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# The dendritic and T cell response to herpes simplex virus-1 is modulated by dietary vitamin E

**Patricia A. Sheridan*** and **Melinda A. Beck**
*Department of Nutrition, University of North Carolina, Chapel Hill, NC 27599*

## Abstract

Previous studies from our laboratory have shown that dietary α-tocopherol (vitamin EVE) is essential for regulating the cytokine and chemokine response in the brain to herpes simplex virus-1 (HSV-1) infection. The timing of T cell infiltration is critical to the resolution of central nervous system HSV-1 infections. Specifically, the appearance of "neuroprotective" $CD8^+IFN\text{-}\gamma^+$ T cells is crucial. During CNS infection, $CD8^+$ T cell priming and expansion in the draining lymph node, followed by recruitment and expansion occurs in the spleen with subsequent accumulation in the brain. Weanling male BALB/cByJ mice were placed on VE deficient (Def) or adequate (Adq) diets for 4 weeks followed by intranasal infection with HSV-1. VE Def mice had fewer $CD8^+IFN\text{-}\gamma^+$ T cells trafficking to the brain despite increased $CD8^+IFN\text{-}\gamma^+$ T cells and activated dendritic cells in the periphery. VE Def mice had increased T regulatory cells in the periphery and brain and the increase in Tregs decreases $CD8^+$ T cell numbers in the brain. Our results demonstrate that adequate levels of VE are important for trafficking antigen-specific T cells to the brain and dietary VE levels modulate T regulatory and dendritic cells in the periphery.

### Keywords

Neuroimmunology; HSV-1; T cells; Dendritic Cells; Vitamin E

## Introduction

Herpes simplex virus (HSV)-1 is a common human pathogen. It is the primary causative agent of HSV encephalitis (HSE); the most common fatal sporadic encephalitis in humans [1,2]. Ninety percent of all HSE cases are caused by HSV-1 [3]. HSE is a significant problem for the immunosuppressed, including people with HIV and those undergoing chemotherapy [4,5].

Intranasal (i.n.) infection with HSV-1 in mice results in viral entry to the CNS along neuronal pathways of the olfactory and trigeminal nerves[6] and mimics the hypothesized route of human HSE. [1,7].

$CD8^+$ T cells play a critical role in the response to HSV-1. While the entrance of lymphocytes into the brain parenchyma is tightly controlled by an intact blood brain barrier (BBB), insults,



*Address correspondence to: Dr. Patricia A. Sheridan Dept. of Nutrition Schools of Public Health and Medicine 2215 MHRC, CB# 7461 University of North Carolina at Chapel Hill Chapel Hill, NC 27599 Phone: (919) 966-7162 Fax: (919) 843-0776 Email: E-mail: patricia_sheridan@med.unc.edu.

such as a viral infection, allow the BBB to become permeable and circulating lymphocytes can enter the brain parenchyma [8-10]. After HSV-1 infection of the CNS, $CD8^+$ lymphocytes can be found in the brain [7,11-13]. T cells are responsible for controlling viral replication, but are also partially responsible for the neuropathogenesis associated with HSE[14-16]. The timing of T cell infiltration into the brain is critical for controlling the spread of HSV-1 through the brain, but also correlates with the onset and extent of encephalitic symptoms [12]. T cell production of IFN-γ has been demonstrated to be critical for the resolution of both peripheral and central HSV-1 infections [17-20] and inhibits apoptotic neuronal cell death during HSV-1 infection [20]. These studies suggest that while inflammation associated with T cell infiltration can cause encephalitic symptoms, IFN-γ production is important for both clearing the infection and inhibiting neuronal cell death.

T cells circulate through the brain via the vasculature, and monitor the brain at the BBB, however, resident lymphocytes are not a feature of the brain [21]. Antigen or virus travels via the cerebral spinal fluid to the lymph nodes in order to initiate the T cell response [22]. Dendritic cells that cross the endothelium, as well as microglia, may also function as antigen presenting cells at the BBB[23,24]. Intracerebral infection of mice with murine hepatitis virus results in $CD8^+$ T cell priming and expansion in the draining lymph node (DLN), followed by recruitment and expansion in the spleen with subsequent accumulation in the brain[25]. Data from both the ocular and foot pad models of HSV-1 infection demonstrate that activation of T cells occurs in the DLN and that Ag-specific T cells can be found not only at the site of infection, but also in the spleen [19,26]. During i.n. HSV-1 infection, antigen-specific $CD8^+$ T cells are found in the superficial cervical lymph node[27].

In addition to antigen-specific $CD8^+$ T cells, T regulatory cells (Tregs) are induced during HSV-1 infections [28-32] and function to limit antigen specific $CD8^+$T cell responses [32-34]. Originally identified by their role as immune suppressor cells important for maintaining tolerance and limiting autoimmune disease, Tregs are characterized by the co-expression of CD4 and CD25 and the presence of the forkhead/winged helix transcription factor 3 (FoxP3).

The lipid soluble antioxidant vitamin E is found in cellular membranes and prevents lipid peroxidation by scavenging free radicals [35]. VE Def mice are more susceptible to HSV-1 infection, exhibiting greater symptoms of encephalitis and higher viral titers [36]. The production of cytokines, chemokines and adhesion molecules was also significantly increased in HSV-1 infected VE Def mice compared to mice on a VE Adq diet. Studies in both humans and rodents have demonstrated effects of VE supplementation and deficiency on T cells[37, 38]. Therefore, we designed these studies to determine the impact of VE deficiency on the development and trafficking of the antigen specific $CD8^+$ T cell response to HSV-1 and ultimately, their role in clearing HSV-1 in the brain.

VE deficiency resulted in alterations in HSV-specific $CD8^+$ T cells and Tregs. VE deficiency increased the number of activated DC in both the DLN and the spleen following infection. We demonstrate that the increase in Tregs in the periphery inhibits $CD8^+$ T cell migration to the brain. This study suggests that the level of dietary VE can affect the T cell mediated immune response during a primary HSV-1 infection.

