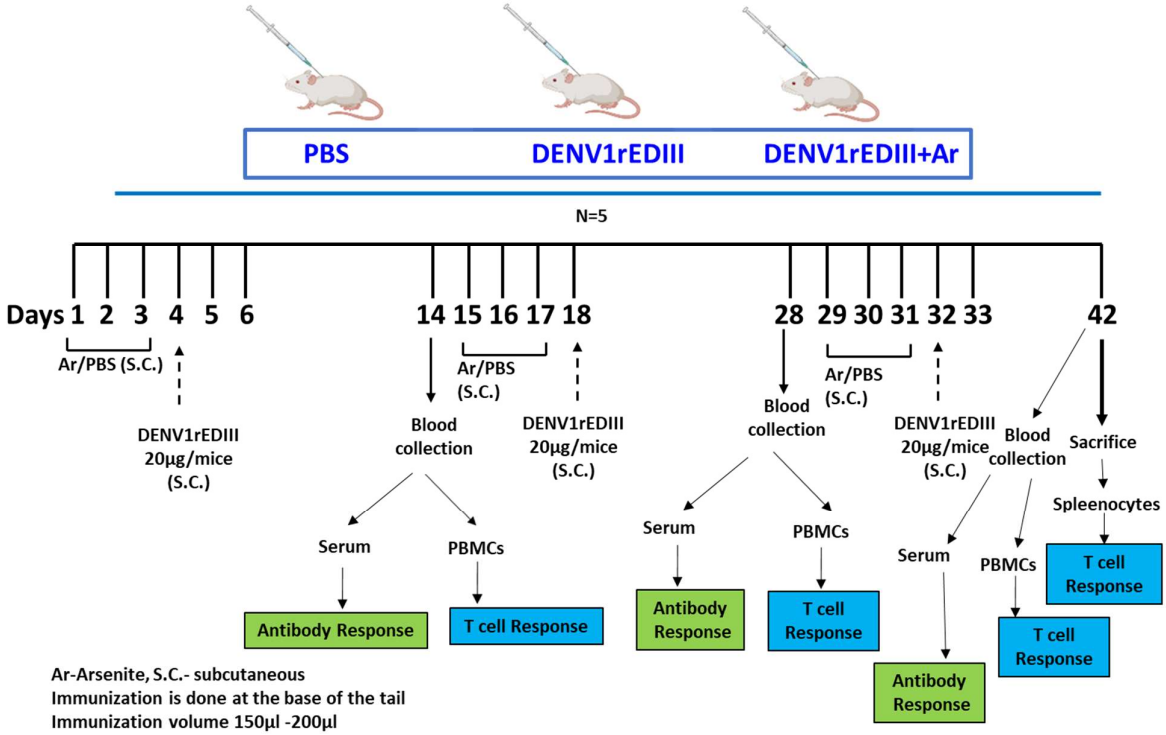


Figure 8: Immunization Schedule of mice for DENV1rEDIII protein

3.13.3 DENV2rEDIII (Envelope domain III protein of dengue virus 2) antigen

The mice (Balb/c), aged 6-8 weeks were primed in various groups: 1) PBS, 2) DENV2rEDIII 3) DENV2rEDIII+Ar by the subcutaneous route. Mice were primed with the arsenite 5 µg/mice for three days. On the 4th day, mice were immunized with the DENV2rEDIII 20 µg/ mice. On the 14th day, mice blood was collected and isolated serum for the antibody response and PBMCs for the T cell response. From day 15th to 17^{th,} mice were primed with arsenite 5 µg/mice. On the 18th day, mice were immunized with the booster dose of DENV2rEDIII 20 µg/mice. 28th day after immunization, mice blood was collected, and serum was isolated for the antibody response and PBMCs for the T cell response. From the 29th to 31st day, mice were again primed with the arsenite and immunized on the 32nd day with a booster dose of DENV2rEDIII of 20 µg/ mice. On the 42nd day, mice were euthanized and collected serum for the antibody response and PBMCs for the T cell response. Splenocytes were also collected for the T-cell response.

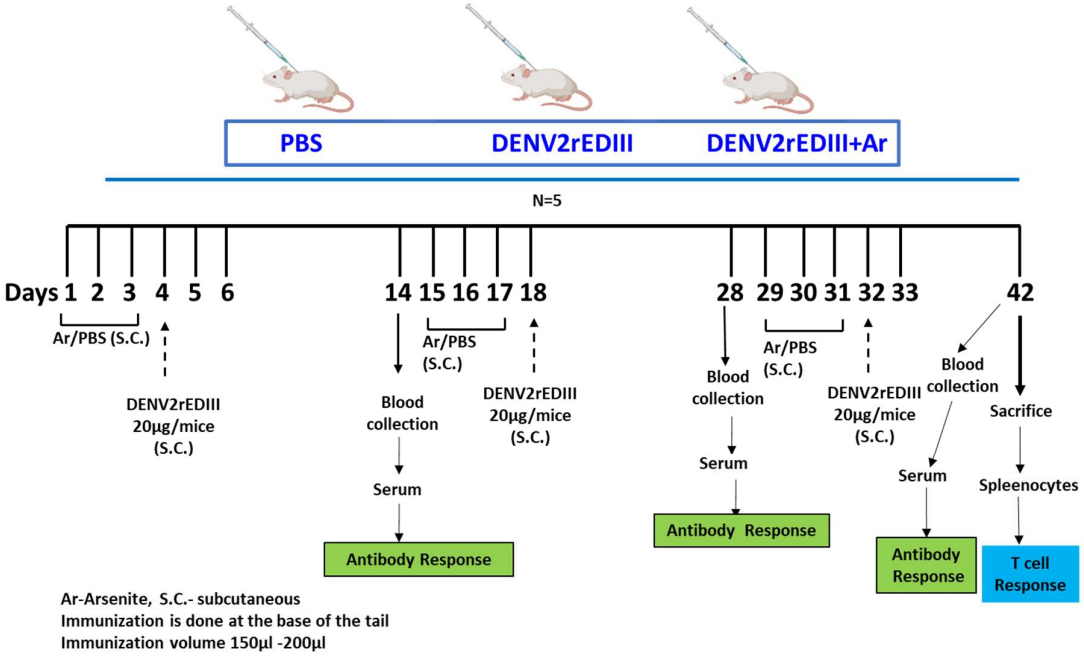


Figure 9: Immunization Schedule of mice for DENV2rEDIII protein

3.13.4 tDENVrEDIII (Tetravalent combination of all the four serotypes of the EDIII antigen of dengue) antigen.

The mice (Balb/c), aged 6-8 weeks, were primed in various groups: 1) PBS, 2) tDENVrEDIII 3) tDENVrEDIII +Ar by the subcutaneous route. (tDENVrEDIII antigen formulation was made

by physically mixing the EDIII domain antigen from all the four serotypes of dengue virus containing 10 µg each of the EDIII domain) Mice were primed with the arsenite 5 µg/ mice for three days. On the 4th day, mice were immunized with the tDENVrEDIII 40 µg/mice. On the 14th day, mice blood was collected and isolated serum for the antibody response and PBMCs for the T cell response. From day 15th to 17th, mice were primed with arsenite with 5 µg/ mice. On the 18th day, mice were immunized with the booster dose of tDENVrEDIII at 40 µg/mice. 28th day after immunization, mice blood was collected, and serum was isolated for the antibody response and PBMCs for the T cell response. From the 29th to 31st day, mice were again primed with the arsenite and immunized on the 32nd day with a booster dose of tDENVrEDIII of 40 µg/ mice. On the 42nd day, mice were euthanized and collected serum for the antibody response and PBMCs for the T cell response. Splenocytes were also collected for the T-cell response.

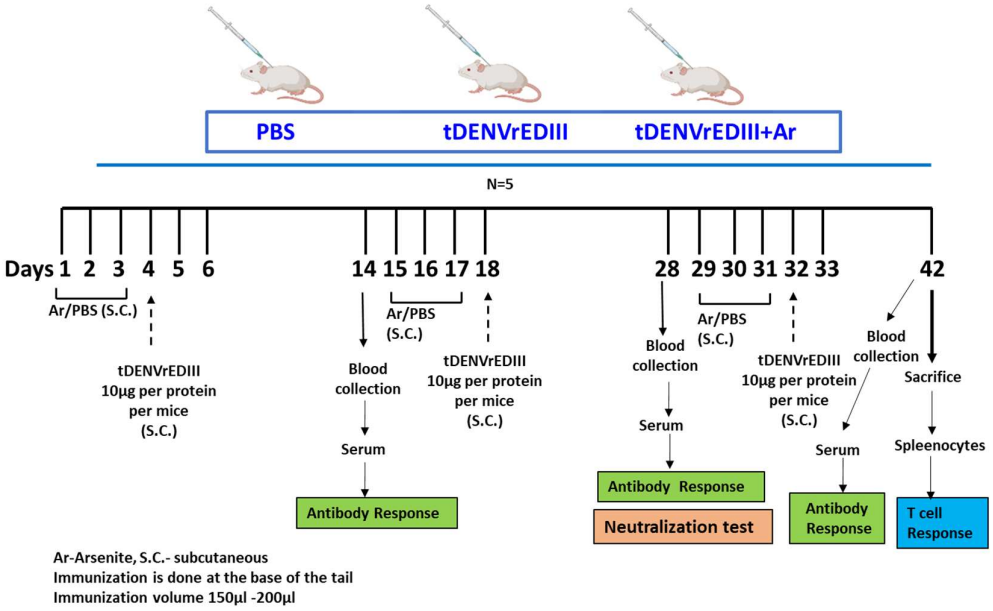


Figure 10: Immunization Schedule of mice for tDENVrEDIII protein

3.14 Antigen-specific T-Cell recall assay

T-cell was examined on the splenocytes and PBMCs obtained from the mice of various immunization groups. Mice's spleen was extracted, single cells were recovered by mechanical disruption, filtration with a 70 µm filter, and pelleted at 350Xg for five minutes. Cells were lysed using 1X red blood cell (RBC) lysis buffer for 10 minutes. For the PBMCs isolation, blood was diluted 2-4 times with the buffer (buffer containing phosphate buffer saline pH 7.2 and 2 mM EDTA) and layered over the 1 ml Ficoll gradient in the 15 ml falcon. Centrifuged

the sample at 400xg without a break for 40 min @ 20°C in a swinging bucket rotor. Collect the middle white layer containing mononuclear cells in the fresh 15 ml falcon. Add 3 ml of the buffer to the collected cells and spin down at 300Xg @ 20°C for 10 minutes. Remove the supernatant, add 3ml of buffer to the pellet, and centrifuge it at 200Xg for 10 minutes at 20°C. Resuspend the pellet in the RPMI medium for the experiment. In the 96-well U-shaped bottom plate, splenocytes and PBMCs (1X 10^6 cells /well) were seeded with RPMI 1640 and stimulated with a pure antigen such as the DENV1rEDIII (10µg/ml), DENV2rEDIII(10µg/ml). In the case of Ova immunized mice, splenocytes restimulated with the SIINFEKL peptide. The cells were stimulated in the presence of GolgiStop (BD Biosciences) for 8 hours @ 37°C. After treatment, cells were centrifuged at 2000rpm for 10 min and washed once with FACS buffer (FBS 3%, 1XPBS and 200mM EDTA). Now, cells were stained with surface T-cell markers such as CD4 tagged with FITC and CD8 with PerCP (BD Biosciences) for 60 min @ 4°C. Further cells were washed with the FACS buffer thrice and fixed with 4% paraformaldehyde @ RT for 20 min. After fixation, cells were subjected to permeabilization for intra-cellular staining by adding into 1X Perm Wash Buffer (BioLegend) for 20 min @ RT. Now permeabilised cells were stained with APC-labeled anti-mouse IFN-γ and PE-labelled IL-2 antibody (BD Biosciences) at room temperature for 60 min. Finally, cells were washed and resuspended in the FACS buffer. The data was recorded in BD LSR Fortessa cytometer and examined using FlowJo (Tree Star Inc.).

3.15 Indirect ELISA for Serum IgG and subtypes.

ELISA plate (96 well) coated with EDIII protein for all four DENV serotypes separately with 10 µg/ml of each protein overnight in the carbonate buffer with a volume of 100 µl each well in duplicate. In the case of Ova, 20 µg/ml protein was used to coat the plate. The next day plate was washed thrice with 1XPBST (1X PBS + 0.05% Tween 20) with 300 µl for each well. After which plate was blocked with the 4 % skim milk prepared in the 1XPBS for the 2 hrs Later, the plate was washed four times with 1X PBST, and 100 µl of diluted serum (1:100) was added to each well prepared in the 0.1 % skim milk and incubated for 2 hrs. After which, the plate was washed four times with 1X PBST and incubated with the HRP conjugated anti-IgG (1:5000), and anti-IgG subtypes antibodies, i.e., IgG1 (1:5000) and IgG2a (1:2000), IgG2b (1:2000) for the 1 hr @ RT. Then, wash with 1X PBST six times and add the TMB substrate 50 µl/well, which allowed the reaction to develop to specific points, stopped with 2 N H_2SO_4. Finally, absorbance was recorded at 450 nm with reference 570 nm. Graphs were plotted by taking the O.D. on the Y-Axis.

3.16 Germinal Centre B-cell staining employing flow cytometry.

To analyse the germinal centre-specific B-cells, isolated lymph nodes from immunized mice were crushed and passed through a 40 μm cell strainer. The cells were centrifuged at 1500 rpm for 5 min, and the obtained cells were resuspended in 1XRBC lysis buffer for 10 min @ RT. Finally, cells were collected and washed with FACS buffer, and surface markers such as B220 tagged with Alexa Fluor 488, IgG tagged with Alexa Fluor 647, and biotin labelled anti-GL7 were stained for 1 hour at 4°C. Cells were washed and incubated with Alexa Fluor 555–labelled streptavidin to tagged anti-GL-7 antibody for 1 hour @ RT. The cells were fixed with 4% paraformaldehyde and washed with FACS buffer; data were recorded in BD Fortessa LSRII and obtained data were analysed using FlowJo.

3.17 Quantitative RT PCR (qRT-PCR)

Macrophage cells were seeded with the density (1 X 10^6 /well) in the six-well plate, and cells were treated with varied concentrations of arsenite and a single concentration of LPS (500ng/ml). Media was withdrawn following treatment with or without arsenite, followed by rinse with 1X ice-cold PBS. Cells were then scraped and centrifuged for 5 minutes at 1200 rpm @ 4^0 C. Trizol (300μl/sample) was added to the pellet and properly mixed before being left at room temperature for 5 minutes, followed by chloroform treatment on ice for 15 minutes. The aqueous phase was then separated by centrifugation, which was carried out for 15 minutes at 4°C and 2000 rpm. The aqueous phase was correctly mixed with 500μl of isopropanol before being incubated for 15 minutes on ice. Again, centrifugation was carried out for 30 minutes at 4°C at 12000 rpm. The supernatant discarded RNA pellet visible at the bottom of the tube was washed and kept for air drying. RNA concentration was determined using a nanodrop after an air-dried pellet was reconstituted in TE buffer. A similar RNA isolation protocol was used to isolate the RNA from the cells of lymph nodes. The cDNA was synthesised by Takara (PrimeScript 1st strand cDNA Synthesis Kit) by converting RNA into cDNA (1000 ng). qPCR was carried out using an AB biosystem thermal cycler (Thermos Fisher Scientific) with gene-specific primers (Supporting Information Table 1). The thermal cycler parameters: one cycle of denaturation at 94°C for 2 min followed by 40 cycles of amplification. The amplification parameters were denaturation at 94°C/30 s, annealing at 58°C/30 s, and extension at 72°C/40 s. Each sample's relative mRNA expression was calculated compared to the housekeeping gene GAPDH.

Table 1: Gene-specific primers for qRT-PCR.

Genes		Primers 5'-3'
Pax5	FP	CCATCAGGACAGGACATGGAG
(Mouse)	RP	GGCAAGTTCCACTATCCTTTGG
Bcor1	FP	CTTTCTGCAACCCCTCTGTATG
(Mouse)	RP	CTTTCTGCAACCCCTCTGTATG
IL-17ra	FP	CATCAGCGAGCTAATGTCACA
(Mouse)	RP	AGCGTGTCTCAAACAGTCATTTA
Il-18r1	FP	TCTCAAGTCGGAAATGATCGTCG
(Mouse)	RP	ATCTCTGCTTGTCACGAGCTT
Ifngr2	FP	TCCTCGCCAGACTCGTTTTC
(Mouse)	RP	GTCTTGGGTCATTGCTGGAAG
Irf7	FP	GAGACTGGCTATTGGGGGAG
(Mouse)	RP	GACCGAAATGCTTCCAGGG
Ikzf1	FP	AGACAAGTGCCTGTCAGACAT
(Mouse)	RP	CCAGGTAGTTGATGGCATTGTTG
Xbp1	FP	GACAGAGAGTCAAACTAACGTGG
(Mouse)	RP	GTCCAGCAGGCAAGAAGGT
Tank	FP	AGACATAGTCTGCGAAGGAACG
(Mouse)	RP	ATGCTCTATTGAGTTGCTCACC
Gapdh	FP	AAGGTCATCCCAGAGCTGAA
(Mouse)	RP	CTGCTTCACCACCTTCTTGA
IL-6	FP	CTGCAAGAGACTTCCATCCAG
(Mouse)	RP	AGTGGTATAGACAGGTCTGTTGG
TNF-α	FP	CCTGTAGCCCACGTCGTAG
(Mouse)	RP	GGGAGTAGACAAGGTACAACCC
IL-1β	FP	GAAATGCCACCTTTTGACAGTG
(Mouse)	RP	TGGATGCTCTCATCAGGACAG

3.18 Proliferation of DENV in C6/36 cells

C6/36 cells were allowed to grow fully confluent in the T75 (75-$cm^{2)}$ flask. Discard the used growth medium from 75-cm^2 flask containing C6/36 cells and wash the flask twice with PBS. Added 1.5 ml of 0.05% trypsin-EDTA and incubated at 33˚C for 2 min. Gently tap the flask to

separate the cell monolayer, add complete DMEM medium, collect the cells in the 15 ml centrifuge tube, and centrifuged at room temperature at 5 min at 500 × g. Discard the medium, resuspend the cell pellet in fresh DMEM complete medium, and incubate in the new T75 culture flask for 24 hrs. On the next day-old culture medium is removed from the flask. Cells were rinsed with PBS, removed the remaining PBS, and the calculated inoculum was added to the flask.

Formula of Inoculum: (cells in one flask) (MOI)/ (virus titer in pfu) × (1000 µl) = virus inoculum (µl).

Bring the flask volume to 3 ml with DMEM without FBS. Incubate the flask for one hour at 33°C. Every 15 minutes, gently rock the flask to disperse the virus uniformly and stop the monolayer from drying out. Added the 7 ml of DMEM with the 2% of FBS, bringing the final volume up to 10 ml. Incubate for three to four days at 33°C. To confirm cell mortality, examine cells under a microscope every day.

3.19 Serotyping of the DENV

following the manufacturer's instruction, viral RNA was isolated from the culture supernatant of DENV-infected cells from all four serotypes (DENV 1-4) using RNA isolation kit (Macherey Nagel Germany). RNA extracted is further used for the generation of cDNA using the kit from (Takara Bio Inc. in Japan). cDNA synthesized using a D2 reverse primer (Lanciotti et al., 1992). Obtained cDNA amplified by using D1 as forward and D2 as reverse primer using the PCR. The obtained product was again amplified by another round of PCR reaction in which the initial amplification product acts as a template and D1 forward primer and serotype-specific reverse primers (Table 2) TS1, TS2, TS3, and TS4 were used as earlier described by (Afroz et al., 2016).

