Studying the potential of Poly(I:C) and R848 in generation of B cell response and T cells to protein antigen

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IN
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AHMEDABAD

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NIRMA UNIVERSITY
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NAAC ACCREDITED 'A+' GRADE

CERTIFICATE

This is to certify that the thesis entitled ***"Studying the potential of Poly(I:C) and R848 in generation of B cell response and T cells to protein antigen"*** submitted to the Institute of Science, Nirma University in partial fulfilment of the requirement for the award of degree of M.Sc. (Biochemistry / Biotechnology), is a faithful record of bonafied research work carried out by **Hirva Fultariya (20MBC003), Mayurdhvajsinh Jadeja (20MBT017), Mili Mehta (20MBT028), Nishchay Bhatt (20MBC007) and Sakshi Trivedi (20MBT056)** under the guidance of **Prof. Sarat K. Dalai**. No part of the thesis has been submitted for any other degree or diploma.

Prof. Sarat K. Dalai
(Dissertation guide)

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Prof. Sarat K. Dalai
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Director
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Declaration

We, hereby declare that the dissertation work entitled **"*Studying the potential of Poly(I:C) and R848 in generation of B cell response & T cells to protein antigen*"** submitted to Institute of Science, Nirma University, is a record of the original work done by us under the guidance of **Prof. Sarat K. Dalai**. The work incorporated in the thesis has not been submitted for the award of any other degree, diploma, associateship, fellowship, titles in this or any other University or other institution of higher learning. We further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: 9th MAY 2022
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List of abbreviations

ACK RBC Lysis buffer: Ammonium-Chloride-Potassium Red Blood Cells Lysis buffer

Amp: Adenylsuccinate lyase

APC: Antigen presenting cells

BD: Becton, Dickinson and Company

CD: Cluster of Differentiation

CMI: Cell mediated immunity

CP: Circum-sporozoite protein and Poly(I:C)

CPCSEA: The Committee for the Purpose of Control and Supervision of Experiments on Animals

CPR: Circum-sporozoite protein, Poly (I:C) and R848

CR: Circum-sporozoite protein and R848

CSP: Circum-sporozoite protein

CTLs: Cytotoxic T Lymphocytes

DCs: Dendritic cells

DMEM: Dulbecco's Modified Eagle Medium

DOTAP: 1,2-dioleoyl-3-trimethylammonium propane

dsRNA: Double stranded ribonucleic acid

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FMDV: Foot-and-mouth disease virus

GST: Glutathione S-Transferase

HBSS: Hank’s balanced salt solution

IAEC: Institutional Animal Ethical Committee

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL: Interleukin

MDA5: Melanoma differentiation-associated gene 5

MHC: Major histocompatibility complex

MPL: Mono-phosphoryl lipid A

NK Cells: Natural Killer Cells

nm: Nanometre

PAMPs: Pathogen-associated molecular pattern(s)

PBS: Phosphate buffer saline

Poly (I:C): Polyinosinic-polycytidylic acid

PRR: Pattern recognition receptors

R848: Resiquimod

rCSP: recombinant Circum-sporozoite Protein

RIG 1: Retinoic acid-inducible gene 1

RPM: Revolutions per minute

RPMI: Roswell Park Memorial Institute Medium

SLTRiP: Sporozoite, liver stage tryptophan-rich protein

T_{CM}: Central memory T Cells

T_{EM}: Effector memory T Cells

Th1/Th2:T Helper 1/T Helper 2

TLR: Toll like receptors

TMB: Tetramethylbenzidine

TNF-α: Tumour necrosis factor

TRAP: Thrombospondin-related anonymous protein

µg: Microgram

µl: Microlitre

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Abstract

Our Immune system has its own blueprint to fight against various range of pathogen but there are certain microorganisms that escape our prompt immune system. With the advancements in technology, the interaction between host and pathogen has been well studied although the mechanism remains poorly understood. So, to combat this bleak situation, vaccination is the gold-standard approach aiming to achieve protective and sterile immunity. However, activating and licensing the activity of both B and T cells is a major hurdle in successful vaccination. To enhance immune response, scientific community has tried using different adjuvants and at certain stage, success has been achieved, however, further validation is ongoing. With respect to this, here we are trying to explore the role of two different adjuvants: Poly(I:C) and R848, in line of sub-unit vaccine approach. Poly(I:C), a TLR 3 agonist, is well known for activating antigen-specific CMI response. On the other hand, R848, a TLR 7/8 agonist, is well investigated for its ability to generate high antibody titres. Moreover, protein antigen along with Poly (I:C), R848 and its combination shown the elevated antigen specific IgG levels and proliferative function of $CD4^+$ and $CD8^+$ T cells after *in-vitro* peptide specific stimulation. In addition to that, repeated immunization has enhanced the production of cytotoxic T cells which may provide us protective immunity. In nutshell, both the adjuvant may aid in activating and boosting the immune system alongside protein antigen and could be the part of vaccination with prior detailed investigation.

1. Introduction

We co-exist in a world congested with microbes and tend to have a natural defence against these living beings but occasionally we get exposed to a few of them which has an adverse effect on us so to mitigate this hurdle, usage of vaccines is one of the gold-standard approach among all the available options.

Our ultimate goal is to induce antigen-specific humoral as well as Cell mediated Immunity for any type of pathogen [1]. Recent advances in this domain have led to 2nd generation of vaccines, which includes the sub-unit part of a protein, for example, CSP (circum-sporozoite protein) which is a part of the RTS, S vaccine against the malaria and is able to provide sterile protection as it has B cell and T cell epitope [2-5]. The low efficacy rate of RTS,S vaccine in its final phase III study conducted in African region led us to think about further to achieve the highest protection. Further, latest developments have led to the discovery of SLTRiP, another malaria liver-stage protein which has shown the promising the results in activating the immune system and reduction in the liver-stage burden during the sporozoite challenge study, indicates as a probable vaccine candidate for this protozoa infection [6].

Many vaccines make use of adjuvants which help them drive the route of response by activating the pattern recognition receptors (PRR) leading to the production of cytokines, engaging antigen presenting cells (APCs), bridging the gap between innate and adaptive immunity, ultimately dispensing appropriate amount of B and T cell response and eventually to a memory bank [7, 8]. There are distinct lineages of these memory T cells: Central memory (T_{CM}) which are $CD62L^{high}$ and effector memory (T_{EM}) which are $CD62L^{low}$ [9]. These two subsets have a very critical role upon antigen exposure, while the T_{EM} are typically found in circulation and readily reach the site of infection, T_{CM} are positioned in draining lymph node but are highly proliferative and both provide long lasting protection for future infections [10]. For B cell memory, the sub-types are generally divided into: Unswitched Memory B cells (similar to Marginal zone B cells) $CD19^{+}CD27^{+}IgM^{+}$ and Switched Memory B cells which are $CD19^{+}CD27^{+}IgM^{-}IgG^{+}$ and upon re-exposure these cells participate in providing quicker and stronger response as compared to their naïve precursors [11].

Since the beginning, the most widely used adjuvant has been the derivatives of Aluminium which mainly includes ‘alum’- aluminium salts. It is renowned for producing high antibody titres but lacks the ability to generate CMI and Th1-type immunity [12]. Consequently, for a response that focuses on Th1 and Th2 immunity, combinations of adjuvant have been an area of focus. Recently,

combination of alum, DOTAP (1,2-dioleoyl-3-trimethylammonium propane), D35 were used against a protein antigen which was able to induce strong T cell and antibody response [13].

TLR3 agonist- Poly(I:C), a synthetic analogue of viral dsRNA and activates multiple PRRs, namely- TLR3, RIG-I (retinoic acid-inducible gene 1) and melanoma differentiation-associated gene 5 (MDA5) which is significant in eliminating the pathogen, Poly(I:C) is able to generate Th1immune response and $CD8^+$ T cell [14].

On the other hand, R848- an imidazoquinoline compound and a TLR7/TLR8 agonist which functions via MyD88-dependent pathway is found to be a potent activator of B cells and had depicted a great ability to enhance the antibody titre [15]. Given these properties, we hypothesized that the combination might have a synergistic effect nourishing the CMI response along with a strong memory T cell response.

This study focuses on the potential of Poly(I:C) and R848 in generation of antibody producing cells (B cells) and the $CD8^+$ T cells to protein antigen- rCSP (recombinant CSP) and SLTRiP protein.

2. Literature Review

Vaccination is still a significant public health strategy for disease/infection prevention. Live vaccinations are known to elicit strong immune responses and provide adequate protection. Live vaccinations, on the other hand, have been linked to a variety of safety problems, therefore a significant trend in vaccine development has been to employ subunit vaccines, which are made up of highly purified proteins which can be addressed by the immune system. However, subunit vaccines have a fundamental disadvantage that they are minimally immunogenic, resulting in low or inadequate vaccination effectiveness. Subunit vaccinations must be given with adjuvants in order to be effective [16].

2. 1 Adjuvants: A crucial player in vaccines

Adjuvants are substances that boost the immune system's response to co-inoculated antigens. The word adjuvant comes from the Latin word *adjuvare*, which means "to aid" or "to enhance [17]."

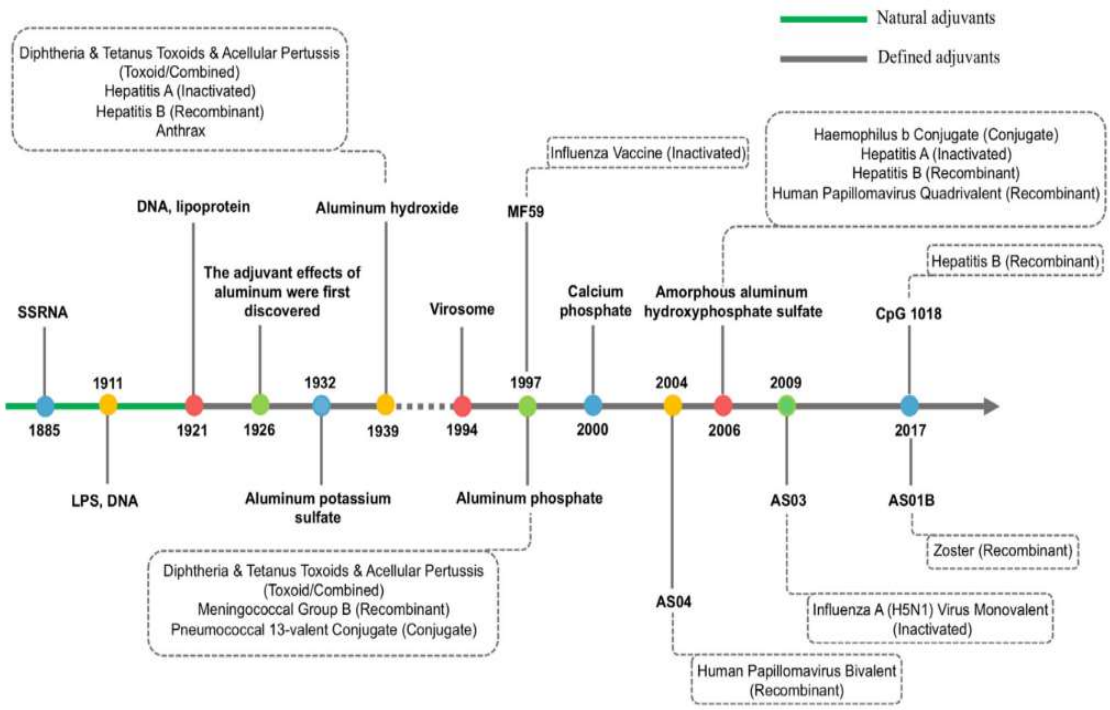


Figure 1: Timeline of Adjuvants (adapted from [18])

2.2 Role of adjuvants

Adjuvants have typically been used to boost the degree of an adaptive response to a vaccination, depending on antibody titre or capacity to prevent the infection, but they have recently taken on a new role; directing the kind of adaptive response to generate the most efficient forms of protection for each pathogen. As a result, there are two unique reasons why an adjuvant should be added in a vaccination. The first reason is to improve the general population's response to a vaccine by increasing mean antibody titres and/or the fraction of subjects who become protectively immunized; improve seroconversion rates in peoples with lowered sensitivity due to age, illness, or therapeutic interventions and allow immunization with low dosage of vaccine. The demand of several immunizations for many injections creates compliance concerns as well as considerable logistical obstacles in many parts of the world. Adjuvants can help to cut down on the number of doses needed to achieve protection.

The second reason to include an adjuvant in a vaccination is to change the immune response's quality in following ways: (1) provide functionally appropriate types of immune response (e.g., T helper 1 cell-Th1 versus Th2 cell, $CD8^+$ versus $CD4^+$ T cells and specific antibody isotypes); (2) increase the generation of memory—especially T cell memory; (3) increase the speed of initial response, which may be critical in a pandemic outbreak of infection; and (4) change the breadth, specificity, or affinity of the response [7].

2.3 Drawbacks of single adjuvant and new direction towards combination approach:

All the vaccines which are currently available contain a single adjuvant, with a few exceptions. Although certain vaccines with a sole adjuvant give optimal protection, in many cases, the adjuvant has a number of drawbacks, such as inducing low-potency or low-quality immune responses. This is especially true with human vaccinations, since the number of clinically authorised adjuvants is restricted. Alum, which was the sole clinically utilised adjuvant in humans until recently, often promotes Th2- but not Th1-type immune responses, and hence does not provide enough protection against diseases like influenza. Even the most promising experimental adjuvants have drawbacks. These limits can be solved by combining various adjuvants [16].

A number of vaccinations using various adjuvant combinations have been tested. This method appears to be promising, and it has the potential to improve the efficacy of a variety of currently available vaccinations. This method could be very useful in the development of a malaria vaccine. Aluminium salts (alum), MF59 (an oil-in-water emulsion) (Switzerland), MPL (mono-phosphoryl

lipid A; a glycolipid), virus-like particle, immune-potentiating reconstituted influenza virosomes, and cholera toxin are examples of adjuvants and delivery systems that have been approved for human clinical trial testing or are components of licensed vaccines and have been used in malaria vaccines.

To uncover innovative combinations of adjuvants and formulations capable of producing significant, long-lasting humoral and cellular immune responses in people, new adjuvant development is required for the development of vaccine against the malaria and other intracellular parasites. These novel adjuvants and formulations should, in theory, produce a protective immune response with fewer administrations [19]. So, looking forward in the same direction; we have used Poly(I:C), R848 and combination of both.

2.4 Poly(I:C)

Poly-IC**:** Polyinosinic-polycytidylic acid (poly(I:C)) is a synthetic dsRNA that, through a pattern known as pathogen-associated molecular patterns (PAMPs), can activate numerous aspects of the host defence. PAMPs are stand-alone immunomodulators or "danger signals" that are being widely recognised as important components of many current vaccines. It can be used as a PAMP-adjuvant when correctly coupled with an antigen, resulting in the modulation and optimization of the antigen-specific immune response [20]. It was also reported to be a Toll-like receptor (TLR) agonist, and it was discovered that poly(I:C) activates TLR3 by analyzing its effects on B cell activation. It improves B cell activation by increasing the expression of surface receptors, cytokine secretion, and proliferation [21]. Poly(I:C) has been also found to induce the production of cytokines such as IL-12, IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) [21].

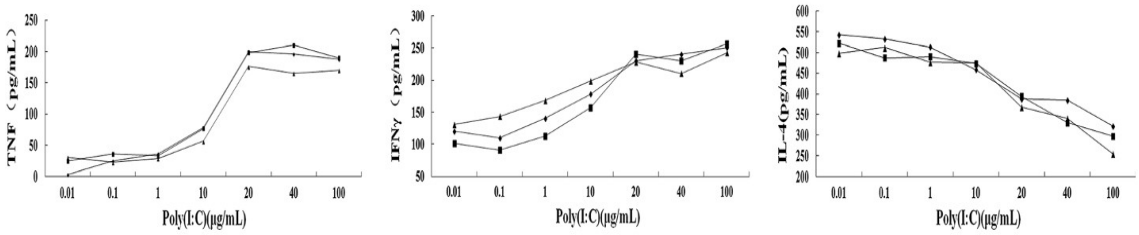


Figure 2: In vitro immune activation of BALB/c mice splenocytes by poly(I:C) (Adopted from [21])

Poly(I:C) potentially enhance Th1 cytokines and type I IFNs by stimulating innate immunity via TLR3 and other signal transduction pathways. The maturation of DCs and the activation of B cells are then induced [22]. As a result, potent $CD4^+$ T cell and humoral immune responses can be elicited.

