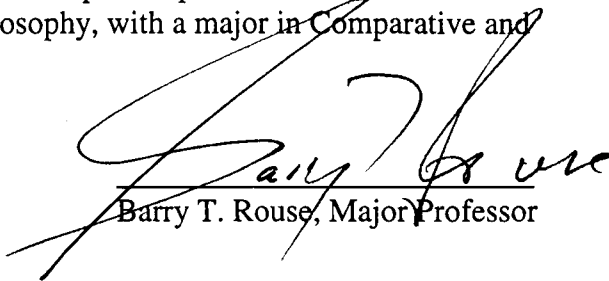


To the Graduate Council:

I am submitting herewith a dissertation written by Shivaprakash Gangappa entitled "On the Role of CD4+ T Cells and Pathogenic Mechanisms in HSV-1 Induced Ocular Disease: Herpetic Stromal Keratitis". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.



Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Thomas T. Schick
Philip M. Borish
Robert N. Moore

Accepted for the Council:



Laurie Mink
Associate Vice Chancellor and
Dean of The Graduate School

**On the Role of CD4+ T Cells and Pathogenic Mechanisms in HSV-1-
Induced Ocular Disease: Herpetic Stromal Keratitis**

A

Dissertation Presented

for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Shivaprakash Gangappa

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Dedication

This dissertation is dedicated to my parents, wife, and family members, without whose help and self less devotion to me I could never have achieved my goals

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Abstract

Experimental infection of mice by scarification of cornea with HSV exhibits ocular disease pattern similar to the histopathological manifestations seen in man. An experiment in athymic mice that did not succumb to herpetic stromal keratitis (HSK) showed that HSK is an immunopathological manifestation of host T cell mediated immune response. It was confirmed later that following infection of corneas in the murine model with RE strain of HSV-1, the immunopathology is manifested by CD4+T lymphocytes that exhibit the Th1 cytokine profile. A prominent cell type present in HSK lesions is the neutrophil (PMN), the infiltration of which is a biphasic event following HSV infection of immunocompetant mice, an initial transient infiltration appeared to be triggered by replicating virus and a second more intense invasion along with other inflammatory cells including CD4+ cells. This secondary PMN invasion episode, seemingly orchestrated by CD4+T cells, is called HSK and is not apparently triggered by replicating virus which remains undetectable at this stage. By taking advantage of cell depletion and adoptive transfer studies, the type of cells involved and sequence of cellular events in initiation and effector phase have been addressed. While some of these approaches could explain one facet of HSK immunopathology, identifying the antigen(s) (viral and/or host) to which T cells respond is another interesting field. Some of the recent observations lend credence to the growing notion that HSK represents an autoreactive inflammatory reaction set off by HSV that unmasks sequestered corneal antigens is normally accessible to the immune system. In this study, experiments were designed to address such issues and hence primarily focussed on elucidating the mechanisms which drive the inflammatory responses in HSV-infected murine cornea.

Experiments described in this dissertation are aimed at T cell specificity, role of viral antigens in lesion development and mechanisms that drive the inflammatory response in the infected cornea. The series of experiments described here and their results indicate 'nonspecific activation of CD4+T cells' as a novel mechanism in herpes-induced stromal keratitis.

Following an overview of herpes induced ocular disease in part 1, part 2 in addition to supporting HSK as a T cell-mediated immunoinflammatory disease, the results also show the potential for controlling the disease by coreceptor modulation. In part 3, characterization of a unique mouse model with skewed T cell repertoire (Tg-SCID) has been described which is useful for studying antigen specificity in T cell-mediated immunopathological disorders. Results of experiments described in part 4 propose nonspecific activation of T cells and hence the cytokine milieu by virus replication as a novel mechanism by which CD4+ T cells could orchestrate a virus induced ocular immunopathology. Finally, using adoptive transfer protocols, part 5 examines whether lesion development in Tg-SCID mice is virus replication, persistence and CD4+ T cell dependent. Additionally, in part 5, the differential role of HSV-specific antiserum and CD8+ T cells on virus dissemination was analyzed in the infected cornea.

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Part 1: General Introduction and Overview

Chapter 1: Herpes simplex virus –1 induced corneal disease

Herpes simplex virus-1 (HSV-1) induced stromal keratitis is the major cause of non-traumatic corneal blindness in USA and other countries. Infection of the corneal stroma in humans is likely to result during bouts of recurrent disease and appears as an immunopathological reaction to the virus. Thus the lesions in the corneal stroma are characterized by inflammatory cell infiltrates, cloudiness leading to loss of keratinocytes and scar tissue deposition which finally results in vision impairment. In United States alone, 20,000 new cases of ocular herpes have been projected each year and one fifth of patients with ocular herpes develop some form of stromal disease (1).

Corneal stromal pathology associated with HSV-1 is due to immunological mechanisms (2) where most investigators have favored a T cell mediated pathogenesis (3). Although it is established that immune response plays important role in the pathogenesis of HSK, the specific mechanisms remain to be resolved (4). Upon HSV infection, the immune response elicited restricts the spread of HSV and also may facilitate the establishment of latency.

There are three general types of herpes simplex virus corneal diseases namely: 1. Epithelial keratitis- involves lytic viral infection of the epithelium which is self-limiting and effects no permanent damage to the cornea. 2. Disciform endothelitis – with accompanying stromal edema. This is thought to be due to viral infection of corneal endothelium resulting in endothelial lysis by viral infection or in combination with immune mechanisms. Resultant endothelial dysfunction lead to stromal edema (5). 3. Stromal keratitis – a form of stromal opacification produced by immunologically

mediated mechanisms (6). Recurrent episodes of viral production lead to stromal inflammation and progressive corneal scarring with possible loss of vision. Detection of viral profiles and antigens in keratoplasty buttons have been variable (7). Events in HSK are set off by infection with HSV-1. Replication of virus is a necessary event in the induction process because some of the replication defective mutant viruses fail to induce corneal pathology (8).

Chapter 2: Immunopathology in the corneal stroma – herpetic stromal keratitis

The infiltrates in inflamed cornea are composed of polymorphonuclear cells (PMN), macrophages, lymphocytes, and plasma cells (9,6,10). Quantitation of cells has shown that majority are macrophages or CD4 T cells. Studies have indicated that cells isolated from patients with HSV keratitis are capable of both HSV specific cytotoxic activity (11,12,13) (mediated by T lymphocytes) and HSV-nonspecific cytotoxic activity (mediated by natural killer cells) (11). Due to cytokines that are released, there is increased expression of cell surface molecules (ICAM-1, HLA-DR) and cytokines are known to regulate the activity and function of immunocompetent cells (14).

The mechanism that is responsible for the immunopathology seen in human herpes simplex keratitis is unknown, although both delayed type of hypersensitivity (DTH) and cytotoxic T lymphocytes (CTL) responses may be involved differentially in response to various strains of HSV. Typically, DTH responses are mediated by T cells that express the CD4+ phenotype, where as CTL activity is most often mediated by cells that expresses the CD8+ phenotype (15). Some studies have indicated that the primary T cell involved in lysis of HSV-1 infected targets is a MHC class II restricted T cell that is CD4+,CD8- (16). Studies designed to directly measure the production of cytokines have found that HSV-1 can weakly induce peripheral blood lymphocytes to produce the proinflammatory cytokines IL-1 β , TNF- α , and IL-6 (17). Also, Oakes and associates (18) have reported that HSV-1 induces IL-8 when corneal keratocytes, but not corneal epithelial cells, are infected with HSV-1. IL-8 is particularly interesting in that it is known to be an important proinflammatory cytokine that acts as a

potent chemoattractant for neutrophils and lymphocytes (19). In addition to acting as a chemoattractant, IL-8 also activates neutrophil, which could play a role in the damage associated with this disease.

Several laboratories have demonstrated persistent HSV DNA in humans and animals during quiescent stromal keratitis (20,21,22), however, LAT (latency associated transcripts) transcription or lytic transcription has not been demonstrated (21). Thus persistent HSV DNA in corneal tissue differs from neuronal latency in that it does not appear to be transcriptionally active for LATs (at the limits of detection of in situ hybridization) (21). This theory is consistent with the finding that the LAT's promoter appears to be neuron specific (23). One explanation of the data, suggested by Hill and Blyth, is that infectious particles are periodically produced in the trigeminal ganglia and transported to the cornea via retrograde axoplasmic flow (24,20).

Chapter 3: Mouse model and critical events in herpes induced stromal keratitis

Although the rabbit model is useful for studying the basic histopathology of primary and recurrent disease, and testing the effects of various drug treatments on the course of the disease, it is more difficult to study immunopathology of herpetic eye disease in outbred rabbits. Therefore investigators have turned to murine models, which, because of the existence of genetically defined strains and the ready availability of immunologic reagents, provide an excellent model system to determine the role that immune responses play in the development of HSV-1 mediated tissue damage. As is the case for many diseases, certain mouse strains are more susceptible for developing herpetic keratitis (BALB/c, A/J, NIH), where as other strains tend to resist the development of disease (C57Bl/6, C3H) (25). The majority of reports examining HSK in mice has focussed on the events following primary HSV infection. Recently, however, investigators have developed and characterized a murine model for recurrent herpes keratitis using NIH strains of mice (26,27,28). As is the case in human disease, the precise mechanism for primary herpetic keratitis (primary HSK) in mice has not been conclusively determined. It is believed, however, that the disease is predominantly the result of the interaction of virus and host immune cells and components and not the result of direct viral cytolysis of corneal cells (9). Evidence supporting the role that immunologic responses play in HSK includes the fact that neither SCID nor nude mice develop the disease following corneal infection with the virus (29,30). When these immunodeficient mice are reconstituted by adoptive transfer of spleen cells (from mice exposed to HSV) and then infected intraocularly with HSV, they develop signs

characteristic of primary HSK (29). These studies clearly demonstrated the requirement of immunocompetent cells for the development of HSV-induced corneal keratitis. These findings were further extended by Russell and associates (31), who deleted T cells from the HSV immune spleen cells prior to adoptive transfer in to nude mice. Their results indicated that, where as all those mice that received unfractionated immune spleen cells developed disease, only one mouse in seven developed disease in the T cell deleted transfer group. As a result of this and other studies, most investigators agree that primary HSK is a T cell-mediated disease. It is generally held, though there are many exceptions, that CD8+ T cells are the primary mediators of cytotoxic T lymphocytes (CTL) activity, and that CD4+ T cells, by the release of various cytokines, mediate delayed type of hypersensitivity (DTH) responses (15).

The concept that CD4+ T cells are the primary mediators of primary herpetic keratitis has been put forward by many investigators. The studies that have supported this belief have used an adoptive transfer model (30,31) or SCID mice (29) that are reconstituted with either CD4+ or CD8+ T cells prior to infection with HSV-1. These studies demonstrated that only those mice reconstituted with CD4+ T cells developed primary HSK. Likewise, when normal mice were depleted of CD4-bearing T cells and then challenged with HSV-1 (RE strain), HSK immunopathology was either markedly reduced or eliminated. Conversely, when these mice were treated with anti-CD8 antibody, HSK was more severe and the incidence of encephalomyelitis increased. It was concluded from these experiments that CD4+ T cells are responsible for HSK, and CD8+ T cells appear to play a protective role against viral infections (32,33,34). Also

when corneal sections were immunochemically stained for T cell markers or mononuclear cells removed from the cornea were subjected to microfluorimetric analysis, only CD4+ T cells could be detected (32).

The role that cytokine production plays in primary herpetic keratitis has been addressed using a variety of techniques. Studies have been performed to evaluate the cytokine profile of ocular cells removed following infection with HSV-1. It is generally held that there are two types of T helper cells. These cells are differentiated on the basis of types of cytokines they produce. Those that produce IFN- γ , IL-2, and TNF- α or TNF- β are considered Th1 cells and mediate responses such as DTH, whereas those T cells that produce IL-4, IL-6 and IL-10 are designated Th2 cells and favor certain antibody responses (35). Niemialtowski and Rouse (36) discovered that the predominant cytokines produced were IFN- γ , IL-2, and TNF- α or TNF- β . At the same time they were unable to detect the production of IL-4 or IL-10, suggesting that major CD4+ T cell in the eye is of the Th1 type. Similar conclusions were derived when Hendricks and associates (37) treated mice with antibodies directed against IFN- γ , IL-2, and IL-4. Their results indicated that mice, which were treated with anti-IFN- γ and anti-IL-2 antibodies, which would block a Th1-mediated response, did not develop primary HSK disease. However, those mice that were treated with anti-IL-4 antibodies, which will inhibit Th2-mediated responses, demonstrated a normal disease course. Also, administration of exogenous IL-10, a pleomorphic cytokine, ameliorated the onset of herpes simplex stromal disease (38).

Chapter 4: Pathogenic mechanisms of HSK in mice.

Over the past decade, atleast five different pathogenic mechanisms have been described in the syndrome of HSK in mice.

1. *HSK due to HSV- specific Th1-type cells*

Most investigators now agree that HSK results from CD4+ DTH cells that secrete IFN- γ and other lymphokines typically produced by Th1 cells (39,40,41,42,43). IL-12, a cytokine closely linked to activation of Th1 cells, is upregulated very early in HSV-infected corneas and in the draining cervical lymphnode (44). Also, T cells harvested from eyes with HSK are primarily CD4+ and secrete IFN- γ , TNF- α , and IL-2 but not IL-4 or IL-10 (41). Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of cytokine gene expression in eyes with HSK reveals IL-2 mRNA, far greater levels of mRNA for IFN- γ than IL-4, but little mRNA for IL-5 or IL-10 (41). Systemic treatment of mice with neutralizing anti-IFN- γ or anti-IL-2 antibodies significantly reduces HSK risk by ocular infection with the RE-strain of HSV-1 (42). IFN- γ mediates corneal inflammation by facilitating PMN infiltration. This is accomplished by upregulating the platelet endothelial cell adhesion molecule(PECAM-1) on vascular endothelial cells in HSV-1 –infected corneas (43). However, experiments performed using mice with a defective gene encoding IFN- γ acquired typical HSK when infected with HSV on scarified cornea (45). The time course and severity are virtually identical to wild-type mice, indicating that HSK lesions must be produced by cytokines other than IFN- γ .

2. HSK due to HSV-specific CD4+ Th2-type cells.

Although most investigators agree with predominant role of Th1 cells in pathogenesis of HSK, confusion surrounds the possible pathogenic roles of Th2 cells, which secrete IL-4, IL-5, IL-6 and IL-10. Foster and colleagues (46,47) using C.AL 20 (HSK-susceptible) and CB-17(HSK-resistant) reported that, 1. Corneas of C.AL-20 mice become infiltrated primarily with CD4+ T cells, most of which contain T- cell receptors (TCRs) using V β 8; 2. A long term cultured CD4+ T cell line and clone, prepared from mice immunized with an immunodominant of gD of HSV, exacerbated HSK in recipient mouse with ocular HSV infection; 3. the cloned T cells were found to secrete IL-4, and to use this cytokine as an autocrine growth factor ; 4. Transfer of this Th2 clone in to C.B-17 SCID mice resulted in severe HSK when the ocular surface of the mice was infected with HSV. Thus HSV-specific Th2 cells appear to be quite capable of producing HSK.

3. HSK due to HSV-specific CTLs.

It was suspected early on that CTLs might participate in HSK (48). If A/J mice are chosen as experimental subjects for HSK study, ocular infection with the RE strain of HSV-1 produces disease that is mediated primarily by CD4+ T cells (49). However, if the same strain of mice is infected ocularly with the KOS strain of HSV, the stromal keratitis that develops is dependent up on CD8+ T cells. Similar results were obtained when mice were depleted of either CD4+ or CD8+ T cells and then infected intracorneally with the KOS strain of HSV-1. Depletion of CD8+ T cells resulted in

reduced incidence and severity of HSK. These experiments implicated CD8+ CTLs in the pathogenesis of HSK.

4. HSK due to HSV-specific antibodies

In murine model of HSK, experimental support for this pathogenic mechanism remains scant. In rabbits, while live virus could not be found in corneas at the peak intensity of HSK, trace amounts of viral antigens were detected in the stroma (50). It was speculated that viral antigens were trapped in the stroma in antigen-antibody complexes, and that these complexes activated complement, leading to an inflammatory process that compromised corneal endothelium. One other pathway by which anti-HSV antibodies could cause HSK is by antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells (51). NK cells display surface receptors for the Fc component of IgG molecules (FcγR) and, if anti-HSV antibodies bind viral antigens on corneal cells and then afix NK cells via FcγR, lysis of corneal cells could occur. As an indirect evidence for this idea, depletion of NK cells from HSK-susceptible mice renders the mice resistant to ocular infection with HSV (51).

5. HSK directed at corneal autoantigens

Certain observations although as indirect evidence laid the foundation for speculating involvement of non-viral antigens in HSK. Firstly, the lesions in HSK susceptible immunocompetent mice manifests in the apparent absence of viral antigens (44). HSV infected and naïve CD4+ T cell reconstituted SCID mice show corneal

pathology with no reactivity to HSV (52). Direct evidence comes from Avery and associates (53) who reported that adult naïve C.AL-20 mice can be rendered tolerant of allelic determinants on IgG2a^b allotype molecules by injecting the molecules intravenously; mice made tolerant in this fashion fail to develop HSK when infected intrastromally with HSV-1 (KOS strain). Cells from such IgG2a^b-tolerant C.AL-20 mice fail to transfer HSK. Extracts of cornea, but not other tissues, stimulate HSK-producing T cells that recognize IgG2a^b (292-308), implying that cornea contains a molecule that happens to include a sequence of amino acids identical to peptide sequence 292-308. Thus, in this model, even though the investigators implied that HSK arises from an autoimmune attack directed at unique antigens expressed on corneal tissues, their data provided no direct proof that HSK develops when corneal autoantigens are exposed by corneal inflammation. Recently, a report from the same laboratory attributed molecular mimicry as one of the mechanism involved in pathogenesis of HSK in mice (54). In this report they showed that two keratogenic T cell clones reactive against IgG2a^b (292-308) and corneal lysate of mice infected with HSV respectively, also reacted to stimulation with UL6 peptide (299-314), a virion associated core protein of HSV. In addition, tolerization with UL-6 peptide and challenge with HSV-KOS failed to show HSK in C.AL-20 mice. Also, deletion of HSV-1 (KOS) protein UL-6 eliminated the ability of this virus to induce HSK in mice. They interpreted their finding as viral determinants that mimic host antigens trigger self-reactive T cell clones to destroy host tissue.

Specific aims and rationale

Main objective of the present study is to understand the mechanism by which CD4+ T cells get activated, enter the cornea and drive effector functions in the cornea that culminates in blebbing and ulceration. Experiments were designed to understand whether or not virus specific responses are critical in manifestation of disease. Primary guiding hypothesis is that HSK is the end result of a complex multistep process (viral replication, biphasic PMN invasion, angiogenesis, CD4+T cells infiltration, blebbing and necrosis) in the cornea. As the target antigens recognized by the CD4+ T cells in HSK pathogenesis are not known, experiments were directed to define the means by which the cells involved are contributing to lesion development and its regulation. To address the mechanisms by which CD4+ T cells get activated and mediate corneal pathology, experiments were based on the following ground work;

1. Viral antigens and viral mRNA are not demonstrable after 5-7 days post infection and the principal lesions are not evident at this time.
2. Adoptive transfer of naïve CD4+ T cells to HSV infected SCID mice causes HSK
- 3 Adoptive transfer of clones reactive against corneal autoantigens causes HSK
- 4 Autoreactive T cell clones with no HSV reactivity caused HSK when transferred to nude mice.

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**Part 2: Inhibition of T cell activation by blocking costimulatory
molecules with fusion protein, CTLA4Ig, prevents HSV induced
immunopathology
(*Clinical Immunol. Immunopathol.* 86: 88-94, 1998)**

Chapter 1: Abstract

Herpetic stromal keratitis (HSK) is an immunoinflammatory lesion in the cornea of the eye set off by infection with herpes simplex virus (HSV). The disease appears to be orchestrated by CD4⁺T cells of the Th1 phenotype but the identity of target antigens involved in HSK remains unknown. In this proposal, we investigated if the inhibition of T cell activation with the fusion protein CTLA4Ig, would abrogate the disease process when administered systemically. BALB/c mice infected with HSV-1 (RE strain) by corneal scarification were injected intraperitoneally on a single occasion with CTLA4Ig or L6 control (IgG Fc) given on day 2 or day 5 or day 8 post infection. Lesions in CTLA4Ig treated mice showed markedly reduced severity judged by both slit lamp biomicroscopy and histopathology if treated on day 2 or day 5. Treated animals also expressed minimal HSV-specific splenic T cell and humoral antibody responses. Judged by the profile of T cell and IgG subset responses, inhibition by CTLA4Ig appeared more directly on the HSV-specific Th1 response, correlating with the known role of such cells in HSK. Delay of treatment until the time of disease onset (day 8) had marginal or negligible effects. The results indicate that blockade of coreceptor interaction between T cells and antigen-presenting cells during the induction phase of immune response significantly impairs onset and severity of herpetic stromal keratitis.