Table 2: Serotyping Primers

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

3.20 FNT (Flow cytometry-based neutralization test)

Serum from the immunized mice was analyzed for its neutralizing potential against the DENV virus utilising the FNT. Vero cells were employed in this procedure. In the FNT, 25000 cells/well were seeded in a 96-well plate. These cells were incubated in the previous stage with a mixture of virus and heat-inactivated serum collected from 5 mice from each of the three groups. Triplicates of each serum dilution were analysed. The plates were rocked every 15 minutes for an hour, incubating at 37°C in a 5% CO_2 incubator. At the end of incubation, 200 µl of DMEM (2%FBS) media was added to each well incubated for 24 hr in an incubator containing 5% CO_2. After which, cells were washed with the 150 µl of 1xPBS and trypsinised. Cells were collected by centrifugation at 1500rpm for 5 min @ RT and fixed with 4% paraformaldehyde for 10 min @ RT. Following this, cells were washed and allowed to permeabilize for 15 minutes and further cells were stained by the anti-NS1 antibody (GENETEX). Finally, samples were stained with a secondary antibody (Alexa Flour 488). Data were recorded in the flow cytometry (BD Accuri C6). In each sample, about 10,000 to 20,000 cells were analyzed. The per cent reduction in the number of infected cells were calculated for each serum dilution. The titers were calculated as the reciprocal of the serum dilution that reduced 50% of the virus infectivity (FNT50).

3.21 Statistical analysis

The results of two or three independent experiments are used to display all data as mean ± SEM. P values lower than 0.05 were regarded as significant. Unpaired student's t-Test was applied to calculate the significance of difference amongst groups. The analysis is done by the GraphPad Prism software.

4. RESULTS

4.1 Objective 1

To elucidate the role of ISR pathway activation in the modulation of innate effector function using an in-vitro macrophage stimulation system

4.1.1 Arsenite shows no cytotoxicity in macrophages

To determine the nontoxic concentration of arsenite, MTT assay was performed. Cell damage or cytotoxicity can be detected by monitoring cellular activity. In the MTT assay, there is utilization of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). This yellow dye that is water soluble is absorbed by living cells and reduced by mitochondrial dehydrogenases. The reduction product is water-insoluble and dissolved for colourimetric measurement (Morgan, n.d.). Concentrations of arsenite from 0.1 to 1000 µM were used to treat the macrophages. Arsenite treatment is given for 24 and 48 hrs, showing no cytotoxicity up to 100 µM (Figure 11).

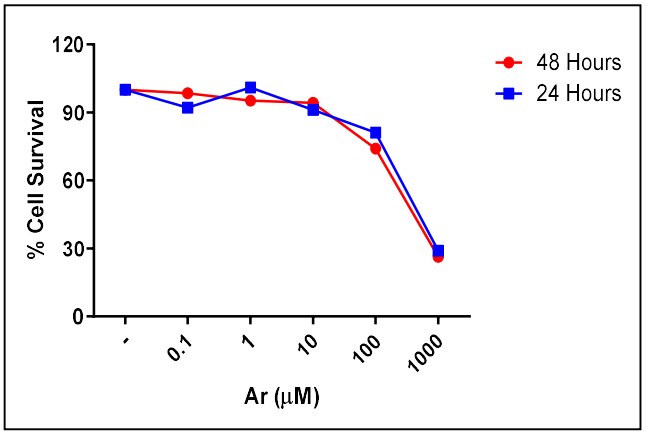


Figure 11: cytotoxicity assay

MTT assay to evaluate the cytotoxicity of the arsenite (concentration varies from 0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM) by treating J774A.1 macrophages cells.

4.1.2 Arsenite increases the expression of P-eIF2α and is responsible for the formation of stress granules in macrophages.

Immune cells programme their cellular machinery by activating the ISR pathway (Naz et al., 2019). eIF2 kinases, including GCN2, PERK, HRI, and PKR, activate the ISR pathway. (Pakos-Zebrucka et al., 2016). Each can sense and respond to distinct stress signals, including amino acid starvation, ER stress, heme deficiency, and viral infection, respectively (Pakos-

Zebrucka et al., 2016b). Upon activation, eIF2α kinases phosphorylate alpha subunit of eIF2 on serine 51 (Ron, 2002a), which results in the attenuation of active preinitiation translational complex eIF2-GTP-tRNA^{Met} assembly and polysome formation (Dey, Trieselmann, et al., 2005) thus, leading to the global shut down of general protein synthesis (Donnelly et al., 2013). ISR expression increases the recruitment of RNA-binding proteins TIA1 (T cell restricted intracellular antigen-1) and TIAR (TIA-1 related) (Naz et al., 2019). Macrophages were treated with varying concentrations of arsenite from 0.1 to 10 µM. As the concentration of arsenite increases, the expression of P- eIF2 increases (Figure 12 A and B). Additionally, increased concentration triggers the development of stress granules in macrophages. TIA1 and TIAR protein expression confirmed the formation of stress granules (Figure 12 C).

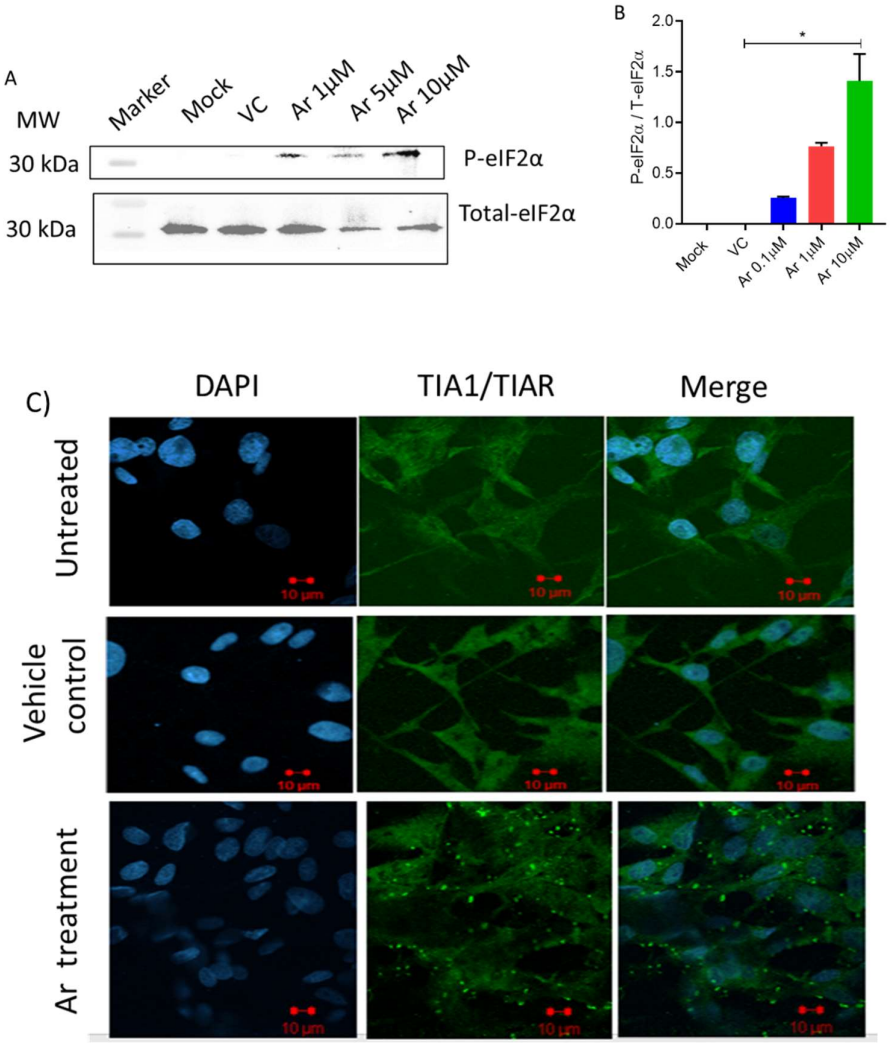


Figure 12: Arsenite increases the expression of P-eIF2α and is responsible for the formation of stress granules in macrophages

(A) Immunoblot analysis of the P- eIF2α and total- eIF2α were J774A.1 macrophages treated with arsenite concentrations 1 μM, 5 μM and 10 μM. Total- eIF2α used as control. (B) Densitometry plot of the P-eIF2α/Total-eIF2α (C) Stress granules formation in J774A.1 macrophages confirmed with RNA binding protein TIA1 and TIAR after being treated with arsenite 5 μM concentration. By employing the student t-test, statistical significance was calculated, and value *P≤0.05 was considered significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates

4.1.3 Arsenite decreases the TNF-α, IL-6, and IL-1β expression in LPS induced Macrophages.

Cytokines are secreted proteins released by cells (J.-M. Zhang & An, 2007), proinflammatory predominately secreted by the activated macrophages involved in the upregulation of TNF-α, IL-6, and IL-1β (J.-M. Zhang & An, 2007). IL-1β is released by the monocytes and macrophages in response to cell damage, infection, invasion, and inflammation. (J.-M. Zhang & An, 2007). IL-6 mediates host defense by activating acute phase response, hematopoiesis, and immune reactions (Tanaka et al., 2014). The monocyte or macrophages secrete TNF-α during acute inflammation and are responsible for many functions, such as necrosis and apoptosis. It is also responsible for resistance to infection and cancer (Idriss & Naismith, 2000). Relative mRNA expression for TNF-α (Figure 13 A), IL-6 (Figure 13 B), and IL-1β (Figure 13 C) decrease with the increase in the concentration of arsenite in LPS-primed macrophages checked by using the RT-PCR.

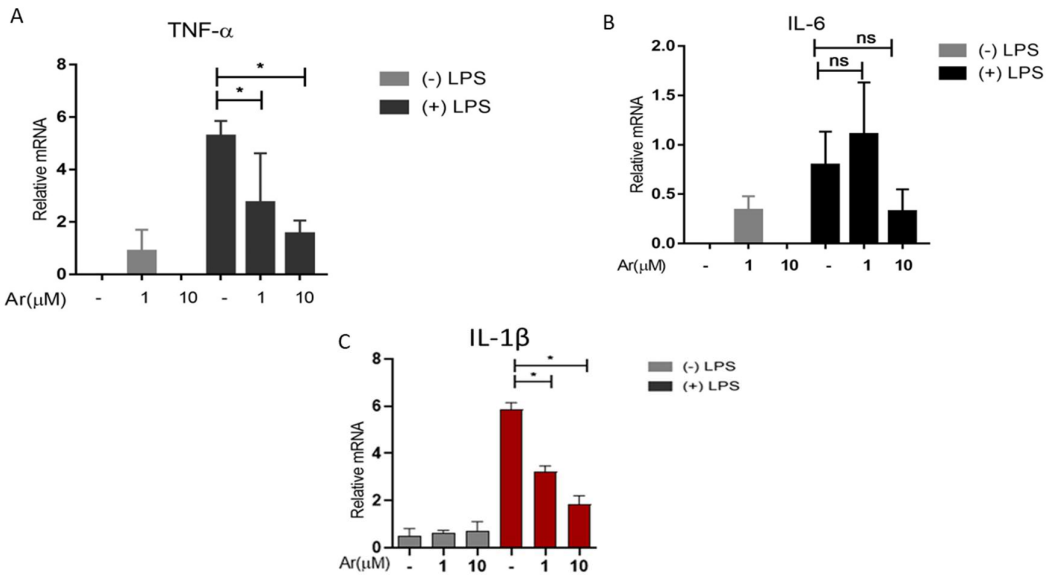


Figure 13: Ar decreases the TNF-α, IL-6 and IL-1β expression in LPS induced Macrophages: Macrophages were first primed for 3 hr of LPS and later treated with 3 hr of increase in the concentration of arsenite for the (A) TNF-α, (B) IL-6 and (C) IL-1β. By employing the student t-test, statistical significance was calculated, value *P≤0.05 was considered significant, and ns = not significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates.

4.1.4 Arsenite reduces the reactive oxygen species in macrophages

Reactive oxygen species (ROS) act as a cell signalling molecule for biological processes. However, ROS generation damages different cellular organelles, which can disturb normal physiology. The improper balance between ROS production and antioxidant defences leads to various diseases (Auten & Davis, 2009). The effect of arsenite on LPS-induced J774A.1 macrophage cells was studied (Chakravortty et al., 2001). Reactive oxygen species concentration decreases as the concentration of arsenite increases in the LPS-induced macrophages (Figure 14 A and B).

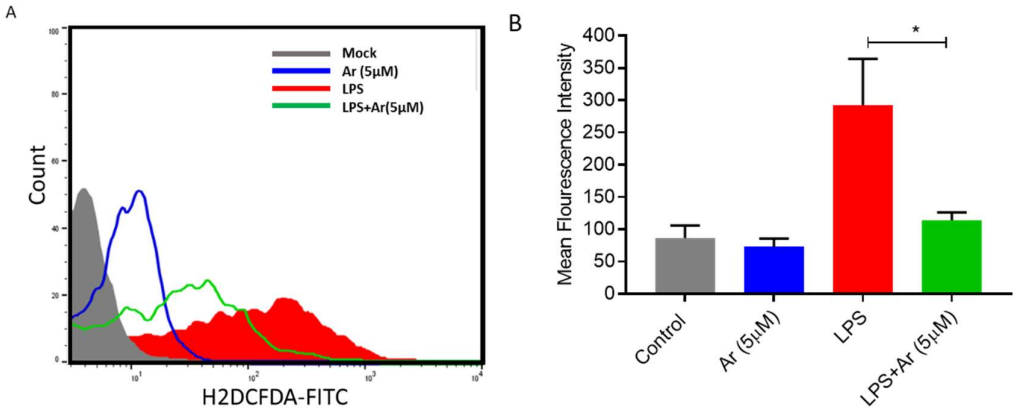


Figure 14: Ar treatment reduces the reactive oxygen species in macrophages

(A) Macrophages primed with the LPS for 3 hr and later treated with arsenite for 3 hr (B) Mean fluorescence Intensity Plot. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates

4.1.5 Arsenite increases Ova antigen uptake in macrophages

Macrophages are the professional APC (BARKER et al., 2002). For effective antigen presentation, APCs need to uptake antigen efficiently. APC use a different mechanism for antigen presentation (Lanzavecchia, 1996). Ova uptake was studied by treating macrophages with arsenite at 37°C (Figure 15) (Ravindran et al., 2014a). Ova uptake increases in macrophages after treatment with Ar at 37°C.

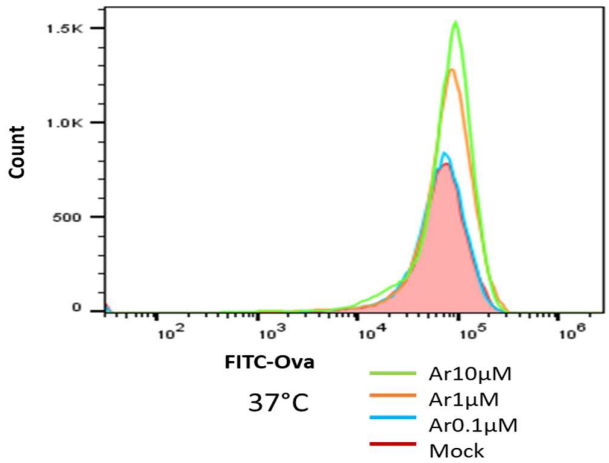


Figure 15: Ova uptake increased in macrophages treated with Ar

J774A.1 macrophages treated with the increase in the concentration of arsenite and Ova uptake confirmed by the FITC-Ova at 37°C

4.1.6 Arsenite inhibits Nitric oxide production in LPS-primed macrophages.

Nitric oxide production has an essential function *in vivo*, such as vasodilation, neuronal messenger molecule and potent immune system molecules, such as cytokine mediators. Mice macrophages provide the best-studied NO production examples (MacMicking et al., 1997). The LPS enhances NO production *in vitro* (Chakravortty et al., 2001). Arsenite inhibits nitrite production in macrophages treated with the different concentrations of arsenite and induced with the LPS (Figure 16). As the concentrations of arsenite increase, expression of nitrite decreases in LPS-induced macrophages.

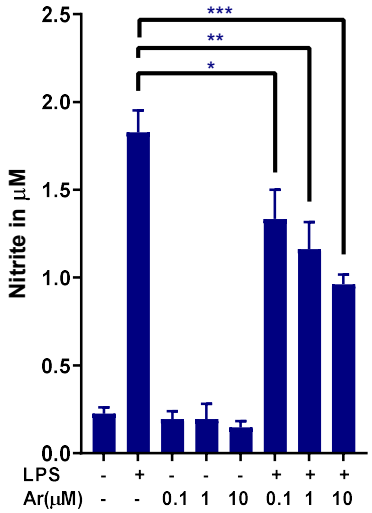


Figure 16: Arsenite inhibits Nitric oxide production in LPS-primed Macrophages

Macrophages treated with the increase in the concentration of arsenite where macrophages already primed with LPS. An experiment was performed for 18 hr. Expression of the Nitrite measured in the µM concentration. By employing the student t-test, statistical significance was calculated; value *P≤0.05, **P ≤ 0.005, ***P ≤ 0.0005 was considered significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates.

4.1.7 Arsenite enhances the MHC-I expression in macrophages.

APC engage T cells in interaction, connecting innate immunity to adaptive immunity (Gaudino & Kumar, 2019). Antigen presentation is when an antigen engulfs macrophages using a specific

receptor and presents outside the cell surface (Nesmiyanov, 2022). When the macrophages are treated with an increasing concentration of arsenite, antigen presentation increases, which is confirmed by the increase in the % of MHC I cells (Figure 17 A and B).

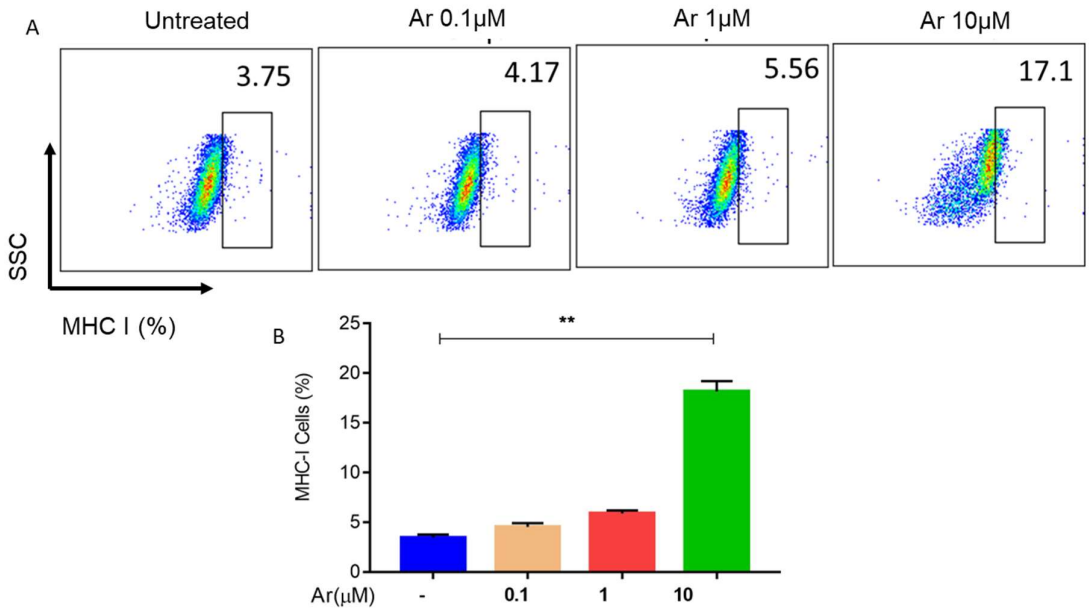


Figure 17: Arsenite enhances the MHC-I expression in macrophages

(A) representative FACS plot for MHC I cells, macrophages treated with the increasing concentration of arsenite. (B) the average percentage of the MHC I cells. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates.