## Materials and Methods

### Mice, diets and infection

Weanling BALB/cByJ male mice (Jackson Labs, Bar Harbor, ME) were fed *ad libitum*: 1) VE deficient diet (TD 88163) or 2) VE (dl-α-tocopheryl acetate) adequate diet (Harlan Teklad, Madison, WI). After 4 weeks on the diets, mice were lightly anesthetized with a ketamine

(0.6mg/kg)/xylazine (0.35mg/kg) solution and infected i.n. with $1.5\times10^6$ PFU HSV-1 in 10μL total volume. Mice were housed in the University of North Carolina Animal Facility, an AAALAC accredited facility. Animals were maintained under protocols approved by the Institutional Animal Use and Care Committee.

### HSV-1 virus stocks and inactivation

HSV-1 McIntyre (ATCC, Manassas, VA) stocks were propagated in Vero cells (ATCC), collected, centrifuged, stored at -80°C and inactivated as previously described [36] [39].

### Lymphocyte isolation and flow cytometry

Mice were perfused with 30mL PBS and the brain, cervical lymph nodes and spleen were collected. The tissues were gently dissociated into single cell suspensions with a Seward Model 80 Stomacher (Tekmar, Cincinnati, OH) and filtered through 70μ nylon mesh. For isolation of parenchymal lymphocytes from the brain, cells were pelleted by centrifugation, resuspended in 70% Percoll (Sigma, St. Louis, MO) and overlayed with 35% Percoll [12]. The suspension was then centrifuged for 20 min at 2000×g. The mononuclear cells were collected from the 35%/70% interphase, washed 2X with HBSS, and resuspended in RPMI. All cell suspensions were then counted using a hemocytometer.

Cells ($5\times10^5$ total) were incubated with anti-CD16/32 to block non-specific binding to Fc receptors. The number of $CD8^+$ lymphocytes was determined by incubating $1\times10^6$ cells with a FITC-conjugated anti-CD3 mAb, APC-conjugated anti-CD4 mAB and PerCp-conjugated anti-CD8α mAb (BD Biosciences) following the manufacturer's protocol.

For IFN-γ staining, lymphocytes were incubated for 4 hrs with HSV-infected P815 antigen presenting cells or UV-inactivated HSV-1 at an MOI of 10 PFU/ cell in the presence of Bredfilin A (Golgiplug, BD Biosciences). P815 cells (ATCC) were maintained in RPMI-1640. Surface staining was performed as stated above, cells were fixed, permeabilized and incubated with anti-IFN-γ (PE) antibody (BD Biosciences). Splenocytes from uninfected mice incubated in the same culture conditions served as negative controls for IFN-γ staining. Cells from infected mice were also incubated with uninfected P815 cells as a second control. Because HSV-1 tetramers are not available for BALB/c mice, this method allows us to identify antigen specific IFN-γ producing $CD8^+$ T cells.

DC were identified by their expression of CD11b and CD11c. Activation was assessed using anti- CD40, CD80 and CD86 antibodies (eBiosciences).

For the identification of T regulatory cells, cells were incubated with anti-CD4 and anti-CD25 antibody (BD PharMingen), permeabilized, fixed and incubated with anti-FoxP3 antibody and IL-10 or the appropriate isotype control (eBioscience, San Diego, CA). Cells for IL-10 staining were restimulated *ex vivo* with UV-inactivated HSV and described above. Cells were analyzed using a FACSCalibur (BD Biosciences, MountainView, CA) or Accuri C6 flow cytometer (Ann Arbor, MI) and data analysis was performed using WinMDI 2.9 (Joseph Trotter, Scripps) or CFlow software (Ann Arbor, MI).

### T Cell Depletion

The anti-CD25 antibody (Rat IgG1 Clone PC61) or irrelevant IgG (Rat IgG1 to horseradish peroxidase) (BioXCell, New Lebonan, NH) were administered by i.p. injection 2 days prior to infection and on d 6 p.i. at 0.5 mg/mouse in 200 ul of PBS . To confirm depletion, splenocytes were stained with anti-CD25 clone 7D4, CD4 and FoxP3 Ab (BD Biosciences). For CD8 depletion, anti-CD8α antibody 53-6.7 (eBioscience) or irrelevant IgG were administered by i.p. injection 2 days prior to infection and on d 2, 4 and 6 p.i. at 0.1 mg/mouse in 200 ul total

volume . To confirm depletion, splenocytes were stained with anti-CD8β, CD3, CD4 and CD8α Ab(BD Biosciences).

### HSV-1 Titers

For viral titers from the forebrain and brainstem, DNA was extracted (DNeasy, Qiagen), quantified and HSV-1 genome was determined by qPCR with primers and probe specific for the HSV-1 ICP0 gene as previously described [36]. DNA from uninfected tissue was extracted in parallel and served as a negative control.

### Statistics

Data were analyzed by two-tailed ANOVA. Post-hoc analyses were performed using the Fisher's PLSD post-hoc. Statistical analyses were performed with JMP 6 software (SAS Institute Inc., Cary, NC). Data were considered statistically significant if $P<0.05$.

## Results

### VE Def mice have decreased IFN-γ-producing Ag-specific, CD8+ T cells at d9 p.i.

To determine if VE influences either the trafficking of T cells or the number of antigen specific $CD8^+IFN\text{-}\gamma^+$ T cells, we identified the T cell subsets infiltrating the brain. VE Def mice had significantly fewer $CD8^+$ T cells (Fig. 1A) at d9p.i. Additionally, VE Def mice had significantly fewer $CD8^+IFN\text{-}\gamma^+$ T cells at d9 p.i. (Fig. 1B).

