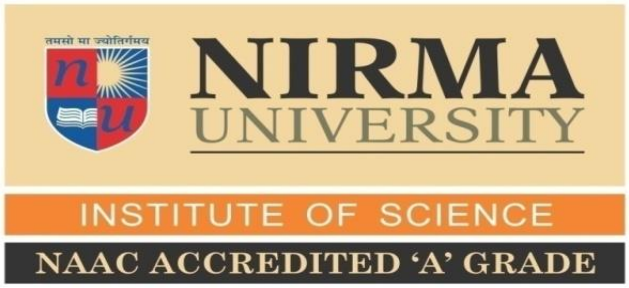
Infectious status of sporozoites evoke Innate response, leading to Cell-mediated protective CD8^{+}T cell response against *Plasmodia* infection

In Partial fulfillment of requirement for
The Degree of

MASTERS OF SCIENCE
IN
BIOTECHNOLOGY/MICROBIOLOGY



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First of all we thank **almighty** for showering his blessings on us and providing strength and courage at all time.

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experiments. We would also like to thank our friends **Shrusti Dave** and **Milan Patel** for being our "coordination compounds" supporting us through the entire dissertation period and fulfilling our requirements for tea, food and refreshments at all the times we had to stay up late.

We are also grateful to the staff members of Maharaja Sayajirao University (MSU), Baroda for providing access to Flow Cytometer.

And last but not at all the least; we would like to thank our lovely parents for understanding us and encouraging us to pursue our dreams. Without their blessing we would not have reached where we are today.

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Thank You!

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Ritika Parikh Khyati Patel

Contents

ABBREVIATIONS

Ab	Antibody
APC	Antigen Presenting Cell
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
DC	Dendritic Cells
DEPC	Diethyl Pyro Carbonate
DNA	Deoxyribonucleic Acid
DAMPs	Danger Associated Molecular Patterns
EtBr	Ethidium Bromide
FACS	Fluorescence Activated Cell Sorter
IFN-γ	Interferon gamma
IL	Interleukin
IRF	Interferon Regulatory Factor
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
PAMPs	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffer Saline
RAS	Radiation attenuated sporozoites
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TAE	Tris-Acetate-EDTA
TNF-α	Tumor Necrosis Factor alpha

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INTRODUCTION

General overview on malaria

Malaria, a vector-borne infectious disease, is currently a universal concern with a significant social, economic, and human cost, mainly in developing countries. Malaria still remains a serious challenge to human health despite tremendous development in healthcare system. (WHO 2015). The genus *Plasmodium*, the causative agent of malaria, is a member of the Apicomplexa phylum of protozoa that contains more than 5000 named species (spp.), mostly obligatory intracellular parasites, and all with a complex of organelles at the apical end of the invasive stages. Different *Plasmodium* spp. can infect different vertebrates, including human, other primates, rodents, birds, and even reptiles. Four distinct *Plasmodium* spp. are known to be pathogenic to man, *Plasmodium falciparum, P. malariae, P. vivax* and *P. ovale*. A significant amount of research has been made in *Plasmodium* spp. that infects rodents; these are *P. berghei* and *P. yoelii*.

Attempts to control the spread of the disease have been severely impeded by the emergence of drug-resistant parasites as well as insecticide-resistant mosquitoes. Therefore, a safe and effective anti-malarial vaccine is required to be developed to win the combat against malaria.

Plasmodium life cycle and immune response

The *Plasmodium* life cycle is quite complex and comprises two hosts: (i) the definitive host, which is the female Anopheles mosquito, and (ii), a vertebrate host. Malarial life cycle begins with the bite of an infected female *Anopheles* sp. mosquito, initially having symptomless pre-erythrocytic stage followed by the invasion of mature erythrocytes by merozoites and the initiation of pathogenicintra-erythrocytic stages (Fig. 1).

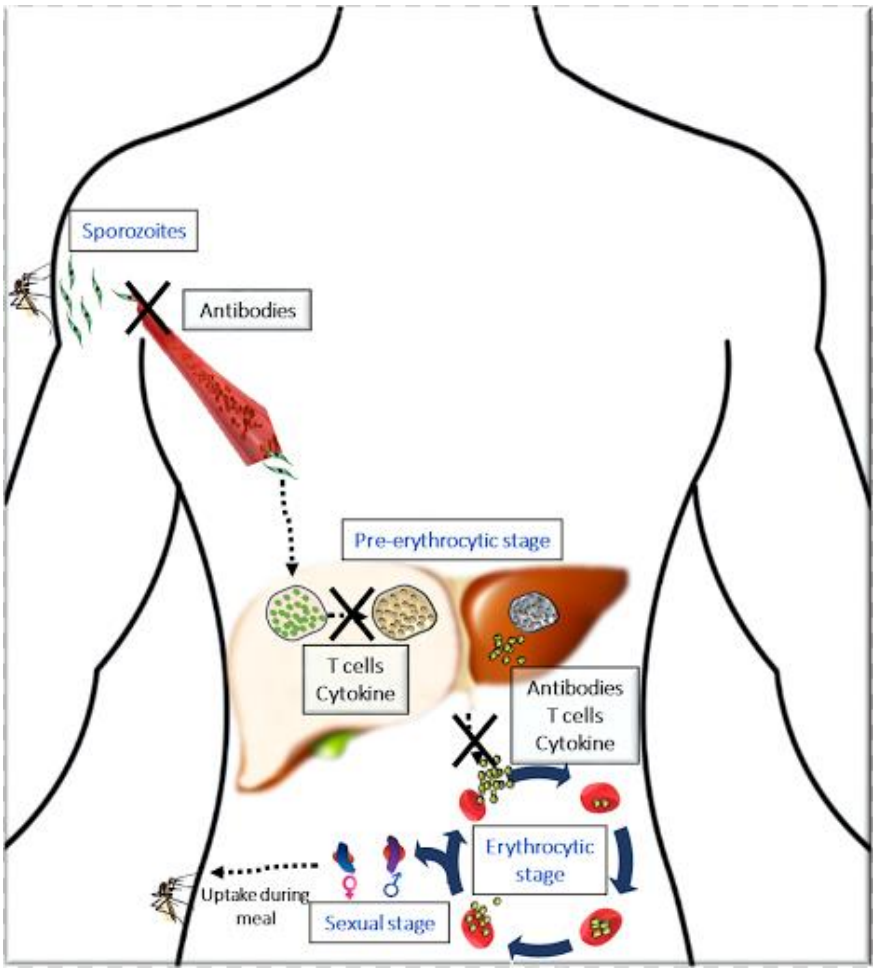


Figure 1: Life cycle of malaria parasite and possible immune mechanisms involved at various stages of the *Plasmodium* life cycle for interruption of infection**:** Infected female *Anopheles* mosquito delivers the sporozoites into the bloodstream. They pass quickly into the human liver where they multiply asexually as merozoites, causing no symptoms. Parasite stages in the liver are clinically silent. Fever and severe malaria are associated with the parasite cycle in the blood, as well as adherence of infected RBCs to blood vessel endothelium and to each other. Then merozoites invade erythrocytes and multiply until the cells ruptures releasing merozoites and infect mature erythrocytes. This cycle is repeated, causing fever each time parasites break free. Some merozoites develop sexually to form gametocytes, which are infective to mosquito. In the liver stage it is possible that protective immune response is generated by $CD8^{+}$T cells and cytokine. At the blood stage, antibodies and $CD4^{+}$T cells are responsible for the controlling the *Plasmodium* infection.

Liver stage vaccines against malaria

Although, use of anti-malarial drugs has helped reduce the mortality but emergence of drug resistant parasite demands for an effective vaccine to control malaria. But such malaria vaccine development is critical due to the complexity of the parasite and its life cycle [1, 2], antigenic variation [3], and a poor understanding of the interaction between *P. falciparum* and the human immune system [4]. Tremendous research is undergoing for development of effective vaccine but only RTS, S a pre-erythrocytic subunit vaccine has gone through phase-III clinical trials by inducing antibody and $CD4^+$ T cells response against surface protein the CSP (Circum sporozoite protein), expressed on the surface of sporozoites [5]. But protection generated by RTS,S was very short lived last upto 6 months [6]. Various studies have suggested that in malaria endemic area protective immunity is achieved in individuals who have lived there for 6-7 years due to repeated exposure of malarial parasites. However, they lost protection, if they stay away from malaria endemic areas for 2-3 years. This indicates that repeated exposure to malaria parasite is critical to maintain the immunity (Fig 2).

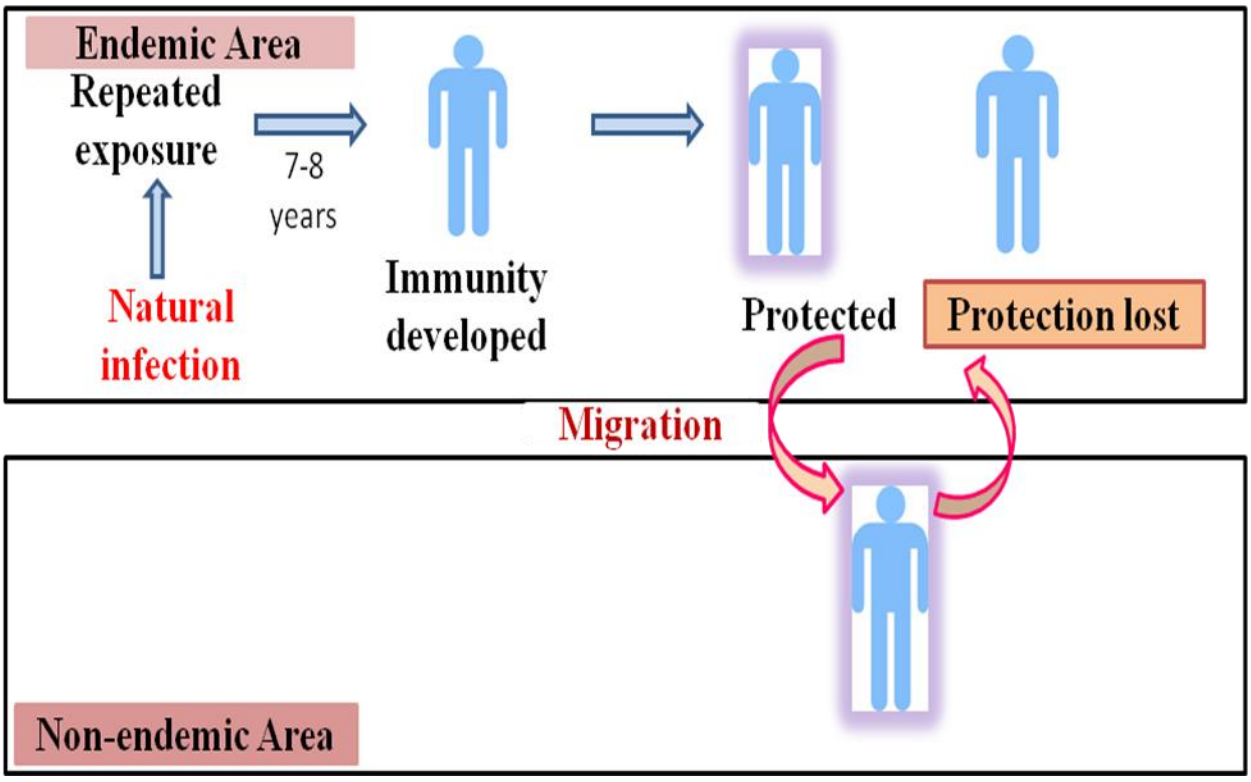


Figure 2:- Maintenance of protective immunity.

