

The role of CD8⁺ T cells during the pre-clinical phase of corneal HSV-1 infection.

Submitted by
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THESIS MENTOR FORM

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MENTOR STATEMENT

I, Dr. Susmit Suvas, hereby certify that Ms. Ashima Vohra carried out her Honors College undergraduate thesis project under my guidance. She independently carried out the experiments and has generated interesting publishable results. These studies are of high significance in the field of pathogenesis of ocular HSV-1 infection.

ABSTRACT

Approximately 90% of world's population is seropositive for Herpes Simplex Virus-1 (HSV-1). However, most individuals show no symptoms because HSV-1 largely remains in its non-replicating mode inside the neurons. If the virus is activated, it can cause cold sores around the mouth. The virus can also migrate to the eye, where it causes infection and chronic inflammation in the cornea of the eye. The corneal inflammation induced by HSV-1, if not effectively controlled by steroids and anti-viral drugs, will then give rise to herpetic stromal keratitis (HSK). HSK leads to permanent corneal damage and is the leading cause of infection-induced blindness in the United States. If HSV-1 replication in the cornea continues to remain uncontrolled, the virus may spread to the brain and cause encephalitis (brain fever).

In terms of HSV-1, CD8⁺ T cells are known to mediate viral latency in the neurons, but the role of this immune cell type in regulating corneal inflammation after ocular HSV-1 infection is not well characterized. In this study, we worked in a mouse model to decipher the role of CD8⁺ T cells in corneas infected with HSV-1. First, we measured the influx of CD8⁺ T cells in the cornea at different time points after ocular HSV-1 infection. Our preliminary data showed that CD8⁺ T cells are indeed present in HSV-1 infected corneas, with maximum levels on day 8 post-infection. Accordingly, we carried out localized depletion of CD8⁺ T cells in the cornea via subconjunctival administration of anti-CD8⁺ antibodies on day 5 and day 7 post-infection. The depletion of CD8⁺ T cells was confirmed by flow cytometry on day 9 post-infection. Interestingly, the loss of CD8⁺ T cells resulted in the development of encephalitis in approximately 50% of the treated mice, suggesting that the virus spread to the brain.

To determine exactly how the loss of CD8⁺ T cells affects immunoinflammatory events in corneas infected with HSV-1, we carried out protein arrays for cytokines and angiogenic factors

in the CD8⁺ depleted and non-depleted corneas. Our results indicated that, in the absence of CD8⁺ T cells, the levels of many pro-inflammatory cytokines, chemokines, and angiogenic factors were up-regulated in the cornea. As expected, the results of our experiments demonstrate the involvement of CD8⁺ T cells in the regulation of immunoinflammatory events in HSV-1 infected corneas. Future studies will be focused on determining how CD8⁺ T cells affect the development of HSK lesions.

I. INTRODUCTION

Herpes simplex virus I (HSV-1) spreads through contact with mucosal linings, which include the eye, the nose, and the mouth. From the mucosal surface, the virus migrates to the trigeminal ganglia (TG) of the central nervous system; the TG is a bundle of dense nerve fibers located behind the eye in the brain. This is where HSV-1 establishes latency, which means the virus DNA is simply present in the central nervous system and is not replicating or causing infection. Approximately seventy percent of children under the age of five and ninety percent of adults are seropositive for HSV-1 (Reinhard 2008). Seropositive means that HSV-1 DNA has established latency in the central nervous system. However, only twenty to thirty percent of those who are seropositive for HSV-1 actually develop the clinical disease, indicating that the virus usually remains in its latent form (Reinhard 2008).

If the virus is activated, HSV-1 can travel via nerves from the TG to the peripheral tissues, where it then causes viral infection. In severe cases, the virus travels to the brain, where it causes encephalitis, commonly known as brain fever. HSV-1 can also infect the mouth, where it causes herpes labialis, commonly known as cold sores, and gingivostomatitis, which is swelling of the gums (Carr et al., 2006). The virus can also migrate to the eye, where it causes herpetic stromal keratitis (HSK), which leads to blindness.

This paper focuses on HSV-1 infection in the eye. HSV-1 infects the cornea, which is the clear layer covering the front of the eyeball. The cornea has three major layers: the top layer is the epithelium, the middle layer is the stroma, and the inner layer is the endothelium (Figure 1.1). Specifically, HSV-1 infects the epithelial layer of the cornea of the eye (Carr et al., 2006). Recurrent HSV-1 infection can cause the development of HSK in the eye. This condition is characterized by chronic inflammation, opacity (clouding), angiogenesis (vascularization), and scarring, all of which eventually cause blindness (Carr et al., 2006). The progression of HSV-1 in humans is similar to its progression in mice. In both species, HSV-1 infection in the eye is characterized by corneal opacity, which is the clouding of the clear layer covering the eye, and by angiogenesis, which is the movement of blood vessels into the clear layer of the eye. Hence, the mouse model was used for this study, and all further discussion will be in terms of HSV-1 infection in the corneas of mice.

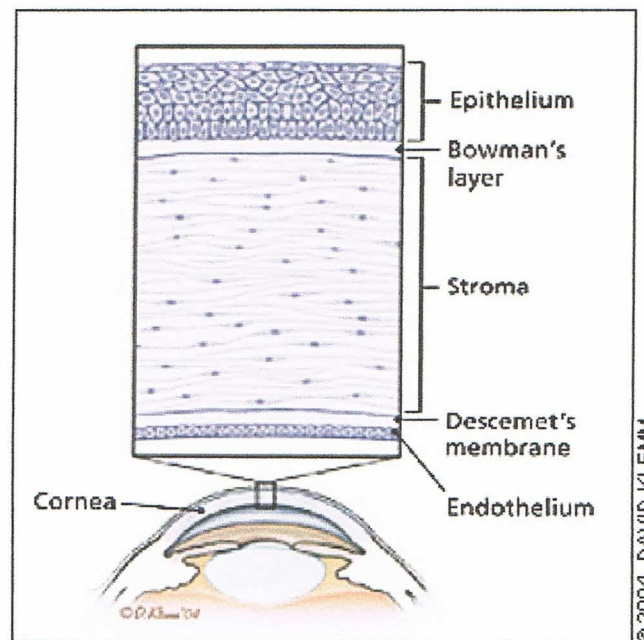


Figure 1.1: A schematic of the cornea. HSV-1 infection primarily takes place in the epithelial layer (top layer) of the cornea. HSK is an inflammatory response triggered by the spread of HSV-1 to the stromal layer (middle layer) of the cornea (Carr et al., 2006).

