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Homeostatic expansion of CD4⁺ T cells upregulates VLA-4 and exacerbates HSV induced corneal immunopathology

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Abstract

Using a viral induced immunopathology model, we showed that when CD4⁺ T cells were allowed to undergo homeostatic expansion prior to ocular HSV infection, mice developed more severe inflammatory lesions with the increased severity associated with enhanced effector function of ocular CD4⁺ T cells and blocking their functional activity reduced the lesion severity. Additionally, homeostatically expanded CD4⁺ T cells upregulated VLA-4, and in vivo administration of anti-VLA-4 mAb significantly decreased the homeostatic proliferation. Furthermore, blocking of VLA-4 interaction also diminished the infiltration of CD4⁺ T cells into the cornea and decreased lesion severity. Our results imply that homeostatic expansion of T cells, as could occur in a virus induced lymphopenia, may generate cells with enhanced effector function that can contribute to tissue damage.

Keywords

Inflammation; viral; T lymphocytes; adhesion molecules

In healthy animals, the peripheral lymphocyte pool size remains quite constant. Conditions of lymphopenia usually induce lymphocytes to proliferate so as to restore the pool size [1]. This event, referred to as homeostatic proliferation, involves naïve and memory lymphocytes and various factors such as self peptide reactivity [2,3], clonal competition [4,5] and different cytokines regulate their cell division [6–8]. These homeostatically dividing cells express many activation markers characteristic of memory cells and are functionally active [9,10]. Recently, it has been argued that lymphopenia may result in the homeostatic expansion of autoreactive T cells that mediate some autoimmune diseases [11,12]. In addition, T cell homeostatic expansion (HE) may also result in the enhanced anti-tumor activity of CD8⁺ T cells [13]. Several viral infections induce a temporary or long term lymphopenia [14–19]. It is not clear what role, if any, the vigorous compensatory lymphocyte expansion that may follow plays in anti-viral immunity and pathology. Certain reports claim that these events may explain the association of some viruses with autoimmunity [12]. However, autoimmune disease expression

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is a very rare event after any human virus infection and no single human virus, whether causing lymphopenia or not, has regularly been associated with human autoimmunity [20]. A common event, however, during recovery from many virus infections is collateral tissue damage caused by activated T cells [20]. In some locations, such as the brain, lung or the eye, this can result in significant morbidity. It is not clear if the T cells involved in the tissue damage represent viral antigen specific, homeostatically expanded autoantigen specific, or nonspecific cells that react with neither viral nor autoantigens.

Herpes simplex virus -1 (HSV-1) infection of the mouse cornea can lead to the development of an immunopathological lesion, termed herpetic stromal keratitis (HSK). The pathogenesis of HSK, as studied in the mouse, involves numerous components, but CD4⁺ T cells appear to be the principal orchestrators of the immunopathology [21]. One of the possible ways by which CD4⁺ T cells can get activated in the eye involves bystander activation of T cells after ocular HSV-1 infection [22]. Once activated, CD4⁺ T cells secrete Th1 specific cytokines that play an important role in orchestrating immunoinflammatory reaction in the cornea [23]. In the present communication, we determined that homeostatically expanded CD4⁺ T cells causes more tissue damage to virus infected cornea when compared to infected control animals. Our results demonstrated that homeostatic expansion of CD4⁺ T cells enhanced their effector functions that lead to increased corneal lesion severity. Interestingly, homeostatically expanded transgenic T cells, not demonstrably reactive with either viral or autoantigens, significantly contributed to lesion expression but they were less pathogenic when unable to secrete IFN- γ . Additionally, cells undergoing homeostatic proliferation upregulated VLA-4 and in adoptive transfer experiments blocking VLA-4 with specific anti-sera significantly decreased the ocular infiltration of CD4⁺ T cells and the consequent severity of ocular lesions. Our results support the idea that tissue damage evident during recovery from viral infections may be the consequence of homeostatically expanded cells which in the stromal keratitis (SK) model can be mediated by cells of non-viral antigen specificity. Whether such a mechanism occurs as a result of homeostatic expansion in natural infection models that cause lymphopenia requires further evaluation.