Chapter 2: Introduction

Stromal keratitis resulting from ocular infection with herpes simplex virus is a frequent cause of vision impairment (1). Lesions are suspected to represent largely immunopathological responses to viral or host antigens and management includes the use of immunosuppressive drugs (2,3). The pathogenesis of herpetic stromal keratitis is best understood from studies in a mouse model. Murine HSK is clearly a T cell- orchestrated immunopathological syndrome (4), but the nature of antigens involved in the reaction remain uncertain (5,6).

The CD4+ T cells, which are the principal mediators of HSK express the Th1 cytokine-producing phenotype (7,8). Furthermore, during recovery the balance of Th1-Th2 cells appears to shift towards a Th2 pattern (9). Conceivably, control of HSK might be achieved by measures which minimize the induction and expression of CD4+ Th1 lymphocytes. In some systems, T cell induction *in vivo* can be impaired by obstructing certain molecules essentially involved in costimulation (10,11,12). One such measure is the use of the soluble fusion protein CTLA4Ig that binds to B7 (ligand for CD28) and in so doing interferes with costimulator signals transmitted by that coreceptor (10). This effect appears to be of more consequence to CD4+ Th1 induction than it is to the Th2 lymphocyte subset (13,14). In consequence, it was hypothesized that the administration of CTLA4Ig following HSV infection might prevent T cell commitment towards Th1 and provide a means of modulating HSK development. Our results show that a single administration of CTLA4Ig given 2 days post infection almost abolished HSK lesion development. Diminished severity occurred if treatment was begun at 5

days, but delay until day 8 (the time of disease onset) provided minimal or zero protection. Our results show the potential for controlling HSK by coreceptor modulation and also add further support to the contention that HSK is indeed a T cell mediated immunoinflammatory disease.

Chapter 3: Materials and Methods

Mice

Four to five week old BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were used. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All ocular experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals.

Virus

HSV-1 RE strain used was propagated on Vero cells and stored as infectious cell preparations at -70° C.

Corneal infection

The corneal surfaces of deeply anaesthetized mice (methoxy flurane; Pittman-Moore, Mondelein, IL) were scarified with a sterile 27-gauge needle and 1×10^6 TCID₅₀ HSV-1 RE strain was applied in a 4.0 μ l volume and gently massaged onto the eyelids. Six animals were used in each group.

Injections

Forty-eight hours or 5 or 8 days following corneal scarification (infection), mice were injected i.p. with a single dose of 200 μ g of either the soluble recombinant fusion protein {murine or human CTLA4Ig (consisting of the CTLA4 extracellular domain and Fc portion of IgG1)} or L6 mAb (IgG1 Fc) or hIgG control. This dose of the fusion protein was chosen based on a preliminary study that compared results of therapy with four dose (75, 150, 200 and 300 μ g per mouse) levels.

Clinical evaluation

Mice were scored by a person who was blinded to the experiment design according to their clinical severity as follows; Score 0, normal cornea; Score 1, Neovascularization at periphery, iris visible; Score 2, partial opacity, iris not visible; Score 3, neovascularization at center, opacity; Score 4, bleb formation; Score 5, necrosis. The data were plotted as the mean daily clinical score for all animals in a particular treatment group.

Immunoglobulin ELISA

Serum collected at the end of the experiment (day 20 post infection) was analyzed for HSV-specific total IgG, IgG1, and IgG2a using standard ELISA as described previously (15). Basically, ELISA plates were coated with 100 μ l of HSV antigen in carbonate buffer (pH 9.8), and after overnight incubation at 4⁰ C, the plates were washed three times in PBS containing 0.05% tween 20, pH 7.2 (PBST) and then blocked using

PBS (pH 7.2) with 3% dehydrated milk for 2h at 37⁰ C. A total of 200µl of serially diluted serum samples (prediluted in PBST) were added in duplicate, and washed wells coated with 0.25 µg/ml goat anti mouse IgG were treated with serially diluted standard mouse IgG or mouse IgG1 or mouse IgG2a. Plates were incubated for 2h at 37⁰ C. After three washes, 100µl of goat anti-mouse IgG HRP or goat anti-mouse IgG1 HRP or goat anti-mouse IgG2a HRP was added. After three washes, ABTS substrate (Sigma, St.Louis, MO, no. A1888) was added. The concentration of the antibodies in the serum samples was determined from the standard curve.

HSV-specific proliferation assay

Splenocytes from all treated mice were collected at day 20 post infection and pooled. Single cell suspensions were stimulated with HSV-1 KOS or Con A exactly as described in detail elsewhere (16). A range of stimulator to responder ratios were used to define optimal condition for lymphoproliferation during a 5-day assay.

Cytokine assays

Splenocytes from treated and control mice were suspended in 10% RPMI 1640 and 10⁶ cells in 1 ml were stimulated *in vitro* with 1.5 m.o.i. (multiplicity of infection, prior to inactivation) of UV- inactivated HSV-1 (KOS strain). Similar number of cells were treated as unstimulated or Con A-stimulated (5 µg/10⁶ cells/ml) in 12-well culture plates. Plates were incubated at 37⁰ C for 72 h. The supernatant fluid was collected and stored

at -20°C until use. These supernatants were screened for the presence of IFN- γ and IL-4 by ELISA as described previously (15).

Histopathology

Twenty days after infection, sections of eye were prepared for histopathology according to standard procedures. Briefly, at the termination of experiment whole eyes were fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Sections were observed for the thickness of corneas; presence of inflammatory infiltrates, neovascularization, epithelial erosions, superficial or deep ulcers; and for evidence of corneal perforation.

Statistics

Wherever specified, data obtained were analyzed for statistical significance by Student's t test.

Chapter 4 : Results

Attenuation of HSK by CTLA4Ig Given Intraperitoneally

Groups of mice were infected topically by administering HSV to the lightly abraded cornea and animals were evaluated periodically for the clinical severity of their HSK lesions. At the end of the experiment, sample eyes were fixed and stained for histological evaluation. Fig. 1 records the clinical severity of HSK in treated and control mice in 3 separate experiments in which a single administration of CTLA4Ig was given at 2 days post infection (p.i). The single administration of CTLA4Ig profoundly inhibited the expression of HSK. Upon examination at day 20 p.i., 11 of the 18 animals had negative or negligible lesions and the other 7 animals had only minimal responses. Sample eyes from the minimal response animals were examined histologically to record lesion severity. As can be seen in Figs. 2a and 2b, mild pathological changes were evident. These consisted of some cellular infiltrates in the stroma but no stromal thickening or any histopathological lesions in the cornea. Histological sections of the corneas of uninfected control animals are included for comparison (Figs. 2c and 2d).

Whereas lesions were mild or inapparent in CTLA4Ig-treated animals, the majority of control infected animals, either untreated or treated with control Ig, developed severe lesions (4+ or greater) (Figs. 2e and 2f). The eyes from such animals showed profound histopathological reactions; the stroma was thickened and was densely infiltrated with inflammatory cells, and the epithelium showed erosion.

In other experiments, the effect of a single administration of CTLA4Ig given at either 5 or 8 days p.i. was evaluated. Usually in untreated animals replicating virus is no longer present in the corneal epithelium by 5 days p.i. (17). As is recorded in Fig. 3, treatment at 5 days with CTLA4Ig inhibited the severity of clinical HSK especially when compared with controls at day 14. The beneficial effects of treatment were less evident when measured at day 20 (and in fact not statistically significant). The data are consistent with the interpretation that day 5 treatment slows the development of HSK rather than preventing its ultimate expression. Delay of treatment until day 8, the approximate time of onset of clinical lesions, was without effect either in terms of rate of progression or severity of HSK.

Effect of CTLA4Ig Administration on Immune Response to HSV

To understand possible mechanisms by which CTLA4Ig treatment modified herpetic ocular lesions, the nature of various immune responses were measured at the 20-day termination point of experiments. The antigen-driven lymphoproliferation assay with splenocytes was used which for HSV-specific responses is mainly a measure of CD4+ T cells (4). Markedly suppressed responses to HSV occurred in the pooled splenocytes from animals treated with CTLA4Ig on day 2 as well as on day 5. The responses of day 8 treated animals were lower than controls but nonetheless positive. It was evident from measurements of cytokines induced by antigen stimulation of splenocytes that IFN- γ was absent in cultures from mice treated at day 2 with CTLA4Ig (Table 1) and was present in lower levels than the control in cultures from mice treated

on day 5. CTLA4Ig treatment has no effect on IL-4 levels. In fact, splenocytes from mice that received CTLA4Ig treatment on day 2 exhibit the highest IL-4 levels among control and other groups. As humanCTLA4Ig was readily available and also has been shown to bind murine B7 with lower affinity than murine fusion protein, in other experiments, the effects of CTLA4Ig treatment with the human fusion protein was studied. Treatment at day 2 p.i. also inhibited the expression of HSK. Furthermore, in two separate experiments, measurements of splenic cytokine responses to HSV stimulation showed diminished HSK yet elevated IL-4 levels (data not shown).

The above results are consistent with the idea that the effects of CTLA4Ig treatment is on CD4 T cell function, in particular the subset producing Th1 cytokines. Further support for this notion came from measurements of the HSV-specific isotype representation in sera from treated and control animals. As shown in Table 2, sera from animals treated on days 2 and 5 p.i. showed a shift in the ratio of IgG2a:IgG1 response to HSV that is typical of a diminished Th1 response (18). The effect on antibody production appeared both as a reduction in the IgG2a response and as an increase in IgG1 antibodies.

Chapter 5: Discussion

Our results demonstrate that coreceptor blockade using the fusion protein CTLA4Ig provides a valuable means of minimizing the immunoinflammatory lesions caused by HSV infection of the cornea. In fact, all animals given CTLA4Ig 2 days following infection with HSV had negligible or minimal lesions during the 20 day observation period. In contrast, 80% of untreated controls developed severe lesions. Delay of CTLA4Ig treatment until day 5 p.i. also provided some benefit although the effect was more to slow the progression of HSK rather than to totally prevent disease expression. The use of CTLA4Ig at the time when inflammatory lesions first became evident at day 8 failed to control disease expression. The efficacy of CTLA4Ig treatment appeared to result from an inhibition of Th1 T cell activation.

In human HSK, and certainly in its mouse model, the pathogenesis is suspected to be largely immunopathological (1) and later on perhaps involves autoreactivity (5). In the mouse model, the T cells involved in orchestrating the HSK lesions are CD4⁺ T cells which largely generate Th1 cytokines (7). Some have demonstrated that certain Th1 cytokines, especially IFN- γ and IL-2, may contribute directly to lesion expression (19). In the HSK model CTLA4Ig treatment was likely successful since the balance of T cell induction was changed and included a suppressed Th1 response. The *in vitro* data provide support for this notion. Antigen-specific proliferative responses, normally a measure of CD4⁺ T cell recognition for HSV (4), were markedly diminished and the cytokines induced from antigen-stimulated splenocytes reflected a suppressed Th1 and unaltered, or even slightly elevated, Th2

pattern. The CTLA4Ig treatment failed to abrogate HSV recognition since all animals developed good antibody responses. However, the pattern of IgG subclass antibody to the virus in treated animals reflected a shift towards the Th2 pattern (20). Accordingly, CTLA4Ig administration represents a means of modulating the nature of the subsequent immune response to HSV infection and it directs it towards a nonimmunopathological Th2 pattern.

The realization that T cell activation can be manipulated by targeting coreceptor function has opened up a convenient means of immunomodulation (21). The approach promises to be of particular value in disease situations where modulation of the TCR signal is impractical either because the antigens involved are unknown or are numerous and complex. Autoimmune diseases (AID) and immunoinflammatory disease resulting from infections with complex pathogens such as HSV represent examples. Indeed, the development of experimental AID has been successfully controlled using CTLA4Ig to block T cell activation (21, 22). In some instances, expression of on-going AID was inhibited following CTLA4Ig administration (23). The HSK disease has also been shown to include autoreactivity in its pathogenesis particularly in the late phase when the disease progresses in the apparent absence of viral antigens in the cornea (24). However, treatment with CTLA4Ig during clinical disease progression was not effective. This observation may mean either that the autoreactive response is too intense to be suppressed perhaps because of inadequate CTLA4Ig access to the inflamed cornea or that autoreactivity hypothesis is flawed. Further experiments are underway to resolve such issues.

It will be important to determine if the pattern of response established during CTLA4Ig modulated primary infection is sustained in the memory situation. Thus although HSV reexpression is difficult to achieve experimentally in the mouse model, recrudescence commonly occurs in humans following virus reactivation from latency (25). Any immune modulation process would be of particular value to control herpes immunopathology if the pattern of events induced proved sustainable. We are currently evaluating the nature of long term memory responses which result in animals whose primary infection with HSV was modulated by CTLA4Ig and other means of coreceptor manipulation.

Figure 1: Clinical score from CTLA4Ig-treated, L6 control antibody treated, and untreated groups of mice. Treatment was given as a single dose i.p. injection on day 2. Top, the clinical score of three separate experiments measured on day8. Middle and bottom, results on days 14 and 20, respectively. Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group. * Differences were statistically significant.

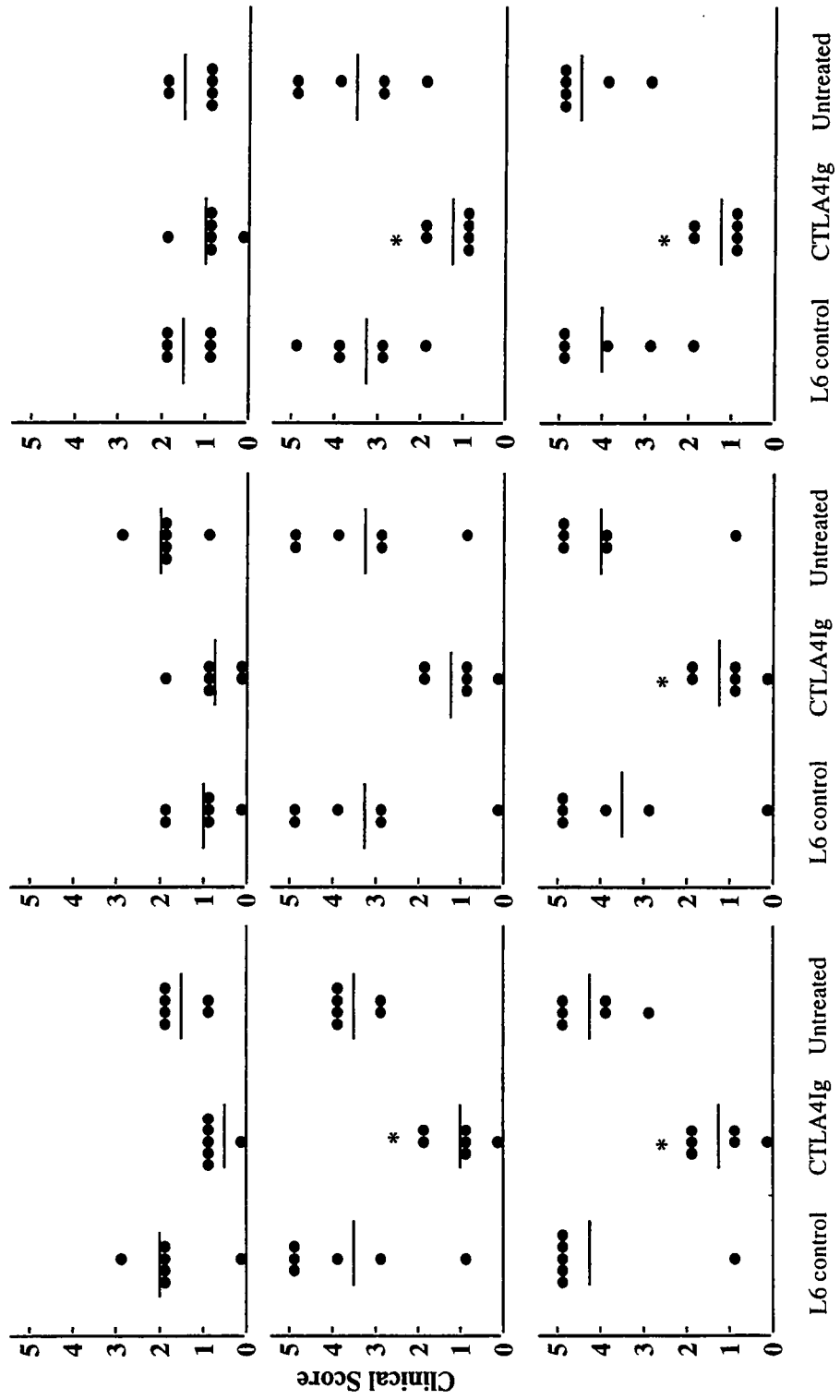


Figure 2: Histopathological sections of corneas from CTLA4Ig treated (day2) animals (a, 200X; and b, 500X), control uninfected animals (c, 200X; and d, 500X), and infected L6 control antibody-treated animals (e, 200X; and f, 500X).

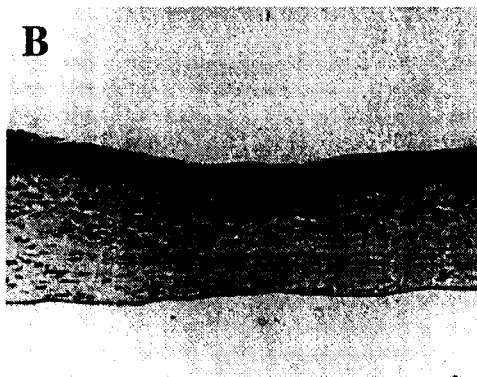
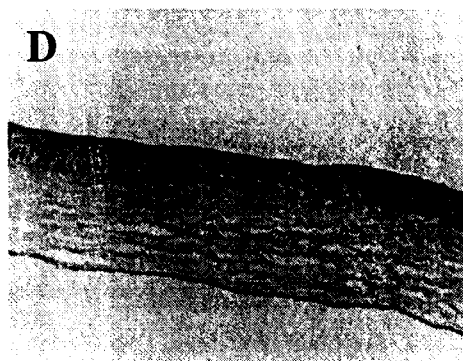
A**B****C****D****E****F**

Figure 3: Clinical score from CTLA4Ig-treated, L6 control antibody-treated and untreated groups of mice. Treatment was given as a single dose i.p. injection on day 5 or day 8. The control antibody, L6, was administered on day 5 post infection. Top, the clinical scores measured on day 8. Middle and bottom, results on days 14 and 20, respectively. Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group.