4.1.8 Arsenite enhances the co-stimulatory signals of macrophages.

For enhanced activation of naïve T cell, co-stimulation is necessary (Lim et al., 2012). When the macrophages are treated with an increasing concentration of arsenite, co-stimulatory signals increases, which is confirmed by the increase in the % of CD86 cells (Figure 18 A and B).

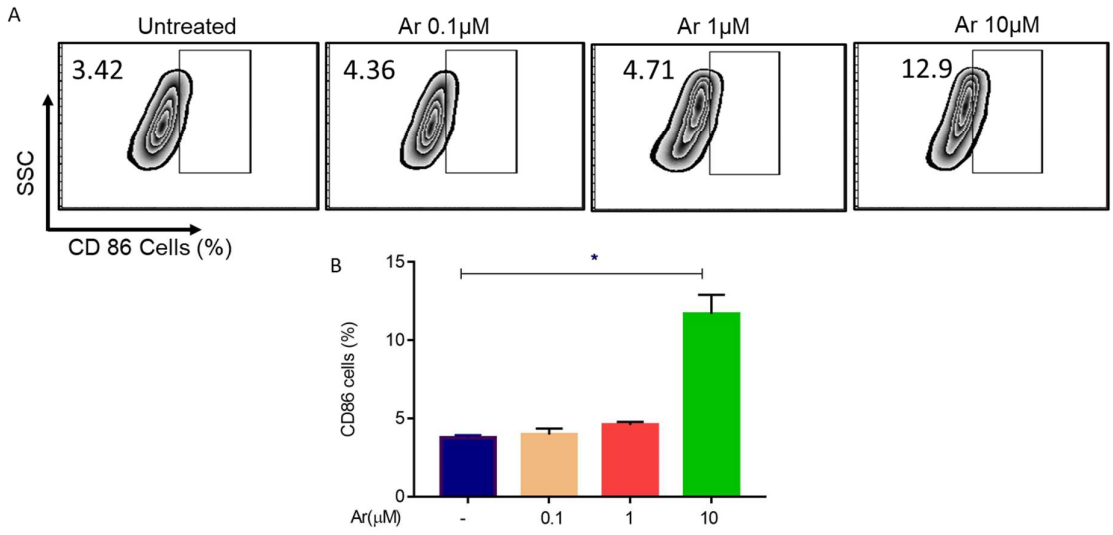


Figure 18: Arsenite enhances the co-stimulatory signal in macrophages.

(A) representative FACS plot for CD86 cells, macrophages treated with the increasing concentration of arsenite (B) average percentage of the CD86 cells. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant. The error bars display the mean +/- SEM. The data represent one of the three independent replicates

4.2 Objective 2

To examine the adjuvanting function of ISR activator, arsenite (Ar) in programming the magnitude of antigen-specific immune responses against various antigens upon immunization in a mouse model

4.2.1 Immunological responses to Ova antigen

4.2.1.1 Mice primed with arsenite and immunized with the Ova showed no change in body weight.

Mice immunized with PBS, Ova, and Ova+Ar showed no difference in body weight up to 42 days (Figure 19).

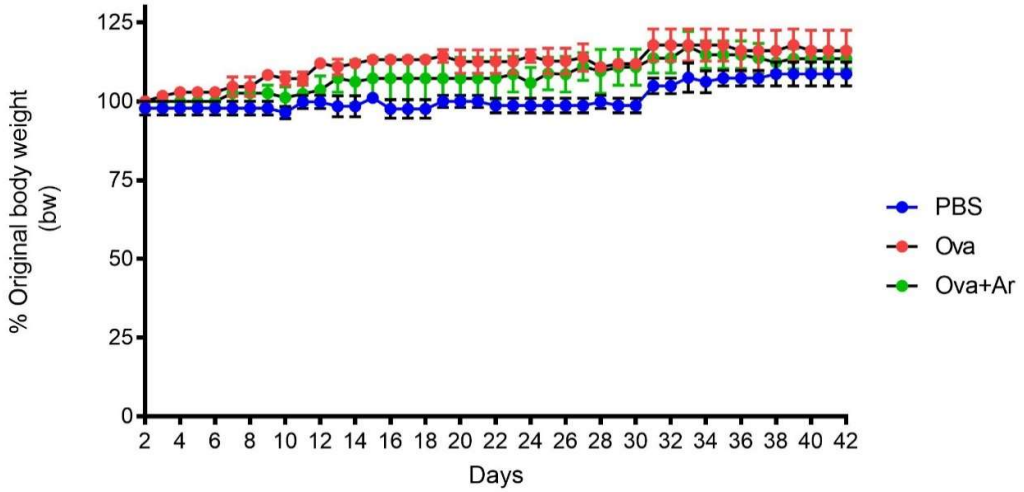


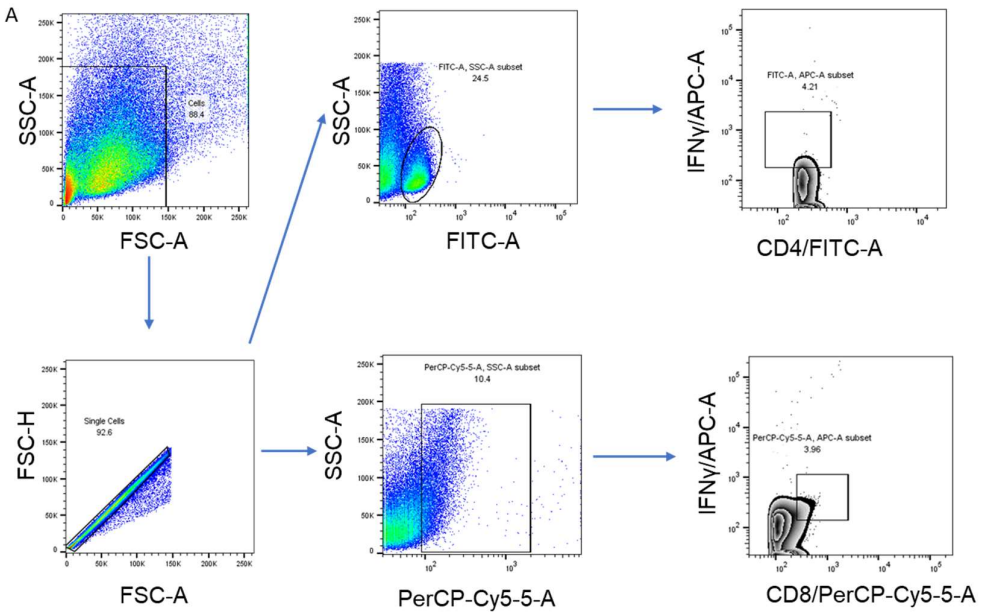
Figure 19: Body weight analysis of mice Immunized with the PBS, Ova and Ova+Ar

The data represent the mean ± SEM of two independent replicates (5X2), and each group contain 5 animals

4.2.1.2 Arsenite induces ISR Pathway that enhances antigen-specific T cell response

ISR (integrated stress response) pathway is present in the eukaryotic cells (Pakos-Zebrucka et al., 2016). We used Ovalbumin as the model antigen and arsenite as the ISR activator to ascertain how the ISR pathway affects antigen-specific T-cell immunity. Ova was immunized subcutaneously after being primed with arsenite, and a group without antigen was kept as a control in which mice were immunized with PBS. Arsenite helps to modulate the immune response by the ISR pathway. After the primary immunization in PBMC restimulated with

SIINFEKL peptide, we checked the T cell response. 42 days after the immunization, it was observed that arsenite treatment enhances the expression of Ova-specific IFN-γ by $CD4^+$ and $CD8^+$ T cells in splenocytes (Figure 20 D and E).



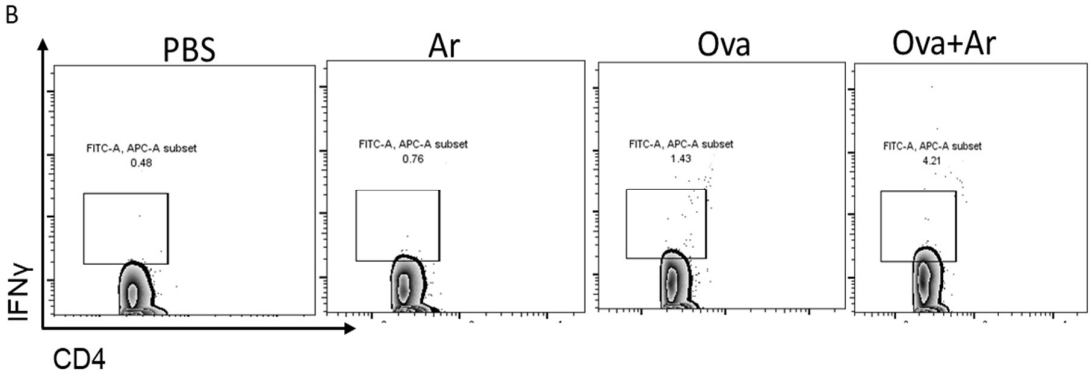


Figure 20 continued on the next page…….

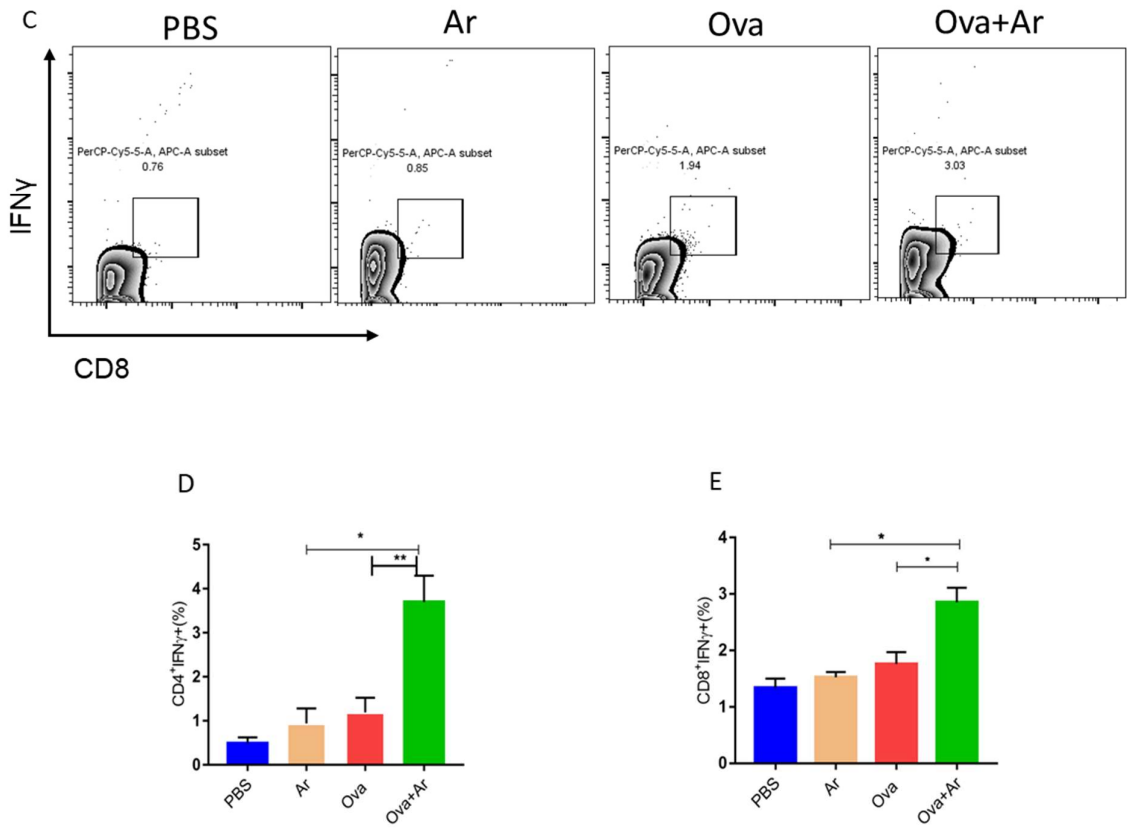


Figure 20: ISR Pathway enhances antigen-specific T cell response

(A) Gating strategies for choosing the $CD4^{+}IFN\gamma^{+}$ and $CD8^{+}IFN\gamma^{+}$ cells. Antigen-specific T-cell response was measured after 42 days of immunization by isolating the splenocytes from the mice. (B and C) representing FACS plot. (D and E) representing the average percentage of the IFNγ producing $CD4^{+}$ and $CD8^{+}$ cells, respectively. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated. Value *P≤0.05, **P ≤ 0.005 was considered significant.

4.2.1.3 Arsenite enhances Ova-Specific antibody response

In the immunological response, $CD4^{+}$ T cells are crucial because they serve to stimulate the B cell and $CD8^{+}$ T cell responses (Swain et al., 2012). Special $CD4^{+}$ T cells called follicular helper cells (Tfh) reside in the B cell follicle (Breitfeld et al., 2000). When immune cells are exposed to antigens, it results in the expansion of immune cells, leading to the formation of plasma and memory cells (Sebina & Pepper, 2018). Plasma cells secrete antibodies specific to the antigen (Pioli, 2019). We observed the $CD4^{+}$ specific antibody response against the mice immunized with the Ovalbumin antigen was confirmed by the formation of IgG and its

subtypes IgG1, IgG2a and IgG2b antibodies in the serum after the 14, 28 and 42 days of immunization. It has been observed that the level of total IgG (Figure 21 A), IgG1 (Figure 21 B), IgG2a (Figure 21 C) and IgG2b (Figure 21 D) is higher in the mice group, which is immunized with Ova and primed with arsenite, compared to the mice group, which is only immunized with Ova. These results establish a link between the ISR pathway and antigen-specific antibody response.

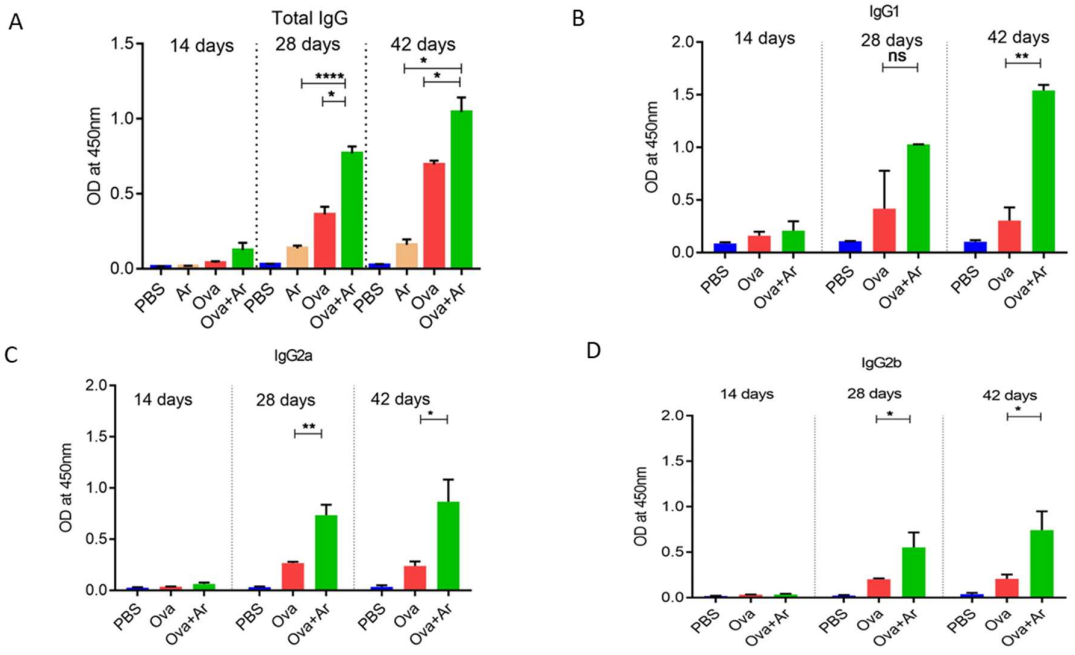


Figure 21: Arsenite enhances Ova Specific antibody response

Indirect ELISA was used to determine antigen-specific IgG and IgG subclasses in serum. Indirect ELISA was done for 14,28, and 42 days after immunization. (A) IgG, (B) IgG1, (C) IgG2a, and (D) IgG2b. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2), and each group contain 5 animals. By employing the student t-test, statistical significance was calculated. Value *P≤0.05, **P ≤ 0.005 and ****P ≤ 0.0001 was considered significant.

4.2.1.4 Arsenite promotes the development of the germinal centre in the lymph node of mice primed with arsenite and immunized with Ova

The secondary lymphoid tissue contains a unique sort of structure called the germinal centre that aids in the production of memory B cells and antibody-secreting plasma cells, both of which aid in the defence against reinfection (Stebegg et al., 2018). Here, we examined the draining lymph node cells by staining them with germinal centre markers $B220^{+}$, $GL\text{-}7^{+}$, and

IgG^{+} and analysed them by Flow cytometry. It was found that the percentage of GC-B cells (B220^{+}GL-7^{+} IgG^{+}) is significantly elevated in the group primed with the arsenite and immunized with Ovalbumin as compared to the group that was only immunized with the ovalbumin (Figure 22 B). The lymph node was isolated after 42 days by euthanizing after the primary immunization. The formation of the germinal centre promotes the shape of the total IgG and other IgG subtypes such as the IgG1, IgG2a and IgG2b.

