Myeloid Derived Suppressor Cells and HSV-1 Latency

Chahat Badhan

MS16058

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

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Certificate of Examination

This is to certify that the dissertation titled “Myeloid Derived Suppressor Cells and HSV-1 Latency” submitted by Ms Chahat Badhan (Reg. No. MS16058) for the partial fulfilment of BS-MS dual degree programme of the Indian Institute of Science Education and Research Mohali 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. Indranil Banerjee Dr. Mahak Sharma Dr. Sharvan Sehrawat

(Supervisor)

Dated: 30 April 2012

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.

Chahat Badhan

(Candidate)

Dated: 30 April 2021

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)

Dated: 30 April 2021

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ABBREVIATIONS

1. PFU: Plaque Forming Units
2. PBS: Phosphate - buffered Saline
3. RBC: Red Blood Cells
4. FACS: Fluorescence-activated Cells Sorting
5. DMEM: Dulbecco's Modified Eagle Media
6. FBS: Fetal Bovine Serum
7. MEM: Modified Eagle Media
8. RNA: Ribonucleic Acid
9. EDTA: Ethylenediaminetetraacetic acid
10. DPI: Days Post Infection
11. IFN-gamma: Interferon-gamma
12. TNF-alpha: Tumor Necrosis Factor
13. GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor

Abstract

We established that MDSCs are important players in HSV-1 latency and reactivation. The project focused on looking at the effects of MDSCs in HSV-1 latency, both *in vivo* and *in vitro*. Initial experiments focused to analyse the kinetics of immune cells in correlation with LAT expression in various organs. It was observed that $CD8^+$ T cell infiltration and LAT expression go hand in hand, indicating that $CD8^+$ T cells have a role in establishment and maintenance of latency. Based on these findings, the next set of experiments aimed at finding the changes in reactivation pattern of HSV-1 in TG by culturing them *in vitro* in the presence and in absence of MDSCs. It was seen that when the TG cells were cultured with MDSCs, virus reactivation was enhanced which was confirmed using plaque assay. To verify if the same is happening is *in vivo, in vitro* generated MDSCs were transferred in infected animals and the LAT expression was used to determine any changes in the latent state. A decrease in LAT expression was seen in the TGs of animals that received MDSCs which indicated viral reactivation in the presence of MDSCs. All the results collectively show that MDSCs enhance reactivation of HSV-1 in TG both *in vivo* and *in vitro*. Future experiments would be in line to identify the exact mechanism by which MDSCs affect latency which so far is suspected by suppressing the function of $CD8^+$ T cells which are essential for establishment and maintenance of latency.

Chapter 1

Introduction

HSV-1 typically causes infection on the mucosal surfaces and attains lifelong latency in sensory ganglia neurons. Its reactivation due to certain stimuli causes nerve damage and severe immunopathological disorders like herpes stromal keratitis. Thus, it is important to understand the factors that cause it to reactivate. The host immune system is suspected to play a crucial role in maintaining and establishment of latency (Theil et al., 2003). This project focuses on understanding the role of one such class of immune cells, Myeloid Derived Suppressor Cells that are immunoregulatory in nature.

1.1 Herpes Simplex Virus

Herpes Simplex Virus belongs to the human *Herpesviridae* family, a set of viruses that produce infection in the majority of human beings. They are categorised into two types: HSV-1 which causes cold sores and HSV-2 which causes genital herpes. Both the viruses are highly contagious and are transmitted through physical contact with infected people.

1.1.1 Virology

HSV-1 is an enveloped virus which has a double stranded linear DNA genome. About 80% of the population is seropositive for HSV-1 infection. Basic structure of this virus includes an icosahedral capsid surrounding the genome. Around the capsid is a tegument that is mainly composed of mRNA and proteins. This is further surrounded by a lipid bilayer envelope which also consists of various proteins and glycoproteins that are essential for viral entry into epithelial cells (Sari et al., 2013). HSV-1 infection symptoms may include watery blisters on skin or mucosal membranes of mouth, nose, lips, eyes etc. It is also known to cause genital herpes but in rare conditions. The virus can undergo both lytic and latent

infection. If the virus is in lytic phase, the infected cells are lysed leading to exposure of nearby cells to the virus and spreading of infection. Non neuronal cells such as epithelial cells and mucosal cells are common targets for viral replication. Primary infection is mostly mild and subclinical and most of the individuals remain asymptomatic. However, there could be several complications in some individuals such as severe corneal lesions that could ultimately lead to blindness and serious neuronal damage.

1.1.2 Herpes Stromal Keratitis

Herpes Stromal Keratitis (HSK) is a potentially blinding immunoinflammatory reaction caused due to HSV-1 corneal infection. The virus enters the corneal epithelial cells and engages in lytic replication. When the lysis of epithelial cells, virus spreads to neighboring cells, giving rise to corneal lesions (Farooq et al., 2010; Koujah et al., 2019). These lesions can last upto a couple of weeks and are generally resolved by the immune system with minimal damage. Despite being cleared from cornea, the virus manages to establish latency in the TG and can get reactivated due to various environmental and physiological factors (Rohman & Sears, 1987; Daheshia et al., 1998).

The main orchestrators of corneal lesions are $CD4^+$ T cells (Knickelbein et al., 2010). Initial immune response is mediated by innate cells such as neutrophils, macrophages and natural killer cells, which is followed by infiltration of $CD4^+$ T cells. $CD4^+$ T cells infiltrate another wave of innate immune cells, mainly neutrophils. Innate cells, in response to this, secrete various kinds of cytokines and chemokines that attract more immune cells and enhance adaptive immune cell response and also secrete pro-angiogenic factors. This ultimately results in neovascularization, neo-lymphangiogenesis, opacification and scarring of the cornea (Koujah et al., 2019) (Figure 1.1). Basically, herpetic lesions, associated immune responses and neovascularization cause scarring and corneal damage leading to complete vision loss during HSK (Biswas & Rouse, 2005).

So far antiviral drugs are used as treatment to control HSV mediated corneal scarring. These drugs target viral entry and lytic cycle. They’re effective against

the virus while it's in the lytic phase but fail to stop it from going into latent phase. HSK severity can be controlled by regulating the immune response and inflammation thus, there are certain drugs that target VEGF availability and work in a direction to regulate inflammatory responses (Chang et al., 2012). Another strategy would be to enhance the regulatory cells mediated response such as Tregs and MDSCs. It has been shown that promoting MDSC response could reduce HSK severity. They work by suppressing effector $CD4^+$ T cell function and promoting endogenous $Foxp3^+$ Tregs. Transfer of *in vitro* generated MDCSs showed a significant reduction in HSK score (Sarkar et al., 2019).

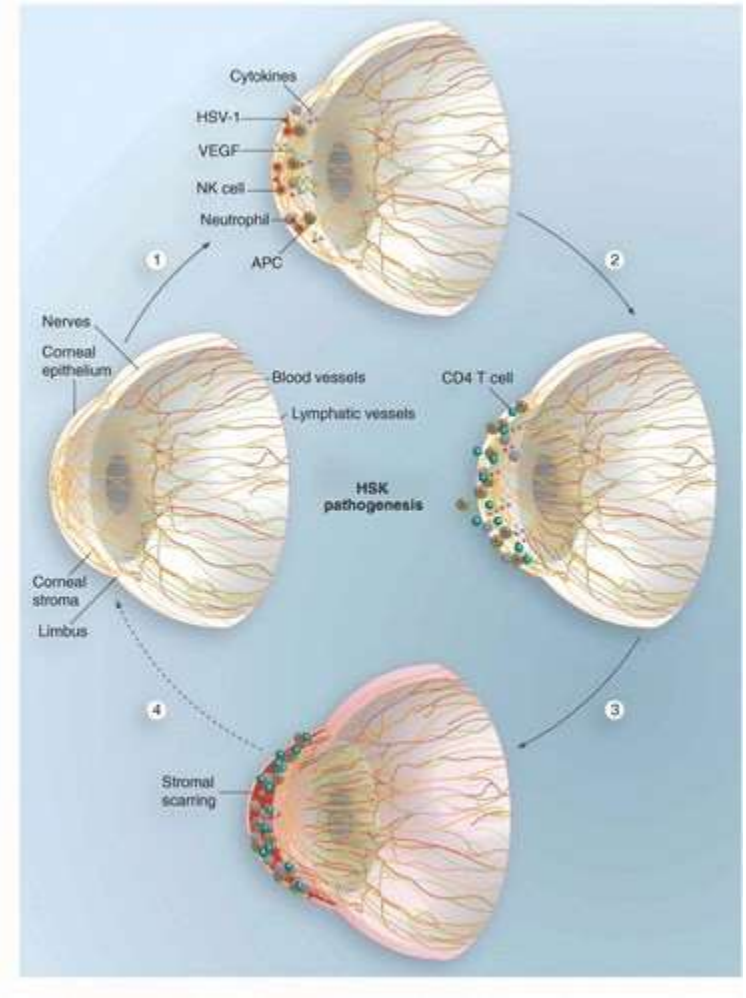

Figure 1.1: Herpes Stromal Keratitis immunopathogenesis progression.

[Source: Knickelbein et al., Future Virology, 2010; Volume 5(6)]

1.1.3 Latency and Reactivation

Viral infections are detected only when they cause, or are suspected to cause visible infections. If a virus doesn't show infection, it's hard for the immune system of the host to detect and eliminate the virus. This proves to be a potent mechanism of evading the immune system to continue living in the host body. HSV has evolved with one such evading mechanism where it enters latency and remains undetectable. Latency is defined as persistence of viral DNA in the absence of a detectable infection. It is a state of tight viral gene repression where most of the lytic genes are silent. After infection, some of the virions can enter the sensory nerve endings and can reach the sensory ganglion by retrograde transport to acquire a latent mode of life cycle (Koganti et al., 2019) (Figure 1.2).