Experimentally, it has been shown that by using radiation attenuated sporozoites (RAS), sterile immunity is developed by three immunizations with RAS in biweekly time, but the protection lasts only about 6 months. But interestingly the protection is prolonged to 18-months or beyond when infectious sporozoite challenge was given before 6 months, reflecting the condition in malaria endemic areas where people get repeated exposure to parasite and maintain their anti-malarial immunity [7]. Therefore one can suggest that repeated exposure to antigens might be responsible for development of long lived immunity.

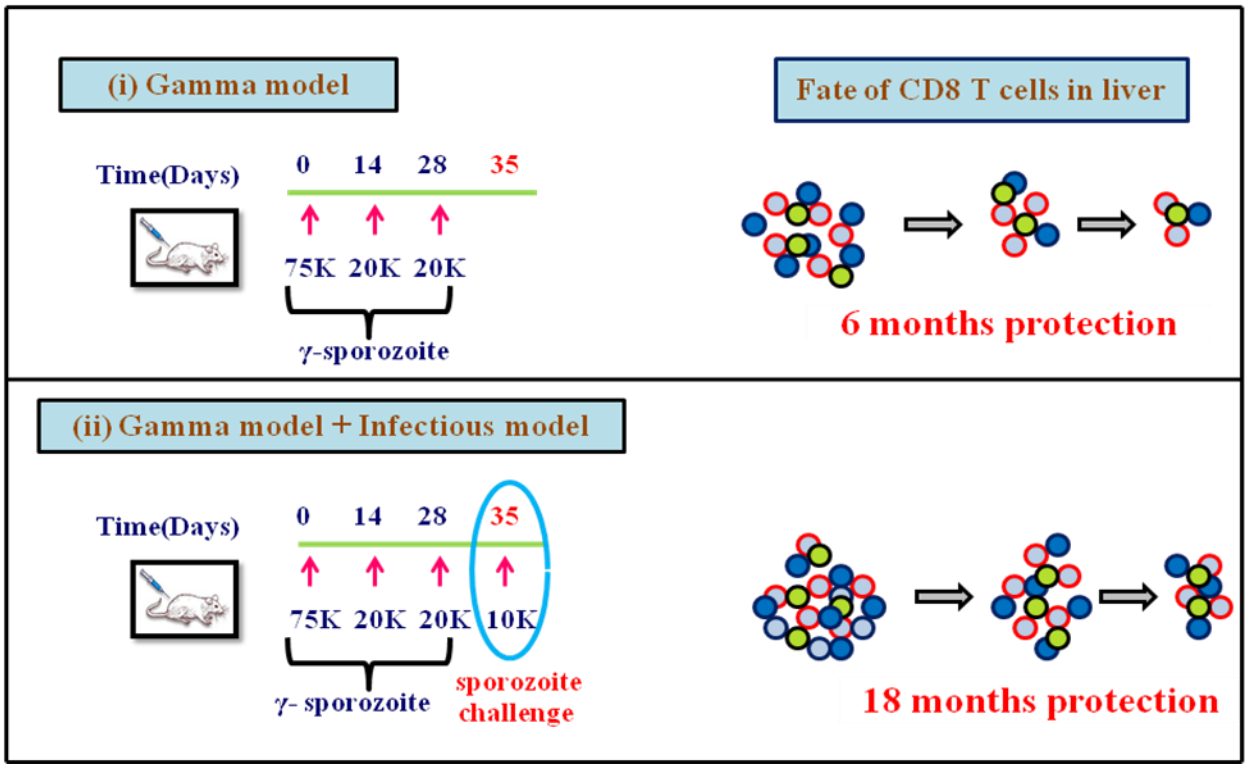


Figure 3: Whole sporozoite vaccination model.

Natural immunity against malaria still poorly understood but there are evidences for the involvement of antibodies as well as effector $CD8^+$ T cells in protection [8]. Subsequently, it has been shown that either loss or the maintenance of protective immunity against *Plasmodium* infection correlates with lower frequency of effector $CD8^+$ T cells in the liver [9]. Thus, it can be assumed that infectious status of *Plasmodium* sporozoite might be playing a key role in the outcome of $CD8^+$T cell response in conferring protection. Now, the question arises "how infectious sporozoite challenge

brings changes in quality of T cells that ensue long-lived protection" to liver-stage infection. The infectious sporozoite challenge might induce host innate response. It is known that innate immune response is linked to adaptive immunity and nature of this innate immune response is determined by various kinds of signals host receives from pathogen. Hence, it might be possible that the nature of innate immune response generated by RAS is different from infectious sporozoite challenge because development of RAS is arrested at early liver stage development while infectious sporozoites would complete liver-stage development. So from this it can be assumed that the profiles of PAMPs (pathogen associated molecular patterns), DAMPs (danger associated molecular patterns) as well as cytokines profile might be different during development of parasite in the liver. It induces difference in signaling for the activation of APCs and generates differential innate response which in turn would modulate $CD8^{+}$T memory cells, which in turn leads to better $CD8^{+}$T cells and hence better protection against Plasmodium.

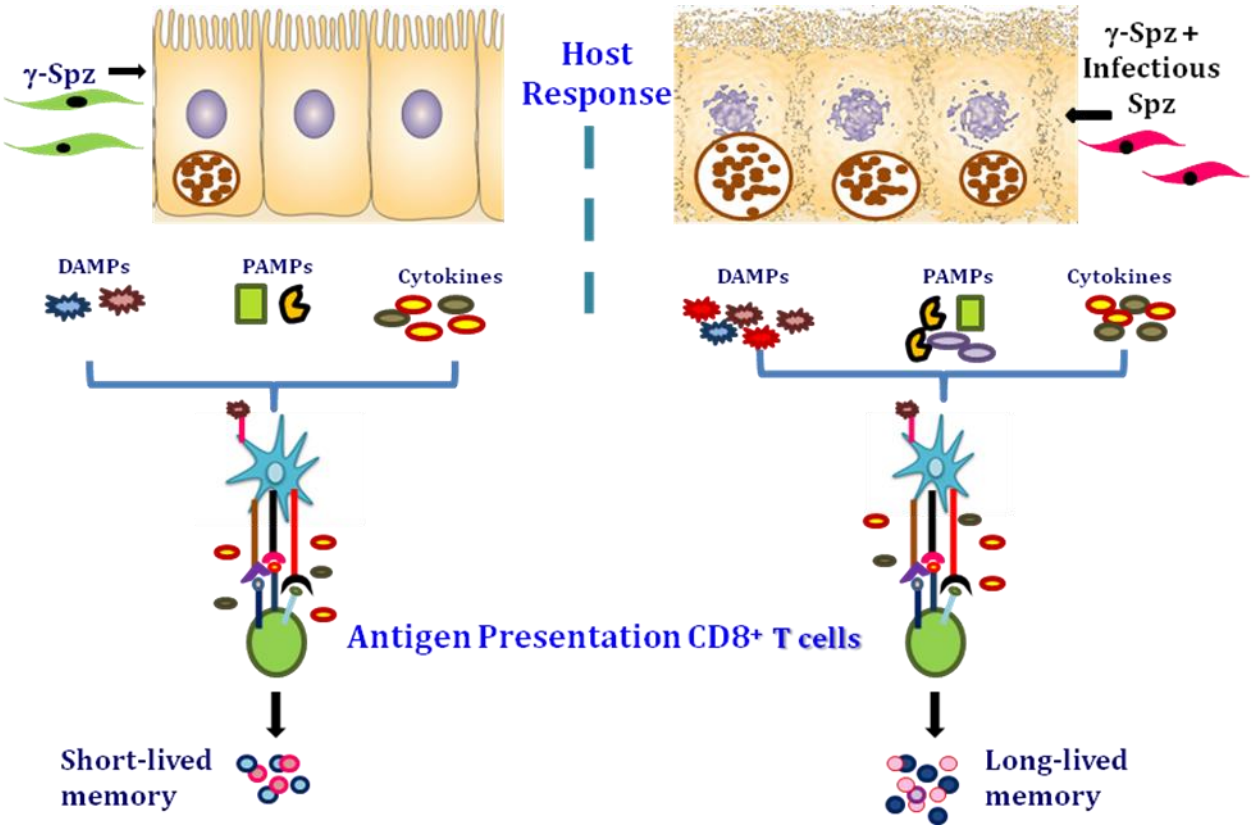


Figure 4: Schematic of host response to RAS v/s infectious sporozoites.

Innate immune response triggering during infectious immunization plays a critical role in inflammatory response, subsequently sensed by adaptive immune system. The adaptive immune response is characterized by specificity developed by clonal gene rearrangements from a broad repertoire of antigen-specific receptors on lymphocytes, the innate immune response is mediated primarily by phagocytic cells and antigen presenting cells (APCs), such as granulocytes, macrophages, and dendritic cells (DCs), and has been regarded as relatively nonspecific.

The question arises how the infectious-sporozoite helps maintain the T-cell immunity, while the γ-sporozoites fails to do so. It seems response of host to infectious sporozoites play a critical role in determining longevity of specific immunity. It is well known that innate immune response determines the fate of adaptive immunity, and thus understanding the potential of innate immune mechanisms directed against *Plasmodium* parasites that contribute to protection as well as to modulate adaptive immunity is very important. The key questions to be resolved are which *Plasmodial* components are responsible for activating the innate immune response and which receptors are engaged and responsible for inflammation. The proposed study is step in direction of defining the protective immunity by addressing the problem through alternative ideas. Better knowledge of innate immune responses to malaria may lead to the development of an effective malaria vaccine.