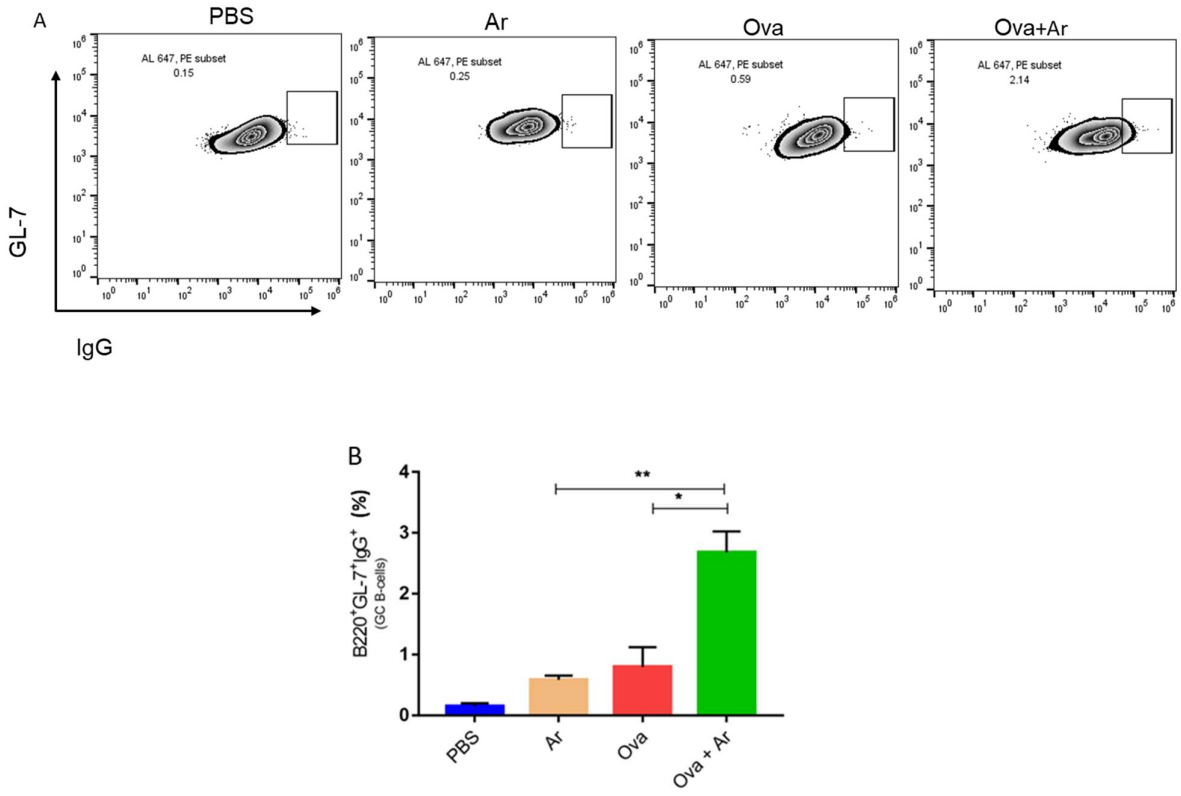


Figure 22: Arsenite promotes the development of the germinal centre in the lymph node
(A) Best representative FACS plot for the data of GC-B cells (B) analysis of the germinal centre formation by calculating the frequency of the triple-positive cells (B220^{+}, GL7^{+} and IgG^{+}) using a flow cytometer. These triple-positive cells are considered GC-B cells. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated. Value *P≤0.05, **P ≤ 0.005 was considered significant.

4.2.1.5 Arsenite enhances GC formation of mice primed with arsenite and mice immunized with Ova

The lymph node sections H and E staining (haematoxylin and eosin) confirm whether the ISR pathway enhances the germinal centre formation. Arsenite enhances GC formation in mice immunized with Ova and primed with arsenite (Figure 23 B). It was observed that mice primed with arsenite only had the GC formation, while the other immunized group didn't have any GC formation.

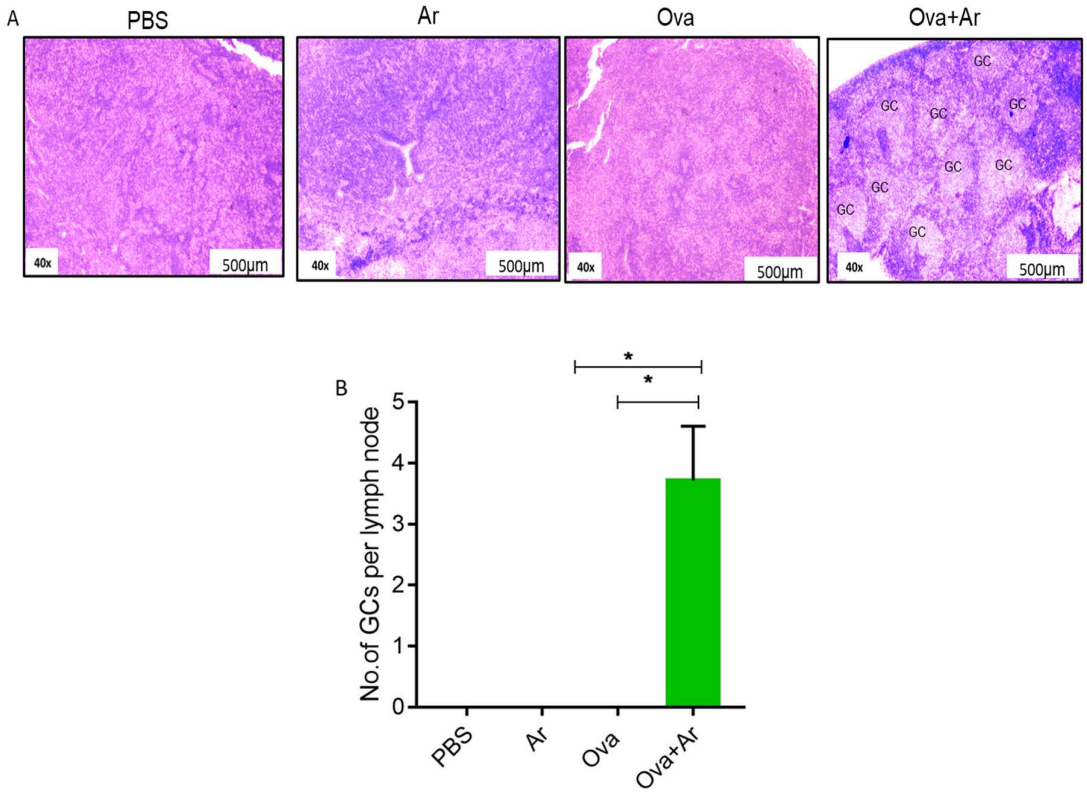


Figure 23: Arsenite shows GC formation in mice immunized with Ova and primed with arsenite

(A) As indicated, lymph node tissue sections were stained with haematoxylin and eosin after 42 days of immunization. (B) The number of GCs per lymph node was calculated as 5 mice per group. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant.

4.2.2 Immunological responses to DENV1rEDIII antigen

4.2.2.1 Expression and purification of the EDIII from the DENV 1 serotype

EDIII from the DENV1 serotype was purified and confirmed through western blot analysis and coomassie staining of SDS gels. The band size for EDIII of the DENV1 was around 14.5 kDa (Figure 24 A and B). It was also confirmed by probing it with an anti-Histidine antibody by western blotting (Figure 24 C).

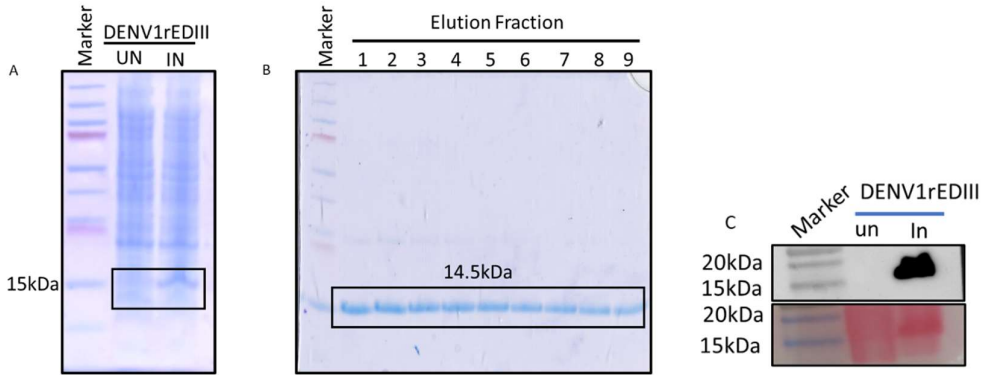


Figure 24: Expression and purification of the EDIII from the denv1 serotype

(A) Coomassie blue-stained SDS PAGE gel image of DENV1rEDIII protein overexpression in *E. coli* Rosetta. (B) The DENV1rEDIII protein affinity chromatography elution fractions are shown in lanes 1 to 9. (C) Purified DENV1rEDIII protein probed with the anti-Histidine antibody confirmed by western blot.

4.2.2.2 No change in body weight was observed as mice immunized with the DENV1rEDIII antigen and primed with arsenite

Mice immunized with PBS, DENV1rEDIII, and DENV1rEDIII+Ar showed no difference in body weight up to 42 days (Figure 27).

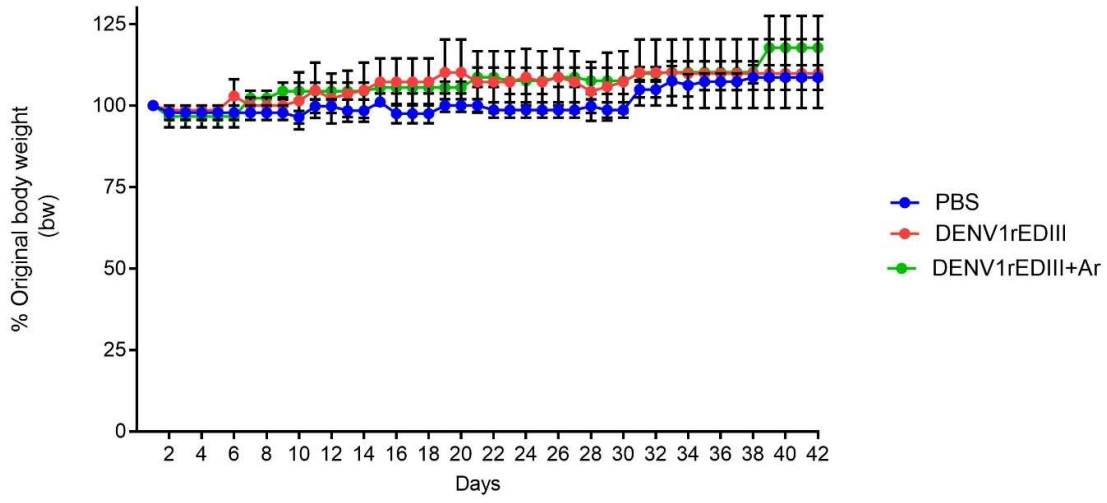


Figure 25: Body weight analysis of mice Immunized with the PBS, DENV1rEDIII and DENV1rEDIII+Ar

The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2), and each group contain 5 animals.

4.2.2.3 ISR Pathway improves antigen-specific T-cell response in mice immunized with DENV1rEDIII and primed with arsenite

We have employed the ISR activator arsenite and DENV1rEDIII antigen as possible vaccine candidates to investigate the impact of the ISR pathway on antigen-specific T-cell immunity (Fahimi et al., 2018). We purified 6X-His-tagged recombinant DENV1EDIII protein. Protein was confirmed through molecular weight by the coomassie-stained SDS PAGE electrophoresis and further confirmed by Immunoblotting. DENV1rEDIII was immunized subcutaneously after being primed with arsenite, and a group without antigen was kept as a control in which mice were immunized with PBS. We checked for the T cell response after the primary immunization in PBMC restimulated with DENV1rEDIII (10 µg/ml). Arsenite treatment was observed to enhance the expression of DENV1rEDIII-specific IFN-γ by $CD4^+$ (Figure 26 A) and $CD8^+$ T (Figure 26 C) cells and IL-2 by $CD4^+$ (Figure 26 B) and $CD8^+$ T (Figure 26 D) cells 14,28 and 42 days after immunization in PBMCs. The expression of IFN-γ and IL-2 decreases after 28 days due to the contraction phase (Harty & Badovinac, 2008).

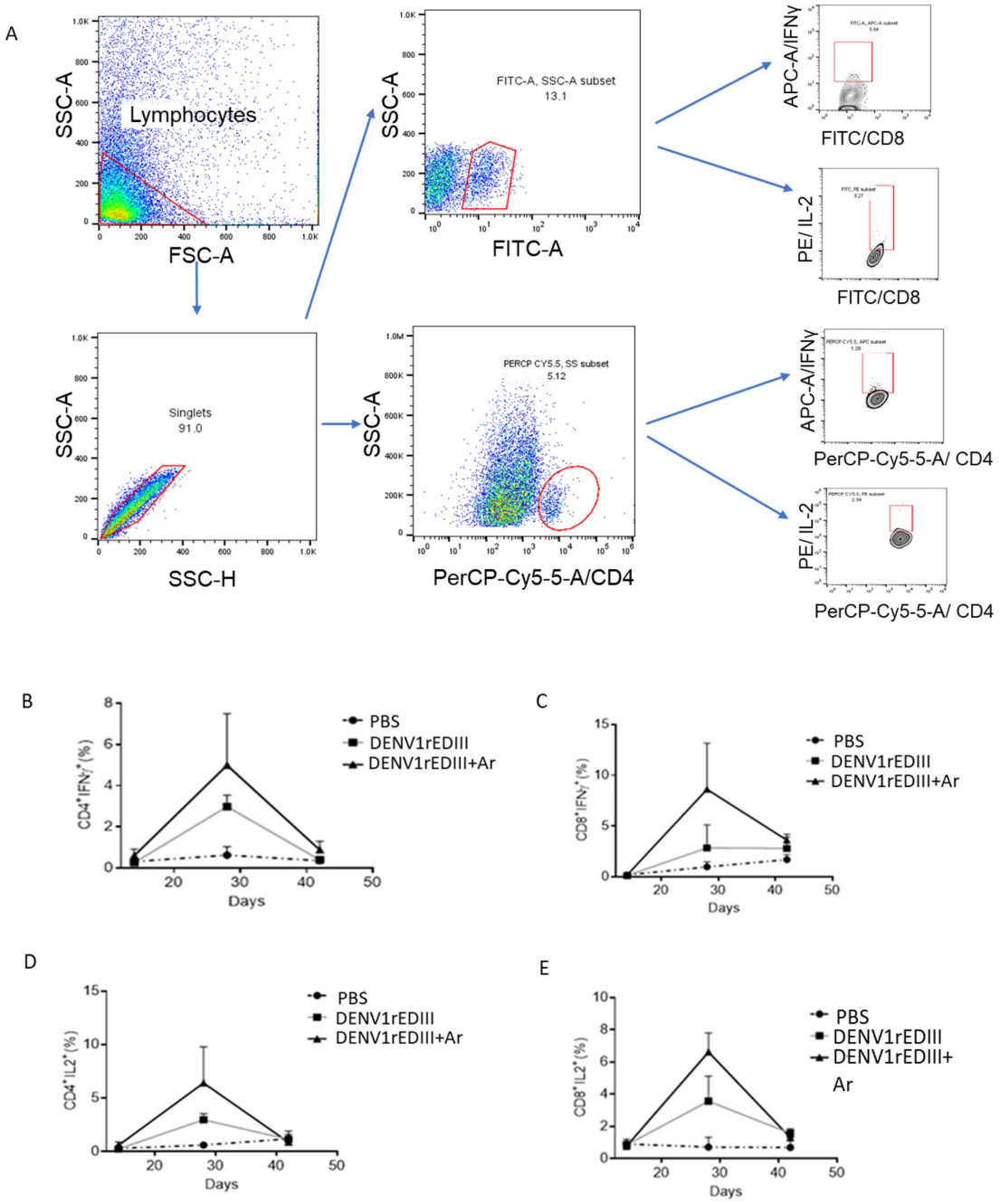


Figure 26: ISR Pathway improves antigen-specific T-cell response in mice immunized with DENV1rEDIII protein and primed with arsenite

(A) gating strategies for the choosing the $CD4^+$, $CD8^+$ cells secreting IFNγ and IL-2. The T-cell response was measured after the 14, 28, and 42 days of the immunization by isolating the PBMCs from the immunized mice. (B to E) T cell kinetics of the PBMC isolated from the immunized mice after 14, 28 and 42 days. Graphs show the frequency of the $CD4^+$ and $CD8^+$

cells secreting IFNγ and IL-2. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2), and each group contain 5 animals

4.2.2.4 Arsenite (Ar) enhances DENV1rEDIII specific antibody response.

We observed that the $CD4^+$ specific antibody response against the mice immunized with the DENV1rEDIII antigen was confirmed by the formation of IgG and its subtypes IgG1, IgG2a and IgG2b antibodies in the serum after the 14, 28 and 42 days of immunization. It has been observed that the level of total IgG (Figure 27 A), IgG1 (Figure 27 B), IgG2a (Figure 27 C) and IgG2b (Figure 27 D) is higher in the mice group, which is immunized with DENV1rEDIII and primed with arsenite, compared to the mice group, which is only immunized with DENV1rEDIII. These results establish a link between the ISR pathway and antigen-specific antibody response.

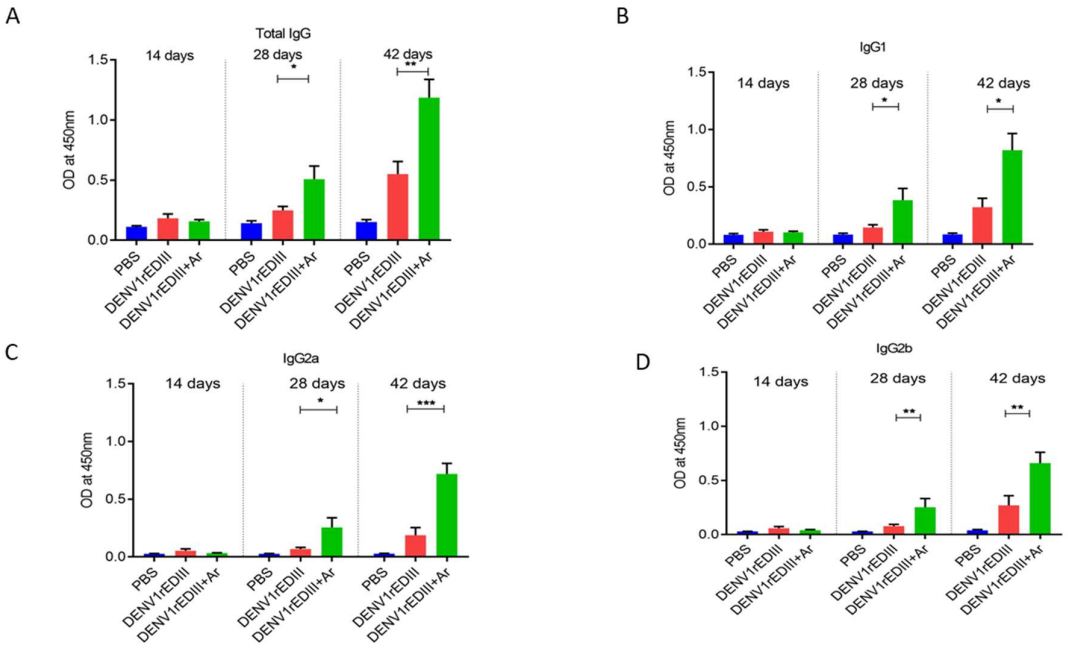


Figure 27: Arsenite (Ar) enhances antigen-specific antibody response in mice

Indirect ELISA was used to determine antigen-specific IgG and IgG subclasses in serum. Indirect ELISA was done for 14,28, and 42 days after immunization. (A) IgG, (B) IgG1, (C) IgG2a, and (D) IgG2b. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated. Value *P≤0.05, **P ≤ 0.005 and ***P ≤ 0.0005 was considered significant.

4.2.2.5 Arsenite-mediated ISR activation augments germinal centre formation in lymph node

Here, we examined the draining lymph node cells by staining them with germinal centre markers $B220^+$, $GL\text{-}7^+$, and IgG^+ and analysed them by Flow cytometry. It was found that the percentage of GC-B cells ($B220^+GL\text{-}7^+ IgG^+$) is significantly elevated in the group primed with the arsenite and immunized with DENV1rEDIII protein compared to the group which was immunized only with the DENV1rEDIII antigen (Figure 28 C). The lymph node was isolated after 42 days by euthanizing after the primary immunization.