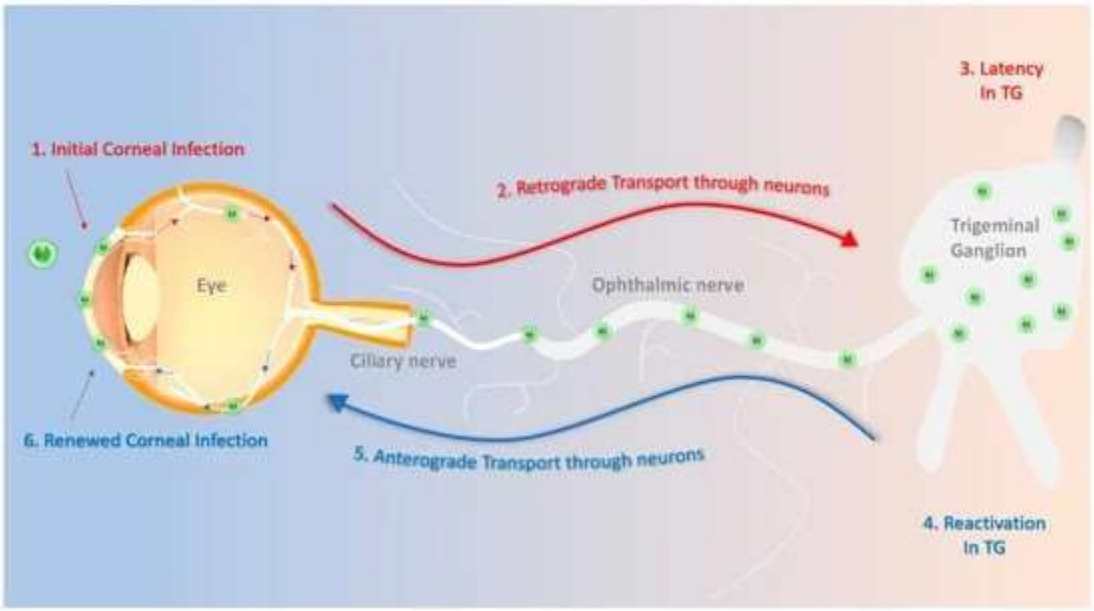

Figure 1.2: Corneal infection to latency

[Source: Koganti et al., Microorganisms, 2019]

HSV can maintain a lifelong latency and no effective vaccine is currently available to mitigate its consequences. Most of the lytic genes are silenced during this period, except for the Latency Associated Transcripts (LATs). LATs are not known to encode for any protein but their role has been identified in establishment and maintenance of latency by regulating neuronal survival and anti-apoptosis (Perng et al., 2000). In the absence of LAT expression, there is a remarkable reduction in the establishment of latency and a massive loss of neurons in trigeminal ganglion because of the lytic activity (Thompson & Sawtell, 2001). Since they are the only genes that are expressed during the latent infection, they can be used as a proxy for determining latency in animals that once showed signs of infection. LAT levels have been used to assess the extent of latency of the virus.

1.1.4 Immune Response to Latency

During latency there are no detectable viral proteins and the neurons in which the virus is latent remains undamaged. And it is well established that TG is an immune privileged site. This means that it is less subjected to immune responses as compared to other sites in the body. But it has been observed that TGs which have latent HSV-1 show infiltration of immune cells like $CD8^+$ T cells and macrophages (Hüfner et al., 2006; Theil et al., 2003). It was also found that the levels of cytokines such as IFN-gamma and TNF-alpha were also elevated in TGs with latent HSV-1. IFN-gamma and TNF-alpha have been demonstrated to block HSV-1replication *in vitro* and *in vivo* (Feduchi et al., 1989). An elevation in levels of chemokines that attract immune cells such as RANTES and IP-10 were also seen. HSV-1 induces expression of RANTES in macrophages. On the basis of this finding, high levels of RANTES and IP-10 was observed in microglia of humans with HSV-1 infection. IP-10 is known to show direct antiviral properties in neurons (Melchjorsen et al., 2002).

All these findings together point in the direction that inflammation helps establish and maintain latency and they could be potential players in reactivation. $CD8^+$ T cells can be narrowed down as key immune players in maintenance of HSV-1

latency as shown by Kanna et. al. They have shown that HSV-1-specific memory/effector $CD8^+$ T lymphocytes that are retained in the ganglion in close apposition to the neurons to prevent full reactivation and virion formation through IFN-γ production and an additional undefined mechanism(s) (Khanna et al., 2004). So, a homeostasis between $CD8^+$ T cells and infected neurons is necessary to maintain the latency. Any perturbation in this coordinated balance could lead to reactivation of the virus. This could mean that immunosuppression could potentially result in reactivation of the virus. To assess this hypothesis, many drugs such as cyclophosphamide and dexamethasone have been assessed. Changes in gene expression in TG previously infected with HSV have been reported (Higaki et al., 2002). A similar observation was made when latently infected mice showed presence of anti-HSV antibodies after immunosuppression by thymectomy (Kastrukoff et al., 1981).

Another way to target immunosuppression could be by making use of regulatory cells such as Treg or MDSCs. The function of immune regulatory cells is to regulate the action of immune cells and their reactions such as inflammation. They have the capability to suppress the activity of immune cells like lymphocytes and thus, can be suspected to have a direct or indirect role in latency and reactivation. So far, very little information is available on the role of immune regulatory cells in HSV-1 latency and reactivation. Tregs have been shown to be critical promoting factors for HSV-1 latency establishment and reactivation. An increase in Tregs increased the viral replication in TG due to reduction of immune surveillance by inhibiting the activity of CD8 T cells (Yu et al., 2018). A similar idea can be applied using MDSCs which are known to suppress the proliferation and activation of T cells. There is a possibility of their involvement in impacting latency and reactivation of HSV-1.

1.2 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs are heterogeneous populations of immature cells which are the result of altered hematopoiesis and are of myeloid origin. They have a characteristic property of being able to suppress both adaptive and innate immune response (Veglia et al., 2018). In mice,

they are identified as $CD11b^+$ and $Gr1^+$. The expression of Ly-6C and Ly-6G further subdivide murine MDSCs into two different subsets: monocytic-MDSCs (M-MDSCs, $CD11b^+Ly6G^-Ly6C$ high) and polymorphonuclear or granulocytic-MDSCs (PMN/G-MDSCs, $CD11b^+Ly6G^+Ly6C$ low). Human G-MDSCs are defined as $CD11b^+CD15^+CD14^-$ or $CD11b^+CD14^-CD66^+$ cells and M-MDSCs are defined as $CD11b^+CD14^+$HLA-DR low/$^-CD15^-$ cells. These are morphologically similar to neutrophils and monocytes. What distinguishes them is their property of immune suppression (Lv et al., 2019). MDSCs are well studied in the tumour microenvironment where they are shown to suppress lymphocytes and NK cells to prevent anti-tumour immune response. And their presence has also been reported in certain pathological conditions. They suppress lymphocyte function by high levels of Arginase-1 (ARG-1), inducible nitric oxidase (iNOS) and reactive oxygen species (ROS) production or indoleamine 2,3-dioxygenase (2,3-IDO) activity (Mougiakakos et al., 2012). They can be generated *in vitro* from bone marrow cells by culturing in presence of certain cytokines including GM-CSF, IL-6 and IL-4**.**

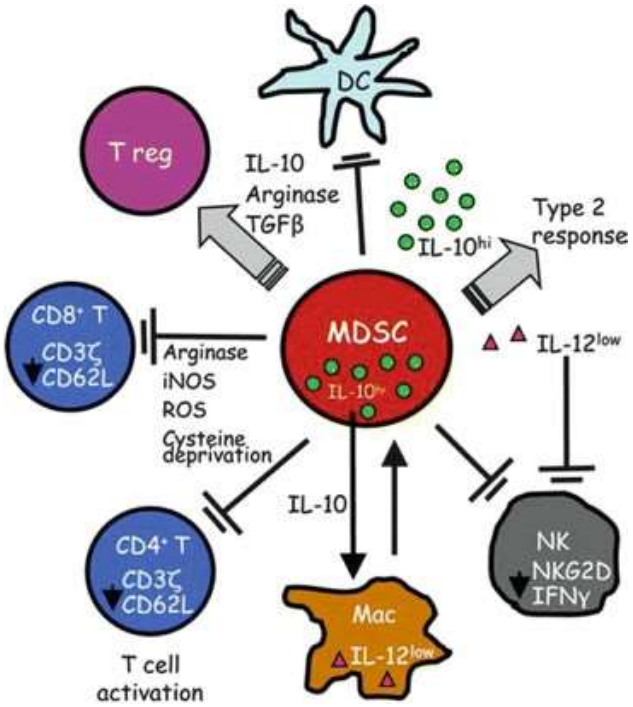

Figure 1.3: Mechanism of action of MDSCs.

[Source: Shibata et al., Immunotherapy of Cancer, 2016]

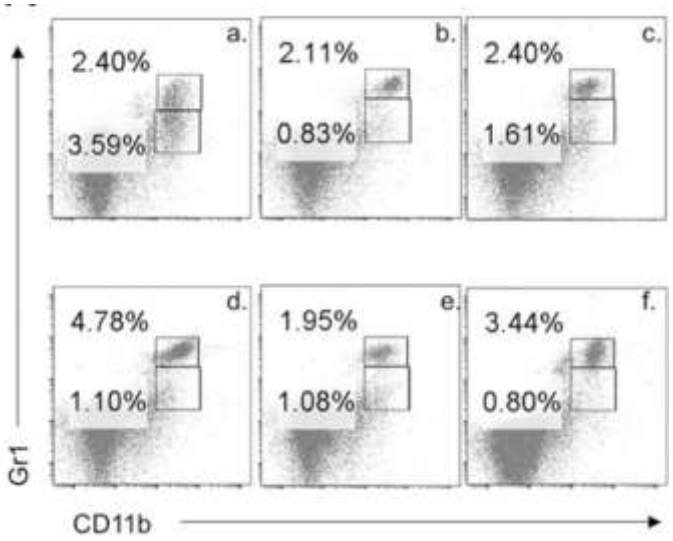

Figure 1.4: Gating for MDSCs based on CD11b and Gr1 in spleen cells

[Source: Sarkar et. al., The Journal of Immunology, 2019]

1.2.1 MDSCs in HSV-1 infection.

MDSCs are majorly known to expand in tumour like conditions but as mentioned above, their expansion is also seen during other pathological conditions such as autoimmunity. The role of MDSCs in viral infection is not widely studied. Very recently, their role in HSV-1 ocular infection was established. It has been shown that *in vitro* generated MDSCs can be used as a cell- based therapy to modulate the severity of HSK. On transfer of *in vitro* generated MDSCs, a reduction in HSK score and angiogenic response was observed in mice infected with HSV-1 (Sarkar et al., 2019). MDSCs enhanced Treg cell response by stabilizing Foxp3 expression and inducing $CD4^+$ T cells to express higher levels of Foxp3. Hence, they have proven to play a crucial role in dealing with HSV-1 mediated corneal scarring. Based on these findings, it can be questioned if MDSCs would have a role to play in maintenance of latency or reactivation of HSV-1.