Furthermore, poly(I:C) could elicit a $CD8^+$ T cell immunological response via a variety of mechanisms [23].

In Malaria, it was found that *E.coli*-derived recobinant circum-sporozoite proteins in combination with poly(I:C)LC induced potent multifunctional (interleukin 2-positive [$IL\text{-}2^+$], tumour necrosis factor alpha-positive [$TNF\text{-}\alpha^+$], gamma interferon-positive [$IFN\text{-}\gamma^+$]) $CD4^+$ effector T cell responses in blood, secondary lymphoid organ- spleen, and particularly in the liver [24].

2.5 R848

Resiquimod (R848) is an imidazoquinoline compound and studies have revealed that it demonstrates potent antitumor and antiviral activity [25-30], and functions via TLR7/TLR8 MyD88-dependent signalling pathway. Toll-like receptors (TLRs) are a type of PRRs preferentially expressed in professional antigen presenting cells such as dendritic cells (DCs) and macrophages which recognizes antigen and plays a significant role in linking the innate and adaptive immune system by having effects on cell maturation, regulatory function in Th1 and Th2 type of responses based on production of proinflammatory cytokines and activating CMI [31, 32]. There is evidence that R-848 (S-28463), induces cytokines such as IFN-α, IL-12 and tumour necrosis factor (TNF)-α in human monocytes, DCs, and mouse macrophages [33-38]. Induction of these cytokines by R-848 has also been observed in other vertebrates such as mice, rats, and monkeys after oral uptake [39].

R848 has been shown to preferentially enhance Th1 immune response through the recognition of TLR7 in antigen-presenting cells in mice, especially DCs [40]. There is combination of adjuvants which when used together can yield specific Th1 or Th2 bias. In a study involving combination of Poly IC, R848 and FMDV antigens formulated with alum revealed that both R848 and poly(I:C) regulated the production of selective cytokines, which shifted its focus on Th1, thus resulting in Th 1 bias [41], by inducing the activation of DCs and upregulating the IFN-γ levels and downregulating IL-4. R-848 indirectly induces IFN-γ production from T cells through an IL-12-dependent mechanism. *In-vivo* studies have revealed that successful enhancement of $CD4^+$ and $CD8^+$ T cells is shown when R848 and Poly(I:C) are co-administered. In a study evaluating the effects of Imiquimod and Resiquimod, on humoral immune response activation, it was shown that R-848 induced B-cell division and differentiation in mouse splenic cultures and purified human B cells. The major biological activity of R848 is associated with activation of different B cell subsets [42]. B cell differentiation is stimulated by mitogens and the activity is checked by measuring the changes in activation of cell surface markers such as MHC class II and B7.2, when expressed on B cells, they function as professional APCs to activate $CD4^+$ T cells. R848 has shown to increase the levels of

surface markers responsible for B cell activation.

R848 has shown to play a role in switch recombination machinery. In a study aimed at understanding the role of R-848 in class switching, enhanced levels of IgE and IgG1(IL-4 dependent) and increased IgG2a via IFN-γ production added to one more characteristic of the TLR7/8 agonist. Furthermore, R848 was shown to be operative at spiking antibody responses by inducing IgM synthesis up to 5 folds. In *in-vitro* studies, R848 when tested with other TLR agonists [43], could significantly induce the production of IL-12 from bone marrow-derived DCs. Among the tested TLR agonists, R848 was the most effective adjuvant in recruiting DCs and NK cells into lymph nodes, and the production of specific anti-OVA IgG2a in *in-vivo* settings [43].

The results on B cell activation and differentiation suggest the potential utility of R-848 to act as an adjuvant to boost antigen-specific immune responses.

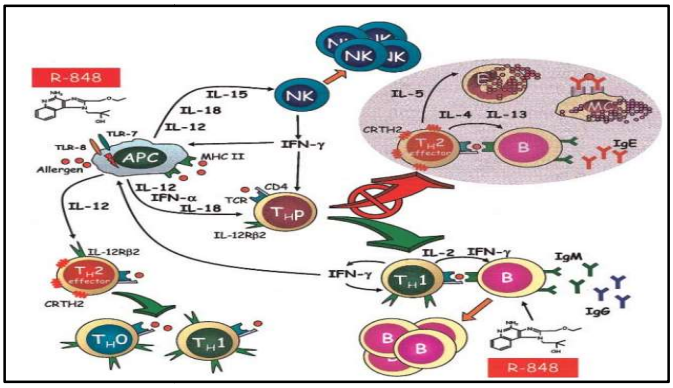


Figure 3: Possible mechanisms of action of R-848 for the modulation of allergen-specific T-cell responses (Adopted from [44])

3. Hypothesis

Repeated immunizations with protein Ag or WSV promoting humoral responses would generate and expand memory B cells whose BCR affinity is expected to improve with each dose of vaccination. It is reasonable to assume that the antigen-specific B cells generated before a given booster dose of vaccine would capture the antigen, bearing epitopes for B as well as T cells, to induce antibody secretion as well as to present the antigen to $CD8^+$ T cells though cross-presentation. Alternatively or simultaneously, the high titer and high affinity antibody produced over the multiple immunizations might form the immune complex (IC) with free circulating antigen or incoming antigen during the repeat dose of vaccination, and be captured by the APCs (DCs, Macrophages) though Fc receptor (FcR), processed and presented to $CD8^+$ T cells. ***Thus, we hypothesize that repeated immunization with vaccines promoting B cell response might generate CD8+ T cells responses to antigens bearing both B cell and T cell (MHC-I restricted) epitopes.***

4. Rationale

Usage of adjuvants in the approach of subunit vaccines has been done since past few years, but there is little success when it comes to activating the cell mediated immunity against the intracellular parasitic infection. Thus, a combination of adjuvants such as Poly(I:C), a $CD8^+$ activator and R848 which is responsible for enhancing the B cell differentiation may increase the threshold of humoral and cell mediated immunity which would ultimately provide sterile protection. Deeper understanding of the mechanism of cross-presentation provides new approaches to look at in vaccine development.

5. Objectives

- Screening of different adjuvants for the generation of B cells and activation of T cell
- Characterization of B and T cell subsets.
- Understand the role of B cells in presentation of malaria liver-stage specific antigen to $CD8^+$ T cells.

6. Materials and methods

6.1 Materials

6.1.1 Animals

Female/male BALB/c and C57BL/6 mice (6 to 8 weeks old) have been procured from the Zydus Research Centre, Changodar, Gujarat. Animals were housed under specific-pathogen-free conditions at animal house, Institute of Pharmacy, Nirma University, Ahmedabad and handled according to CPCSEA guidelines. Autoclaved food and water were provided *ad libitum*. Animals were randomly separated into their respective groups **(Table 1)**. All the protocols were approved by the Institutional Animal Ethical Committee (IAEC), Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat.

6.1.2 Immunization dosage requirement

rCSP: recombinant CSP protein is the major surface protein of the sporozoite and forms a dense coat on the parasite's surface mainly express during the liver-stage infection of malaria infection.

SLTRiP: Sporozoite, liver stage tryptophan-rich protein (SLTRiP) is a conserved tryptophan-rich protein with potential B- and T-cell epitopes. It has also been consider as the one of the potential candidate of malaria liver stage infection.

Poly(I:C) (Cat. code tlrl-picw-250): Polyinosinic-polycytidylic acid (i.e. poly(I:C)) is a synthetic analogue of double-stranded RNA (dsRNA), a molecular pattern associated with viral infections.

R848 (SML0196): Resiquimod belongs to the class of imidazoquinolinamines compounds with immunomodulatory effects. Resiquimod increases the levels of cytokines such as TNF-α, IL-6 and IFN-α.

Table 1A: Immunization dosage

No	Materials	Stock Solution
1.	rCSP Malaria ag*	0.5µg/µL
2.	Poly (I:C)	20µg/µL
3.	R848	10µg/µL
4.	SLTRiP	1.144µg/µL
5.	GST	5.907µg/µL

*Recombinant CSP protein (*Plasmodium falciparum* origin)

Table 1B: Immunization strategy for rCSP antigen

Groups	No. of Mice	Combination of antigen & adjuvant	Amount of Dose
1.	3	CSP	20µg/ mouse
2.	3	CSP + Poly (I:C)	50µg/ mouse
3.	3	CSP + R848	50µg/ mouse
4.	3	CSP + (Poly I:C) + R848	20+50+50µg/ mouse
5.	3	Control- PBS	100µL/ mouse

Table 1C: Immunization strategy for SLTRiP antigen

Groups	No. of Mice	Combination of adjuvant	Amount of Dose
1.	4	SLTRiP –GST + Poly (I:C)	50+20µg/ mouse
2.	3	GST + Poly (I:C)	25+20µg/ mouse
3.	3	Control- PBS	100µL/ mouse

6.1.3 ELISA Requirements:

1. 1X PBS (pH 7.4)
2. Carbonate-Bicarbonate buffer pH 9.6 (Sigma-C3041)
3. Washing Buffer (PBS-T): 10 mM phosphate buffer (pH 7.4), 150 mM NaCl & amp; 0.05% Tween 20 (Sigma-P3563)
4. (OVA/CSP/SLTRiP/TRAP immunized sera)
5. Stopping reagent: 1M HCL or 0.5 M H_2SO_4
6. 96 well microtiter plates
7. Microtiter plate reader

6.1.4 Cell Surface Staining Requirements:

1. RPMI
2. 1X PBS
3. ACK RBC Lysis solution
4. Staining Buffer:
5. Antibody Cocktail

6. 1% Formaldehyde

7. FACS Buffer: FCS in PBS.

6.1.5 Bone marrow isolation:

- Materials and Reagents:
- Sterile paper towel
- Sterile surgical pad (Direct Resource, catalog number: 19015742)
- 23-gauge (or 25-/26-gauge) needle (BD Biosciences, catalog number: 305145)
- 10 ml syringe (BD Biosciences, catalog number: 309604)
- 70 µm nylon cell strainer (Falcon, catalog number: 352350)
- (Note: Currently, it is "Corning, Falcon®, catalog number: 11995-065")
- 50 ml conical tube (Falcon, catalog number: 21008-940)
- (Note: Currently, it is "Corning, Falcon®, catalog number: 21008-940")
- 5 ml syringe plunger (BD Biosciences, catalog number: 309646)
- Adult mice (> 6 weeks, any strain) (e.g.C57BL/6)
- Hank's balanced salt solution (HBSS), no Calcium, no Magnesium, no phenol red (Life Technologies, catalogue number: 14175095) Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalogue number: 14175095".
- DMEM medium, high glucose, pyruvate, L-glutamine (Life Technologies, catalogue number:
- 11995-065) Note: Currently, it is "Thermo Fisher Scientific, Gibco™, catalogue number: 11995-065".
- 70% ethanol
- Fetal bovine serum heat inactivated (FBS) (Sigma-Aldrich, catalog number: F9665)
- Ammonium chloride (NH4Cl) (Sigma-Aldrich, catalog number: 213330)
- Potassium bicarbonate (KHCO3) (Sigma-Aldrich, catalog number: 237205)
- Disodium 12dentate (Sigma-Aldrich, catalog number: D2900000)
- RBC lysis buffer
- DMEM medium

Equipment:

- Blunt-end sterile scissors (Thermo Fisher Scientific, Fisher Scientific, catalog number: 08-950)
- Sharp sterile scissors (Thermo Fisher Scientific, Fisher Scientific, catalog number: 08-940)
- Sterile forceps (Thermo Fisher Scientific, Fisher Scientific, catalog number: 08-890)

- Hausser™ Levy™ Hemacytometer Chamber Set (Thermo Fisher Scientific, Fisher Scientific, catalog number: 02-671-55A) or coulter Z2 cell and particle counter(Beckman Coulter, catalog number: 383550)
- Refrigerated centrifuge
- Sterile culture hood
- CO2 rodent euthanasia chamber

6.1.6 Lysis of whole peripheral blood requirements

1.10X RBC Lysis Buffer (Multi-species) (Invivogen-Cat. No. 00-4300)

2. 12x75 mm round bottom test tubes (BD)

3. Primary antibodies (directly conjugated)

4.Flow Cytometry Staining Buffer (Cat. No. 00-4222)

6.2 Methods

6.2.1 Immunization Dosage

For Immunisations, CSP (candidate malaria liver-stage antigen) and SLTRiP (candidate malaria liver/sporozoite-stage antigen) were given alongside with their respective adjuvants. Mice were primed subcutaneously followed by homologous boost immunizations given 14 days apart. For every booster dose, the concentrations of each antigen-adjuvant group were reduced to half. Two days prior to the booster dose, blood samples were collected and serum were separated and stored at -80°C till further use. Also, flow-cytometry experiments were carried out from the immunized mice to study the phenotypic nature of B and T cells after each immunization.

6.2.1.1 Immunization strategy for CSP immunized mice

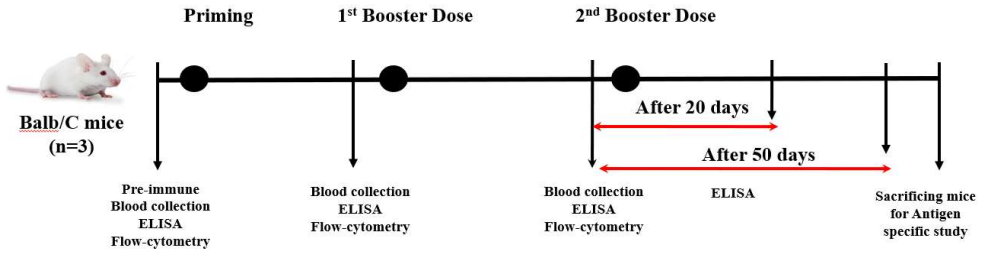


Figure 4: Immunization strategy for CSP antigen. A total of 3 doses were given of the combination of different adjuvants with CSP antigen in five groups namely: 1) Control 2) CSP 3) CP 4) CR 5) CPR. After 50 days of the third dose, one mouse from each group was sacrificed for in vitro study

6.2.1.2 Immunization strategy for SLTRiP immunized mice

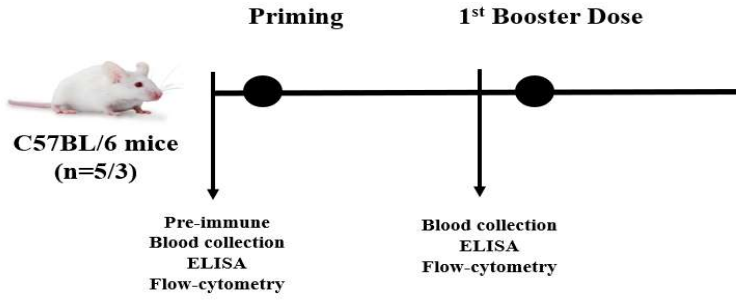


Figure 5: Immunization strategy for SLTRiP antigen. A total of 2 doses were given of the combination of Poly IC with SLTRiP antigen in three groups namely: 1) Control 2) GST and Poly(I:C) 3) SLTRiP and Poly(I:C)

6.2.2 Blood collection:

The blood collection was done 2 days prior to the booster doses from the retro-orbital site of the experimental mice. The blood was collected in eppendorf and anti-coagulating tube (EDTA) for performing ELISA and Flow Cytometric analysis, respectively.