HYPOTHESIS

"Infectious parasite might be activating differential host innate response, which leads to qualitative and quantitative changes in $CD8^+$T cells, making them more protective in nature and survive longer"

In support of this hypothesis we have developed two objectives to study the role of APCs that depicts innate and adaptive immune responses.

- **Objective 1**: To study the modulation of APCs by γ-sporozoites v/s infectious sporozoite inoculation**.**
- **Objective 2**: To determine the Cytokine profiles in liver in response to sporozoite inoculation.

MATERIALS AND METHODS

1. Mice

The experiments on animal as described in this study were performed under the regulations of the Institutional Animal Ethics Committee (IAEC), and by the approval of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Male and female C57BL/6 mice (8-12 weeks old) were obtained from Zydus pharmaceuticals, Ahmedabad, India. These animals were housed in Animal house facility, Institute of Pharmacy, Nirma University. Food and water was available *ad libitum.*

2. Mosquito rearing, dissection and sporozoite isolation

2.1 Maintenance cycle

The larvae of *Anopheles stephensi* were obtained from the National Institute of Immunology, New Delhi, which were reared in the Insectarium facilities at Institute of Science, Nirma University, Ahmedabad. Mosquitoes were fed with a mixture of wheat germ, Kellogg's K special and yeast extract in a ratio of 4:4:1. Then within a month the larvae matured into pupae and they were transferred into cages where they transformed into mosquitoes in 48 hrs. In every 4 days these mosquitoes were fed with uninfected mice blood and they lay eggs within 2 days after the feeding. These eggs hatched into larvae and the cycle was repeated.

2.2 Infectious cycle

P. berghei (ANKA strain) sporozoites were obtained by cyclical transmission in mice and *Anopheles stephensi* mosquitoes. 0.05ml of blood with a 10% parasitemia (i.e., 10^7–10^8 parasitized red blood cells [RBC]) injected intraperitoneally into mice. 2 to 5 days old mosquitoes were fed on infected mice with 4-5% parasitemia. The parasite was then allowed to develop in the

mosquitoes and sporozoites were obtained by dissected salivary glands of mosquitoes 18-20 days after an infective blood meal.

2.3 Isolation of sporozoites

Female *A. stephensi* mosquitoes were sucked out of the cages by using vacuum pump and anaesthetized by keeping on ice (4ºC) for 5 minutes. They were surface sterilized by 70% ethanol and then mounted for dissection with serum free RPMI media on dissecting microscope. The salivary glands were then crushed and centrifuged, from which sporozoites were obtained. The similar process was carried out for non-infected mosquitoes and the salivary glands were injected into mice (Sham dissected).

2.4 Attenuation of sporozoites

For immunization, sporozoites were attenuated by exposure to 12,000 rad of radiation using a cesium-137 source (Bhabhatron, BV Patel PERD centre, Ahmedabad), counted, and adjusted to a given concentration in RPMI 1640. For challenge, sporozoites were used immediately after dissection to ensure maximal infectivity. Sham-dissected salivary gland preparations obtained from non-infected mosquitoes were treated according to the same procedure as described for sporozoites.

3. Immunization for objective 1

3.1. To study the modulation of APCs by γ-sporozoite v/s Infectious sporozoite immunization.

In this study we had characterized APCs in which we had specifically focused on dendritic cells. Groups of four mice inoculated intravenously with 10K RAS and infectious-sporozoites in each group. Organs (Liver, spleen and liver draining LNs) were collected 48 or 72 hrs post inoculation of γ-sporozoites or infectious-sporozoites.

3.2. Liver perfusion and Cells collection

Mice were sacrificed after 48 or 72 hrs for the collection of liver, spleen and liver draining lymph node. The catheter was inserted into the portal vein and liver was slowly perfused with 10ml of PBS to flush out circulating blood. The gall bladder was removed, and the liver was taken in 2ml of 1% RPMI media. For single cell suspension spleen and lymph node were minced while for the isolation of monocytes from the liver, perfused liver were collected and passed through a 70µ nylon cell strainer with the help of 10ml syringe filter. Intra hepatic mononuclear cells (IHMCs) were isolated from the liver cells suspension on a 33 % percol gradient (GE) and RBCs were lysed by RBCs lysis solution.

In spleen and liver suspension RBCs were lysed by RBCs lysis solution for 3 minutes. After isolation cells were washed and re-suspended in the complete RPMI media. Cells were counted by using trypan blue exclusion test on haemocytometer.

3.3. Cell staining for flow cytometry

For cell surface staining, cells (0.5-0.7 million cells per well) were seeded in each well of 96-well plate, in 200µl complete media. The samples were treated with F_c-block diluted normal mouse serum. Further 10µl of antibody cocktail was added and kept in dark for 30 min. For surface staining, performed by using fluorescently antibodies anti-CD11c, anti-IA/IE, anti-CD8α, anti-CD80, anti-CD86, and anti-CD40. F_cR was blocked by using F_c-block. Then, 200µl staining buffer was added and centrifuged at 300-400g for 3-4 minutes. Pellet obtained was re-suspended in 400µl of staining buffer with 1% formaldehyde for fixing the cells up to 48 hrs at 4°C. Flow data were acquired on the BD FACS Aria III and BD FACS Calibar and analyzed by using FlowJo (TreeStar) software.

Antibodies used were:

Marker	Fluorochrome	Company
CD11c	PE	BioLegend
CD8α	APC-H7	BD Biosciences
CD86	PerCP	BioLegend

CD80	V450	BD Biosciences
IA/IE	FITC	BioLegend
CD40	PE-Cy7	BD Biosciences
F4/80	APC	BioLegend

Table: 1 Abs for surface staining

4. Immunization for objective 2

To determine the Cytokine profiles in liver in response to sporozoite encounter.

- To understand the expression of cytokines during infection groups of mice were inoculated intravenously with 10K RAS or infectious sporozoites. Study was carried out after 72 hrs of administration of sporozoites by isolating liver following RNA isolation, cDNA preparation and PCR.

- Comparison of the expression of cytokines was done in infectious group post 24 hrs, 48 hrs and 72 hrs of administration of sporozoites. In this also three groups were made and mice were inoculated intravenously with 10K infectious sporozoites.

4.1 Gene Expression Analysis

Mice were sacrificed from each group at particular time interval and lower left lobe of liver was collected and stored in RNA later solution (Chromos Biotech) at -80°C. RNA was isolated from TRIzol by using chloroform, iso-propanol and ethanol. cDNA was prepared by cDNA synthesis kit (First strand cDNA synthesis kit, #K1612, Fermentas) using Normalized RNA.

Normalization of RNA for comparative study of naive, RAS and infectious samples				
RNA	**O.D**	**Concentration**	**Average (µg/ml)**	**RNA Used (µl)**
Naïve	0.909	1818	1.8	0.83
	0.909	1818		
	0.911	1821		
Infectious sporozoite	0.834	1669	1.6	0.937
	0.836	1673		
	0.837	1674		
RAS (10k)	0.920	735.6	0.735	2.04
	0.921	736.6		
	0.918	734.3		

Normalization of RNA for comparative study of infection group at 24 hrs, 48 hrs and 72 hrs using infectious RNA				
RNA	**O.D**	**Concentration**	**Average (µg/ml)**	**RNA Used (µl)**
24 hrs	0.969	775.1	0.74	2.02
	0.922	737.4		
	0.922	737.4		
48 hrs	1.124	899.0	0.90	1.66
	1.122	857.9		
	1.130	904.1		
72 hrs	0.834	1669	1.6	0.937
	0.836	1673		
	0.837	1674		

Table: 2 Normalization table of RNA

The primers for genes of IL-10, IL-5, IRF-7, IFN-γ and TNF-α were designed using PrimerQuest tool (Integrated DNA Technology).

Table: 3 Primer details

S.No.	Cytokines	Length	Amplicon Length	T_m	GC %
1	**IL-10**	1306	402		
	Forward-CTATGCTGCCTGCTCTTACTG			55.4°C	52.4%
	Reverse CTCCACTGCCTTGCTCTTATT			54.9°C	52.4
2	**IRF 7 (For Type I IFN)**	1876	395		
	Forward CACACTGCATCTTGGCTTTG			54.9°C	50%
	Reverse GCTGTTGCTGAAGAAGGTAGTA			55.1°C	52.4%
3	**TNF-α**	1332	254		
	Forward CGTCAGCCGATTTGCTATCT			55.0°C	50%
	Reverse TGAACACCCATTCCCTTCAC			55.0°C	50%
4	**IFN-γ**	1207	308		
	Forward CACACTGCATCTTGGCTTTG			54.8°C	50%
	Reverse GCTGTTGCTGAAGAAGGTAGTA			54.6°C	50%
5	**IL-15**	1287	342		
	Forward GGTACCAACTGGATAGATGTAAGATATGACCTGGAG			61.1	44.4%
	Reverse TAGGATCCGGACGTGTTGATGAACATTTGG			61.7	46.7%
6	**β-actin**	1889			
	Forward GGAATCCTGTGGCATCCATGAAAC			58.4	50%
	Reverse TAAAACGCAGCTCAGTAACAGTCCG			59.1	48%

Qualitative gene expression analysis was carried out by using cDNA synthesis kit (Thermo Scientific) and cDNA was further amplified by using PCR master mix (Takara) by using specific primers for the specific cytokines as mentioned above. Amplification of each gene that is IL-10 IL-15, IRF-7, IFN-γ and TNF-α was done for identifying the

annealing temperature by gradient PCR. After confirming the annealing temperature we proceeded for gene expression study for different cytokines. Results were analyzed by using 1.5 % agarose gel electrophoresis.

RESULTS & DISCUSSION

Modulation of APCs by γ-sporozoite v/s infectious sporozoite immunization.

Dendritic cells are professional APCs which play central role in the induction of protective immune response against various pathogens. DCs prime T-cells via MHC-peptide complexes (signal 1), in conjunction with the various co-stimulatory molecules (signal 2) and cytokines such as IL-10 and IL-12 (signal 3).

Various studies reported that RAS of *Plasmodium* berghei induces protection against liver-stage *Plasmodium* infection that is mediated primarily by intrahepatic IFN-γ producing effector/memory $CD8^+$T cells. However, the mechanisms involved in activation of $CD8^+$T cells in the liver require further investigation. The APC (antigen presenting cell) that process and present liver-stage antigens for the induction of the $CD8^+$TEM cells is yet to be fully understood.

