Immunomodulatory studies during viral infections

Amal Mathew

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science

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Certificate of Examination

This is to certify that the dissertation titled "***Immunomodulatory studies during viral infections***" submitted by **Mr. Amal Mathew** (Reg. No. MS12084) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Mahak Sharma Dr. Rajesh Ramachandran Dr. Sharvan Sehrawat
(Supervisor)

Dated: 21st April 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Sharvan Sehrawat at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Amal Mathew
(Candidate)

Dated: 21st April, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Sharvan Sehrawat
(Supervisor)

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List of Figures

Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
VEGF	Vascular endothelial growth factor
PFU	Plaque forming units
RBC	Red blood cells
FACS	Fluorescence activated cell sorting
CFSE	Carboxyfluorescein succinimidyl ester
DENV	Dengue Virus
IFN	Interferons
PCR	Polymerase chain reaction
IgG	Immunoglobulin G
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay

Contents

ABSTRACT

Immunomodulation refers to the process of altering immune responses to a desired level. We attempted to modulate the immune system of mice and zebrafish for studying host pathogen interaction during viral infections. Studies in mice focused on elucidating the role of Myeloid Derived Suppressor cells (MDSCs) in HSV1 induced immunoinflamatory reaction in cornea. Initial experiments were aimed at understanding the kinetics of MDSCs in lymphoid and non-lymphoid organs. We found cell populations phenotypically similar to MDSCs in cornea and spleen during the course of infections. Experiments were also carried out to generate MDSCs *in vitro* and to check whether such cells can control the corneal inflammatory reactions caused by CD4 T cells. We were able to show that *in vitro* generated cells were able to control the proliferation of CD4 T cells in an antigen specific manner. Future experiments could be performed to establish the therapeutic value of *in vitro* generated MDSCs in controlling the immunoinflamatory reactions in cornea.

My second project was aimed at generating a novel model for studying Dengue viral pathogenesis. We tried to generate a type I interferon receptor knockout zebrafish using CRISPR/Cas tool. We targeted CRFB5, which is a common receptor chain in two groups of type I interferon signaling in zebrafish. We generated gRNAs against CRFB5 and showed that indeed the gRNA is able to cause double strand breaks in the CRFB5 amplicon. Next we have to microinject gRNA and Cas9 mRNA into oocytes of zebrafish, to generate knock out fishes.

CHAPTER I

INVESTIGATING THE ROLE OF MYELOID DERIVED SUPPRESSOR CELLS DURING HERPES VIRUS INDUCED CORNEAL IMMUNOINFLAMATORY REACTION

1.1 INTRODUCTION

The cornea has an immune privileged status since transparency is required for optimal vision. Any inflammatory reaction in cornea leads to impairment of vision, therefore several mechanisms are put in place to ensure excessive inflammation does not proceed. These include existence of several anti-inflammatory molecules and a lack of vascularization (1). Infection with Herpes Simplex Virus type 1 (HSV-1) depletes the immunoprivilaged status of cornea and further lead to a potentially blinding immunopathological condition known as Herpes Stromal Keratitis (HSK). HSV-1 is a very common human pathogen and close to 80 percent population is seropositive for HSV I infection. Upon infection of orofacial region, the virus first replicates at the site of infection and through retrograde transport migrates to trigeminal ganglia where it establish life long latency. Several mechanisms play critical role in the maintenance of latency and include the balance of proinflammatory and anti inflammatory cell activity (2). One of the main reasons for HSK is the reactivation of virus from latency, which leads to recurrent episodes of corneal inflammation eventually leading to scarring. Current treatment for HSK is based on a combinational therapy with topical antivirals to inhibit viral replication and corticosteroids to prevent infection and inflammation but both these approaches are not optimally effective.

1.1.1 Virology

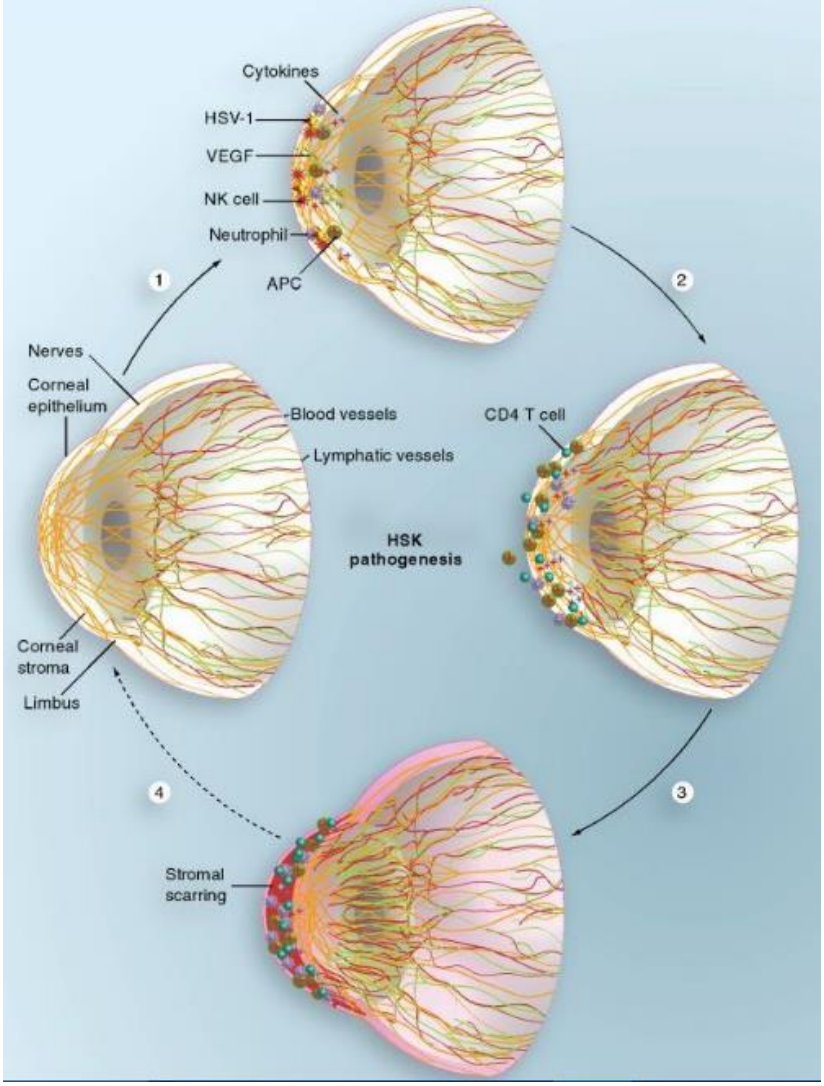
HSV-1 is a common human pathogen which belongs to the family Herpesviridae. It is an enveloped DNA virus. It gains entry into host cells by the binding of certain

glycoproteins on its membrane to their transmembrane receptors, which include heparin sulphate receptors. HSV-1 commonly causes sores around the mouth and rarely cause genital herpes. It is transmitted through oral to oral contact. A cell infected by HSV-1 can undergo several modifications including modifications in nucleolus, cytoskeletal and matrix abnormalities and plasma membrane alterations (3-5). It can establish both latent and productive infections. When the virus enters the lytic phase there is cell lysis and virus spreads to nearby cells. Non neural cells like epithelial cells are more susceptible to lysis. The virus establishes latency in ganglion and the ganglionic sites to harbor latent virus are decided by the primary infection site.

1.1.2 HSK Pathogenesis

Cornea lacks blood vessels as it would interfere with its normal function since transparency is required for optimal vision. Eye uses various mechanisms to prevent inflammation, immune responsiveness and development of neovascularization. Development of neovascularization can be particularly damaging to vision as new blood vessels can diffract light and also damage the structural integrity of the cornea by depositing proteins and also recruiting inflammatory cells. When this occurs cornea loses its 'immunoprivilaged' status. Once virus infects the ocular surface of cornea, it replicates in the epithelial cells in the cornea for about 6 days after which the replicating virus is not found in the cornea. But in case of immunocompromised animals virus can penetrate even further. As a consequence of infection by virus several cytokines and chemokines are upregulated which includes VEGF, IL-1, IL-6, CCL2, CXCL1 and CXCL2. This results in the recruitment of several inflammatory cells into the stroma of cornea. The infiltrating cells include NK cells, gamma delta T cells, dendritic cells, macrophages and neutrophils. Even though innate immune cells are recruited soon after corneal infection the main players of stromal lesions and subsequent immunopathological events are T cells. The CD4 T cells become evident in the stroma at seven days post infection and the number peak at around 14-21 days post infection. The CD4 T cells induce further infiltration of neutrophils and dendritic cells, which leads to enhanced and a protracted inflammatory lesion. The role of T cells in manifesting inflammation have been well elucidated with studies conducted in animals that lack T cells (6). Even though the role of T cells have been elucidated, their antigen

specificity remains unclear. Different theories have been proposed to explain the source of stimulus for the activation of CD4 T cells. One theory suggests a bystander activation by cytokines which activates CD4 cells of any specificity to mediate HSK. The second theory suggests that HSK is a consequence of auto reactive T cells that arise as a result of molecular mimicry by a common peptide in HSV and corneal protein. The third theory suggests that HSK is a result of HSV specific CD4 T cells.



Future Virology. 2010;5(6):699-708

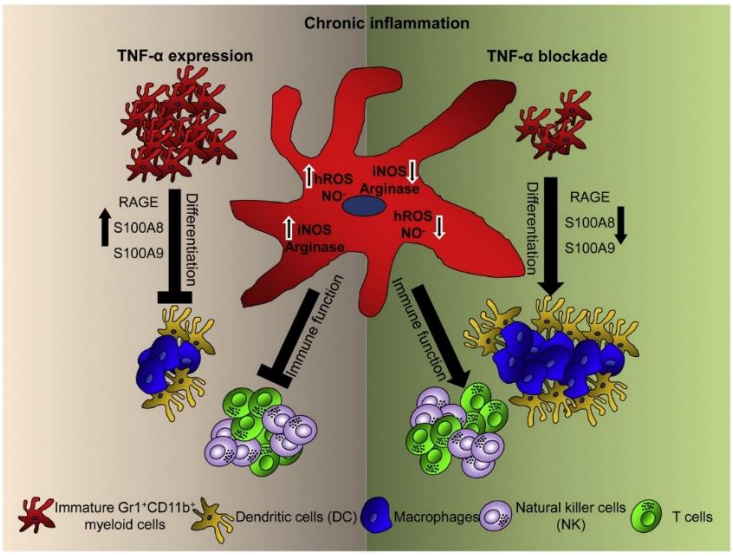
Fig 1.1: Progression of HSK immunopathogenesis

The figure represents the progression of disease starting from entry of HSV and recruitment of neutrophils (1) to further infiltration of CD4 cells (2) and stromal scarring (3) which is caused by inflammation.

The main features of Herpes stromal keratitis are lesions and angiogenesis which marks the disease. Controlling disease severity can be mainly achieved by dampening the immune responses or by controlling the inflammatory reactions. Various drugs have shown to control the immunopathological lesions in eye by controlling the availability of VEGF and also controlling inflammatory reactions (7). Other solution would be to enhance the function of regulatory cells (8,9). Studies focusing on the innate immune regulatory cells such as Myeloid Derived Suppresser cells (MDSCs) are lacking and hence could provide insight in to the pathogenesis of disease. Through this project we are trying to understand the role of MDSCs during HSK.

1.1.3 Myeloid Derived Suppresser Cells (MDSCs)

Myeloid Derived Suppressor cells (MDSCs) are a heterogeneous group of cells from myeloid lineage. MDSCs are shown to expand during pathological condition as a result of aberrant myelopoieses (10). Their differentiation is arrested during such conditions. MDSCs are well studied in tumour microenvironment, where they are shown to suppress T cells and NK cells to prevent anti-tumour immune responses.