6.2.3 ELISA Protocol:

With repeated immunization (rCSP & SLTRiP antigen) in mice, the collected serums were checked against the formation of antibodies. In general, the whole IgG used to be found in major amount, we have done the indirect ELISA testing to study the generation of antibody. Below mentioned protocol was followed.

1. A 96 well plate was coated with CSP (1 µg/well) or SLTRiP (0.5 µg/well) and incubated overnight at 4^{o}C (100µl/well).
2. Next day after overnight incubation, the unbound antigen was decanted by inverting the plate followed by blocking with 2% BSA in PBS (200µL/well). The plate was further incubated for 2 hours at room temperature.
3. The block solution was decanted and diluted serum samples (1: 500 to 1: 16000) were added into the respective wells (100µl/well) in duplicates and further incubated for 2 hours at room temperature.
4. After the incubation, washing was performed thrice using PBS-T washing buffer (200µl/well)
5. The antibody dilution (IgG-1:2000) was added into each well (100µl/well) and incubated for 1 hour in dark at room temperature. After the incubation the wells were again washed thrice with

PBS-T washing buffer (200µl/well).

6. Further, the substrate TMB-H_2O_2 (100µl/well) was added into each of the wells followed by addition of the stop solution 1 N HCL, and the absorbance was read at 450 nm in the microplate ELISA reader with the reference at OD 655 nm

6.2.4 Cell surface and Intracellular staining protocol:

To study and compare the profiling of immune cells (B and T cells), blood was collected from immunized and control group of mice. Further, the cell surface staining protocol was followed (standardized in our laboratory) and different cell population was monitored throughout the procedure. The protocol is mentioned below.

1. The harvesting of cells from a whole spleen/bone marrow from a "normal" strain of mouse (e.g., BALB/c orC57Bl/6) was done.

2. Spleen was minced in sterile 1X PBS solution in 15 ml tube. The cells were pellet down by spinning at 1000 rpm for 10 min at 10^{o}C.

3. The cells were resuspended in minimum volume and then treated with 2ml ACK RBC lysis solution and kept for 3 min. Repeated the washing step using 1X PBS (or 1% RPMI) (For spleen cells-2 times RBC lysis washing step was performed).

4. The pellet was dissolved in chilled complete RPMI, and cells were counted under a microscope.

5. Antigen stimulation: Suspended appropriate number of cells (0.5-0.7 million cells per well) in each well of 96-well plate, in 200µl of complete media, prepared desired number of well according to need.

Note: While giving antigen stimulation *in-vitro* makes dilution of antigen in complete media and makeup 200µl in each well (100 µl cell+100 µl antigen). In antigen stimulation, incubate the cells at37^{o}C and 5%CO_2 for the required period of time (2 hours for intracellular cytokine staining and for cell proliferation 50-72 hours) according to need.

6. Incubated, centrifuged the plate at 300-400g for 3-4 min, the media was discarded directly by inverting the plate without disturbing the pellet (pellet was clearly visible).

7. Added 200 µl staining buffer in one well pulled the cells in one well and centrifuged the plate at 300-400g for 3-4 min, discarded the media directly by inverting the plate without disturbing the pellet.

8. Resuspended the pellet in minimum volume (approx.10µl), then 5-10 µl of Fc-block diluted normal mouse serum was added, final concentration of Fc-block was 1:100. Mixed by gently tapping the plate, incubated the plate on ice for 10minutes.

Note: Fc-block blocks the immunoglobulin's Fc-receptors.

9. Added 10µl of antibody cocktail and tapped the plate gently and kept on ice for 30 minutes in the dark.

Note: Ensure that the Fc -block and antibodies are bound well, for this reason pipetting and tapping the tube are crucial steps that would give the better separation of cells in results.

Dilutions are made in such a way that Fc-block is eventually diluted1:100 times and antibodies are diluted 1:150 times in the suspension of cells.

10. Incubated and added 200 µl staining buffer and centrifuged at 300-400g for 3-4 minutes. Pellet obtained was washed once again with a staining buffer centrifuged as above.

11. Finally the pellet was resuspended in 400 µl of staining buffer with 1 % Formaldehyde for fixing the cells up to 48 hours at 4^{o}C.

12. Samples were acquired using a flow-cytometer. The acquired results were analyzed by using the FlowJo software.

Staining buffer/FACSbuffer:1% FCS in PBS

Intracellular Cytokine Staining

1. Steps (1-5) were repeated as above mentioned in cell surface staining protocol.
2. After antigen stimulation, addition of Brefeldin A 1000X solution (5.0mg/ml stock): Required concentration range 0.5g/200lor 1X solution. The 96-well plate was incubated at 37^{o}C in 5% CO_2 for 2 hours.
3. Cell surface staining was performed according to the protocol.
4. After final wash of cell surface staining, supernatant was aspirated, and the 96-well plate was agitated.
5. 100 µl of Cyto-fix/Cyto-permBuffer was added to each sample well. Plate was incubated for 20 minutes at room temperature (This step fixes the mouse cell morphology and permeabilizes the activated cells for subsequent intracellular staining.)
6. 100 µl of 1× Perm/Wash buffer was added to each sample well and the plate was centrifuged at 400-500×g for5 minutes at 10°C.

7. Supernatant was aspirated from each sample well and plate was agitated to disrupt cell pellets.
8. Step 6 and 7 were repeated.
9. Purified blocking Antibody Cocktail (Fc-Block) was added to the desired sample wells in 20µl aliquots. 30µl of Perm/Washbuffer was added to the same sample wells.
10. Isotype Control Cocktail and conjugated anti-cytokine antibodies were added to the desired sample wells in 20 µl aliquots. Additionally, 30 µl of Perm/Wash buffer was added to the same sample wells.
11. 50 µl of BD Perm/Wash Buffer was added to the sample wells designated as auto-fluorescence controls.
12. The 96-well plate was incubated for 30-35 minutes at 4°C.
13. 100 µl of BDPerm/Wash buffer was added to each sample well and the plate at 400-500×g for 5 minutes at10°C.
14. Supernatant was aspirated and plate was agitated to disrupt the cell pellets.
15. Step18-19 was repeated.
16. Contents of each well were transferred to a correspondingly numbered sample tube using 200 µl of FACS Buffer (1% FCS + PBS). To fix the cells FACS Buffer with 1% formaldehyde was added in each tube till it gets to 400 µl.
17. Samples were analyzed on a flow-cytometer for data acquisition.

6.2.5 Antigen Specific Response for CD8+ T Cells

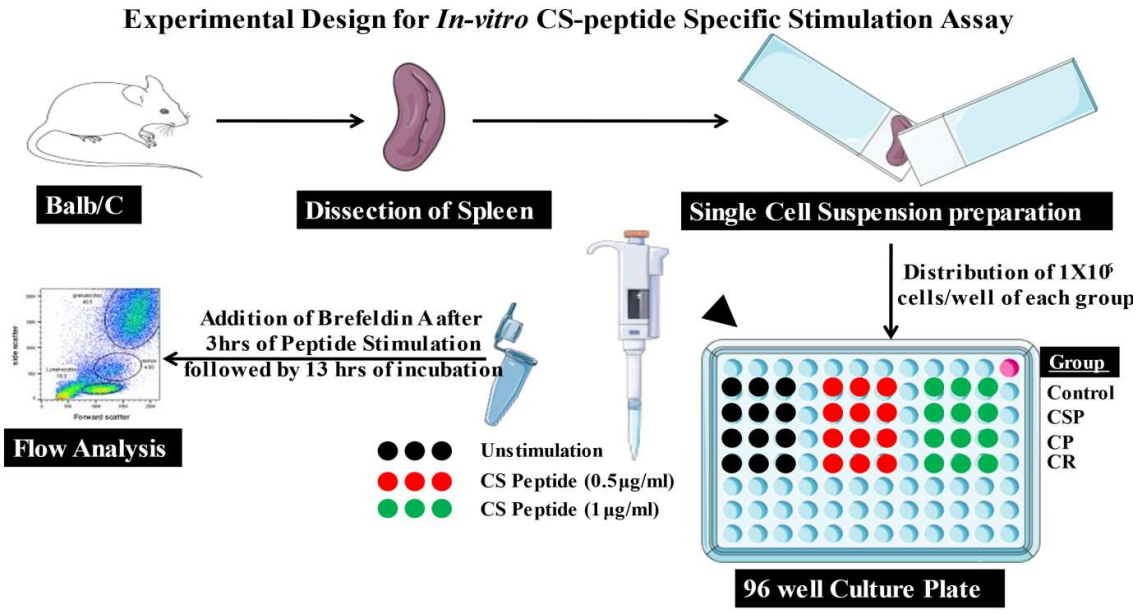


Figure 6: Strategy for *in-vitro* CS peptide stimulation assay to study Ag specific T cell response from the spleen of adjuvant-based CSP immunized mice

6.2.5.1 Immune cell isolation from Mouse femur bone marrow (adapted and modified from [45])

1. The mouse was euthanized with CO_2 and placed onto a sterile surgical pad in a sterile hood. Sterilization of the mouse abdomen area and skin of hind limbs with 70% ethanol was done **(Fig 7).**

Figure 7: Sterilization of mouse abdomen area and skin of hind limbs

2. The abdominal cavity was opened with blunt end sterile scissors and the surface muscles were removed to find the pelvic-hip joint **(Fig 8)**

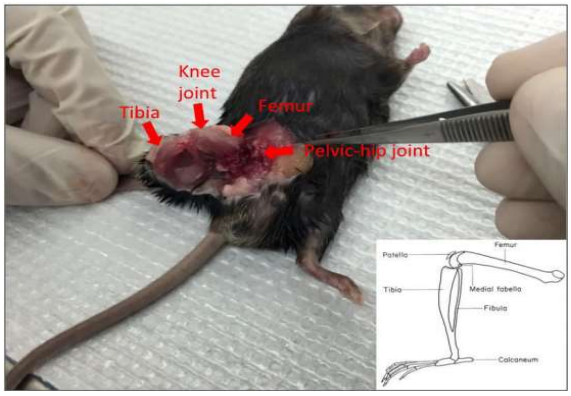


Figure 8: Find the pelvic-hip joint

3. The hind leg above the pelvic-hip joint was cut with sharp sterile scissors. The tibia from the hind leg below the knee joint was cut with sharp sterile scissors **(Fig9 & 10).**

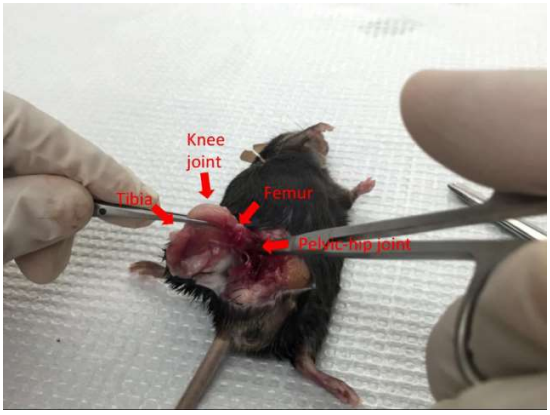


Figure 9: Cut off the hind leg above the pelvic-hip joint

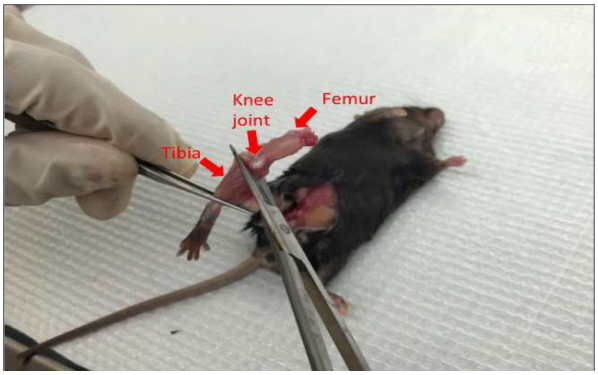


Figure 10: Cut off the tibia at knee joint

4. (Optional: If higher yield of bone marrow cells is needed, tibia can also be used for

bone marrow cell isolation. Cut at the tibia ankle joint to dissect the tibia. The following procedures can be applied to both femur and tibia)

5. The muscles and residue tissues surrounding the femur were removed with sterile forceps and scissors **(Fig 11)**

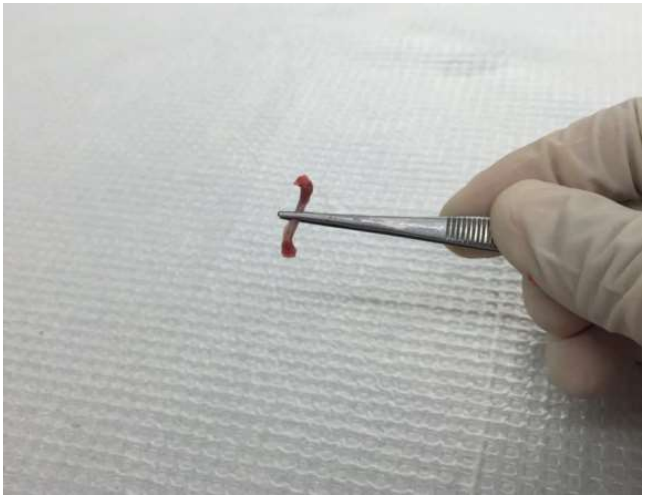


Figure 11: Remove the muscles and residue tissues surrounding the femur

6. The femurs were cut at both ends with sharp sterile scissors. Using a 23-gauge (some literature suggests 25-or 26-gauge) needle and a 10cc syringe filled with ice-cold HBSS to flush the bone marrow out onto a 70 μm nylon cell strainer placed in a 50 ml Falcon conical tube. Usage of all the 10ml HBSS was done or until the flowthrough turned white **(Fig12 and 13)**

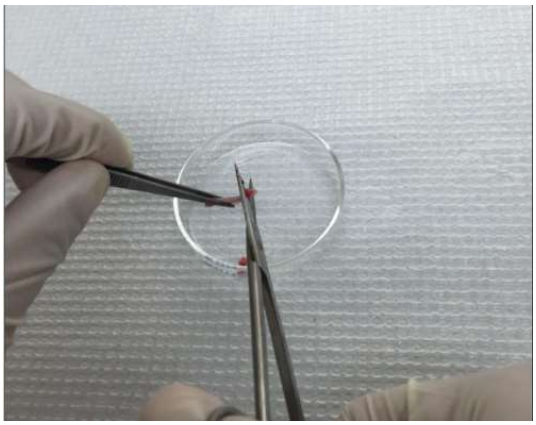


Figure 12: Cut femurs at both ends

Figure 13: Flush the bone marrow onto the cell strainer with HBSS

7. (Optional) In case some residue bone marrow cells could not be flushed off (very few bone marrow visible in the flow through or the yield is significantly less, e.g.< 1 x 10^7 cells),scraping the inner surface of the femur with the needle and flushing with extra ~5 ml HBSS can be done **(Fig 14).**

Figure 14: (Optional) Scrape the inner surface of femur with needle

8. The bone marrow was smashed through the cell strainer with a 5ml plunger **(Fig 15)**. And washing was done with another ~5 ml HBSS.

Figure 15: Smash the bone marrow cells through the cell strainer with a 5 ml plunger

9. The cells were centrifuged at 1,500 rpm for 7 min at 4 °C and the supernatant was discarded.

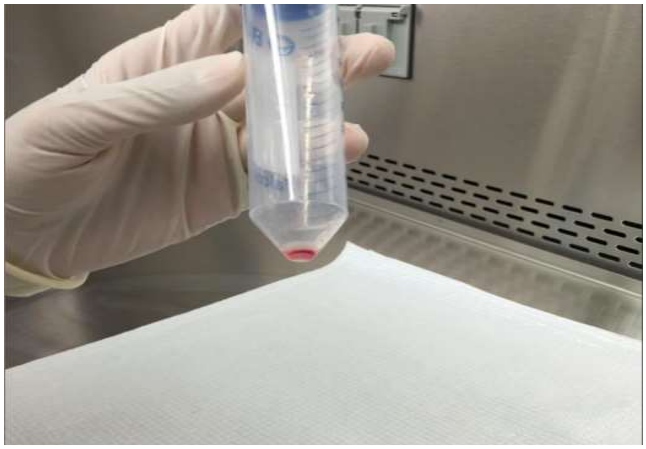


Figure 16: Cell pellet before RBC buffer resuspension

10. The cell pellet was resuspended with 1 ml RBC lysis buffer (for each mouse). Incubation for 5 min at room temperature or 2 min at 37°C was done followed by neutralization of the lysis buffer by adding 5ml FBS **(Fig 16)**

11. The cells were centrifuged at 1,500 rpm for 7 min at 4 °C and the supernatant was discarded. The cell pellet was resuspended with appropriate media for the next step of assay such as 5 ml DMEM medium containing 10% FBS. Cells were then placed on ice.