Materials and Methods

Mice and virus

Female 6 to 8-wk-old BALB/c SCID (H-2^d), mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and DO11.10 (OVA-TCR Tg mice), DO11.10 IFN- γ $-/-$ mice were housed and bred in the animal facilities at the University of Tennessee. The DO11.10 mice were kindly provided by C. Weaver (University of Alabama, Birmingham, AL). Approximately 70% of CD4⁺ T cells in the DO11.10 mice were KJ1.26.1 (anti-OVA-TCR Ab) positive. SCIDS were kept in our special pathogen free facility. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE was propagated and titrated on Vero cells (ATCC CCL81) using standard protocols. The virus was stored in aliquots at -80°C until use.

T cell purification, adoptive transfer and virus infection

DO11.10 CD4⁺ T cells were purified by positive selection with CD4 microbeads as per manufacturer's protocol (Miltenyi Biotec, Auburn, CA). The purified CD4⁺ T cells were incubated with anti-CD25 mAb (10 μg /10⁸ cells) at 4 $^{\circ}\text{C}$ for 30 minutes. This was followed by the addition of baby rabbit complement (BRC) into the cell culture at 1:200 dilution and cells were kept at 37 $^{\circ}\text{C}$ for 45 minutes (Cedarlane laboratories). Next, CD4⁺CD25⁻ T cells obtained after BRC killing were labeled with 2.5 μm CFSE (Molecular probes, Eugene, OR) as reported by others [24] and suspended in PBS for injection. Each mouse received 10⁷ CFSE labeled

CD4⁺ T cells via lateral tail vein injection. In test group of animals, CD4⁺ T cells underwent homeostatic expansion for 7 days prior to ocular HSV infection while control group of animals received CD4⁺ T cells 24 h before ocular infection. To enumerate the infiltration of CD4⁺ T cells into the diseased corneas, mice from both test and control groups were terminated at different time-points post infection and liberase digested corneal samples were stained for CD4⁺ T cells by flow cytometry. When indicated, some mice received intra-peritoneal ammonium sulfate cut anti-VLA-4 mAb (1mg/mouse) post adoptive transfer. In certain experiments, role of OVA-specific T cells in determining the corneal lesion severity after homeostatic expansion was ascertained in SCID recipients. Since, approximately 70 % of CD4⁺ T cells in the DO11.10 mice are KJ1.26.1 (anti-OVA-TCR Ab) positive, CD4⁺KJ⁺ T cells purified from DO11.10 IFN- γ ^{-/-} mice were mixed with CD4⁺KJ⁻ T cells from DO11.10 mice in 70 : 30 ratio (7×10^6 : 3×10^6) and adoptively transferred into BALB/c SCID. SCID mice were ocularly infected with HSV-1 RE at a dose of 10^4 PFU/eye 7 days post-adoptive transfer and clinical scoring of the eyes was done as described earlier [22].

Flow cytometric analysis

Cell preparation—Single-cell suspensions were prepared from corneas, draining lymph nodes (Cervical and submandibular) and spleen of recipient mice at different time points post-infection. Corneas were digested with Liberase (Roche Diagnostics). Briefly, a small incision was made at the junction of the limbus and the corneal cap was carefully removed. The pooled corneas were incubated with 60 U/ml Liberase for 60 min at 37°C in a humidified atmosphere of 5% CO₂. After incubation, the corneas were disrupted by grinding with a syringe plunger on a stainless steel mesh and a single-cell suspension was made in complete RPMI 1640 medium. Cells were counted with trypan blue exclusion with high viability.