When HSV-1 is activated and moves into the eye to cause infection, the mouse's immune system will also be triggered and will try to clear the infection. The immune system is regulated by a variety of immune cells and the proteins those immune cells secrete. The immune cells can be divided into subgroups like T cells, macrophages, and neutrophils. Their secreted proteins can be further divided into subgroups like interleukins, leukocytes, and chemokines. In addition to clearing the infections, the secreted proteins often cause inflammation and tissue damage at the site of infection (Carr et al., 2006).

In mice, the HSV-1 infection consists of two phases (Twardy et al., 2011). The first phase is the pre-clinical phase, which lasts from day zero to day eight post-ocular infection (POI). During this period, the virus is actively replicating and the immune response is trying to clear the virus from the cornea. The second phase is the clinical phase, which lasts from day eight to day eighteen post-ocular infection. During this period, the virus has been cleared by the immune response. However, the immune response persists and is now causing corneal tissue damage and lesions in the cornea. In other words, HSV-1 does not directly cause blindness. The virus does so indirectly by initiating a strong immune response; it is this immune response that causes the corneal tissue damage, and this tissue damage leads to blindness.

Both phases of the infection involve a variety of immune cells and molecular events (Figure 1.2). Specifically, this paper will discuss the role of CD8⁺ T cells during HSV-1 infection in the corneas of mice. Studies carried out in mouse models have shown that, upon initial infection of the cornea with HSV-1, inflammatory cells such as neutrophils and macrophages enter into the infected cornea to control the spread of virus (Tumpey et al., 1996). In other words, mice with suppressed neutrophils were more susceptible to increasingly severe HSV-I infections like encephalitis (brain fever). However, it has now been demonstrated that the monoclonal

antibodies used to deplete the corneal tissue of neutrophils could have led to the depletion of other significant immune cell types including CD8⁺ T cells (Wojtasiak et al.). Therefore, CD8⁺ T cells may play a role in controlling HSV-1 infection in the cornea.

Pre-clinical phase	Clinical phase
[Day 0 to day 8 post-ocular infection]	[Day 8 to day 18 post-ocular infection]
Influx of: • Neutrophils • Macrophages • NK cells • Dendritic cells • CD8⁺ T cells	Influx of: • Neutrophils • Dendritic cells • CD4⁺ T cells • CD4⁺CD25⁺ cells • Regulatory T cells
Molecular events: IL-1, IL-6, VEGF-A, IFN-γ, MCP-1, IL-17, IL-12, and TNF-α	Molecular Events: IL-1, IL-6, VEGF-A, MMP-9, IL-2, IL-17, IL-12, and TNF-α

Figure 2.2: The table above lists some the cells and pro-inflammatory protein molecules involved in each phase of the corneal HSV-1 infection (Carr et al., 2006). Notice that there is some overlap between the pre-clinical and clinical phases of HSV-1 infection. Neutrophils are secreted in both phases, and IL-1 molecules are secreted in both phases as well.

CD8⁺ T cells are well reported to keep HSV-1 latent in the neuronal cell bodies of the trigeminal ganglia, thereby preventing the virus from migrating to the peripheral tissues where it causes infection. In fact, when CD8⁺ T cells were suppressed in the trigeminal ganglia, it led to HSV-I activation (Liu et al., 2000). In addition to keeping the virus latent in the central nervous system, if HSV-1 is activated and causes infection, CD8⁺ T cells will move to the site of infection and help clear the virus. Experiments have demonstrated that, during the pre-clinical phase of HSV-1 infection, the versatile CD8⁺ T cells enter the periphery and contribute to the clearing of the infection (Banerjee et al., 2005). Hence, we hypothesized that CD8⁺ T cells are

directly involved in clearing HSV-1 and mediating immunoinflammatory events that take place in the cornea during the pre-clinical phase of the infection. We expect CD8⁺ T cells to be present in the cornea after ocular HSV-1 infection. In addition, we expect that localized depletion of CD8⁺ T cells in the cornea will delay the clearance of HSV-1 from the eye.

II. METHODOLOGY

The objective was to determine the role of herpes-specific CD8⁺ T cells in mediating HSV-1 viral infection in the corneas of mice. These studies were carried out under already approved IACUC protocol 09051-R3 and IBC application 1508. We used the C57BL/6 mouse model. To determine the kinetics of HSV-1-specific CD8⁺ T cells in the cornea at regular intervals during the post-infection period, mice were infected ocularly with 1×10^6 plaque forming units of HSV-1. Then, mice were killed at day 8, day 21, and day 60 post-infection. The corneas excised from mice were pooled and collagenase digested to obtain a single cell suspension. They were then stained by immunofluorescence for flow cytometry. The results of the above-mentioned experiment were also qualitatively confirmed using confocal microscopy. Eyes were frozen and ten-micron sections were mounted on a slide. The sections were then stained for CD8⁺ T cells by immunofluorescence and the slides were visualized under a confocal microscope.

To determine if the depletion of CD8⁺ T cells in the cornea after ocular HSV-1 infection will delay HSV-1 clearance from the eye, subconjunctival CD8⁺ depletions were carried out. Again, C57BL/6 mice were infected ocularly with 1×10^6 plaque forming units of HSV-1. On day five and day seven post-infection, mice received subconjunctival injections of anti-CD8⁺ antibodies to locally deplete CD8⁺ T cells in the cornea. The control groups of mice were given subconjunctival injections of normal saline. On day nine post-infection, the corneas of the mice

were excised and the extent of the CD8⁺ T cell depletion was determined by flow cytometry as described earlier.

The effect of CD8⁺ depletion on molecular events during corneal HSV-1 infection was determined using two types of protein arrays: cytokine arrays and angiogenic factor arrays. These arrays allowed us to semi-quantitatively compare the levels of various pro-inflammatory molecules in the CD8⁺ depleted and the control group of infected mice. An enzyme-linked immunosorbent assay (ELISA) was carried out for the molecules identified by the protein arrays whose levels changed drastically between the control and treated groups of infected mice. This assay used color change to accurately quantify protein levels, thereby confirming the results of the protein arrays.

III. RESULTS AND DISCUSSION

Kinetics of CD8⁺ T cells in the cornea after ocular HSV-1 infection

On day eight, day 21, and day 60 post-ocular infection (POI), corneas were excised from mice and the levels of CD8⁺ T cells were quantified using flow cytometry (Figure 3.1). The highest level of CD8⁺ T cells was present on day eight POI and decreased as the infection progressed (Figure 3.2).