12. The bone marrow cells were counted with a haemocytometer or a Beckman Z2 coulter-counter. Cells were now ready for assays or culture and can stay viable on ice for at least 5 h.

6.2.5.2 Lysis of mouse spleen and bone marrow cells

- The mouse spleen is harvested, and a single-cell suspension is prepared.
- The cells are pellet down by centrifugation at 500xg for 5 minutes at room temperature and the supernatant is aspirated.
- The pellet is resuspended in 3-10 mL of prepared 1XRBC Lysis Buffer (Multi-species) followed by incubation for 4-5 minutes at room temperature.
- After lysis, centrifugation immediately at 500xg for 5minutes at room temperature was performed and the supernatant decanted.
- The pellet was resuspended in 2mL of flow-cytometry staining buffer and centrifuged again, the supernatant decanted, and a cell count was performed under the microscope.

6.2.5.3 Antigen Specific Assay

(Before using, Thermo Fisher 10X RBC Lysis Buffer (Multi-species) must be diluted1:10 with room temperature reagent grade water.)

- Antibody staining followed by lysis of whole peripheral blood
- Aliquots of a sample of whole blood into a tube were prepared. (For mice, use 50-100 µl of blood) Note: The ThermoFisher 10XRBC Lysis Buffer (Multi-species) has been shown to work equivalently in blood collected with either heparin or EDTA as the anticoagulant.
- Addition of the antibody(s) needed for staining (in a volume of greater than 50µl) is done and mixed thoroughly.
- Incubate for 30 minutes in the dark (if staining with flurochrome-conjugated antibodies) at room temperature.
- Addition of 2mL of room temperature prepared 1XRBC Lysis Buffer (Multi-species), and then either pulse vortex or inversion was done to mix. Incubation at room temperature in the dark was done for 4 – 10 minutes.

Note: Turbidity can be observed to evaluate red blood cell lysis. Once the sample becomes clear, lysis is complete.

- After lysis, centrifugation at 500 x g for 5 minutes at room temperature was performed and the supernatant was decanted.
- (Optional) The samples can again be incubated with an additional 1X RBC Lysis

Buffer (Multi-species) (1mL for 3minutes) if further removal of red blood cells is needed. However, this step is not typically necessary since small numbers of residual red blood cells do not interfere with subsequent assays and can be gated out during flow-cytometric analysis.

- The pellet was resuspended in 2 mL of Flow Cytometry Staining Buffer and centrifuged again. The supernatant was decanted, and the pellet was resuspended in 200 µl of flow-cytometry staining buffer. The samples were analyzed by flow-cytometry

Table 2A: Markers for cell surface and intracellular staining of T cells

Anti- mouse antibody CD3	**Per CP CY 5.5**
Anti -mouse antibody CD4	**V_{500}**
Anti -mouse antibody CD8	**Alexa 700**
Anti -mouse antibody CD44	**APC CY 7**
Anti -mouse antibody CD62L	**V_{450}**
Anti -mouse antibody CD107A	**FITC**
Anti -mouse antibody IFN γ	**APC**

6.2.6 Antigen Specific Response for B cells

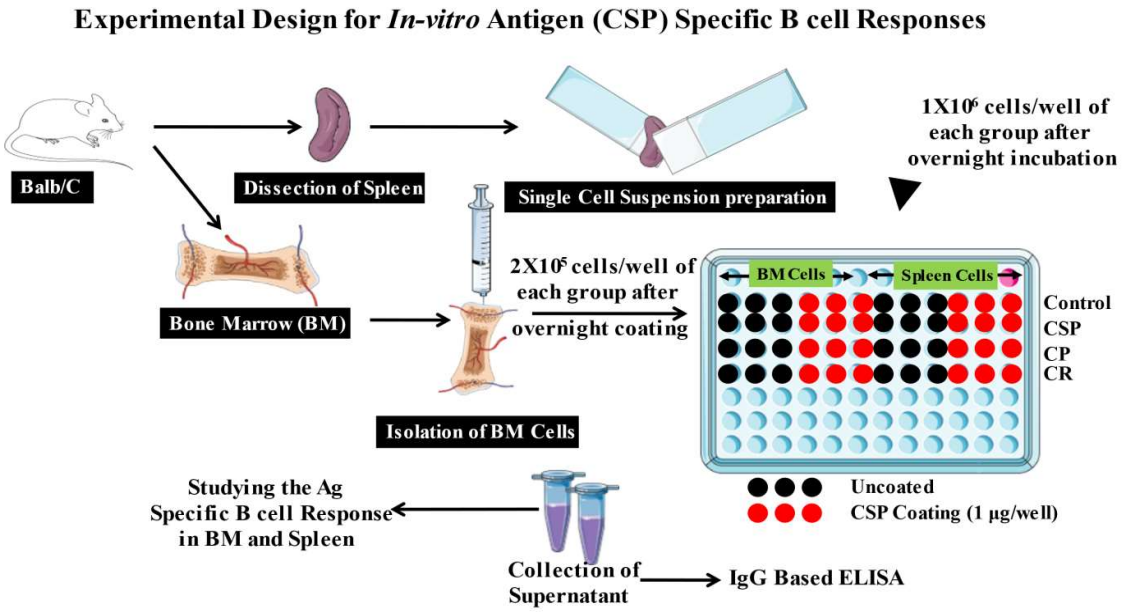


Figure 17: Strategy for *in-vitro* CSP antigen stimulation assay to study antigen specific B cell response from spleen and bone marrow of adjuvant-based CSP immunized mice

Antigen Specific Response for B cells

- Lysis of mouse spleen and bone marrow cells was done as mentioned previously
- To measure the antigen-specific antibody production and memory B cell response, the cell culture plates were coated with CSP (1µg/well) and incubated overnight. The plates were washed and blocked with 10% complete media (200µl/well) for 2h at room temperature, before adding the cells (Both coated and uncoated wells were utilized)
- Spleen and bone marrow cells, harvested from the Boost 2w mice, were seeded at a density of 2×10^6 cells/mL onto the plates and incubated at 37 °C for 1, 3 and 5 day and 1 d, respectively.
- Anti-mouse IgG antibodies were used to detect Ag-specific antibodies produced by the cells (The cell surface staining as well as intracellular staining was performed as mentioned previously).

Table 2B: Markers for cell surface and intracellular staining of B cells

Anti-mouse antibody CD19	PE CY 7
Anti-mouse antibody CD73	PE
Anti-mouse antibody CD80	APC
Anti-mouse antibody CD86	V_{450}
Anti-mouse antibody IgM	BV_{510}
Anti-mouse antibody IgG	Alexa 700
Anti-mouse antibody CD138	BV_{510}

(All the antibodies were obtained from BD Biosciences.)

7. Results and Discussion

7.1 CSP immunization and immune response

Immunological memory represents a concept wherein individuals, once encountered a disease remains protected upon a second time exposure by a combination of lasting antibody titers in conjunction with memory B cells and T cells. Vaccines have shown to be successful in achieving promising humoral immune responses, but to elucidate cytotoxic T lymphocytes remains a challenge. Here, we have used rCSP protein (malaria liver-stage vaccine candidate) for immunization in BALB/C mice along with a combination of adjuvants (Poly(I:C) and R848) to study the humoral and CMI responses. To study the status of different immune sentinels ($CD4^+$, $CD8^+$ T cells and B cell population) in circulation, cell surface staining was performed from the whole blood sample collected on every 2 days prior to next. A total of 3 doses were given (**Fig 4**). The analysis for acquired cells (using AttenuX machine) was performed via FlowJo software. After acquiring the samples, compensation was applied to all the samples and using the common gating strategy **(Fig 18)**.

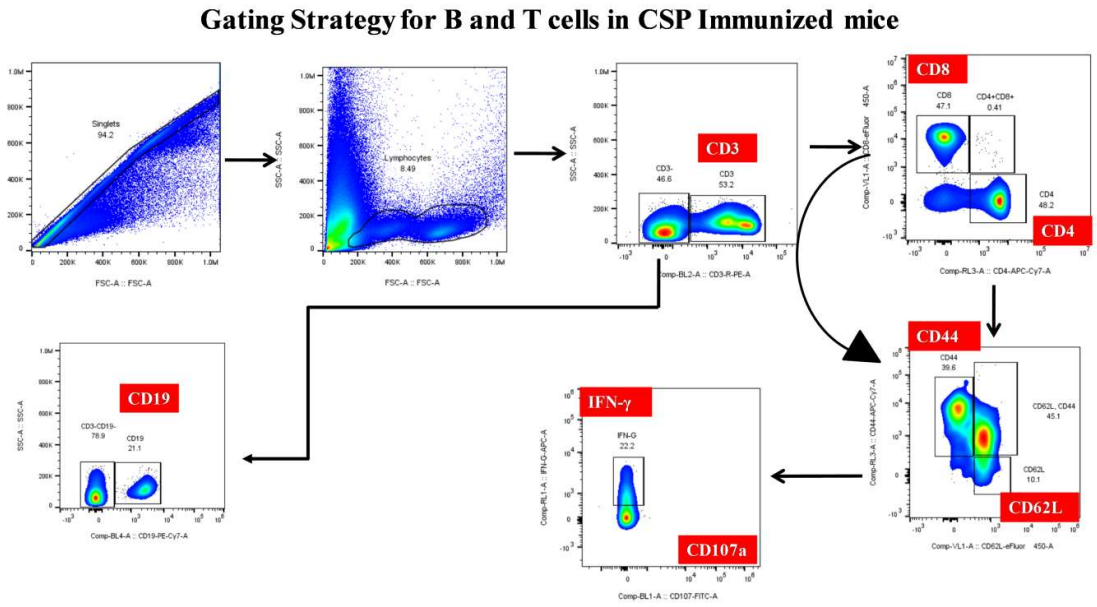


Figure 18: The common gating strategy to analyze B and T cells ($CD4^+$ and $CD8^+$T cells) response in blood samples from adjuvant based-CSP immunized mice

The figure 18 shows the common gating strategy for adjuvant-based CSP immunized mice and applied to all samples to study the pattern of B and T cell population. The main aim of the study is to look for the generation of B and T cell population under the influence of CSP administered along with the adjuvants. As seen in the figure, the lymphocyte population was selected by keeping the FSC-A (forward scatter) on X axis indicating the size of the cell and

the SSC-A (side scatter) on Y axis which demonstrates the intracellular complexity of the cell **(Fig 18)**. The more the forward scatter, larger the cell similarly more the side scatter, higher the complexity of the cell. Lymphocytes are smaller in size and have less internal complexity, based on this the lymphocytes were identified.

Further, $CD3^+$ cells were gated from the lymphocytes which represent the T cell population since CD3 is a costimulatory molecule which helps the T cell in antigenic recognition by the MHC-TCR signalling. $CD3^-$ cells were further gated to differentiate for B cell ($CD3^-$ $CD19^+$) **(Fig 18)**. The major subsets of T cells i.e., T helper cells distinguished by $CD4^+$ and $CD8^+$ as cytotoxic T cells were gated from $CD3^+$. Further, Central memory T cells ($CD44^+$ $CD62L^+$) and effector memory T cells ($CD44^+$ $CD62L^-$) were separated from both the T cells population. $CD8^+$ T cell activation is characterized by the 2 intracellular markers namely CD107a which is a degranulation marker that gets upregulated upon $CD8^+$ T cell activation and IFN-γ production releases perforin and granzyme which creates pores and mediates direct killing in the target cells respectively. Thus, CD107a was gated from $CD8^+$ which represented killer cells and IFN-γ production was checked from both the $CD4^+$ and $CD8^+$ T cells **(Fig 18)**.

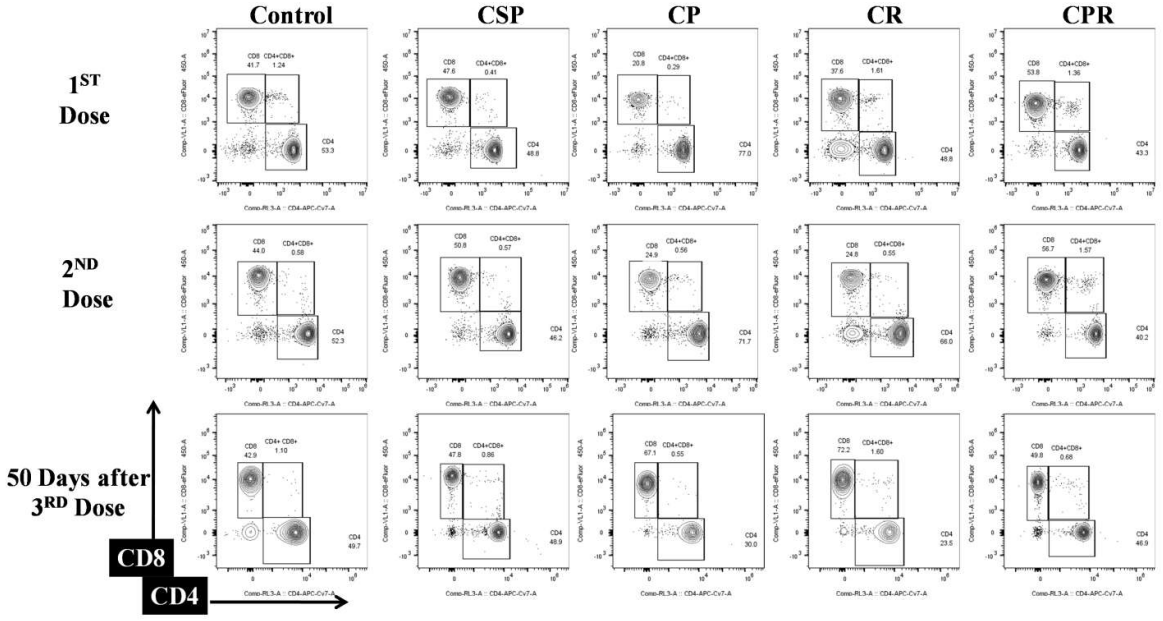


Figure 19: Status of $CD4^+$ and $CD8^+$ T cells in adjuvant-based CSP immunized mice (Immunization groups= **Control (**PBS), **CSP** (CSP protein only), **CP** (CSP protein and Poly(I:C)), **CR** (CSP protein and R848), **CPR** (CSP protein, Poly(I:C) and R848)) ($CD4^+$ and $CD8^+$ T cells on X and Y axis are separated from CD3, respectively, individual gate depicts the number of cells from parent one and each box is the representation of individual group

where N=3 mice/group; Upper, middle and lower panel represent the data after 1st, 2nd, and 50 days after 3rd dose of adjuvant-based CSP immunization)

It shows the $CD4^+$ and $CD8^+$ T cell population in the five different groups 1) Control 2) CSP 3) CP 4) CR 5) CPR. It has been noticed that with compare to control all the other groups have shown the production of $CD4^+$ and $CD8^+$ T cells are obtained after each dose of immunization **(Fig 19)**. However, CP group have shown the highest frequency of $CD4^+$ T cells whereas CR group represent for the higher $CD8^+$ T cells production **(Fig 19)**. Interestingly, the frequency of $CD8^+$ T cells were found to be moreover similar to CR group and slightly higher than CP group which may suggest the role of both adjuvant in activating $CD8^+$ T cells.