To determine if removal of $CD8^+$ T cells from mice results in increased HSE, VE Adq mice were treated with anti-$CD8^+$ antibody or an irrelevant IgG. At the time of infection the percentage of $CD8\beta^+$ T cells in the spleens of antibody treated mice was reduced from 27.6% (±2.2) to 18% (±1.9%). Continued depletion of the $CD8^+$ T cells was confirmed by the expression of CD8α and CD8β on $CD3^+$ cells (Fig. 2A). At d9 p.i. the percentage of $CD3^+CD8\beta^+$ in both the brain and the spleen were significantly reduced in the antibody treated mice (Fig. 2A and B). Anti-CD8 treated mice have a significant increase in $CD4^+$ T cells in the brain and in the spleen after infection (Fig. 2A and B). The depletion of CD8 T cells results in a trend toward an increase in HSE pathology (Fig 2C).

### VE Def mice have increased DLN and splenic IFN-γ-producing Ag-specific, CD8+ T cells

As the decrease in T cells in the brain of VE Def mice may be a result of a failure of the cells to develop in the periphery, we examined the number of $CD8^+$ T cells in the DLN of the infected mice at d7 and d9 p.i. The VE Def mice had an increase percentage of $CD8^+IFN\text{-}\gamma^+$ T cells at d7 and 9 p.i. and an increased number at d9 compared to VE Adq mice (Fig. 3A and B).

To determine if this pattern was also present in the spleen, the numbers $CD8^+$ T cells (Fig. 3C) and $CD8^+IFN\text{-}\gamma^+$ T cells (Fig. 3D) were examined. Although the total number of $CD8^+$ T cells was not different between the mice on the two diets, at d7 p.i, the number of HSV-1 specific IFN-γ-producing $CD8^+$ T cells was significantly higher in the VE Def compared with VE Adq mice.

### Increased DC numbers and activation in spleen of Def mice

Dendritic cells present antigen to naïve T cells and are required for the expansion of antigen-specific $CD8^+$ T cells[40]. The expression of CD40, CD80 and CD86 indicates the activation of DC and expression of co-stimulatory molecules that are required for optimal T cell activation [41,42]. We examined $CD11c^+CD11b^{int}$ cells in the DLN and spleen of HSV-1 infected mice. Compared to VE Adq mice, VE Def mice had an increase in the percentage of activated DC in the DLN (Fig 4A) and spleen (Fig 4C) as determined by an increase in CD40, CD80 and

CD86 expression (Fig 4E, F, and G). In the spleen the number of activated DC was significantly increased in the Def mice and a trend toward an increase in the DLN was also noted (Fig 4B and D).

### No difference in CCR5 expression on CD8$^+$IFN-γ$^+$ T cells in spleen and DLN of VE Adq and VE Def HSV-1 infected mice

CCR5 is required for T cells to cross the BBB [43]. We hypothesized that CD8$^+$IFN-γ$^+$ T cells in DLN and spleen may have decreased CCR5 in Def mice and therefore inhibit their trafficking to the brain. However, the number of CD8$^+$IFN-γ$^+$CCR5$^+$ cells was increased in the DLN and spleen in VE Def mice (Fig 5A and 5B). Additionally, the mean fluorescence intensity (MFI) for CCR5 on CD8$^+$IFN-γ$^+$ T cells was not different between the groups in the spleen (Fig 5C) or DLN (data not shown).

### Regulatory T cells increased in the spleens of VE Def and VE Adq after HSV-1 infection and are significantly higher in the brains of VE Def mice

It has been hypothesized that Tregs may limit the migration of effector cells from the secondary lymphoid organs to the site of inflammation/infection [44]. We sought to determine if the level of VE altered the numbers of Tregs in the spleen after HSV-1 infection. By d9 p.i., Tregs were significantly higher in percentage and number in the spleen of VE Def compared to VE Adq mice (Fig. 6 A and B). In the brain, the number of Tregs at d9 was significantly higher in VE Def mice (Fig 6C). Following infection, cells that were FoxP3$^+$ also produced IL-10 (Fig 6D). Thus, there was a clear difference in splenic and brain Treg numbers dependent on VE levels.

### PC61 treatment restores Ag-specific CD8$^+$ T cell trafficking to brain of VE def mice

To test the hypothesis that the increase in peripheral Tregs may inhibit T cell trafficking to the brain, we depleted Tregs by treating the VE Def mice with the anti-CD25 Ab PC61. Previous studies in HSV-1 infected VE Adq mice have demonstrated that treatment with PC61 results in an increase in HSV-1 specific CD8$^+$ T cells [28,29] and are more effective at controlling HSV-1 replication [32]. At the time of infection the percentage of CD4$^+$CD25$^+$ cells in the spleen was reduced by PC61 treatment (from 6.9% to 2.1%). In VE Def mice, PC61 treatment abrogated the increase in Tregs in both the spleen (Fig 7A) and DLN (data not shown) following HSV-1 infection. Following PC61 treatment, Tregs were roughly equivalent between the VE Adq and VE Def groups (Fig 7A). At d9 p.i., VE Def mice had 68% fewer CD8$^+$IFN-γ$^+$ T cells (Fig 7B) compared to VE Adq mice. Depletion of Tregs from the VE Def mice by PC61 treatment resulted in a restoration of CD8$^+$IFN-γ$^+$ T cells in the brain.

### Restored CD8$^+$ IFN-γ$^+$ T cell trafficking to brain of VE def mice fails to significantly decrease HSV titer and encephalitic symptoms

At d7 p.i. brain stem and forebrain were removed from VE Adq-IgG, VE Def-IgG and VE Def-PC61-treated mice. In the VE Def mice, restoring CD8$^+$ T cell trafficking by PC61-treatment failed to significantly decrease viral titers (Fig 8A). In addition, VE Def-PC61-treated mice showed similar encephalitic symptoms as the VE Def mice (Fig 8B).