1.3 Hypothesis of this project

As mentioned above, it has been seen that despite being an immune privileged site, TG shows infiltration of immune cells during HSV-1 infection. These infiltrated cells mainly comprise of $CD8^+$ T cells which play a crucial role in maintenance of latency. In this project we have tried to establish a relationship between MDSCs and HSV-1 latency. Since MDSCs have been shown to control HSK progression, we think that they could have a role in disrupting the latency and causing viral reactivation. We hypothesize that since MDSCs are potent regulators of CD8 T cell function, their presence in TG at the time of latency should disturb the local CD8 T cell functioning leading to reactivation of virus. Thus, treatment with MDSCs should not only reduce HSK score, but should also show changes in reactivation pattern once the latency is achieved.

Chapter 2

Material and Methods

2.1 Animal model and Virus

6-8 weeks old C57BL/6 mice and BALB/c mice were used for experiments. HSV-1 was used for corneal infections in mice. IAEC (Institutional Animal Ethics Committee) approved protocols were used for conducting animal experiments.

2.2 Corneal infection and disease scoring

Mice were anesthetized by i.p. (intraperitoneal) injection of 2,2,2-Tribromoethanol (Avertin) before infection. The cornea was scarified by a 32-gauge needle and a 3µl drop containing 0.5×10^{5} PFU of the virus was put on eye. The eyes were observed at regular intervals for 30 days under a slit-lamp bio-microscope and disease progression was observed. Corneal lesions (Table 1) and angiogenesis were scored according to the following.

Table 1. Herpes Stromal Keratitis Scoring in mice

Score	Meaning
0	Normal cornea
1	Mild corneal haze
2	Moderate corneal opacity/scarring
3	Severe corneal opacity but iris visible
4	Opaque cornea and corneal ulcers
5	Ruptured cornea and necrotising keratitis

Angiogenesis scoring was as follows: A grade of 4 was given to one quadrant of eye lobe representing a centripetal growth of 1.5 mm toward the center. The scores of all four quadrants were summed to derive the angiogenesis index. Therefore, angiogenesis was scored from 0-16 where 0 represents normal cornea and 16 represents maximum vascularisation.

2.3 Isolation of Bone Marrow

1. C57BL/6 mice (males) were euthanized in an euthanasia chamber and sprayed with 70% ethanol.
2. Skin covering the legs along with muscles were cut to expose the hindlimb bones.
3. Hindlimb bones (Femur and Tibia) were procured by dislocating them from the hip joint and knee joint.
4. Extra muscles were removed and bones were washed in 70% ethanol, followed by multiple washes with ice cold PBS.
5. Washed bones were placed in ice cold 10% RPMI in a petri-dish. The ends of bones were chopped off and bone marrow (BM) was flushed with the same media using a 2ml syringe.
6. The flushed BM cells were passed through the syringe multiple times to make a single cell suspension.

2.4 *In vitro* generation of MDSCs

1. BM cells isolated from animals by above mentioned protocol were incubated with 5ml RBC lysis buffer (Table 2) at room temperature to lyse RBCs.
2. The cells washed with PBS twice and resuspended in complete RPMI media.
3. Cells were counted using a hemocytometer and 3×10^{6} cells were added to petri-dish containing 10ml RPMI supplemented with IL6, IL4, and GM-CSF cytokines, 40ng/ml each.
4. After 5 days, cells were washed and resuspended in PBS. Cells were used for further experiments and the remaining cells were stained for CD11b and Gr1 for characterization.

Table 2. RBC Lysis Buffer composition

Components	Molarity
NH_4Cl	155mM
$NaHCO_3$	12mM
$EDTA$	0.1mM

2.5 Cell preparation for Surface staining

1. Mice were euthanized using CO_2 in euthansia chamber.
2. Trigeminal ganglion, cornea, cervical lymph nodes and spleen were isolated at different time points during the course of infection.
3. Cornea and TGs were digested with liberase (2.5mg/ml) for 45 minutes at 37℃ in a humidified atmosphere with 5% CO_2. After digestion with liberase, the organs were broken down by reverse pipetting. The cells were passed through an insulin syringe to make a single cell suspension.
4. Single cell suspension from lymph nodes and spleen was prepared by straining the organs through a 70-micron cell strainer. Spleen cells were treated with 1ml RBC lysis buffer for 5 minutes at room temperature.
5. All the cells were washed with PBS twice.
6. Cells were counted using a hemocytometer and $1x10^6$ cells of each sample were used for surface staining.

2.6 Surface Staining

1. All the staining steps were performed at 4℃. Cells were taken in a 96 well plate and were incubated in antibodies diluted in the FACS buffer (2% FBS in PBS). Different dilutions of antibodies were used (Table 3). Each sample was incubated in 40µl of antibody solutions for 30 min.
2. After incubation with antibody solutions, cells were washed thrice with FACS buffer.
3. Cells were resuspended in 200µl FACS buffer and acquired in BD FACSAria cell sorter and analysed using Flowjo software v10.
4. Cells were fixed with 0.25% PFA (diluted in FACS buffer) stored in 4 ℃ for acquiring at later time point.

Table 3. Antibody Dilutions

ANTIBODY	**DILUTIONS**
CD11b - FITC	1:300
Gr1 - APC	1:200
CD45 - PerCP Cy5.5	1:400
CD8 - FITC	1:400
SSIEFARL - PE	1:200

2.7 Antibodies Used

All antibodies were purchased from BD. All antibodies were diluted in the FACS buffer. List of antibodies used is as follows: CD11b – FITC, Gr1 – APC, CD45 - PerCP Cy5.5 and SSIEFARL-H-2K^{b} tetramer- PE.

2.8 *In vitro* HSV-1 Reactivation Assay

1. Mice infected with 50,000 PFU HSV-1 were euthanized using CO_2 in euthansia chamber.
2. Trigeminal Ganglions were isolated from the animals and were digested with liberase (2.5mg/ml) for 45 minutes at 37℃ in a humidified atmosphere with 5% CO_2. This was followed by single cell suspension preparation by trituration.
3. TG cells were plated in a 96 well plate (flat bottom) such that there is approximately 1 TG per well. For some experiments, the cells were pooled before plating.
4. The cells were cultured in DMEM (200µl) containing 10% FBS in a 5% CO_2 humidified incubator at 37℃ for 10 days.
5. Approximately 150µl supernatant was collected after every 24 hrs period and replaced with fresh DMEM with 10% FBS in the culture. The collected supernatant was stored at -80° C until further use.
6. Presence of the infectious virions in the supernatant was detected using standard Plaque Assay.

2.9 Plaque Assay for virus titration

1. Collected supernatants from reactivation experiments were serially diluted in serum free DMEM.
2. 250µl of these dilutions was put on a monolayer of Vero E6 cells in 24 well plates and incubated in a 5% CO_2 humidified incubator at 37℃ for 4 hours.
3. After incubation the media was removed and cells were washed with PBS once. After washing the monolayer was covered with 0.8% Low melting Agarose mix (1:1 of LME Agarose and MEM)
4. The plates were incubated in a 5% CO_2 humidified incubator at 37 ° C for 4-5 days.
5. After 4-5 days when the plaques are seen under the microscope, the cells were fixed with 4% PFA overnight at 4℃.
6. Cells were stained with 200µl of 2% w/v crystal violet for 15 minutes and were

washed with tap water. The plates were left to dry at room temperature and the number of plaques were counted.

7. The following formula was used to determine the number of infectious virions per sample.

$$No.\ of\ plaques \times 10^{3} / dilution \times 250 = PFU/ml$$

2.10 RNA isolation

1. Trigeminal ganglions were isolated from infected and uninfected mice at different days during the time of infection. Tissues were homogenized by treating with liberase and passing through a syringe multiple times with PBS (200µl per sample).
2. Then 800µl RNA lysis buffer (Table 4) was added and the mixture was vortexed multiple times to break the cells.
3. 800µl Phenol chloroform was added to the mixture and everything was mixed properly by invert mixing.
4. After this the samples were centrifuged at 13,000 RPMI at 4℃ for 20 minutes and the aqueous layer was collected.
5. The aqueous layer was mixed with 500µl isopropanol and 200µl 1.2M NaCl solution. Everything was mixed thoroughly by invert mixing.
6. The samples were incubated at -20℃ overnight.
7. After incubation the samples were centrifuged at 13,000 RPMI at 4℃ for 20 minutes and the pellet was taken.
8. The pellet was dissolved in 200µl ethanol (80%) by tapping on the micro centrifuge tube and centrifuged at 13,000 RPMI at 4℃ for 20 minutes.
9. The pellet was taken and air dried at room temperature.
10. After air drying the pellet was dissolved in 50µl nuclease free water at 65℃.
11. The RNA was stored in -80℃ until further use.

Table 4. RNA lysis buffer composition

Tris (pH 8.0)	100mM
LiCl	500mM
EDTA (pH 8.0)	50mM
SDS	2%
2-mercaptoethanol	3%

2.11 cDNA Preparation

1. A master mix for all the samples were prepared using the following reagents (Table 5).

Table 5. Sample preparation for cDNA synthesis

Reagent	**Volume**	**Final Concentration**
5X cDNA synthesis buffer	4µl	1X
dNTP mix	2µl	500µM each
RNA primer (random hexamer)	0.75µl	
Anchored OligoDT	0.25µl	
Verso enzyme mix	0.5µl	
RT enhancer	1µl	
Template (RNA)		1ng
Nuclease free water	Volume make up	
Total volume	20µl	

2. The RNA purity and concentration of all the samples were taken. Based on the concentration of RNA, 1-5µl of sample RNA is taken and volume make-up is done using nuclease free water.
3. The mixture was incubated at 30 ℃ for 10 minutes, followed by 42 ℃ for 60 minutes and finally at 72 ℃ for 15 minutes.
4. Prepared cDNA was stored in -20 ℃ until further use.