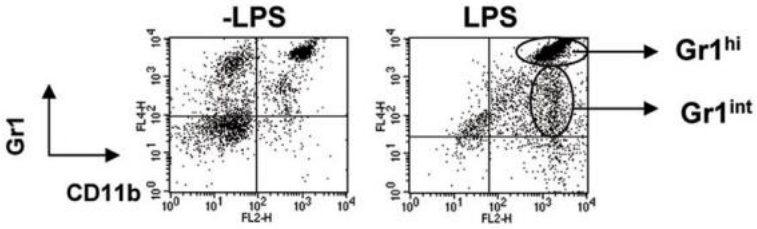


Immunity 38, 541–554, March 21, 2013

Fig 1.2 : Formation of MDSCs

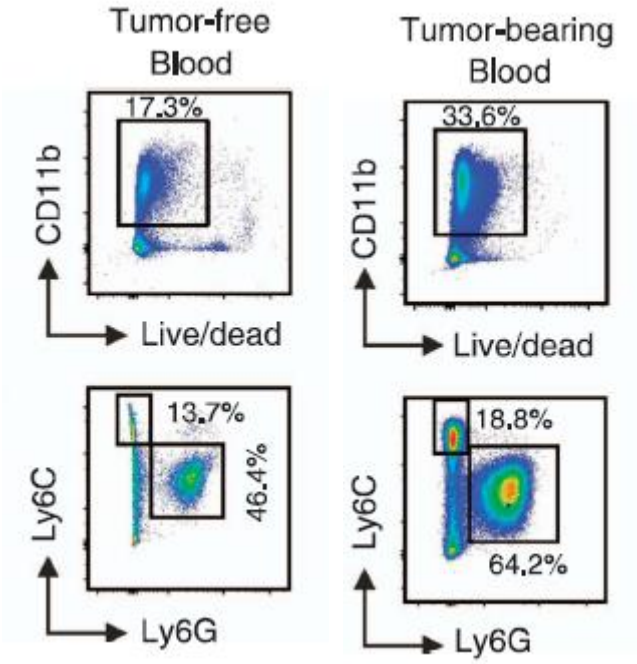
During pathological conditions and cancers, terminal differentiation of myeloid cells are blocked and suppressor cells are formed.

In mice, MDSCs are phenotypically characterized as $CD11b^{+}$ $Gr1^{+}$cells. There are distinct subsets that are identified within this population and are mainly divided into monocytic and granulocytic MDSCs. Gr1 antibodies binds to two epitopes Ly6C and Ly6G. Based on the relative expression of these molecules, MDSCs are classified into different subsets. $CD11b^{+}$ $Gr1^{hi}$ and $CD11b^{+}$ $Gr1^{int}$ cells are shown to have suppressive function. When analyzed on the basis of CD11b, Ly6C and Ly6G, $CD11b^{+}Ly6C^{hi}$ $Ly6G^{-}$ and $CD11b^{+}$ $Ly6C^{low}Ly6G^{hi}$ cells are shown to have suppressive activity.



Mucosal Immunol. 2010 Nov; 3(6): 578–593

(a)



Nature Communications 7,12150 (2016)

. (b)

Fig 1.3 : Gating strategy for MDSCs based on different markers.

Panel (a) shows the gating of MDSCs based on CD11b and Gr1. Paanel (b) shows the gating of MDSCs based on CD11b, Ly6C and Ly6G.

However, a clear distinction and identification of MDSCs based on phenotypic expression of these markers is challenging.. Expansion of myeloid compartment under pathological condition is a common phenomenon and neutrophils as well as monocytes are identified by the same set of markers. Therefore, identification of MDSCs is usually accompanied by their functional ability to inhibit responses. Since MDSCs are known to suppress T cell proliferation, an in vitro proliferation assay of T cells in presence of MDSCs is used to designate MDSCs. Along with functional assays expression profile of various molecules can also help in identifying MDSCs. These include suppressive molecules like arginase 1(ARG1), nitric oxide synthase 2 (NOS2) and reactive oxygen species. Detection of these molecules by RT-PCR can give an idea of the suppressive environment.

Even though MDSCs are known to suppress immune responses in cancers and pathological conditions, recent studies have shown that they could help maintain homeostasis and the outcome of transplantation (11).

Since the lesions during herpes stromal keratitis are mainly caused by activated CD4 T cells, a logical therapy to control the disease would to suppress the function of activated T cells and over represent regulatory cell populations. Regulatory cells consist of both adaptive and innate immune origin. Extensive studies have been performed on the role of adaptive immune regulatory cells (Tregs) on controlling HSK. Here we are investigating the role MDSCs on modulating viral induced inflammatory lesions.

We are mainly trying to address two questions,

1. Are MDSCs involved in disease progression during HSK?
2. Can MDSCs modulate viral induced inflammatory lesions and control disease progression?

1.2 MATERIAL METHODS

1.2.1 Mice and viruses

BALB/c female mice of 4-8 weeks old were used for the experiments. HSV-1 RE strain of virus was used for infections. Institutional animal ethics committee (IAEC) approved protocols were used for conducted animal experiments.

1.2.2 Corneal HSV infection and clinical scoring

Mice were anesthetized by i.p (intra-peritoneal) injection of tribromomethanol (Avertin) and the corneal infections were conducted. The cornea was scarified using a 32 G needle and a 2 µl drop containing 1 x 10^5 PFU of virus was applied to the eyes. The eyes were examined for scoring for lesion severity and angiogenesis on different days by a slit-lamp bimicroscopre. The scoring of lesions are as follows: 0 : normal cornea, +1 : mild corneal haze, +2 : moderate corneal opacity or scarring, +3 : severe corneal opacity but iris visible, +4 : opaque cornea and cornel ulcer, +5 cornea rupture and necrotizing keratitis. The scoring for angiogenesis is as follows: a grade of 4 was given to quadrant of a circle representing a centripetal growth of 1.5mm towards the center. The scores of all 4 quadrants were summed to derive the angiogenesis index.

1.2.3 Preparation of samples for Flow Cytometry

Mice were euthanized and experiments were performed in accordance with protocols approved by IAEC. Cornea, spleen, cervical lymph node and trigeminal ganglia were isolated at different time points post infection for immune analysis. HSV infected cornea were harvested from infected mice and digested with liberase (2.5 mg/ml) for 45 min at 37^{o}C in a humidified atmosphere of 5% CO_2. After incubation, the cornea was grinded and passed through insulin syringe multiple times for preparing single cell suspension. Trigeminal ganglia was also treated with liberase (2.5 mg/ml) and processed similarly. Single cell suspension was prepared from cervical lymph node (draining lymph node) by straining through a 70-micron cell strainer. Similarly single cell suspension was prepared from cell strainers and spleen samples were

treated with RBC lysis buffer (2 ml) to eliminate RBCs. Cells were counted for all the samples using a hemocytometer. One million cells were taken from each sample for surface staining.

RBC Lysis Buffer (1X)	
NH_4Cl	155mM
$NaHCO_3$	12mM
EDTA	0.1mM

.

1.2.4 Surface staining and Flow Cytometry

All staining steps were conducted at 4^{o}C. Antibodies used were diluted to a 1:100 ratio in FACS buffer and 50 µl of antibody solutions were added to each sample. The samples were incubated in ice for 30 min and 3 washings were performed in FACS buffer after the incubation. After washings, samples were resuspended in 200 µl of FACS buffer and was acquired on a BD Accuri Flow cytometer and analysed using Flowjo or Cell quest pro softwares.

FACS Buffer
2% FBS in PBS

1.2.5 Antibodies Used

All the antibodies used were purchased from BD. The antibodies used are CD 11b – FITC, CD4 –PE, Ly6c-PerCP and Gr1-APC. The channels for flurochromes are as follows : FITC-FL-1, PE- FL-2, Per-CP-FL-3 and APC-FL-4. All antibody dilutions were prepared in FACS Buffer.

1.2.6 Isolation of Bone Marrow cells

1. Mice were euthanized and sprayed with 70% ethanol.
2. The skin covering the legs were removed and muscles were cutoff using scissors.

3. The leg was dislocated from hip joint avoiding breakage of femur head.
4. Remaining muscles on femur and tibia was removed and the femur was separated from the tibia at knee joint.
5. The bones were washed with 70% ethanol and placed in a petri dish containing ice cold RPMI supplemented with 10% FBS and 1% penstrep.
6. The epiphyses of the bones were cut-off and bone marrow was flushed using a 2 ml syringe. Complete RPMI medium was used for flushing.
7. The flushed BM cells were passed multiple times through syringe for making a single cell suspension.

1.2.7 In vitro generation of MDSCs

1. Bone marrow cells isolated from BALB/c female mice using the protocol mentioned above.
2. BM cells were incubated with 1ml of RBC lysis buffer form 5 minutes at room temperature.
3. The cells were washed in PBS twice and resuspended in complete RPMI medium.
4. The cells were counted using a hemocytometer.
5. After cell were counted, $3x10^6$ cells were added to a $25mm^2$ flask containing 5 ml RPMI supplemented with IL-6, IL-4 and GM-CSF at 40 ng/ml .
6. Some cells were also grown in RPMI without any cytokine treatment.
7. After 4 days, cells were washed and some cells were stained for CD11b, Gr1 and Ly6C for characterization.
8. Remaining cells were used for down stream experiments.

1.2.8 CFSE labeling of Lymph Node cells

1. Cervical lymph node (CLN) of a day 15 infected HSV 1 infected mice was isolated and single cell suspension was prepared.
2. Cells were counted using a hemocytometer and 10 million cells taken and were resuspended in 1 ml PBS.
3. 10 µM CFSE solution of 1 ml was prepared and was added drop wise to CLN cells in PBS.

4. After addition the sample was mixed thoroughly and incubated at room temperature for 5 minutes.
5. After incubation, FBS (2ml) was added and the sample was centrifuged at 1500 rpm at room temperature for 5 minutes.
6. Next, the supernatant was thrown and the cells were resuspended in 10% FBS in PBS solution and again centrifuged at 1500 rpm at room temperature for 5 minutes.
7. After centrifugation the supernatant was thrown and the cells were resuspended in RPMI.

1.2.9 *In-vitro* suppression assay

1. The culture was prepared such that the number of MDSCs/BM cells to CLN cell are in a ratio of 1:1, 1:2, 1:4, and 1:8 respectively in a 24 well plate in 1 ml media.
2. The cells were stimulated with HSV-1 RE strain with MOI -1.
3. The cells were co-incubated for 3 days, and in the 3rd day the cells were harvested for FACS analysis.
4. On 3rd day, cells were stained for CD4 marker and the CFSE dilution of CD4 cells were analyzed.

1.3RESULTS AND DISCUSSION

1.3.1 Disease Scoring

In order to understand the role of MDSCs in during disease progression, animals were infected via ocular route and analysed for disease development. Simultaneously we also performed the kinetic analysis of $CD11b^{+}$ $Gr1^{+}$ population, which corresponds to MDSCs. The disease was analysed based on the angiogenesis and the lesion scores in the cornea (Fig 1.4).

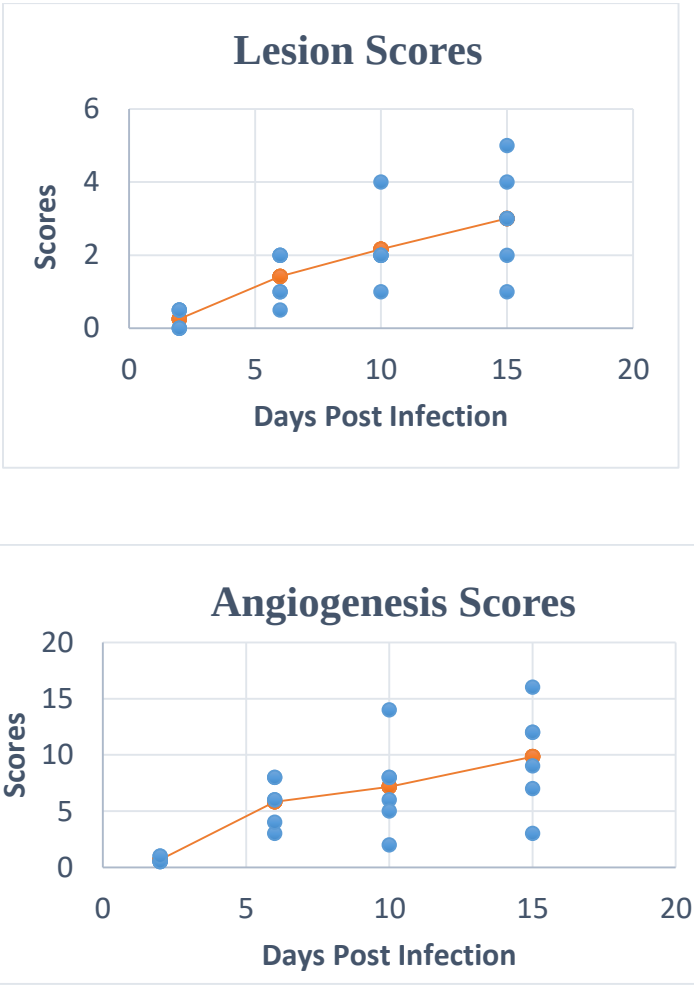


Fig 1.4 : Disease scoring during the course of infection.

It was observed that the mice were developing disease during the course of infection. Now tried to see how different cell populations that corresponds to MDSCs varied over the course of infection. Analysis were mainly done on cornea and spleen.

1.3.2 MDSC kinetics in spleen

After the disease was characterized, the animals were euthanized and different organs were collected. Surface staining of single cell suspension was performed as described. The time course change of $CD11b^+$ $Gr1^+$ cells were analysed during the course of infection for 15 days (Fig 1.5).