## Discussion

The timing of T cell infiltration is critical to the resolution of CNS HSV-1 infections. The appearance of "neuroprotective" CD8$^+$IFN-γ$^+$ T cells is crucial for the resolution of infection [12,20,27]. In the present study, VE Def mice had significantly fewer brain parenchymal CD8$^+$ T cells, as well as fewer CD8$^+$IFN-γ$^+$ T cells at d9 p.i. Decreased CD8$^+$IFN-γ$^+$ T cells in HSV-1 infected restraint-stressed mice resulted in increased mortality [12,27]. Previous studies from our lab have demonstrated increased HSE symptoms mortality in HSV-1 infected

VE Def mice emphasizing the functional significance of decreased $CD8^+IFN\text{-}\gamma^+$ T cells in the brain[36]. The removal of $CD8^+$ T cells from HSV-1 infected mice by genetic knockout, antibody depletion or the elimination of $CD8^+$ T cell function via lymphotoxin-α or granzyme A knockout results in an increase in the severity of HSV-1 [19,45-47]. In the current study, depletion $CD8^+$ T cells resulted in a trend toward more significant HSE pathology, similar to the VE Def mice. Anti-CD8 treated VE Adq mice had a significant increase in $CD4^+$ T cells that infiltrate the brain. This compensatory increase, which is not seen in VE Def mice (data not shown), may protect the anti-CD8 treated mice from significant mortality [45].

In VE Def mice it appears that $CD8^+$ cells are accumulating in the DLN and spleen and do not traffic to the brain. It is likely that the increase in $CD8^+IFN\text{-}\gamma^+$ T cells in the DLN is due to increased antigen-bearing activated DC as a result of increased viral titers in the brains of VE Def mice [36]. However, it is not clear if the increase in $CD8^+IFN\text{-}\gamma^+$ T cells in the spleen is a result of antigen presentation by activated DC draining larger amounts of virus from the brain, or simply a result of proliferation by $CD8^+IFN\text{-}\gamma^+$ T cells. We were unable to find genomic DNA or mRNA of HSV-1 in the spleen, suggesting that the virus is not actively replicating in the spleen (data not shown). In the ocular model of HSV-1 infection, DLN and spleen have been identified as locations for the proliferation of HSV-1-specific $CD8^+$ T cells [19] and similar to our findings, no replicating virus was found in the spleen. Thus far, HSV-1 antigen from CNS infections has not been identified in the spleen. When fluorescent beads are injected into the cerebral hemisphere of mice, the majority of bead-positive DC are found in the cervical and submandibular LN, however, a small percentage of DC in the spleen are bead positive [48]. These data suggest that HSV-antigen from the brain may be presented in the spleen to induce antigen-specific T cells.

The decrease in $CD8^+$ T cells in the brain of VE Def mice is particularly surprising in light of the increase in $CD8^+IFN\text{-}\gamma^+$ T cells in both the DLN and spleen. Data from our lab indicate that both chemokines and adhesion molecules are increased in the brains of VE Def mice [36], suggesting that the failure of the T cells to traffic to the brain is not due to a lack chemokine and adhesion molecule expression in the brain.

CCR5 promotes T cell trafficking across the blood brain barrier [43]. While CCR5 is increased on T cells in humans following VE supplementation [49], little is known about the effect of VE Def. In the current model, the failure of $CD8^+IFN\text{-}\gamma^+$ T cells to traffic to the brain is not a result of CCR5 down-regulation, as the expression of CCR5 on these cells in both the DLN and spleen was similar between diets.

Tregs function to maintain immunological tolerance as well as reducing pathological immune responses, which may occur with viral infections [34]. Recently, many studies have demonstrated that Tregs are increased during both HSV-1 and HSV-2 infections [28-32]. It is hypothesized that Tregs are upregulated during infection in order to limit damage to tissues by cytotoxic cells [34]. In our study, Tregs were significantly increased in the spleens and brains of VE Def mice compared to VE Adq mice. The role of Tregs in the ocular/stromal keratitis model of HSV-1 has been extensively studied [28,50,51]. Previous studies have demonstrated that VE Adq mice depleted of Tregs prior to HSV-1 infection are more effective at controlling the virus by increasing cytotoxicity and proliferation of $CD8^+$T cells and increasing the frequency of $CD8^+IFN\text{-}\gamma^+$ T cells [32]. However, the induction of Tregs in the periphery and CNS of HSV-1 infection with the i.n. model has not been studied. In the current study, the VE Def mice have a high frequency of Tregs and lower $CD8^+IFN\text{-}\gamma^+$ T cells. However, in the spleens of VE Def mice there was a high frequency of $CD8^+IFN\text{-}\gamma^+$ T cells, while also increasing Tregs in the spleen. These findings seem paradoxical, however, we only measured the numbers of Tregs, not their suppressive capacity. Tregs isolated from HSV-infected rabbit conjunctiva are effective in inhibiting the proliferation of anti-CD3-stimulated $CD8^+$ T cells,

but not $CD8^+$ T cells re-stimulated with HSV[52], while studies in mice of the HSK model have demonstrated that Tregs form the spleen have significant suppressive effect on HSV-peptide stimulated CD 8 T cells[32]. In VE Def mice, it appears that Tregs at the site of infection are functioning differently than those in the spleen. It remains to be determined if Tregs isolated from the site of infection/inflammation are functionally different from those isolated from secondary lymphoid organs.