2.12 RT-qPCR

RT-qPCR was carried out using 2x-DyNamo ColorFlash SYBR Green qRT-PCR kit (ThermoFisher) as per the manufacturer's protocol in a QuantStudio Real Time PCR system (ThermoFisher). To calculate fold change in gene expression, we first normalized the values with the internal control (18s rRNA) to obtain △Ct values. These △Ct values were then normalized with the experimental control to obtain △(△(tainzeds)). The relative expression for each gene was then calculated by using $2^{-\triangle(\triangle Ct)}$ values as described in (Sharma et al., 2021). Following primers were used (Ramakrishnan et al., 1994):

GAC-AGC-AAA-AAT-CCC-GTC-AG (5'primer)

ACG-AGG-GAA-AAC-AAT-AAG-GG (3' primer)

2.13 Statistical Analysis

To measure the level of statistical significance, the data was subjected to analysis using ANOVA, or Student t test as indicated in the figure legends. Graph Pad Prism v8.0.2 was used for such analysis. The level of significance was determined as $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$***, $P < 0.0001$**** and ns for $P > 0.05$.

Chapter 3

Results

3.1 HSK and Angiogenesis score

First of all, it is important to understand the progression of disease over time. So we monitored the development of immunoinflammatory disease at different time points. The difference between an infected and uninfected cornea was quite distinctive as seen in Figure 3.1. The disease score of cornea of the infected animals were scored for keratitis and angiogenesis at about every alternate day throughout the course of infection Figure 3.2 and Figure 3.3.

Uninfected **Infected**

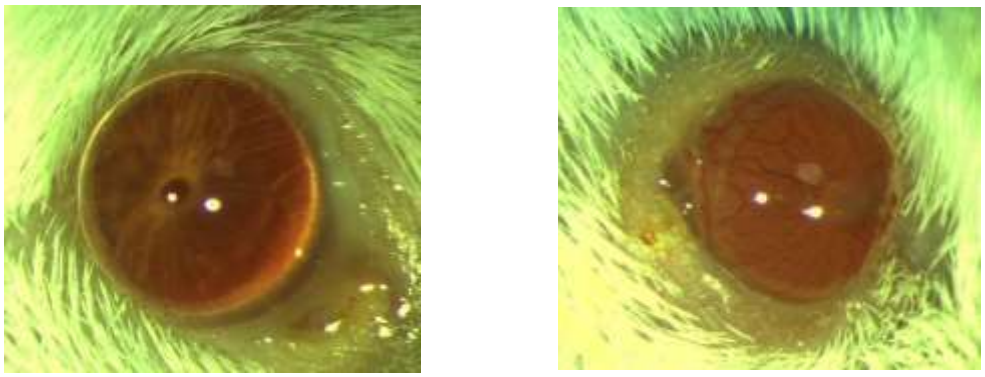

Figure 3.1: HSV-1 infected and uninfected cornea.

It was observed that the cornea develops opacity and neovascularization after ocular infection with HSV-1 after day 5 of infection. Most animals developed the peak of infection between 15-20 dpi. The disease score remained the same after 20 dpi.

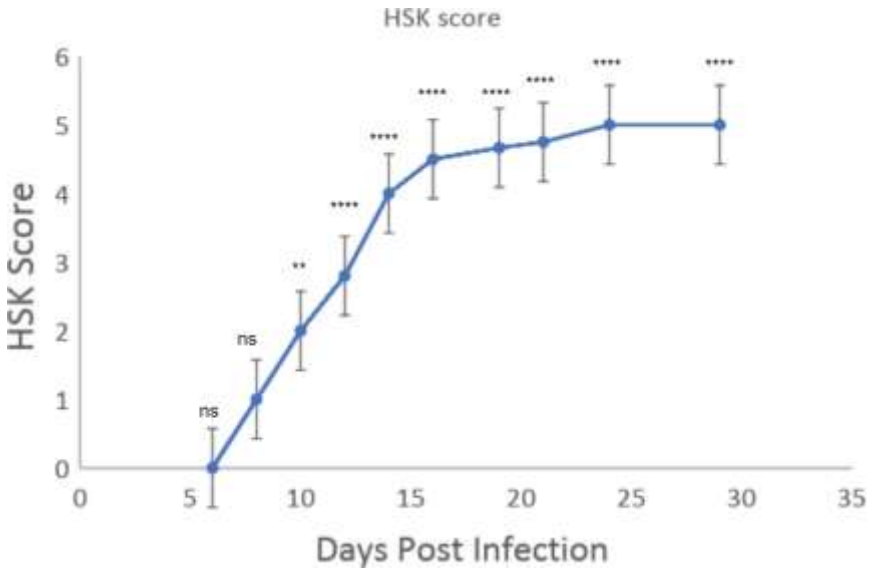

Figure 3.2 HSK score over the course of infection: Cornea of animals were observed under a slit-lamp bio-microscope and animals were scored from 0-5. One way ANOVA was performed for determining the significance levels in control and infected groups. $p < 0.01$ ** $p < 0.0001$ **** and ns for not significant.

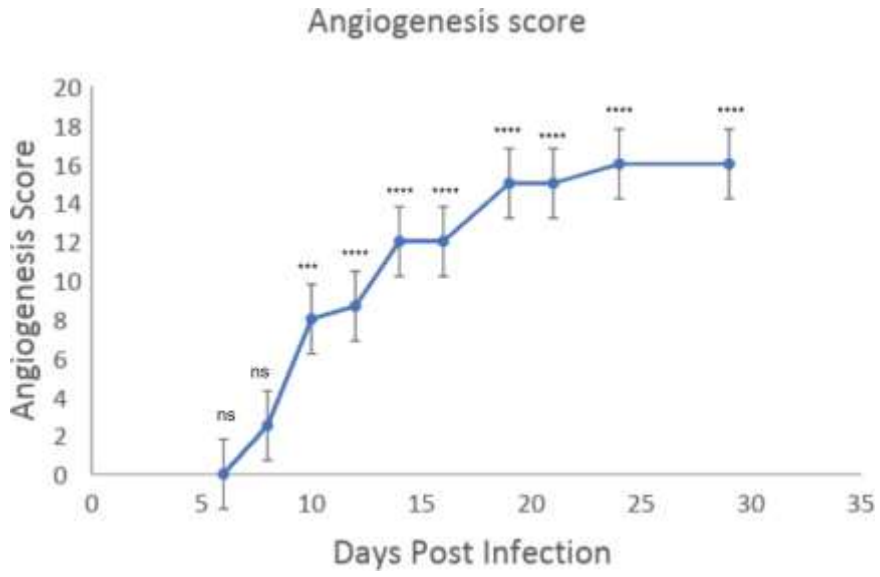

Figure 3.3 Angiogenesis score over the course of infection: Cornea of animals were observed under a slit-lamp bio-microscope and animals were scored from 0-16. One

way ANOVA was performed for determining the significance levels in control and infected groups. $p < 0.001$ *** $p < 0.0001$ **** and ns for not significant

3.2 Immune cell dynamics in Spleen, Cornea, TG and CLN

Along with the progression of HSK in the cornea of mice, immune cell dynamics were also measured in different organs and tissue sites. To understand the correlation between latency and immune cells, it is important to understand the kinetics of immune cells in different organs, especially trigeminal ganglion (TG) where HSV1 persists in latent stage. Thus, animals were sacrificed at three different time points over the course of infection (6dpi, 15dpi, and 30dpi) for 30 days and cornea, spleen, cervical lymph nodes (CLN) and TG were isolated. Surface staining was performed on their single cell suspensions as described earlier. The kinetics of HSV-1 specific $CD8^+$ T Cells (SSIEFARL-tetramer positive CD8 T cells) were assessed. Figure 3.4, 3.5, 3.6 and 3.7 show the percentage of tetramer positive CD8 T cells in gated $CD45^+$ cells.

It can be seen that as the disease progresses the kinetics of CD8 T cells are similar in spleen, CLN and cornea but a bit different in TG. It was also observed that CD8 T cells specific to HSV-1 increases at 15 dpi when the disease score is maximum. The infiltration of antigen-specific CD8 T cells goes down after 15 dpi in spleen and CLN and the disease is being resolved. But in TG, the levels of virus specific CD8 T cells remain high even after 30 dpi which might indicate that the virus is still present in TG even after it is cleared from cornea and other organs. The presence of such cells at 30dpi indicates that the virus is not completely out of the system. The next question to address here is whether latency

has any correlation with these immune kinetics or not. To find that out, LAT expressions were checked at different days post infection using RT-qPCR.