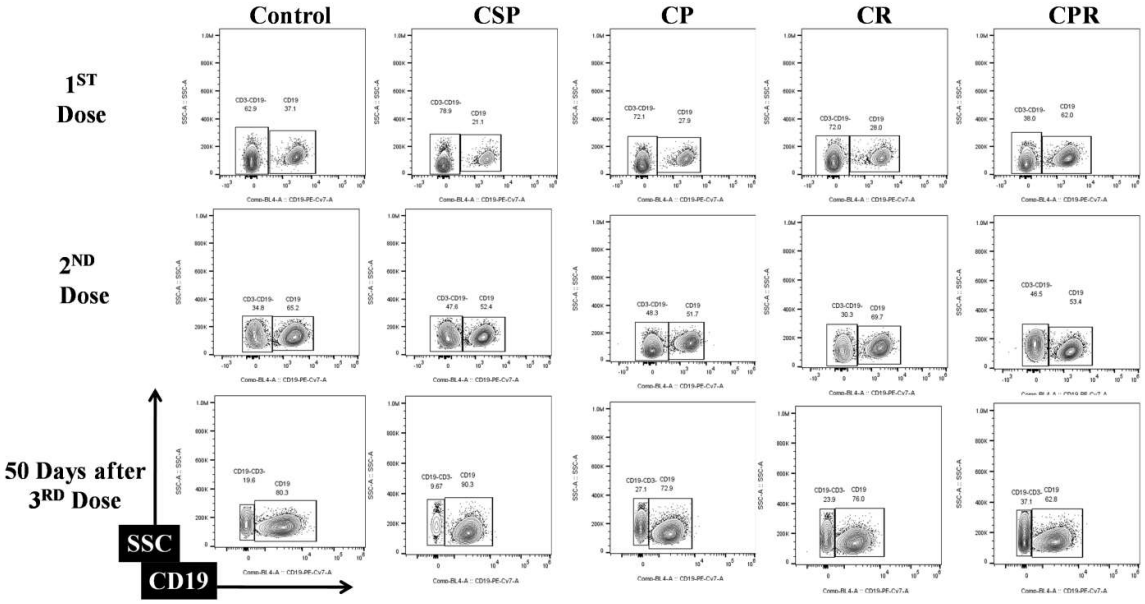
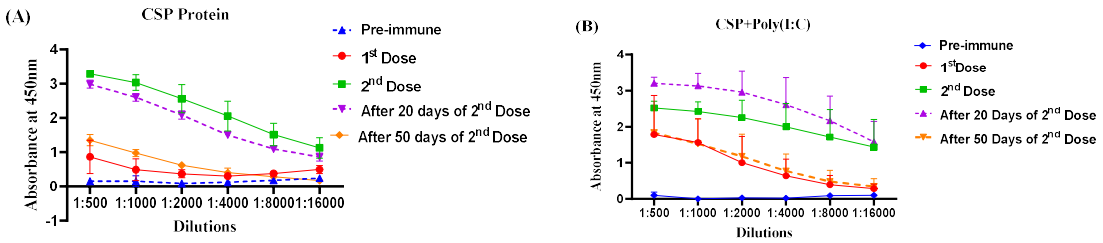


Figure 20: Status of B cells in adjuvant-based CSP immunized mice (Immunization groups= **Control (**PBS), **CSP** (CSP protein only), **CP** (CSP protein and Poly(I:C)), **CR** (CSP protein and R848), **CPR** (CSP protein, Poly(I:C) and R848)) ($CD19^+$ B cells are separated from $CD3^-$ population on X axis, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper, middle and lower panel represent the data after 1st, 2nd, and 50 days after 3rd dose of adjuvant-based CSP immunization)

The above given figure depict the status of B cell population in the five different groups 1) Control 2) CSP 3) CP 4) CR 5) CPR. In both the control and CSP, homogenous levels of B cells are observed after each dose. Among all, CP group have shown the gradual increase in the B cell population with each dose of immunization with compare to CR and CPR group **(Fig 20)**.

Helper T cells recognized by the $CD4^+$ lineage marker, produce cytokines which function in direct lysis of target cells or activates other functions such as the effector abilities of T cells and increases antibody production from plasma B cells. Cytotoxic T cells are effective especially in the case of intracellular pathogens such as parasites to exhibit killing of infected cells. As shown in the **figure 19**, the percentage of $CD4^+$ and $CD8^+$ T cells were shown to have increased in all the experimental groups. After the second dose as compared to the 1st dose, all the groups show that with the repeated immunization the cell mediated immunity can be enhanced. A peculiar observation of 10-12 % increment in $CD4^+$ and $CD8^+$ T cells was observed even after 50 days of the third dose that depicts the efficacy of the CSP as an antigen as well as the combination of different adjuvants, especially the combination of R848 and CSP antigen was proved to give the elevated helper T cell response as compared to other groups. The synergistic effect of Poly (I:C), R848 along with the antigen was able to elicit the highest $CD8^+$ T cell response after the second dose of CSP [46]. Different B cell subsets unveil different functions such as the plasma B cells manifests the protective aid by secreting antigen specific antibodies whereas the memory B cells illustrates the reactive aid by rapid differentiation into plasma cells in secondary germinal centers upon reencounter of the cognate antigen. As shown in **figure 20**, the B cell population was seen to be in high numbers even after 50 days of 3rd dose which shows the long-lasting humoral immune response with the given combination of adjuvants, especially the CP group was giving the highest B cell response, CR group was also successful in rising decent number of B cells as well. Alongside, the flow analysis of B and T cells, we have also measured the whole IgG response against the CSP protein using ELISA **(Fig 21)**.



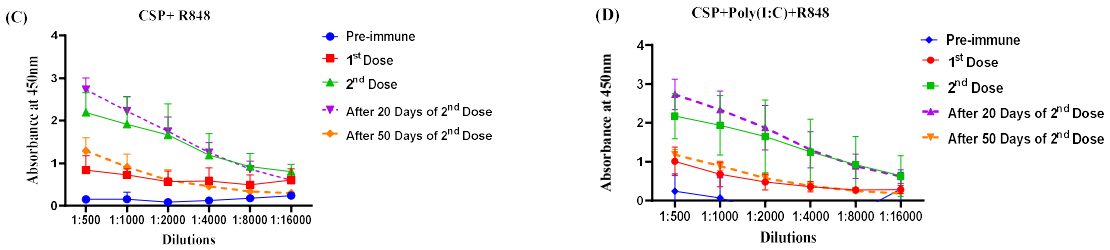


Figure 21: Antibody titer in different adjuvants-based CSP immunized mice (Immunization groups= (A) **CSP** (CSP protein only (upper left), (B) **CP** (CSP protein and Poly(I:C)) (upper right), (C) **CR** (CSP protein and R848) (bottom left), (D) **CPR** (CSP protein, Poly(I:C) and R848)) (lower right) (N=3 mice/group and bar indicates data in the form of mean ± SD of in each dose of group)

Here we have checked the whole IgG response as it constitutes more than 75% of serum via indirect ELISA. We have given two repetitive doses on the regular intervals. With the number of doses administered at every interval, the antibody titer in the serum increased concurrently. Similarly, as the dilutions of the serum increased, the decline in absorbance was observed accordingly. The increase in Ab titers upon repeated immunization, suggest the formation of plasma cells and long-lived memory B cells [46].

As illustrated in **figure 21A**, in case of CSP immunization, the breakpoint was achieved at 1:2000 showing that at this dilution it has the highest antibody titer. 1:2000 was observed to be the breakpoint in both **figure 21B and 21C**, whereas CPR showed a breakpoint at 1:1000 dilutions [46]. In CSP immunization, the antibody titers were observed to be highest after 2^{nd} dose; the antibody levels even after 50 days were higher than the 1^{st} dose showing the persistence of antibody secreting long lived plasma B cells. **Figure 21B, C and D** showcases the antibody levels of CSP administered with Poly(I:C), R848 and Poly(I:C) + R848, respectively which demonstrates a similar trend of antibody profile wherein the antibody levels after 20 days of 2^{nd} dose are higher than the 2^{nd} dose itself, and even after 50 days of the second dose the antibody levels are almost similar with the 1^{st} dose. As opposed to a steep decline in antibody levels after 20 days of 2^{nd} dose in **figure 21C** and **21D**, **figure 21B** manifests a gentle decline, from which inference can be made that Poly (I:C) along with CSP was successful in giving highest antibody response. After analyzing the trend of antibody response, we have measured the titer for each group and depicted in **figure 22.**

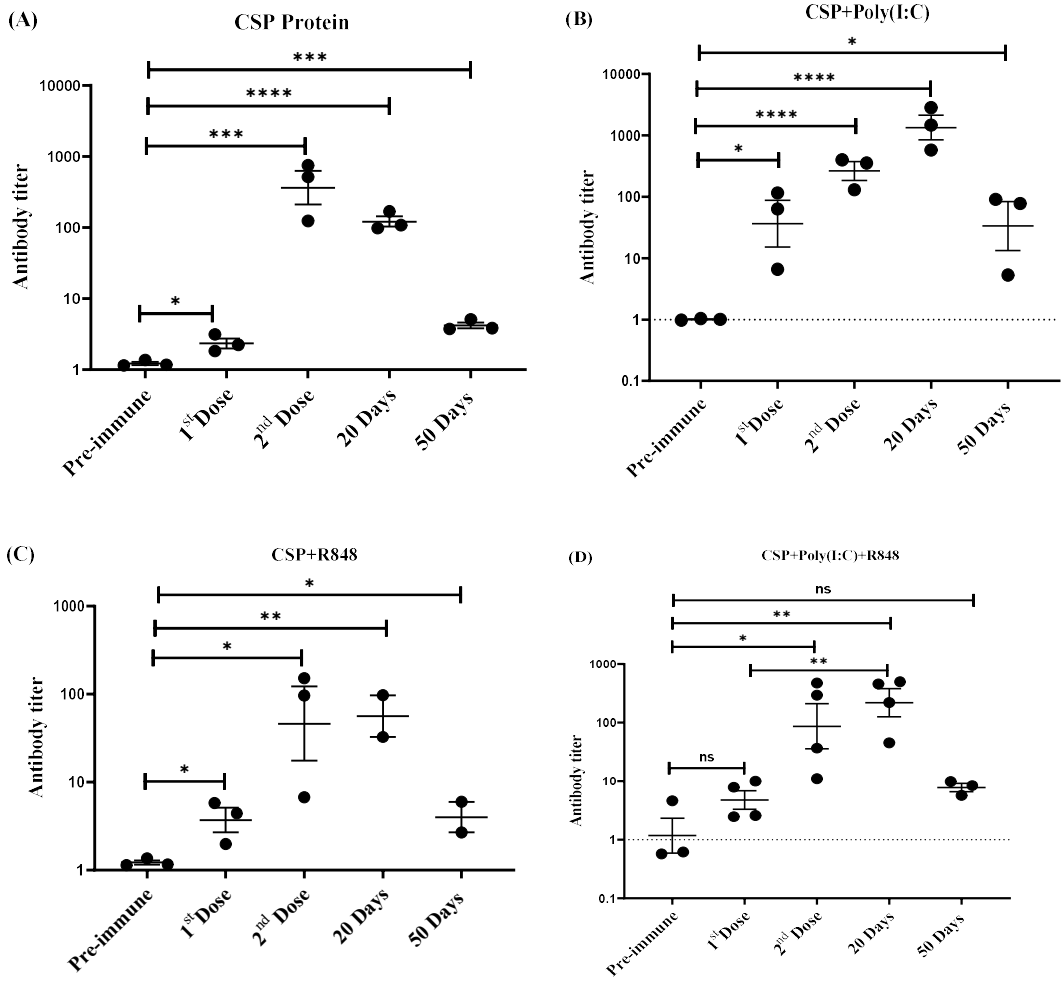


Figure 22: Antibody endpoint in different adjuvants-based CSP immunization (Immunization groups= (A) **CSP** (CSP protein only) (Upper left), (B) **CP** (CSP protein and Poly(I:C)) (Upper right), (C) **CR** (CSP protein and R848) (bottom left) and (D) **CPR** (with CSP protein, Poly(I:C) and R848)) (bottom right) (Baseline counted by calculating the breakpoint in pre-immune sera in each group and compared with respective dosage, each dot point represent the data of single mice where N=3 mice/group, values are represented in mean ± SEM) (two-tailed t-test *, **, ***, **** $P<0.05$, ns= non significant)

Dilutions upto 1:16,000 showed good antibody levels (**Fig 22**) indicating that further dilutions were required to obtain end point titer. We further checked how many number of times the dilutions are required for each dose as compared to the pre immune sera of every particular group to get an endpoint titer to avoid any erroneous results as shown in **table 3**.

Table 3: Comparison between antibody titer after each dose of CSP immunization

Groups	After 1st Dose	After 2nd Dose	After 20 days of 2nd Dose	After 20 days of 2nd Dose
CSP	4	30	24	7
CP	400	6440	830	400
CR	6.5	19	20	7
CPR	9	27	32	13

(Note: The numbers mentioned in the table represents the number of times dilution needs to be done to get an endpoint titer pre immune serum)

The data represent the generation of B and T cells against the CSP protein immunization. However, it is more of interest to know the antigen specific B and T cells against the protein antigen. Therefore, one mouse from each group have been sacrificed and tested against the CS-peptide which is specific for the CD8 T cells.

After CSP immunization, to check whether the B and T cell repertoire generated was antigen specific or not, *in-vitro* stimulation of CS peptide was given and compared with the unstimulated control. The CS peptide stimulation was given in two concentrations which are 1) 0.5 µg/well and 2) 1 µg/well (**Fig 23 and 24**).

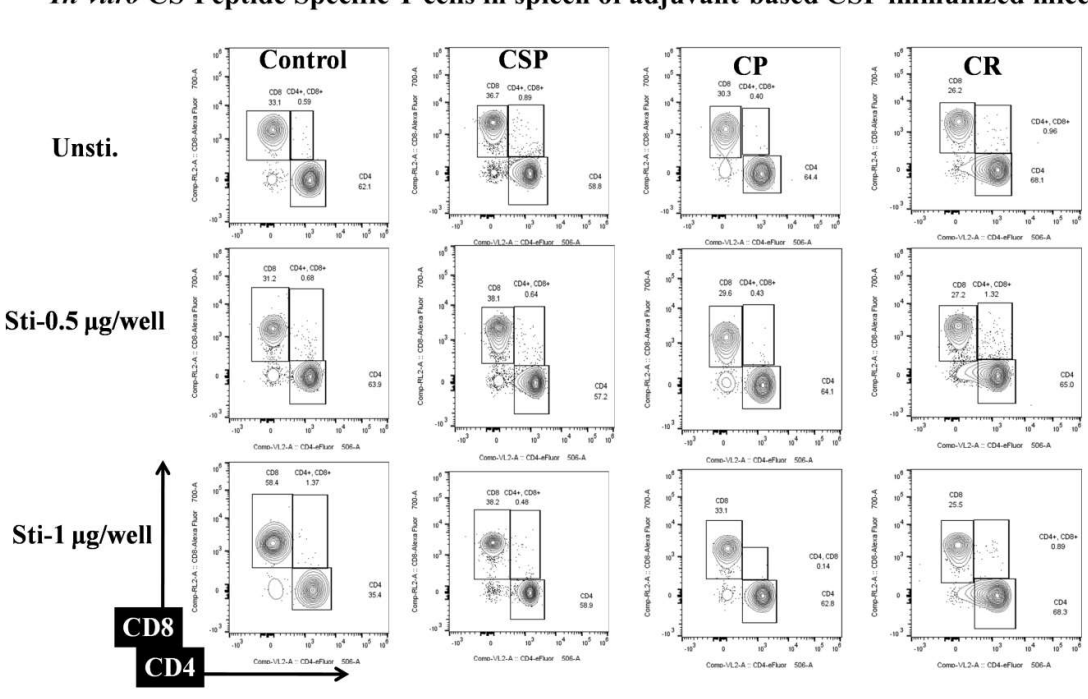


Figure 23: Generation of CS peptide specific $CD4^+$ and $CD8^+$ T cells against the *in-vitro* CS-Peptide stimulation (Immunization groups= **Control (**PBS), **CSP** (CSP protein only),

CP (CSP protein and Poly(I:C)), **CR** (CSP protein and R848), **CPR** (CSP protein, Poly(I:C) and R848)) ($CD4^+$ and $CD8^+$ T cells on X and Y axis are separated from CD3, respectively, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper, middle and bottom panel represent the data from unstimulated, stimulated with 0.5 µg/well and stimulated with 1 µg/well of CS-peptide specific to $CD8^+$ T cell epitope of CSP protein to the splenocytes of each group) (in Balb/C mice-H_2K^d)

It shows the activation pattern of $CD4^+$ and $CD8^+$ T cells in four different groups. In CSP group, levels of both helper T cell and cytotoxic T cells are almost similar in all the three cases. In CP and CR group, almost similar $CD4^+$ and $CD8^+$ cell population has been obtained with minute changes in concentration. Alongside, study the CS-peptide specific T cells; the generation of central and effector memory T cells was also monitored **(Fig 24)**.