Inducible Tregs (iTregs) are induced from conventional $CD4^+CD25^-$ cells in the presence of DC-presented antigen and TGF-β in peripheral lymphoid organs[53,54]. The development of natural Tregs (nTregs) vs. IL-2/TGF-β inducible Tregs in HSV infection is unclear[55]. However, Tregs induced *in vitro* from conventional $CD4^+$ T cells are effective in suppressing $CD8^+$ effector cells when transferred prior to HSV-1 infection[28]. VE deficiency increases TGF-β and ROS, both of which have been shown to convert precursor cells to become Tregs [56,57], while Toll-like receptor (TLR) signaling and ROS induce effector T cells [58,59]. We are unaware of other studies which have examined the simultaneous development of $CD8^+$ T cells and Tregs in a VE Def host. It is possible that the environment in the VE Def host alters the homeostatic regulation of Tregs and T cell effectors. DC-Ag-stimulation in VE Def mice in conjunction with TGF-β, TLR and ROS could lead to both increased $CD8^+IFN\text{-}\gamma^+$ T cells and Tregs. Studies addressing this possibility are underway in our lab.

Removal of Tregs by PC61 treatment restored $CD8^+IFN\text{-}\gamma^+$ T cells in the brain. These data suggest that Tregs are acting either to inhibit $CD8^+IFN\text{-}\gamma^+$ T cell function in the brain or limit their ability to traffic to the brain. We are unaware of any studies that demonstrate Treg cell ability to inhibit $CD8^+$ T cell trafficking during a viral infection. However, several studies have demonstrated that Treg cells can inhibit the migration of $CD4^+$ T cells to the antigen containing tissue [44,60,61].

The restoration of $CD8^+IFN\text{-}\gamma^+$ T cells trafficking to the brains of VE Def mice failed to significantly decrease viral titers and encephalitic symptoms as an increase in the number of $CD8^+IFN\text{-}\gamma^+$ T cells did not occur until d9 p.i.. At 7 d p.i the brains of BALB/c mice infected i.n. with HSV-1 have significant increases in 8-isoprostane, DNA damage and nitric oxide mRNA.[62]. In addition, VE deficiency alone results in the death of neurons[63] and amplifies neurotoxicity in the brain[64]. Studies are underway in the lab to address earlier events in the brains of VE Def mice related to oxidative stress and microglia activation which may contribute more directly to HSE symptoms and mortality.

A second possibility suggests that the inability of VE Def mice to control HSV-1 infection is more complex than the failure of $CD8^+IFN\text{-}\gamma^+$ T cells to traffic to the brain. T cell activation, proliferation and function, including the production of perforin and granzyme, are important for clearing viral infections from neurons [47,65].

In summary, VE deficiency changes the dynamics of the development of the antigen-specific $CD8^+$ T cell response both in the periphery and the CNS via a combination of dendritic cell activation in the periphery and development of T regulatory cells.

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## Abbreviations

Adq, adequate
Ag, antigen

APC, antigen presenting cells
DC, dendritic cells
Def, deficient
DLN, draining lymph node
FoxP3, forkhead/winged helix transcription factor 3
HSE, herpes simplex virus encephalitis
HSV-1, herpes simplex virus
IFN, interferon
i.n., intranasal
iTregs, inducible Tregs
p.i., post infection
α-TOC, α-tocopherol
Tregs, regulatory T cells
VE, vitamin E

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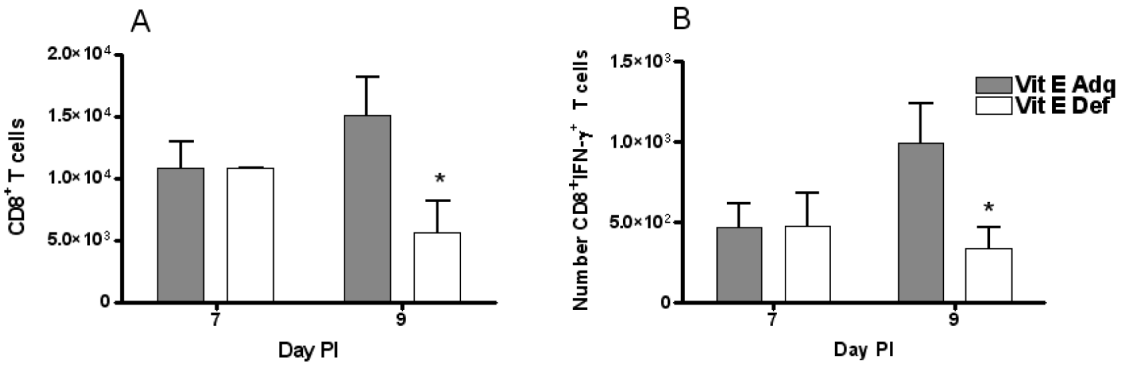


**Figure 1.**

VE Def mice have decreased $CD8^+IFN\text{-}\gamma^+$ T cells at d9 p.i. Mice were infected i.n. with HSV-1, sacrificed and perfused at d7 and 9p.i. Brain mononuclear cells were recovered and incubated with APCs as described in the Methods. Number of A) $CD8^+$ T cells and B) $CD8^+IFN\text{-}\gamma^+$ T cells. Data are the mean +/- SEM of n=6 mice/group/day. *$p<0.05$ compared to VE Adq mice at that time point.