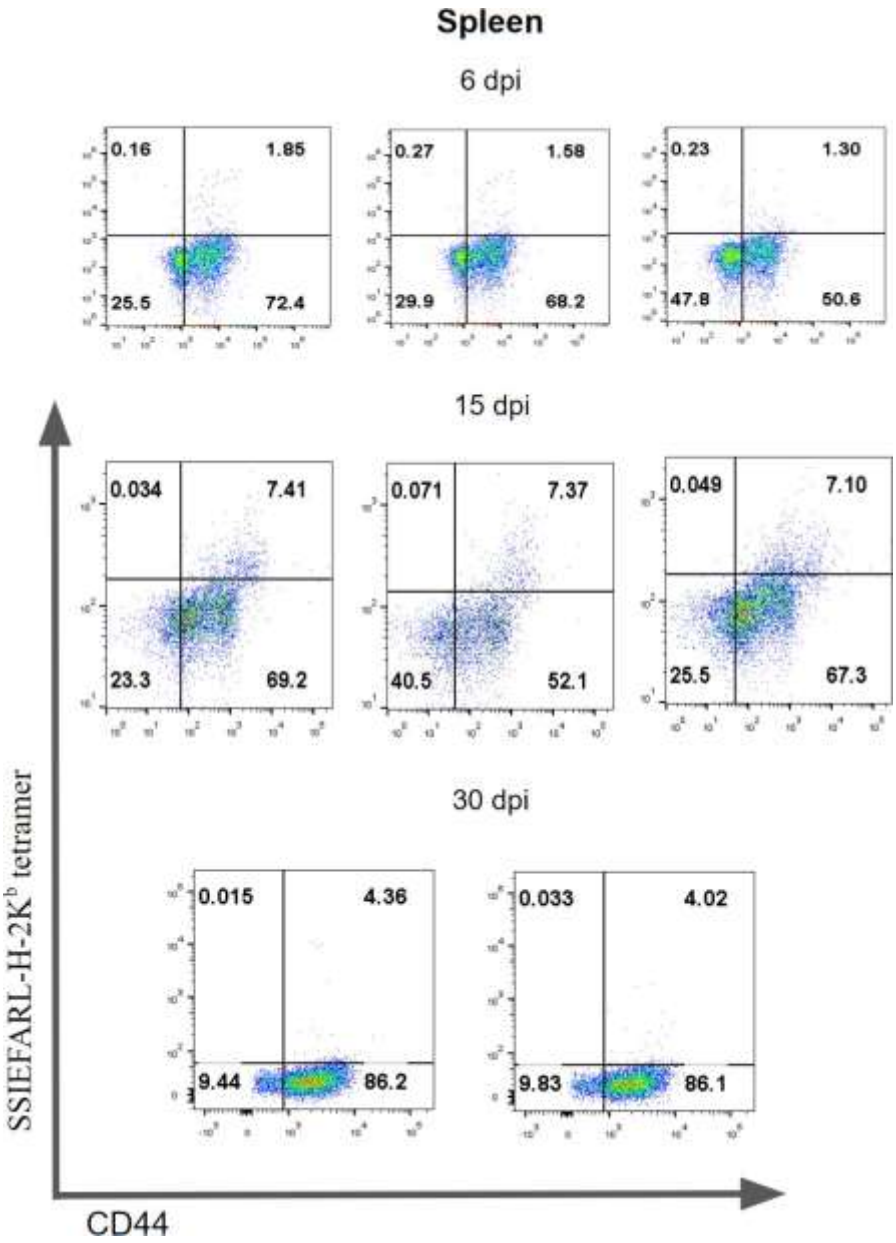

Figure 3.4: Representative plot from spleen

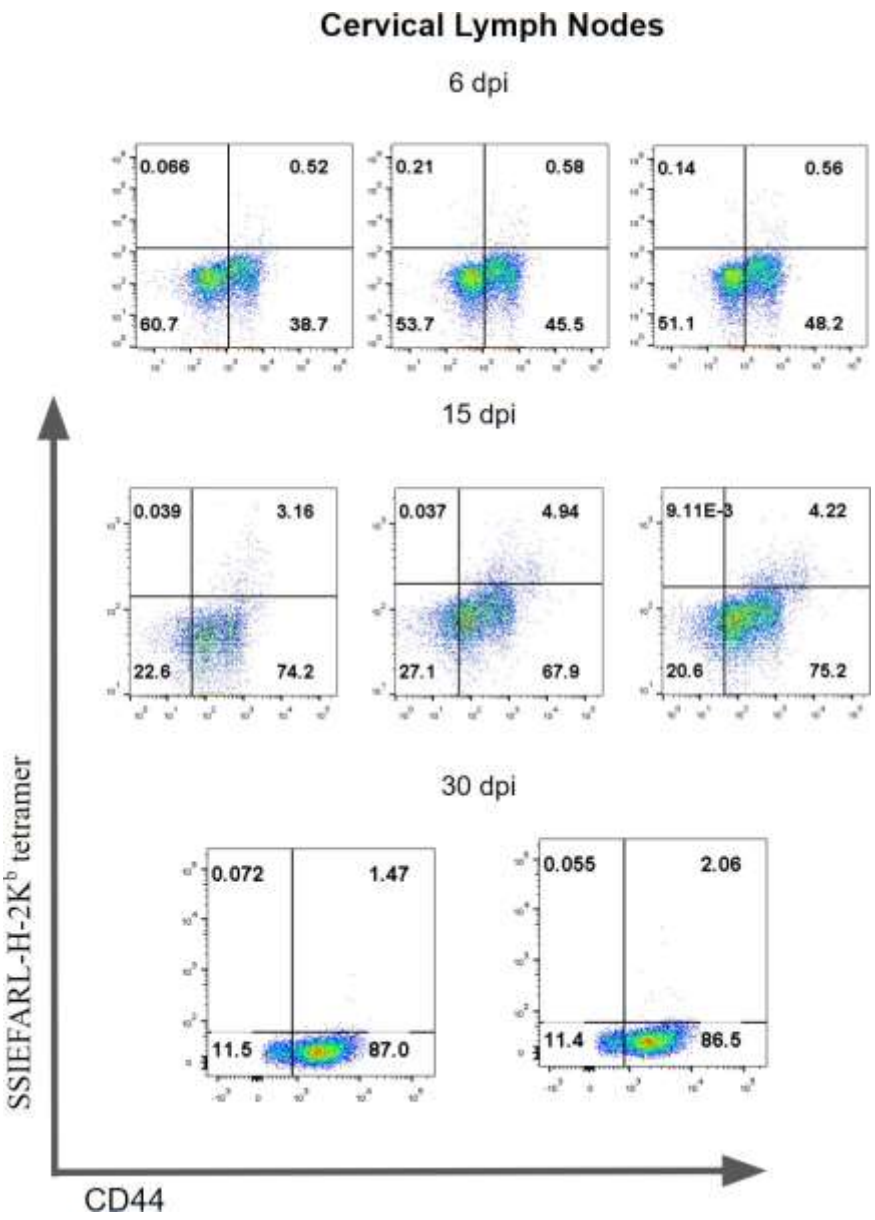

Figure 3.5 Representative plots from Cervical Lymph Nodes

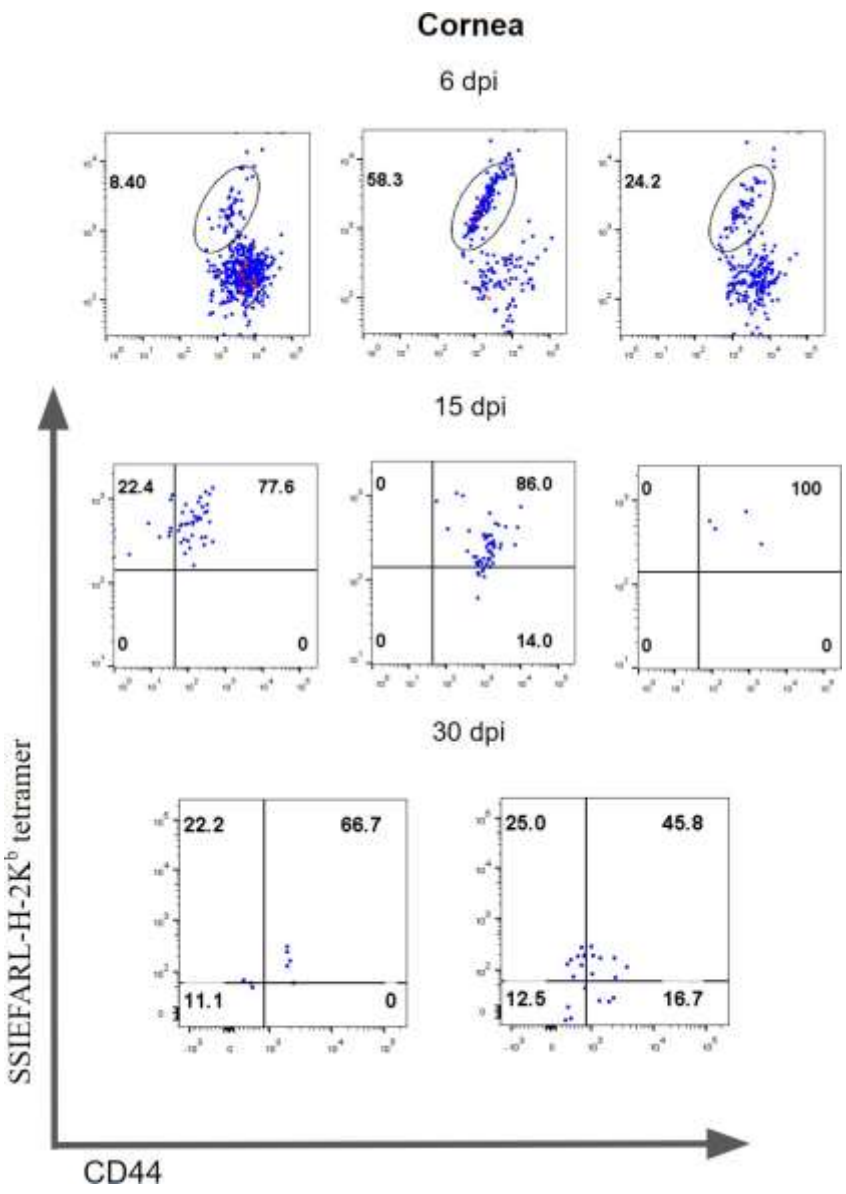

Figure 3.6 Representative plots from cornea

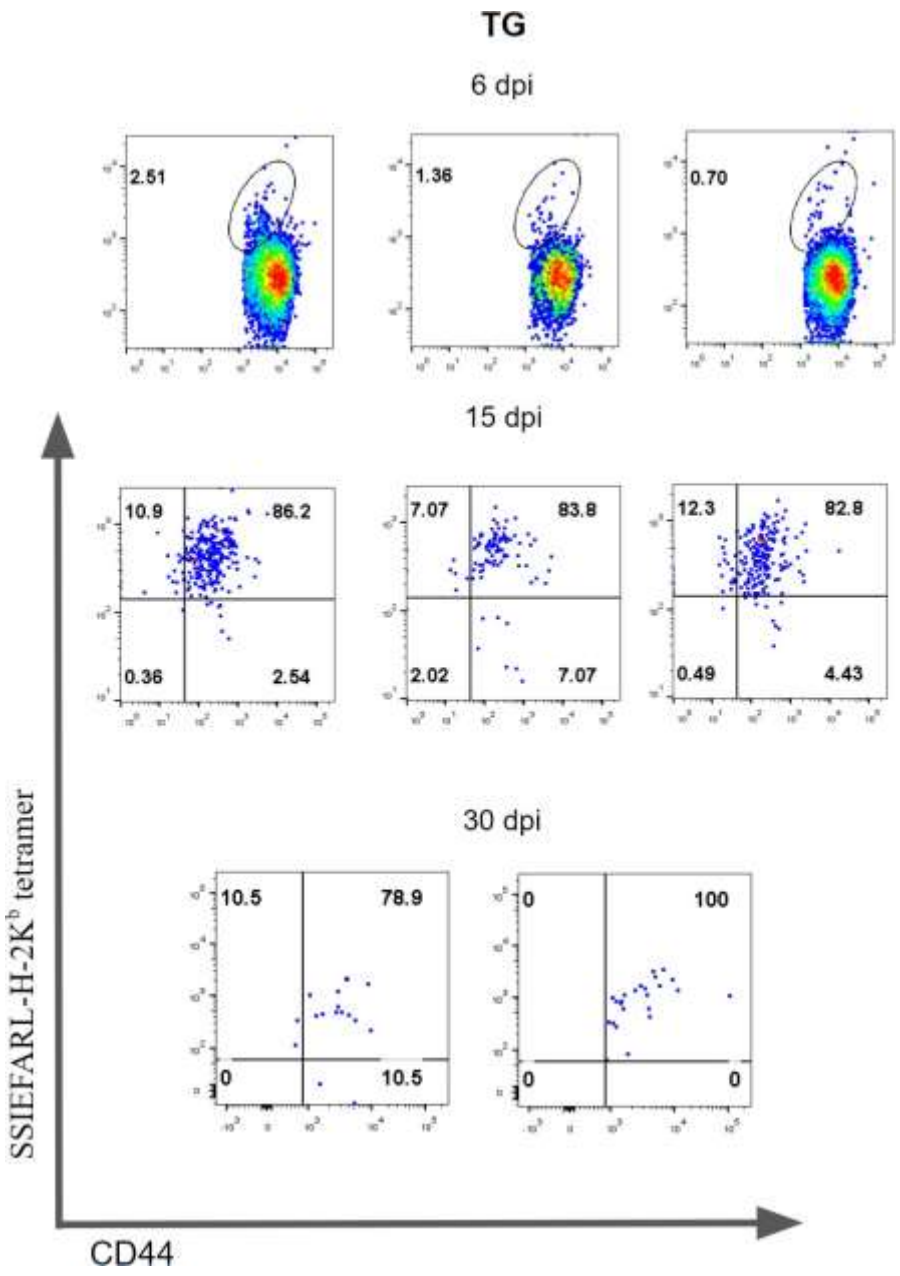

Figure 3.7 Representative plots from TG

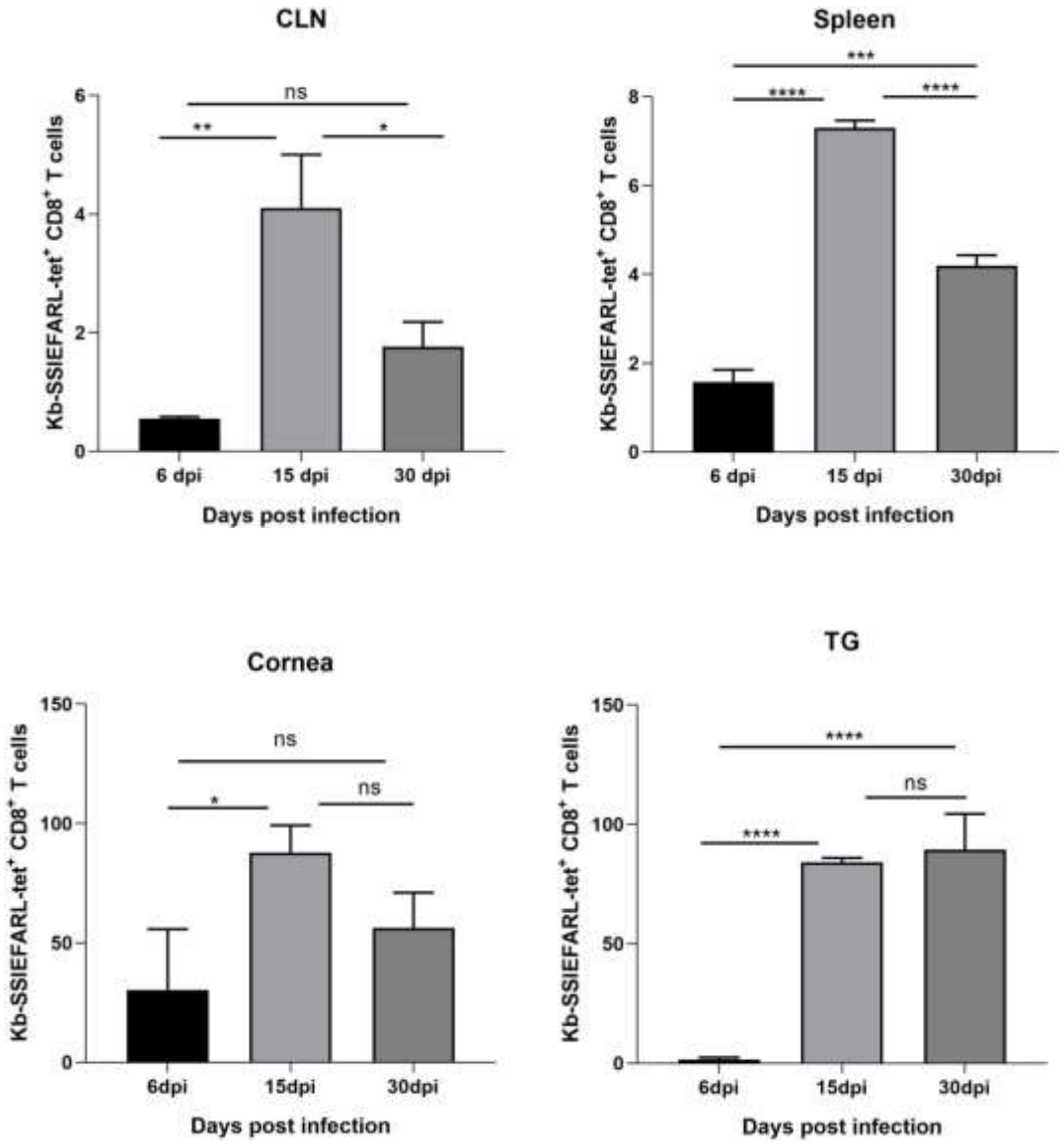

Figure 3.8 Virus specific CD8+ T cells kinetics in spleen, CLN, Cornea and TG: Mice were sacrificed at different days (6dpi,15dpi, and 30dpi). Spleen, CLN, cornea and TG cells were surface stained with CD45, CD8, and SSIEFARL-H-2K^b tetramer. One way ANOVA was performed for determining the significance levels in control and infected groups. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$***, $P < 0.0001$**** and ns for $P > 0.05$.