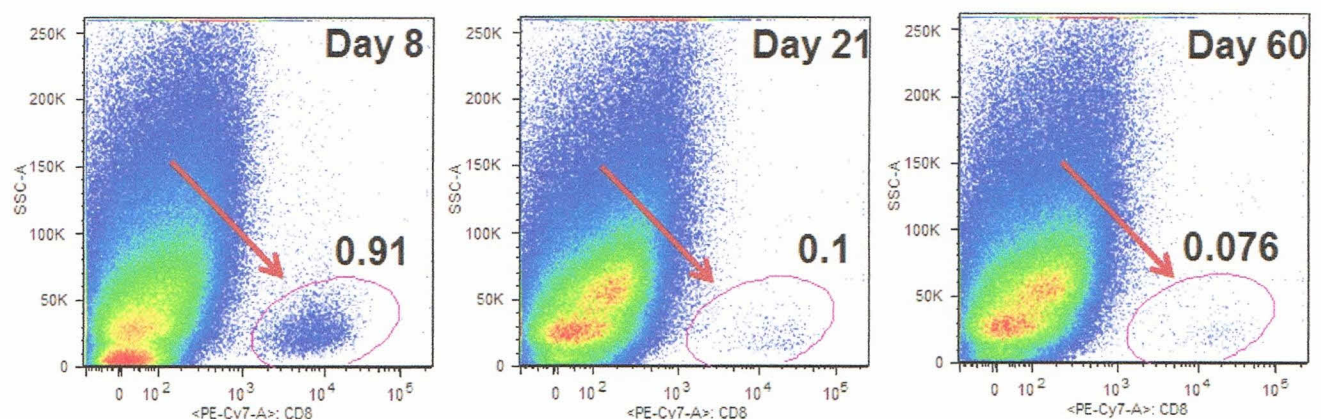


Figure 3.1: The population of CD8⁺ T cells identified using flow cytometry has been gated by the circle. Notice the decreasing CD8⁺ levels. The percentage of cells in the cornea that are CD8⁺ T cells decreases from a high of 0.91% on day 8 POI (far left) to 0.1% on day 21 (middle) and 0.076% on day 60 (far right).

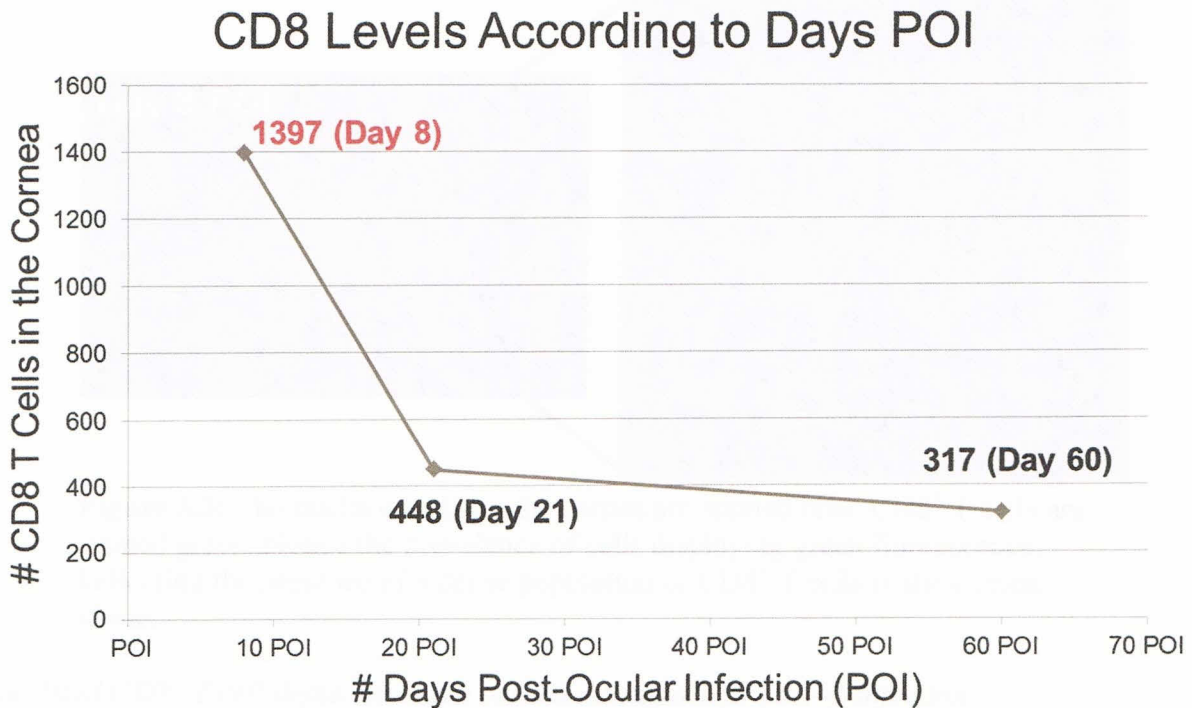


Figure 3.2: Based on total cell populations of each cornea and the percentage of CD8⁺ T cells (Figure 3.1), the exact number of CD8⁺ T cells present in the cornea was determined for each day POI. The downward trend in CD8⁺ levels is evident.

The quantitative results of flow cytometry were qualitatively confirmed using confocal microscopy. Sections of a day eight POI cornea were stained for CD8⁺ T cells and visualized under a confocal microscope. The images obtained confirmed a substantial population of CD8⁺ T cells in the stroma of the cornea on day eight POI (Figure 3.3). Therefore, CD8⁺ T cells migrate into the cornea during the pre-clinical phase of HSV-1 infection in the cornea. It is likely that they play a role in clearing the virus from the cornea. If that is the case, then locally depleting CD8⁺ T cells from the cornea during the pre-clinical period of ocular HSV-1 infection should delay viral clearance in the cornea.