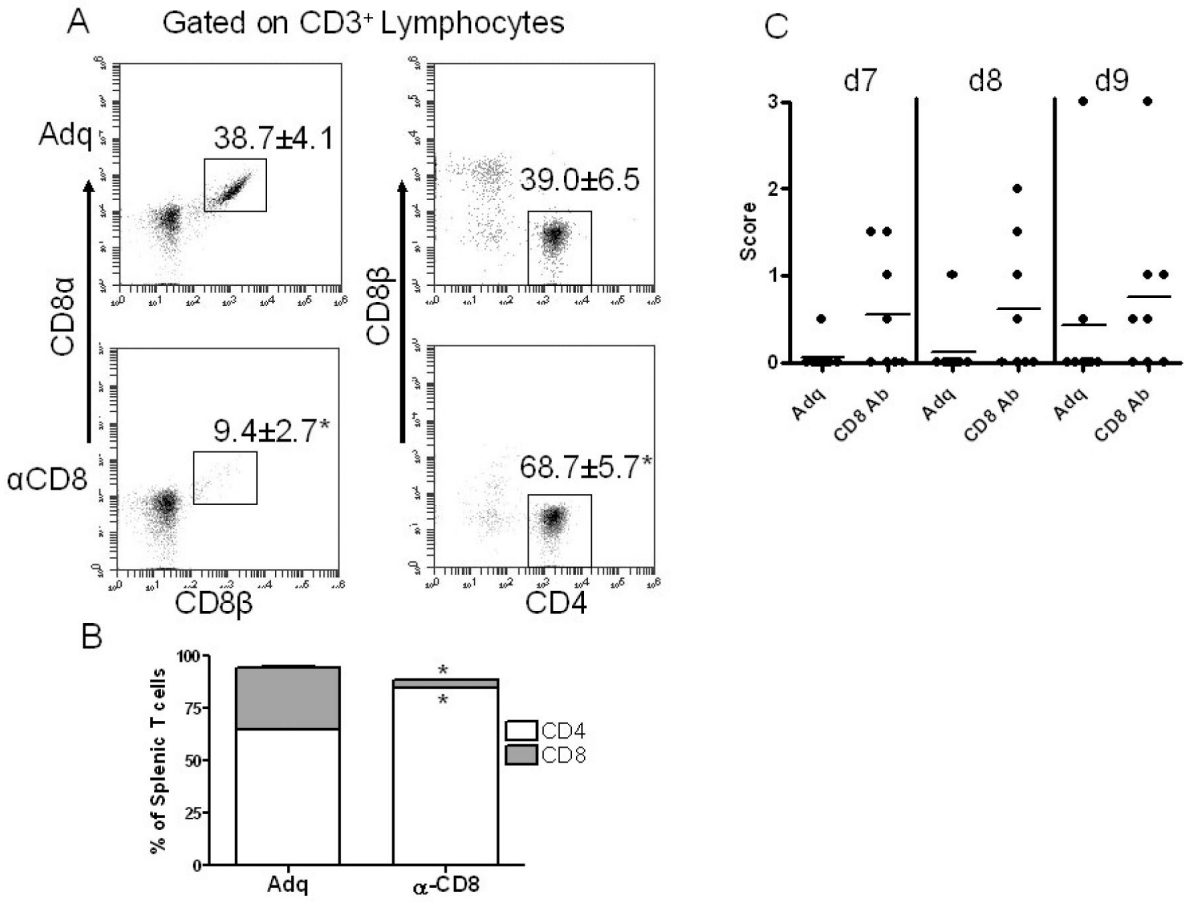


**Figure 2.**

Treatment with anti-CD8 antibody increases CD4 T cell infiltrate and symptoms of HSE pathology. Mice were treated with either α-CD8 antibody 53-6.7 or IgG as described in the methods. A) Representative density plots from the brains of IgG-treated or 53-6.7 Ab-treated mice and the mean percentage of $CD8\alpha^{+}CD8\beta^{+}$ T cells and $CD4^{+}CD8\beta^{-}$ T cells at d9 p.i. B) Percentage of $CD4^{+}$ and $CD8^{+}$ T cells in the spleens of VE Adq and α-CD8-treated mice at d9 p.i. C) HSE pathology scores. Data are the mean +/- SEM of n=8 mice/group. *$p<0.05$ compared to VE Adq mice.