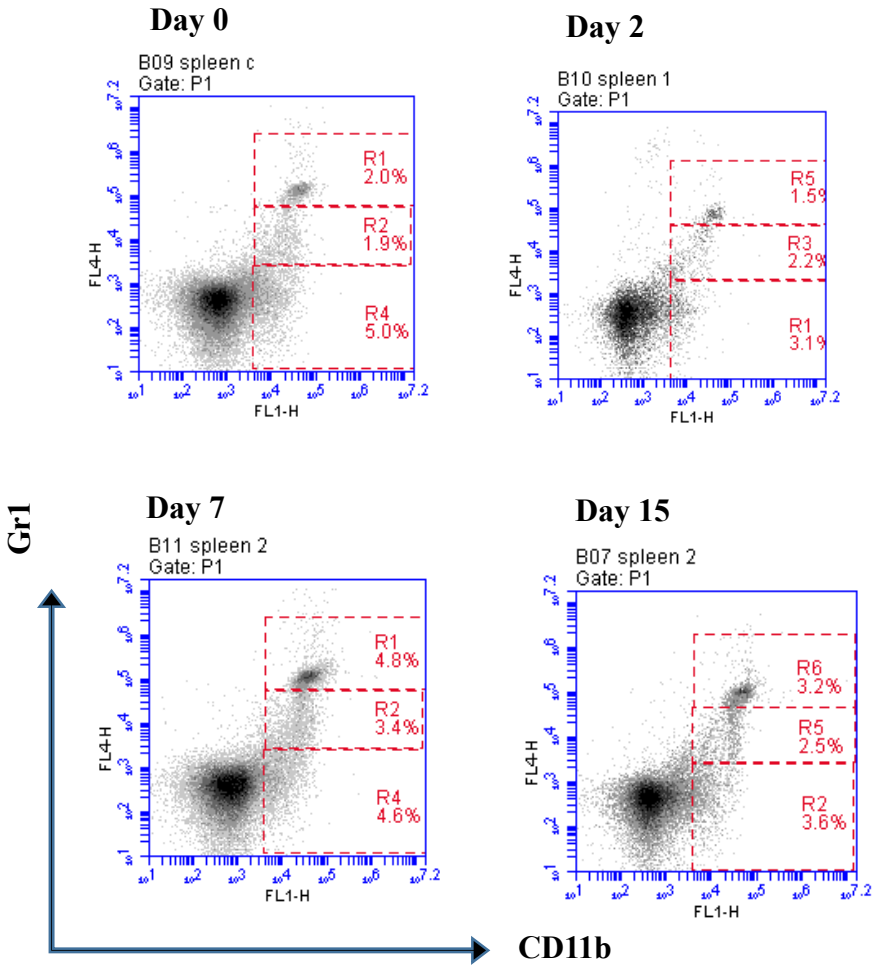


Fig 1.5 : Representative plots from spleen

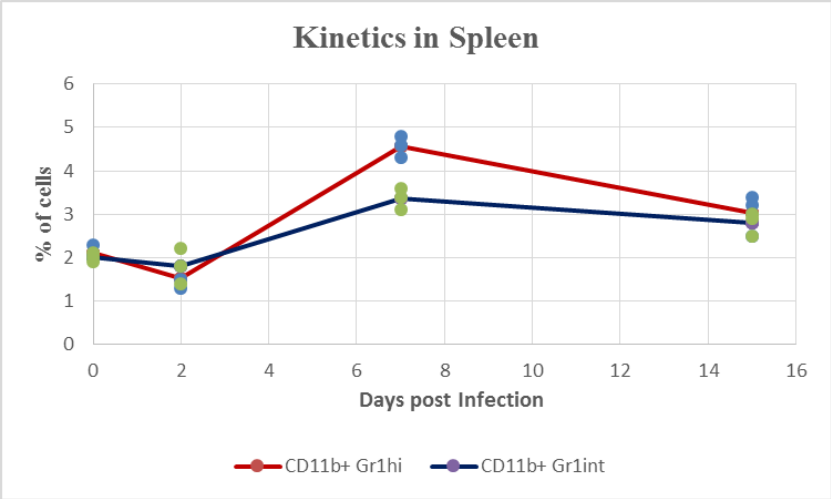


Fig 1.6 : CD11b$^+$ Gr1$^+$ cells in spleen during the course of infection

In spleen it was observed that the percentage of CD11b$^+$ Gr1^{hi} and CD11b$^+$ Gr1^{int} cells increased at day 7, showing that as a result of inflammation, the population that phenotypically corresponds to MDSCs have infiltrated into spleen (Fig 1.6). However, the functionality of such cells was not ascertained using in vitro or in vivo assays.

1.3.3 MDSC kinetics in cornea

A similar analysis was carried out in cornea, where the CD11b$^+$ Gr1$^+$ cells were analysed during the course of infection. Here cornea was isolated from infected and control mice, it was treated with liberase and then single cell suspension was prepared. Each time before analyses disease scores were calculated (Fig 1.7).

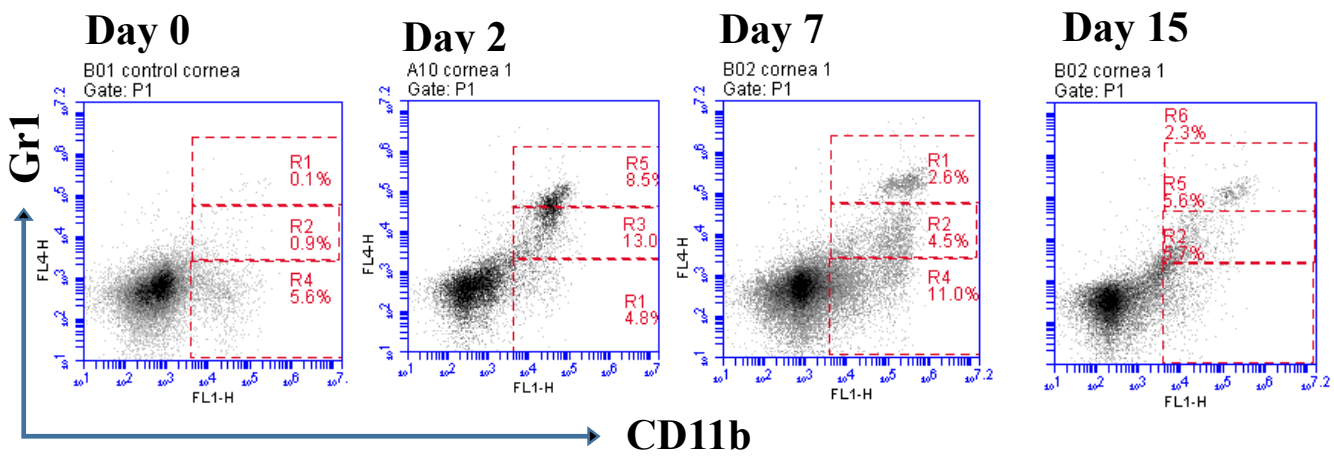


Fig 1.7 : Representative plots from cornea

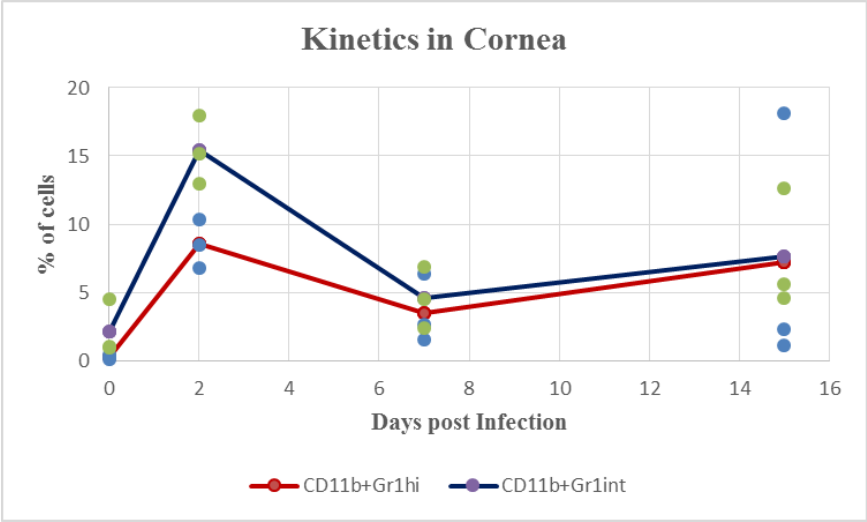


Fig 1.8 : CD11b$^+$ Gr1$^+$ cells in cornea during the course of infection

In cornea we found that the cell population representing CD11b$^+$ Gr1$^+$ inversely correlated with disease (day 7 and 15). Thus, when the disease severity was less the infiltration of such cells was reduced. Since CD4$^+$ T cells are the main orchestrators of this corneal disease, the inverse kinetics of disease progression and MDSC population infiltration suggests that the cell numbers of MDSCs in cornea would be too less to control the inflammation induced by CD4$^+$ T cells.

1.3.4 *In vitro* generation of MDSCs

Next we wanted to investigate whether MDSCs can modulate viral induced inflammatory reactions and control disease progression. So we tried to generate MDSCs *in vitro* and did a phenotypic characterization of the generated cells. Here we isolated bone marrow cells and treated them with IL-6, IL-4 and GM-CSF. 4 days after treatment with cytokines, we harvested the cells and surface staining was carried out (Fig 1.9).

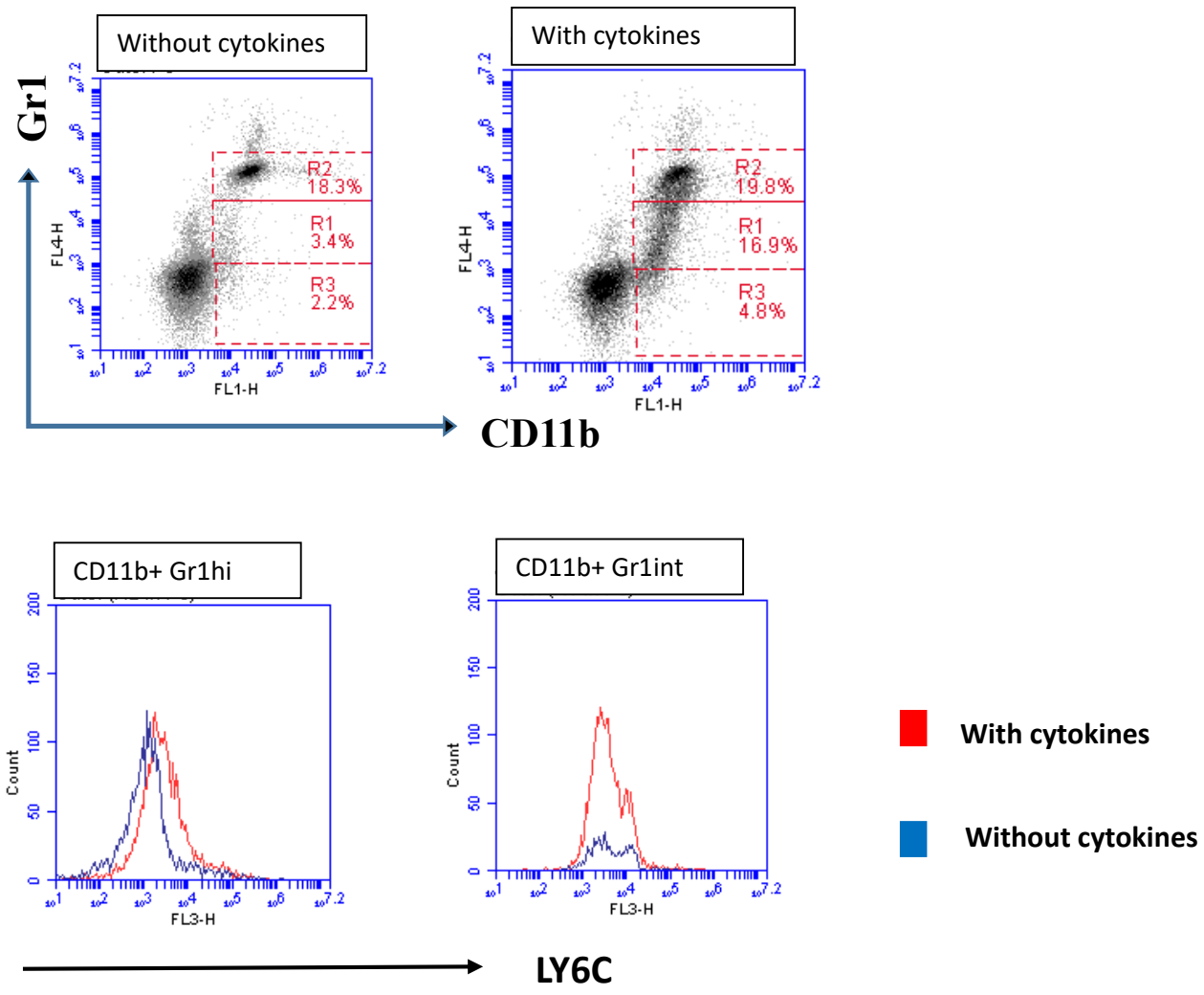


Fig 1.9 : Phenotypic characterization of *in vitro* generated MDSC.