***In-vitro* CS-Peptide Specific T_{EM} & T_{CM} $CD8^+$ T cells in spleen of adjuvant-based CSP immunized mice**

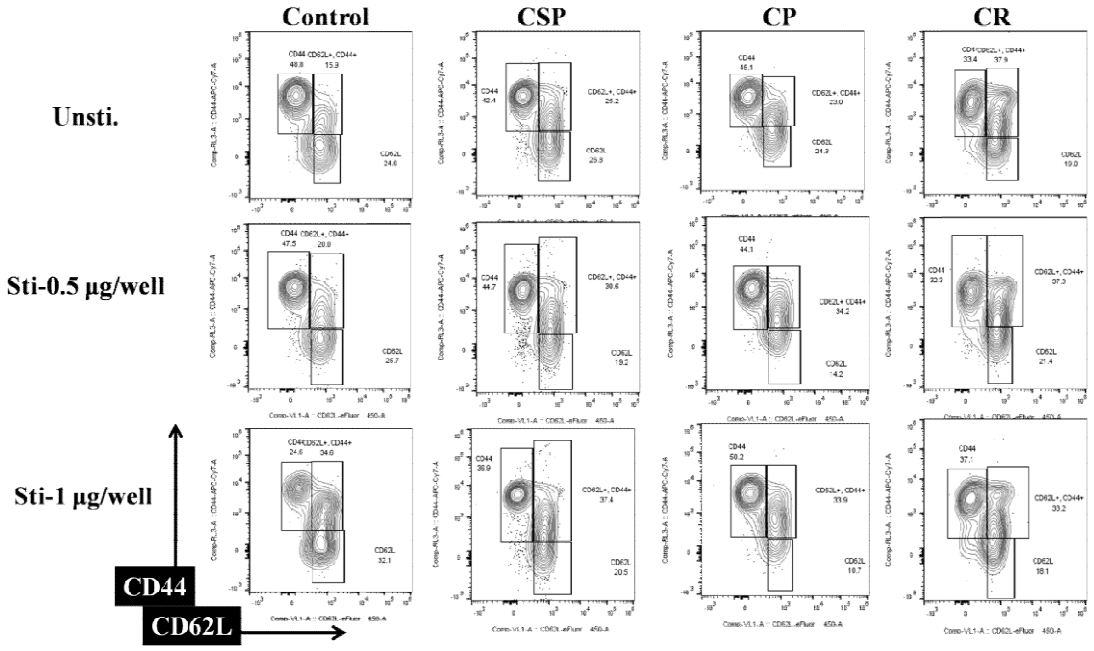


Figure 24: Generation of CS peptide specific effector (T_{EM}) and central memory (T_{CM}) $CD8^+$ T cells against the *in-vitro* CS-Peptide stimulation (Immunization groups= **Control (**PBS), **CSP** (CSP protein only), **CP** (CSP protein and Poly(I:C)), **CR** (CSP protein and R848), **CPR** (CSP protein, Poly(I:C) and R848)) ($CD44^+$ and $CD62L^+$ T cells on Y and X axis are separated from $CD8^+$ T cells, respectively, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper, middle and bottom panel represent the data from unstimulated, stimulated with 0.5 µg/well and stimulated with 1 µg/well of CS-peptide specific to $CD8^+$ T

cell epitope of CSP protein to the splenocytes of each group) (in Balb/C mice-H_2K^d) (Phenotype of T_{EM}= $CD44^+CD62L^-$ & T_{CM}= $CD44^{hi}CD62L^{hi}$)

Figure 24 shows the T_{CM} and T_{EM} cell population in the four groups 1) Control 2) CSP 3) CP 4) CR after flow cytometric analysis in *in-vitro* stimulation study. In control and CSP group, levels of T_{CM} are increasing with increasing concentration of peptide stimulation whereas the T_{EM} at 1 µg concentration. In both the CP and CR group the 0.5 µg concentration gives good central memory response whereas 1 µg concentration gives good effector memory T cell response suggesting that with increasing concentration of CS peptide, the conversion of central memory into effector memory may take place. Further, out of peptide specific T cells, IFN-γ producing $CD8^+$ T cells were also measured in terms of effector memory T cells **(Fig 25).**

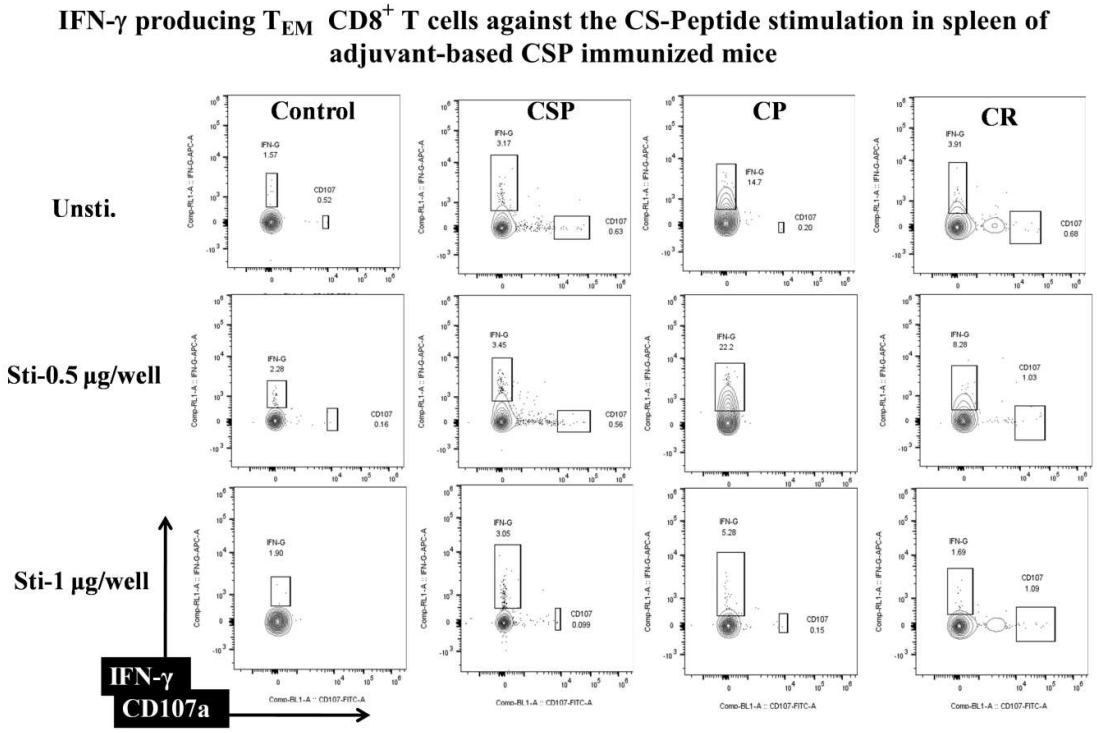


Figure 25: IFN-γ producing CTLs from CS peptide specific T_{EM} in experimental groups after *in-vitro* peptide stimulation (Immunization groups= **Control (**PBS), **CSP** (CSP protein only), **CP** (CSP protein and Poly(I:C)), **CR** (CSP protein and R848), **CPR** (CSP protein, Poly(I:C) and R848)) (CD107a and IFN-γ producing $CD8^+$ T cell specific T_{EM} cells were gated on X and Y axis and separated from T_{EM} of $CD8^+$T cells, respectively, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper, middle and bottom panel represent the data from unstimulated, stimulated with 0.5 µg/well and stimulated with 1 µg/well of CS-peptide

specific to $CD8^+$ T cell epitope of CSP protein to the splenocytes of each group) (in Balb/C mice-H_2K^d) (Phenotype of cytotoxic $CD8^+$ T cells= $CD8^+CD44^+CD107a^+IFN\text{-}\gamma^+$)

CD107a is an intracellular marker of cytotoxic T lymphocytes which protects the killer cells from death and thus supports direct killing. Figure 25 shows the IFN-γ production in T_{EM} cells, wherein in both the control and CSP group the similar levels of IFN-γ have been produced. CP shows the highest interferon gamma level at 0.5 μg concentration whereas CR shows the highest CD107a marker detection indicating the presence of killer cells. In the T cell wing, memory T cells circulate in lymphoid as well as non-lymphoid organs and based on the surface markers are characterized as central memory and effector memory T cells. The memory response is carried out by effector memory T cells (T_{EM}) which migrates towards the inflamed/injured tissues and mount the specific immune response. On the other hand, reactive memory is governed by central memory T cells (T_{CM}) which remains in the vicinity of the secondary lymphoid organs specific to T cells.

In general, T_{CM} do not have any specific function, however during the antigen encounter it rapidly proliferates and differentiates into effector cells. Therefore, during the immunization strategy we have checked the T_{CM} & T_{EM} response after each booster dose generated by $CD4^+$ and $CD8^+$ T cells. The concentration of 0.5 μg showed indistinguishable or homogenous pattern of generation of $CD4^+$and $CD8^+$T cells as compared to unstimulated control by both the Poly(I:C) and R848, indicating that the T cells were antigen specific in previous studies. In case of central and effector $CD4^+$ memory T cells, the central memory was seen to be high in numbers in case of Poly(I:C) and CSP combination especially at 1 μg concentration. Effector memory T cells were seen to be highest in case R848+CSP at 0.5 μg concentration.

In our immunization strategy we checked the T_{CM} & T_{EM} response after each booster dose generated by $CD4^+$ and $CD8^+$ T cells. The percentage of T cell population i.e., naïve T cells, central memory T cells and effector memory T cells are represented as shown in the **figure 26A**. As compared to the unstimulated control, the 0.5 μg concentration showed almost similar percentage of cells in all the three T cell subsets indicating the antigenic specificity of generated T cells in previous CSP immunization study. A peculiar observation with respect to the concentration of CS peptide indicated that with the increase in concentration the T_{CM} cells decreased and the T_{EM} cells increased thus showing that the central memory T cells may convert into effector memory T cells which mediates direct killing of target cells upon antigen exposure. Further IFN-γ production by $CD4^+$ T cells was checked for which in both the adjuvants, 0.5 μg concentrations showed dominance in producing high IFN-γ levels **(Fig 26B)**. **Figure 26 C** shows IFN-γ production from $CD8^+$ effector T cells, the 0.5 μg

concentration in all the 4 groups produces higher amounts of IFN-γ. CP has a highest production of IFN-γ, suggesting that 0.5 µg can be optimized further for future experiments.

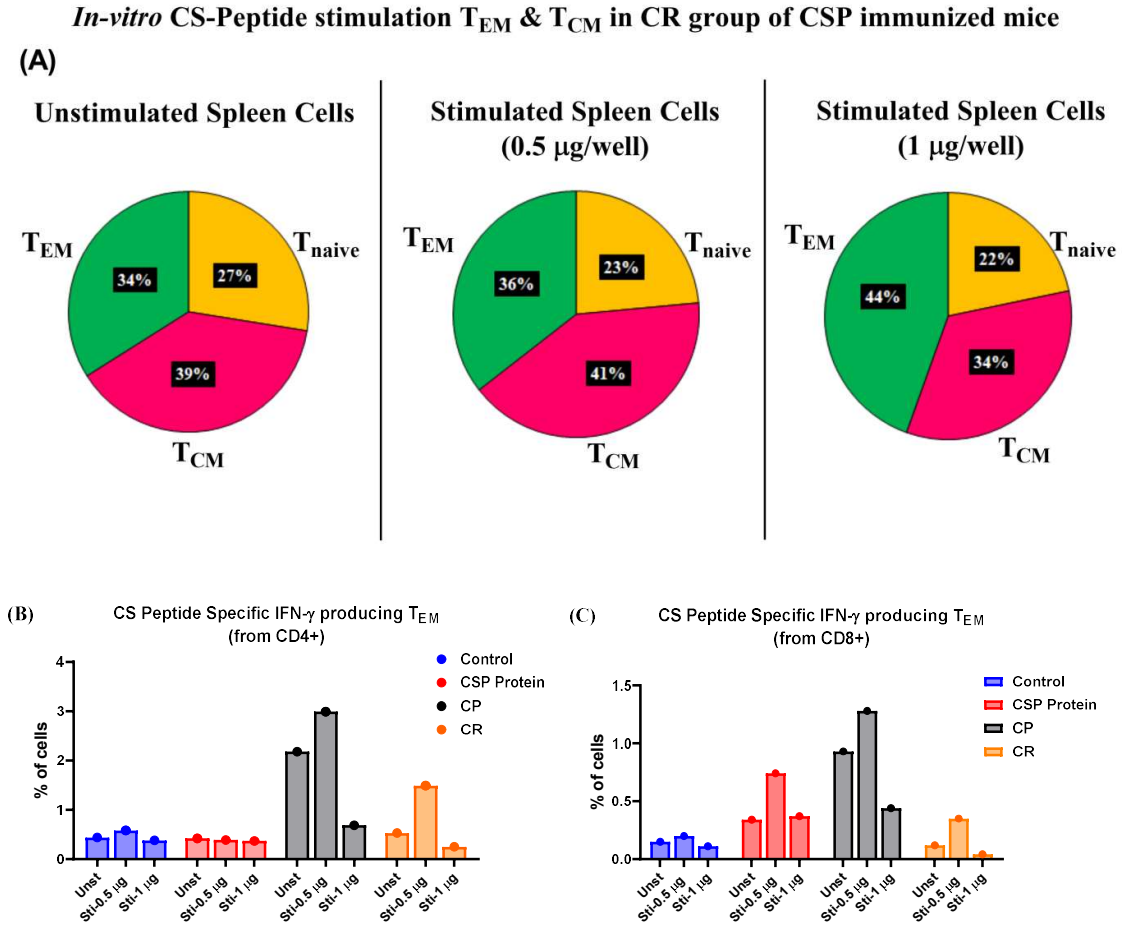


Figure 26: Status of CS-Peptide specific CD8$^+$ T cells in splenocytes of different adjuvants-based CSP immunized mice which were subjected to 3 different conditions of (I) Unstimulated (II) CS Peptide stimulation of concentrations 0.5µg and (III) 1µg. **(A)** CS Peptide specific CD8$^+$ T cells in CR group; unstimulated (left), stimulated with 0.5 µg/ml (middle) and stimulated with 1.0 µg/ml (right) splenocytes **(B)** IFN-γ CD4$^+$ T_{EM} in adjuvant-based CSP immunized mice **(C)** IFN-γ CD8$^+$ T_{EM} in adjuvant-based CSP immunized mice

After analysing the blood cells of CSP immunized mice, we wanted to scrutinize if these cells were antigen specific thus, to identify CD19$^+$ population, the protocol to harvest splenocytes was executed and these cells were bifurcated into unstimulated and stimulated (1 µg/ well) population. These cells were further analyzed on Day 1, 3 and 5 of stimulation with the help of flow cytometer. Increasing population of B cells was to be observed upon the above-mentioned days; however R848 was giving the highest peaks of B cells among all the other groups (data not shown).