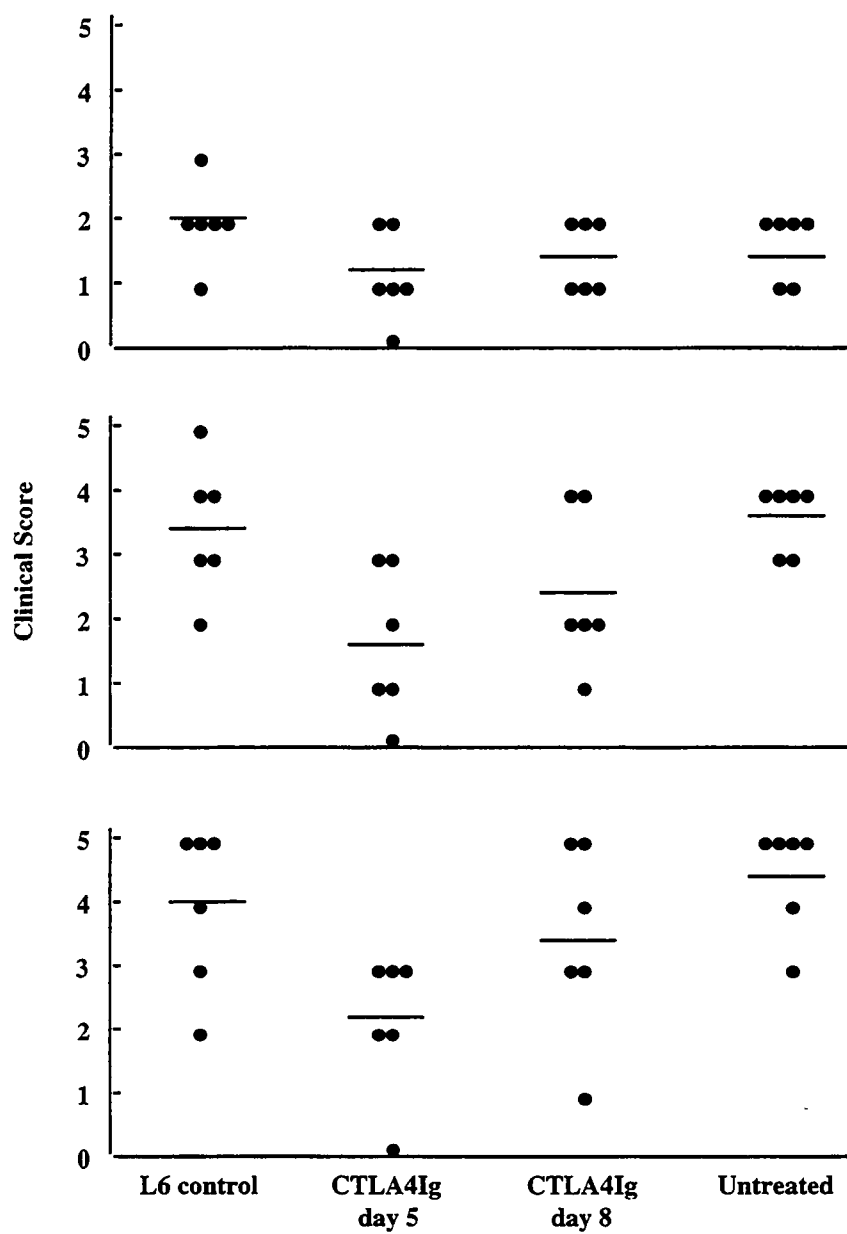


Table 1: In vitro T cell proliferative and cytokine responses in splenocyte populations from CTLA4 Ig-treated and control groups of mice^a

Test group	lymphoproliferation(cpm)			IFN- γ (ng/ml)		IL-4 (pg/ml)		
	HSVinfected stimulators	Con A stimulation	uninfected stimulators	HSV stimulation	none stimulation	HSV stimulation	none stimulation	Con A stimulation
untreated	2456 $\pm$ 238	2553 $\pm$ 584	175 $\pm$ 146	22 $\pm$ 5.1	<1	39 $\pm$ 5.5	ND ^b	ND 467 $\pm$ 50
L6 control treated-	1870 $\pm$ 466	3485 $\pm$ 191	107 $\pm$ 34	13 $\pm$ 2.7	<1	34 $\pm$ 14	200 $\pm$ 100	ND 450 $\pm$ 212
CTLA4 Ig- day2	103 $\pm$ 31 ^c	3414 $\pm$ 458	56 $\pm$ 14	<1 ^d	<1	20 $\pm$ 11	800 $\pm$ 150 ^e	ND 900 $\pm$ 135
CTLA4 Ig- day5	154 $\pm$ 59	9050 $\pm$ 576	62 $\pm$ 12	7 $\pm$ 3.3	<1	34 $\pm$ 3.8	500 $\pm$ 325	ND 350 $\pm$ 141
CTLA4 Ig- day8	1131 $\pm$ 365	3220 $\pm$ 226	99 $\pm$ 16	14 $\pm$ 2.8	<1	39 $\pm$ 4.6	200 $\pm$ 100	ND 450 $\pm$ 70

^a On day 20 post infection mice were sacrificed and their splenocytes (responders) were analysed for in vitro Tcell proliferation and cytokine production as described in 'Materials and methods'. The SD is based on three replicates per group. ^b ND, not detectable within the limits of this assay. ^c significantly different from values obtained for mice treated with control Ab (p<0.005), ^d significantly different from values obtained for mice treated with control Ab (p<0.0003), ^e significantly different from values obtained for mice treated with control Ab (p<0.001)

Table 2: Antibody response against HSV after CTLA4Ig-treated and control groups of BALB/c mice ^a

Test group	HSV-specific antibody			
	Total IgG ($\mu\text{g/ml}$)	Ig G1 ($\mu\text{g/ml}$)	IgG2a ($\mu\text{g/ml}$)	IgG1:IgG2a ratio
Untreated	39.3 $\pm$ 1.7	1.0 $\pm$ 0.1	23.9 $\pm$ 11.2	1: 23
CTLA4Ig- day 2 p.i	20.5 $\pm$ 1.4	3.4 $\pm$ 0.1 ^b	10.8 $\pm$ 0.7	1: 3
CTLA4Ig- day 5 p.i	20.1 $\pm$ 7.6	3.0 $\pm$ 0.7 ^c	12.2 $\pm$ 1.4	1: 4
CTLA4Ig- day 8 p.i	38.5 $\pm$ 0.9	2.0 $\pm$ 0.2	20.3 $\pm$ 2.4	1: 10
L6 control- day 5 p.i.	41.4 $\pm$ 2.9	0.6 $\pm$ 0.1	19.7 $\pm$ 3.3	1: 29

a BALB/c mice infected with HSV-RE by corneal scarification received no treatment or single injection of 200 μg of CTLA4Ig or L6 control on day 2, day 5 or day 8 post infection. On day 20 the mice were bled and serum was separated, pooled, and screened by ELISA for isotypes IgG1 and IgG2a. Based on the values obtained from the standard curves, the antigen-specific IgG1 and IgG2a was represented as IgG1: IgG2a ratio. The SD is based on three replicates per group.

^b significantly different from values obtained for mice treated with control Ab ($p < 0.0002$)

^c significantly different from values obtained for mice treated with control Ab ($p < 0.0003$)

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**Part 3: α β TCR transgenic-SCID mice: a unique mouse model to study
CD4⁺ T cell antigen specificity**

Chapter 1: Abstract

The transgenic mouse line DO10 expresses the $\alpha \beta$ T-cell receptor (TCR) from the T cell hybridoma DO11.10, reactive to chicken ovalbumin (OVA, 323-339) peptide. The majority of CD4+ T cells (about 70%) from this mouse line express the antigen-specific TCR clonotype from DO11.10 and can be identified with the anti-clonotypic monoclonal antibody KJ1-26. Remaining 30% of CD4+ T cells, which are not specific to OVA, but could recognize other antigens, are the result of incomplete allelic exclusion with respect to TCR-V α chain that leads to endogenous V α rearrangement. In order to eliminate endogenous V α chain rearrangement so that a mouse model with single antigen specificity could be generated, we repeatedly back crossed DO11.10 mice with SCID mice for four generations. The resultant mice lymphocyte populations consisted of only CD4+ T cells and were without any B cells and CD8+ T cells. Also, almost all of the CD4+ T cells (99%) were found to be OVA-specific (KJ1-26+). Such mice with a skewed Tcell repertoire and no ability to mount humoral response to antigens are of great value in studying T cell mediated immune disorders in a mouse model.

Chapter 2: Introduction

In thymocytes developing along the T cell receptor (TCR) $\alpha\beta$ lineage, the rules governing regulation of α and β chain gene rearrangements are distinct. While the presence of a productively rearranged β chain gene generally prevents further β chain rearrangement (1), both alleles of the α chain gene rearrange until a productive TCR $\alpha\beta$ is formed and undergoes positive selection (2). Cell surface expression of two α chains has been detected in TCR transgenic mice (3) and in peripheral T cells (4). DO11.10 mice are no exception to this rule. Virtually all thymocytes and peripheral T cells in these mice express the transgenic TCR from a T cell hybridoma, DO11.10, that recognizes chicken ovalbumin (OVA) in the context of I-Ad (5). The transgenic TCR expressed in this system is detected by the clonotypic monoclonal antibody (mAb), KJ1-26 and by the mAb V β 8, F23.1(6). Thymocytes expressing this TCR develop through an immature CD4+CD8+TCR^{lo} stage comprising 70% thymocytes and, in the H-2d haplotype, progress to mature CD4+CD8-TCR^{hi} thymocytes and are exported to the periphery as OVA-reactive mature CD4+ T cells (5).

In order to eliminate endogenous V α rearrangement in the DO11.10 transgenic mice, so that a mouse model with specificity to single antigen could be raised, we sought for the use of SCID mice. Studies in SCID mice, which are impaired in their ability to productively rearrange and express TCR genes (7,8,9), have revealed that SCID thymocytes are arrested at the CD4- CD8- stage of development (10,11). In the present study we examined whether it is possible to eliminate endogenous V α rearrangement in DO11.10, so that such mice could be used for addressing antigen specificity in HSK

where antigens involved and CD4+ T cell specificity are not defined. We approached this issue by breeding an already rearranged TCR α β Tg into SCID mice. Offspring resulting from this were characterized for expression of transgene and circulating immunoglobulins. We found that lymphocyte population in resultant Tg-SCID mice were comprised of only CD4+ T cells, with no B cells and CD8+ T cells. Also almost all the CD4+ T cells in Tg-SCID mice were found to be OVA-specific. Additionally, compared to DO11.10 mice where most of the V α chains present in the BALB/c mice were observed (18 of the 20 analyzed), the Tg-SCID mice expressed only 4 of the V α TCR chain mRNA and cell surface expression of such dual V α TCR was minimal (<1%).

Chapter 3: Materials and methods

Mice

Four to five week old BALB/c mice, SCID mice (Harlan Sprague Dawley, Indianapolis, IN), OVA-TCR transgenic mice (DO11.10 mice- kindly provided by Dr. Dennis Loh ,Washington University School of Medicine, St. Louis, MO and Dr. Casey Weaver, University of Alabama, Birmingham, AL) were used. All food, water, bedding, and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. All experiments were conducted in compliance with the guide for the care and use of Laboratory Animal Resources, Commission Life Sciences, and National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care.

Breeding protocol

Transgenic mice (DO11.10) were crossed to SCID mice for four generations and with brother-sister mating, it was possible to obtain Tg-SCID mice (as screened by PCR-tail DNA, and serum IgM-ELISA). In each of the breeding cages, two female DO11.10 mice and a male SCID mouse were housed. The offsprings were weaned at 3 weeks of age and screened for presence of transgene and SCID phenotype.

Screening for Transgene and SCID phenotype

Parent and offspring mice were screened for transgene and SCID phenotype. Presence of transgene was analyzed by PCR(polymerase chain reaction) and serum IgM levels by ELISA assay.

PCR: In order to confirm the genotype of the offsprings, genomic DNA was isolated from tail tissue using Proteinase K digestion buffer (0.5 mg/ml) and phenol-chloroform (1:1 mixture equilbrated with 100mM Tris, pH 8.0) extraction. A 35 cycle PCR was performed on an MJ Research PTC-100 thermocycler (Watertown, MA) using the following primer sequence and the program repeated 34 times.:

Primer sequence

TCR V α 13.5: 5'- CAG GAG GGA TCC AGT GCC AGC -3'

J α 5'- TGG CTC TAC AGT GAG TTT GGT -3'

Denature: 94⁰ C for 1 min

Anneal : 55⁰ C for 1 min

Extension: 72⁰ C for 1 min

The PCR products were loaded on to 1.5% agarose gels and electrophoresed to generate adequate band separation of 320 base pair product.

Immunoglobulin ELISA

Total IgM was determined from serum samples using sandwich ELISA described elsewhere (12). Briefly, coating antibody (goat anti-mouse IgM, Jackson Immuno Research, West Grove, PA) was diluted in carbonate buffer, pH 7.9. Standard antibody

of known concentration (10 μ g/ml, mouse myeloma IgM, Sigma) and samples were analyzed in duplicate and diluted in 1X PBS as were secondary (1:2000, HRP-rabbit anti-mouse IgM, Zymed) antibody. Samples and standards were incubated overnight at 4⁰ C. Secondary antibody was incubated for 2 h at 37⁰ C. Plates were developed using ABTS (2,2'-azino-di-(3-ethylbenzthiazoline) sulfonic acid, Sigma) solution (4.4 mg ABTS in 20 ml 0.1M citric acid plus 20 ml 0.1 M dibasic sodium phosphate and 4 ml 30% H₂O₂). All samples were run in duplicate and average absorbance was read at 405 Δ on an automated plate reader (Spectra Max 340, Molecular Devices, Sunnyvale, CA). Titers are expressed as a group mean, in μ g/ml.

Flow cytometric analysis for cell surface expression of OVA-TCR+ (KJ1-26.1+) cells

Isolated cervical lymph node cell populations were analyzed for cell surface markers by flow cytometry. Viable cells were blocked with heat inactivated FBS, washed three times with FACS buffer (1 X PBS with 1% BSA and 0.05% NaN₃). For DO11.10 mice and Tg-SCID mice characterization, cells from cervical lymph nodes were stained with anti-CD4-FITC, anti-CD8-PE. For transgenic TCR positive T cells, KJ1-26.1 (anti-OVA TCR Ab, a kind gift from Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO) antibody was used and detected by adding goat -anti-mouse IgG2a-FITC antibody. Events were collected and analyzed using a Becton-Dickinson FACScan analyzer.

Chapter 4: Results

Characteristics of DO11.10 (Tg) mice

PCR analysis of genomic DNA from Tail tissue of DO11.10 mice showed presence of transgene (Fig 1). Such mice also showed mean serum IgM level of 902 $\mu\text{g/ml}$ compared to naïve BALB/c mice that had 1158 $\mu\text{g/ml}$ levels and SCID mice where IgM level was $<1 \mu\text{g/ml}$ (Table 1). Flow cytometric analysis of lymph node cells from DO11.10 mice revealed that not all of the CD4⁺ T cells stained for OVA-TCR (KJ1.26.1⁺). Only 70% of the CD4⁺ T cells stained for OVA-TCR(CD4⁺,KJ1.26.1⁺), and the rest of the CD4⁺ T cell population showed CD4⁺KJ1-26.1⁻ (Fig 2). Although there was not a significant population of CD8⁺ T cells (3-4%), B cell population (B220⁺, 42%) was comparable to BALB/c mice. In order to determine the pattern of V α and V β chain of TCR, V α and V β mRNA analysis by RT-PCR was performed. As shown in Fig. 3, DO11.10 mice had all of the V α chains that were present in BALB/c mice but only had one V β chain that was introduced as the transgene (V β 8.2). Analysis of cell surface expression of dual V α TCR, by FACS analysis, revealed that dual V α expression (V α 2 and V α 13.1) was up to 3% of the lymph node cell population (Fig. 4).

Characteristics of Tg-SCID mice

All of the offsprings that were generated after back crossing DO11.10 mice with SCID mice were positive for transgene as determined by PCR (Fig 5), and also none of these mice had detectable levels of serum IgM (Table 1). Tg-SCID mice thus obtained by several back crossing of DO11.10 mice with SCID mice, by FACS analysis had only

CD4⁺ T cells and few or no CD8⁺T cells (Fig 6) and were without any functional B cells as screened by serum IgM ELISA. Also, almost all (>99%) of the CD4⁺ T cells were KJ1.26.1 (anti-OVA-TCR antibody) positive T cells (Fig 6). Analysis of V α and V β mRNA revealed 4 other V α chains in addition to V α 13.1 transgene and single rearranged V β chain in splenocytes of Tg-SCID mice (Fig 7). Cell surface expression of V α 2 and V α 13.1(KJ1-26.1) on CD4⁺ Tcells showed that less than 1% of cells had dual V α TCR in splenocytes of Tg-SCID mice (Fig 8).

Part 5: Discussion

The results of this study describes an animal model (Tg-SCID) with restricted T cell repertoire where almost all of the CD4⁺ T cells (99%) were found to have specificity to OVA peptide as analyzed by FACS analysis for OVA-specific TCR. Additionally there are no CD8⁺ T cells and functional B cells in these mice. Also, analysis of T cells for various V α and V β chain of TCR showed that, where as the wild type DO11.10 mice had most of the V α chains (18 out of 20 analyzed) that were present in BALB/c mice, Tg-SCID mice had only 4 of the V α chains in addition to the rearranged V α transgene, V α 13.1. However because of complete allelic exclusion with respect to β chain of TCR, only one V β chain (V β 8.2) was detectable in both DO11.10 and the Tg-SCID mice.

In the present study we bred into SCID mice the OVA-specific $\alpha\beta$ TCR transgenic mice (DO11.10) that possessed an already rearranged TCRV α (V α 13.1) and V β transgene (V β 8.2) (5). A similar approach has already been described by Tonegawa and associates (13), where they generated TCR transgenic mice specific for myelin basic protein (MBP) and crossed them to RAG-1-deficient mice to obtain mice that have T cells expressing the transgenic TCR but no other lymphocytes. Another study by Barrett et. al. showed that DO11.10 mice crossed to RAG-1 knock out mice resulted in offsprings that had CD4⁺ T cells all of which were specific to OVA-peptide (14). In addition, they also found that number of CD4⁺ T cells with dual-V α TCR were minimal. Other reports have verified the expression of two functionally intact TCRs by peripheral T cells in human T cell clones (15) and in up to 15% of T cells isolated from peripheral

blood (16). Von Boehmer and colleagues (17) suggested that dual TCR expression usually resulted from incomplete exclusion of TCR V α gene rearrangement and allowed developing T cells to test various TCR combinations during positive selection. The potential for dual V α TCR-expressing cells to recognize an increased diversity of environmental antigens was highlighted by Balomenos et al. (18), who found that dual TCR-expressing cells expressed activated phenotypes and expanded in the periphery of older TCR Tg and normal mice.

The mouse model described here is unique in that it's TCR repertoire has been restricted to recognize a single antigen and hence could be a very useful model to study T cell development (19) and T cell specificity in various T cell driven inflammatory diseases (20). This mouse model described here was used to address viral antigen-specificity and role of T cell in mediating HSV induced ocular immunopathology in mice.