In the liver and various lymphoid organs, the distribution of DCs is different and phenotypes of the DC subsets are heterogeneous in nature. Various investigators have identified a number of subsets of DCs on the basis of their expression of CD8α. $CD8\alpha^+$ DCs and $CD8\alpha^-$ DCs are also known as conventional DCs, which perform various unique functions. $CD8\alpha^+$ DCs play major role in cross presentation of antigen to the $CD8^+$ T cells, whereas $CD8\alpha^-$ DCs are known to induce $CD4^+$ T cells [10].

As we have proposed that the infectious status of sporozoite plays critical role in development of long lasting protective sterile immunity, we have characterized the antigen presenting cells in liver as well as in different lymphoid organs (i.e. spleen and liver draining LNs) and looked for their activation status of dendritic cells in response to infectious sporozoites that brings in changes in expression of activation markers on DCs, at 48 hrs and 72 hrs post immunization.

Increase in accumulation of CD8α⁺ DCs in the liver following RAS or infectious sporozoites.

Induction of T cell response starts with accumulation of APCs at the site of infection to capture the antigen followed by migration to the nearest draining lymph nodes. We wanted to test whether CD8α DCs are accumulated in liver and different lymphoid organs. C57BL/6 mice were immunized with 10K RAS and infectious sporozoites via intravenous immunization and sham (salivary gland debris of normal mosquitoes) as a baseline control; organs were taken after 48 and 72 hrs post immunization. Hepatic monocytes were stained with Abs specific for CD11c and CD8α and analyzed by flow cytometry to reveal 2 subpopulations of DC.

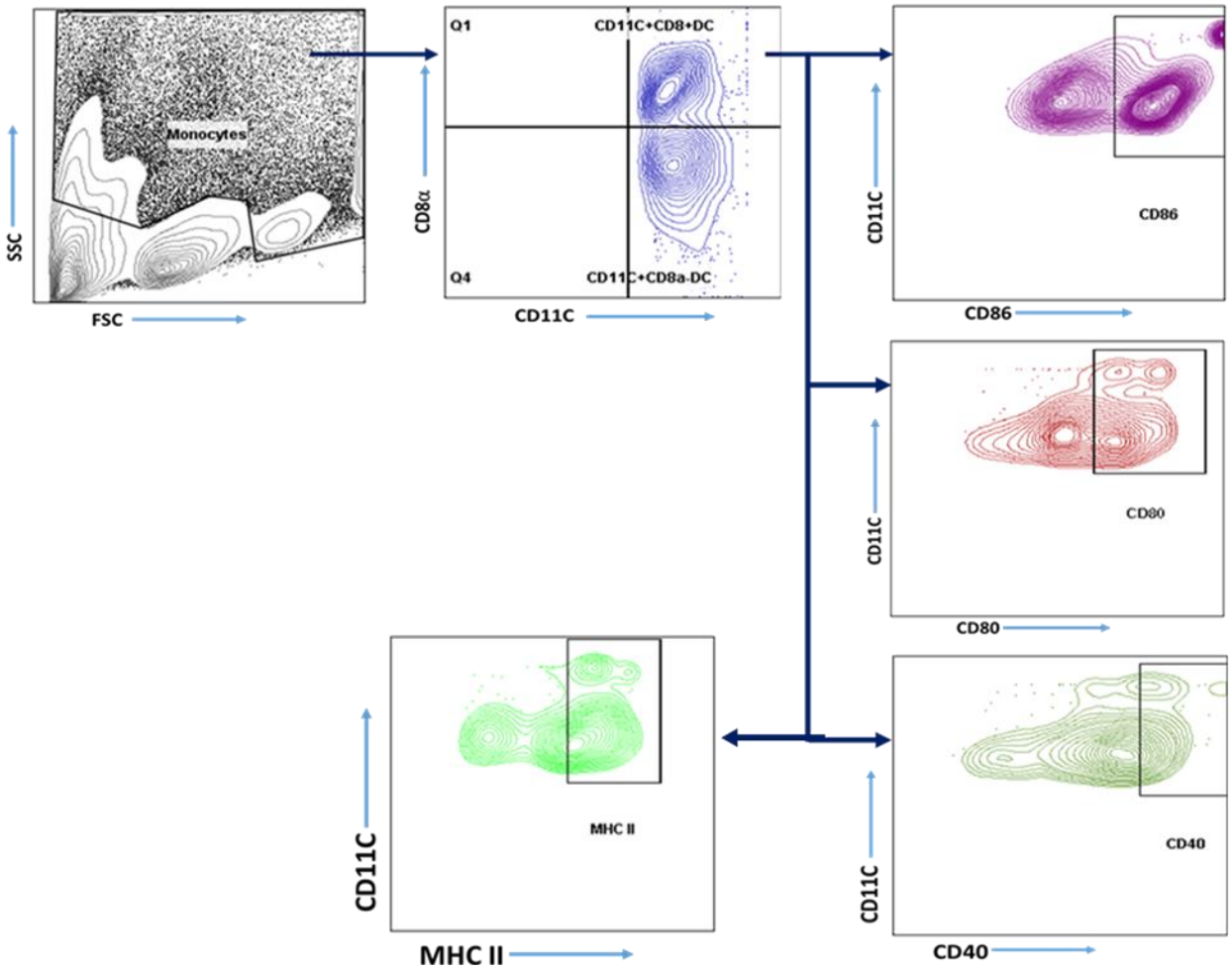


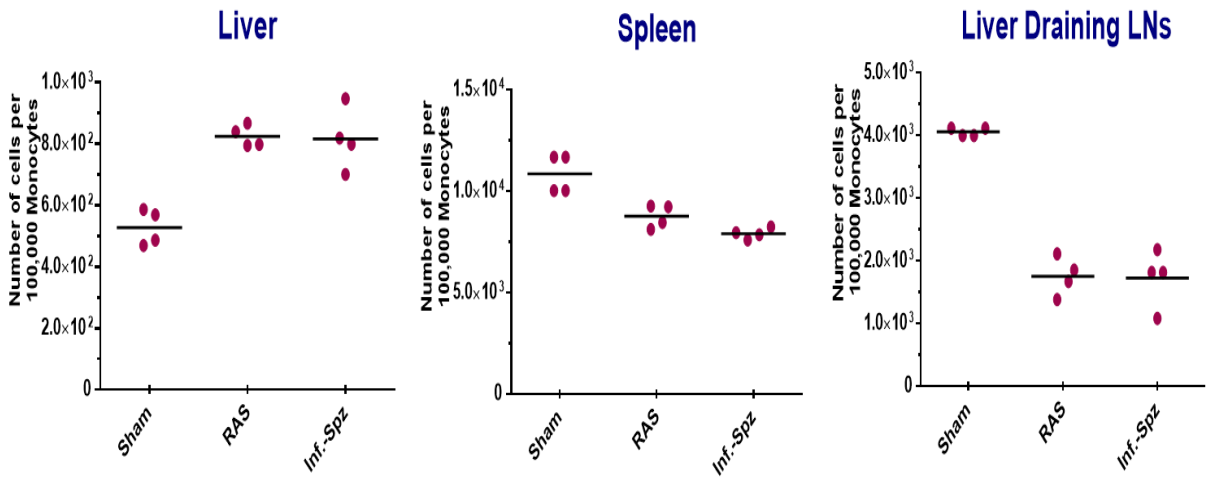
Figure: 5 Schematics for flow cytometer data analysis

We have characterized the DCs in the liver and different lymphoid organs in response to RAS as well as to infectious sporozoites on basis of CD8α expression. Firstly, we looked for the accumulation of $CD11c^{+}CD8\alpha^{+}$ DCs in the liver and different lymphoid organs. Then we looked for activation status of $CD11c^{+}CD8\alpha^{+}$ DCs by various activation markers on the surface of DCs required for the induction of memory $CD8^{+}$ T cells against *Plasmodia*.

(a) Accumulation of $CD11c^{+}CD8\alpha^{+}$ DCs in different organs at 48 hrs

Accumulation of $CD8\alpha^{+}$ DCs increased significantly in infectious and RAS immunized mice compared to sham immunized mice. But results are not significant for spleen and liver draining lymph node as $CD8\alpha^{+}$ DCs relatively increases in sham immunized group as compared to infectious and RAS immunization.

(i)



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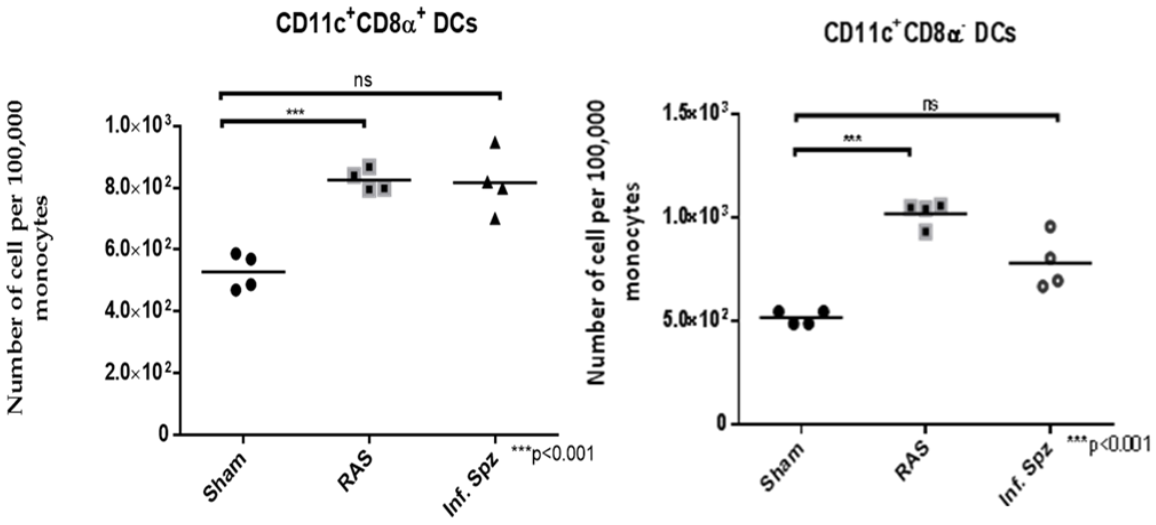


Figure: 6 Difference in accumulation of DCs in liver, spleen and liver draining lymph node at 48 hrs. Each shape in data shown is individual group of mice. (i) Accumulation of $CD11c^{+}CD8\alpha^{+}$ DCs in response to RAS and infectious sporozoites; (ii) Comparison between $CD8\alpha^{+}$ DCs and $CD8\alpha^{-}$ DCs in liver. Each shape in data shown is individual group of mice. (**p<0.001)