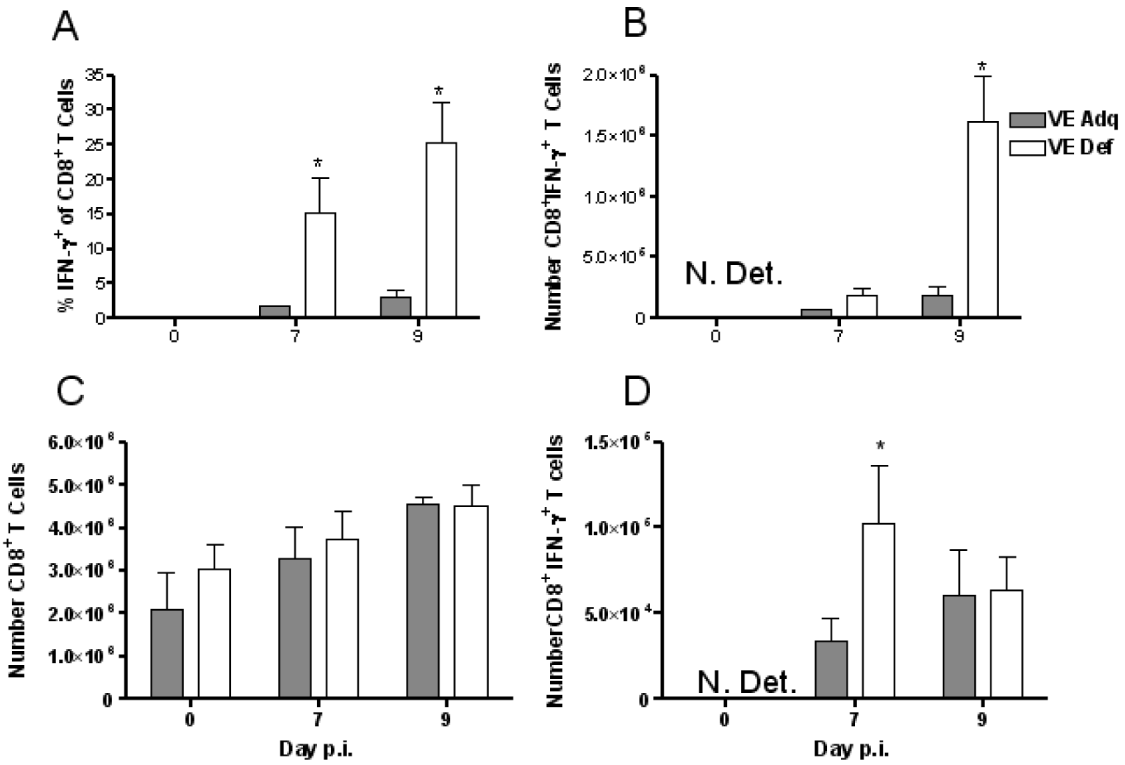


**Figure 3.**

VE deficiency increases the number of $CD8^+$T cells in DLN and spleen of HSV-1 infected mice. At d 7 and 9 p.i. cells from DLN and spleen were incubated with APCs as described in the Methods. A) Percentage of $CD8^+$ T cells producing IFN-γ in the DLN. B) Number of $CD8^+IFN\text{-}\gamma^+$ T cells in the DLN. C) Number of $CD8^+$ T cell in the spleen. D) Number of $CD8^+IFN\text{-}\gamma^+$ T cells in the spleen. Data are the mean +/- SEM of n=6 mice/group/day. *$p<0.05$ compared to VE Adq mice at that time point. N.Det.-Not detected.

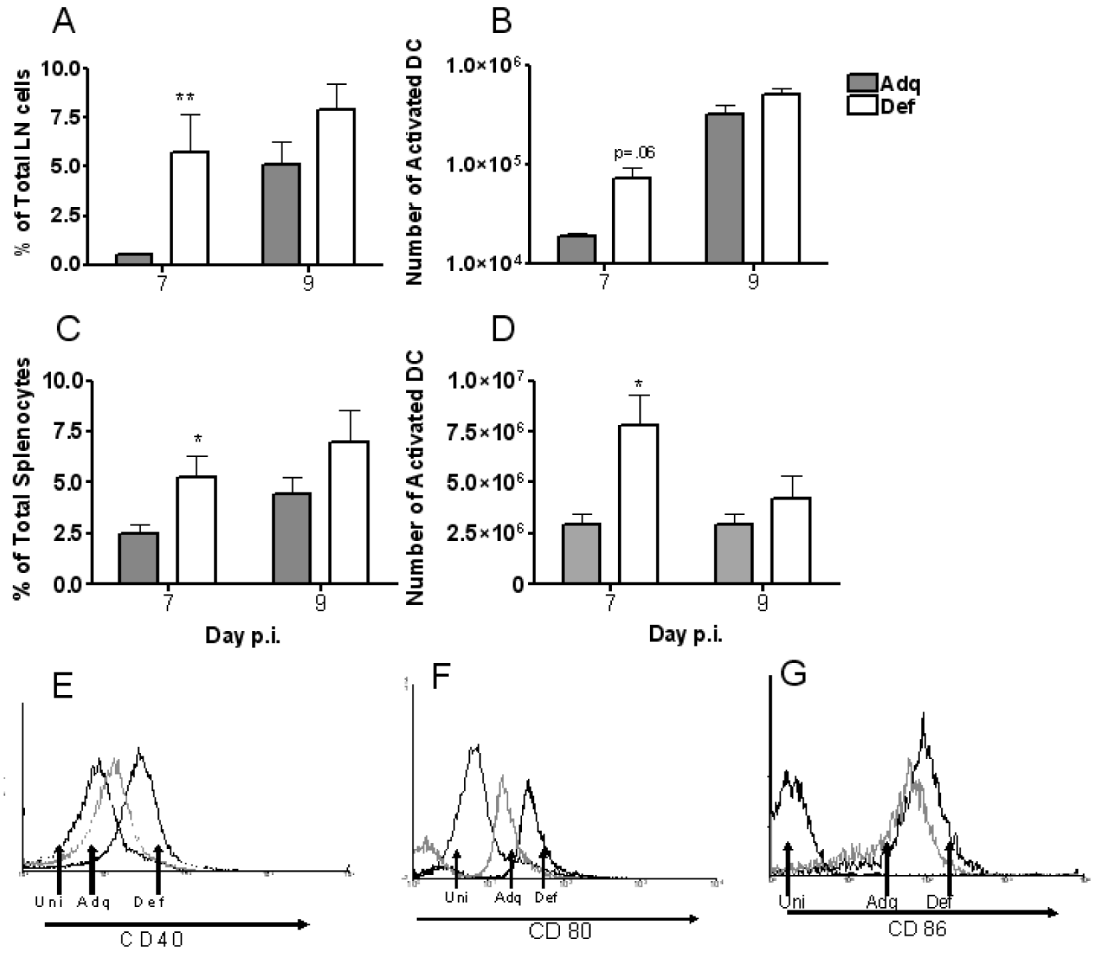


**Figure 4.**

Increased percentage and number of activated DC in DLN and spleen of VE Def mice. Dendritic cells were identified by their expression of CD11c and CD11b. A and C) Percentage of total cells that are activated DC in the DLN and spleen respectively. B and D) Number of activated DC in DLN and spleen. Data are the mean +/- SEM of n=6 mice/group/day. **, $p<0.01$ and *$p<0.05$ compared to VE Adq mice at that time point. E, F, G) Representative histograms for CD40, CD80 and CD86 on DC in the spleen of uninfected VE Adq mice, VE Adq and Def mice at d7 p.i.

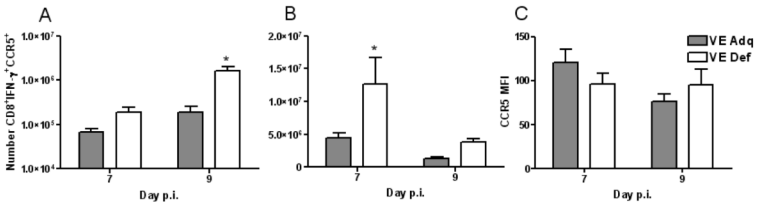


**Figure 5.**

CCR5 is not decreased on $CD8^+IFN\text{-}\gamma^+$ T cells in the DLN or spleen of VE Def mice. At d 7 and 9 p.i. DLN and spleen were removed and incubated with UV-HSV as described in the methods. A) Number of $CD8^+$ $IFN\text{-}\gamma^+$ $CCR5^+$ T cells in the DLN. B) Number of $CD8^+IFN\text{-}\gamma^+CCR5^+$ T cells in the spleen. C) MFI of CCR5 expression on $CD8^+IFN\text{-}\gamma^+$ T cells from the spleen. Data are the mean +/- SEM of n=6 mice/group/day. *$p<0.05$ compared to VE Adq mice at that time point.