Figure1: PCR analysis of genomic DNA from tail tissue of DO11.10 mice

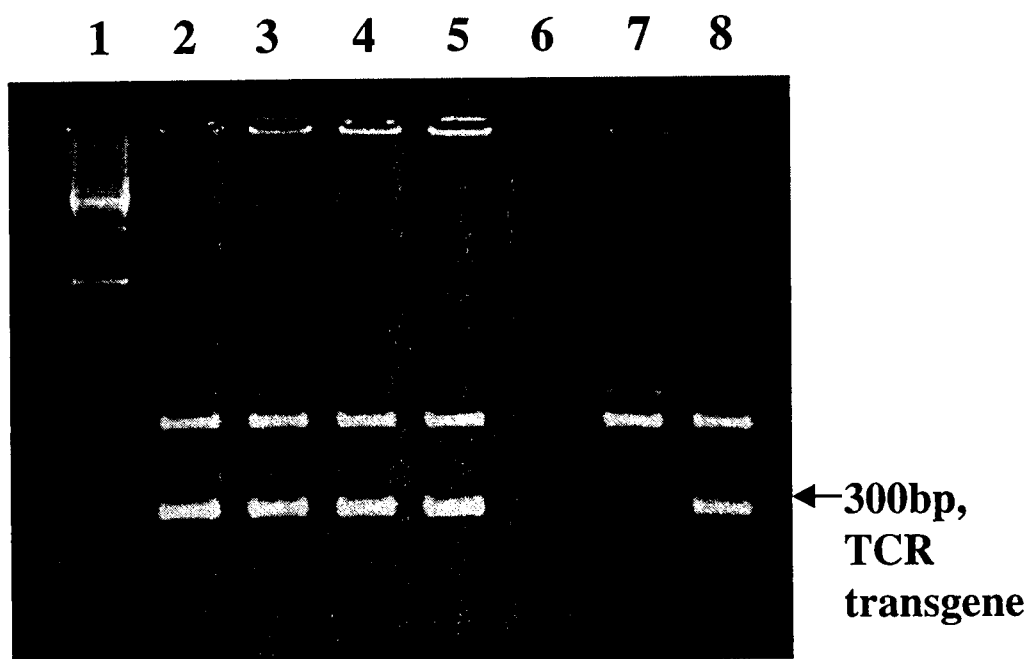


Figure 2: Flow cytometric analysis of cervical lymphnode cells from BALB/c and DO11.10 mice

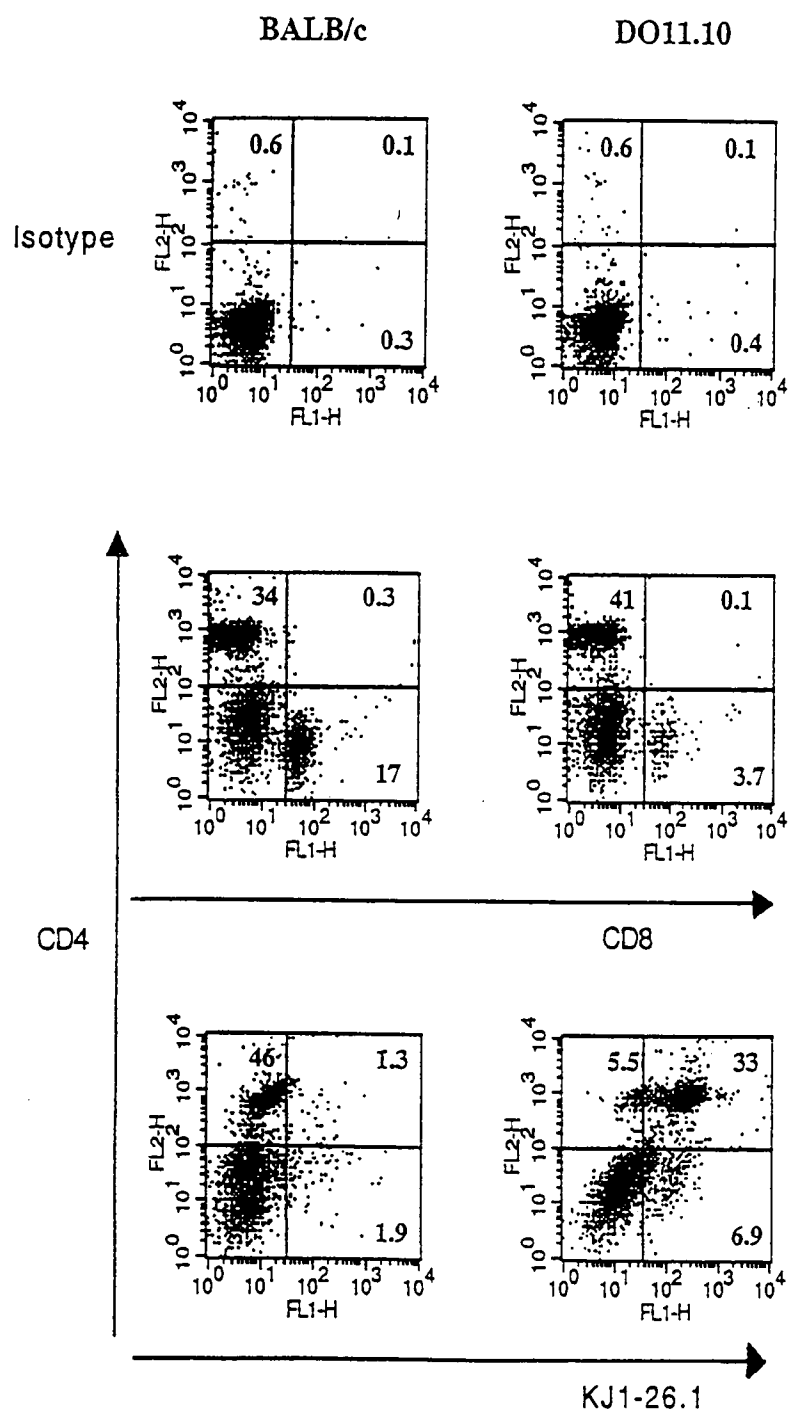


Figure 3: Pattern of V α and V β TCR mRNA analyzed by RT-PCR
on splenocytes from DO11.10 mice and BALB/c mice.

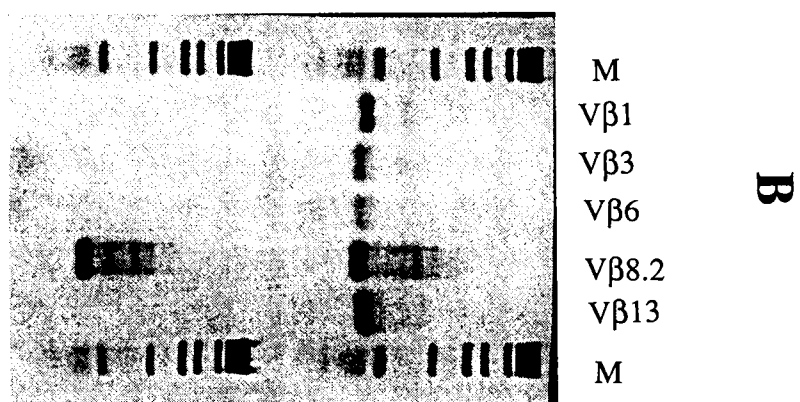
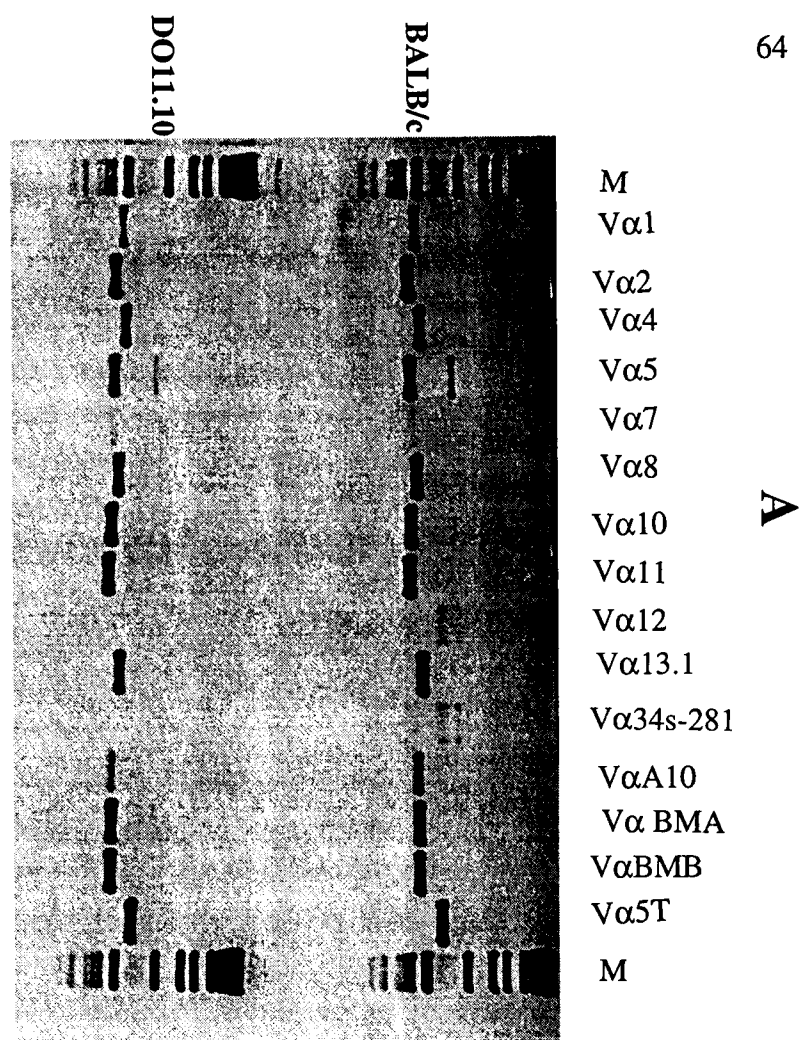


Figure 4: Flow cytometric analysis of cell surface expression of dual

V α TCR+ T cells in cervical lymph nodes of BALB/c and DO11.10
mice.

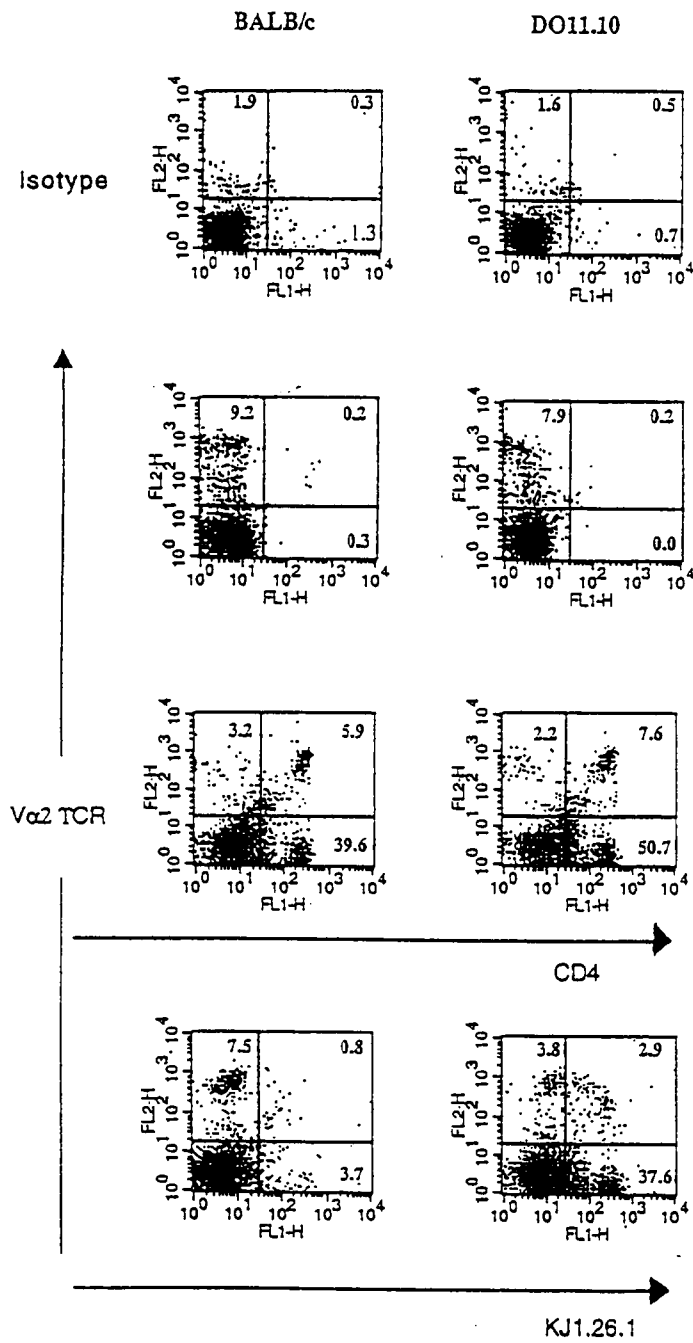


Figure 5: PCR analysis of genomic DNA from tail tissue of Tg-SCID mice.

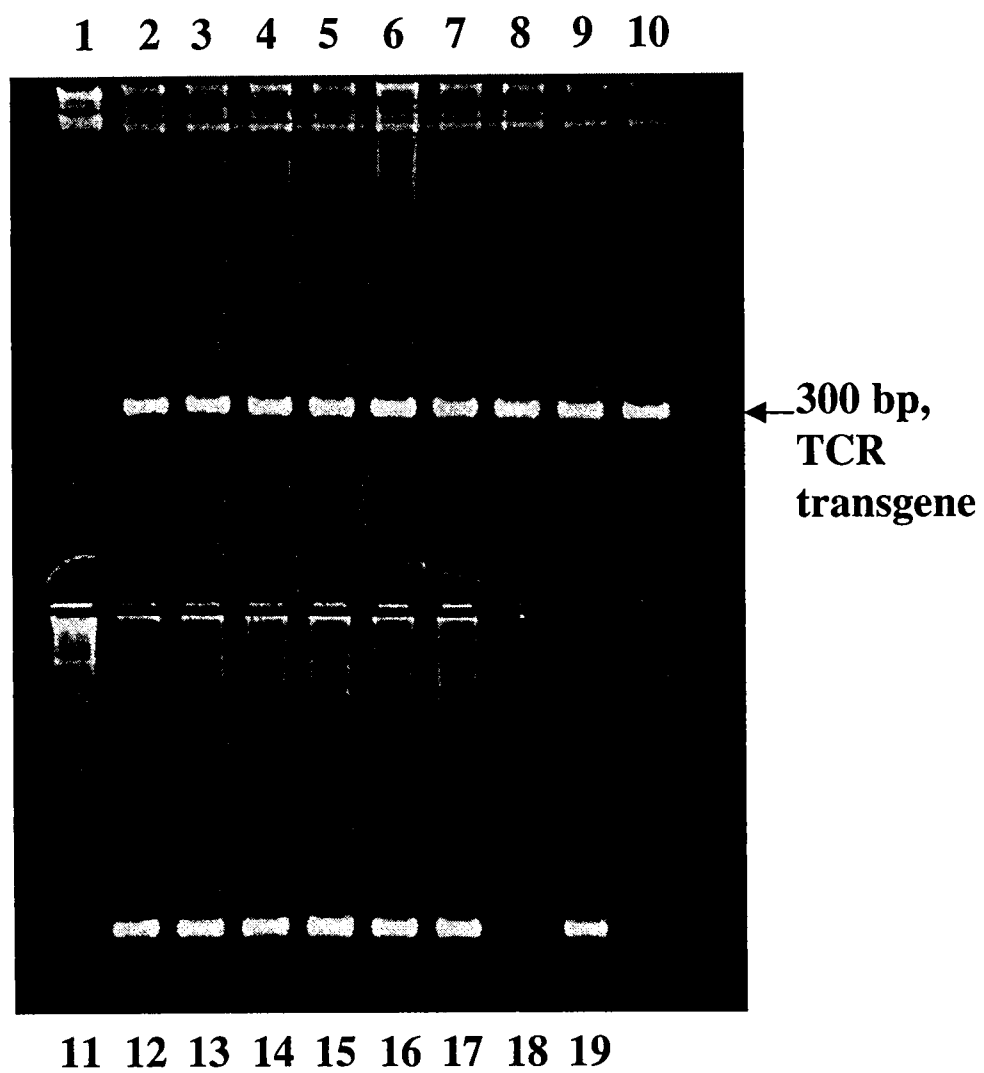


Figure 6: Flow cytometric analysis of lymph node cells from
BALB/c, DO11.10 and Tg-SCID mice.

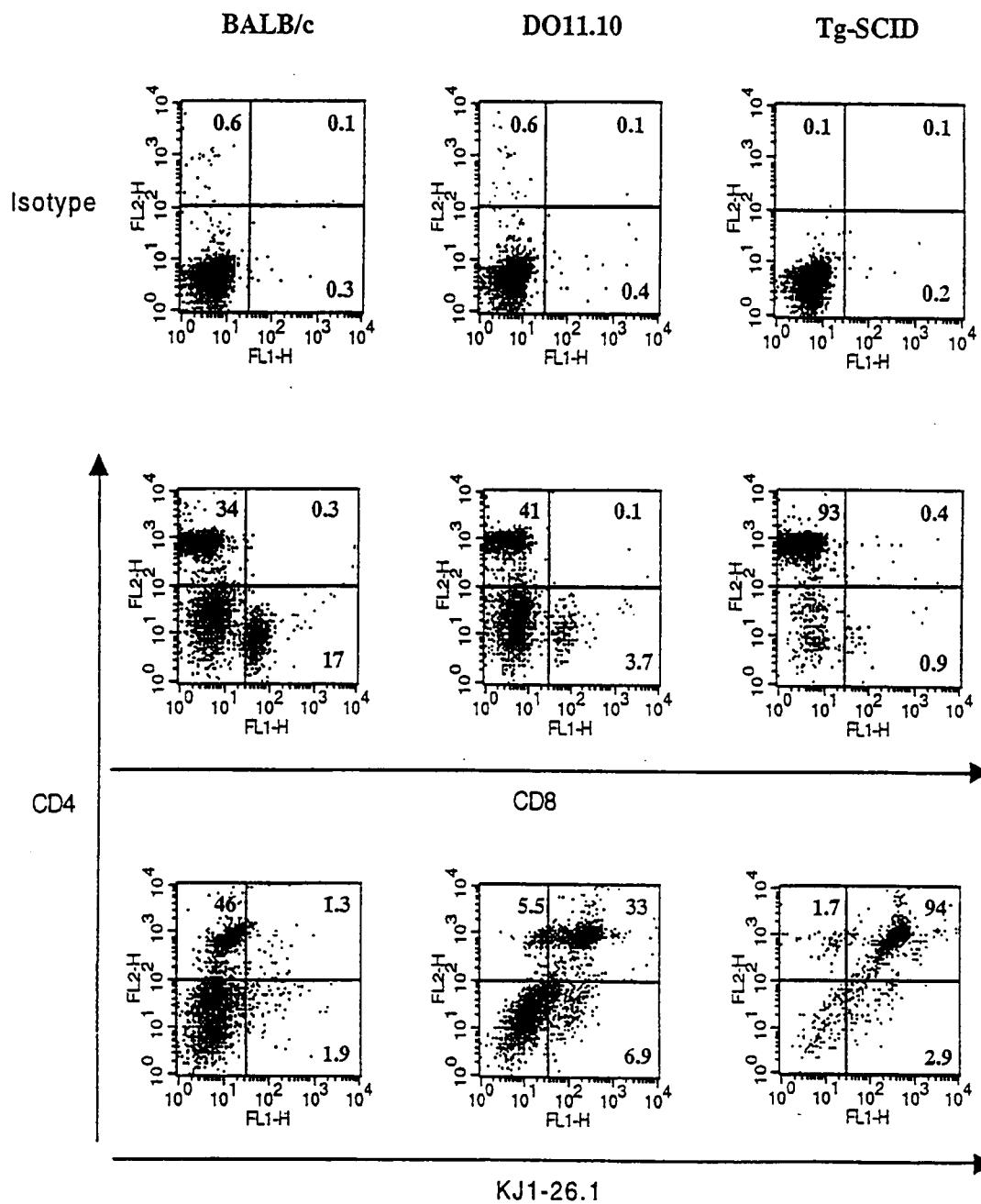


Figure 7: Pattern of V α and V β TCR mRNA analyzed by RT-PCR
on splenocytes from Tg-SCID mice.

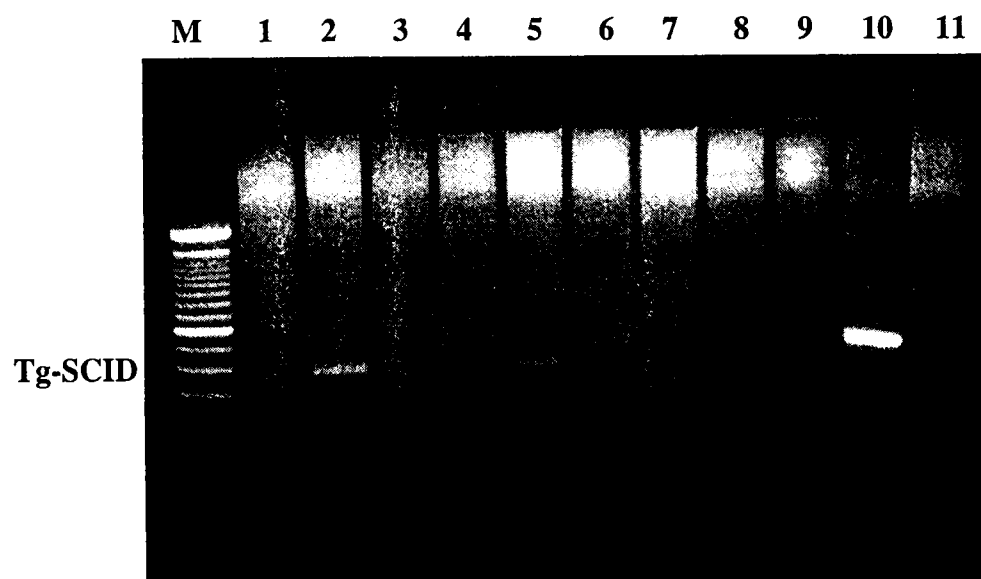
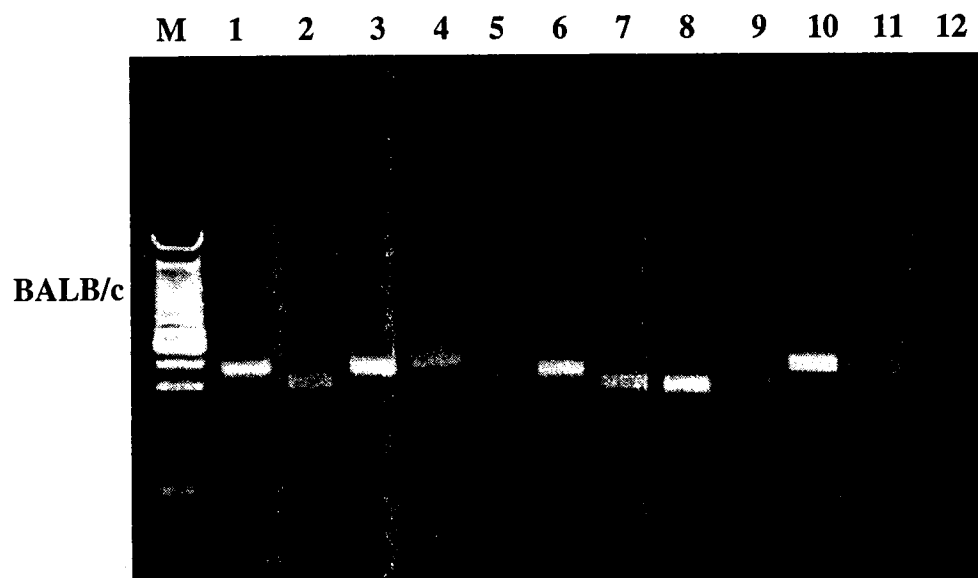


Figure 8: Cell surface expression of dual V α TCR+ T cells from splenocytes of Tg-SCID mice.