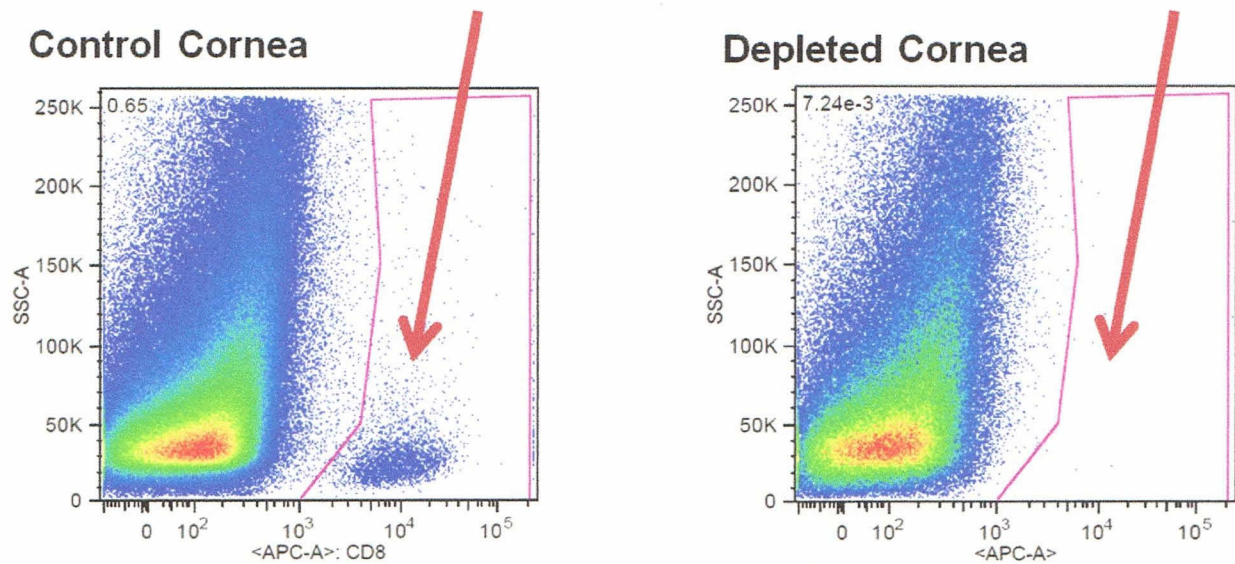


Figure 3.4: The population of CD8⁺ T cells was identified using flow cytometry and has been gated in the figure above. Notice that the depleted cornea (on the right) lacks a distinct CD8⁺ T cell population.

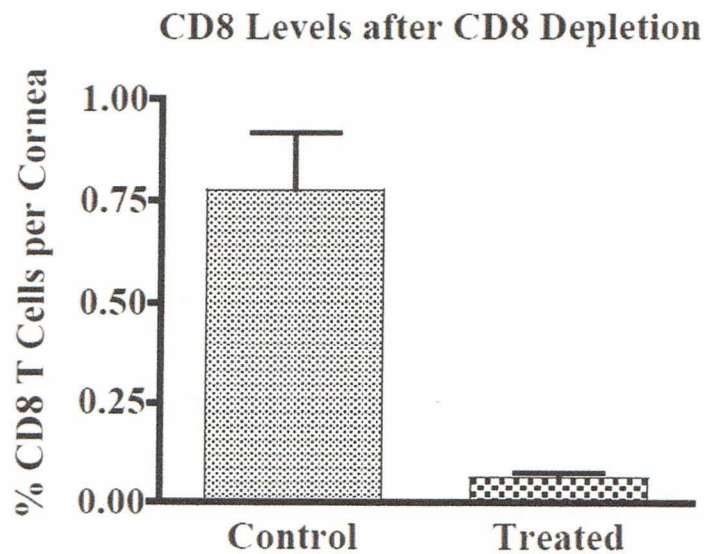


Figure 3.5: The levels of CD8⁺ T cells in control and treated mice were compiled and analyzed using a one-sided t-test. It indicated that there was a statistically significant decrease in CD8⁺ T cell levels in the treated group. Therefore, the localized CD8⁺ depletion was successful.

The effects of CD8⁺ depletion on cellular events during corneal HSV-1 infection

Neutrophils are phagocytic immune cells that help clear the virus from the cornea. However, neutrophils are also pro-inflammatory and cause corneal damage, angiogenesis, and the formation of reactive oxygen species. Therefore, their actions need to be heavily mediated so that extensive tissue damage does not occur. In the mice that underwent CD8⁺ depletion, a higher neutrophil population was observed in the cornea (Figure 3.6). Because CD8⁺ depletion in the cornea led to a statistically significant increase in the level of pro-inflammatory neutrophils (Figure 3.7), it can be concluded that the loss of CD8⁺ T cells leads to increased corneal inflammation.

Macrophages assist in phagocytosis and in controlling viral replication. They also secrete pro-inflammatory molecules and recruit neutrophils to the site of infection. In the mice that underwent CD8⁺ depletion, higher macrophage levels were observed in the cornea (Figure 3.8). Because CD8⁺ depletion in the cornea led to a statistically significant increase in the level of macrophages (Figure 3.9), it can be concluded that CD8⁺ T cells mediate viral replication in the cornea and that their depletion leads to an increase in corneal inflammation.

Inflammatory monocytes (IMs), like macrophages, are responsible for phagocytosis and help control viral replication in the cornea. According to flow cytometry, the loss of CD8⁺ T cells seems to result in a slight increase in the levels of IMs (Figure 3.10). However, the CD8⁺ depletion did not lead to a statistically significant increase in the level of IMs. This data may indicate that CD8⁺ T cells do not greatly influence the levels of IMs in the cornea during infection. The lack of statistical significance could be due to some mouse-to-mouse variation as well. Repeat experimentation is required to accurately determine the effect of CD8⁺ depletion on IM levels.

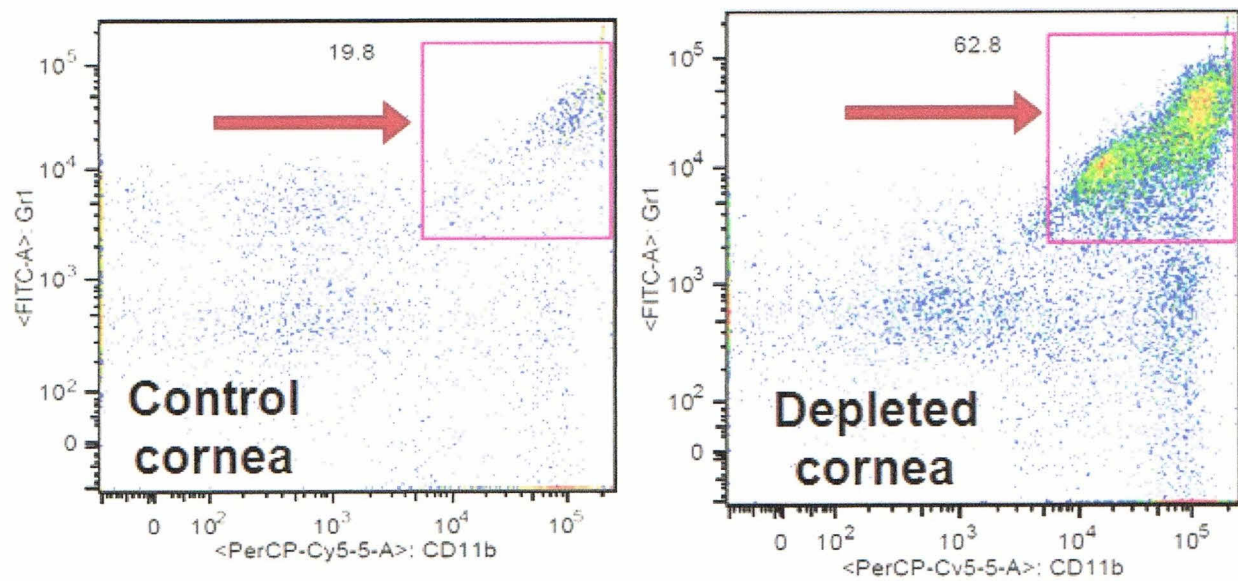


Figure 3.6: The neutrophil levels in each cornea were measured using flow cytometry. The neutrophil population has been gated in the graphs above. Notice the substantial increase in the neutrophil population in the CD8⁺ depleted cornea.