3.3 LAT expressions in TG over the course of infection (30 days)

As mentioned before, Latency Associated Transcripts (LATs) are the only genes that are expressed when the virus is in the latent stage. So to determine any change in the latent state, LAT expressions were measured as an indicator of latency. LAT mRNA levels were checked in TG samples at six different days over the course of infection. Each day 3 mice were sacrificed for the analysis.

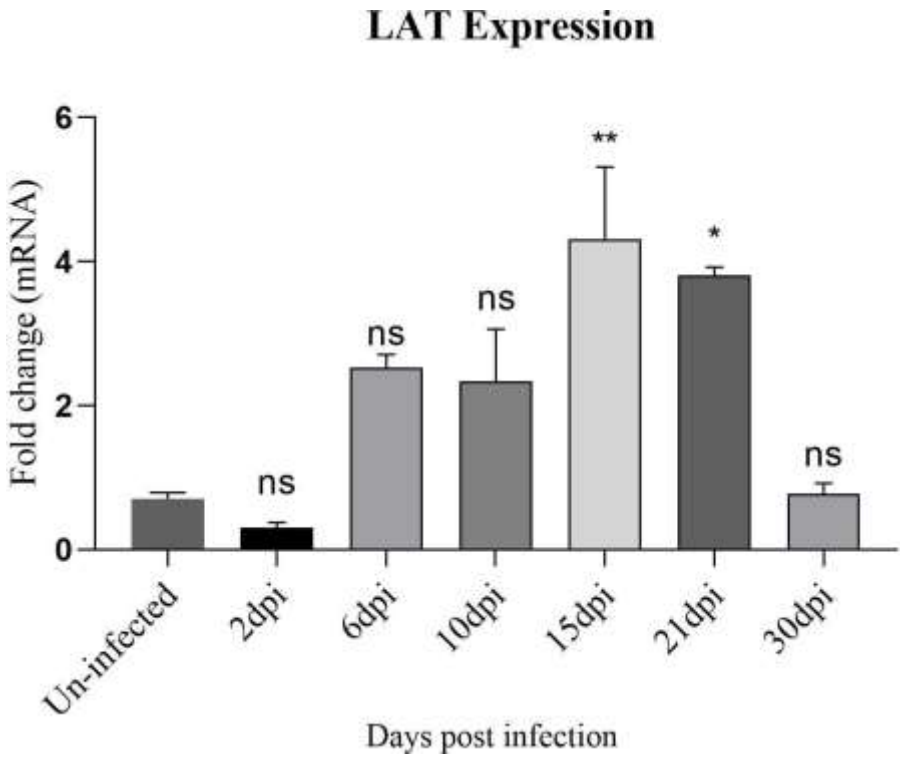

Figure 3.9: LAT expression levels over the course of infection (in comparison with uninfected): Mice were sacrificed on different days post infection and their TGs were tested for LAT mRNA expression using RT-qPCR. One way ANOVA was performed for

determining the significance levels in uninfected and infected groups. $P < 0.05$ *, $P < 0.01$ **, and ns for $P > 0.05$.