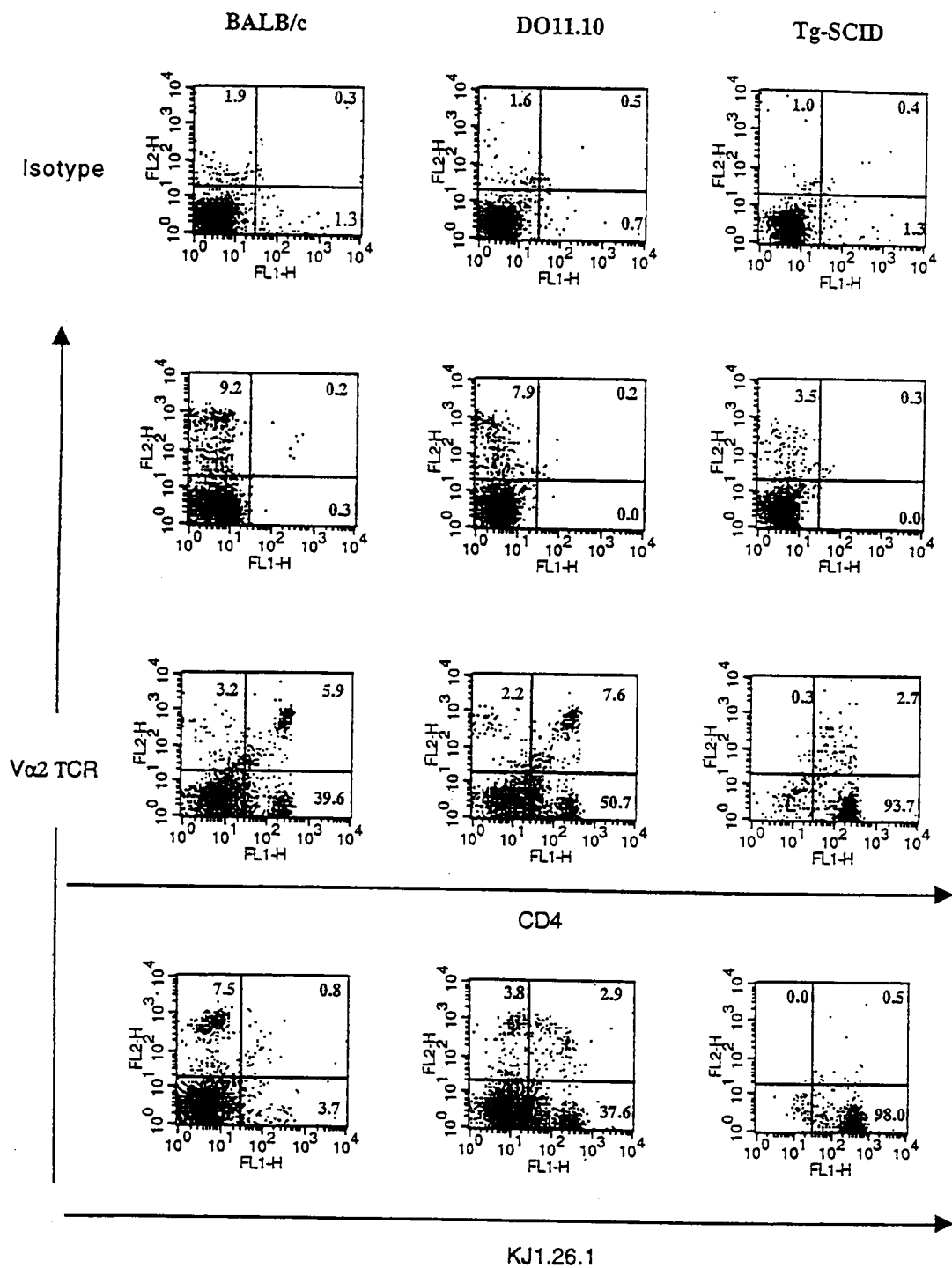


Table 1: Serum IgM levels in BALB/c, DO11.10, Tg+SCID-, Tg+SCID+, and SCID mice^a

Mice (n=6)	IgM (μ g/ml)
BALB/c	1158 $\pm$ 221
DO11.10	902 $\pm$ 117
Tg+ SCID-	205 $\pm$ 56
Tg+ SCID+	<1
SCID	<1

^a Serum was separated from blood collected from six week old BALB/c, DO11.10, Tg+SCID-, Tg+SCID+, and SCID mice and IgM levels were analyzed as described in 'Materials and methods'. The values represent the mean $\pm$ SD

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**Part 4: Virus induced immunoinflammatory lesions in the absence of
viral antigen recognition**

(*J. Immunol.* 161: 4289-4300,1998)

Chapter 1: Abstract

Herpetic Stromal Keratitis (HSK) is a CD4⁺ T cell-controlled immunopathological lesion in the eye that results from infection with Herpes Simplex Virus (HSV). Target antigens involved in HSK remain undefined. In this study, we determined if HSK could be induced in animals genetically incapable of generating HSV antigen-specific CD4⁺ T cells. Mice bearing transgenic TCR specific to OVA-peptide 323-339 (DO11.10) were crossed to SCID mice whose offspring (Tg-SCID) possessed CD4⁺ T cells, >98% of which expressed the OVA peptide specific TCR. HSV infection of Tg-SCID mice was lethal, and mice failed to generate detectable T cell responses even after repeated immunization with a mutant avirulent virus (AN-1). Immunization with AN-1 virus followed by ocular challenge with HSV resulted in ocular inflammation before encephalitis, in contrast to the protection conferred in the control BALB/c and DO11.10 mice. These results indicate that clinical HSK may not require viral antigen recognition by CD4⁺ T cells and that T cells of irrelevant specificity can be recruited, activated, and driven into effector function in the HSV-infected cornea. This is suggested to represent a bystander activation effect resulting from the presence of proinflammatory mediators resulting from HSV replication.

Chapter 2: Introduction

Herpes simplex viruses infection is a common cause of ocular disease and can result in a chronic inflammatory reaction that impairs vision (1). This latter manifestation is called herpetic stromal keratitis and usually occurs as a consequence of virus reactivation from latency in the trigeminal ganglion (2). HSK in man, and certainly its murine experimental model, appears to be an immunopathological disease process (3,4). The crucial cell type orchestrating the inflammation is a CD4+ T cell that has a Th1 cytokine-producing profile (5,6). Other cell types also play important roles in the pathogenesis of HSK. These include Langerhans cells, macrophages and perhaps surprisingly, polymorphonuclear neutrophils (PMN) (7-9).

Among many unsolved issues regarding the pathogenesis of HSK is the identity of antigens responsible for driving the immunopathologic process and subsequently its resolution. One might expect that virus-derived antigens should be primarily involved and that the CD4+ T cells that recognize such antigens in the corneal stroma recruit and guide the inflammatory process. This notion, however, is confounded by the observation that neither replicating virus, viral mRNA nor viral proteins, is usually detectable during the clinically apparent progressive stage of HSK (10). Viral DNA can be demonstrated throughout the disease process, but this DNA does not appear to be expressed (11). Viral peptides could conceivably persist in the cornea for long periods in association with Langerhans cells, but no direct evidence supports this possibility.

An alternative source of antigen to drive the HSK could derive from corneal tissue perhaps being unmasked and altered as a consequence of HSV replication. This idea

received support from the observations of Avery et al. which showed that HSK could be transferred to virus-infected athymic mice with a cell line reactive with an autoantigen expressed by an Ig isotype (12). We have also shown adoptive transfer of HSK with cell lines of uncharacterized autoreactivity, but which do not additionally express detectable HSV reactivity (13). Unfortunately, in the adoptive transfer models, recipient mice are immunocompromised and virus replicates for longer periods, including the time during HSK expression. Thus, viral antigens are present and cross reactivity with such antigens or bystander activation by virus-derived superantigens might cause infiltrating T cells to emanate inflammatory cytokines.

In the present report, we have approached the question of the role of nonviral antigens in HSK using animals susceptible to HSV but unable to develop immune responses against HSV proteins. For such studies, we selected TCR $\alpha\beta$ OVA-transgenic mice, that were backcrossed to SCID and shown to possess CD4+ T cells, that were almost exclusively $\alpha\beta$ OVA-TCR positive. Moreover the mice were unable to develop detectable T cell responses to HSV even when repeatedly immunized with a live but replication-defective mutant HSV. Such mice still developed HSK upon ocular infection with HSV. We interpret our results to indicate that the CD4+ T cells, shown present in lesions, must be mediating inflammation either by recognizing nonviral autoantigens in the cornea or perhaps more likely because of nonspecific bystander activation by virus- or host-derived components.

Chapter 3: Materials and Methods

Mice

Four-to five-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN), OVA-TCR- transgenic mice (DO11.10 mice- kindly provided by Dr. Dennis Loh ,Washington University School of Medicine, St. Louis, MO and Dr. Casey Weaver, University of Alabama, Birmingham, AL) were used. Transgenic mice were crossed to SCID mice for two generations, and, with brother - sister mating, it was possible to obtain Tg-SCID mice (as screened by PCR-tail DNA, and serum IgM-ELISA). Tg-SCID mice thus obtained had only CD4+ T cells and few or no CD8+T cells as tested by FACS analysis, and were without any B cells, as screened by serum IgM ELISA. Also, almost all (>98%) of the CD4+ T cells were KJ1.26.1 (anti-OVATCR antibody) - positive T cells. Transgenic mice (DO11.10) and HSK susceptible BALB/c groups of mice were used as the control animals. All food, water, bedding, and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. To prevent bacterial superinfections, all Tg-SCID and SCID mice received prophylactic treatment of sulfatrim pediatric suspensions (Barre-National Inc., Baltimore, MD) at the rate of 5 ml per 200 ml of drinking water. Antibiotic treatment was started 1 day before the beginning of the experiments. All experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animal Resources*, Commission Life Sciences, National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All ocular

experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals.

Virus

HSV-1 RE strain used was propagated on vero cells and stored as infectious cell preparations at -70°C . Replication-defective virus, AN-1 was kindly provided by Dr. Sandra K. Weller, University of Connecticut Health Center, Farmington, CT.

Corneal infections, Immunizations, and Passive transfer

In each experiment, control eyes were scarified and treated with 4.0 μl of PBS or tissue culture extracts. The corneal surfaces of deeply anaesthetized mice (methoxy flurane; Pittman-Moore, Mondelein, IL) were scarified with a sterile 27-gauge needle, and 5×10^5 TCID-50 HSV-1 RE strain was applied in a 4.0 μl volume and gently massaged on to the eye lids. For immunization with AN-1 virus or UV-inactivated HSV-1, anesthetized mice were injected with 5×10^6 TCID-50 in 50 μl volume on day 0 and day 14 through the footpad route (25 μl per foot pad) of injection. To assess the role of replication in lesion development, mice were infected with virus on the cornea and 24h later given 300 μl of anti-HSV serum (40 $\mu\text{g}/\text{ml}$ HSV-specific IgG as determined by ELISA). In such experiments, controls were infected and given normal mouse serum.

Clinical evaluation

Mice were scored according to their clinical severity by a person who was blinded to the experimental design using a slit lamp biomicroscope (Keeler Instruments, Broomall, PA) as follows; Score 0, normal cornea ; Score 1, Neovascularization at periphery, iris visible ; Score 2, partial opacity, iris not visible ; Score 3, neovascularization at center, opacity ; Score 4, bleb formation ; Score 5, necrosis. The data were plotted as the mean daily clinical score for all animals in a particular treatment group.

Immunoglobulin ELISA

Serum collected was analyzed for HSV-specific total IgG, using standard ELISA as described previously (14). Basically, ELISA plates were coated with 100 μ l of HSV antigen in carbonate buffer (pH 9.8), and, after overnight incubation at 40⁰ C, the plates were washed three times in PBS containing 0.05% Tween 20, pH 7.2 (PBST) and then blocked using PBS (pH 7.2) with 3% dehydrated milk for 2 h at 37⁰ C. A total of 200 μ l of serially diluted serum samples (prediluted in PBST) were added in duplicate, and washed wells coated with goat anti mouse-IgG (0.25 μ g/ml) were treated with serially diluted standard mouse IgG. Plates were incubated for 2h at 37⁰ C. After three washes, 100 μ l of goat anti-mouse IgG HRP (Horse Raddish Peroxidase) was added. After three washes, 2, 2-azino-bis 3-ethyl benz-thiazoline- 6 -sulfonic acid substrate (Sigma, St. Louis, MO, no. A1888) was added. The concentration of the antibodies in the serum samples was determined from the standard curve.

Cytokine assay

For cytokine (IFN- γ) assay, splenocytes from mice were suspended in 10% RPMI 1640, and 10^6 cells in 1 ml were stimulated *in vitro* with 1.5 m.o.i. (multiplicity of infection, before inactivation) of UV-inactivated HSV-1 (KOS strain). Similar number of cells were Con A-stimulated ($5 \mu\text{g}/10^6$ cells/ml) in 12-well culture plates. Plates were incubated at 37°C for 72 h. The supernatant fluid was collected and stored at -20°C until use. These supernatants were screened for the presence of IFN- γ by ELISA as described previously (14).

Histopathology

Eleven days after infection, sections of eye were prepared for histopathology according to standard procedures. Briefly, at the termination of experiments whole eyes were fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin. Sections were observed for thickness of cornea, presence of inflammatory infiltrates, neovascularization, epithelial erosions, superficial or deep ulcers, and corneal perforation.

Proliferation assay

Splenocytes (responders) at day 11 post infection were collected and restimulated *in vitro* with HSV-1 KOS (m.o.i. of 1.5 before UV irradiation) or OVA peptide (Research Genetics, Huntsville, AL). The HSV-1-specific lymphoproliferation was measured as described previously (15). Briefly, responders : stimulator ratios tested

ranged from 10:1 to 0.625:1. Responders + stimulator mixtures were incubated at 37⁰ C for 4 days. [3H] TdR (1.0 µCi/well) was added, harvested 18 h later, and read using a beta scintillation counter (Trace 96, Inotech, Lansing, MI). The results were expressed as mean±SD.

RNA isolation and RT-PCR

On day 11 post infection, splenocytes and cornea were collected and transferred to Tri-reagent (Molecular Biology Inc., Cincinnati, OH). The corneas were teased with sterile 21-gauge needle and titrated with a sterile 1 ml syringe plunger to expose the cells to lytic action of the Tri-reagent. The total cellular RNA was isolated from the Tri-reagent cellular lysate by adding chloroform and centrifugation followed by ethanol / isopropyl ethanol precipitation of the aqueous RNA solution according to the manufacturer's instructions. The RNA thus obtained was reverse transcribed using oligo (dT) primers and super script (Life Technologies, Bethesda, MD) according to standard protocol (16). The cDNA thus obtained was used as a template in subsequent qualitative and quantitative polymerase chain reactions. Primers for different Vα , Cα and Vβ , Cβ were used to check various segments of TCR (17). For semiquantitation of the leaky Vα chain of TCR, equal amounts of cDNA samples were amplified with Vα 1, Vα 2, Vα 5, Vα 8, Vα 11 primers and compared with Vα 13.1 expression in Tg-SCID and BALB/c samples.

Flow cytometric analysis.

Isolated cervical lymph node cell populations were analyzed for cell surface markers by flow cytometry. Viable cells were blocked with heat inactivated FBS and washed three times with FACS buffer (1 X PBS with 1% BSA and 0.05% NaN₃). For Tg-SCID mice characterization, cells from cervical lymph nodes were stained with anti-CD4-FITC and anti-CD8-PE. For transgenic TCR-positive T cells, KJ1-26.1 (anti-OVA TCR Ab , a kind gift from Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO) antibody was used and detected by adding goat -anti-mouse IgG2a-FITC antibody. To check for activation markers on CD4+ T cells, lymph node cells were blocked with FBS and double stained with FITC or PE-labeled anti-CD4 and anti-CD45 RB-PE or anti-L-Selectin-PE. Dual V α TCR analysis was done using TCR V α 2-PE and KJ1-26.1 antibody. Events were collected and analyzed using a Becton-Dickinson (Mountain View, CA) FACScan analyzer.

Immunohistochemical staining

At the termination of experiments, eyes were nucleated and snap frozen in OCT compound (Miles, Elkhart, IN). Eight-micron- thick sections were cut, air dried, and fixed in cold acetone for 5 min. The sections were then blocked with heat inactivated goat serum and stained with biotinylated anti-CD4, anti-Thy1.2 (Pharmingen, Sandiego, CA) or biotinylated KJ1-26.1 antibody (biotinylated anti-OVA TCR, a kind gift from Dr. Jerold Woodward, University of Kentucky, Lexington, KY). Sections were then treated with HRP-conjugated streptavidin (1:1000) and 3,3'-diaminobenzidine (Vector,

Burlingame, CA) and counterstained with hematoxylin. For OVA-TCR (KJ1-26.1) staining, sections were also treated with a tyramide signal amplification kit (TSA Indirect, Dupont NEN, Boston, MA) before treatment with diaminobenzidine.

Delayed-type hypersensitivity (DTH) assays

On the day of testing, each mouse was injected with 20 μ l of 10^5 UV-inactivated HSV-1 KOS (10^6 PFUs before inactivation) in the right ear and the same volume of vero cell extract in the left ear. The right and left ear thickness of each mouse were measured with a screw gauge Odimeter (Oditest, H.C. Kroeplin Schuechtern, Germany) and recorded individually. The thickness was measured at 1 h before ear injection, then at 24, 48, and 72 h after injections, and the values were represented as $n \times \text{mm}^{-2}$. The mean ear thickness of each ear from each group of animals was calculated, and the mean increase between before and 48 h after injections were compared among BALB/c mice, DO11.10 mice, and Tg-SCID mice.

Virus recovery

At various time points p.i., eye swabs were taken using sterile swabs soaked in McCoy medium containing 100 IU/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY). The swabs were then placed in tubes containing 500 μ l of the above mentioned transport medium and stored at -80°C . For detection of HSV in swabs, the samples were thawed and vortexed, and 100 μ l of each sample from

individually marked mice was used for quantification of virus recovery by standard PFU assay on vero cell cultures as described elsewhere (18).

Statistical analysis

Wherever specified, data obtained were analyzed for statistical significance by Student's *t*- test.

Chapter 4: Results

Stromal Keratitis in BALB/c and DO11.10 mice

As reported previously, infection of the scratched cornea of BALB/c mice with HSV-1 RE is followed after 8-10 days by the development of a clinically evident inflammatory response in the cornea (19). Typically, responses are at their peak around 3 weeks p.i., and they may persist for several weeks. A similar response profile is recorded in Fig 1. In addition, figure 1 shows the response pattern that occurred in DO11.10 mice. It is apparent that the clinical pattern was much the same. Sample BALB/c and DO11.10 mice were killed at 14 days p.i. and their eyes examined histologically and additionally analyzed by immunohistochemistry for the presence of CD4+ T cells (Fig. 2). Essentially no differences were observed between the two animal strains.