Humoral immunity upon re-exposure to pathogens relies on the two arms of B cell family- LLPCs (long lived plasma cells) and Memory B cell that come from GCs (germinal centre) which is subjected to primary response. Following the previous B cell population trail, we wanted to inspect if within this pool of population, were $CD73^{+}CD80^{-}$ memory B cell population. Adjuvant R848, upon stimulation increased the proliferative capacity of these cell types, as compared to other groups (data not shown).

B cell memory pool is a network of multiple subsets of this species which can include unswitched and unmutated cells, in order to do so, we analyzed the unswitched memory B cell population, i.e., the triple positive (antigen experienced) cells: $CD19^{+}CD73^{+}IgM^{+}$, which share the attributes of functional memory B cells. Poly (I:C) was able to give rise to high number of these cells, however upon stimulation, a decrease in the population was observed which in turn suggest that these cells might have switched. (Data not shown) To satisfy our speculation, we examined Switched memory B cell niche, i.e., $CD19^{+}CD73^{+}IgG^{+}$ cells. Antigenic stimulation increased the proliferative rate of these cells in all groups, CR group showed potential in the increase of cell population upon stimulation. (Data not shown)

To characterize memory B cell pool, CD73, CD80, CD273 have been used to denote this population into 'less mature' and 'mature' groups. We looked for $CD73^{+}CD80^{+}$ double positive MBCs, each group from our data showed adequate number of cells, R848 was able to maintain this population, however, the cells started to dwindle upon antigenic stimulation in the Poly (I:C) group (Data not shown). To look further into the data, we wanted to investigate $CD19^{+}CD73^{+}$ cells for IgG and IgM production. Upon antigenic stimulation the proliferative rate of $CD19^{+}CD73^{+}$ cells elevated by 20% in CR group. We wanted to investigate further, for triple positive $CD19^{+}CD73^{+}IgM^{+}$ cells, which represent unswitched memory phenotype; our data suggests that upon stimulation, the percentage of this phenotype increased by 8%**.** We wanted to look further in our data, so we examined cells positive for $CD19^{+}CD73^{+}IgG^{+}$ and our data suggests that there was 77% of IgG secreting cells upon antigenic stimulation and unstimulated B cells had 85% of IgG producing cells.**(Figure 27)**

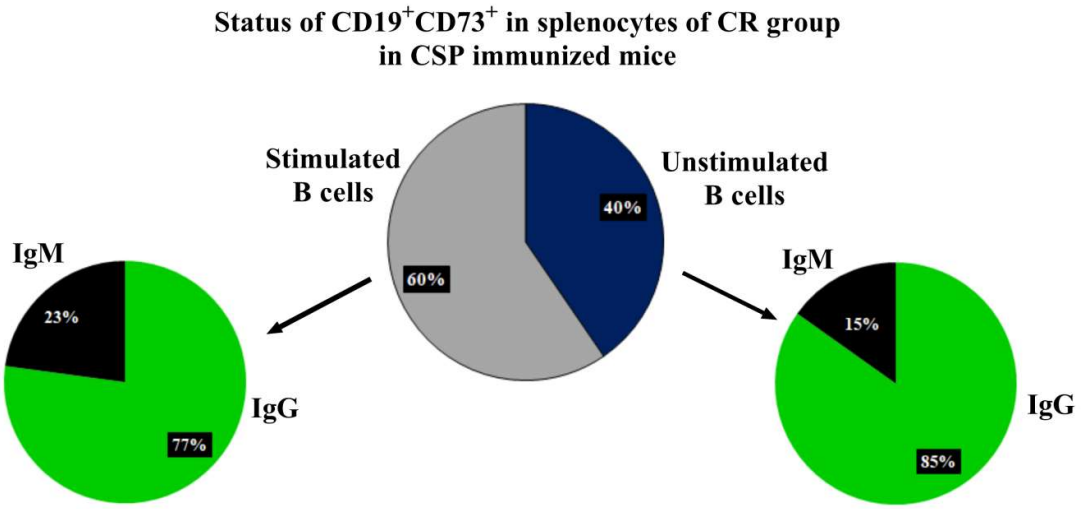


Figure 27 Status of antigen specific $CD19^{+}CD73^{+}$ B cells in splenocytes of CR group in CSP immunized mice. Two different conditions of (I) unstimulated (II) stimulated (CSP antigen-1µg/well). IgM and IgG producing B cells from CSP stimulated (left side) and unstimulated population (right side).

We also demonstrated antigen specific B cell response in bone marrow of different adjuvant-based CSP immunized mice. To isolate bone marrow cells protocol 6.2.5.1 was followed and the cells were subjected to antigen specific assay, which was followed by protocol 6.2.6. Bone Marrow is the major organ for production, development, and maturation of B cells which derive from HSCs (hematopoietic stem cells) before they are released into peripheral blood to reach the secondary lymphoid organs. Here, we first sorted the $CD19^{+}$ population from BM, Poly I:C and R848 gave relatively the same results, however a decline in trend was noticed upon antigenic stimulation (data not shown).

One of the hallmarks of our immune system is its ability to 'recall' from previous exposures to pathogens, this complex machinery entwines the fate of future infections. Although humoral immunological is mediated by serum antibody titre which is secreted by short lived plasma cells (SLPCs) and long-lived plasma cells (LLPCs), however, humoral immunity is not limited to Ab titres, it includes Memory B cells which constitute as a critical arm of the humoral response, however, they are subjected to class switch upon antigen re-exposure and ultimately secrete antibodies. Our data depicts the double positive CD19/CD27 cells in BM, a similar trend is observed in Poly(I:C) and R848; with antigenic stimulation, the percentage of cells have increased, although R848 group had a towering number of MBCs (data not shown).

B cells that depart from GCs can either meet the memory B cell pool or the plasma cells which reside in the BM or the gut. As mentioned earlier PCs can be subdivided into SLPCs or LLPCs and are renowned for secreting high amounts of antibody upon exposure to pathogen. To further investigate, the diverse population of Bone Marrow, we looked for plasma cells that are $CD19^{-}CD138^{+}$, upon stimulation Poly (I:C) had shown rapid proliferation of PCs (data not shown).

PCs are the key for robust antibody production and they may secrete up to 10^3 antibody per second, they maybe SLPCs (which have the proliferative capacity up to only 3-5 days) and LLPCs (which are 'non-proliferative and may live till several months) and these all phenotypes can be termed as ASCs (Antibody Secreting Cells). To further decipher the data, we looked into $CD19^{-}CD138^{+}IgG^{+}$ cell type; each group had IgG producing PCs, upon stimulating each group, it was observed that IgG levels were decreased, on the contrary IgG levels in the adjuvant R848 were quiet high compared to other groups.

7. 2 SLTRiP Immunization

Total lymphocytes were first gated on a side scatter area (SSC-A)/ forward scatter area (FSC-A) plot and then gated on the $CD3^{+}$ and $CD3^{-}$ population. The $CD3^{+}$ plots were then gated into $CD8^{+}$ and $CD4^{+}$ T cell populations, these two populations were then gated for IFN-γ **(Fig 28)**. A population of $CD44^{+}$ was separated from $CD3^{+}CD8^{+}$ phenotype. Later the $CD3^{-}$ cells were gated for the B cell population in the peripheral blood, i.e., $CD19^{+}$ and along with $CD3^{-}$ $CD19^{-}$, which may probably be NK (natural killer) cells. These $CD19^{+}$ cells were then further gated for the subsets of interest, namely, $CD73^{+}$ and $CD80^{+}$ to analyze the memory B cell population **(Fig 28).**

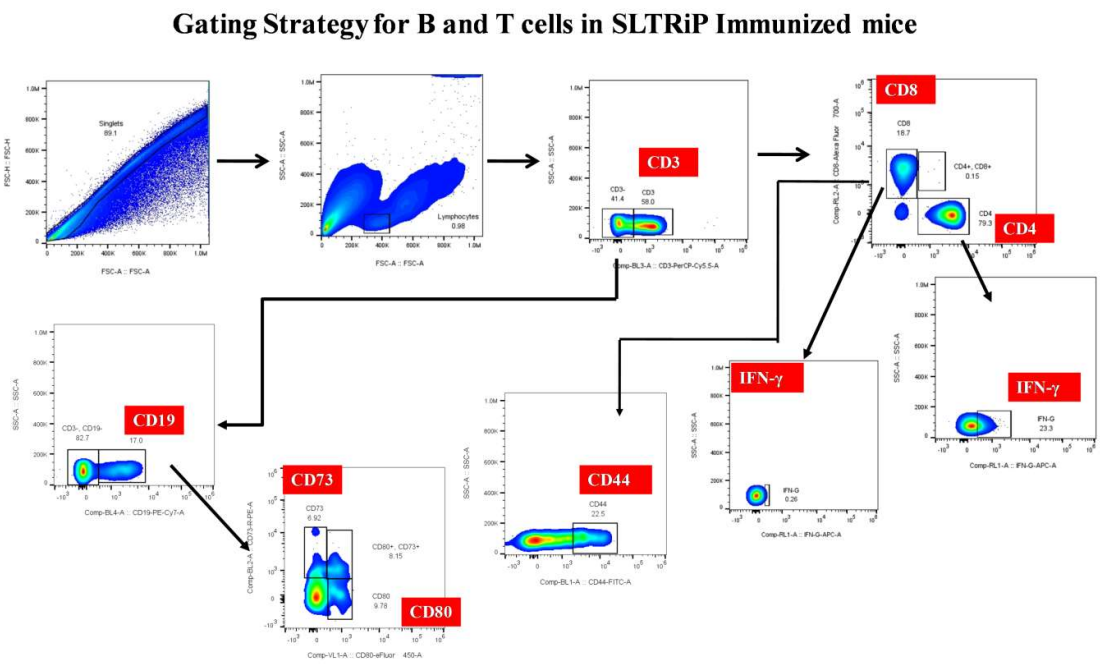


Figure 28: The common gating strategy to analyze the T effector, T memory, and cytotoxic T lymphocytes from $CD4^+$ and $CD8^+$ T cells and memory B cells from $CD19^+$ cells in SLTRiP immunized mice

$CD8^+$ and $CD4^+$ T cell populations were observed in all the mice groups. However, SLTRiP immunized mice showed elevated levels of T cell subsets upon first and second immunization as compared to the control and GST groups **(Fig 29)** [47].

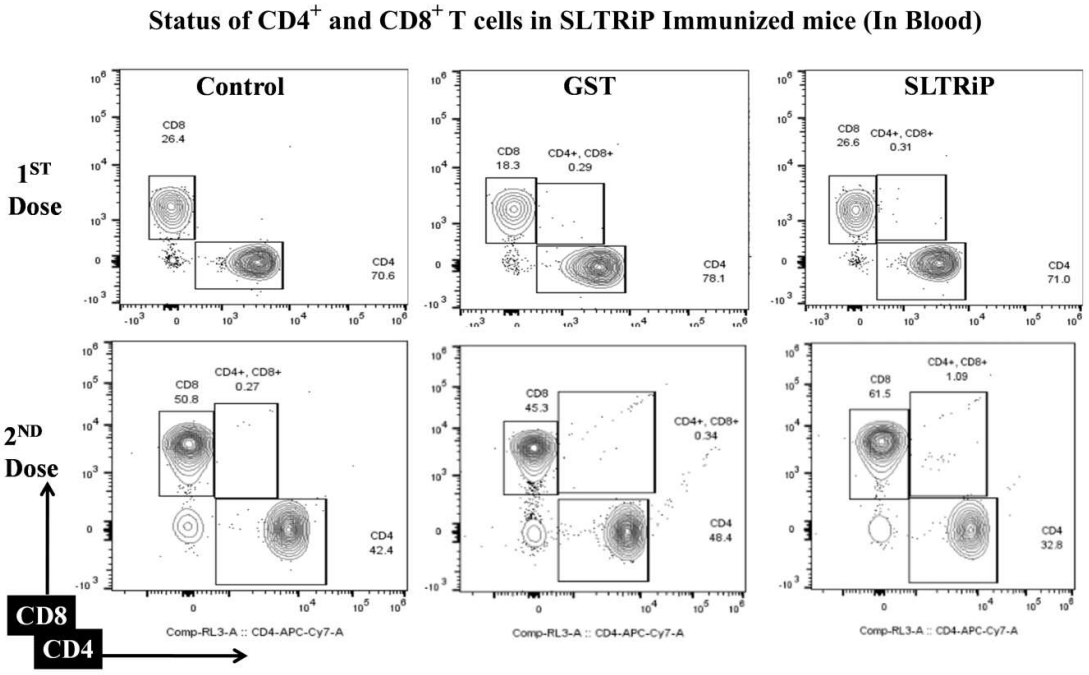


Figure 29: $CD4^+$ and $CD8^+$ T cells in experimental groups of mice after two doses of SLTRiP Immunization (Immunization groups= **Control** (PBS), **GST** (GST & Poly(I:C)) **and SLTRiP** (SLTRiP protein and Poly(I:C)) ($CD4^+$ and $CD8^+$ T cells on X and Y axis were gated from CD3, respectively, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper and bottom panel represent the data after 1st and 2nd dose of SLTRiP immunization)

The T cell subsets, namely, $CD8^+$ and $CD4^+$ T cells were subjected to their cytotoxicity levels in the first round of immunization. As compared to $CD8^+$, $CD4^+$ T cells were observed to be more cytotoxic in all the groups **(Fig 30)**.

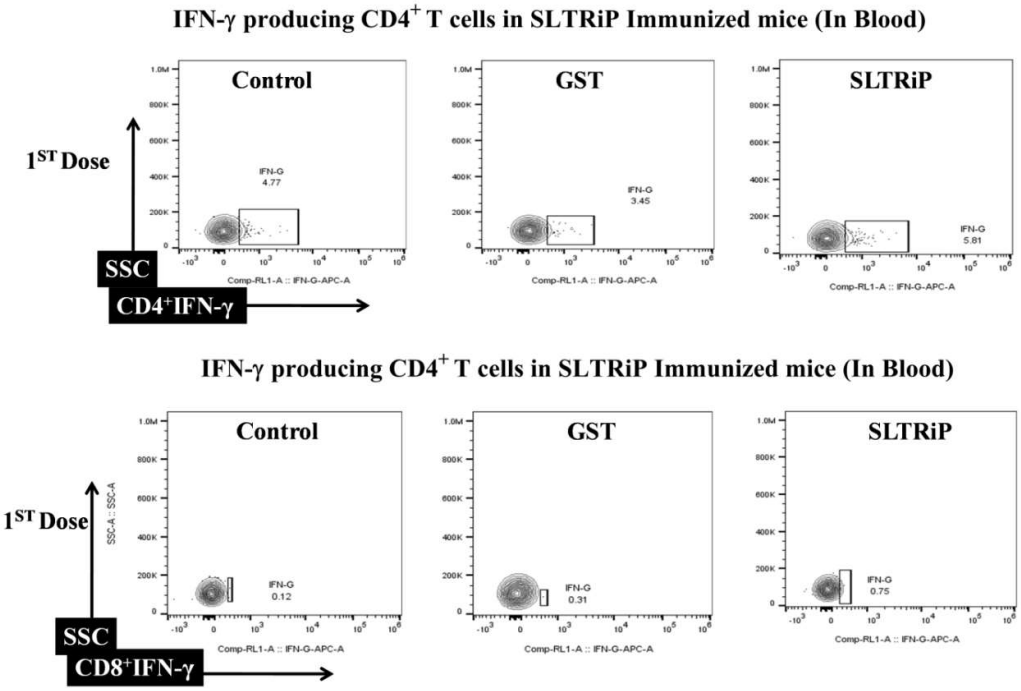


Figure 30: IFN-γ producing $CD4^+$ (upper panel) and IFN-γ producing $CD8^+$ T cells (lower panel) in SLTRiP immunized mice after 1st doses (Immunization groups= **Control** (PBS), **GST** (GST & Poly(I:C)) **and SLTRiP** (SLTRiP protein and Poly(I:C)) (IFN-γ producing $CD4^+$ and $CD8^+$ T cells on X axis were gated from $CD4^+$ and $CD8^+$ T cells, respectively, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper and bottom panel represent the data after 1st dose of SLTRiP immunization)

$CD19^+$ cell population was analyzed with the help of a flow cytometer upon priming and the subsequent dose of immunization. Comparing the 1st and 2nd dose, a distinguished population of B cells was observed after 14 days of 2nd immunization, along with that, the B cell population proliferated in higher numbers as compared to the first dose **(Fig 31)**.