Staining for flow cytometry—The single-cell suspension obtained from draining lymph nodes (DLN) and corneal samples were stained for cell surface CD4 molecules by FACS. Briefly, a total of 1×10^6 cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min. in FACS buffer followed by addition of FITC-labeled anti-CD4 mAb for an additional 45 min. For intracellular staining, surface-labeled cells were permeabilized with permeabilization buffer (BD Biosciences, Mountain View, CA) and PE-labeled anti-IFN- γ mAb was added. Finally, the cells were washed three times and samples were acquired on a FACScan (BD Biosciences). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

Reagents and intracellular detection of IFN- γ —CRL 1911 (anti-VLA-4) hybridoma was purchased from American Type Culture Collection (Manassas, VA) and antibody was grown in tissue culture roller bottles. For all injections, an ammonium sulfate cut of VLA-4 mAb (1.0 mg/mouse) was used. Cells obtained from recipients were stained with different antibodies at 4°C and the antibodies purchased from BD Pharmingen (San Diego, CA) were PE labeled CD49d, $\alpha 4\beta 7$, CD103 and IFN- γ . For Intracellular IFN- γ staining, X-ray irradiated DO11.10 T depleted splenocytes (TdSPC) pulsed with OVA_{323–339} were used as stimulators for KJ⁺CD4⁺ T cells while for polyclonal activation tissue culture plates were coated with 1.0 μ g/ml of anti-CD3 ϵ mAb (145-2C11; BD Pharmingen) or were left untreated. Liberase (200 U/ml) digested total corneal cells were cultured in the respective plates for 18h in RPMI 1640 +10% FBS and Brefeldin A was added at a concentration of 1 μ l/well for the last 6h of culture. For intracellular detection of IFN- γ , the intracellular staining kit from BD Pharmingen (San Diego, CA) was used.

Cytokine ELISA—Single cell suspension obtained after liberase digestion of 4 corneal samples on day 15 post-infection from both control and test group of animals were either unstimulated or stimulated with plate bound anti-CD3 mAb (1.0 μ g/ml) or OVA_{323–339} peptide

(10.0 µg/ml) for 48 h in complete RPMI medium. The culture supernatants from the cell cultures were screened for the presence of IFN-γ. Briefly, ELISA plate was coated with capture anti-IFN-γ mAb (2.0 µg/ml) in 0.1 M Na₂HPO₄ (pH 9.0) and incubated at 4°C overnight. The plate was washed with PBS-Tween 20 and blocked with 3% BSA for 2 h at room temperature. After washing, serially diluted samples and standards were added to the plate and incubated at 4°C overnight. The plate was washed followed by the addition of anti-IFN-γ biotin labeled detection Ab for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was added at 1/1000 dilutions. The color was developed by adding the substrate (ABTS) solution (Sigma-Aldrich, St. Louis, MO) and the concentration was calculated with an automated ELISA reader (SpectraMAX 340; Molecular Devices, Sunnyvale, CA).

Statistical analysis

All analysis for statistically significant differences were performed with a student's paired t test. $p < 0.01$ were considered statistically significant. Results are expressed as mean $\pm$ S.D.

Results

Homeostatic CD4⁺ T cell expansion enhances their effector activity and increases the lesion severity caused by ocular HSV infection