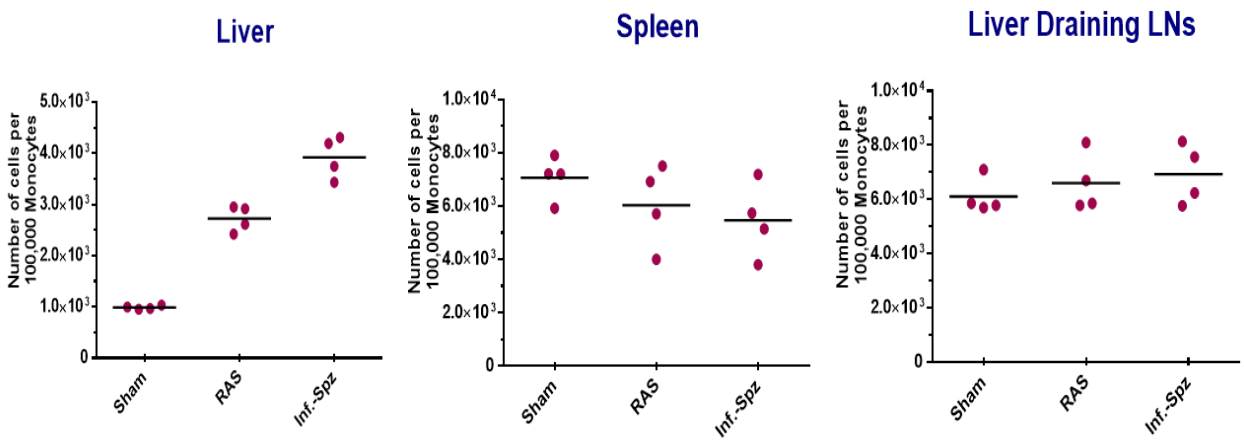
The number of $CD8\alpha^{+}$ DCs and $CD8\alpha^{-}$ DCs per 100,000 total live cells recovered from liver, spleen and liver draining lymph node has been shown in the figure (6). The accumulation of $CD8\alpha^{+}$ DCs and $CD8\alpha^{-}$ DCs is relatively higher as in the case of infectious and RAS immunized mice as compared to sham negative control. More specifically, the accumulation of $CD8\alpha^{+}$ DCs and $CD8\alpha^{-}$ DCs significantly higher as in the case of infectious sporozoite followed by RAS compared to sham group in response to RAS and infectious sporozoites.

(b) Accumulation of $CD11c^{+}CD8\alpha^{+}$ DCs in different organs at 72 hrs

$CD8\alpha^{+}$ DCs significantly increased in infectious and RAS immunized mice while accumulation of $CD8\alpha^{+}$ DCs relatively lowers in the livers of sham mice as compared to infectious and RAS. For spleen and liver draining lymph node results are not significant

as CD8α$^+$ DCs relatively higher in sham immunized group as compared to infectious and RAS immunized group.

(i)



(ii)

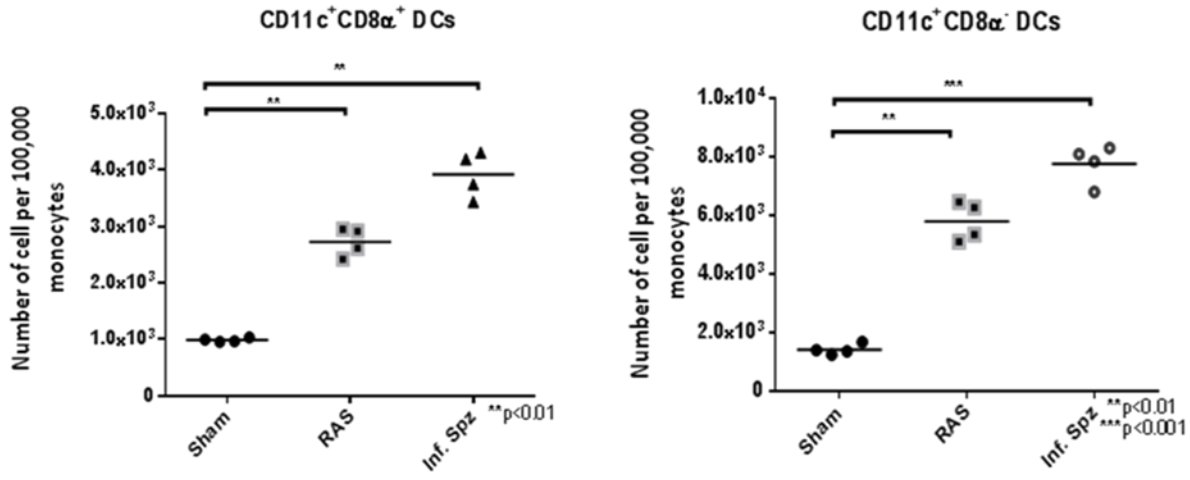


Figure: 7 Difference in accumulation of DCs in liver, spleen and liver draining lymph node at 72 hrs. Each shape in data shown is individual group of mice. (i) Accumulation of CD11c$^+$CD8α$^+$ DCs in response to RAS and infectious sporozoites; (ii) Comparison between CD8α$^+$ DCs and CD8α$^-$ DCs in liver. Each shape in data shown is individual group of mice. **p<0.001 and ***p<0.001.

The number of CD8α^{+} DCs and CD8α^{-} DCs per 100,000 total live cells recovered from liver, spleen and liver draining lymph node has been shown in the figure 7. The accumulation of CD8α^{+} DCs and CD8α^{-} DCs is relatively higher as in the case of infectious and RAS immunized mice as compared to sham negative control. More specifically, the accumulation of CD8α^{+} DCs and CD8α^{-} DCs significantly higher as compared to sham immunized group in response to RAS as well as infectious sporozoites.

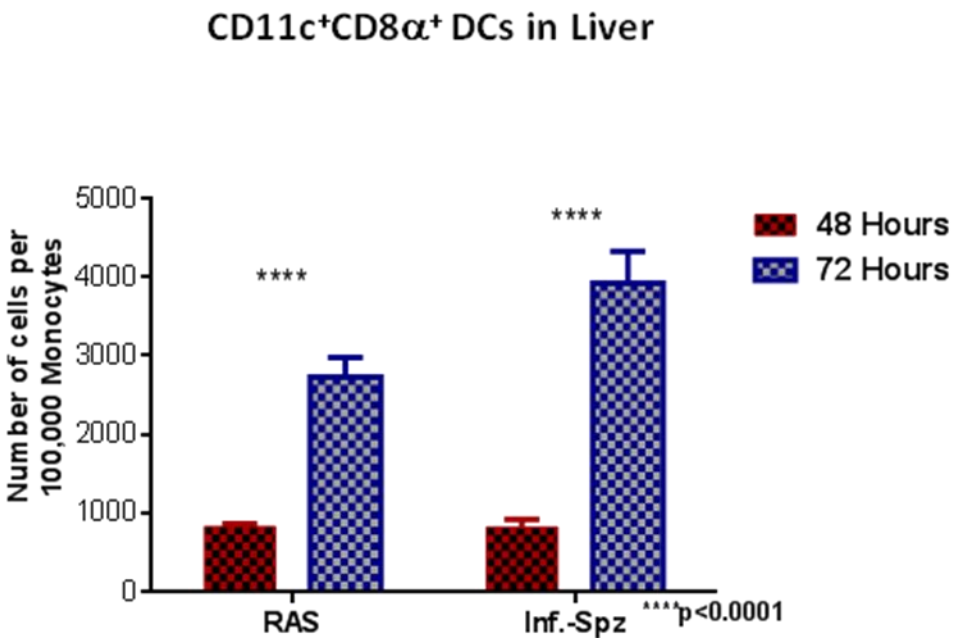


Figure: 8 Comparison of CD11c^{+}CD8α^{+} DCs at 48 and 72 hrs: The data shown is mean of four individual mice. ****p<0.0001

Comparison of the number of CD8α^{+} DCs and CD8α^{-} DCs per 100,000 total live cells recovered from liver, spleen and liver draining lymph node has been shown in the figure 8. Number of monocytes is increases with time and significantly higher at 72 hrs in the case of infectious and RAS immunized mice compared to 48 hrs immunized mice.