Initially, we had expected that the DO11.10 mice might fail to develop HSK since their limited TCR repertoire might compromise their development of an immune response to the virus. Accordingly, if HSK was the consequence of a CD4+ T cell antiviral response, lesions would be absent in TCR-transgenic mice. However, as recorded in Table 1, the DO11.10 mice responded immunologically to HSV and developed both T cell and antibody responses. Moreover, the DO11.10 mice recovered normally from infection and did not succumb to viral encephalitis. The explanation for such patent anti-HSV responses and protective immunity was presumably because of the extensive TCR α -chain leakage evident in the DO11.10 mouse (17). Thus, as shown in Figure 3, the DO11.10 mice expressed most of the TCR α -chains expressed by BALB/c

at least as detected by RT-PCR for α -chain and β -chain mRNA. Quantitative comparisons of α - and β -chain expression in transgenic and BALB/c animals were not made.

HSK in Tg-SCID mice

In an attempt to reduce or perhaps eliminate TCR α -chain leakage in the transgenic mice, DO11.10 mice were back crossed to SCIDs, and the offspring selected that were TCR Tg+ and SCID+ (Tg-SCID). Such mice were shown by FACS analysis to possess T cells, all of which were CD4+ and expressed the KJ1.26.1 idiotype marker (Fig. 4). The mice possessed no B cells and failed to generate IgM antibody to environmental antigens (data not shown). These Tg-SCID mice were highly susceptible to HSV infection and rapidly succumbed to encephalitis following ocular infection with HSV-1 RE at 4-5 weeks of age. Animals infected via the cornea at 7-8 weeks of age survived for as long as 13 days before dying from lethal encephalitis. Notably, however, most animals of this age groups expressed mild ocular lesions (clinical score 3 at day 10) that were approximately equal to those observed in BALB/c and DO11.10 mice at the same time frame (Fig 5). Analysis of lesions by histopathology as well as for the presence of CD4+ T cells by immunohistochemistry revealed a situation comparable to that observed in BALB/c mice (Fig 6). In addition, the T cells in the cornea were clonotype positive (KJ1-26.1+). Accordingly, we judge that the Tg-SCID mice had lesions of HSK, although all animals died of HSV encephalitis within 4-5 days of their lesions being manifest. Control SCIDs lacking T cells failed to express lesions when

examined around day 10 p.i. (Fig 6) and also died of encephalitis by 14 days. Analysis of virus recovery from the infected eyes of Tg-SCID (Table 2) revealed that infectious virus was demonstrable up to day 11 whereas, in the BALB/c mice, infectious virus was not demonstrable beyond 6 days post infection. In another set of experiments to determine whether the disease was indeed a result of HSV replication, Tg-SCID mice were infected with HSV and, after 24 h, given anti-HSV antiserum. This procedure not only protected mice from death by encephalitis, but also, as shown previously by Pepose and colleagues (20), prevented the development of primary HSK. These data are shown in Figure 7, as are the results in Tg-SCID mice that received normal mouse serum. The latter animals developed HSK and died of encephalitis.

Immune responsiveness of Tg-SCID mice

The fact that Tg-SCID mice could express HSK, even though they failed to survive HSV infection, could mean that they too had sufficient V α -chain leakage and developed anti-HSV CD4+ T cells that in turn orchestrated the ocular inflammatory response. Alternatively, it could be that the HSK responses in the Tg-SCID mice were the consequence of a nonvirus-specific, perhaps autoreactive response, set off in the cornea as a consequence of HSV infection.

Following HSV ocular infection, evidence was sought for lymphoid expansion in Tg-SCID mice. Mice with HSK had enlarged and more cellular cervical lymph nodes (Table 3) and, upon analysis by FACS for activation markers, showed a higher percentage of CD4+ T cells expressing the activated phenotype, i.e CD45RB low

and L-selectin low, than cells from uninfected mice (Fig 8). To assess whether Tg-SCID mice could develop anti-HSV immunity, animals with early HSK were killed at day 11 p.i., and their spleens were tested for HSV-specific lymphoproliferation and cytokine secretion. As controls, infected BALB/c and DO11.10 splenocytes were tested at the same time p.i.. The results shown in Table 4 indicate that the Tg-SCID mice failed to respond to HSV, but they did respond to OVA stimulation. In addition, mice were infected in the food pad with UV inactivated HSV-1 KOS and tested 2 weeks later for HSV-1-specific DTH response. Such DTH responses were lacking in Tg-SCID mice, but were present in similarly immunized BALB/c control mice (Table 5).

To further evaluate the HSV-specific immune competence of Tg-SCID mice, 5- to 6-week-old mice were immunized with a replication-competent but package-defective HSV mutant (AN-1) virus, known to induce excellent immune responses in BALB/c mice, but neither lethal nor in fact capable of inducing HSK lesions (21). Animals were immunized 2 times at 14-day intervals, and, after a further 8 days, animals were sacrificed and their spleens were tested for HSV-specific T cell immunity. It is evident from Table 6 that the Tg-SCID mice still failed to develop HSV-specific immunity detectable either *in vivo* by DTH or by *in vitro* antigen stimulation of their splenocytes. Further attesting to the failure of such AN-1 immunized mice to generate immune responses to HSV was the fact that the mice still succumbed to encephalitis upon ocular infection with HSV-1 RE. Before death, however, the immunized and ocularly infected mice all developed HSK lesions (Table 7).

Repertoire expression and evaluation of dual TCR-containing T cells in Tg-SCID mice

The above results indicate that Tg-SCID mice fail to recognize HSV antigens, yet such animals still expressed HSK lesions. Virus infection did result in an increase in T cell activation, but the issue arose as to how the repertoire-restricted Tg-SCID T cells could mediate HSK. One possibility was that Tg-SCID T cells showed V α or V β leakage and that such dual V α TCR+ T cells recognized corneal antigens and mediated HSK. Evidence for V α segment leakage was searched for by RT-PCR using splenic RNA collected from both uninfected and HSV- infected Tg-SCID mice. As is apparent in Fig 9 and Table 8, evidence for V α (but not V β) leakage was present in splenic samples in both groups of animals. Interestingly, examination of individual animals showed that all of them expressed V α 1, V α 2, V α 5, and V α 11 in addition to the V α 13.1 transgene. We obtained no evidence that HSV infection changed the nature of the V α leakage, but the expression level appeared increased in samples from HSV- infected Tg-SCIDs (data not shown). By semi-quantitative PCR, V α 2 showed the most leakage of the 4 extra V α segments expressed. Evidence for cells in the draining lymph node of HSV- infected mice that expressed surface V α 2 in addition to V α 13.1 was sought for by FACS analysis. Such dual V α -positive T cells were present in DO11.10 samples (but the 2.9% value is likely not significant) but were not detectable in either Tg-SCID or BALB/c samples (Fig 10). Furthermore, perhaps arguing against a pathogenic role for V α dual-positive cells in mediating HSK, we were unable to find evidence for leaked V α expression in corneal samples from Tg-SCID mice with early HSK (Fig 11).

Chapter 5: Discussion

This report analyzes the pathogenesis of the ocular inflammatory lesion, herpetic stromal keratitis, a sequel to corneal infection with HSV and an important vision-impairing lesion in mankind. HSK is a CD4+ T cell dependent immunoinflammatory process presumed to involve recognition of HSV derived antigens (13). The data presented in this report, however, indicate that, whereas HSK results from HSV infection and requires the presence of CD4+ T cells, the lesion occurs regularly in animals in which T cell response to HSV antigens could not be demonstrated. Our results are interpreted to support the notion that effector CD4+ T cells, perhaps of any specificity, entering the proinflammatory environment of the HSV-infected cornea may receive bystander activation and further contribute to the orchestration of an immunoinflammatory lesion. We consider an alternative idea that T cells of Tg-SCID mice cross-react with an HSV-induced autoantigen in the eye as less likely, given the marked repertoire handicap of Tg-SCID mice.

That HSK represents an immunopathological lesion in both man, the natural host, and in the murine experimental counterpart are widely accepted (2,4,13). However, the identity of antigens involved as targets for the immunopathological response remains to be defined. Characteristically, a virus- induced immunopathologic disease occurs in situations where virus persists in the presence of an ongoing immune response (22, 23). Most commonly, either CD8+ or CD4+ T cells are the cells involved in controlling lesion development (23). In HSK, the primary cell type that orchestrates lesions, are CD4+ T cells of the Th1-cytokine-producing profile (5,6). However, during disease

expression, it is usually not possible to demonstrate either replicating virus or viral gene expression (24). Although it is difficult to exclude the possibility that viral antigen-derived peptides persist in association with corneal antigen presenting cells and that these drive the inflammatory process, other mechanistic explanations have emerged. Some have advocated that the principal cognate antigens recognized by CD4+ T cells are host antigens unmasked in the normally avascular cornea as a consequence of HSV replication (1, 12), or by inflammatory products released from the rapidly responding neutrophil response (9, 13). The host antigen target hypothesis receives strongest support by studies of Avery et al (12) who investigated the basis for the marked differences in susceptibility between two congenic mouse strains. Their observations were interpreted to indicate that HSV infection caused the availability of a corneal antigen, that cross-reacted, with the IgG2a isotype of immunoglobulin. Other more indirect lines of evidence supporting a role for autoantigens in HSK include the observance of antiself peptide-reactive T cells following HSV infection and the adoptive transfer of HSK to infected SCIDs with an autoreactive cell line (25, S.J. Babu and B.T. Rouse, unpublished observations).

Our observation that viral antigen unreactive Tg-SCID mice still develop HSK could be explained by their T cells recognizing non-viral antigens, perhaps even host derived autoantigens expressed as a consequence of virus replication in the cornea. Corneal Langerhans cells presenting non-viral autoantigens which may be cross-reactive to the OVA-specific TCR may present such a possibility. Although we can not formally reject the autoantigen hypothesis, we consider the explanation as unlikely. Thus it is

difficult to accept the idea that a T cell repertoire-handicapped mouse would still be able to recognize an autoantigen but not one or more of the abundant antigens derived from a virus that encodes more than 70 proteins (26). Alternatively, some viral or virus-induced self proteins could act as a superantigen stimulating CD4+ T cells via binding to V β region of TCR (27). We, in fact, observed a mild lymphocytosis in mice following HSV infection, and showed that lymphocytes expressing activation markers were more abundant in infected mice. HSV itself is not known to express superantigen activity, so the explanation for the lymphocyte stimulation remains unknown. Possible sources of stimulation are host heat shock proteins (HSP), some of which are up regulated as a sequel to HSV infection (28). There is some evidence that host HSPs may act as cognate antigens in certain autoimmune diseases and some of them may act as polyclonal activators (29, 30). Conceivably, T cells expressing more than a single TCR could exhibit loose recognition characteristics that includes virus induced heat shock proteins. Indeed some have advocated that T cells with multiple V α expression could be involved in mediating certain autoimmune diseases (31). In our study, we demonstrated V α leakage in lymphoid tissue in Tg-SCID mice and noted that the extent, although not the spectrum of leakage, appeared elevated after HSV infection. However, arguing against the pathogenic role of dual V α cells in HSK was our failure to demonstrate V α leakage in corneal tissue taken from Tg-SCID mice with HSK. In addition, the lymphoid tissue from such mice did not contain significant number of cells with dual V α expression.

An alternative mechanism by which CD4+ T cells became activated to mediate immunoinflammatory lesions in Tg-SCID mice could involve a process akin to bystander activation. Accordingly, in the presence of a 'cytokine storm', cells with appropriate receptors may become activated and in turn contribute to the inflammatory response. This is thought to be a mechanistic event in the disease Dengue hemorrhagic fever (32). This bystander activation is usually consisted to largely involved mononuclear phagocytes (33), but it has also clearly been shown to apply in some circumstances to CD4+ T cells (34, 35). Since we did not observe any detectable anti-HSV reactivity even after AN-1 immunization, it is tempting to speculate that CD4 T cells in the cornea are activated in to effector function by mechanisms other than those involving TCR occupancy. One prominent consequence of HSV replication in the mouse eye is the expression of many chemokines and cytokines, contributed by corneal epithelium and perhaps by recruited inflammatory cells (19, 21, 36). Therefore, we speculate that, in the HSV- infected eye, this proinflammatory microenvironment could activate ingressing CD4 T cells. Indeed, it has been reported previously that resting naive human CD4+ T cells could be activated to proliferate by a cytokine combination consisting of IL-2, TNF- α , and IL-6 (34). Such a combination also caused effector function in resting memory CD4 T cells, as measured by lymphokine synthesis and providing help for Ig production by B cells (34). Alternatively, antigen independent T cell activation could also occur through accessory molecules on the T cells. One such mechanism includes CD2- mediated interactions on the T cells with ligands such as LFA-3 and CD59. Indeed, it has been reported that LFA-3 and CD59 expressed on rat

retinal pigment epithelium could trigger syngeneic T cells to proliferate and produce IL-2 in the absence of any exogenous antigen (37). LFA-3 and CD59 are reported to be expressed by corneal epithelium, stromal keratocytes and retinal pigment epithelium (37, 38). While the role of these molecules in non-specific T cell activation in the murine cornea is not clear, it is tempting to speculate that such possibilities could occur in our mouse model. According, we propose that CD4+ T cells drawn in to the HSV- infected cornea, become activated either by the proinflammatory environment of the cornea and/or by activation through accessory molecules such as CD2 in a TCR-independent manner. Such nonspecifically activated CD4+ T cells generate effector molecules that could recruit and activate PMNs and macrophages, which further contribute to the inflammation process in the eye. Additional experiments are currently underway to test these ideas.

Figure 1: Clinical score from HSV-1 RE infected BALB/c mice and DO11.10 mice on day 8 (left panel), day 12 (middle panel) and day 16 (right panel). Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group. Each group of mice consisted of six animals and the results shown here represent one of the three experiments performed.

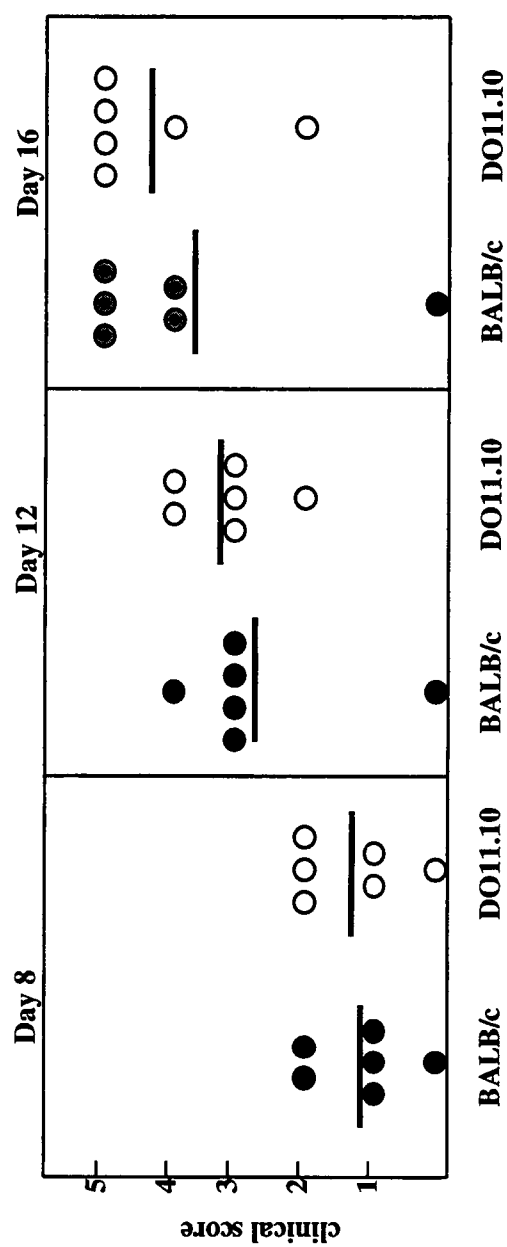


Figure 2: Histopathological sections of corneas at day 11 p.i. from HSV-1 infected BALB/c mice (A, B) and DO11.10 (D,E) showing inflammatory infiltrates. Immunoperoxidase staining demonstrating CD4+ T cells at day 11 p.i. in the cornea of HSV-1 infected BALB/c mice (C) and DO11.10 mice (F). Magnifications, X200 (A, D), X400 (B, E), and X800 (C, F).

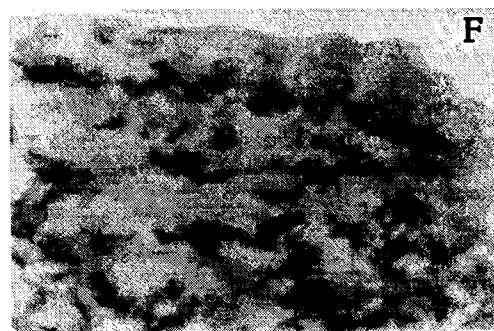
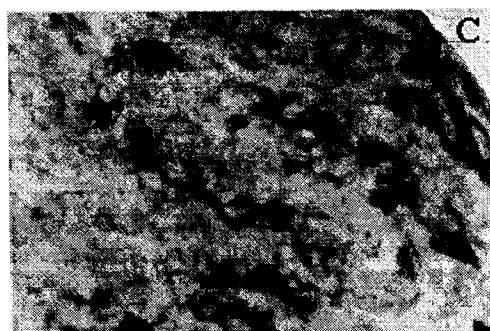
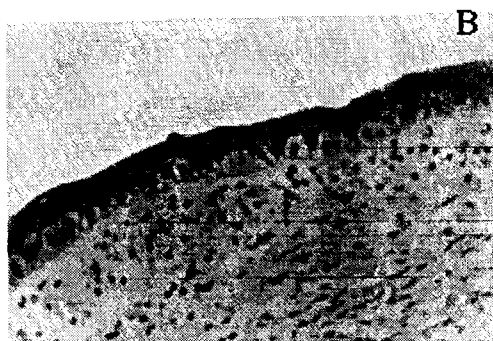
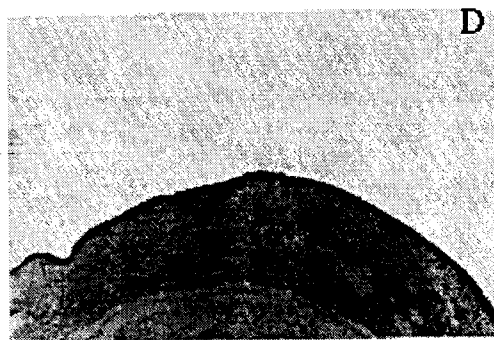


Figure 3: TCR V α and V β analysis in splenocytes of DO11.10 and BALB/c mice. Total RNA was isolated from the splenocytes and reverse transcribed as described in Materials and Methods. Equal amounts of cDNA was used for analysis of different V α (A) and V β (B) using specific primers corresponding to variable regions along with a unique primer corresponding to either constant region of C α or C β .