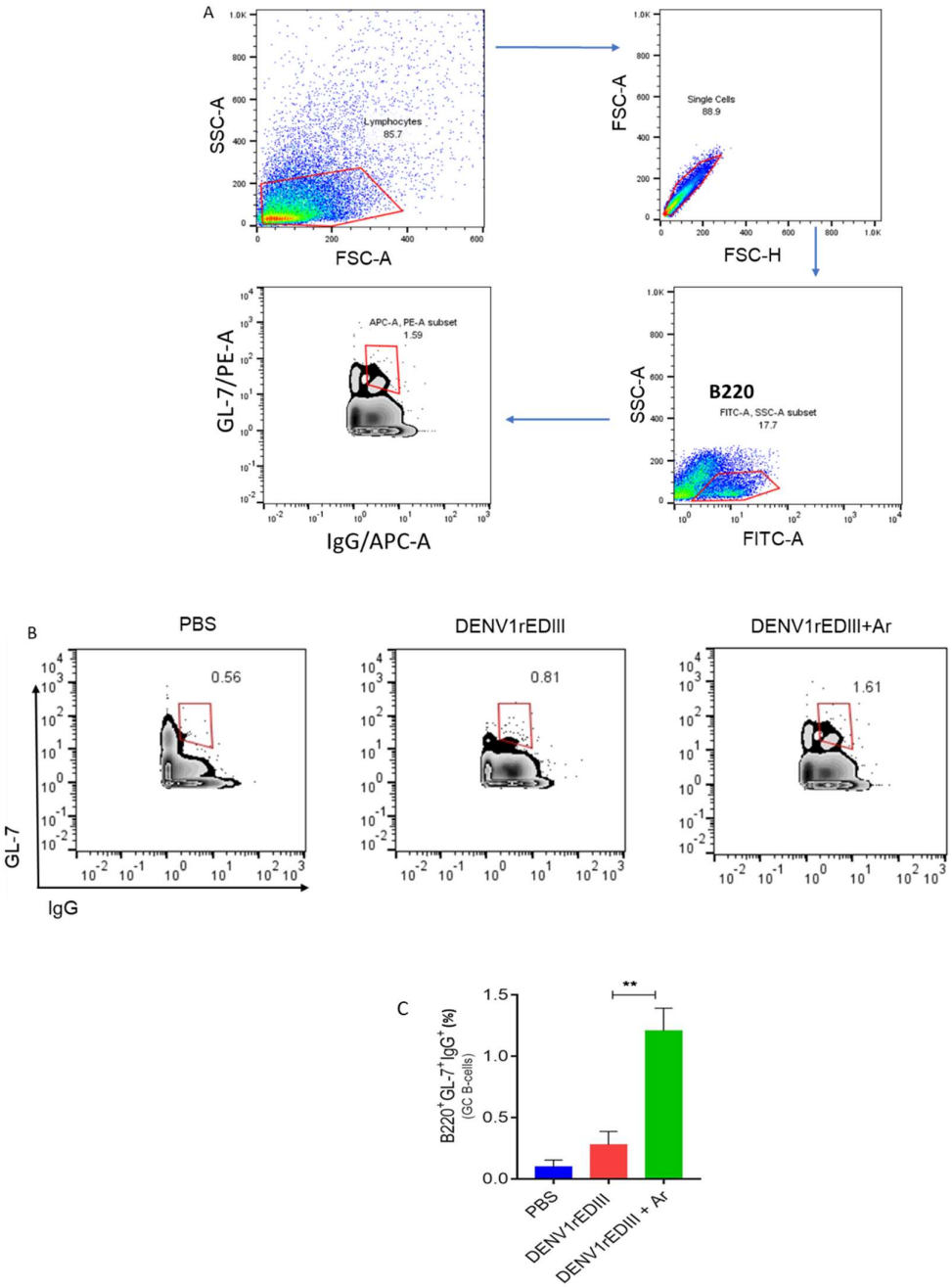


Figure 28: Arsenite-mediated ISR activation augments germinal centre formation in lymph node

(A) Gating strategies for selecting GC-B cells (B) Best representative FACS plot for the data of GC-B cells (C) Analysis of the germinal centre formation by calculating the frequency of the triple-positive cells ($B220^+$, $GL7^+$ and IgG^+) using a flow cytometer. These triple-positive cells are considered GC-B cells. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, value **$P \leq 0.005$ was considered significant.

4.2.2.6 Arsenite enhances GC formation of mice primed with arsenite and immunized with DENV1rEDIII

Arsenite enhances GC formation in mice primed with arsenite and immunized with DENV1rEDIII (Figure 29 B). It was observed that mice primed with arsenite only had the GC formation, while the other immunized group didn't have any GC formation.

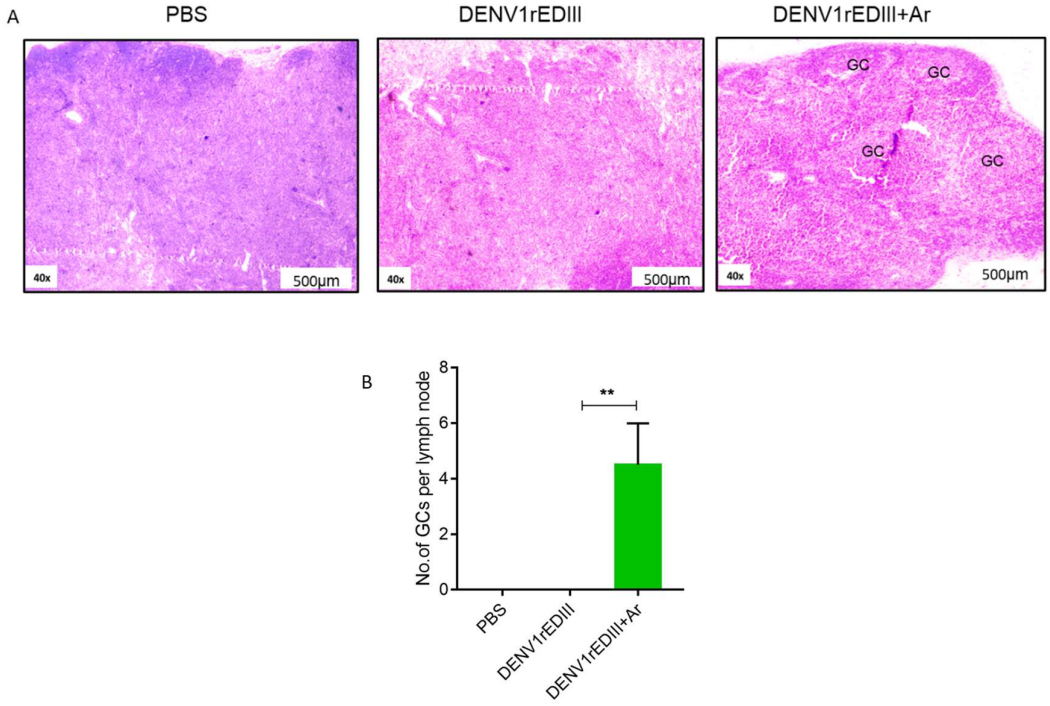


Figure 29: Arsenite shows GC formation in mice immunized with DENV1rEDIII

(A) As indicated, lymph node tissue sections were stained with haematoxylin and eosin after 42 days of immunization (B) The number of GCs per lymph node was calculated as 5 mice per group. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2);

each group contains 5 animals. By employing the student t-test, statistical significance was calculated, value **P ≤ 0.005 was considered significant.

4.2.3 Immunological responses to DENV2rEDIII antigen

4.2.3.1 Expression and purification of the EDIII from the DENV 2 serotype

EDIII from the DENV2 serotype was purified and confirmed through western blot analysis and coomassie staining of SDS gels. The band size for EDIII of the DENV2 was around 14.5 kDa (Figure 30 A and B). It was also confirmed by probing it with an anti-Histidine antibody by western blotting (Figure 30 C).

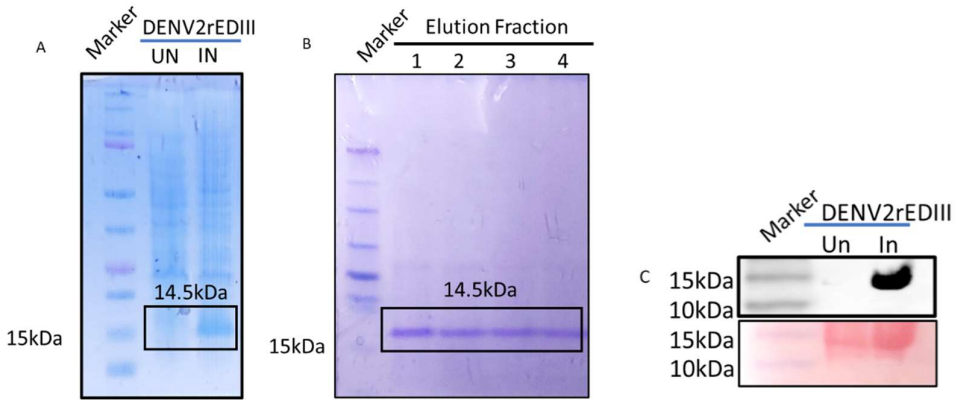


Figure 30: Expression and purification of the EDIII from the DENV 2 serotype

(A) Coomassie blue-stained SDS PAGE gel image of DENV2rEDIII protein overexpression in *E. coli* Rosetta. (B) The DENV2rEDIII protein affinity chromatography elution fractions are shown in lanes 1 to 4. (C) Purified DENV2rEDIII protein probed with the anti-histidine antibody confirmed by Western blot.

4.2.3.2 Arsenite (Ar) enhances DENV2rEDIII specific T-cell response

DENV2rEDIII was immunized subcutaneously after being primed with arsenite, and a group without antigen was kept as a control in which mice were immunized with PBS. We checked for the T cell response 42 days after the primary immunization in splenocytes restimulated with DENV2rEDIII antigen. Arsenite treatment was observed to enhance the expression of DENV2rEDIII -specific IFN-γ (Figure 31 A) and TNF-α (Figure 31 B).

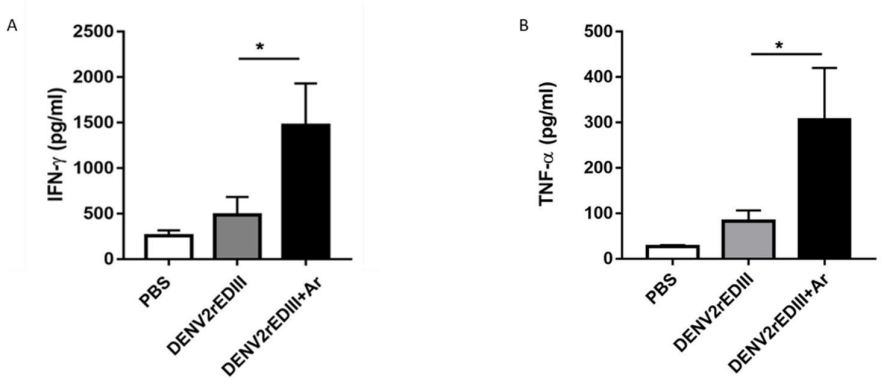


Figure 31 Arsenite (Ar) enhances DENV2rEDIII specific T-cell response.

The secretion of TNF-α and IFN-γ cytokines in the culture supernatant of DENV2rEDIII (10μg/ml) protein restimulated splenocytes from DENV2rEDIII protein immunized mice with or without arsenite. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant.

4.2.3.3 Arsenite enhances DENV2rEDIII specific B-cells response

We observed that the $CD4^+$ specific antibody response against the mice immunized with the DENV2rEDIII antigen was confirmed by the formation of IgG and its subtypes IgG1, IgG2a and IgG2b antibodies in the serum after the 14, 28 and 42 days of immunization. It has been observed that the level of total IgG (Figure 32 A), IgG1 (Figure 32 B), IgG2a (Figure 32 C), and IgG2b (Figure 32 D) is higher in the mice group, which is immunized with DENV2rEDIII and primed with arsenite, compared to the mice group, which is only immunized with DENV2rEDIII. These results establish a link between the ISR pathway and antigen-specific antibody response.

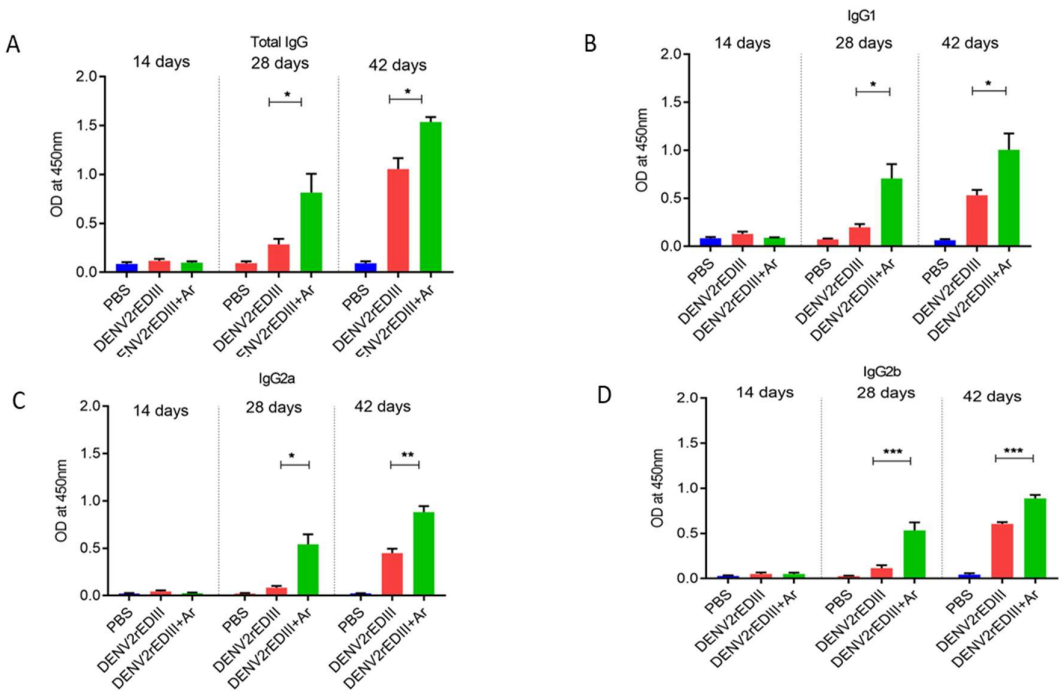


Figure 32: Arsenite (Ar) enhances DENV2rEDIII specific B-cells response

Indirect ELISA was used to determine antigen-specific IgG and IgG subclasses in serum. Indirect ELISA was done for 14,28, and 42 days after immunization. (A) IgG, (B) IgG1, (C) IgG2a, and (D) IgG2b. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated. Value *P≤0.05, **P ≤ 0.005 and ***P ≤ 0.0005 was considered significant.

4.2.3.4 Arsenite-mediated ISR activation augments germinal centre formation in lymph node

We examine the draining lymph node cells by staining them with germinal centre markers $B220^{+}$, $GL\text{-}7^{+}$, and IgG^{+} analysed using FlowJo. The percentage of $B220^{+}GL\text{-}7^{+}IgG^{+}$ GC B cells was found to be high in the group immunized with the DENV2rEDIII and primed with arsenite compared to the group only immunized with the antigen (Figure 33 B). The lymph node was isolated after 42 days by euthanizing after the Primary Immunization.

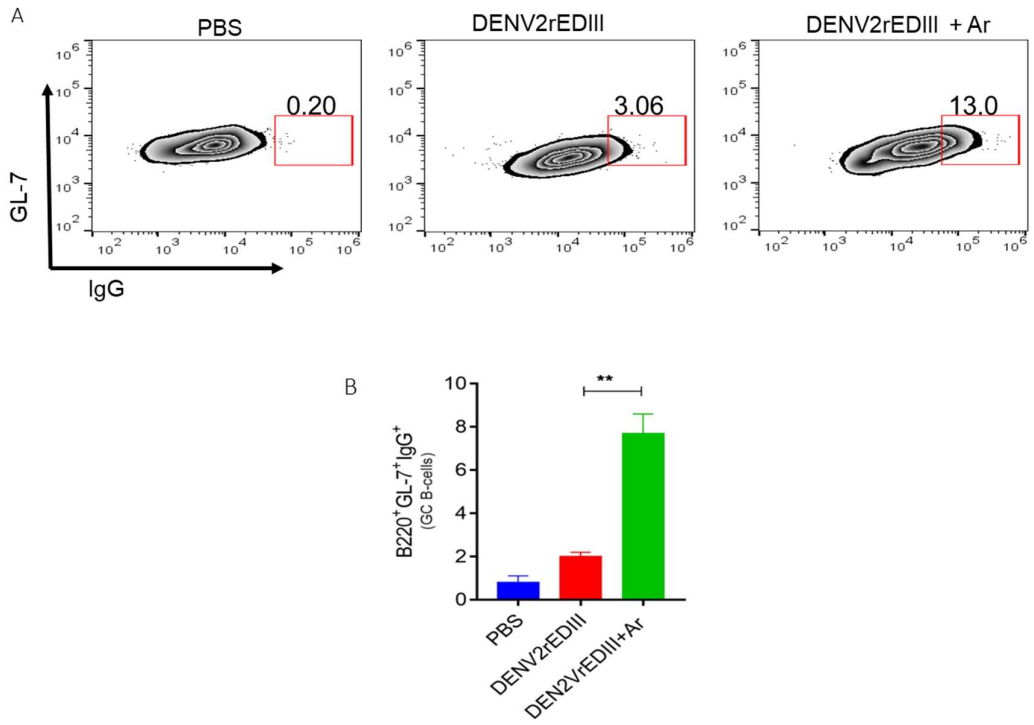


Figure 33: Arsenite-induced ISR activation drives the B-cell response towards the germinal centre pathway.

(A) Gating strategies for selecting GC-B cells (B) Analysis of the germinal centre formation by calculating the frequency of the triple-positive cells ($B220^+$, $GL7^+$ and IgG^+) using a flow cytometer. These triple-positive cells are considered GC-B cells. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, value $^{**}P \leq 0.005$ was considered significant.

4.2.3.5 Arsenite activates germinal centre formation in mice primed with arsenite and immunized with DENV2rEDIII

The germinal centre formation is also confirmed by the enhancement of the expression of genes (Figure 34) such as PAX5 (Revilla-i-Domingo et al., 2012) and BCOR1 (Yang et al., 2015) are master regulators of germinal centre B cells. IL17ra (Ding et al., 2013), IL18R1, IKZF1, IRF7, and XBP1 have roles in the proliferation, Survival and differentiation of GC B cells. While the IFNGR2 has a role in the differentiation of B cells. It suggests that the ISR pathway plays an essential role in the enhancement of antigen-specific antibody responses, such as neutralizing antibody IgG and programming the memory B cell formation.

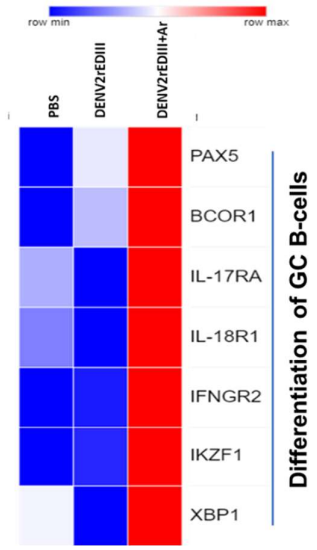


Figure 34: Arsenite pretreatment increases GC formation in DENV2rEDIII-immunized mice.

Gene expression analysis from lymph nodes by qRT-PCR study, where lymph node cells were restimulated for 24 hours with DENV2rEDIII protein. The heat maps showing statistically significant changes were created by analysing two independent replicates (5X2), and each group contain 5 animals.