Homeostatically expanded cells have previously been shown to actively participate in autoimmune, tumor specific and transplantation responses [12,13,25]. To characterize the role of such cells in virus induced immunopathology, a model of HSV induced corneal lesions was used [20]. As shown in Figure 1a, adoptively transferred CFSE labeled CD4⁺ T cells underwent several rounds of divisions in the SCID mice and a significantly ($p=0.000337$) more severe lesions were observed in the mice where adoptively transferred cells underwent homeostatic expansion prior to ocular infection (test group) (Figure 1b). Ocular CD4⁺ T cells recovered at day 15 post-infection from homeostatically expanded (test) and control group of animals were determined for their functional activity. As is evident in Figure 1c, restimulation in vitro, either with anti-CD3 mAb (1.0 µg/ml) or OVA₃₂₃₋₃₃₉ (10.0 µg/ml) for 18 h in the presence of IL-2, revealed significant levels of intracellular IFN-γ protein. However, no IFN-γ production was observed in the absence of T cell stimulus (Fig. 1c). Furthermore, by day 15 post-infection majority of ocular CD4⁺ T cells in both groups had lost their CFSE, but about two fold more CD4⁺ T cells in the homeostatically expanded (test) group secreted IFN-γ both in response to polyclonal or antigen specific T cell stimulation compared to the control animals ($p=0.000318$) as determined by flow cytometry. In addition, polyclonal as well as antigen specific stimulus of corneal CD4⁺ T cells lead to higher production of IFN-γ (Figure 2) from the test group of animals as measured by ELISA. Such results indicated that homeostatic expansion of CD4⁺ T cells prior to virus infection enhanced their effector function and resulted into more tissue damage (Figure 2b). However, enhanced corneal lesion severity observed in test group of animals could also be the effect of stronger influx of CD4⁺ T cells into the diseased cornea when compared with control group of animals. As is evident in Figure 2c and Table 1, no statistically significant differences ($p > 0.05$) in the number of infiltrating CD4⁺ T cells into virus infected corneas were obtained, when compared between test and control group of animals at different time-points post-infection.

Homeostatically expanded CD4⁺KJ⁺ T cells actively participate in HSV induced corneal immunopathology and enhance lesion severity

CD4⁺ T cells that induce HSK lesions are predominantly of Th1 phenotype and might not be viral antigen specific [23,26]. Thus, experiments were designed to observe if homeostatically expanded IFN-γ secreting non-viral antigen specific (Ova-specific) T cells significantly contribute to tissue damage in corneal lesions. As is evident in Figure 3a, mice in group A, given a mixture of KJ⁺ cells (Ova-specific) from DO11.10 IFN-γ^{-/-} mice and KJ⁻ T cells

(non-Ova-specific), at all measured time-points had significantly less ($p=0.00016$) clinical scoring of SK lesion severity when compared with group B animals that received a KJ⁺KJ⁻ T cell mixture from wild type DO11.10 animals. Cells isolated from liberase digested corneas were assessed for any functional differences in CD4⁺ T cells. A similar level of CD4⁺ T cell infiltration was observed in the corneal samples of both the groups on day 15 p.i. as measured by flow cytometry (Fig. 3b). However, in group A, very few CD4⁺ T cells present in the Liberase digested corneal samples expressed intracellular IFN- γ , when stimulated in vitro with T-depleted splenocytes pulsed with OVA₃₂₃₋₃₃₉ peptide (Fig. 3b). In contrast, in group B about 68% of ocular CD4⁺ T cells expressed IFN- γ when stimulated in vitro with OVA₃₂₃₋₃₃₉ peptide. Interestingly, a substantial number of ocular KJ⁺ T cells in group B expressed IFN- γ even in the absence of in vitro OVA₃₂₃₋₃₃₉ stimulation (data not shown). The small amount of OVA₃₂₃₋₃₃₉ induced IFN- γ expressing CD4⁺ T cells in group A could probably have come from contaminating KJ⁺ cell population while purifying KJ⁻CD4⁺ T cells from the wild type DO11.10 mice. Since both groups of animals received equal number of adoptively transferred KJ⁺ and KJ⁻ cells and were equally allowed to undergo HE, decreased intensity of SK lesion severity measured in group A animals was attributed to the inability of ocular KJ⁺ cells (Ova-specific) to secrete IFN- γ . Thus, our results emphasized that homeostatically expanded non-viral antigen specific functional CD4⁺ T cells induced significant corneal tissue damage after ocular HSV infection.