It was seen that LAT expression gradually increased till 15 dpi. The expression levels were highest at 15 dpi and the levels started to go down afterwards. This high expression level at 15 dpi with maximum disease score around the same time indicates that latency is achieved. It attains full latency at around 15 dpi which matches with the peak of infection. At 30dpi, the LAT expressions have gone down. This could either mean that the virus might have reactivated from latency due to some stressful episode.

3.4 *In vitro* reactivation assay of infected TG

To check for reactivation kinetics of HSV1 in latently infected TGs, *in vitro* reactivation assays were performed. Mice were infected with 50,000 PFU of HSV-1. TGs were collected at different days post infection and single cell suspensions were prepared. These cells were cultured in different groups in DMEM with 10% FBS in 96 well plates such that each well contained approx. 1 TG. These cells were cultured in the presence or absence of *in vitro* generated MDSCs for 7-10 days and supernatants were collected after every 24 hrs. The collected supernatant was tested for viral titer at different days using plaque assay (Figure 3.10).

The purpose of this experiment was to see if there is enhanced reactivation of virus in presence *in vitro* generated MDSCs. For some cases TG cells were sorted to remove $CD11b^+$ and $Gr1^+$ cells with the intention to remove MDSCs from TG

cells. Different groups were made and each group had different conditions. The cells in the control group were cultured in absence of *in vitro* generated MDSCs and the experimental group that had added MDSCs.

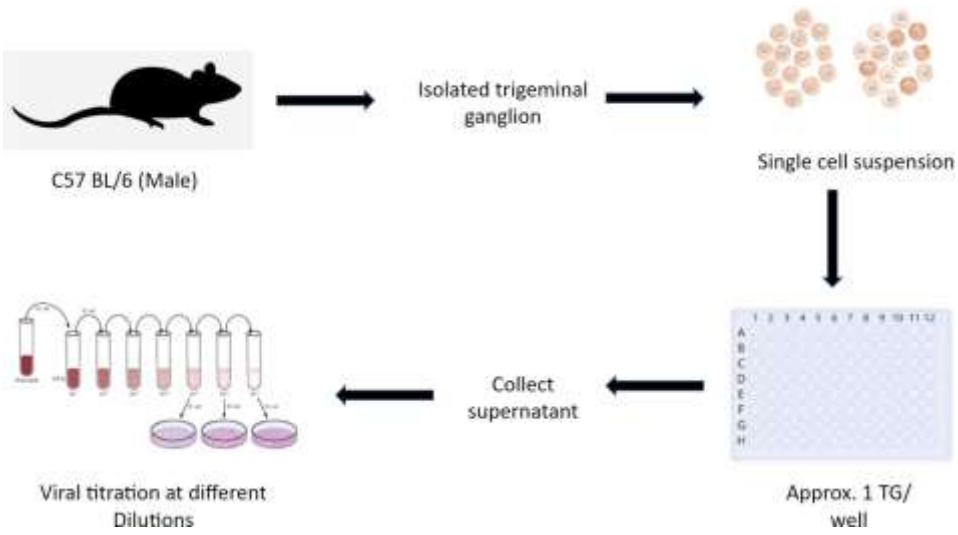

Figure 3.10: Experimental setup for reactivation assay

It was seen that the virus titer was more in the TG cells when they were cultured in presence in MDSCs as compared to the control group after 30 dpi (Figure 3.11). The infectious virions were barely detectable in the supernatant when cells were cultured in absence of MDSCs upto 10 days in control animals. An efficient reactivation was observed in cells cultured in the presence of MDSCs after day 5 in culture. Viral titers were low after day five but the overall levels were more for the MDSCs group as compared to the control group. It could be clearly seen that the presence of MDSCs promotes the reactivation of HSV1 from TG when cultured *in vitro*. A similar reactivation pattern was seen in TG cells isolated from

mice after 120 dpi (Figure 3.12). At 120 dpi TG cells were further divided in two groups: one with MDSCs sorted out (sorted group) and one without sorting (unsorted group which had resident $CD11b^{+}Gr1^{+}$ cells). Thus, it could be seen that the presence of MDSCs enhanced reactivation in infected TGs which were cultured *in vitro* for 10 days. However, the differences were only statistically different at earlier time points. One obvious point of divergence between 30 and 120 dpi TG was that in 30dpi we did not observe reactivation of HSV1 in the control animals but at 120 dpi the control TG also showed reactivation of the virus. This could relate to the overall abundance of virus specific $CD8^{+}$ T cells that were more in 30dpi as compared to those at 120 dpi. In 120dpi sorted TG cells, the differences in the virus activation was upto two log different in control and MDSCs supplemented cultures but in unsorted cells such differences were approximately 6log different. This could be attributed to the mechanical stress caused by the sorting process that might have induced killing of some TG cells.

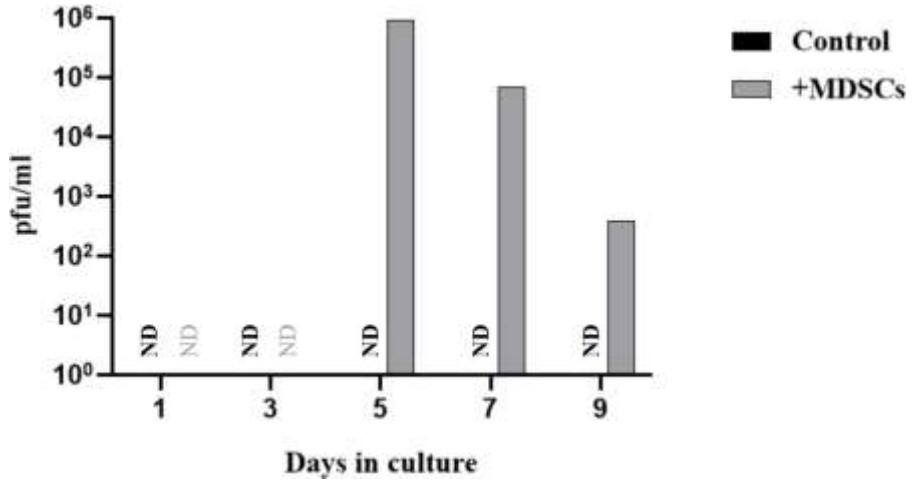

Figure 3.11 Reactivation pattern of 30 dpi TG culture.

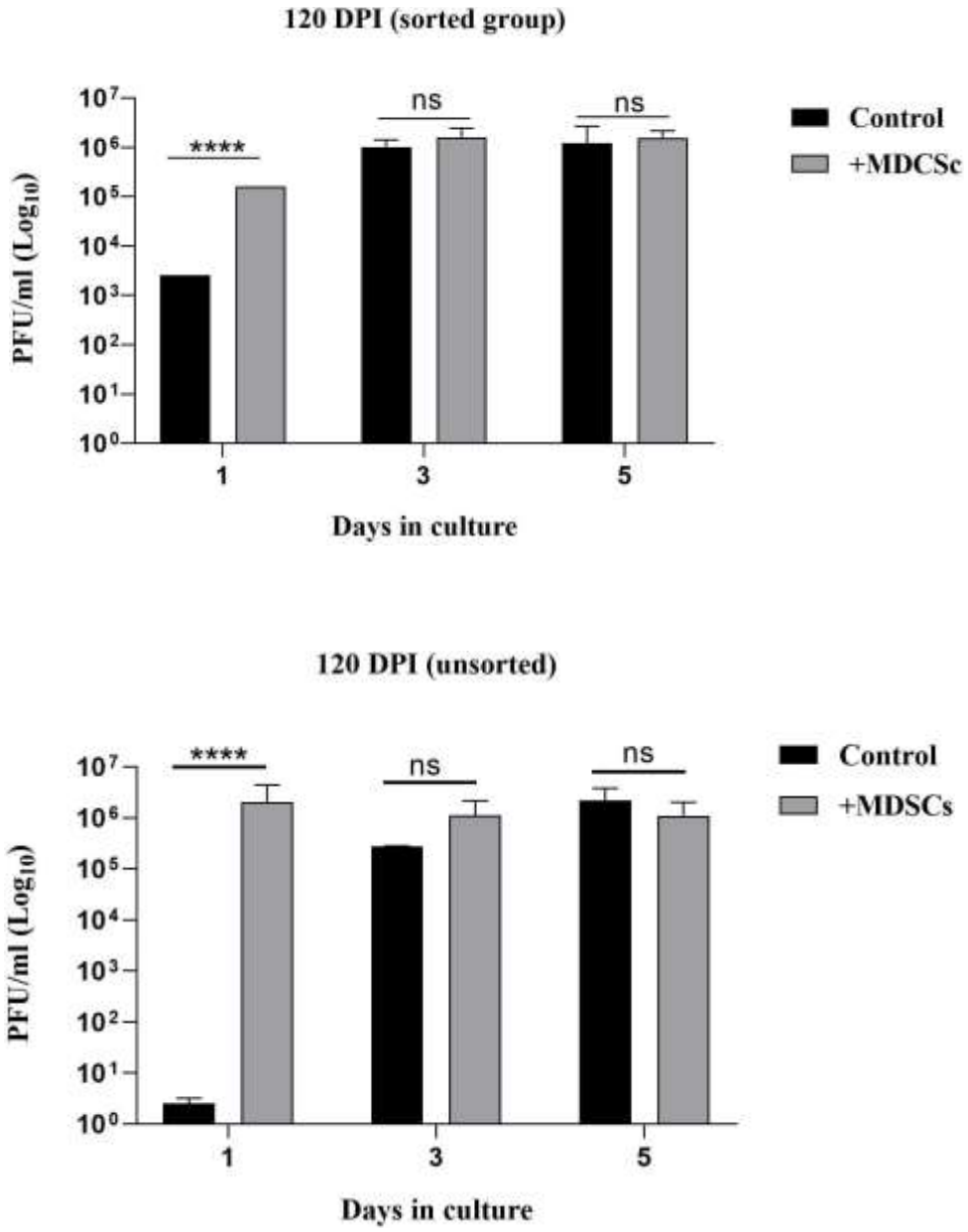

Figure 3.12 Reactivation patterns in 120 dpi TG culture: Plaque Assay was performed on collected supernatant to determine viral titer. Two way ANOVA was performed for determining the significance levels in control and +MDSC groups. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$***, $P < 0.0001$**** and ns for $P > 0.05$.