4.2.4 tDENVrEDIII as a candidate antigen to assess the immunological responses in a mouse model system when administered with Arsenite

4.2.4.1 Expression and purification of the EDIII from the DENV 3 serotype

EDIII from the DENV3 serotype was purified and confirmed through western blot analysis and coomassie staining of SDS gels. It was found that the band size for EDIII of the DENV3 came to around 15 kDa (Figure 35 A and B). It was also confirmed by probing it with an anti-Histidine antibody by western blotting (Figure 35 C).

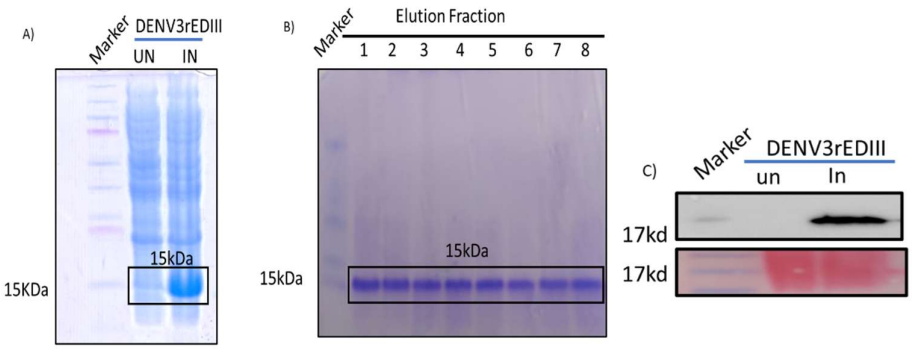


Figure 35: Expression and purification of the EDIII from the DENV 3 serotype

(A) Coomassie blue-stained SDS PAGE gel image of DENV3rEDIII protein overexpression in *E. coli* Rosetta (B) The DENV3rEDIII protein affinity chromatography elution fractions are shown in lanes 1 to 8. (C) Purified DENV3rEDIII protein probed with the anti-histidine antibody confirmed by Western blot.

4.2.4.2 Expression and purification of the EDIII from the DENV 4 serotype

EDIII from the DENV4 serotypes was purified from the DENV4 and confirmed through western blot analysis and coomassie staining of SDS gels. It was found that the band size for EDIII of the DENV4 came to about 14.5 kDa (Figure 36 A and B). It was also confirmed by probing it with an anti-Histidine antibody by western blotting (Figure 36 C).

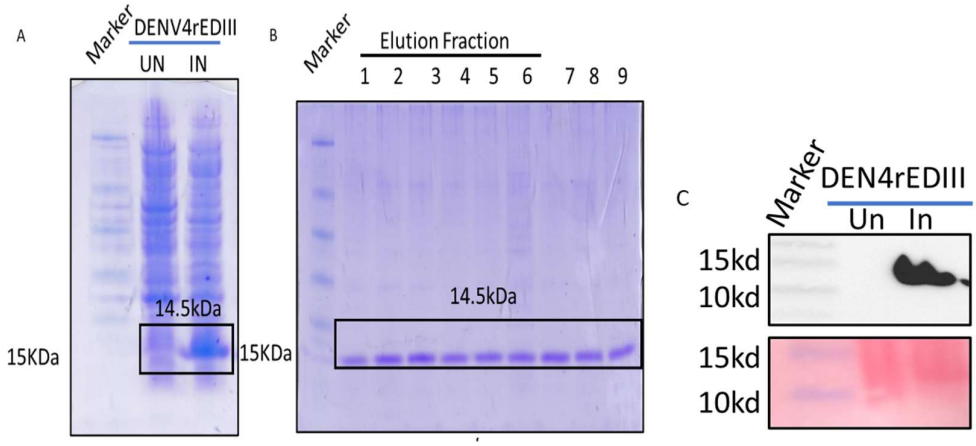


Figure 36: Expression and purification of the EDIII from the DENV4 serotype

(A) Coomassie blue-stained SDS PAGE gel image of DENV4rEDIII protein overexpression in *E. coli* Rosetta. (B) The DENV4rEDIII protein affinity chromatography elution fractions are shown in lanes 1 to 9. (C) Purified DENV4rEDIII protein probed with the anti-histidine antibody confirmed by Western blot analysis

4.2.4.3 Arsenite (Ar) enhances antigen-specific T-cell response

tDENVrEDIII was immunized subcutaneously after being primed with arsenite, and a group without antigen was kept as a control in which mice were immunized with PBS. We checked for the T cell response 42 days after the primary immunization in splenocytes restimulated with DENV2rEDIII (10µg/ml). Arsenite treatment was observed to enhance the expression of IFN-γ by $CD4^+$ (Figure 37 D) and $CD8^+$ T (Figure 37 E) cells, which are DENV2rEDIII-specific.

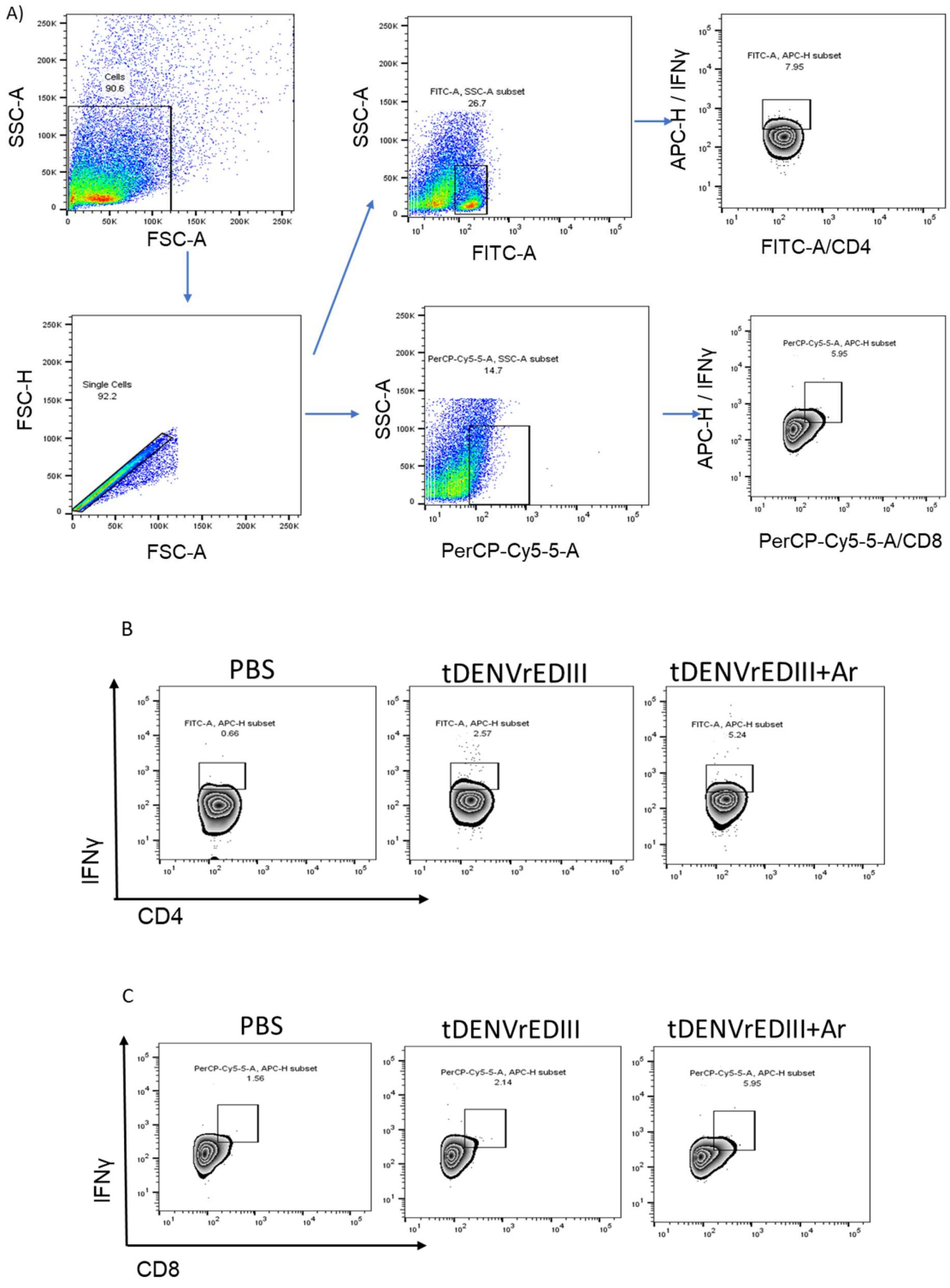


Figure 37 continued on the next page…….

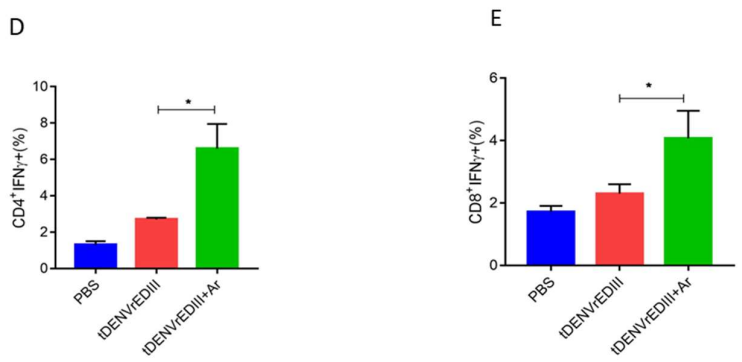


Figure 37: Arsenite (Ar) enhances antigen-specific T-cell response

(A) Gating strategies for choosing the $CD4^+$ and $CD8^+$ cells secreting IFNγ. The T-cell response was measured after 42 days of immunization by isolating the splenocytes from the immunized mice. (B and C) shows the FACS plot. (D and E) Graphs show the frequency of the $CD4^+$ and $CD8^+$ cells secreting IFNγ. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, value **$P \leq 0.005$ was considered significant.

4.2.4.4 Arsenite-induced ISR activation triggers enhanced antigen-specific antibody production

We observed that the $CD4^+$ specific antibody response against the mice immunized with the tDENVrEDIII antigen was confirmed by the formation of IgG and its subtypes IgG1, IgG2a and IgG2b antibodies in the serum after the 14, 28 and 42 days of immunization. It has been observed that the level of IgG and its subtypes IgG1, IgG2a and IgG2b is higher for mice group, which is immunized with tDENVrEDIII and primed with arsenite, compared to the mice group, which is only immunized with tDENVrEDIII. These results establish a link between the ISR pathway and antigen-specific antibody response. (Figure 38 A-I)

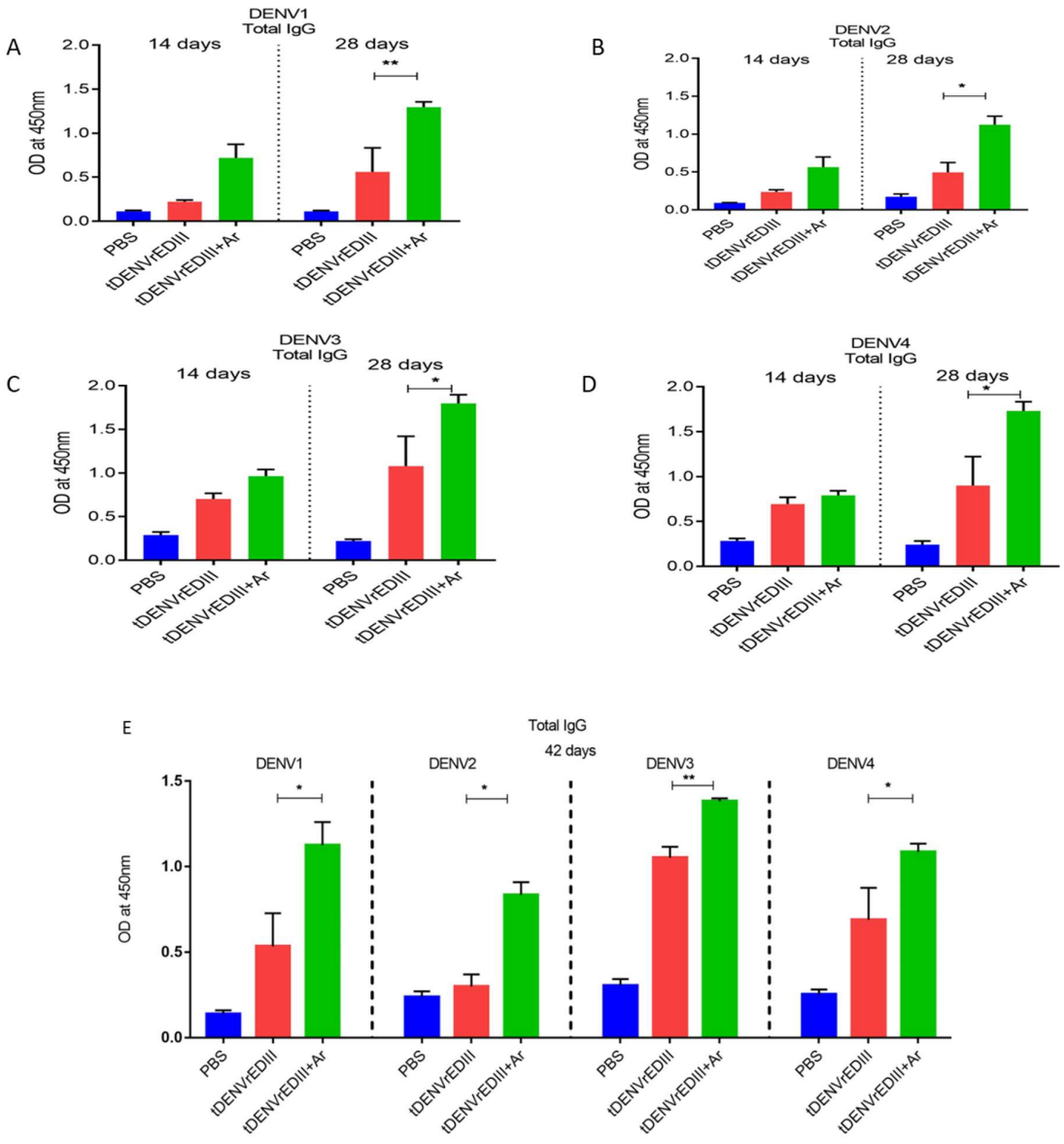


Figure 38 continued on the next page…….

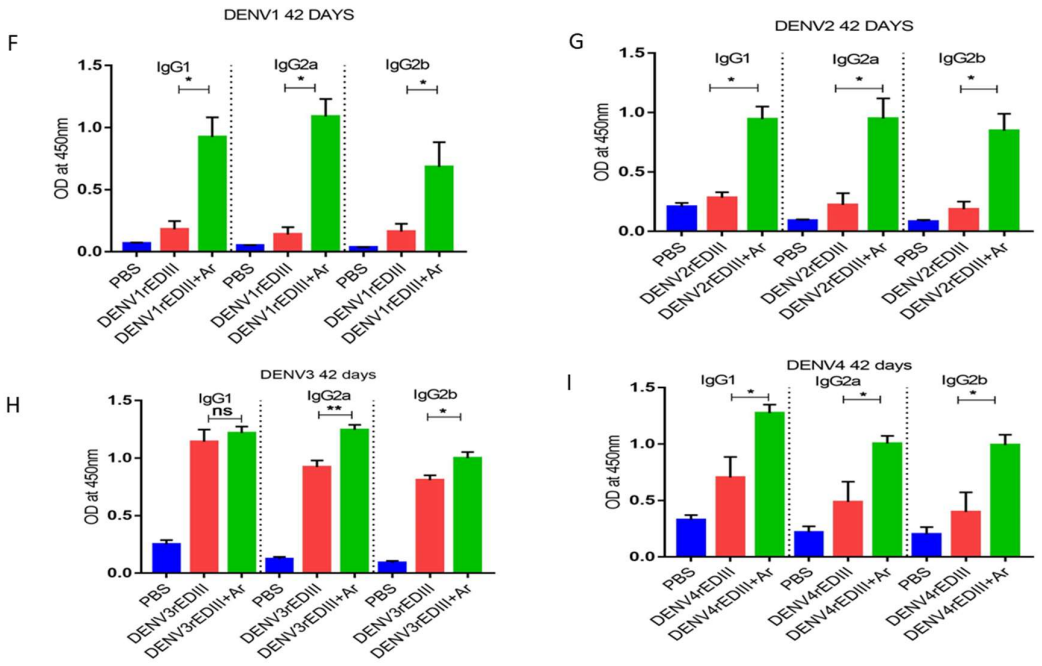


Figure 38: Arsenite-induced ISR activation triggers enhanced antigen-specific antibody production

Total IgG response compared for the 14 and 28 days against all four antigens of dengue virus: (A) DENV1, (B) DENV2, (C) DENV3 and (D) DENV4. (**E)** Total IgG response after 42 days of immunization against all four dengue virus antigens. **(F to I)** IgG1, IgG2a, and IgG2b response after the 42 days after immunization against all four antigens of dengue virus (F) DENV1 (G) DENV2 (H) DENV3 (I) DENV4. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, ns = not significant, value *P≤0.05, **P ≤ 0.005 was considered significant.

4.2.4.5 Arsenite promotes the development of the germinal centre in the lymph node

we examine the draining lymph node cells by staining them with germinal centre markers $B220^{+}$, $GL\text{-}7^{+}$, IgG^{+} and analysed by Flow cytometry. The percentage of ($B220^{+}GL\text{-}7^{+}IgG^{+}$) cells was found to be high in the group immunized with the tDENVrEDIII and primed with arsenite compared to the group only immunized with the antigen (Figure 39 C). The lymph node was isolated after 42 days by euthanizing after the Primary Immunization.