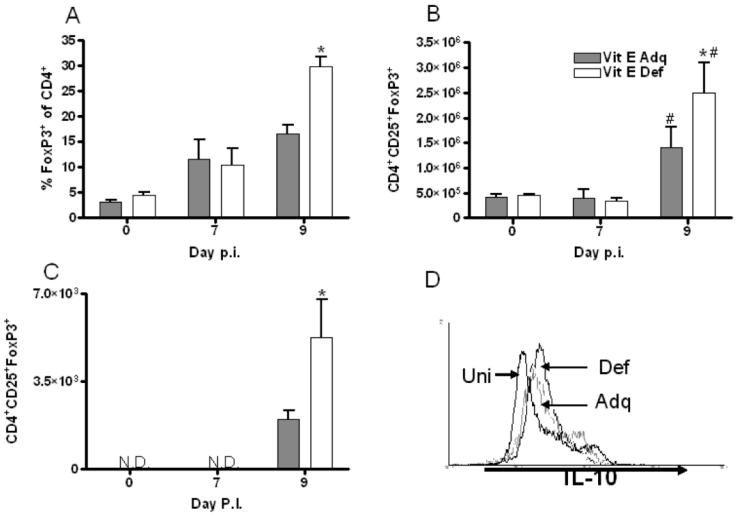


**Figure 6.**

T regulatory cells ($CD4^+CD25^+FoxP3^+$) are increased in the spleen following HSV-1 infection and are augmented by VE deficiency. A) Percentage of $CD4^+$ T cells that express FoxP3 in the spleen, B) Number of Tregs in the spleen at d0, 7, and 9 p.i. C) Number of Tregs in the brain at d9 p.i. Data are the mean +/- SEM of n=6-7 mice/group/day. *$p<0.05$ compared to VE Adq mice at that time point, #$p<0.05$ compared to uninfected control. D) Representative histogram of IL-10 by intracellular staining in of $CD4^+CD25^+FoxP3^+$ spleen cells stimulated ex vivo with UV-HSV from d0 (uninfected), VE Adq and VE Def mice at d9 p.i.

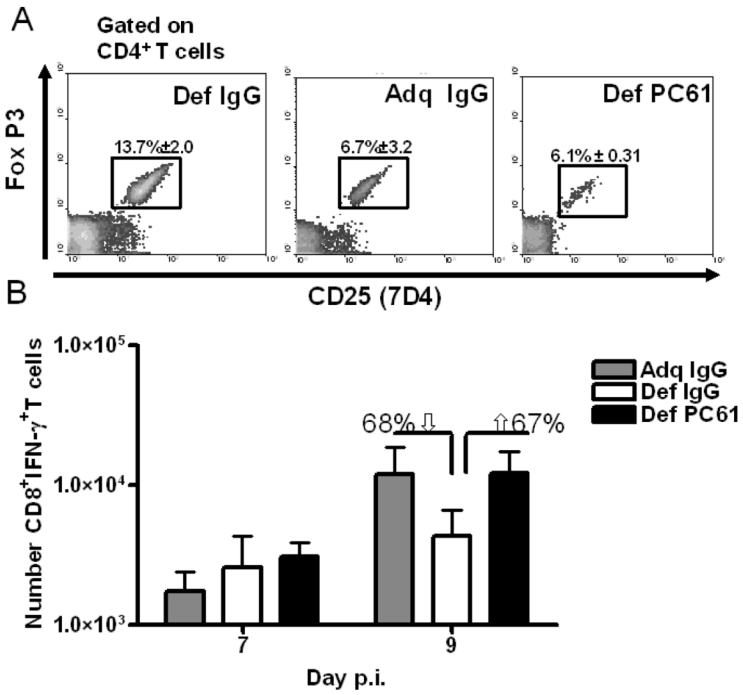


**Figure 7.**

Treatment with PC61 eliminates up-regulation of Tregs and restores $CD8^+IFN\text{-}\gamma^+$ T cell trafficking to brain of VE Def mice. Mice were treated with either PC61 or IgG as described in the methods. For IFN-γ staining, splenocytes were restimulated with UV-HSV as described in the methods. A) Representative density plots from the spleens of IgG-treated VE Def and VE Adq mice and PC61-treated VE Def mice. B) $CD8^+IFN\text{-}\gamma^+$ T cells from brain. Data are the mean +/- SEM of n=5 mice/group/day.

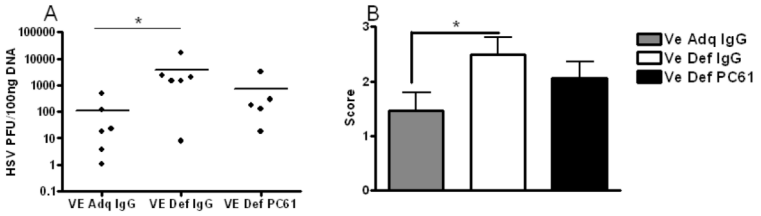


**Figure 8.**

Restored $CD8^{+}IFN\text{-}\gamma^{+}$ T cell trafficking to brain of VE Def mice does not decrease viral titer and encephalitic symptoms. A) HSV DNA in the brain stem and forebrain of VE Def-IgG, VE Adq-IgG and VE Def-PC61 treated mice. B) Encephalitis symptom score for these mice at d9 p.i. Data are from 5-6 mice/group for the HSV DNA and n=8 mice/group for HSE score.