3.5 LAT expression after transferring MDSCs to animals infected with HSV-1 (>30dpi)

After observing increased reactivation in MDSCs supplemented group, the next question was to check if the same is observed *in vivo* or not. To this end, >30 dpi animals were taken which developed HSK. About 5 million *in vitro* generated MDSCs were transferred to each animal by intravenous injections. These animals were sacrificed after 3 days and 6 days post transfer and their TGs were collected. Isolated TGs were checked for LAT expression using RT-qPCR and were *in vitro* cultured in 96 well plate in DMEM with 10% FBS. LAT expression was compared with uninfected and infected animals without receiving MDSC.

We observed reduction in LAT expression in TG in MDSCs recipient animals (Figure 3.13). The LAT expression was as low as seen in uninfected animals. As mentioned earlier LAT expression directly correlates with latency. A reduction in LAT expression could indicate that the virus is no longer in a latent state and is reactivated. Thus, looking at the LAT expression in comparison with uninfected and untransferred groups, it can be inferred that treatment with MDSCs enhances reactivation of the virus.

To further assess the amount of virions present in the system after reactivation, plaque assay could be performed on collected supernatant from TG cultures.

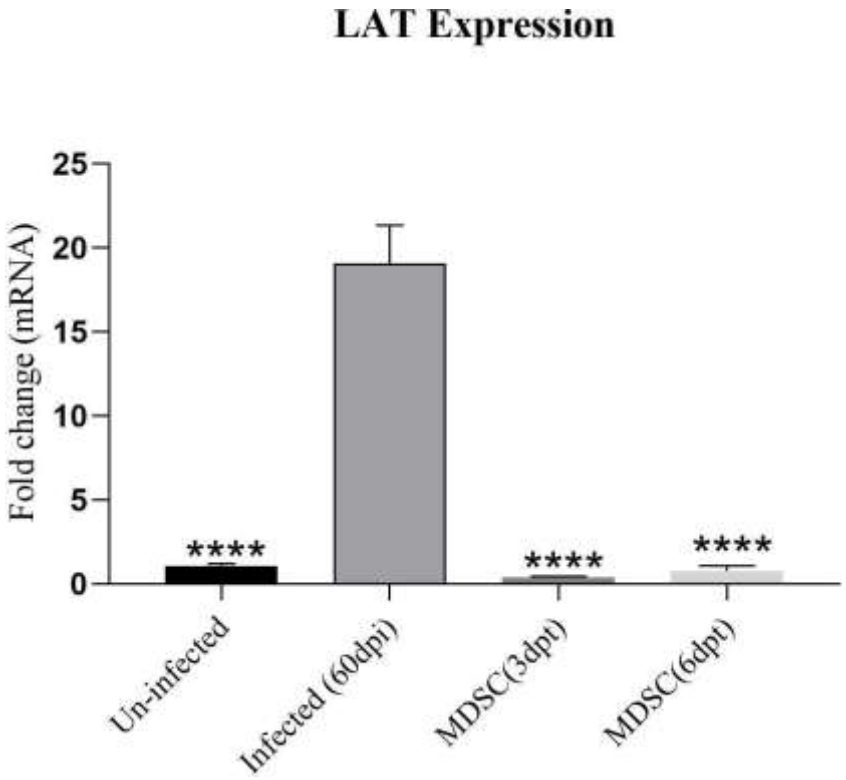

Figure 3.13: LAT expression after transferring *in vitro* generated MDSCS to infected animals (in comparison with infected): 5 million *in vitro* generated MDSCs were transferred to each animal and their TGs were checked for LAT expression using RT-qPCR after 3- and 6-days post transfer. One way ANOVA was performed for determining the significance levels in control and +MDSC groups. $P < 0.05$ *, $P < 0.01$ **, $P < 0.001$***, $P < 0.0001$**** and ns for $P > 0.05$.

Chapter 4

4.1 Conclusion

The obtained data suggested that latent HSV-1 reactivation is enhanced in presence of MDSCs both *in vitro* and *in vivo*. Infiltration of tetramer positive $CD8^+$ T cells which reactive to HSV-1 antigens was seen in TG around 15dpi and such cells were detectable till 30 dpi. These consistent levels of CD8 T cells could indicate that the virus is present in TG even after the disease is resolved in the cornea and the resident virus specific CD8 T cells helped in maintaining the latent state. The levels of these cells in spleen, CLN and cornea peaked around 15 dpi and then reduced by 30 dpi which could suggest that the inflammation receded after 15 dpi. This could also mean that the virus was cleared from cornea but the levels of these cells in TG indicated its presence in TG. This is in agreement with the well-established fact that HSV-1 attains latency in attained in TG. To verify this, LAT mRNA levels were checked over different days and it was seen that the LAT expression was highest at 15dpi but drastically reduced afterwards. It was expected that LAT levels would be maintained till 30 dpi but that did not seem to happen here. This reduction could be due to two possible reasons, first, that the virus needs to reactivate after a regular interval which explains the high levels of CD8 T cells in TG at 30 dpi. Another reason could be that there was an unexpected stimulus such as stress that resulted in the reactivation of the virus giving us lower LAT levels at 30dpi.

It is already well established that CD8 T cells are responsible to maintain latency and stabilize LAT expressions. While their major function is to control the virus

reactivation, such cells could kill precious neurons in so doing. Therefore, A regulated activity of effector cells could help preserve the neuronal functions. To test this, when infected TGs were cultured in the presence of MDSCs, it could be seen that the reactivation was enhanced as compared to culturing TGs in absence of MDSCs. MDSCs inhibit CD8 T cell activation and proliferation and thus block their normal functioning and their ability to maintain latency. To further check this effect, $CD11b^{+}$ $Gr1^{+}$ were sorted out of the TG using FACS to deplete most of the innate cells such as monocytes, macrophages and dendritic cells that have the property of antigen presentation. Depleting these cells would inhibit the suppressor function of MDSCs against CD8 T cells and might kill neurons. In addition to that when these cells are cultured in presence of MDSCs, they would block the activity of CD8 T cells. So, the reactivation in sorted TG cells which were cultured in presence of MDSCs should have been more than the reactivation observed in unsorted TG cells that were cultured in presence of MDSCs. But such a trend was not observed. Nevertheless, it was still observed that the reactivation of HSV-1 in presence of MDSCs were higher as compared to when they were cultured without MDSCs *in vitro.*

The next question that arises here is that do we see the same phenotype *in vivo*? To address this question, 5 million *in vitro* generated MDSCs were transferred to each animal that was infected with HSV-1. LAT expression was used to determine if the virus was reactivated or not. The LAT expressions were drastically reduced when MDSCs were transferred to infected animals. Their levels were as low as seen in uninfected animals. This result was consistent with our observation of

MDSCs' effect on latency *in vitro*. Along with reduction in LAT expressions, signs of HSK were also seen to develop which included facial swelling and corneal lesions (data not shown).

From all these experiments, it can be concluded the MDSCs have a role to play in the reactivation of HSV-1. Their presence enhances viral reactivation both *in vitro* and *in vivo.* It can be suspected that they do so by inhibiting the function of CD8 T cells that are essential for maintaining the latency without damaging the neurons.

4.2 Discussions

Dealing with HSV-1 is challenging because of the ability of this virus to remain in a dormant state of regulated gene expression known as latency. As mentioned earlier, the virus remains latent in the TG which is an immune privileged site until some kind of stimulus such as exposure to UV, stress, fever, etc. causes it to reactivate. The results show that virus specific CD8 T cells infiltrate the TG and remain there in dormant stage as seen by LAT expression on the isolated TG and virus titration from TG cells culture *in vitro.*

The function of MDSCs is widely seen in cancer progression. They are associated with poor patient prognosis and resistance to cancer therapies (Anani & Shurin, 201). Their function in regulating autoimmune responses is also well established. Recently their role in dampening HSV-1 ocular infection was established where repletion of HSV-1 infected mice with *in vitro* generated MDSCs controlled HSK progression (Sarkar et al., 2019). In this project it has been demonstrated that

MDSCs not only just control HSK progression but also are responsible for affecting the latent state of the virus. It is seen to disrupt the latency and enhance the reactivation of HSV-1. So far the treatment of HSV1 includes antiviral drugs that mostly target viral replication and viral entry (Chang et al., 2012). Since HSV-1 mediated diseases such as HSK are mainly caused due to hyperactive immune response, MDSCs can be thought as a potential therapy for controlling HSV-1 infections. Not only because of its immunosuppressive nature but also because it promotes an immunosuppressive response by promoting the expansion of Tregs. Since MDSCs inhibit HSV-1 latency, it means that the virus could not attain the latent state and are cleared from the host. It would remain in a lytic phase and cause damage to the host body. MDSCs can cause reactivation of the latent virus as it suppresses T cells. Reactivating the virus using MDSCs could cause neuronal damage that can ultimately lead to encephalitis. Thus MDSCs therapy alone is not enough to tackle HSV-1 infection. Along with preventing it from going latent, it is important to prevent it from causing further infection. Thus, a combination of antiviral drugs along with MDSCs can manage HSV- 1 infection in better ways.

4.3 Future Directions

Here, we have suspected that MDSCs mediated reactivations are due to regulation of CD8 T cells in TG. It is also possible that MDSCs can have a direct effect on latency and reactivation which has not been addressed in this study.

We have seen that reactivation is happening at a faster rate in the presence of MDSCs *in vivo* by seeing a decrease in the LAT expressions in TG. But these results don't tell whether the reactivated virus is infectious in nature or not. It is possible that MDSCs are causing a reduction in LAT expression but that does not mean that it is resulting in infectious virions that are capable of causing further damage to the host. Thus to address this problem, it is necessary to observe symptoms of HSK in animals transferred with MDSCs. Along with that, it would also be useful to quantify the amount of virus that is reactivated by virus titration and plaque assays.

Another possibility that can be thought of is what happens to latency if MDSC treatment is given since the beginning of the disease, i.e., as soon as the virus reaches TG and before the inflammation reaches to maximum. It can be thought that MDSCs can prevent infiltration of CD8 T cells in TG which will stop the virus from attaining latency in the first place and can be cleared from the system.

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