Homeostatically expanded CD4⁺ T cell upregulates $\alpha 4\beta 1$ (VLA-4) with each division and in vivo blocking of VLA-4 interaction decreases lesion severity

VLA-4 was recently shown to be a key integrin molecule expressed by ocular CD4⁺ T cells involved in SK lesions [27]. Thus, expression of VLA-4 as a result of homeostatic expansion was determined on CD4⁺ T cells. As is evident in Figure 4a CFSE labeled CD4⁺ T cells upregulated $\alpha 4\beta 1$ (VLA-4) integrin with each successive division as a result of homeostatic proliferation and blocking of VLA-4 interactions while using anti-VLA-4 mAb significantly decreased the homeostatic expansion of CFSE labeled CD4⁺ T cells (Fig. 4b) when compared to the Rat Ig treated control group. In addition, anti-VLA-4 mAb treated group had higher number of undivided CFSE^{hi} CD4⁺ T cells both in the DLN and spleen when compared to Rat Ig treated group (Figure 4c). Since, homeostatic expansion upregulate VLA-4, its role in modulating the SK lesion severity was determined. As shown in Fig. 5a, blocking of VLA-4 interaction significantly reduced ($p=0.0038$) the percentage of CD4⁺ T cells in liberase digested ocular cells isolated 15 days p.i. In addition, SK lesion severity in VLA-4 treated animals were significantly decreased ($p=0.000325$) compared to the Rat Ig treated control group (Fig. 5b). We may conclude from our results that VLA-4 interactions not only regulate homeostatic proliferation but could also be an important homing molecule on CD4⁺ T cells that enter into the cornea and blocking their interactions decreased SK lesion severity.

Discussion

This report demonstrates that homeostatic expansion of CD4⁺ T cells increased their effector function and aggravated lesion severity induced by ocular HSV infection. Using a mouse model of herpetic stromal keratitis, we showed that when cells were allowed to undergo homeostatic expansion prior to ocular infection, animals developed more severe inflammatory lesions with the increased lesion severity associated with higher levels of CD4⁺ T cell effector function in the cornea. Our results also showed that proliferating CD4⁺ T cells in the lymphopenic environment upregulated VLA-4 and that in vivo administration of anti-VLA-4 mAb significantly decreased homeostatic expansion. Furthermore, in vivo blocking of VLA-4 interaction diminished the infiltration of CD4⁺ T cells into the cornea and decreased the lesion severity. Interestingly, in this model the major proportion of T cells involved in the tissue

damage were homeostatically expanded T cells that react with neither viral nor corneal autoantigens and blocking their functional activity reduced the severity of SK lesions.

Recent reports have documented that when T cells undergo extensive proliferation under conditions of lymphopenia they may elicit autoimmunity, effective anti-tumor immune response or show dominant resistance to tolerance [12,13,25]. Infection by several viral pathogens may result in temporary or long term lymphopenia. Examples in human include HIV, measles and influenza virus [15–18]. It is conceivable that when lymphocyte numbers rebound, as a result of homeostatic proliferation some may be involved in tissue damage as recently hypothesized by the Savernick group [12]. Our observations support this hypothesis, although in our model the level of lymphopenia was complete exceeding that which usually follows acute virus infection. In addition, in the model we used, the ocular lesions set off by HSV infection were shown to be mediated by bystander CD4⁺ T cells that appear not to cross-react with viral or host autoantigens [22,28]. Our results clearly show that homeostatic expansion of CD4⁺ T cells prior to ocular infection aggravated the lesion severity and the expanded population of cells showed enhanced polyclonal as well as OVA_{323–339} peptide specific induced cytokine responses. However, an important question is whether T cells undergoing homeostatic proliferation and actively participating in an effector immune response are predominantly self or foreign antigen specific.

Homeostatic proliferation is governed by several different factors [29] and one of them is TCR interaction with self-peptide MHC complex [2]. Although, several different groups have shown that homeostatic expansion of both CD4⁺ and CD8⁺ T cells is inhibited in conditions when TCR engagement with relevant self-peptide MHC complex is prevented, some reports argue that homeostatic proliferation can also occur even in the absence of MHC ligands [30]. In fact, a recent report showed that under lymphopenic conditions alloantigen specific T cells undergo homeostatic expansion and become a barrier to transplantation tolerance [25]. In this report, we have demonstrated that after homeostatic expansion, a major proportion of CD4⁺ T cells involved in the corneal tissue damage are homeostatically expanded OVA-specific T cells that react with neither viral nor corneal autoantigens and inhibiting the IFN- γ secretion from these cells significantly reduced the stromal keratitis. IFN- γ expression was shown previously to be involved in SK lesion severity [23,26]. However, we can also not rule out the active participation of homeostatically expanded CD4⁺KJ⁻ T cells in influencing the lesion severity.