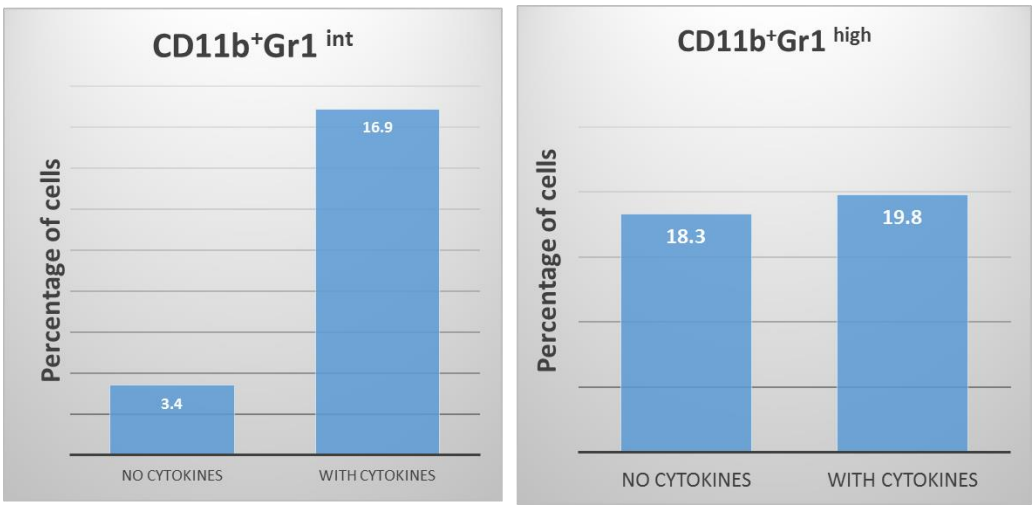


Fig 1.10 : Change in different subsets of CD11b$^+$ Gr1$^+$ cells after cytokine treatment

After surface staining of the *in vitro* generated cells, we found that there was an increase in the CD11b$^+$ Gr1$^+$ cells (Fig 1.10). The increase was more prominent in CD11b$^+$ Gr1^{int} cells. In case of CD11b$^+$ Gr1^{hi} cells, there was an increase in the Ly6C (monocytic marker) expression after treatment with cytokines showing that these cells are entering differentiation. Finally, these cells were having phenotypic attributes of MDSCs, but the question remains on whether they have the suppressive functions as of MDSCs. To answer this question we carried out an *in vitro* suppression assay on CD4 T cells to check whether the in vitro generated cells do have suppressive activity or not.

1.3.5 *In vitro* Suppression Assay

To check the functionality of the generated MDSCs an *in vitro* suppression experiment was carried out. We isolated cervical lymph node (CLN) from an HSV infected mice and labelled the cells with CFSE. We then co-incubated the CLN with either MDSCs or bone marrow cells and stimulated them with virus. 3 days after incubation, CFSE dilution of CD4$^+$ T cells were analyzed in a flow cytometer.

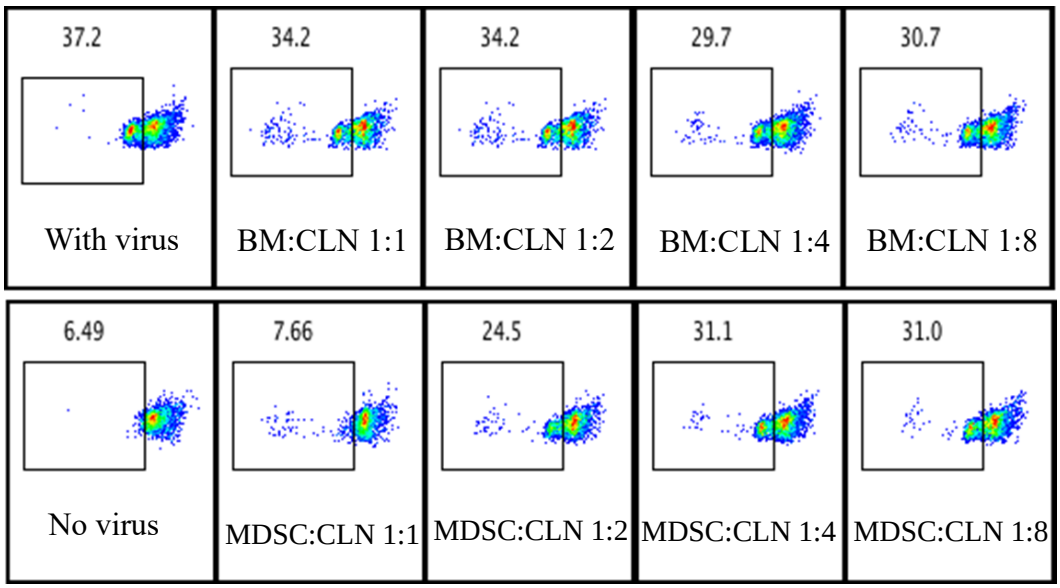


Fig 1.11 : CFSE dilution of CD4 cells from CLN.

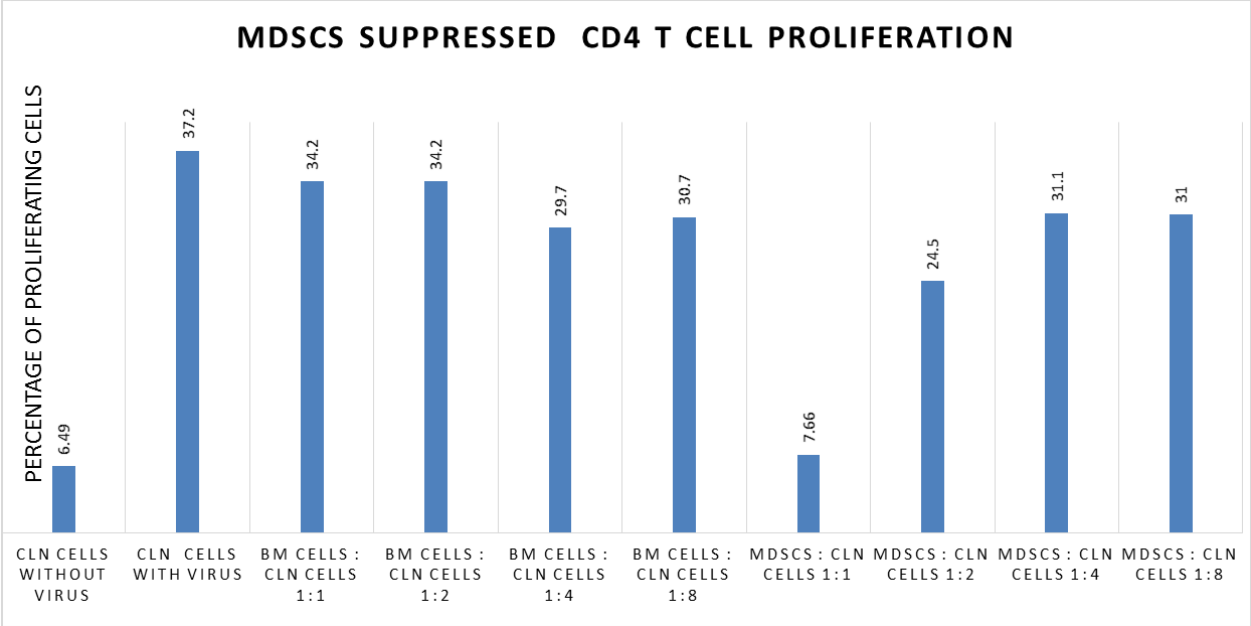


Fig 1.12 : Proliferation of CD4 T cells

Since CLN cells were isolated from a 15 day HSV infected mice, there would be antigen specific T cells which would have undergone clonal expansion since the mice was previously infected by HSV 1 virus. When we re-stimulate them with HSV 1 virus the already committed $CD4^+$ T cells would further undergo proliferation. As is shown in figure Fig 1.11, when cells were not stimulated with virus the proliferation of CD4 T cells was around 7%. But when the cells were stimulated with virus the proliferation increased to 37%. *In vitro* generated MDSCs were co incubated with CLN cells stimulated with virus. As a control bone marrow cells from naïve mice were also co incubated with CLN cells. When the samples were analyzed after 3 days we found that MDSCs were able to inhibit the proliferation of CD4 cells in a dose dependent manner. Moreover the inhibition was in an antigen-specific manner since CLN cells were stimulated with HSV-1 virus. This experiment suggests that the *in vitro* generated cells have the potential to inhibit the proliferation of CD4 T cells which expand after HSV 1 infection.

1.4 CONCLUSION AND FUTURE DIRECTIONS

First of all we tried to see whether MDSCs have any role during HSK disease progression. We found that, during higher disease the $CD11b^+$ $Gr1^+$ populations that corresponds to MDSCs were less in cornea. Since MDSC population is less in cornea more CD4 mediated pathology occurs. But the evidence is inconclusive for saying MDSCs do have a role during disease progression and future studies would focus on depleting such cells before infection with HSV1 and monitoring disease progression. In addition the functional mediators of such cells would be analyzed during infection. In order to demonstrate whether or not MDSCs can modulate HSV 1 induced immunopathological lesions caused by CD4 T cells, we performed preliminary experiments to measure the suppressive activity of *in vitro* generated MDSCs. We were able to show that *in vitro* generated MDSCs were able to suppress the proliferation of CD4 T cells in an antigen specific manner. But, the main idea was be to show that MDSCs are able to control the lesions caused by HSV infection in cornea and for that adoptive transfer experiments have to be carried out. Currently adoptive transfer experiments are underway which would give a better idea about the immunomodulatory role of MDSCs during HSK.

CHAPTER II

IMMUNOMODULATION IN ZEBRAFISH TO STUDY DENGUE VIRAL PATHOGENESIS

2.1 INTRODUCTION

2.1.1 Zebrafish as an Immunological model system

Zebrafish has been used as a model organism since 1930's. Early stages of zebrafish are optically transparent and facilitates development and regeneration studies. The ease of applying genome editing tools and technologies in zebrafish allows deciphering pathways and molecular signaling events in greater detail. Zebrafish has also been used to study genes and molecular species involved in the induction or the progression of different diseases since forward genetic screens can be performed in a much easier manner (12). The genome of zebrafish has already been sequenced; therefore phenotypic association with genotypes can be established. Invertebrate model organisms like *Caenorhabditis elegans* and *Drosophila melanogast*er have provided substantial information about the mechanisms that control various developmental pathways, but how hematopoiesis and differentiation of adaptive immune cells take place during homeostatic as well as diseased conditions is impossible to investigate in these models. This is because of the reason that cells of same blood lineage are either not present in other vertebrates or not defined or in these models. Zebrafish has conserved genetic program that underlie vertebrate blood development. Moreover they are known to possess most cell types, cellular products or genes known for human innate and adaptive immune system. They have both B cells and T cells which allows for studying lymphoid populations and also how such cells respond during pathophysiology of diseases. High fecundity of zebrafish allows using multiple replicates for experimentation to reveal even subtle differences, a feature that is not so easily provided by some of the favored models such as rodents that are frequently used for carrying out preclinical immunological studies. Even though zebrafish offers a lot of advantages for studying immunological phenomenon, certain drawbacks also exist. One of the major drawbacks includes a poorly characterized lymphatic system, which some believe might

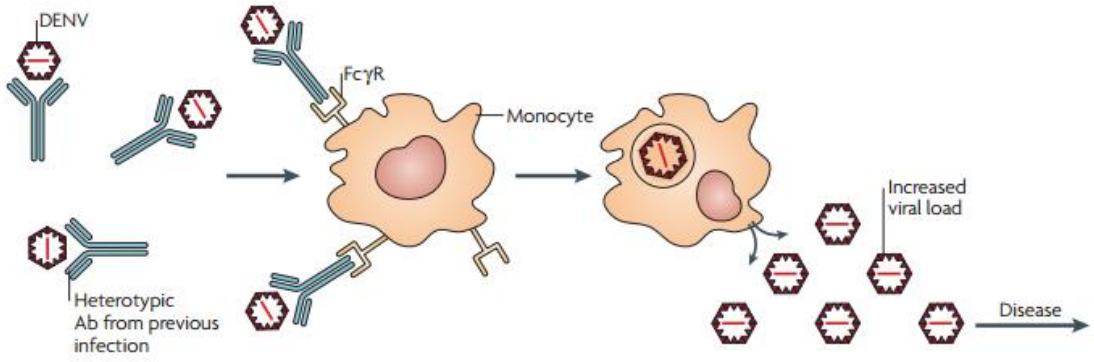
be missing altogether. However such a possibility is least likely. Although B cells have been identified in zebrafish, the diversity in immunoglobulins repertoire and whether or not a class switching does exist is not clearly defined (13). This is complicated by the fact that immunological tools such as antibodies for cell surface markers and established well-characterized cell lines are lacking.