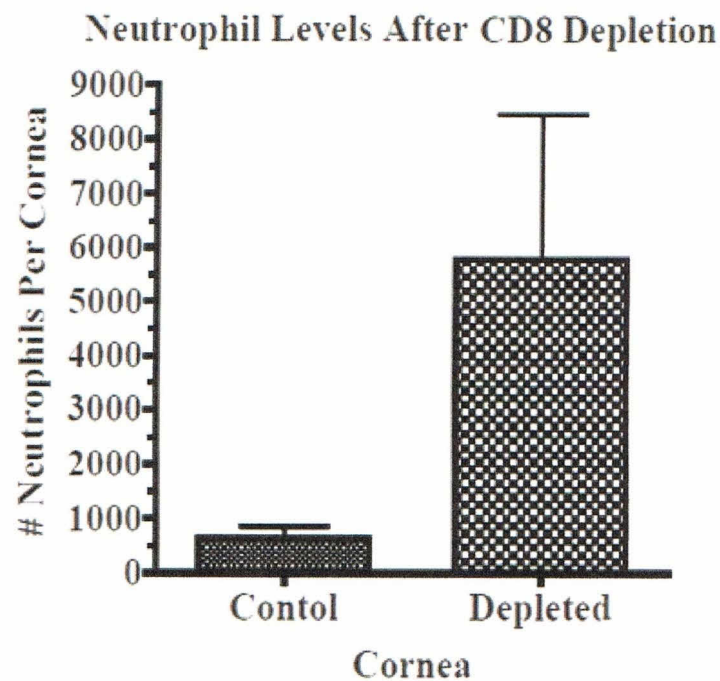


Figure 3.7: The depletion of CD8⁺ T cells led to a statistically significant increase in neutrophil level. This indicates that the loss of CD8⁺ T cells leads to increased inflammation in the cornea.

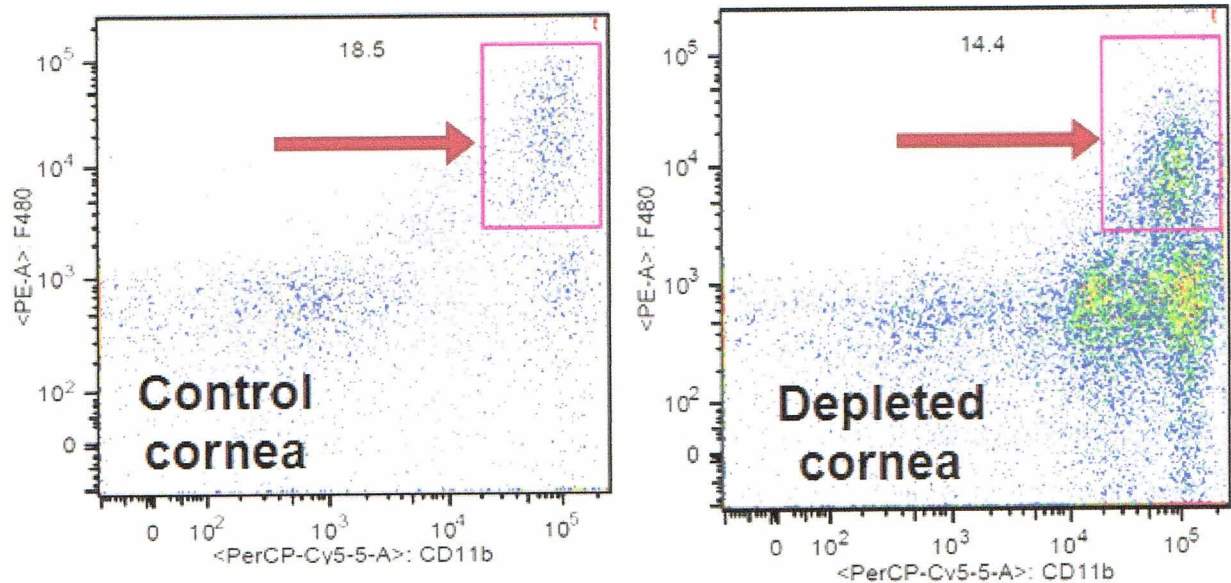


Figure 3.8: The macrophage levels in each cornea were measured using flow cytometry. The macrophage population has been gated in the graphs above. Notice that the loss of CD8⁺ T cells led to an increase in the corneal levels of macrophages.

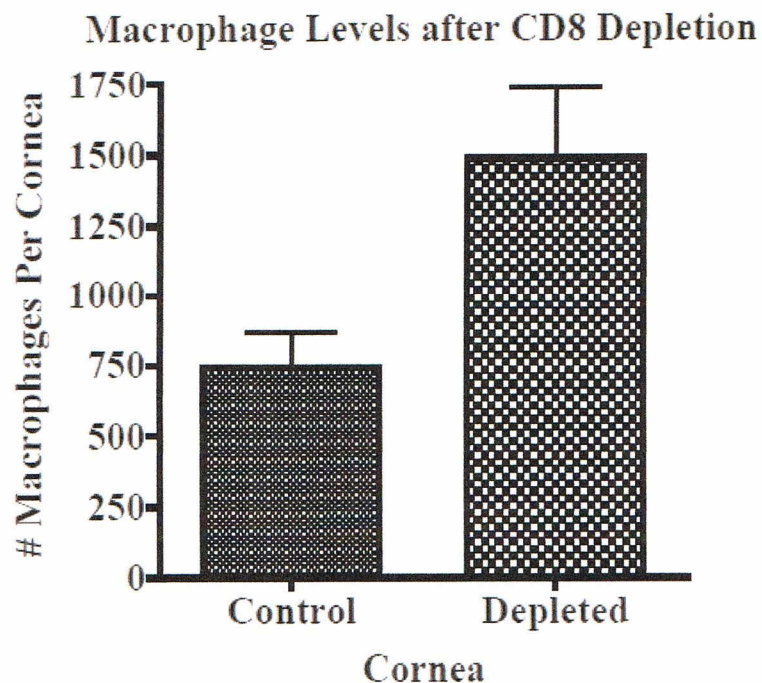


Figure 3.9: The depletion of CD8⁺ T cells led to a statistically significant increase in macrophage levels. This indicates that CD8⁺ T cells control viral replication in the cornea and that the loss of CD8⁺ T cells increases cornea inflammation.