An additional reason that enhanced lesion severity occurred after homeostatic expansion could be a change in the expression of homing receptors on expanded T cells. In fact, homeostatic expansion was reported to cause significant changes in the expression level of certain homing and migratory receptors on T cells [1,9]. As a result, expressing cells could migrate to non-lymphoid tissue. $\alpha 4\beta 1$ is an integrin that is involved in the transendothelial migration of T cells under inflammatory conditions [31,32]. Previous reports have shown emphatically that the selectin CD44 is upregulated as a result of homeostatic expansion of T cells [10]. Our result shows that VLA-4, an integrin, is also upregulated as a result of homeostatic expansion. Interestingly, it has been shown recently that CD44 associates with VLA-4 on the plasma membrane of immune cells and possibly the combination of CD44-hyaluronic acid and VLA-4-VCAM-1 interaction can act as a supercode for entry of effector T lymphocytes into the sites of inflammation [33]. In fact, our results demonstrated that in vivo blocking of VLA-4 interactions by using anti-VLA-4 mAb not only significantly decreased the infiltration of effector CD4⁺ T cells into the cornea, but also diminished lesion severity. We also demonstrated that in vivo blocking of VLA-4 interaction retarded, but did not completely inhibited, the homeostatic expansion of CD4⁺ T cells. This could be either because of lack of sufficient antibody to completely block the VLA-4 interactions or the involvement of several additional factors in regulating the homeostatic expansion, such as IL-7 [34]. In fact, IL-7 can also induce and activate the VLA-4 molecule on T cells and thymocytes [35,36] and VLA-4

interactions with its ligands could be one of the mechanisms by which IL-7 controls the homeostatic expansion of naïve CD4⁺ T cells.

Finally, the importance of homeostatic expansion should be tested in natural viral infections that cause lymphopenia, since the process could be one mechanism by which tissue damage is caused during clearance of infection. The model used in the present study was contrived in using transgenic T cells transferred into a completely lymphopenic animal. Moreover, this system may over-emphasize the role of bystander participants in viral induced tissue damage. We are currently evaluating if more modest levels of lymphopenia induced in immunocompetent mice can also result in active participation of homeostatically expanded cells in tissue damage.

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Figure 1. Homeostatic expansion of CD4⁺ T cells aggravated stromal keratitis (SK) lesion severity
a, CFSE labeled splenic CD4⁺ T cells obtained 7 days post-adoptive transfer from a SCID recipient. **b**, Lesion severity was measured at different time-intervals in both test as well as the control group. Each dot represents an individual BALB/c SCID eye and the horizontal line indicates the mean clinical score for each group. Each group of mice consisted of ten animals and the results shown are the representative of three similar experiments. *, $p < 0.0001$ **c**, Liberase digested total ocular cells isolated from both groups on day 15 post ocular infection were either unstimulated or stimulated with anti-CD3 mAb (1.0 μ g/ml) or OVA₃₂₃₋₃₃₉ peptide for 18 h and intracellular level of IFN- γ was measured by Flow Cytometry. Data shown is the

representative of three similar experiments. * $p < 0.0001$ when compared with HSV infected control group.