In spite of all these unaddressed issues, there has been an increase in the use of zebrafish as a model for studying microbial infections and viral pathogenesis in recent times (14,15,16,17). One of the major drawbacks of using higher vertebrate models for disease progression is their inability to track viral particles. Since zebrafish is transparent during early stages of development, usage of fluorescently labelled virus enables investigators to track the virus and also understand the progression of disease in real time. This information can be used for better understanding and devising strategies for various human infections. Till date a few human viruses have been shown to infect zebrafish. Some studies have shown the experimental susceptibility of zebrafish to infection by viruses including infectious pancreatic necrotic virus (IPNV), infectious hematopoietic necrotic virus (IHNV), spring viraemia of carp virus (SVCV), snakehead rhabdovirus (SHRV), viral hemorrhagic septicemia virus (VHSV), Herpes simplex virus type 1 (HSV type 1 and Chikungunya virus. The susceptibility of zebrafish for Dengue virus and subsequent pathophysiological events has not been reported. Insights into the pathogenesis of dengue virus would help better understand the contribution of host and viral factors. We aimed to develop zebrafish as a model for studying dengue viral infection and deciphering the contribution of cellular and molecular events that favor or control the disease development.

2.1.2 Dengue Viral Infections

Dengue virus is an RNA virus belonging to the family Flaviviridae and genus flavivirus. It is transmitted to humans by mosquitoe (*Aedes aeygypti*) bites. Four serotypes of the virus are known. Primary infection with any of these serotypes is usually asymptomatic but can also results in a wide spectrum of clinical symptoms depending on the host factors such as age of infection, any concurrent or previous infection or the immune status. Based on the clinical symptoms the disease can range from Dengue Fever (DF) to Dengue Hemorrhagic Fever (DHF) and later to Dengue Shock Syndrome (DSS). However, in most cases, DHF and DSS

are caused by a secondary heterologous infection with a different serotype of dengue virus. Antibody Dependent Enhancement (ADE) of infection or activation of cross-reactive T cells (as outlined in Fig: 2.1) that confer least protection are known to be the primary cause of enhanced severity of disease (18).

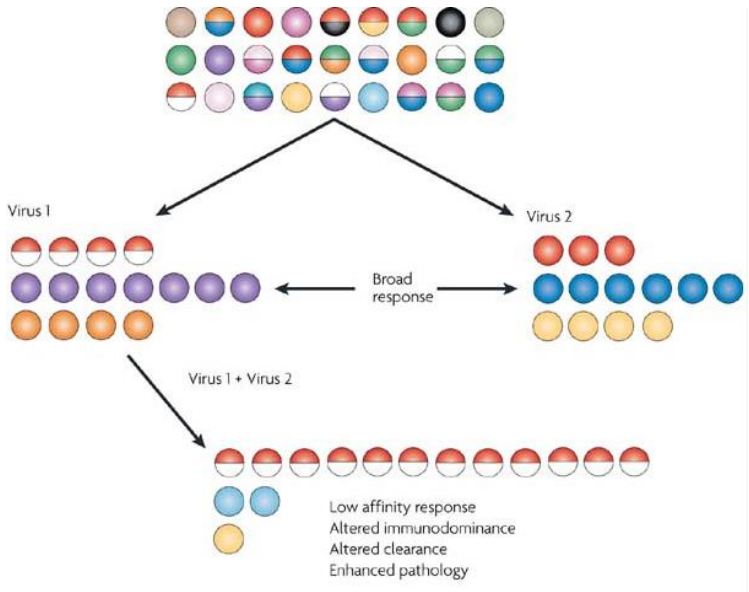


Nature Reviews Microbiology **5**, 518-528 (July 2007) | doi:10.1038/nrmicro1690

Fig 2.1 : ADE by Dengue virus.
Non neutralising antibodies facilitate the entry of Dengue virus into immune cells and allow viral replication

Such antibodies and T cells are not able to neutralize or eliminate the virus but could contribute to immunopathological response nonetheless. As the hyperendemicity of DENV infection is becoming more and more evident, it is likely that multiple serotypes show their prevalence simultaneously leading to enhanced dengue virus related pathologies. Given the fact that in a vast area with predominantly tropical climate, it is not easy to control mosquito population and can indeed favor the proliferation of vectors carrying multiple serotypes. This makes the case for transmitting multiple heterologous strains in susceptible population and thereby enhanced disease prevalence as well as severity. Infection with one of the serotypes leads to the production of neutralizing antibodies that provide immunity against the homologous infecting serotype. Since there exist cross-reactivity between different serotypes, certain cross reactive but non-neutralizing antibodies are also produced particularly during heterologous infection. These non-neutralising antibodies facilitate the entry of Dengue virus into immune cells such as those belonging to innate immune system and allow virus replication. This further increases the viral load and a subsequent severe disease. Similarly, cross reactive CD8 and possibly CD4

T cells expand during heterologous infection (as outlined in Fig 2.2) (19). These cells produce cytokines, chemokines and other mediators such as VEGF but are not able to eliminate virus-infected cells.



Nature Reviews Microbiology **5**, 555-563 (July 2007) | doi:10.1038/nrmicro1709

Fig 2.2 : Narrowing of T cell response by cross reactivity and heterologous immunity
During heterologous viral challenge there is a dramatic narrowing of T cell repertoire. Here there is an expansion of cross reactive T cells.

The mediators cause plasma leakage; a major manifestation of the disease DHF and in most severe cases DSS leading to mortality. Plasma leakage and a loss of fluids can further results in tissue hypoperfusion, lactic acidosis, hypoglycemia, hypocalcaemia and finally multiple organ failure.

2.1.3 Animal models for DENV infections

Even though we understand the contribution of various cellular and molecular mechanisms during disease progression caused by DENV, the exact role of certain critical factors remains unknown. Some of the least described issues include the kinetics of immune induction, the precise contribution of polyfunctional versus monofunctional virus reactive CD8 T cells and

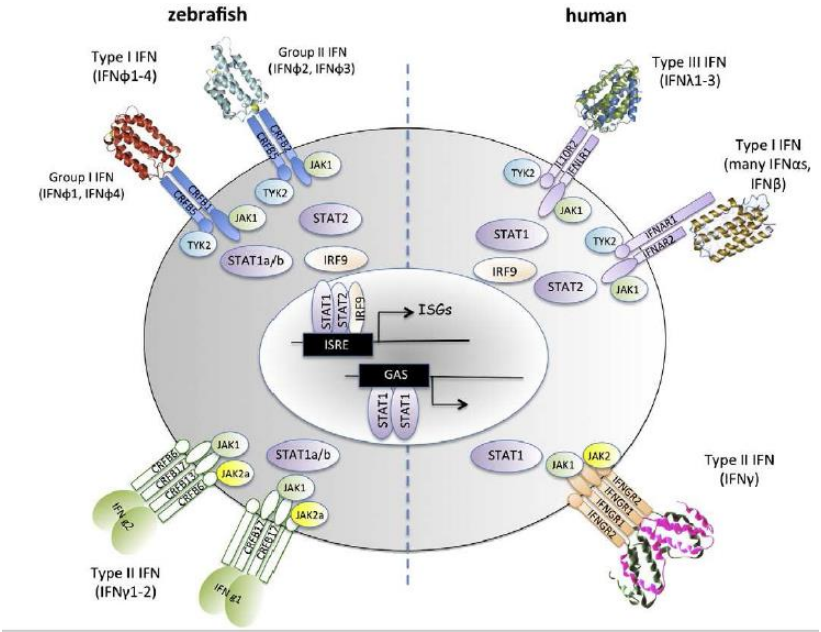
also the magnitude of virus specific antibody responses targeted towards structural and non-structural proteins with respect to their relative contribution in dictating the severity of disease. Additionally the relative contribution of innate and adaptive immune mechanisms in the causation of disease largely remained unexplored. Similarly, whether antibodies play a dominant role in disease pathogenesis or T cells are critical players remains undefined. One of the main reasons is the difficulty in developing animal models for Dengue infection since DENV does not infect non-human primates. Some immunodeficent mice infected with DENV show signs of severe disease similarly seen in humans. Immunomodulation either by drugs or by genetic manipulation is required to establish severe infection and hence study the pathogenesis. One such model is $IFNR^{-/-}$ mice which lacks Interferon α/β or α/β/γ receptors. In these animals a severe diseases was induced by DENV infection and such animals have been used for investigating the potential role of T cells in the pathogenesis and protection. Similarly such animals also have helped facilitate vaccine target identification. Even though mice models offers certain advantages for studying DENV pathogenesis, zebrafish do have unique advantages as discussed earlier. Thus, genetic manipulations can be done in an easier way and viral particles can be tracked in real time enabling us to follow disease progression and interaction of various cells in zebrafish. This can help address many unresolved questions regarding dengue viral pathogenesis. Through this project we attempted generation of zebrafish lines that is susceptible for DENV infection. As explained earlier, signaling through type I interferons is a critical decider whether animal is susceptible or resistant to infection, we focused to eliminate the gene for one of the critical players in Type 1 interferon signaling in zebrafish.

2.1.4 Type I interferons in zebrafish

Innate immunity is the first line of defense against an invading pathogen. Interferons, as the name suggests interfere with viral replication (20). Type 1 interferons are the most critical cytokines of innate antiviral response and are predominantly produced by infected cells as well as innate immune cells that include plasmacytoid dendritic cells. Interferons elicit their function by the induction of specialized effector proteins encoded by IFN stimulated genes (ISGs) that disrupt viral replication cycle. Receptors for type I interferon are present on most cells and its ligation with type I IFN leads to transcription of more than 100 ISGs. The Janus

kinase/ signal transducer and activators of transcription (JAK/STAT) pathway are critically involved in effecting interferon signaling. Binding of interferons to receptor causes receptor dimerization leading to activation of JAKs that are already bound to the receptor. This leads to receptor phosphorylation, which then creates docking sites for STATs. STATs are then phosphorylated by JAKs activity. Phosphorylated STAT form dimers and translocates to the nucleus to cause transcription and translation of numerous genes. Some of these include activation of protein kinase R (PKR), which phosphorylate elongation initiation factor-2 (eIF-2) rendering it nonfunctional and hence protein synthesis in cells is inhibited. The IFN signaling also induces an enzyme 2'5'oligoadenylate synthetase, which promotes ribonuclease L activity. Ribonuclease L degrades mRNA having a poly(A) tail and hence new viral protein is not synthesized. This creates an 'anti-viral state' in the cells.

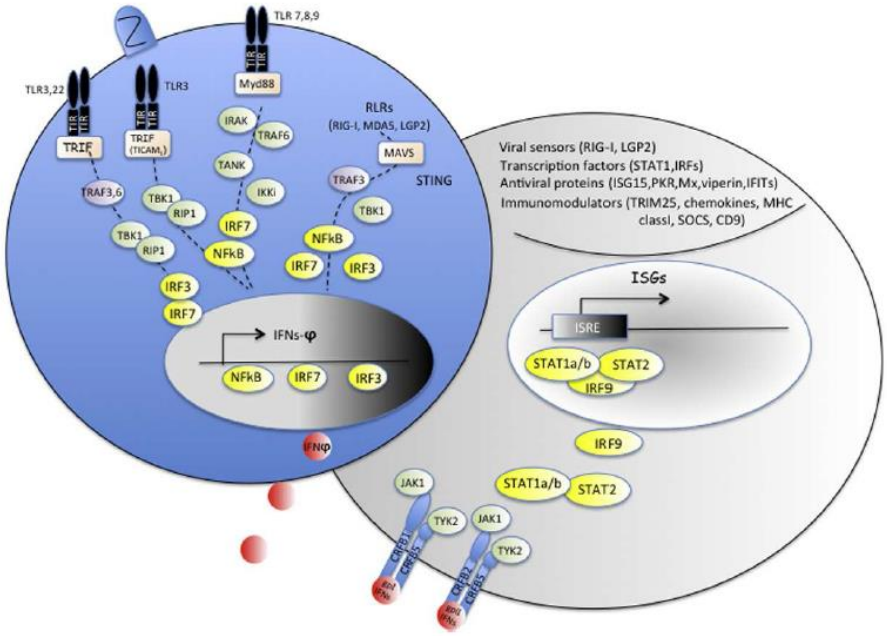
Till date, four virus-induced interferons have been identified in zebrafish and are known as interferon phi's (IFNφs). IFNφs are classified into two groups based on the number of cysteine residues predicted to be engaged in disuphide bridges. IFNφ1 and IFNφ4 belong to Group I and IFNφ2 and IFNφ3 belongs to Group II. The two groups of IFNs were found to signal via two different receptor complexes. Group I Interferons signal through CRFB1-CRFB5 receptor complex and Group II Interferons signal through CRFB2-CRFB5 complex (as outlined in figure 2.3 and 2.4) (21).