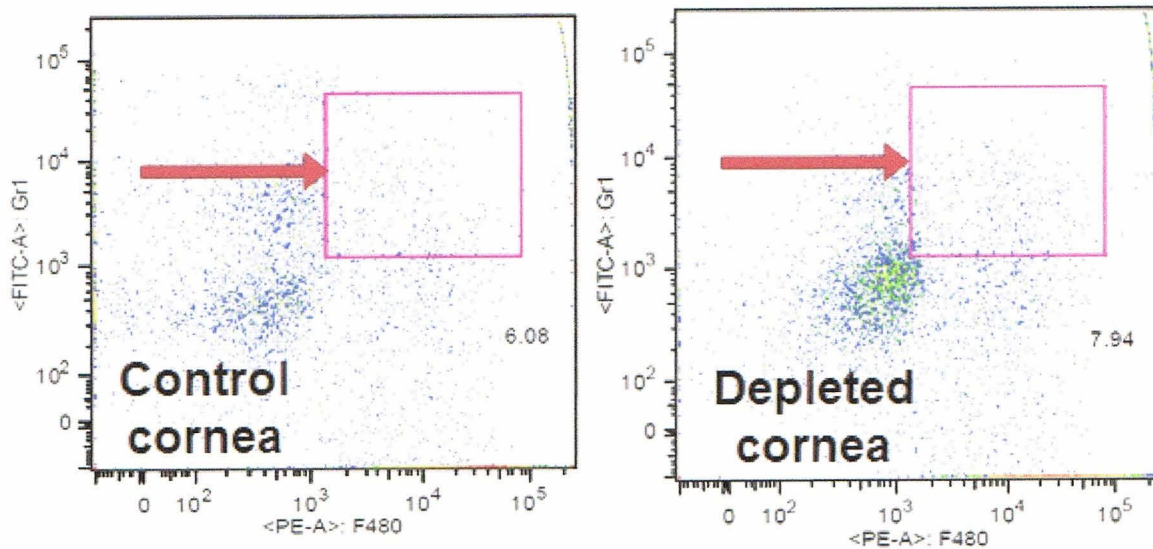


Figure 3.10: The inflammatory monocyte (IM) levels in each cornea were measured using flow cytometry. The IM population has been gated in the graphs above. Notice that the loss of CD8⁺ T cells led to a slight increase in the corneal levels of IMs.

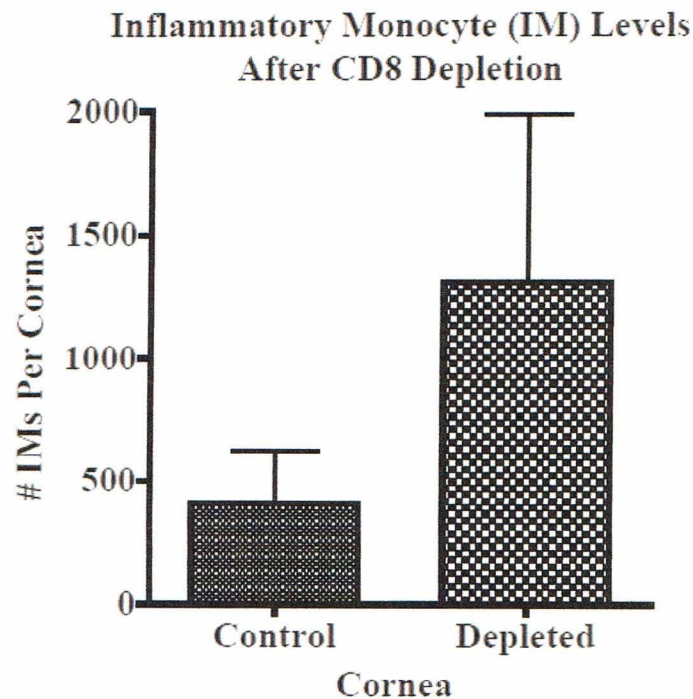


Figure 3.11: The depletion of CD8⁺ T cells did not lead to a statistically significant increase in IM levels. This indicates that CD8⁺ T cells may or may not mediate the influx of IMs into the cornea during the pre-clinical phase of HSV-1. Further experimentation is needed.

Overall, the loss of CD8⁺ T cells did lead to increased levels of pro-inflammatory immune cells like neutrophils and macrophages. Since localized CD8⁺ depletion led to increased levels of neutrophils, which are responsible for corneal scarring and angiogenesis, CD8⁺ T cells must play a role in suppressing corneal inflammation. Since localized CD8⁺ depletion led to increased levels of macrophages, which control viral replication via phagocytosis, CD8⁺ T cells must play a role in controlling viral replication in the cornea.

The effects of CD8⁺ depletion on molecular events during corneal HSV-1 infection

After the role of CD8⁺ T cells in cellular events was determined, their role in molecular events was explored. Various pro-inflammatory proteins are secreted by immune cells during HSV-1 infection in the cornea. These include cytokines, which are cell-signaling protein molecules that enter the cornea to help regulate viral clearance, and angiogenic factors, which are cell-signaling protein molecules that enter the cornea and facilitate vascularization. Protein arrays were used to semi-quantitatively measure the levels of cytokines and angiogenic factors in control corneas and depleted corneas.

The CD8⁺ depleted cornea showed higher levels of various cytokines: TIMP-1, CD54, MCP-1, MIP-2, IL-1F3, CXCL1 (Figure 3.12). The CD8⁺ depleted cornea showed higher levels of various angiogenic factors as well: osteopontin, IGFBP-1, MCP-1, IGFBP-3, MMP8, MMP9, MMP3, Serpin E1, TIMP-1 (Figure 3.13). The depletion of CD8⁺ T cells led to an increase in a variety of pro-inflammatory cytokines and angiogenic factors. Just as the cellular events indicated, the molecular events also indicate that CD8⁺ T cells suppress virus-induced inflammation in the cornea. Therefore, CD8⁺ T cells mediate a range of cellular and molecular events in the cornea in order to suppress the viral replication and the virus-induced inflammation that occurs during HSV-1 infection in the cornea.