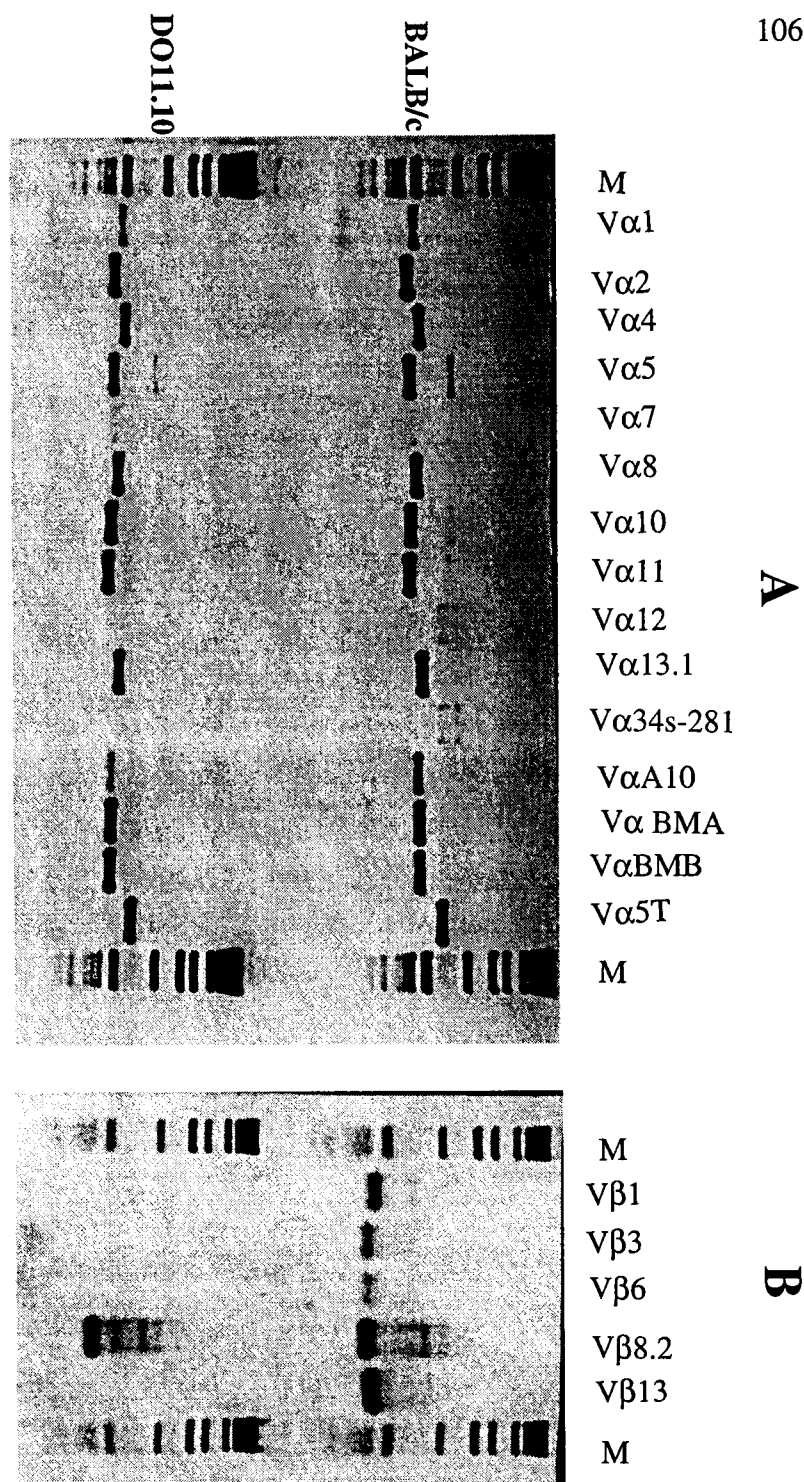


Figure 4: Flow cytometric analysis of draining lymph node cells for CD4+ T cells, CD8+ T cells and CD4+ KJ1.26.1+ T cells from BALB/c mice (left column), DO11.10 mice (middle column) and Tg-SCID mice (right column). Numbers indicate relative percentages of cells with in a quadrant.

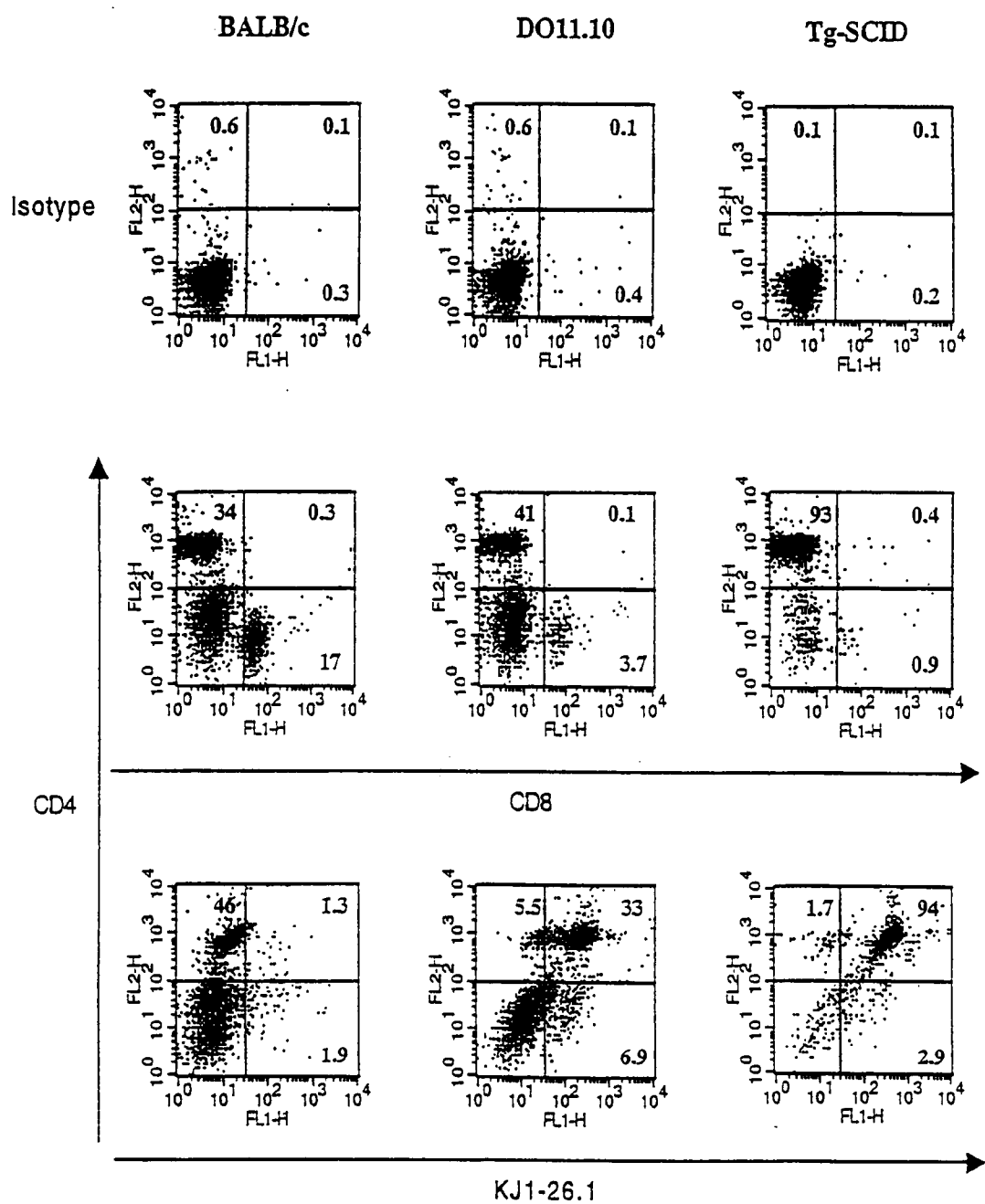


Figure 5: Clinical score from HSV-1 RE infected BALB/c, DO11.10, Tg-SCID mice on day 6 (left panel), day 8 (middle panel) and day 11 (right panel). Each dot represents the value for an individual mouse, and the horizontal line represents the mean clinical score for the group. Each group of mice consisted of six animals and the results shown here represent one of the three experiments performed.

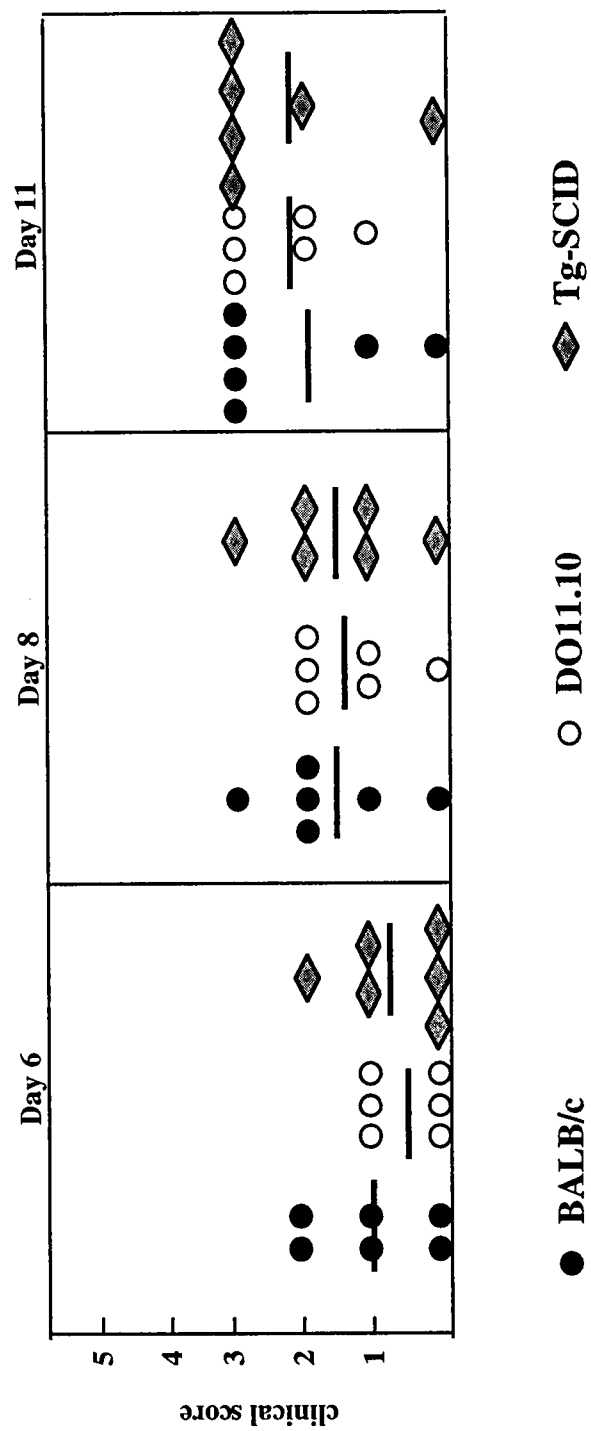


Figure 6: Histopathological sections of corneas at day 11 p.i. from HSV-1 infected Tg-SCID (A, B) and SCID mice (C,D). Immunoperoxidase staining demonstrating CD4+ T cells (E) and KJ1.26.1+ (anti-Ova TCR) cells (F) at day 11 p.i. in the cornea of HSV-1 infected Tg-SCID mice. Magnifications, X200 (A, C), X400 (B, D), and X800 (E, F).

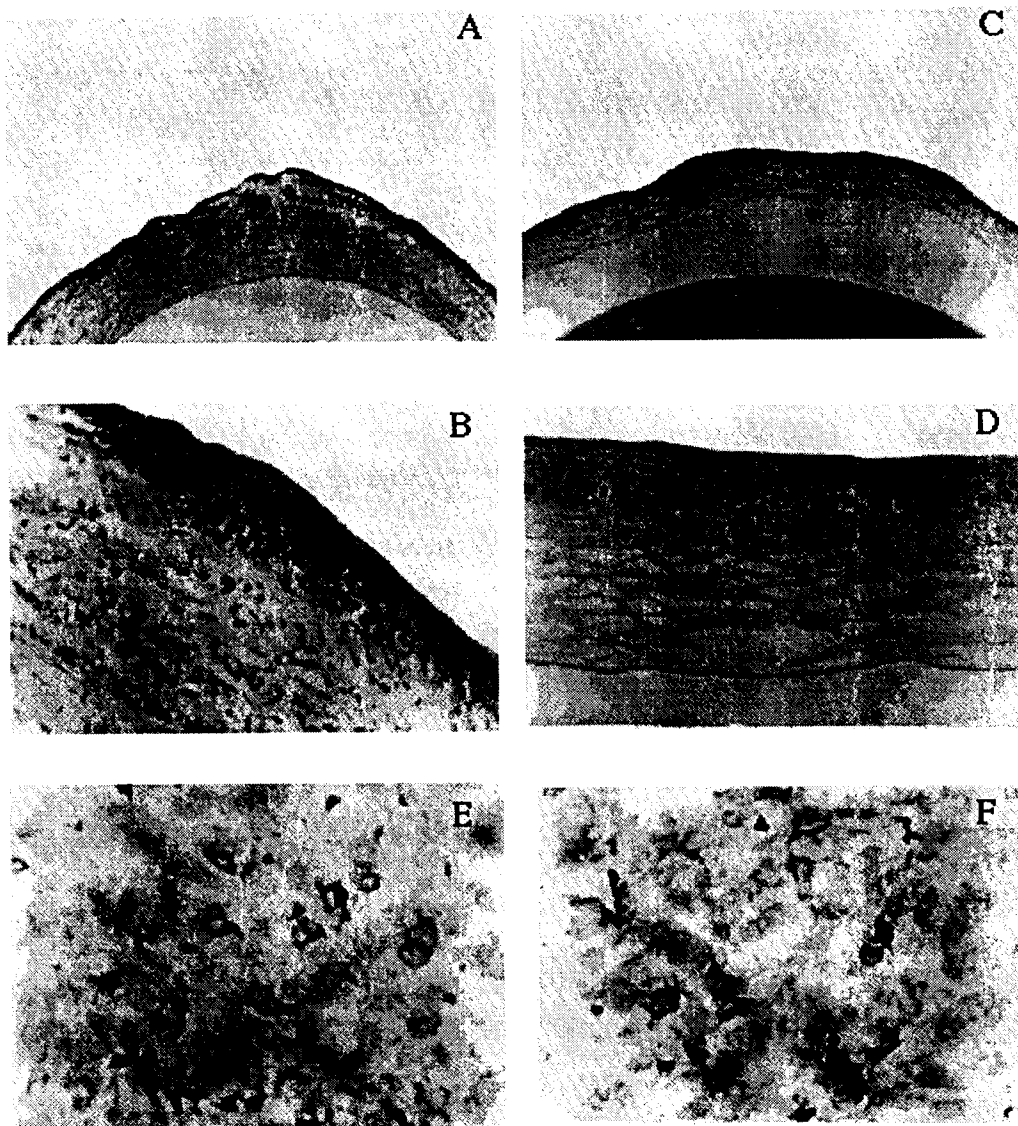


Figure 7: Clinical score from HSV-RE infected Tg-SCID mice and DO11.10 mice on day 6, day 8, and day11 post infection.

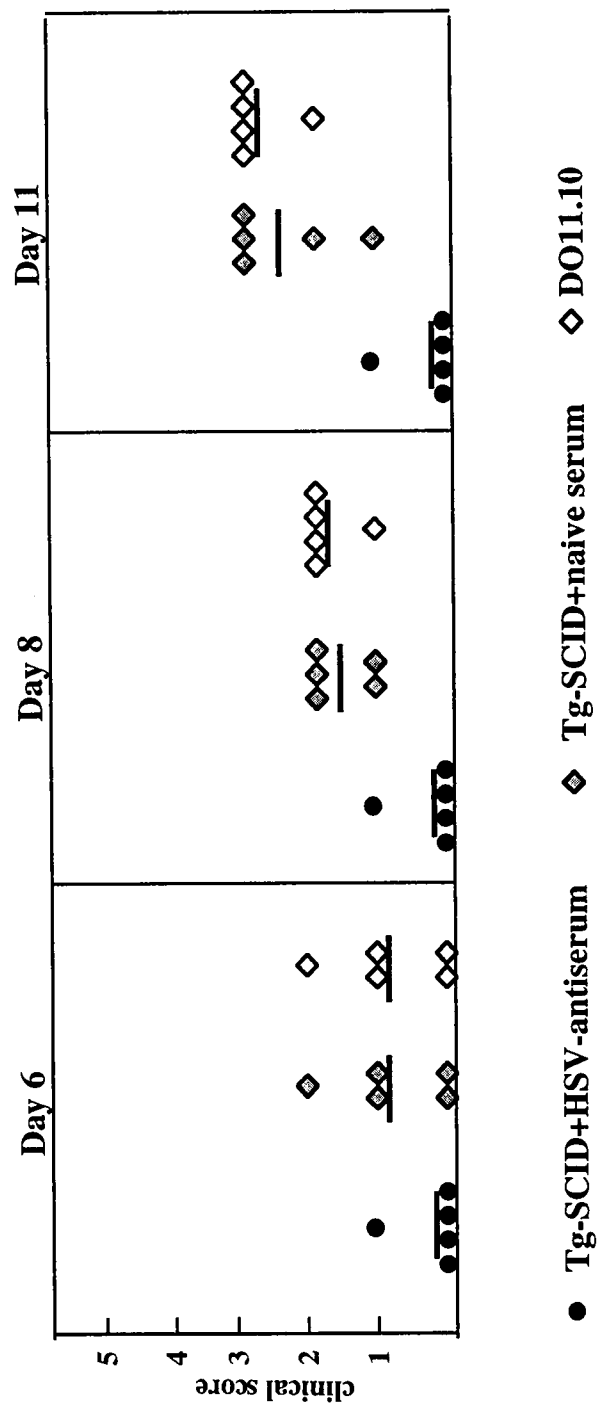


Figure 8: Phenotypic analysis of draining lymph node cells from naive and HSV infected mice. Cervical lymph node cells from BALB/c (upper panel) and Tg-SCID (lower panel) mice were stained with anti-CD4 FITC in combination with either anti-CD45RB PE or anti-L-Selectin PE antibodies and analyzed by flow cytometry. Bold line, profile of the naive mice; shaded area, profile of the HSV infected mice; dotted line, isotype antibody control. Data shown are representative of four experiments.

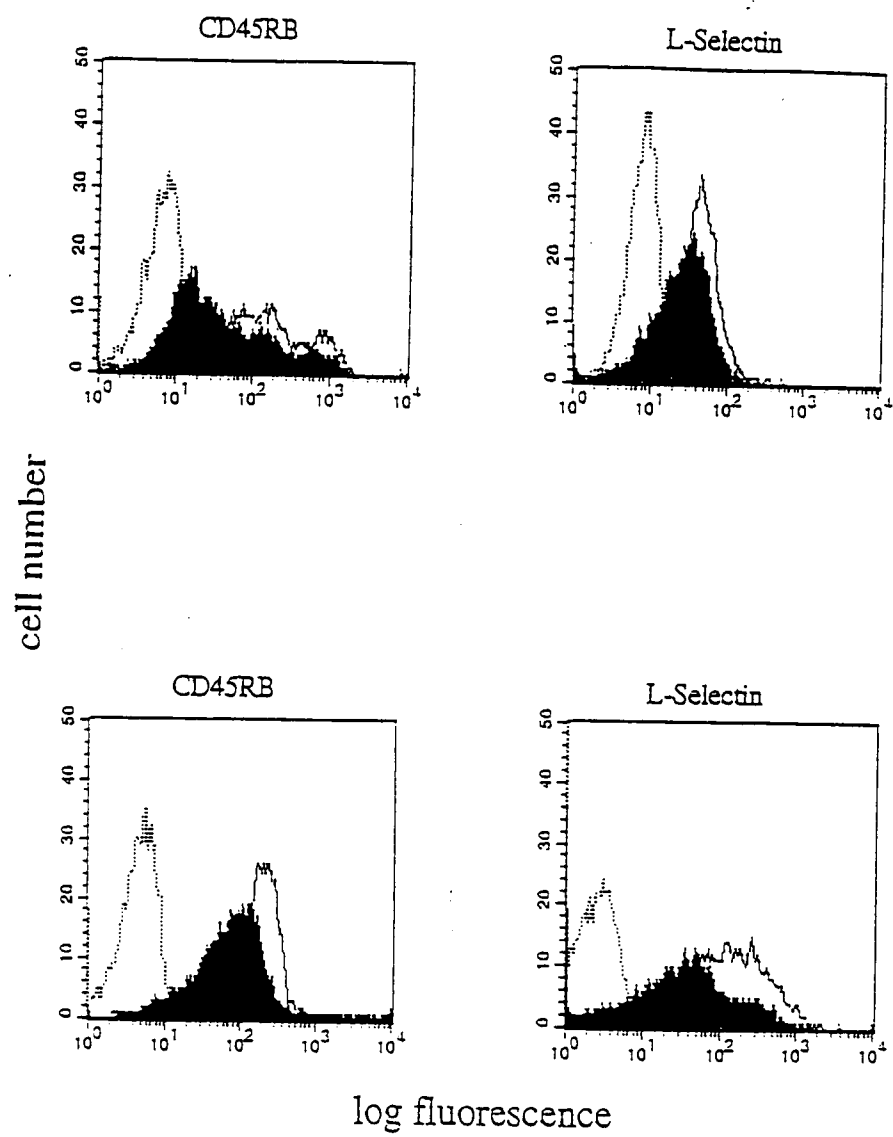


Figure 9: V α expression in BALB/c and Tg-SCID mice splenic T cells. Total RNA was isolated from splenocytes and reverse transcribed as described in Materials and Methods. The nomenclature of V α and the C α primer used for amplification are as mentioned in Reference No.17. Upper panel, Lane 1-13: Marker (100 bp DNA ladder), V α 1, V α 2, V α 3, V α 4, V α 8, V α 10, V α 11, V α 12, V α 13.1, V α 34.S-281, Negative control. Lower panel, Lane 1-11: Marker (100 bp DNA ladder), V α 1, V α 2, V α 3, V α 4, V α 8, V α 10, V α 11, V α 12, V α 13.1, V α 34.S-281. V α 6, V α 7, and V α 9 were also analyzed and found to be not expressed in BALB/c. Therefore they were omitted in re-analysis. Not shown here is the data for V β analysis in BALB/c and Tg-SCID splenocytes and the data for extent of V α expression in HSV infected BALB/c and Tg-SCID mice splenocytes.