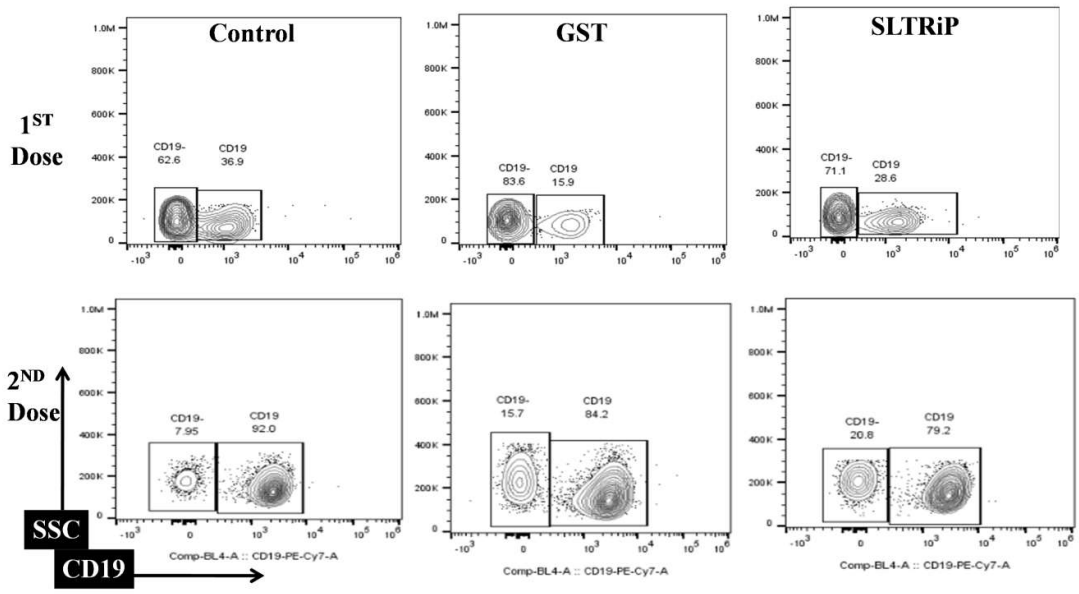


Figure 31: Status of B cells in experimental groups of SLTRiP immunized mice (Immunization groups= **Control** (PBS), **GST** (GST & Poly(I:C)) **and SLTRiP** (SLTRiP protein and Poly(I:C)) ($CD19^+$ B cells are separated from $CD3^-$ population on X axis,, individual gate depict the number of cells from parent one and each box is the representation of individual group where N=3 mice/group; Upper and bottom panel represent the data after 1st and 2nd dose of SLTRiP immunization)

Cellular and humoral immune responses are the key to eradicating any pathogen-affected cell and their persistence in the circulation could potentially provide protective and sterile immunity. Here we checked the immunogenicity of SLTRiP protein along with the adjuvant, Poly(I:C), which gave optimum results from the previous CSP immunization study, hence resulting in the generation of serum antibody as well as induction of cytotoxicity in cells. SLTRiP protein was able to generate both the lineage of T cells, that is, $CD4^+$ and $CD8^+$T cell phenotype, and maintained them during the 2nd dose of immunization as well [47]. However, further analysis of the antigen specificity of the cells needs to be analyzed (data not shown.) Moreover, SLTRiP protein along with Poly(I:C) adjuvant was tested for their B cell response upon two subsequent doses of immunization. **Figure 32** represents the $CD3^-CD19^+$ population, a similar pattern of B cell was observed, with an increase of this population upon 2nd dose in all the groups, although, the SLTRiP group had a pre-eminent population of $CD19^+$ B cell population.

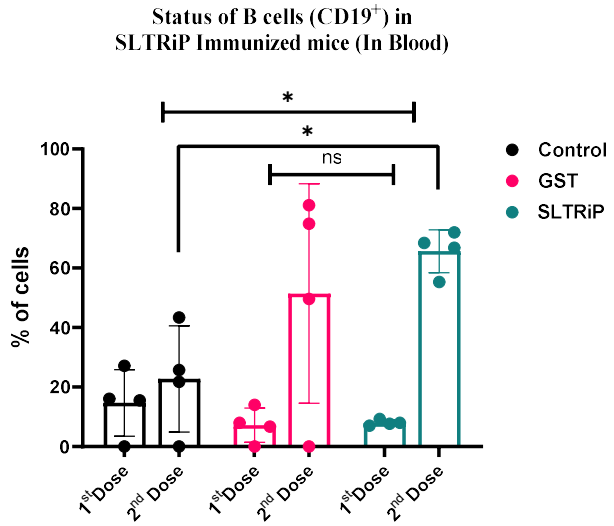


Figure 32: Status of activated B cells in SLTRiP immunized mice (Immunization groups= **Control** (PBS), **GST** (GST & Poly(I:C)) **and SLTRiP** (SLTRiP protein and Poly(I:C)) (N=3 mice/group, each dot represent the value of individual mice and bar indicates the mean ± SD of in each dose of group) (2way ANOVA *P<0.05 and multiple t test; ns= non significant)

Mostly all viruses and bacteria expand within the cytoplasm of the cell and they can only be eradicated by destruction or modification of the infected cell, here Cytotoxic T Cells (CTLs) come to the rescue, these cells release perforin and granzyme B to kill the targeted cell in an MHC class I fashion. IFN-γ production is associated with both innate and adaptive immune responses, and plays a major role in eradication of intracellular pathogens., the naïve $CD4^+$ T cells and $CD8^+$ T cells secretes minute amounts of IFN- γ, however unlike $CD8^+$ T cells which directly are destined to form IFN- γ producing CTLs, $CD4^+$ T cells differentiate into 4 major cell subsets named Th1, Th2, Th17 and Treg cells out of which only Th1 promotes production of substantial amounts of IFN- γ [48]. As shown in figure 33, to check their cytotoxicity, both $CD4^+$ and $CD8^+$ cells were analyzed. $CD4^+$ were found to be more cytotoxic as compared to the $CD8^+$ cells (data of IFN-Ɣ from $CD8^+$ not shown), which could mean that this lineage might provide protective immunity, however, further analysis needs to be performed to establish this suggestion.

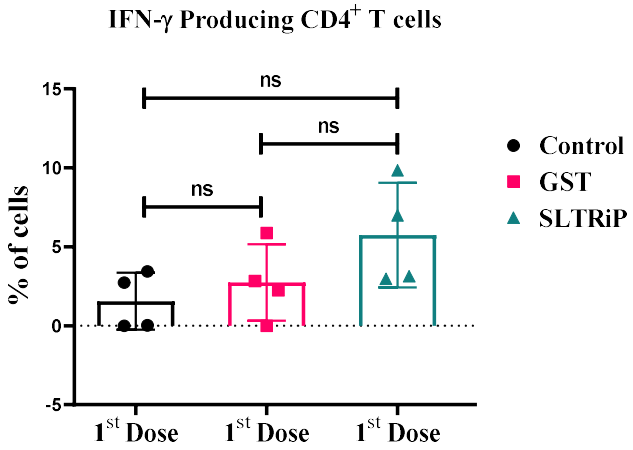


Figure 33: Status of IFN-γ producing CD4$^+$ T cells in blood of SLTRiP immunized mice (Immunization groups= **Control** (PBS), **GST** (GST & Poly(I:C)) **and SLTRiP** (SLTRiP protein and Poly(I:C)) (N=3 mice/group, each dot represent the value of individual mice and bar indicates the mean ± SD of in each dose of group) (multiple t test; ns= non significant)

Persistence of memory B cell is the key feature of long-lasting protection to any future infections. Memory B cell in circulation, upon re-exposure can switch and produce vast numbers of antibody than naïve B cells. Antibody produced by these MBCs have high avidity and affinity as it goes under multiple rounds of somatic hypermutations in the GCs, as they undergo affinity maturation, followed by, V(D)J recombination. Circulating MBCs get activated earliest and are the major players in producing high titer of antibody.

CD73 is an ectoenzyme that catalyzes the conversion of AMP to adenosine, thus playing a role in inflammatory responses. CD73 expression is required for the optimal responses of BM derived plasma cells. The CD73 mediated adenosine signalling plays a prominent function in mature germinal centre and thus have an important role in sustaining the long-lived plasma cells [49].

CD80 belongs to B7 family member which can bind to molecules like CD28, CTLA-4, and PD-L1.CD80 has nonredundant roles in T_{FH} cell development, GC B cell survival, and development of long-lived plasma cells. CD80 is also responsible for regulation of survival and differentiation of both B and T cells within the GC via ongoing B–T interactions [50].

CD73$^+$ CD80$^+$ B cells are known as the differentiated memory B cells which when expressed together on IgM producing or switched memory B cells have been evidently shown to have higher somatic hyper mutations as compared to unmutated MBCs and have greater affinity

for antigen as compared to single positive cells. Upon reinfection, the double positive MBCs show robust ability to differentiate into plasma cells [51].

Memory B cell subsets with CD73, CD80 and CD273 as their surface markers provide a wide range of phenotypic characteristics, on one end having the capability of seeding new germinal centers and on the other end having the potential to differentiate into plasma cells. After 2 doses of SLTRiP immunization, the generation of different memory B cell subset was studied. After the first dose, the $CD80^+$ cells were higher which suggests that these cells had the robust ability to differentiate into plasma cells but lacked the ability to seed secondary germinal centers **(Fig 34)** whereas after the second dose the $CD73^+$ population increased suggesting that upon antigen re-exposure, the formation of mature germinal centers was supported via higher CD73 expression, which in turn also upregulated the survival of antibody secreting long lived plasma cells [49]. The double positive cells capable of both were also seen in lower percentage in both the cases showing the generation of differentiated memory B cells via SLTRiP **(Fig 34)**.

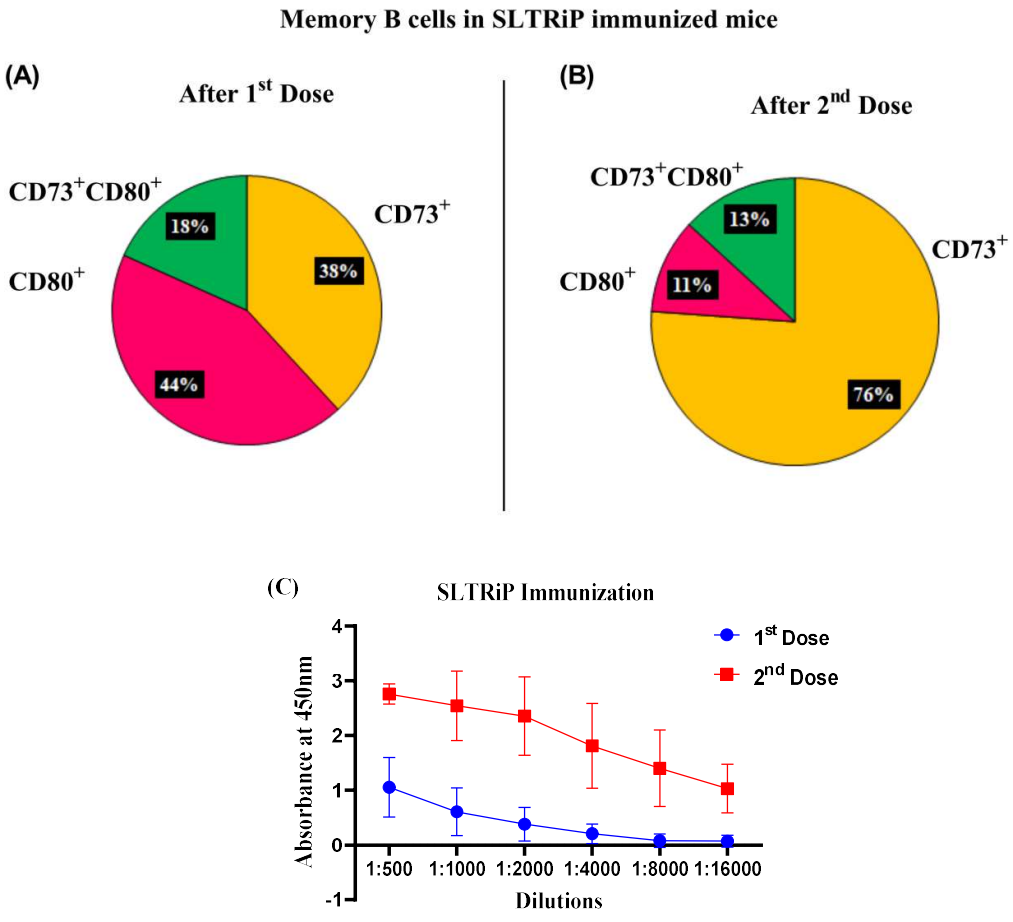


Figure 34: Status of memory B cells and antibody titer in blood of SLTRiP immunized mice. Memory B cells have been identified as $CD73^+$ and $CD80^+$ alongside double positive memory cells as $CD73^+CD80^+$ B cells in SLTRiP immunized mice **(A)** after 1st dose (left side) and **(B)** after 2nd dose (right side) **(C)** antibody titer in SLTRiP immunized mice after two dosage (N=3 mice/group and bar indicates data in the form of mean ± SD of in each dose of group)

The double positive cells were seen in lower percentage in both the doses of immunization which suggests the generation of differentiated memory B cells via SLTRiP along with Poly(I:C). We wanted to scrutinize these cells for antibody production so the mice were subjected to retro-orbital blood collection and ELISA was performed following protocol 6.2.3. **Figure 34C** compares the Antibody (IgG) titre upon first and second dose of immunization. Upon second dose, the IgG production increased immensely which may suggest that Poly(I:C) along with SLTRiP can not only induce CTLs production but also provide robust antibody production, however, antigen specific study could lead us to more promising results.

8. Summary

To date, vaccination is the prime way to protect living beings against invading pathogens and most of the vaccines focus on generating B cell immunity which includes neutralizing antibodies, however, little to none focus is given to CMI response. Therefore, developing an effective adjuvant based vaccine with the focus on both the wings of immune system is required.

In this study, we examined the effects of Poly(I:C), R848 and its combination to protein antigen, namely, rCSP and SLTRiP and we found the pattern of B and T cell response. Both of the adjuvants, along with its combination were able to generate $CD4^+$ and $CD8^+$ T cells population, although Poly(I:C)-adjuvanted rCSP and SLTRiP induced robust antibody (IgG titre) production, suggesting Th-2 influenced response by Poly(I:C). Additionally, it also delineates the conversion of T_{CM} to T_{EM} followed by IFN-γ production which suggests its cytotoxic activity. In continuation, we have observed a pattern of memory B cell population (with IgM and IgG production) in both Poly(I:C) and R848 upon stimulation.

Both R848 and Poly(I:C) used in this study belong to TLR agonists, hence they activate and trigger the TLR signalling pathways which may cause certain level of inflammation either, locally or systematically therefore, lower dosage of the adjuvants were used in this study to curtail down the inflammation induced.

In nutshell, we demonstrated the discrete effects of Poly(I:C) and R848 on the induction of varied population of B and T cells, antigen-specific responses, as well as higher antibody production. Despite the encouraging results of both the adjuvants in activating the immune system, detailed experimentation are required for further validation.

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