(c) Activation status of Hepatic CD11c^{+}CD8α^{+} DCs

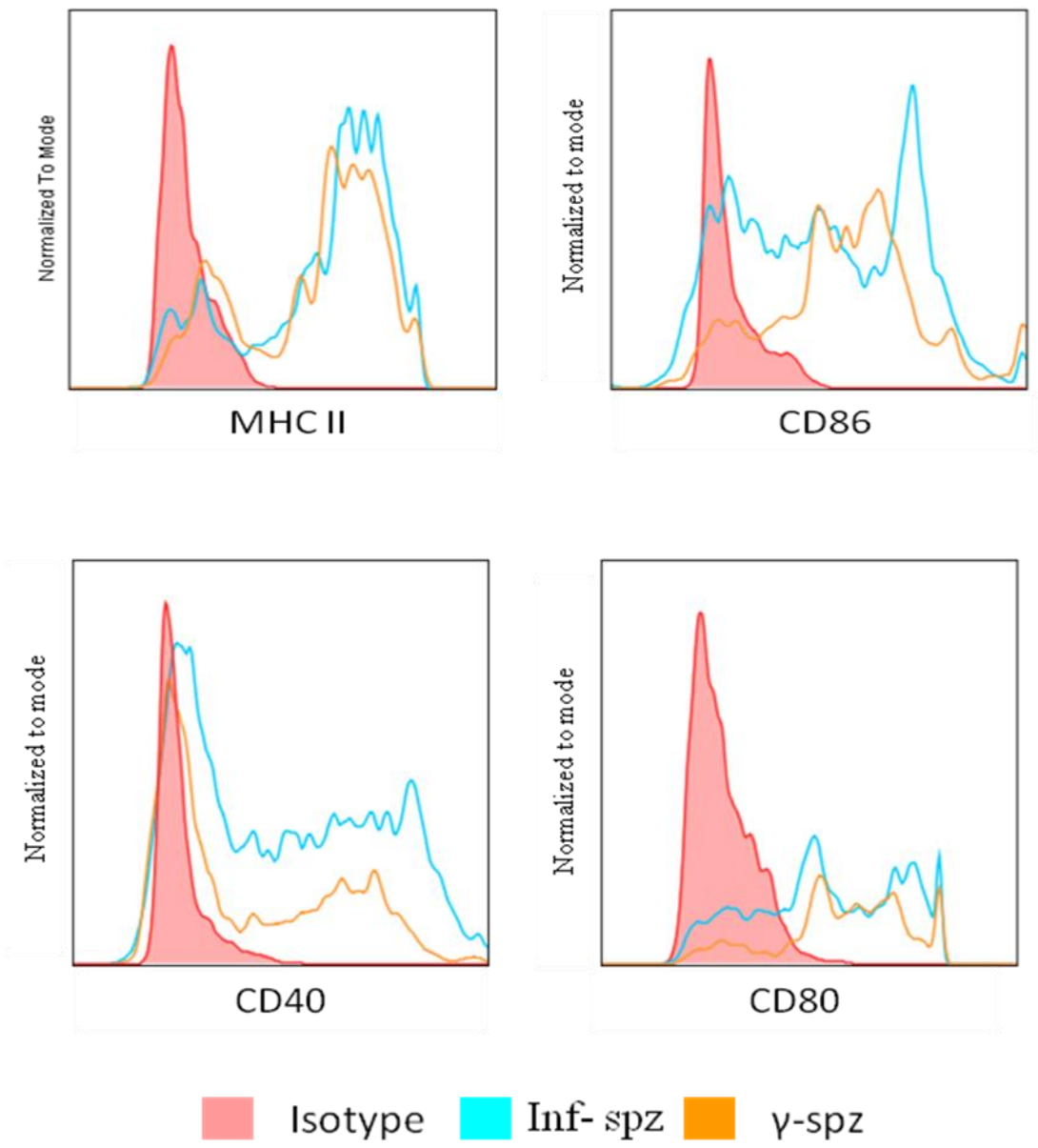


Figure: 9 Expression of MHC class II, CD86, CD80, and CD40. These data are representative one mice of each group.

Changes in the expression of MHC class II and co-stimulatory molecules in RAS and infectious immunized mice has been shown in figure 9. Hepatic $CD11c^{+}CD8\alpha^{+}$ DCs showed up-regulation of MHC class II and co-stimulatory molecules; CD40, CD80, CD86 in case of infectious sporozoite followed by RAS compared to isotype antibody control.

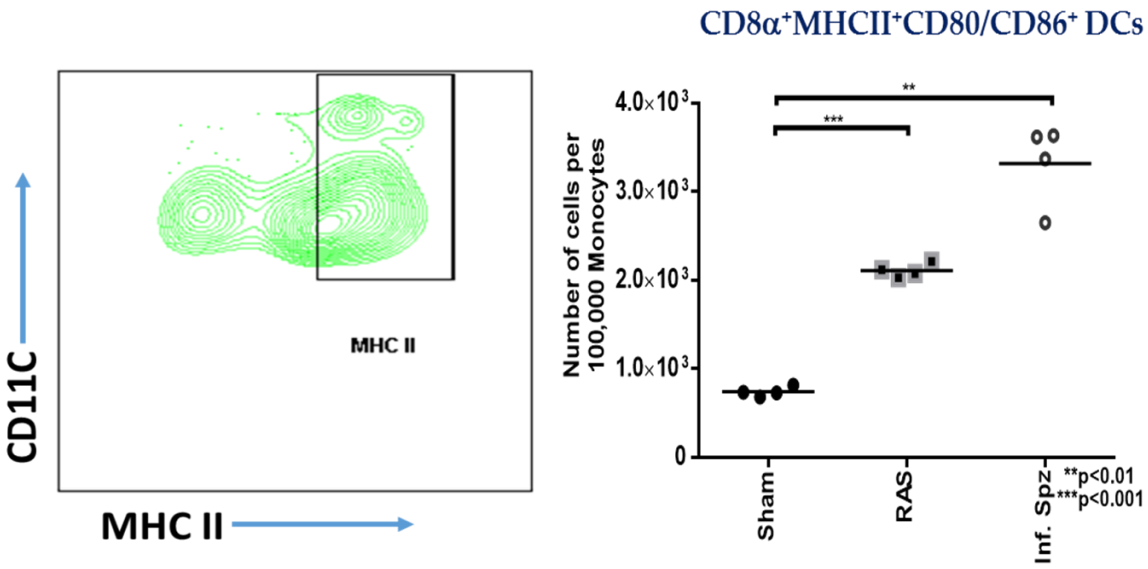


Figure: 10 Accumulation of activated $CD8\alpha^{+}$ DCs in liver with higher expression of MHCII and co-stimulatory molecules (CD80/86). Each shape in data shown is individual group of mice. **p<0.01, ***p<0.001.

Activation of $CD8\alpha^{+}$ DCs is significantly higher in liver of infectious group followed by RAS as compared to sham negative control figure 10. $CD8\alpha^{+}$ DCs is increased in the case of RAS as well as infectious immunization mice. Now we want to look for accumulation of activated $CD8\alpha^{+}$ DCs with higher expression of MHC II. It is higher in the case of RAS as well as infectious sporozoites immunized mice as compared to sham control.

(a) MHC II and co-stimulatory molecule expression on CD11c^{+}CD8a^{+} DCs in terms of MFI (Mean fluorescent intensity)

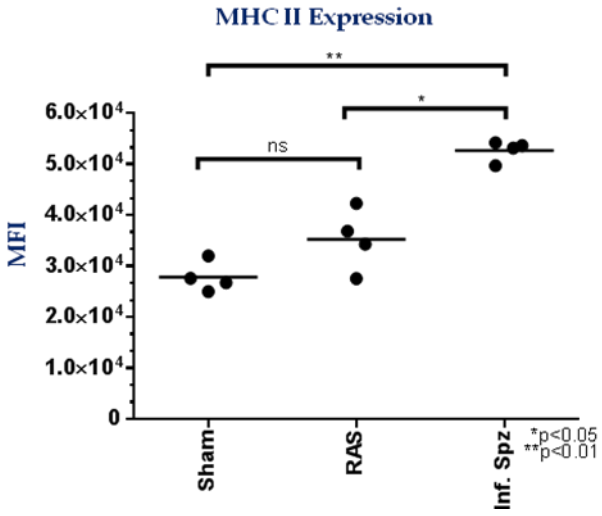


Figure: 11 Expression of MHC II of CD8α^{+} DCs.

The estimation of MHC II mean fluorescent intensity (MFI) on CD8α^{+}DCs accumulated in liver is higher in γ-sporozoites and infectious sporozoites immunization as compare to sham immunized group. The expression of MHC II was determined by flow-cytometry analysis and is expressed as the mean fluorescent intensity (MFI). Each shape in data shown is individual group of mice.

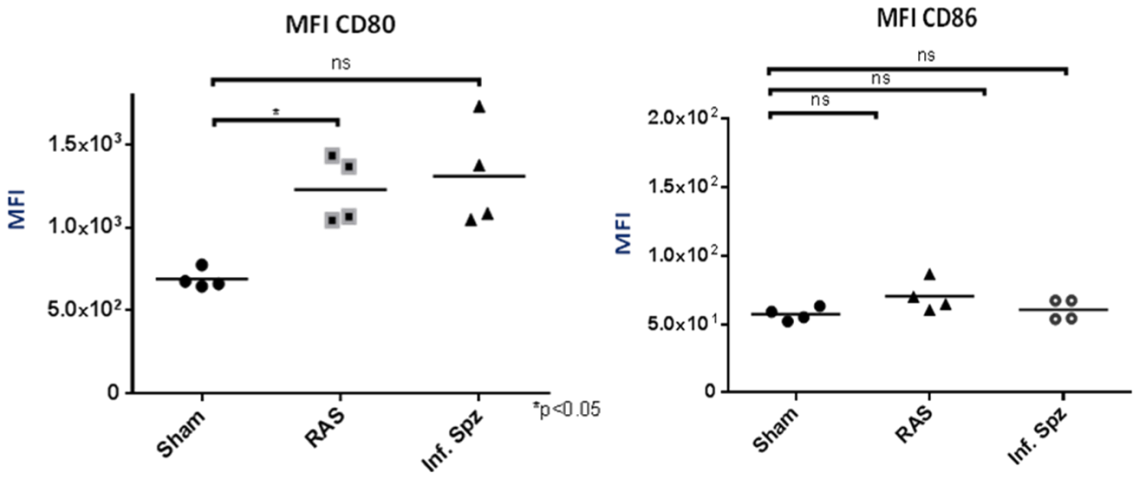


Figure: 12 Expression levels of CD80 and CD86 on the surface of CD8α⁺ DCs presented as mean fluorescence intensity. The estimation of CD80 and CD86 mean fluorescent intensity (MFI) on CD8α⁺ DCs accumulated in liver in response to γ-sporozoites and infectious sporozoites immunization as compare to sham immunized group. CD86 and CD86 are differentially up-regulated on CD8α⁺ DCs in the liver following RAS and infectious-sporozoites. The expression of CD80 and CD86 was determined by flow-cytometry analysis and is expressed as the mean fluorescent intensity (MFI). Each shape in data shown is individual group of mice.

CD80 and CD86 molecules present on DCs it binds with the CD28 on the surface of T cells and provides necessary signal for the activation of lymphocytes resulting in secretion of IL-2 and proliferation of T lymphocytes. Moreover, linking of CD80 with CD28 molecule mainly influences development of T helper type I immune response, whereas CD86 molecule plays pre-dominantly role in the induction of T helper type 2 immune response. We noticed that expression of CD80 molecule was significantly higher on CD8α⁺ DCs accumulated in the liver in response to γ-sporozoites as well as infectious sporozoites immunized mice comparing to the sham immunized group. Increase of CD80 expression on CD8α⁺ DCs could contribute to strong promotion of T cell response and to increase of protective immunity.

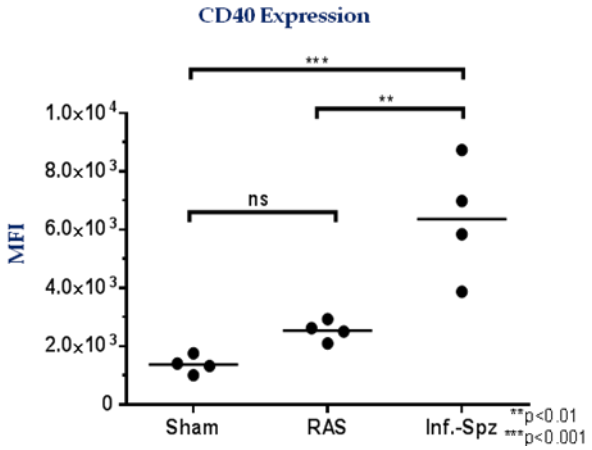


Figure: 13 Expression of CD40 of CD8α⁺ DCs. The estimation of CD40 mean fluorescent intensity (MFI) on CD8α⁺DCs accumulated in liver in response to γ-sporozoites and infectious sporozoites immunization as compare to sham immunized group. The expression of CD40 was determined by FACS analysis and is expressed as d mean fluorescent intensity (MFI). Each shape in data shown is individual group of mice.