Figure 2. Polyclonal or antigen specific T cell stimulus lead to higher level of IFN- γ production from ocular CD4⁺ T cells obtained from test group of animals

a) Liberase digested single cell suspension of corneal samples from both test and control group of animals were unstimulated or stimulated with anti-CD3 mAb or OVA₃₂₃₋₃₃₉ peptide for 48 h in complete RPMI medium. IFN- γ levels in the culture supernatant were estimated by ELISA. Data shown is the representative of two similar experiments. * $p=0.00267$ when compared with control group of animals. **b)** Immunofluorescence staining of CD4⁺ T cells in HSV-1 infected corneas of homeostatically expanded (test) and control group of animals at day 15 post infection.

Figure 3. Homeostatically expanded CD4⁺KJ⁺ T cells functionally participated in HSV induced corneal tissue damage

a, SK lesion severity was determined at different time-points in both group A and B animals. Each dot represents an individual BALB/c SCID eye and the horizontal line indicates the mean clinical score for each group. Each group of mice consisted of ten animals and the results shown are the representative of two similar experiments. *, $p < 0.0001$ **b**, Intracellular levels of IFN- γ was measured 15 days post infection in the liberase digested ocular cells from group A and B when stimulated in vitro with SPC pulsed OVA₃₂₃₋₃₃₉. Values shown in each plot represent the percentage of CD4⁺ T cells expressing IFN- γ . *, $p < 0.001$ when compared with group A animals.

Figure 4. Homeostatic expansion of CD4⁺ T cells upregulated VLA-4

a, Homeostatically proliferating CD4⁺CD25⁻ T cells upregulated VLA-4 with each division. Cells were gated on the lymphocytes and numbers shown in the plot indicate the percentage of cells expressing VLA-4 in each division. Results shown are representative of three similar experiments **b**, In vivo blocking of VLA-4 interactions slowed down the homeostatic proliferation of adoptively transferred CD4⁺CD25⁻ T cells. **c**, anti-VLA-4 mAb treated group had higher percentage of undivided CFSE^{hi} CD4⁺ T cells. The numbers in each plot represent the percentage of gated cells in the first and second division. Data shown is the representative of three similar experiments. *, $p < 0.001$ when compared with Rat Ig treated test group of animals.

Figure 5. In vivo blocking of VLA-4 interaction significantly diminished the lesion severity
a, CD4⁺CD25⁻ T cells purified from DO11.10 mice were adoptively transferred into BALB/c SCID mice. One group was VLA-4 treated whereas the control group received Rat Ig. Four corneal samples from each group were compared for the infiltration of CD4⁺ T cells by Flow Cytometry on day 15 post-infection. Numbers indicated in the each plot represents the mean percentage of three similar experiments. *, p<0.001 compared with Rat Ig treated HSV-infected control group. **b**, SK lesion severity was measured in both groups at different time-interval post-infection. Each group of mice consisted of 10 animals and the results shown are the representative of two similar experiments. *, p<0.001 when compared with control group of animals.

Table 1

Ocular CD4⁺ T cells recovered from control (no homeostatic proliferation before infection) and test group (homeostatic proliferation prior to infection) of animals at different days post-ocular infection.

Days post-infection	% CD4 ⁺ T cells (mean $\pm$ SD)		Number of CD4 ⁺ T cells / eye ^a	
	Control	Test	Control	Test
12	1.5 $\pm$ 0.3	1.6 $\pm$ 0.4 *	1.12 $\times$ 10 ³	1.23 $\times$ 10 ³ *
15	2.1 $\pm$ 0.5	2.3 $\pm$ 0.3 *	1.67 $\times$ 10 ³	1.75 $\times$ 10 ³ *
18	1.2 $\pm$ 0.5	1.4 $\pm$ 0.2 *	0.85 $\times$ 10 ³	0.95 $\times$ 10 ³ *

^a Four corneal samples from each group of animals were liberase digested and stained for CD4⁺ T cells. A total of 1.5×10^5 cells were acquired and FITC- labeled CD4⁺ T cells were gated to determine the percentage of CD4⁺ T cells. The number of CD4⁺ T cells / eye was calculated from total viable ocular cells obtained after liberase digestion from both groups.

* p>0.05 when compared with control group of animals.