J. Mol. Biol. (2013) 425, 4904–4920

Fig 2.3 : Zebrafish and human interferons and their receptors

The figure represents the homology that exist in human and zebrafish interferon signaling. Zebrafish IFN signaling is represented on left and human IFN signaling is represented on right.



J. Mol. Biol. (2013) 425, 4904–4920

Fig 2.4 : IFN signaling pathways in Fish

PRRs recognize the pathogens and stimulate the production of IFNφs. These IFNφs binds to IFN receptor and further stimulate the production of ISGs which helps in creating an 'anti-viral state'.

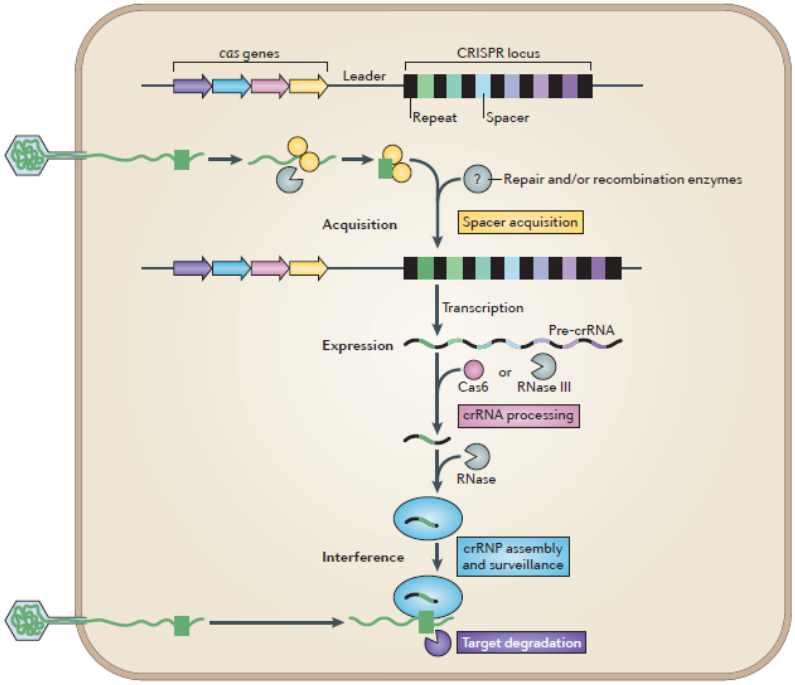
In last decade or so our understanding about virus-induced interferons in zebrafish has increased tremendously but still a lot more needs to be done. Therefore, targeting one or more of the IFNφs won't be a good option as there might be more IFNφs. CRFB5 is a common receptor chain in interferon signaling by both group I and group II IFNs in zebrafish. So, one of the way for targeting type I interferons would be to knockout CRFB5 as this would help achieve a complete disruption of interferon signaling by two groups. This is one of the major reasons why we focused on targeting CRFB5 and not IFNφs for disrupting IFN signaling.

2.1.5 Precise Genome Editing

Generation of precise and targeted alterations in the genome of living cells has enabled us to understand various genes and pathways crucial for development and disease. Most of the genome editing tools makes use of the function of nucleases to make double stranded breaks in DNA. These double strand breaks can be repaired by two major pathways; non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ repairs the breaks by random addition or removal of nucleotides, which can lead to frame shifts. This is a highly error prone repair method (22). HDR recombination makes use of homologous template to repair the breaks and is an error proof mechanism. Recent approaches for targeted genome editing include zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENS) and clustered regulatory interspersed short palindromic repeats and CRISPR associated protein-9 (CRISPR/Cas9). One of the main factors that one needs to consider while selecting genome-editing tools and its application is the efficiency. Among various tools used currently, CRISPR/Cas9 has shown to edit genomes easily and more efficiently when compared to others. Other genome editing techniques like ZFNs, TALENS are shown to have an efficiency less than 50% on most reports. CRISPR/Cas9 mediated gene knockout has shown to be >70% efficient in Zebrafish (23). An ease of performance, high efficiency and versatility make CRISPR/Cas9 the most favorite tool for genome editing.

2.1.6 CRISPR/Cas9 System

CRISPR/Cas was initially discovered to be an essential component of several bacterial and archeal immune system. CRISPR/Cas is an RNA guided endonuclease system in these organisms that helps eliminate invading genetic material (24). The process can be divided into three main process; acquisition, expression and interference (figure 2.5). During acquisition, integration of new spacers that corresponds to foreign DNA occurs. In next phase known as expression phase, the CRISPR locus is transcribed and the small CrisprRNAs (crRNAs) are formed. In the last phase, which is the interference phase, crRNA along with Cas forms a ribonucleocomplex and scans the invading DNA for complementary nucleic acid target. Upon successful recognition Cas proteins eventually degrade the target.



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doi:10.1038/nrmicro3279

Fig 2.5 : Mechanism of CRISPR/Cas in prokaryotes.

CRISPR/Cas system works as an adaptive immune system in prokaryotes. An RNP complex is formed from CRISPR/Cas array, which identifies foreign genetic elements and cleaves it.

After it's discovery in bacteria, CRISPR/Cas has been modified to use it as a tool for genome engineering (13). For genome engineering Cas9 and guide RNA (gRNA) are required. The gRNA is a simplified version of crRNA and has to be complementary to DNA of our interest. One major prerequisite for CRISPR/Cas is the Protospacer Adjacent Motif (PAM) at target DNA which is required by Cas9 nuclease for creating double strand breaks (DSBs) (Fig 2.6). Therefore, the gRNA should be complementary to the sequence, which has a PAM at the end. Once Cas9 nuclease creates DSBs, NHEJ and/or HDR mechanisms are activated. NHEJ being error prone can induce frameshift in the DNA that eventually leads to loss of function. Even though HDR occurs at a low frequency, we can use it for knocking in constructs by providing homologous arms corresponding to the DSBs in the construct.

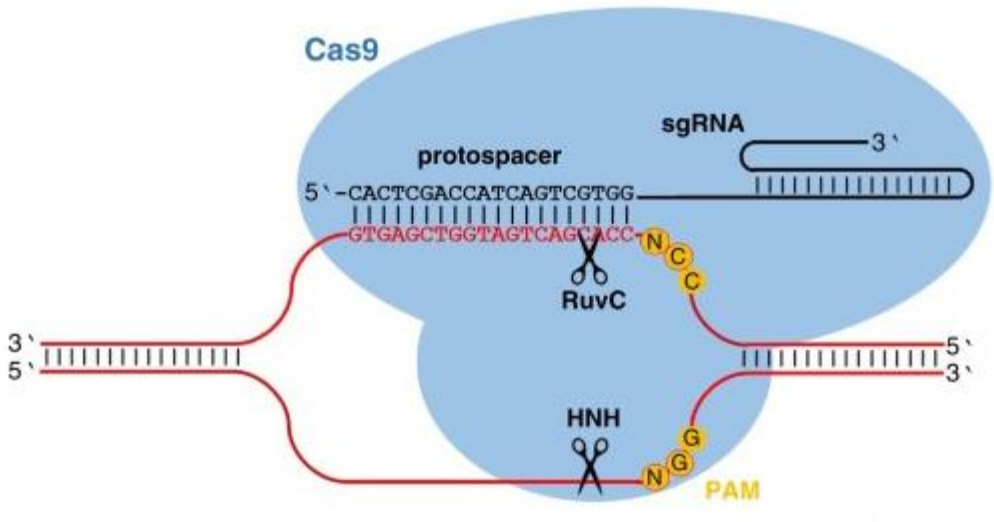

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Fig 2.6. Mechanism of CRISPR/Cas genome editing

The gRNA that is targeted to our gene of interest binds adjacent to PAM motif and creates DSBs 3 nucleotides upstream of PAM motif

2.1.7 Project Goal

We use CRISPR/Cas9 system for disrupting the signaling through type I interferon by targeting CRFB5. As this is a common receptor chain that form the receptor for two groups of type I IFNs in zebrafish. Knocking out CRFB5 would eventually disrupt the virus induced interferon signaling and ISGs rendering these animals more susceptible for DENV infections.

2.2 MATERIAL METHODS

2.2.1 Online Tools

Different online tools are available that predicts gRNA against a target gene. We used two tools: CRISPR DESIGN (http://crispr.mit.edu) and then Agilent CRISPR Tool (https://earray.chem.agilent.com/suredesign/home.htm). Using CRISPR DESIGN we found the best gRNA with maximum scores that target exon 2 of CRFB5. The scoring is mainly based on minimum off target effects on genic regions. Agilent CRISPR tool was used to validate the gRNA sequence obtained by first tool. In addition the sequence of whole gRNA along with T7 promoter upstream sequence was retrieved that consisted of 20 nt complementary sequence upstream of T7 promoter and the gRNA scaffold.

The template for IVT is given below:

5`GCTACATTATGCTGAGTGATATCCTCGCCACTTCGCCTGCGAGCAAAATCTCGA TACGACTTTTCGTATCGTTCAATTTTATTCCGATCAGGCAATAGTTGAACTTTTTC ACCGTGGCTCAGCCACGAA 3`, T7 promoter, complementary gRNA, gRNA scaffold.

2.2.2 RNA isolation from zebrafish

Liver from zebrafish was isolated and used for RNA isolation. Briefly the steps involved are enlisted below:

1. The tissue sample was homogenized in Trizol (200µl) by passing it through insulin syringe multiple times.
2. Then 40µl of chloroform was added and the mixture was incubated at room temperature in a rotator for 15 min at 40 rpm.
3. After incubation the sample was centrifuged at 7000 rpm for 5 minutes at room temperature.
4. Further the aqueous portion was collected and to which 0.4 volume of isopropanol was added and the cocktail was incubated on ice for 15 minutes.
5. The sample was centrifuged at 13,000 rpm for 15 minutes at 4^{o} C.
6. The supernatant was discarded and 200µl of 80% ethanol was added to the pellet. Mixing was done by tapping the micro centrifuge tube.
7. The mixture was centrifuged at maximum speed for 15 min and the supernatant was discarded.

8. The pellet was dried at room temperature for 2 minutes and 50 µl of DEPC treated water was added.
9. The RNA was further stored at -80^{o}C until further use.

2.2.3 cDNA preparation

The reaction mixture with the following composition was prepared in a micro centrifuge tube.

1st reaction:

OligodT Primer (50µM)	-0.5 µl
Random hexamers (50 µM)	-0.5 µl
dNTP mixture (10mM)	-1 µl
Template RNA	-5 µg
RNAse free dH_2O *ad*	-10 µl

This mixture was incubated at 65^{o}C in a heating block to disrupt secondary structure in the template RNA for 5 minutes and then cooled immediately and the second reactions carried out.

2nd reaction :

Template RNA Primer Mixture from reaction 1	- 10 µl
5 x Prime Script Buffer	- 4 µl
RNAse Inhibitor	- 0.5 µl (20 units)
Prime Script Reverse transcriptase (RTase)	- 1 µl
RNAse free water *ad*	- 20 µl

The mixture was incubated at 30^{o}C for 10 minutes and then at 42^{o}C for 1 hour and finally at 72^{o}C for 15 minutes.

2.2.4 Polymerase chain reaction (PCR) to amplify CRFB5

First 4 exons of CRFB5 were amplified using PCR with a set of following set of forward and reverse primers.

Forward Primer (FP): ATG GAG GAG TGT GCC CTG CTG CTG

Reverse Primer (RP): CCG AGT GTC ATC AGC CTG TCT CCA G

The reaction conditions are as follows

2.5mM dNTPS	- 1.6 µl (200nM)
10µM FP	- 1 µl (500nM)
10µM RP	- 1 µl (500nM)
cDNA	- 0.5 µl (2%)
5x Phusion buffer	- 4 µl (1x)
HF Polymerase	- 0.2 µl
$MgCl_2$	- 0.4 µl (1mM)
MQ Water *ad*	- 20 µl

2.2.5 Gel purification of CRFB5

The amplified product was visualized by 1% agarose gel and gel purified using Invitrogen Gel purification kit according to manufacturer's protocol.

2.2.6 *In Vitro* transcription of gRNA and Cas9 mRNA

The gRNA and Cas9 are under T7 promoter and *in vitro* transcription kit from Agilent Technologies which uses T7 RNA polymerase, was used for in vitro Transcription using the manufacturer's protocol.

2.2.*7 In vitro* digestion Assay

The functionality of gRNA was confirmed by performing an *in vitro* digestion of CRFB5 by Cas9 nuclease protein. The reaction mixture and conditions are as follows.