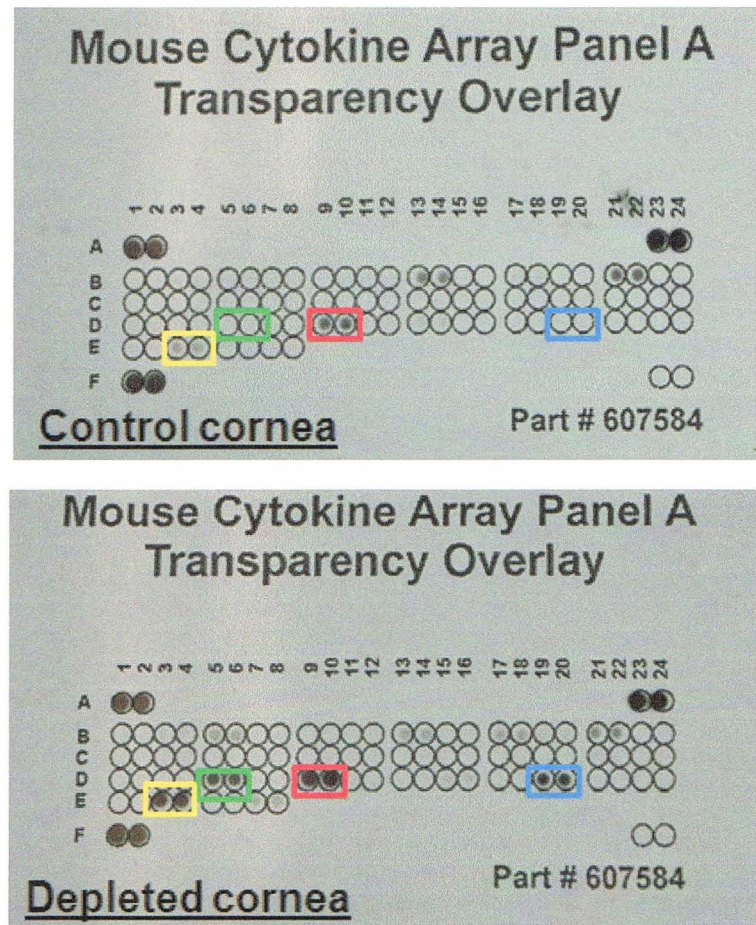
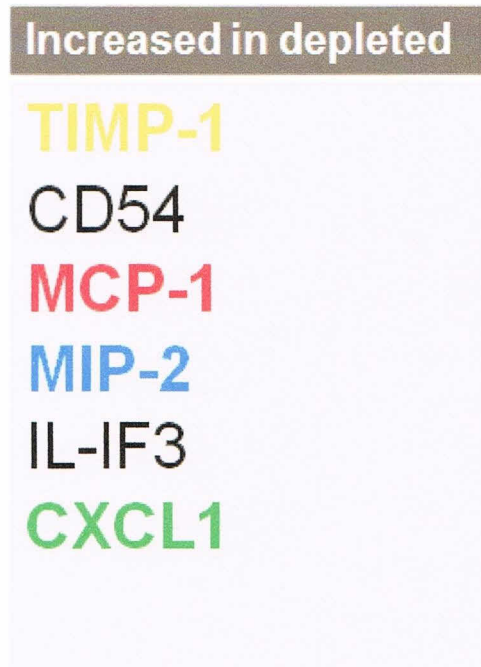


Figure 3.12: The cytokine protein array. Darker spots indicate more of that specific protein is present in the sample. Specific cytokines and their corresponding positions on the protein array have been color coded. The CD8⁺ depleted cornea showed higher levels of numerous cytokines as listed in the table on the left. This indicates that that CD8⁺ T cells suppress virus-induced inflammation in the cornea.

Increased in depleted
<u>Osteopontin</u>
IGFBP-1
MCP-1
IGFBP-3
MMP8
MMP9
MMP3
<u>Serpin E1</u>
TIMP-1

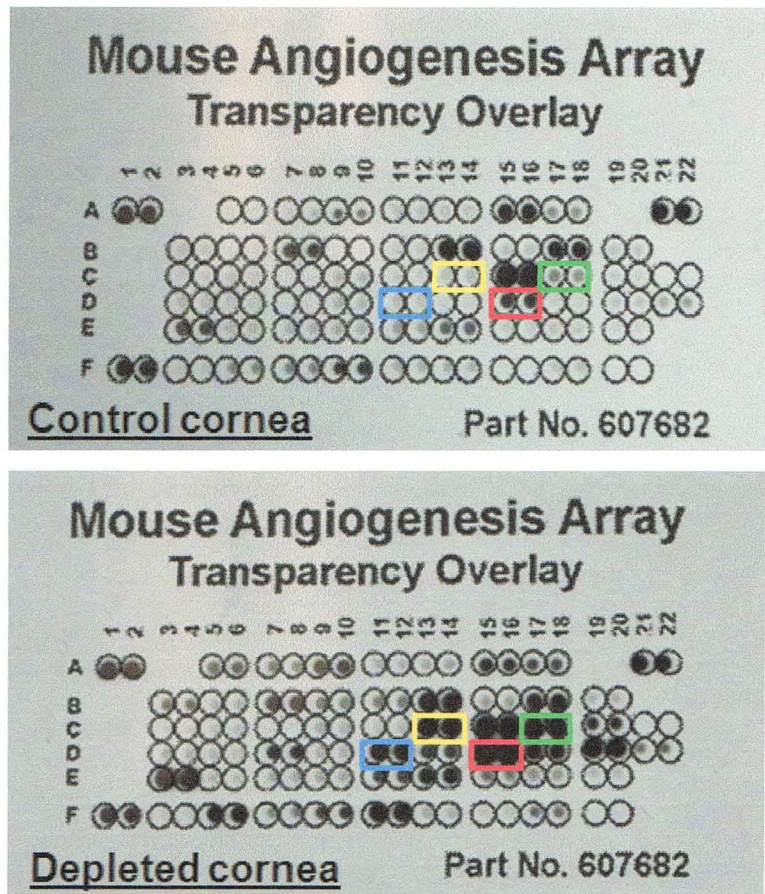


Figure 3.13: The angiogenesis protein array. Darker spots indicate more of that specific protein is present in the sample. Specific angiogenic factors and their corresponding positions on the protein array have been color coded. The CD8⁺ depleted cornea showed higher levels of numerous angiogenic factors as listed in the table on the left. This indicates that that CD8⁺ T cells suppress virus-induced inflammation in the cornea.

The protein arrays were semi-quantitative. Therefore, in order to confirm the pro-inflammatory molecule increase due to CD8⁺ depletion, we ran a fully quantitative ELISA for two of the proteins.

In the cytokine protein array, MIP-2 levels considerably increased in the CD8⁺ depleted cornea (Figure 3.12). MIP-2 is a macrophage inflammatory protein. It is secreted by macrophages and recruits other immune cells to the site of infection (Carr et al., 2006). After running an ELISA, the increase in MIP-2 seems to be related to increased viral load, not CD8⁺ T cell depletion. This is because only the encephalitic mice possessed high amounts of MIP-2 (Figure 3.14). Recall that encephalitis occurs when the viral infection escalates and moves to the brain. Hence, it can be assumed that MIP-2 up-regulation occurred as a result of increased viral load rather than as a result of CD8⁺ depletion.

In the angiogenic factor protein array, MCP-1 levels considerably increased in the CD8⁺ depleted cornea (Figure 3.13). MCP-1 is a monocyte chemo-attractant protein. Like MIP-2, it recruits immune cells to the site of infection. It also promotes vascularization of the cornea, which is the movement of blood vessels into the cornea (Carr et al., 2006). After running an ELISA, there is a distinct upward trend in MCP-1 expression in the CD8⁺ depleted corneas (Figure 3.15). This suggests that CD8⁺ depletion led to the increase in MCP-1 levels.