Cytokine profiles in liver in response to RAS or infectious sporozoite encounter

During *Plasmodium* infection or immunization with RAS, host response to infection is activated as part of the innate immune response including synthesis of a variety of cytokines. Cytokines play an integrative role in the immune response and induce inflammatory as well as anti-inflammatory roles. Cytokines can function either locally in a paracrine or autocrine manner. *Plasmodium* infection is a strong inducer of many cytokines including type-I IFN, tumor necrosis factor α (TNF-α) [11]. Several lines of evidence indicate that these cytokines are important regulators of shaping adaptive immune response and generation of protective immunity.

Plasmodium infection stimulates cytokine expression in classical immune tissues such as the liver. The cytokines that contribute to the shaping adaptive immunity in malaria have not been fully delineated, but potentially could be responsible for the generation of protective immunity against the *Plasmodium* infection in RAS immunized mice and in generating the natural immunity against *Plasmodium*. The current experiments were

designed to test whether infectious status of parasite plays a direct role in stimulating cytokine expression in liver.

Gene expression analysis has been effectively utilized in multiple models to identify innate immune responses to infection. In the present study, we investigated the gene expression profiles in the livers of mice immunized with RAS or infectious sporozoites at 72 hrs post-inoculation. A very limited studies showing histological analysis of innate immune cell responses against *Plasmodium* liver-stage infection have reported that immune cells in filtrate the liver between 48 and 72 hrs post infection with rodent malaria sporozoites. Therefore, we primarily examined the changes in gene expression during liver-stage infection in mice at 72 hrs post last immunization. The wild type *P. berghei* infection in mice progresses to the blood stage after about 48 hrs post infection with sporozoites. In order to ensure that measured responses at 72 hrs post infection are only caused by liver infection and not by the ensuing blood-stage infection, we utilized an attenuated *P. berghei* parasite that develops to late liver-stage forms but does not progress to blood-stage infection.

We have focused on IRF-7, key regulator of Type I, IFN-γ; pro-inflammatory cytokines TNF-α, IL-15 and anti-inflammatory cytokine IL-10. During malarial infection pro-inflammatory cytokines TNF-α and IL-15 released by macrophages play key role in inducing protective immunity. TNF-α is first characterized parasite-induced cytokine while IL-15, a pleiotropic cytokine mainly controls growth and differentiation of T and B lymphocytes, activation of Natural Killer (NK) and phagocytic cells contributing to the homeostasis of the immune system and play role in host defense against infections by inducing activation of effector cells from both innate and adaptive immune system. Type I IFNs have central role to both innate and adaptive immunity which are transcribed by IRF-7.

IRF-7 regulates various immune functions such as MHC expression, antigen presentation, and T cell expansion resulting in driving response in liver stage infection. IFN-γ is involved in innate immune response for the resolution of primary infection by limiting the initial phase of parasite replication. Their expression starts inducing by 24 hrs post

infection, followed by a slow but steady increase in transcript levels until 72 hrs Interferon plays an important role in regulation of T cells during the infection. During infection IL-10 seems to play an important role in defining the T helper cell response to malaria. At the time of acute or chronic stages of the infection, they are highly secreted by macrophages, dendritic cells B cells, and various subsets of $CD4^+$ T cells and $CD8^+$ T cells. Moreover, IL-10 down regulates MHC class II molecules on macrophages, leading to decreased antigen presentation. Thus their expression will help us to determine the level of infection at the liver stage

The expression of cytokines at mRNA level was assessed by qualitative RT-PCR in liver after 72 hrs post immunization by comparing naive, RAS (10K) and infectious (10K) groups. We observed longitudinal changes in cytokine levels in mice with immunization with RAS or infectious sporozoites. IFN-γ, TNF-α and IL-15 being pro-inflammatory are expressed within few hrs of infection. In our study we have seen that TNF- α and IFN-γ expressing in both RAS and infectious sporozoites group. Expression of TNF-α is almost similar in both RAS as well as infectious sporozoites immunized mice group, while IFN-γ expression is higher in the case of infectious sporozoites immunized mice as compared to RAS immunized group. While IL-15 is expressed in group of naïve mice, RAS and Infectious group, but the expression of IL-15 was increased in the case of RAS, and increased significantly in the case of Infectious sporozoites group. IL-15 shows high expression in infection as they play key role in enhancing the innate and adaptive immunity needed to control and clear a primary *Plasmodium* infection in a timely manner.

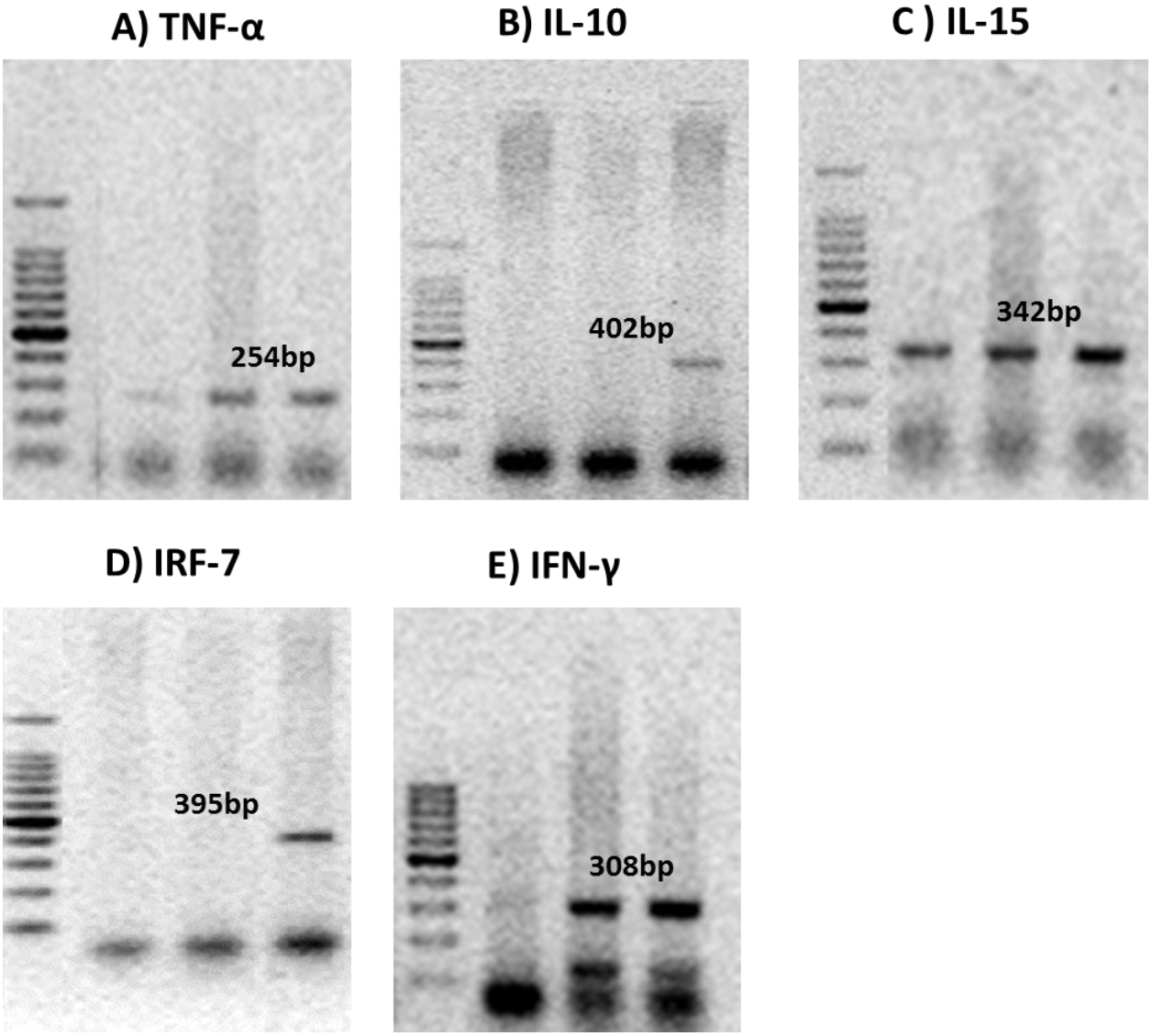


Figure: 14 Cytokine expression of TNF-α (254 bp), IL-10 (402bp), IL-15 (342 bp), IRF-7 (395 bp), IFN-γ (308 bp) in the liver after 72 hrs post immunization of RAS and infectious sporozoite. In each image lane 1 represents DNA 100 bp ladder, lane 2 represents naïve group, lane 3 represents RAS immunized group and lane 4 represents infectious sporozoite immunized group. (A) For TNF-α (amplicon size 254bp) a pro-inflammatory cytokine at an annealing temperature of 53°C. (B) For IL-10 (amplicon size 402bp) an anti-inflammatory cytokine at an annealing temperature of 53°C. (C) For IL-15 (amplicon size 342bp) at an annealing temperature of 53°C. (D) IRF-7 (amplicon size 395bp) an adaptor molecule for type I interferon at an annealing temperature of 53°C. (E)

For IFN-γ (amplicon size 308bp) type II interferon at an annealing temperature of 53.5 ºC.

IL-10 was only expressed in infectious sporozoites immunized group, being anti-inflammatory IL-10 expression decreases and increases according to parasitemia and inflammatory response generated during infection. This negative correlation of IL-10 expression with parasitemia and pro-inflammatory cytokines, suggest IL-10 to be the key cytokine in tilting the balance to an inflammatory response. Same is true also in the case of IRF-7 expression, infectious sporozoite immunized mice has shown expression of IRF-7 mRNA.

Kinetics of cytokines induction with time against infectious sporozoites infection

In order to test whether infectious sporozoites immunization changes cytokines expression with time, we have immunized a group of mice with 10K infectious sporozoites. The expression of various cytokines at mRNA level was assessed in liver in kinetic manner i.e. 24, 48 and 72 hrs post immunization by qualitative RT-PCR and we found that mRNAs were significantly induced with the time as infectious sporozoites.