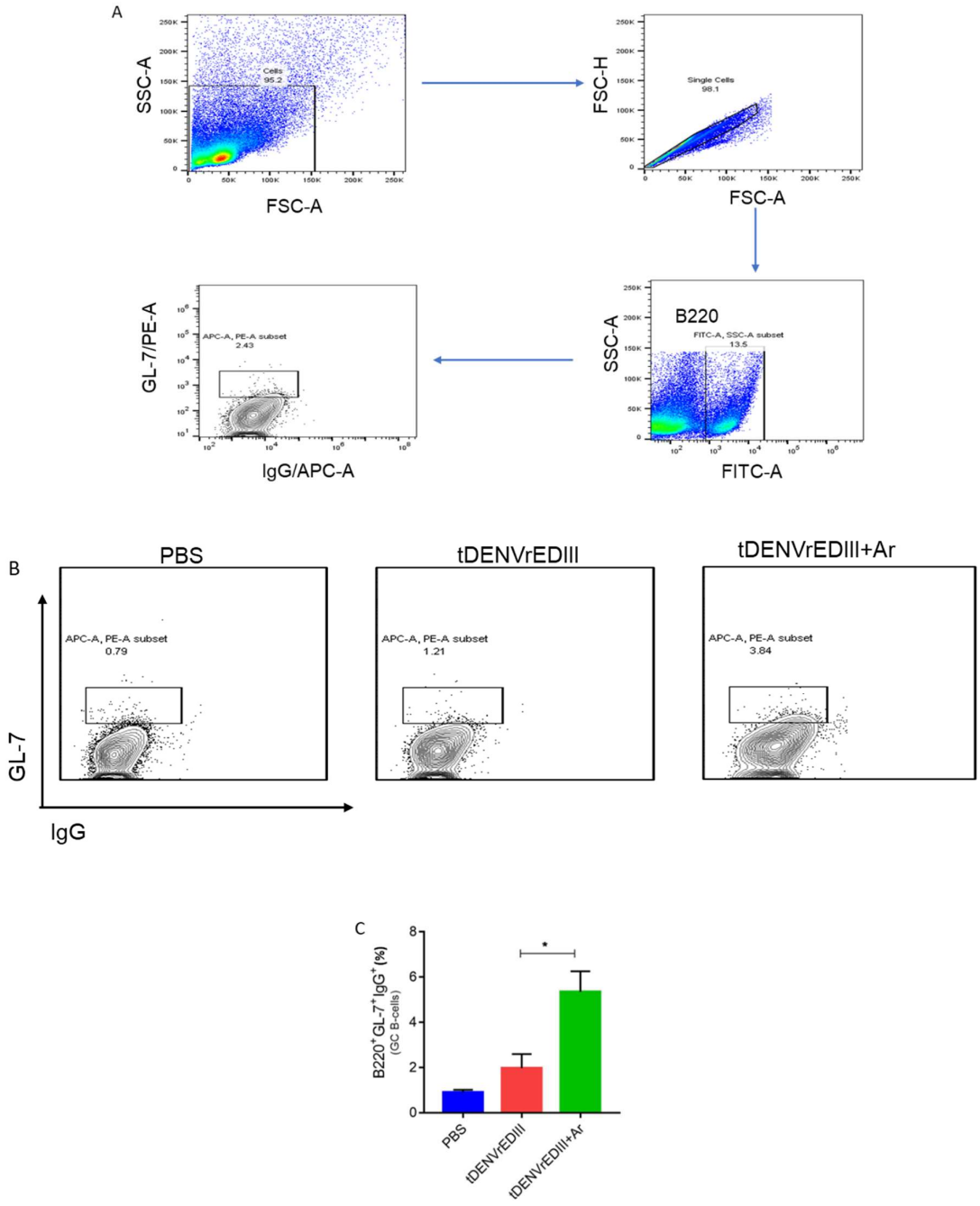


Figure 39: Arsenite promotes the development of the germinal centre in the lymph node
(A) Gating strategies for selecting GC-B cells. (B) Best representative FACS plot for the data of GC-B cells. (C) Analysis of the germinal centre formation by calculating the frequency of the triple-positive cells ($B220^{+}$, $GL7^{+}$ and IgG^{+}) using a flow cytometer. These triple-positive cells are considered GC-B cells. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, and value *P≤0.05 was considered significant.

4.2.4.6 Arsenite activates germinal centre formation in mice primed with arsenite and immunized with tDENVrEDIII

Arsenite enhances GC formation in mice immunized with tDENVrEDIII and primed with Arsenite (Figure 40 A). It was observed that mice primed with arsenite have more GC formation compared to those immunized with only PBS, while the tDENVrEDIII group don't have any GC formation.

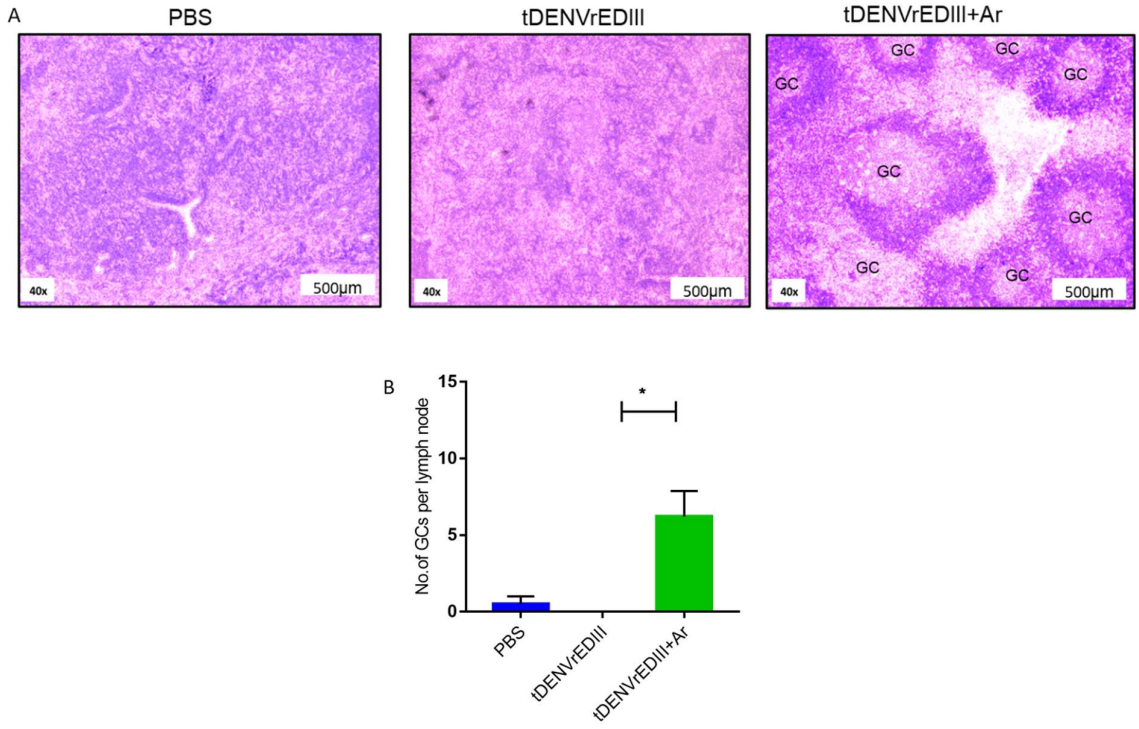


Figure 40: Ar enhances GC formation in mice immunized with tDENVrEDIII

(A) As indicated, lymph node tissue sections were stained with haematoxylin and eosin 42 days after mice were treated with arsenite and immunized with tDENVrEDIII protein. (B)The number of GCs per lymph node was calculated using 5 mice per group. The obtained data is displayed in terms of mean ± SEM of independent replicate (5X2); each group contains 5 animals. By employing the student t-test, statistical significance was calculated, and a value *P≤0.05 was considered significant.

4.3 Objective 3

To dissect the potency of ISR activator arsenite as a Vaccine adjuvant through virus neutralization assay using a Dengue Virus infectious model system.

4.3.1 *In-Vitro* Propagation of the Dengue serotype (1-4) in C6/36 cell line.

Serotypes of Dengue virus, i.e., DENV-1(Hawaii), DENV-2 (TR1751), DENV-3 (Thailand 1973) and DENV-4 (Columbia 1982), propagated successfully in Insect cell line C6/36.

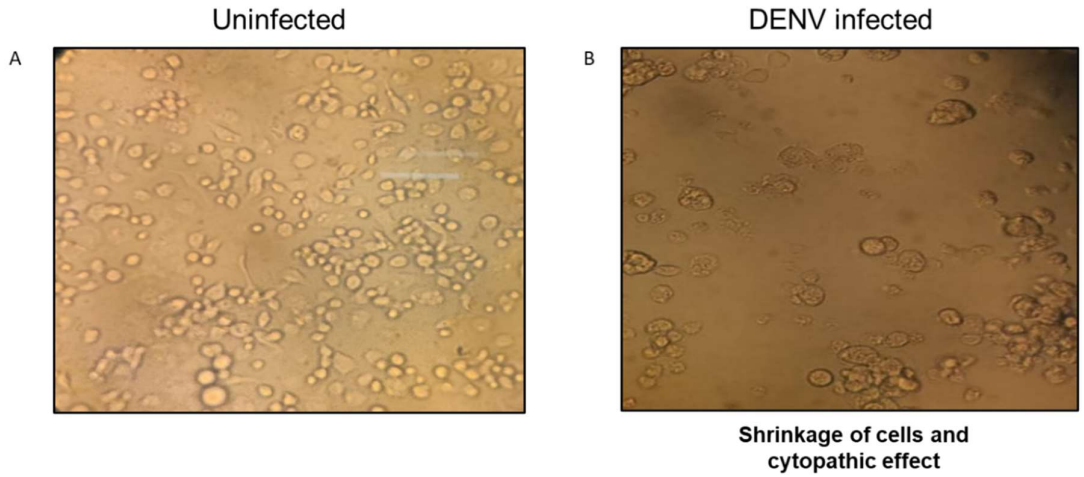


Figure 41: DENV propagation

(A) Uninfected C6/36 cells (B) On day five, a representative image of the CPE effect in DENV 1 infected C6/36 cells

4.3.2 Serotyping of the DENV serotypes (1-4) from the culture supernatant of the DENV-infected C6/36 cell line

First, RNA was isolated for all DENV serotypes, and cDNA was synthesized using the D2 primer. Now, cDNA is amplified Using the D1 forward and D2 reverse primer. Amplified cDNA was confirmed by the agarose gel electrophoresis at the band size observed at about 511 bp (Figure 42 A). The amplified product again undergoes PCR using the D1 forward and TS-specific primer, which gives the band size around 482 bp for the DENV1 and 119 bp for the DENV2, respectively 290 bp for the DENV3 and 392 bp for the DENV4 (Figure 42 B).

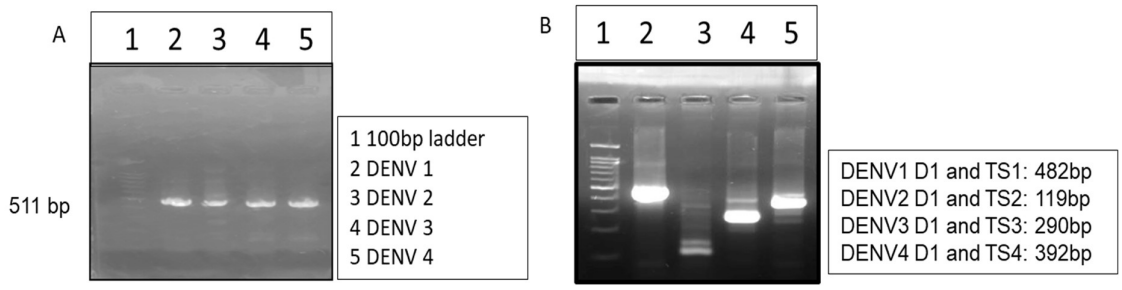


Figure 42: Serotyping of DENV

(A) Gel picture of a 511 bp amplicon generated by semiquantitative RT-PCR. (B) The amplified results of nested PCR employing serotype-specific primers (TS1, TS2, TS3, and TS4) for DENV1, DENV2, DENV3 and DENV4, respectively, shown by an agarose gel.

4.3.3 Arsenite activated ISR pathway augments neutralizing antibodies production which effectively neutralizes all the serotypes of dengue Virus

Virus-neutralizing antibodies are necessary to know the efficacy of vaccine candidates as they are involved in the protective arm of immunity. The virus neutralization antibody is calculated by the FNT_{50} (Khan et al., 2022). Facs neutralization titer (FNT_{50}) is the serum dilution capable of a 50% decrease in DENV-infected cells compared to the control infection performed without any immune serum (Shukla et al., 2018). It has been observed that the mice group immunized with the tDENVrEDIII and primed with arsenite inhibits the virus infectivity more than the mice group, which is immunized with only the tDENVrEDIII antigen. Virus infectivity is expressed in the % normalized infection (Figure 43 A and B) and FNT50 (Figure 43 C and D). These results suggested that activation of ISR pathway-driven B cell response depends on the T cell. That produces neutralizing antibodies that help to neutralize the DENV3 and DENV4 viruses.

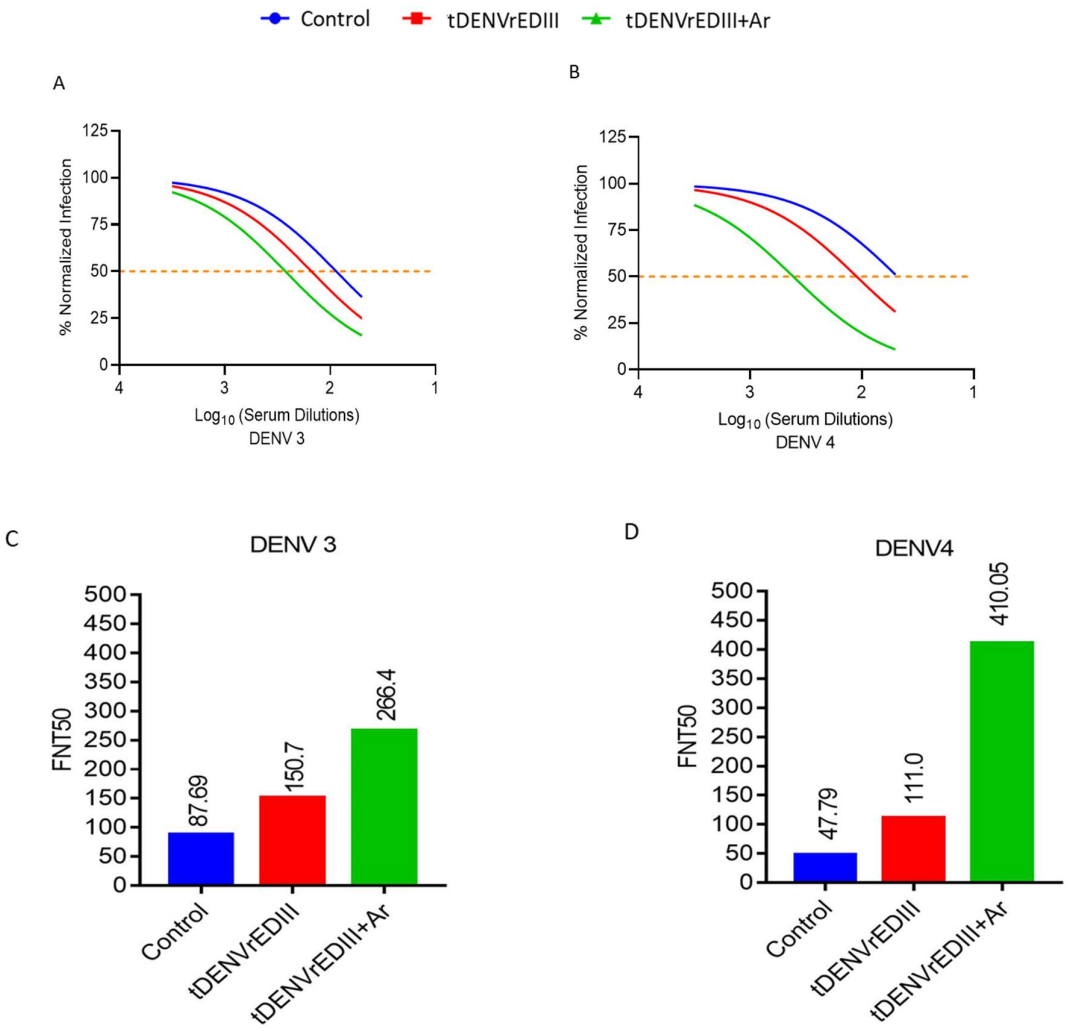


Figure 43: Arsenite activates the ISR pathway that augments neutralizing antibodies and effectively neutralizes DENV serotypes.

The FNT was used to test the *in vitro* virus neutralization capability of vaccinated serum against two serotypes of dengue virus DENV3 (A) and DENV4 (B). By diluting serum until 50% of the virus was neutralized, the FNT50 calculated for all immunized serum was assessed using the FACS neutralization test for DENV3 (C) and DENV4 (D).

5. DISCUSSION

Discussion

Vaccines have significantly lowered the prevalence of infectious diseases. Vaccination campaigns are thought to save more than 3 million lives annually around the globe (Shaw & Feinberg, 2008). A vaccine is made up of different components, such as antigens and adjuvants. Adjuvants are vaccine ingredients that activates, expand, and prolong the immunological response (Pulendran et al., 2021). Besides the advantages, adjuvant also has some disadvantages, thereby there is a requirement for new adjuvants with better immunomodulatory properties and are capable for eliciting a high antigen-specific antibody production, strong immunological memory response, thereby making programming antigen more potent (Bonam et al., 2017). Several strategies are being implemented to develop a new class of adjuvant including understanding the immunobiology of most successful vaccines, such as YF17D (T. D. Querec et al., 2009). YF17D vaccine is the most successful vaccine ever made. However, the mechanism through which the YF17D vaccine works is not studied comprehensively. However, system biology approaches found that it works through the mechanism of the ISR pathway (T. D. Querec et al., 2009).

Immune cells activate the ISR pathway upon stress signals to programme their cellular machinery, thereby modulating various immune signals (Naz et al., 2019). The phosphorylation of the alpha subunit of eIF2 on serine is the site of convergence for all stress events that activate ISR (Ron, 2002b). Having observed that YF17D function through the activation of ISR pathway, tempted us to hypothesize that the small molecule that activate the ISR pathway could be a potential new class of adjuvants. Earlier, it has been shown that Arsenite is a potent activator of ISR pathway. With this observation, we examined whether arsenite enhances the phosphorylation of the alpha subunit of eIF2-alpha in immune cells such as macrophages. Our findings categorically demonstrate that indeed, it enhances the phosphorylation of the eIF2 in J774A.1 macrophages. It has been well reported that Phosphorylation of eIF2-alpha results in blockage of polysomes assembly and recruitment of RBPs at the 3’ UTR of mRNAs transcripts. The mRNA-binding proteins such as TIA-1 and TIAR can aggregate together within granules in response to specific stresses (Gottschald et al., 2010). We checked the formation of the stress granules with TIA-1 and TIAR protein after treatment of the arsenite on macrophages. It confirmed that arsenite enhances the formation of stress granules, confirmed by the mRNA binding protein TIA-1 and TIAR, which signifies post-transcriptional shuttling of various mRNA to regulate gene expression landscape. Further, we investigated whether arsenite-induced stress affects the macrophage's innate effector functions, which eventually shape the adaptive immune response (Deets & Vance, 2021).

Innate and adaptive immune system work cooperatively to mount effective immune response with innate as the first line defense which determine the fate of adaptive immunity by delivering various signals. Predominantly innate cells deliver three signals that that shapes the quality and magnitude of adaptive immune response. The three signals delivered from the APCs to naive T cells are as follows; 1) Antigen processing and presentation by APCs and delivering Cognate MHC-TCR complex signal; 2) Costimulatory signal and 3) effector molecules such as cytokines, chemokines, ROS/RNS etc. Therefore, we examined the antigen uptake, processing and modulation in the effector molecules, such as the ROS/RNS and cytokines, during ISR activation by arsenite. The fundamental components of adaptive immunity are antigen processing and presentation (Pishesha et al., 2022). Keeping this in mind, we analyzed antigen uptake; it was found that macrophages treated with arsenite enhance antigen uptake as the concentration of arsenite increased at 37^0C, clearly indicating the involvement of the active cellular uptake process (Ravindran et al., 2014b). We checked for the antigen presentation with the expression of the MHC I molecule on the macrophages; it was found that macrophages treated with the arsenite show dose-dependent enhancement in the percentage of the MHC-I expression in the macrophages. T- and B-cell antigen-specific receptor signals are necessary to activate adaptive immunological responses. In addition to this antigen-specific signal, activation of naive T lymphocytes requires a second antigen-independent nonspecific signal known as a costimulatory signal. Costimulatory signaling is primarily triggered by interactions between the T-cell integral membrane proteins and their related ligands on the APC. One such signal is the CD86 or B7-2, which interacts with the CD28 of the T-cell (Mir, 2015). Here, we observed a dose-dependent increase in the expression of the costimulatory molecule CD86. We observed that ISR activation programs signal 1 and 2 for proper cell-mediated adaptive immune responses. Next, we examined signal 3, such as the levels of ROS/RNS and cytokines that dictate T cells' effector commitment upon ISR activation in the macrophages. ROS has antibacterial properties and is linked to the rise of inflammatory disorders through increased biological structures and proinflammatory cytokines. However, new research has shown that ROS may also function as an immune system regulator (Tavassolifar et al., 2020). ROS production decreases in the macrophages treated with arsenite and induced with LPS. Proinflammatory cytokines are involved in the up-regulation of inflammatory processes and are primarily produced by activated macrophages (J.-M. Zhang & An, 2007b). TNF-α, IL-6 and IL-1β levels decrease in arsenite-treated and LPS-induced macrophages. The impact of arsenite on LPS-induced NO production in J774A.1 macrophage cells imply an immunomodulatory effect of ISR activation. The above results indicate that ISR activator

arsenite modulates macrophage innate effector functions. It could shape the quality and magnitude of T and B cell responses and have an adjuvanting property. Next, we checked the immunomodulatory role of arsenite in the *in-vivo* mouse model.