Hence, ELISA did confirm the results of the protein arrays. As the protein arrays suggested, the absence of CD8⁺ T cells leads to the up-regulation of pro-inflammatory molecules in the cornea. Further quantification of the molecular events will allow us to learn more about the exact mechanism of regulation CD8⁺ T cells use to suppress virus-induced inflammation in the cornea during the pre-clinical phase of HSV-1 infection.

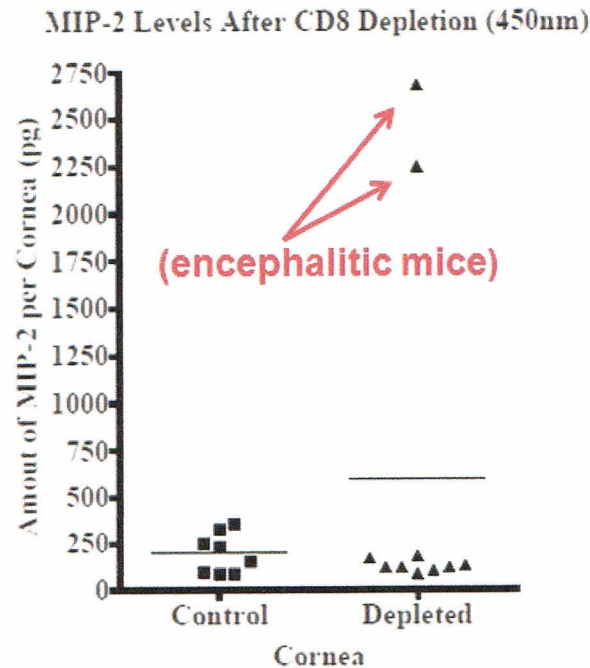


Figure 3.14: The increase in MIP-2 was limited to encephalitic mice. This indicates that the increase in this specific pro-inflammatory cytokine was most likely due to the increased intensity of virus itself, not the depletion of CD8⁺ T cells.

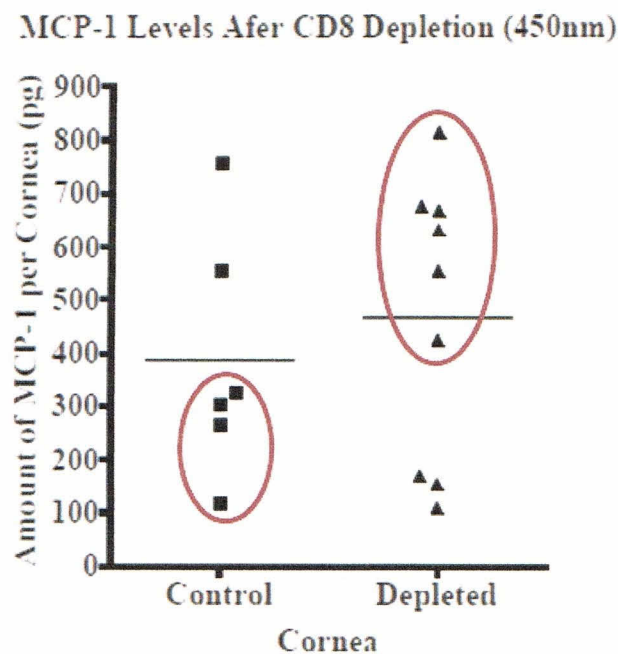


Figure 3.15: There is an upward trend in MCP-1 expression in the CD8⁺ depleted mice. This indicates that the increase in this specific pro-inflammatory angiogenic factor was likely due to the depletion of CD8⁺ T cells.

Overall, CD8⁺ depletion increases corneal inflammation and viral replication in the pre-clinical phase. This was demonstrated by localized depletion of CD8⁺ T cells, which resulted in increased levels of inflammatory cells (neutrophils and macrophages) and molecules (cytokines and angiogenic factors) in the cornea. This indicates that CD8⁺ T cells play an important role in mediating viral clearance in the pre-clinical phase of corneal HSV-1 through a range of cellular and molecular events.

IV. FUTURE EXPERIMENTATION

An individual infected with HSV-1 can develop herpetic stromal keratitis (virus-induced blindness). From the eye, HSV-1 can spread to the central nervous system where it causes herpes simplex encephalitis (brain fever). Moreover, viral shedding caused by an uncontrolled HSV-1 infection in the cornea can spread the infection from one person to another. Therefore, we must obtain a greater understanding of the cellular and molecular events involved in controlling corneal HSV-1 infection. This way, better strategies can be developed to suppress corneal inflammation and clear the actively replicating HSV-1 from the cornea.

Studying the role of CD8⁺ T cells is an important step in learning about the progression of HSV-1 in the cornea. This paper discusses how CD8⁺ T cells suppress the levels of certain immune cells and pro-inflammatory molecules in order to mediate viral replication and inflammation during the pre-clinical phase of corneal HSV-1 infection. Next, we will be looking at how CD8⁺ depletion affects the development of HSK lesions (corneal damage) during the clinical phase of corneal HSV-1 infection. The aim of subsequent experiments will be to reveal even more with respect to the mechanism CD8⁺ T cells utilize to mediate virus-induced inflammation in the cornea. Ultimately, our information may aid pharmaceutical research and could lead to improved anti-viral treatments for HSV-1 infections.

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HONORS COLLEGE THESIS MENTOR FORM

Student's Name: **ASHIMA VOHRA**

Major: **BIOCHEMISTRY**

Tentative Title of Research Thesis: **The role of CD8+ T cells during the pre-clinical phase of corneal HSV-1 infection.**

Mentor's Name: **Dr. Susmit Suvas**

Department: **Department of Biological Sciences**

(Mentor: Please sign and date below AFTER reviewing the final draft.)

Comments:

Ashima is one of the most talented undergraduate student who worked in my lab. She has done an excellent job in carrying out her thesis proposal.

By signing below, I acknowledge that I have read and approved this final thesis for submission to the Honors College Council.

Mentor's Signature



Date

02/28/13

Approved

Yes

Date

02/28/13

The Honors College
Oakland University

Thesis Checklist

Name: **ASHIMA VOHRA**

Date: **03/01/2012**

1. Student's thesis form has appropriate signatures and date [**X**]
2. Mentor's form has appropriate signatures and date [**X**]
3. Thesis includes the appropriate cover page [**X**]
4. Thesis includes the mentor's statement [**X**]
5. Thesis proofread closely for mistakes, errors, and typos [**X**]
6. Thesis pages numbered [**X**]
7. Thesis is clear, readable, presentable [**X**]
8. Student has submitted both a hard and electronic copy of thesis [**X**]
9. Student has written a thank-you note to mentor [**X**]