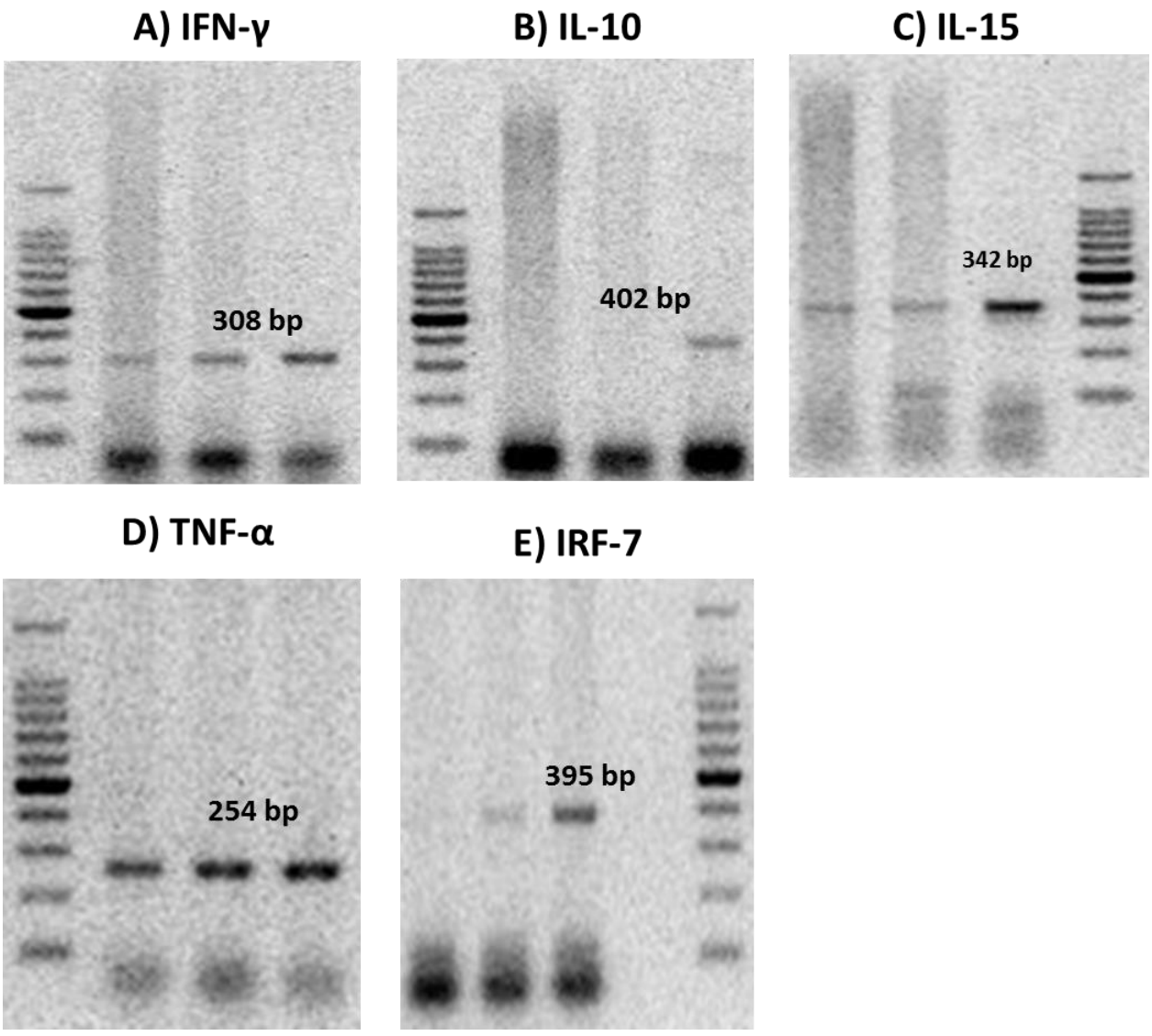


Figure: 15 The comparative study for the mRNA expression of IFN-γ (308 bp), IL-10 (402 bp), IL-15 (342 bp), TNF-α (254 bp) and IRF-7 (395 bp) by qualitative RT-PCR at transcriptional level in the liver after post immunization of infectious sporozoite at 24 hrs, 48 hrs and 72 hrs: Lane 1 represents 24 hrs, lane 2 represents 48 hrs, lane 3 represents 72 hrs PCR product with a ladder besides (100 bp). (A) IFN-γ type II interferon at an annealing temperature of 53.5°C. (B) For IL-10 an anti-inflammatory cytokine at an annealing temperature of 53°C. (C) For IL-15 at an annealing temperature of 53°C. (D) For TNF-α Proinflammatory cytokine at an annealing temperature of 53°C. (E) IRF-7 an adaptor molecule for type I interferon at an annealing temperature of 53°C.

The expression level of IFN-γ was found to be up regulated in 72 hrs as compared to 48 and 24 hrs. IFN-γ is produced exclusively by activated T cells and natural killer cells [12], it increases with time as the infection progresses, further supported by our results that expression of IFN-γ is higher at 72 hrs as compared to 24 hrs and 48 hrs post infection, while the expression of IL-10 only induced at the time of higher level of infection as it is an anti-inflammatory cytokine and thus, its gene expressed at higher level of infection [13]. The IL-15 activates macrophages and is an early host factor that initiates a positive autocrine feedback loop for cytokine expression by their genes [14]. Due to this the genes get expressed at 24 hrs post immunization and increases - with time. This is being supported by our results that cytokine gene expression gets higher at 72 hrs as compared to 48 hrs and 24 hrs. Similar observation was also made the case of TNF-α, a pro-inflammatory cytokine that gets triggered with the onset of infection. It has been clearly shown that the expression increases with the lap of time i.e. 24 hrs, 48 hrs and 72 hrs. IRF-7 which has been found as the adaptor molecule and transcription factor for the type I interferon that eliminates the infection at higher pace. Although IRF-7 expression starts at 48 hrs post sporozoite infection, it increases further at 72 hrs. The findings of others that type I IFN reaches the peak at late stage of infection suggest that IRF-7 controls the production of type I IFN that limits the parasite growth and trigger immune response, protective in nature [15].

SUMMARY

The initial host innate response against the pathogen might dictate the protective memory response, and encompasses an array of innate immune mediators. The infectious status of sporozoite plays an instrumental role in liver stage immunity and impact the host innate response, which leads to $CD8^+$T cell response in rendering sterile-protection. Innate recognition of infection in vertebrates can lead to the induction of adaptive immune responses through activation of innate immune cells especially dendritic cells (DCs). DCs are activated directly by conserved pathogen molecules and indirectly by inflammatory mediators produced by other innate cell types that recognize such molecules. The DCs are an indispensable component of priming and generation of $CD8^+$T cell responses. As $CD8^+$T cells are the major effector cell populations which extends protective immunity against liver-stage infection.

Present study shows that innate immune response induced by infectious sporozoites challenge is qualitatively different, and helps rescue the loss of $CD8^+$T cell response as has been observed in γ-spz immunization without intermittent challenge. The danger signals perceived from the pathogen attack are decisive in establishing the nature of innate immune response, leading to long-term protection. In our results, accumulation of $CD8\alpha^+$ DCs is significantly higher in the case of infectious immunized group as compare to RAS as well as sham immunized group. The higher expression of MHC II, CD80, CD86 and CD40 molecules on the surface of $CD8\alpha^+$ DCs represents the activation status of $CD8\alpha^+$ DCs, reflecting the role of infectious sporozoites in modulating the T cell immune response.

Subsequently, cytokine expression studies in kinetic and independent manner and found that up-regulation of various cytokines i.e. IL-15, IFN-γ, TNF-α, IL-10 and Type I interferon in response to infectious sporozoites immunization. While from this study we also found that the expression of IL-10 got saturated at 72 hrs. By the cytokine study we tried to understand the innate immune mediator's and we would like to have some idea about the PAMPs involved in innate immunity as they are the key to adaptive immune response.

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APPENDIX

Reagent Preparation:

1. **Phosphate buffer saline (1X):-**

NaCl	80 mg
KCl	20 mg
Na_2HPO_4	144 mg
KH_2PO_4	24 mg
Adjust the pH to 7.4 and make up the volume to 100ml.	

2. **RBC Lysis buffer (pH-7.3):-**

15.5 mM NH_4Cl	0.892 mg
0.1 mM EDTA	3.7 mg
12 mM $NaHCO_3$	8.8 mg
pH	7.3

3. **70% iso-propanol (100 ml)**

   Mix 70 ml iso-propanol in 30 ml distilled water.

4. **75% ethanol (100 ml)**

   Mix 75 ml ethanol In 25 ml distilled water.

5. **0.1% DEPC-treated water (100 ml)**

   Add 0.1 ml DEPC in 100 ml distilled water. Stir it with the help of magnetic stirrer

until the globules present in the water dissolve completely. Allow the solution to stand overnight at room temperature. Autoclaved the solution.

Note: - The container used for preparing DEPC water should be autoclaved before use.

6. 50 X TAE buffer(1 liter):-

Tris-base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA	100 ml
Ph	8.3

Mix 242 grams of Tris-base in 750 ml Milli-Q water and stir until all the Tris-base has dissolved completely. Add 100 ml of 0.5 M EDTA and 57.1 ml of glacial acetic acid. Mix the solution again. Adjust the pH to 8.3 if required. Make up the volume to 1000 ml with Milli-Q water. Sterilize by autoclaving.

7. RT–PCR (Reverse Transcriptase-PCR)

First strand cDNA synthesis

The reagents were added in the order mentioned below

Serial No.	**Reagents**	**Volume**
1	Total RNA	1 µg
2	Nucleus free water	10 µl
3	5x reaction buffer	4 µl
4	RiboLock™ Ribonuclease Inhibitor	1 µl
5	10mM dNTP mix	2 µl
6	Oligo (dT) primer (0.5µg/µl) or random hexamer	1 µl

7	M-MuLV reverse transcriptase (20 ug/µl)	2 µl
	Total volume	**20 µl**

Table: 4 Reagents for first strand cDNA synthesis

- **PCR amplification of cDNA**

  The reagents for PCR were added in the order mentioned below

Serial No.	**Reagents**	**Volume**
1	PCR master mix	12.5 µl
2	Nucleus free water	5.5 µl
3	Forward primer	2 µl
4	Reverse primer	2 µl
5	Template	3 µl
	Total volume	**20 µl**

Table: 5 Reagents for PCR