First, we checked the systemic toxicity of the arsenite up to 42 days, and it has been observed no significant difference in body weight; therefore, it is not toxic to the mice. Mice were immunized with the model antigen Ova to check the T and B cell response. The T-cell response was analyzed through IFNγ secreting $CD4^+$ and $CD8^+$ cells (Afroz et al., 2017). It has been observed that mice primed with arsenite have a higher percentage of the IFNγ secreting $CD4^+$ and $CD8^+$ cells than the only Ova and only arsenite group. This indicates that arsenite enhances antigen-specific polyfunctional T-cell response.

Antibody response is checked by the IgG and IgG subtypes (Afroz et al., 2019). It has been observed that mice primed with arsenite and immunized with the Ovalbumin antigen (OVA) have a higher antibody response as compared to the mice immunized with only Ova and only primed with the arsenite. We also checked for germinal centre formation. A specialised substructure called the germinal centre (GC), which develops in secondary lymphoid tissues, produces memory B cells and long-lived antibody-secreting plasma cells that can defend against re-infection. B cell clones that bind antigens with high affinity are developed by somatic hypermutations occurring within the GC in the genes that encode their B cell receptors (Stebegg et al., 2018b). The formation of germinal centres is analysed by the frequency of GC-B cells ($B220^+$ $GL7^+$ IgG^+) in the draining lymph nodes (Afroz et al., 2019). It has been observed that mice primed with arsenite and immunized with Ova have higher germinal centre formation than those immunized with only Ova and primed only with arsenite. The histology of lymph nodes by H & E staining also confirmed that the germinal centre formation occurred only in the group immunized with Ova and primed with arsenite. It affirms that arsenite-induced ISR activation drives the B-cell response towards the germinal centre pathway.

After confirming the immunomodulatory effect of the arsenite on the Ova antigen, we further studied Domain III of the envelope protein of the dengue virus. Dengue is regarded by the World Health Organization (WHO) as a serious worldwide public health threat in tropics and subtropics countries. There are 2.5 billion people who live in dengue-endemic areas, with 400 million infections occurring yearly and a fatality rate that exceeds 5-20% in some places (Hasan et al., 2016b). The RNA virus of the Flaviviridae family, which causes dengue, is transmitted by *Aedes* mosquitoes (Hasan et al., 2016b). The E protein of the dengue virus has an important role; it fuses with the endosomal membranes upon entrance and binds to the cellular receptor to cause viral attachment. Envelope protein has three domains: I, II and III (Crill & Roehrig,

2001). Type-specific and subcomplex-specific epitopes that are major neutralizing determinants are found in domain III (Roehrig, 2003). It has also been observed that recombinant E domain III proteins reduce the infectiousness of viruses (Chin et al., 2007).

The mice group immunized with DENV1rEDIII antigen or DENV2rEDIII antigen and primed with arsenite had a higher antigen-specific T cell response than the mice group, which immunized only with the DENV1rEDIII antigen or DENV2rEDIII antigen. Activation of T cells is required for the more specific antibody response against the antigen (Kasturi et al., 2011) (Haring et al., 2006). Successful immunization creates a protective humoral immunity dependent on generating high-affinity antibodies which have a long life (Lynch et al., 2014). The Mice immunized with DENV1rEDIII or DENV2rEDIII protein and primed with arsenite have higher antibody response than those immunized with only DENV1rEDIII or DENV2rEDIII protein. T-cell activation encourages the synthesis of GC in response to an antigenic challenge, which stimulates the generation of specific antibodies (C. Chen et al., 2018). Our results show that arsenite-induced ISR activation drives the B-cell response towards the germinal centre pathway. Mice group primed with arsenite and immunized with the DENV1rEDIII or DENV2rEDIII protein have a higher percentage of GC B cells than mice immunized only with DENV1rEDIII or DENV2rEDIII protein.

We observed that the ISR activator programs the B and T cell response against DENV1rEDIII and DENV2rEDIII antigens, thereby suggesting the adjuvant function of arsenite. Next, we asked whether arsenite could be used as an adjuvant for developing a tetravalent vaccine formulation against dengue since an effective dengue vaccine should confer a balanced response against all the serotypes of the dengue virus. As of now, a single dengue vaccine has been licensed, Dengvaxia® (CYD-TDV), developed by Sanofi Pasteur with restricted usage (Izmirly et al., 2020). Dengvaxia® has Lower efficacy with dengue 1 and 2 than with dengue 3 and 4 (Tully & Griffiths, 2021). An imbalanced immune response against dengue causes the antigen-dependent enhancement of infection (ADE). Therefore, developing a tetravalent vaccine that induces an effective immune response against all four serotypes is important. Henceforth, mice were primed with arsenite and immunized with tDENVrEDIII protein, which showed a higher percentage of the IFN-γ secreting $CD4^+$ and $CD8^+$ cells. Arsenite has also programmed the magnitude and durability of antigen-specific antibody response against each antigen when immunized in a tetravalent combination. Arsenite with tetravalent antigens demonstrated the activation of the germinal centre that enhances GC-B cells frequency, which might be instrumental in producing high affinity neutralizing antibodies. The enhanced levels of neutralizing antibodies generated during ISR activation using arsenite manifest robust

neutralizing capabilities. Serum obtained from mice primed with ISR activator arsenite can neutralize Dengue virus serotypes effectively, thereby suggesting that it could be a potential vaccine adjuvant.

In conclusion, ISR activator sodium meta arsenite affects the innate effector function of antigen-presenting cells by modulating the antigen uptake and presentation, co-stimulation, and effector cytokines. These innate signals are pivotal for the effective priming of adaptive arms of immunity. We demonstrated that arsenite induces effective antigen-specific polyfunctional T-cell response followed by a strong B-cell response against model Ova antigen. Furthermore, arsenite priming also induces a strong cell-mediated and humoral immunity against individual EDIII dengue antigens, and it shows a similar response against tetravalent EDIII antigen. Notably, arsenite priming induces a significant germinal centre reaction, which is important for the induction of high-affinity antibody and memory B-cell formation for durable immune responses. Finally, arsenite priming generates neutralizing antibody against dengue viruses, which is a correlate of protective immunity. In conclusion the findings of the study highlights the criticality of ISR pathway in programming the quality and magnitude of antigen specific protective immune responses which might open an avenue in developing an ISR activator as a potential vaccine adjuvant, which could overcome some of the current limitations of subunit vaccine.

6. SUMMARY

Summary

Vaccinology has been one of the greatest triumphs of modern medicine with enormous advancement in the area of vaccine discovery, delivery and development platforms. A vaccine comprises different components, such as active ingredients and adjuvants. Active ingredient consists of the harmless form of the pathogen, while the adjuvant acts as an activator of immune response. The vaccine with adjuvant provides a more specific and effective immune response (Pasquale et al., 2015). As the new vaccine develops, new adjuvants with less side effects are always required. A new class of adjuvant having good immunomodulatory capacity can be achieved by studying deeper into the immunological mechanisms triggered during various stimulations including vaccination. Recently multiparametric high through put system biological tools were implemented to dissect the mechanism of yellow fever (YF17D) vaccination. YF17D is a live attenuated one the most successful and effective vaccine ever developed, but the mechanism was not known until recent studies involving s system biology approaches which categorically demonstrated that it works through the activation of the integrated stress response pathway (ISR), which in turn programs various cellular processes such as autophagy to enhance antigen presentation and adaptive immunity (Ravindran et al., 2014a)

The major ISR event includes eIF2 phosphorylation upon sensing the particular stress via eIF2 kinases which in turn dictates the cellular proteome through shutdown of global mRNA translation while increasing the expression of a specific group of genes required to circumvent the particular stress (Derisbourg et al., 2021). We checked for the phosphorylation of eIF2α. Arsenite enhances the phosphorylation of eIF2α as the concentration of arsenite increases, which is confirmed by the western blot analysis. Arsenite also leads to the stress granules formation in macrophages, confirmed through the mRNA binding protein TIA1 and TIAR. TIA1 and TIAR proteins form stress granules (Ganguly et al., 2016); therefore, they could modulate various immune effector pathways. For optimal adaptive immune response, macrophages give three different types of signals to the naïve T cells (Mak & Saunders, 2006). Activation of the ISR pathway using arsenite enhances antigen uptake and MHC expression, thereby suggesting the role of ISR activation in the priming of Signal 1, i.e., MHC, during T cell activation. In addition to signal 1, ISR activation triggers enhanced levels of costimulatory signals (signal 2), which is critical for shaping adaptive immune responses. Apart from enhanced co-stimulation, antigen uptake and MHC expression, it tailors the other innate effector molecules such as ROS, RNS and proinflammatory cytokines (Signal 3). The above

results categorically manifest that ISR activator arsenite modulates macrophage innate effector function.

To examine the adjuvanting function of ISR activator arsenite in programming the magnitude of antigen-specific immune responses against various antigens upon immunization in a mouse model. First, we immunize the mice with Ova antigen and observed the T cell response by $CD4^+$ and $CD8^+$ T cells secreting the IFNγ. It has been observed that mice immunized with Ova and primed with arsenite have a higher T cell response than those immunized only with Ova and only primed with arsenite. Arsenite also enhances antibody-specific immune response, which is analysed by the IgG and IgG subtypes, i.e., IgG1, IgG2a, and IgG2b. We also checked for the germinal Centre formation in the mice; it has been observed that arsenite enhances the germinal Centre formation by activating the ISR pathway. We observed that ISR activation triggers strong Ova-specific T and B cell responses. Next, we checked for the EDIII antigen from the dengue virus.

The mosquito *Aedes aegypti* frequently spreads an arboviral illness called dengue and is hyperendemic in tropical and subtropical climates worldwide. As a result of recent increases in incidence, almost half of the world's population is currently at risk. (Kularatne & Dalugama, 2022). Mature DENV virions are made up of three essential proteins: capsid (C), membrane (M), and envelope (E). The E protein has three different structural domains and is 53 kDa in size (Norazharuddin & Lai, 2018). The most significant properties of E protein in vaccines are associated with domain III (EDIII). This domain appears to be involved in binding host cell receptors for viral entrance; therefore, it could be an optimal vaccine candidate to target and induce long-lasting protective immunity against dengue virus infection. (Fahimi et al., 2014).

EDIIII antigen from the DENV1/DENV2 purified and immunized in mice. The T cell response was checked after immunization. The group primed with arsenite had a higher poly functional T-cell response as compared to the mice group, which was not primed with arsenite. After confirmation of the T cell response, the B cell response was analyzed, which found that arsenite helped to enhance antigen-specific B cell response, which is confirmed through enhancement of the level of the total IgG and its subtypes such as IgG1, IgG2a and IgG2b antibodies. Arsenite also drives the B cell response through the germinal centre pathway in mice immunized with DENV1rEDIII/ DENV2rEDII antigen.

After immunizing the mice individually with DENV1rEDIII and DENV2rEDIII antigens, we further immunized mice with the tetravalent EDIII antigen. We purified the DENV3rEDIII and DENV4rEDIII antigens and confirmed them through the western blot. Forty-two days after immunization, checked for the T cell response, and it was found that mice immunized with the

tDENVrEDIII antigen and primed with arsenite had a higher antigen-specific T cell response as compared to mice immunized only with the tDENVrEDIII antigen. Arsenite-induced ISR activation triggers enhanced antigen-specific antibody production. Arsenite-induced ISR activation directs the B-cell response to the germinal centre of the lymph node. The enhanced frequency of GC-B-cells ($B220^{+}GL\text{-}7^{+}IgG^{+}$) in the arsenite-primed groups substantiates the germinal centre reaction.

We used an *in-vitro* Dengue virus infectious model system to dissect the potency of ISR activator arsenite as a vaccine adjuvant through virus neutralization assay. The enhanced levels of neutralizing antibodies generated during ISR activation using arsenite manifest robust neutralizing capabilities. Serum obtained from mice primed with ISR activator arsenite can neutralize Dengue virus serotypes effectively, thereby suggesting that it could be a potential vaccine adjuvant. We proposed that arsenite-induced ISR activation effectively programmed the magnitude and quality of immune response against different antigens; therefore, it could have possible applications in vaccinology as a vaccine adjuvant for the formulation of new-generation vaccines against emerging and re-emerging diseases.

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8. PUBLICATIONS

VACCINES

Amino acid starvation enhances vaccine efficacy by augmenting neutralizing antibody production

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Specific reduction in the intake of proteins or amino acids (AAs) offers enormous health benefits, including increased life span, protection against age-associated disorders, and improved metabolic fitness and immunity. Cells respond to conditions of AA starvation by activating the amino acid starvation response (AAR). Here, we showed that mimicking AAR with halofuginone (HF) enhanced the magnitude and affinity of neutralizing, antigen-specific antibody responses in mice immunized with dengue virus envelope domain III protein (DENVrEDIII), a potent vaccine candidate against DENV. HF enhanced the formation of germinal centers (GCs) and increased the production of the cytokine IL-10 in the secondary lymphoid organs of vaccinated mice. Furthermore, HF promoted the transcription of genes associated with memory B cell formation and maintenance and maturation of GCs in the draining lymph nodes of vaccinated mice. The increased abundance of IL-10 in HF-preconditioned mice correlated with enhanced GC responses and may promote the establishment of long-lived plasma cells that secrete antigen-specific, high-affinity antibodies. Thus, these data suggest that mimetics of AA starvation could provide an alternative strategy to augment the efficacy of vaccines against dengue and other infectious diseases.

INTRODUCTION

Metabolic regulation of the immune system is evolutionarily conserved and paramount for overall organismal health (*1*, *2*). Nutrient uptake and nutritional status affect immune cell proliferation, differentiation, and survival (*3*). In this regard, epidemiological and clinical studies indicate that malnutrition impairs host immune responses and increases rates of morbidity and mortality after infection (*4*, *5*). Similarly, reduced intake of nutrients without malnutrition, often termed as caloric or dietary restriction (CR or DR), improves metabolic fitness and longevity (*6*) and provides enormous benefits against age-associated disorders such as neurological, cardiovascular, and skeletal problems (*7*, *8*). Exceptions include prolonged energy restriction in small animals, which can reduce growth and development of lymphoid organs and impair antigen-specific immune responses (*9*, *10*). The benefits of CR during viral infections are largely dependent on body weight and may vary between different animals (*7*, *11*–*13*). Small and aged animals subjected to long-term CR are more susceptible to influenza infection because of continuous body mass loss and accompanying energy deficits that impair recovery, despite increased splenocyte proliferation (*11*). However, short-term refeeding after energy restriction in mice restores body weight and fat composition, as well as natural killer cell function required to combat influenza infection (*12*). Emerging evidence suggests that CR has a beneficial impact on various attributes of the immune system. These include enhancing thymopoiesis and maintenance of T cell diversity (*14*), natural killer and $CD4^+$ and $CD8^+$ T cell activity (*15*–*17*), and the apoptotic clearance of senescent T cells in aged mice (*16*, *18*, *19*). Furthermore, CR stimulates adaptive immunity against parasitic infection in experimental cerebral malaria (*20*).

Although the associated benefits of CR are mostly linked to reduced calorie intake, accumulating evidence couples dietary amino acid (AA) restriction to the benefits of CR (*8*). For instance, AA sensing pathways influence both innate and adaptive immunity (*21*). Innate immune cells are auxotrophs for AAs and sense the availability of extracellular AAs through their intrinsic metabolic sensing pathway (*8*, *22*). AA depletion results in the accumulation of uncharged transfer RNAs (tRNAs), which are sensed by general control nonderepressible-2 (GCN2) kinase (*23*) and trigger its activation. Activated GCN2 phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which results in the activation of amino acid starvation response (AAR) pathway that coordinates posttranscriptional and translational immune reprogramming (*24*, *25*). The AAR can also be activated by halofuginone (HF), a derivative of the Chinese herb *Dichroa febrifuga*, which creates a pool of uncharged tRNAs by inhibiting prolyl-tRNA synthetase (*25*), mimicking the effects of AA starvation (*26*). HF has garnered substantial attention in the last two decades owing to its therapeutic potential in various diseases including autoimmune disease, cancer, muscular dystrophy, and hepatic and renal ischemia-reperfusion injury (*25*, *27*–*31*).

Activation of the AAR using HF or amino acid–restricted diet abrogates the production of interleukin-1β (IL-1β) through the GCN2 pathway and provides protection against intestinal inflammation in an experimental mouse model (*24*, *32*). Furthermore, HF inhibits T helper 17 cell differentiation and provides protection against experimental autoimmune encephalomyelitis (*33*). In addition, a molecular signature composed of GCN2 correlates with the $CD8^+$ T cell response to the yellow fever vaccine–17D (YF17D) (*34*, *35*). Furthermore, the GCN2 pathway is crucial for optimal proliferation in response to antigen stimulation in vitro and for the appropriate trafficking of $CD8^+$ T cells to lymphoid organs (*36*). These findings raise the intriguing possibility that the activation of the AAR pathway with HF could enhance the immunogenicity of vaccine antigens.

Although rates of dengue virus (DENV) infection are increasing, there is still no licensed vaccine to date that is effective against all DENV serotypes (*37*). Therefore, there is an urgent need to identify strategies that can enhance the neutralizing antibody response to all the four serotypes of DENV and promote long-term vaccine protection. We used the recombinant envelope protein domain III (EDIII) of DENV as a model vaccine antigen in our study because it is

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9. CONFERENCES

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International Conference On

"Biology And Therapeutics of HIV & Associated Infections"

CERTIFICATE OF PARTICIPATION

This is to certify that Prof//Dr/Mr/Ms Matin Shaikh

participated / ~~delivered invited talk/oral talk~~/presented poster in UH-CDFD joint ***"International Conference On Biology And Therapeutics of HIV & Associated Infections"*** *held from 19th to 21st January 2019 at Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, 500046.*

Prof. Anand K. Kondapi
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International Vaccine Conference
(2017)

This is to certify that .....*Matin Rahim Shaikh*.....of University of Hyderabad, ..Hyderabad... participated and presented a poster in the International Vaccine conference held at ICGEB, New Delhi, from 27th November-29th November, 2017

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*This is to certify that Mr Shaikh Matin Rahim has given oral presentation entitled "**Programming the immunogenicity of vaccine antigens through the activation of Integrated Stress Response Pathway**" in the symposium "**BioAnveshana: Frontiers in Biotechnology and Bioinformatics**" organized by Department of Biotechnology and Bioinformatics (DoBB), School of Life Sciences, University of Hyderabad.*

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Role of Integrated Stress Response (ISR) pathway in programming the immunogenicity of antigens: An approach to develop a novel vaccine adjuvant

by Shaikh Matin

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