Cas9 nuclease	- 1 µl
Target DNA	- 2 µl (100 ng)
gRNA	- 1 µl (1 µg)
10X Cas9 buffer	- 2 µl
RNAse free water *ad*	- 20 µl

2.3 Results and Discussion

2.3.1 Designing gRNAs against CRFB5

Background study was conducted to identify that indeed virus infection of zebrafish primary cells induced interferon response (data not shown). Since CRFB5 is a common receptor chain in the two groups of interferon signaling in zebrafish we considered targeting CRFB5 subunit of the receptor. If the early exons are targeted, a complete disruption of gene function can be achieved. Therefore exon 2 was chosen as a target. Using online tools complementary gRNA with minimum off target effect against zebrafish genome were found. The results for hits against exon 2 of CRFB5 are shown in figure 2.7. The gRNA in box that showed a maximum score was chosen for further experiments.

	score	sequence	
Guide #1	98	GAGCGGTGAAGCGGACGCTC	TGG
Guide #2	94	TGTTGAGCGTCTGAAGCCCG	AGG
Guide #3	93	TGAAGCCCGAGGTTCTGTGG	AGG
Guide #4	93	GTCTGAAGCCCGAGGTTCTG	TGG
Guide #5	93	TGAGTCCTCCACAGAACCTC	GGG
Guide #6	93	GTCCCAGTGCAGCACATACT	GGG
Guide #7	92	AGTCCCAGTGCAGCACATAC	TGG
Guide #8	92	ACATGTACTGAGCGGTGAAG	CGG
Guide #9	92	CTGAGTCCTCCACAGAACCT	CGG
Guide #10	90	AGTATGTGCTGCACTGGGAC	TGG
Guide #11	90	CACCCAGTATGTGCTGCACT	GGG
Guide #12	89	CGCTCTGGTTTACAGCGCTC	TGG
Guide #13	88	TTTACAGCGCTCTGGTTTCC	AGG
Guide #14	82	CTGTGGAGGACTCAGACCTG	TGG
Guide #15	82	GTGCTGCACTGGGACTGGCC	TGG
Guide #16	79	ACACCCAGTATGTGCTGCAC	TGG

Fig 2.7 : Hits from CRIPSR Design tool

2.3.2 Amplification of CRFB5

In order to establish the functionality of gRNA and the CRISPR/Cas9 system, the target template is required. We amplified CRFB5 by PCR. First, RNA was isolated from zebrafish and cDNA was prepared. CRFB5 was amplified from cDNA. PCR product was run on an agarose gel. As is shown in figure 2.8, a specific band of 528 base pair was obtained. The band was gel purified and used for downstream purposes.

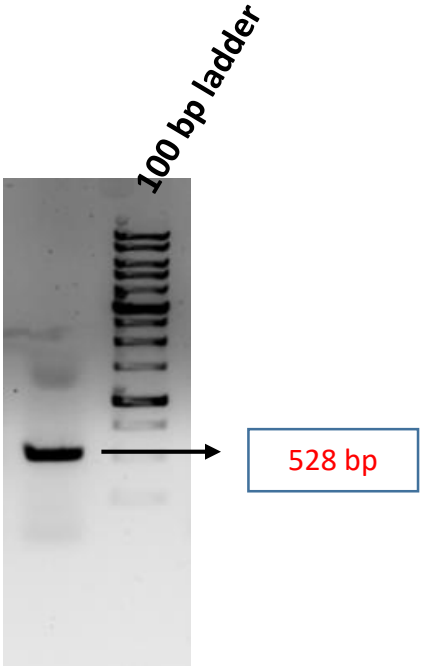

Fig 2.8 : PCR for CRFB5

2.3.3 In Vitro digestion of CRFB5 by gRNA

The functionality of Cas9 protein was evaluated using control template and control gRNA (Fig 2.9 (a)), it was found that the Cas9 protein is functional since cleavage was observed when treated with gRNA. Next, gRNA against CRFB5 was *in vitro* transcribed and the transcript was purified using kit. Then, to check the functionality of gRNA, an *in vitro* digestion of amplified CRFB5 PCR product was performed including gRNA and Cas9 nuclease in the reaction mixture. Since gRNA was specifically binding to the template DNA DSBs were observed and the cleaved product was visualized in agarose gel electrophoresis (Fig 2.9 (b)).

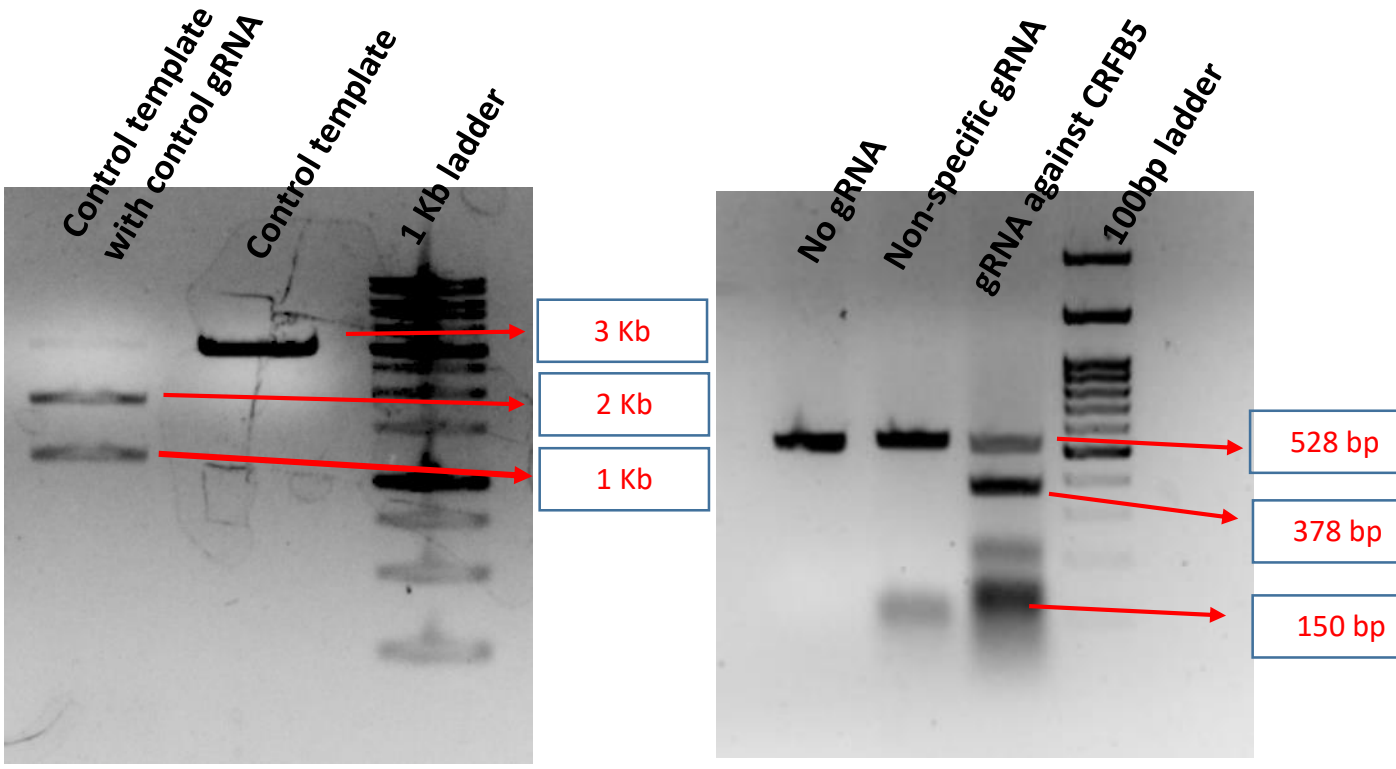

Fig 2.9 : *In vitro* digestion assay

(a) Digestion of control template with cas9 and control gRNA (1st lane) and no gRNA (2nd lane). (b) Digestion of CRFB5 with cas9 and no gRNA (first lane), cas9 and control gRNA (2nd lane), cas9 and specific gRNA (3rd lane) and ladder (4th lane)

2 .4 CONCLUSIONS AND FUTURE DIRECTION

In our initial attempts, we generated functional gRNAs that targets CRFB5 and creates DSBs to yield cleaved products. In order to achieve disruption of CRFB5 in zebrafish, the gRNA along with Cas9 mRNA would be injected in zebrafish eggs and screening would be performed to establish that indeed CRFB5 knocked out animals are obtained. Subsequently studies would be performed to establish zebrafish as a model system for DENV infections.

CHAPTER III

GENERATION OF MICE ANTI GOAT IgG

3.1 INTRODUCTION

Antibodies are glycoproteins that are produced by our immune cells in response to foreign molecules (antigen) that enter our body. Antibodies are secreted by B lymphocytes and they enter in systemic circulation where they bind to the specific antigen and help in clearing it. Antibody production involves the preparation of pure antigenic samples and injecting it into animals along with some adjuvants so as to evoke high secretion of antigen specific antibodies in the serum. Sera consists of polyclonal antibodies (polysera). Monoclonal antibodies are generated by hybridoma technology wherein the B cells are fused with immortal myeloma cells. The culture supernatant of hybridoma would consist of specific monoclonal antibodies. There are various critical steps that has to be taken care of while generating antibodies. Firstly, the purity of antigen determines the specificity of antibodies generated. Moreover before injection, an emulsion has to be prepared by mixing the antigen with an adjuvant. One of the most common adjuvant used is CFA (Freund's adjuvant, complete). The CFA contains *Mycobacterium* in a water-in-oil emulsion. It is capable of causing inflammatory reaction in the site of injection there by recruiting immune cells and also it acts as a depot for the antigen. The next main step is to choose the animal species. Animal species to be chosen should be phylogenetically unrelated and the ease of obtaining blood should also be considered. Next step is the route of injection. The most frequently used route is subcutaneous. Some other factors that have to be taken care of includes the booster dose and volume of blood collected. It is important that not much blood is collected as the hypovolemic shock can occur. The mice anti goat IgG that we are generating can be used in various immune assays were in we can detect the presence of goat IgG. Here were have to have secondary anti mice IgG which is fluorescently labelled for detection.

3.2 MATERIAL METHODS

3.2.1 Salt precipitation

1. A saturated solution of ammonium sulphate prepared.
2. Next, 1.5 ml of goat serum was equally divided into two part and centrifuged at 10,000 rpm for 10 minutes.
3. The supernatant was collected (0.6 ml in two vials) and 0.4 ml of ammonium sulphate was added drop wise in 15 minutes at 4^{o}C.
4. The reaction mixture was kept on rotation in a thermo mixer for 15 minutes at 4^{o}C.
5. After mixing the sample was centrifuged at 10,000 rpm for 10 minutes at 4^{o}C and pellet was collected
6. The pellet was resuspended in 40% ammonium sulphate solution such that the final volume was 2 ml.
7. 1 ml of the resuspended pellet was centrifuged at 10,000 rpm for 10 minutes at 4oC.
8. Obtained pellet was resuspended in 40% ammonium sulphate and final volume was 1 ml.
9. The solution was centrifuged and pellet was obtained was dissolved in 30% Ammonium sulphate.
10. It was centrifuged again and the pellet was resuspended in 30% ammonium sulphate.

3.2.2 Dialysis

1. Dialysis membranes were boiled for 10 minutes in a large volume of 2% sodium carbonate and then washed with water. Next it was boiled in EDTA (1mM concentration).
2. Pellet after ammonium sulphate precipitation was dissolved in 10mM PBS.
3. The sample was filled in dialysis tubes and closed at both ends and kept for dialysis for overnight.
4. PBS was changed three times at fixed intervals. After dialysis the sample was collected from the dialysis tubes.

3.2.3 Seperation of immunoglobulins by gel filtration chromatography

1. For gel filtration column of Hi-trep 16/60 200 HR dimension.
2. It was washed with 1.5 Colum Volume water.
3. The system pumps were washed at higher flow rate.
4. Column was equilibrated with elution buffer.
5. Volume of sample injected was about was about 2% of the column volume.
6. The fractions were collected with 0.5 ml in each fraction.
7. After elution, column was washed with 2cv of water and then preserved in 20% ethanol.

3.2.4 SDS-PAGE to analyze the fractions

1. To analyze the presence of specific antibodies in a particular fraction, SDS PAGE was done.
2. Alternate fraction were taken and 20 µl from each fraction was mixed with SDS loading dye and heated at 95^{o}C C for 5 minutes.
3. The sample was loaded to an SDS PAGE gel.
4. The composition for the respective gels and the buffer used is as follows:

12% Resolving Gel (10 ml)	
Water	3.3 ml
Acrylamide	4.0 ml
Tris HCL buffer	2.5 ml
SDS (10%)	0.1 ml
APS	0.1 ml
TEMED	10 µl

5% stacking Gel (5 ml)	
Water	3.4 ml
Acrylamide	0.83 ml
Tris HCL buffer	.63 ml
SDS (10%)	50 µl
APS	50 µl
TEMED	5 µl

Staining solution (100ml)	
CBB	0.1 g
Water	45 ml
Methanol	45 ml
Acetic Acid	10 ml

De-Staining solution (100ml)	
Water	45 ml
Methanol	45 ml
Acetic Acid	10 ml

10 X SDS PAGE Reservoir Buffer (500ml)	
25nM Tris Base	15.15 g
192mM Glycine	72.05 g
0.1% SDS	5 g

3.2.5 Concentrating and pooling of samples

1. Different fractions were collected and analysed by SDS PAGE.
2. Now only the pure fractions as seen from SDS PAGE was pooled together and was concentrated using a 30 KDa protein concentrator such that the final volume is 1 ml.
3. The concentration of the protein was determined.

3.2.6 Immunization

1. The purified antibody was mixed with CFA (Freund's adjuvant, complete) and an emulsion was prepared.
2. 200 µl of this emulsion (100 µg antibody) was injected sub-cutaneously into mice for the first immunization.
3. After 15 days the first boosting was given, but this time only half the concentration of protein as used.
4. After 1 month the second boosting was given, and finally after 1.5 month the final boosting was given.

3.2.7 Serum collection

1. Pre-Immune serum (PIS) was collected before the immunization, here almost 200 µl of blood was collected and serum was isolated from the blood by centrifugation.
2. Hyper-Immune serum (HIS) was collected at three time points, 15 dys after 1st boosting, 30 days after 1st boosting and final collection.

3.2.8 Determining mice anti goat IgG antibody titers by ELISA

1. ELISA strips were taken and the wells were coated with goat IgG at a concentration of 5 µg/ml in coating buffer.
2. It was incubated at 4^{o}C for overnight.
3. Next day, it was washed 3 times with PBST (200 µl each well for 5 minutes).
4. Further, blocking solution of 3% BSA in PBST was added and was incubated at room temperature for 2 hours.
5. It was washed 3 times in PBST (200 µl each well for 5 minutes).
6. The Anti-sera (HIS and PIS) was added at 50 µl each well with serial dilution and was incubated at room temperature for 1 hour.
7. It was further washed with PBST 3 times and with PBS 2 times.
8. After washing secondary antibody which is conjugated to Alkaline Phosphatase was added (50µl each well).
9. It was incubated at room temperature for 1 hour and was washed 3 times in PBS 50 µl per well for 5 minutes.
10. Next, PNP substrate was added 200 µl each well and it was incubated for 30 minutes at room temperature.
11. The reaction was stopped by adding 50 µl of 3M NaOH to each well.
12. The composition of the solutions used are mentioned below.

Phosphate Buffer Saline	
KH_2PO_4	2mM
Na_2HPO_4	10mM
Nacl	137mM
KCl	2.7mM
Water	

Wash Buffer	0.05% tween 20 in PBS
Blocking Buffer	3% BSA in PBS

Substrate development buffer	
p-nitrophenol	1mg/ml
Glycine	0.1mM
$Mgcl_2$	1mM
$Zncl_2$	1mM

3.2.9 Determination of antibody titers

Antibody titers of the mice anti-goat IgG whole serum was determined from A_{450} values of their log dilutions using the formula:

Log antibody titer = X+ (A-C/A-B) x D

Where

X = Log dil. of the test sample having A_{405} immediately lower than cut-off value.

A = A_{405} of the test sample dil. immediately higher than the cut-off A_{405} value.

B = A_{405} of the test sample dil. immediately lower than the cut-off A_{405} value

C = Cut-off A_{405} value (C = Mean A_{405} of the negative samples + 3x Standard deviation)

D = Log dil. factor i.e. 1

3.3 RESULTS AND DISCUSSION

First of all, IgG molecules in goat serum was salted out using ammonium sulphate precipitation and excess of ammonium sulphate was removed by dialysis. Even though salting out has been carried out, many non specific proteins will also get precipitated. So gel filtration chromatography was done and various fractions were collected (Fig 3.1).

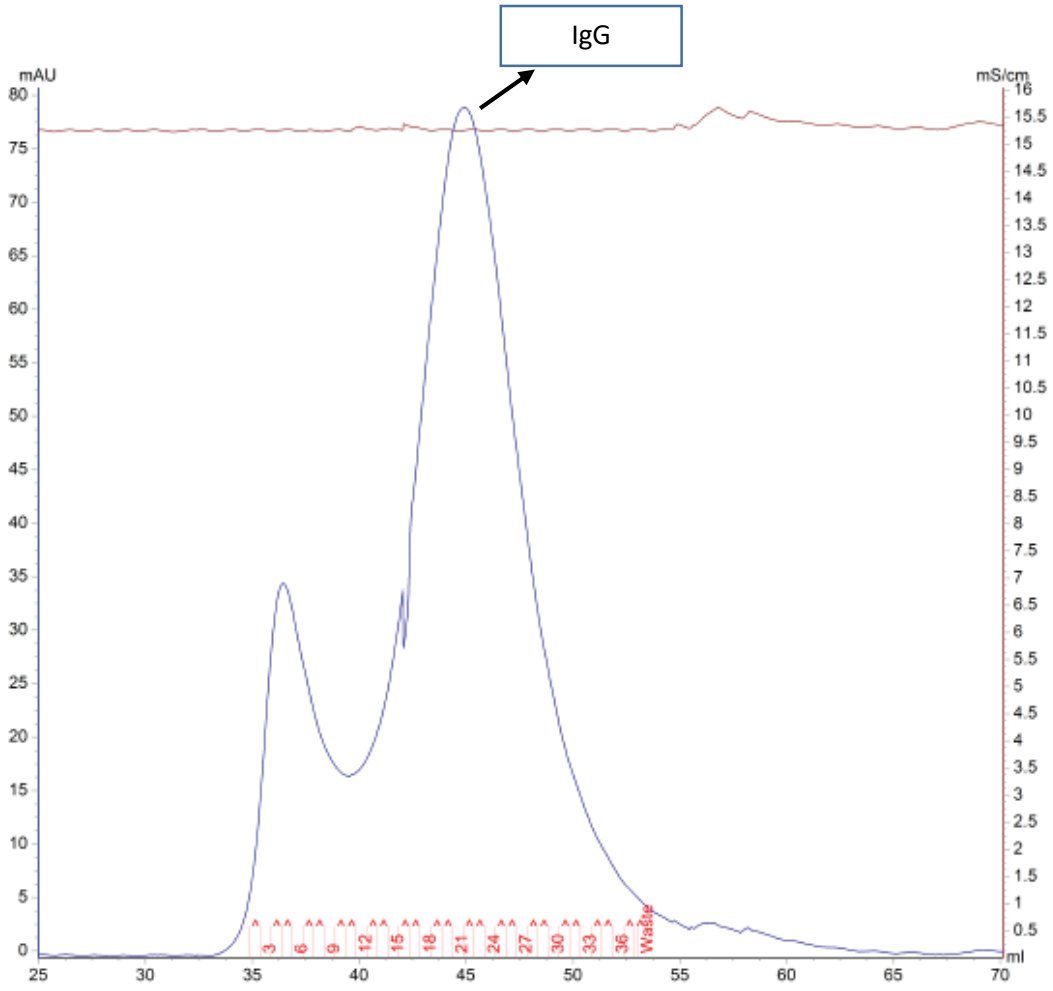

Fig 3.1 : Chromatogram after gel filtration

Different fractions were collected and alternate fractions were loaded on SDS PAGE and purity was checked (Fig 3.2).

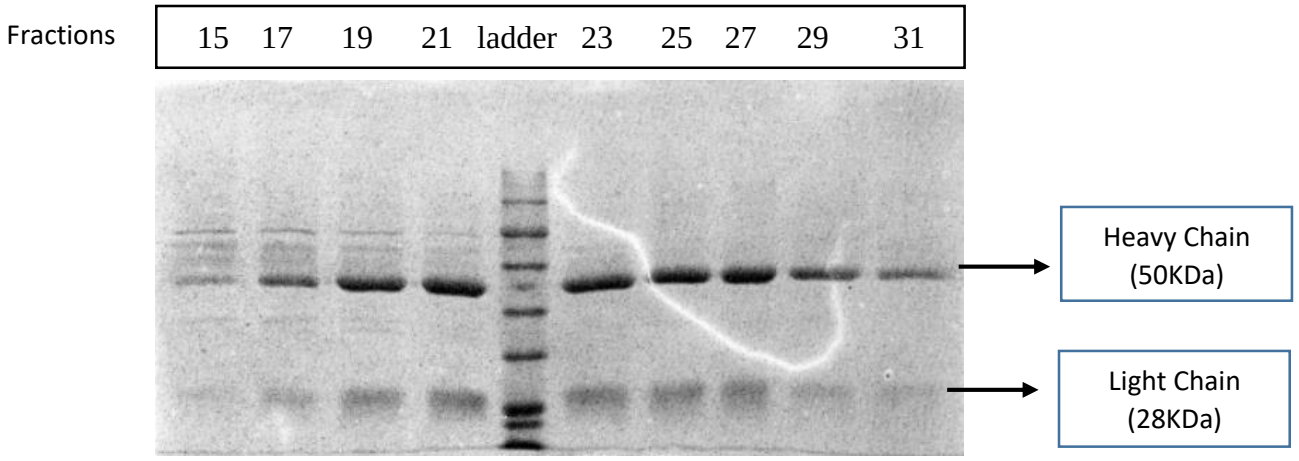

Fig 3.2 : SDS PAGE for different fractions collected

It was found that the initial fractions were impure and contained band other than the light chain and heavy chain on goat IgG. Since the late fractions were pure, these fractions were taken and pooled together. Further the pooled fractions were concentrated using a protein concentrator and pure concentrated goat IgG was obtained. The purity was checked by running the pooled samples on an SDS-PAGE gel (Fig : 3.3).

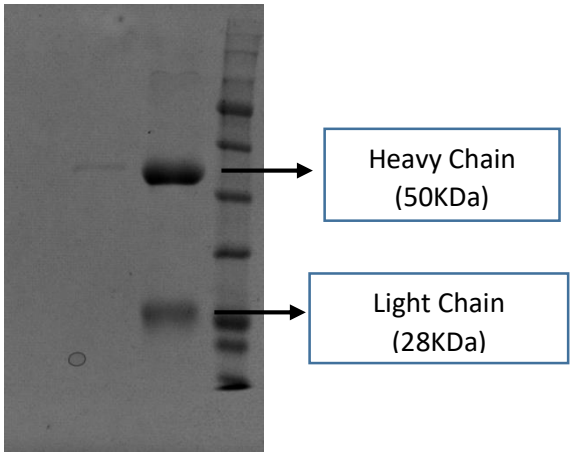

Fig 3.3 : SDS PAGE after pooling pure fractions

Now the goat IgG was concentrated and ready to be injected into mice. Hence an emulsion was prepared using CFA (Freund's adjuvant, complete), and it was subcutaneously injected into mice. The goat antibody will act as an antigen for mice and it will trigger an immune response. Once adaptive immune system gets activated, especially B cells, they start producing antibodies. Now these antibodies can be collected from serum, which is obtained by centrifuging the blood. 2 mice were used for the experiments and the serum was collected at 2 time points, first after the first boosting and the next after second boosting. Now the titers of antibody generated after each immunization can be calculated by ELISA (Fig 3.4). The antibody titers after 2 round of boost dose was calculated (as mentioned in material methods). The titer is 6 x 10^4 for both mice.

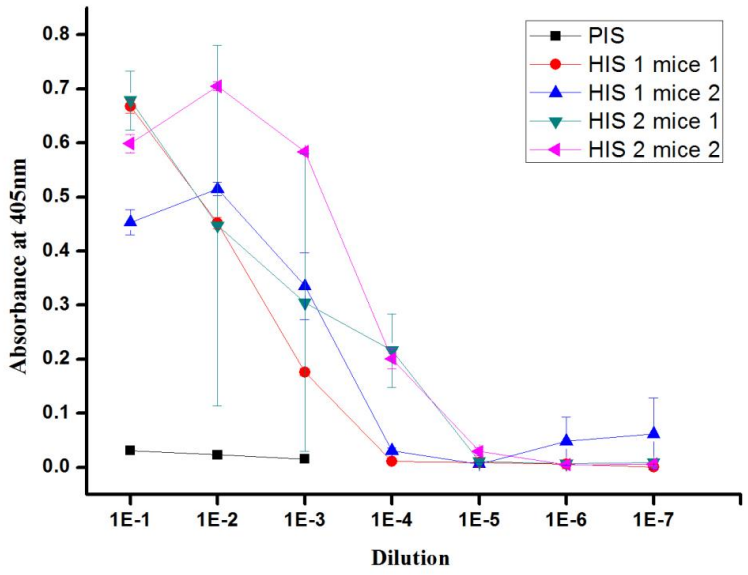

Fig 3.4 : ELISA results for antibody generation

3.4 CONCLUSION

Mice anti goat IgG was generated. The titers were determined by ELISA and was found to be 6 x 10^4 for both mice. Further western blotting has to be done to determine the specificity of antibody generated.

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