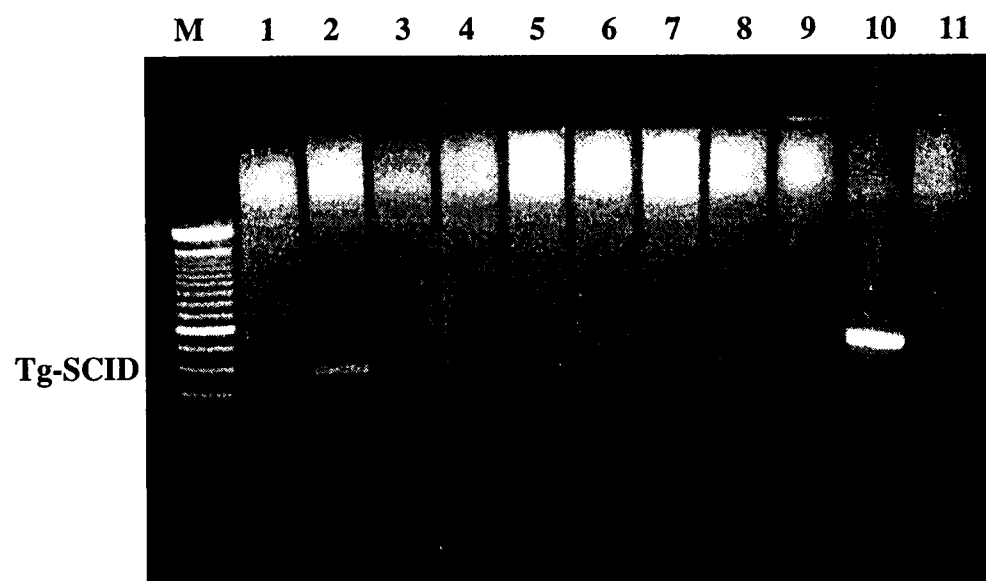
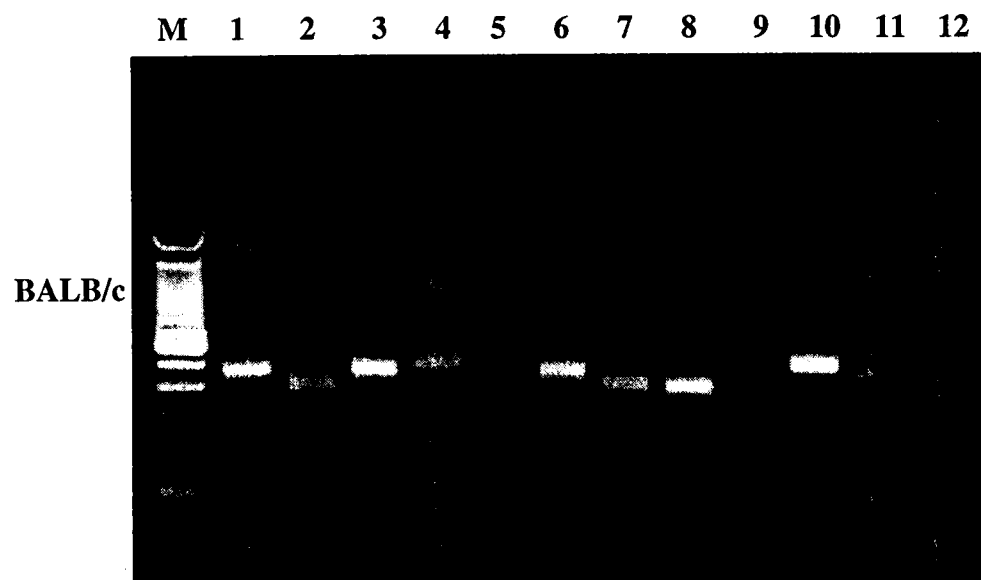


Figure 10: Cell surface expression of dual V α -T cells in Tg-SCID mice. Draining lymph nodes cells from BALB/c (A), DO11.10 (B), and Tg-SCID (C) mice were stained with FITC or PE conjugated mAb for V α 2TCR, CD4, KJ1-26.1 (anti-OVA TCR). Data shown are representative of three experiments.

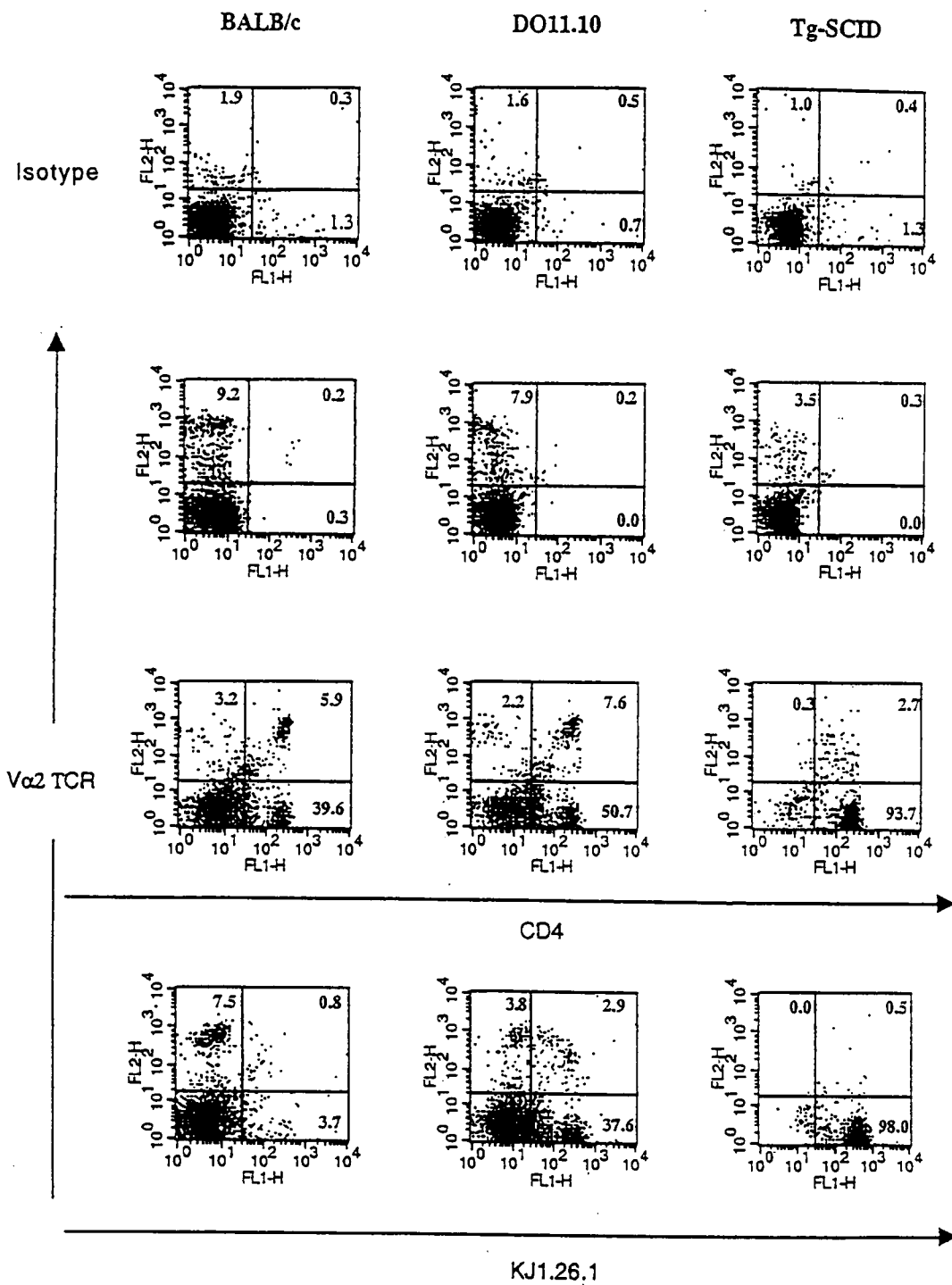


Figure 11: Leaky V α expression in corneal samples of HSK positive BALB/c and Tg-SCID mice. Total RNA was isolated from corneal lysate and reverse transcribed as described in Materials and Methods. Lane 1-7: Marker (100 bp DNA ladder), V α 1, V α 2, V α 5, V α 11, V α 13.1, V α 8.

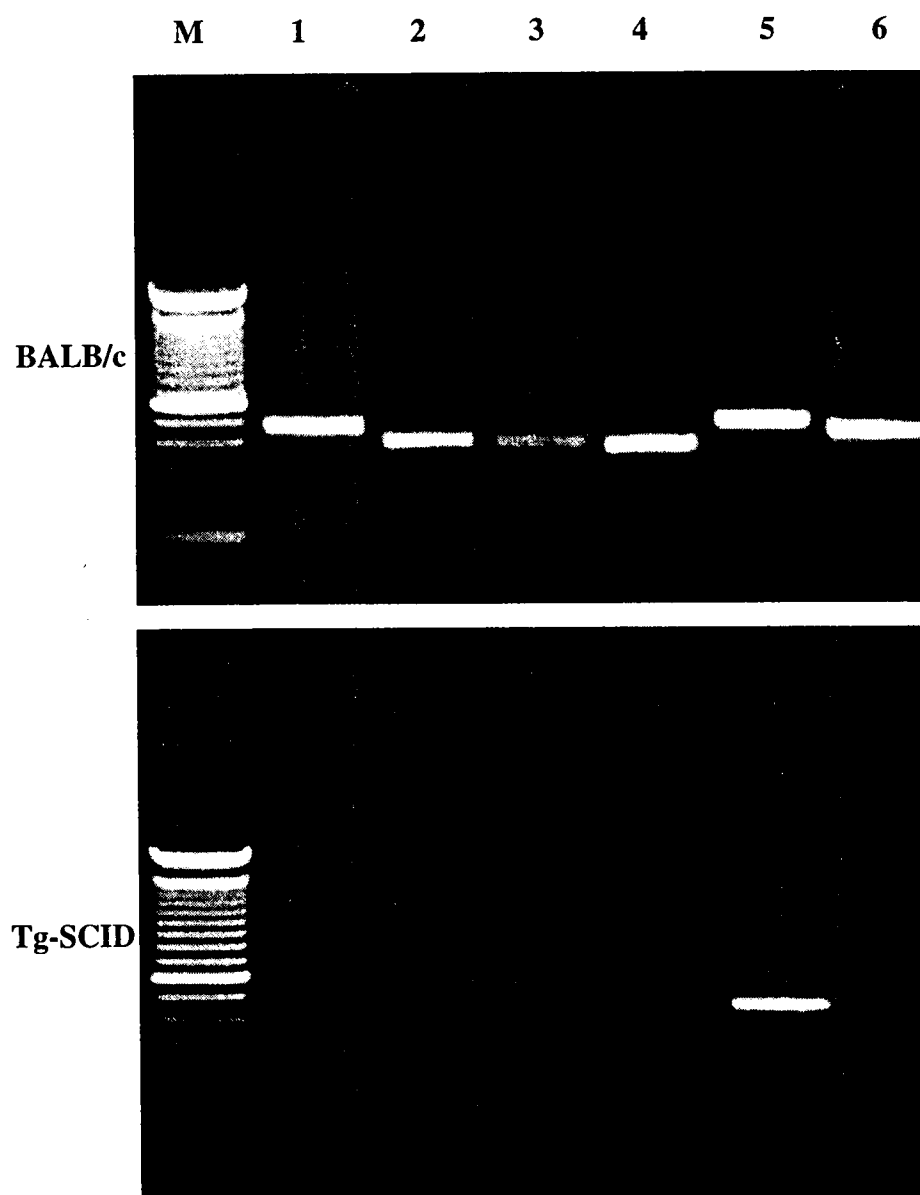


Table 1. Immune responses in HSV-RE infected BALB/c mice and DO11.10 mice.

Mice	Antigen-specific proliferation (cpm) ^a							
	HSV stimulators				OVA stimulators			
	Responders	Responders+ uninfected stimulators	Responders+ HSV stimulators	SI ^b	Responders uninfected stimulators	Responders+ Ova stimulators	SI	HSV-specific Ig G ^c (ng/ml)
BALB/c	200±99	155±60	8159±501	52	309±20	196±134	125±69	1 17427±1646 6691±1394
DO11.10	116±39	43±20	1178±319	27	171±107	192±73	12180±801	63 16198±2429 6797±1442

^a Antigen-specific proliferation in HSV-1 RE infected BALB/c and DO11.10 mice. Five mice/group were individually assayed for proliferation in response to UV-HSV and Ova peptide(323-339) by incorporation of ³[H]. The values represent the mean ± SD.

^b SI, stimulation index, ^c Serum Ig G were measured by standard capture ELISA assay with five mice per group. The values represent the mean ± SD

Table 2: Duration of the presence of infectious virus in the eyeswabs following ocular HSV-1 RE infection ^a

	Days postinfection										
	1	2	3	4	5	6	7	8	9	11	
Mice											
BALB/c	0.38x10 ^{3b}	20x10 ³	0.89x10 ³	11x10 ³	4x10 ³	0.7x10 ³	UD ^c	UD	UD	UD	
Tg-SCID	3x10 ³	10x10 ³	9x10 ³	47x10 ³	38x10 ³	40x10 ³	35x10 ³	50x10 ³	15x10 ³	10x10 ³	

^a HSV-1 RE (5x10⁵ pfu) was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in eye swabs was determined by the agarose overlay method, the number of pfu/ml were estimated. At each time point eye swabs were collected from four mice (n= 4) and viral titers determined.^b pfu/ml, plaque-forming unit per ml, ^c UD, undetectable i.e., below the sensitivity of the assay (<10 pfu)

Table 3: Cell count from cervical lymphnodes of BALB/c and Tg-SCID mice ^a

Mice	Cell count (number of cells/ml)	
	Naive	HSV-immune
BALB/c	2.2×10^6	7.3×10^6
Tg-SCID	1.0×10^6	2.3×10^6

^a HSV-1 RE (5×10^5) was inoculated on scarified cornea of mice and on day 10, draining lymph node cells were counted by trypan blue exclusion method. Data represents one of the four analyses performed.

Table 4: Immune responses in HSV-RE infected BALB/c mice, DO11.10 mice and Tg-SCID mice

Mice	Antigen-specific proliferation (cpm) ^a										IFN- γ ^d (ng/ml)	
	HSV stimulators			OVA stimulators				ConA	HSV specific Ig G ^c (ng/ml)	ConA		
	Responders	Responders+ uninfected stimulators	Responders+ HSV stimulators	SI ^b	Responders	Responders+ uninfected stimulators	Responders+ OVA stimulators					SI
BALB/c	104 $\pm$ 47	62 $\pm$ 19	1283 $\pm$ 378 ^e	21	95 $\pm$ 23	84 $\pm$ 12	44 $\pm$ 24	1	14800 $\pm$ 5144	11706 $\pm$ 1395	14 $\pm$ 3	49 $\pm$ 09
DO11.10	88 $\pm$ 54	33 $\pm$ 08	952 $\pm$ 310 ^f	18	62 $\pm$ 14	111 $\pm$ 61	4520 $\pm$ 588	34	18981 $\pm$ 1914	9842 $\pm$ 1120	9 $\pm$ 4	37 $\pm$ 08
Tg-SCID	63 $\pm$ 18	39 $\pm$ 11	59 $\pm$ 03 ^g	1	67 $\pm$ 35	136 $\pm$ 88	5768 $\pm$ 1264	42	14950 $\pm$ 5826	ND	<1	29 $\pm$ 12

^a Antigen-specific proliferation in HSV-1 RE infected BALB/c, DO11.10 and Tg-SCID mice. Five mice/group were individually assayed for proliferation in response to UV-HSV and OVA peptide(323-339) by incorporation of [³H]. The values (expressed as counts per minute (cpm)) represent the mean $\pm$ SD. ^b SI, Stimulation Index, ^c Serum Ig G were measured by standard capture ELISA assay with five mice per group. The values (expressed as ng/ml) represent the mean $\pm$ SD. ^d Splenocytes from individual mice were restimulated *in vitro* with UV-HSV or ConA and the supernatants were assayed by ELISA and the level of IFN- γ were determined as described in materials and methods. Values represent the mean $\pm$ SD of the concentration of proteins in supernatants, By Student's t-test: ^e versus ^f p<0.00003, ^f versus ^g p<0.0003

Table 5: DTH responses in HSV infected BALB/c and Tg-SCID mice ^a

Mice	Immunized with	Challenged with	
		left ear (vero extract)	right ear (UV HSV-1 KOS)
Tg-SCID	HSV-1 KOS	2.3±0.5 ^b	2.8±2.1 ^c
Tg-SCID	PBS	2.8±1.3	2.5±0.6 ^d
BALB/c	HSV-1 KOS	1.8±1.0	13.5±1.3 ^e
BALB/c	PBS	2.2±1.0	2.0±1.1

^a BALB/c mice or Tg-SCID mice were immunized with 1×10^6 UV-inactivated HSV-1 KOS virus in the hind foot pads. Mice received PBS (pH7.2) served as the negative control. Two weeks after immunization, mice were challenged with 20µl of 1×10^5 UV HSV-1 KOS in the right ear pinna and same volume of vero cell extract was injected in the left ear pinna. Results shown above represent the mean increase in ear thickness after challenge. Values (expressed as nxmm^{-2}) represent the mean $\pm$ SD.

By Student's t-test: ^b versus ^c $p=0.7$, ^c versus ^d $p=0.8$, ^c versus ^e $p<0.001$

Table 6: HSV induced antigen specific proliferative responses of splenocytes and DTH responses of mice immunized with HSV mutant (AN-1).

Mice	Antigen specific proliferation (cpm) ^a				DTH response (mm ⁻²) ^b		
	HSV stimulators		OVA stimulators		Challenged with		
	HSV infected stimulators	Uninfected stimulators	Stimulation index	OVA stimulators	Uninfected stimulators	Stimulation index	
BALB/c	29463±5615 ^c	2908±1215	10	4023±910	3657±1027	1	2.3±0.5
DO11.10	19029±6805 ^d	941±534	20	48340±8532	1438±627	33	2.8±1.3
Tg-SCID	394±89 ^e	387±84	1	81802±12842	999±298	82	1.8±1.0
							2.2±1.5 ^h

Mice were immunized with AN-1 virus (5×10^6 TCID₅₀) through foot pad injection and were boosted with the same dose of virus on day 14. On day 35 post-immunization, splenocytes were individually assayed for antigen specific proliferation as described in materials and methods. The values {expressed as counts per minute (cpm)} represent the mean±SD.

^b Forty eight hours before the termination of the experiment, mice were checked for DTH responses as described in materials and methods.

Results shown above represent the mean increase in ear thickness after challenge. Values (expressed as $n \times \text{mm}^{-2}$) represent the mean±SD.

By Student's t-test: ^c Vs ^e $p < 0.00004$, ^d Vs ^e $p < 0.001$, ^f Vs ^h $p < 0.001$, ^g Vs ^h $p < 0.003$

Table 7: Antibody response, mean clinical score and antigen specific proliferative responses of splenocytes in mice immunized with HSV mutant (AN-1) and challenged ocularly with HSV-1RE.

Mice (n=4)	HSV specific IgG ^b (ng/ml)	Mean clinical score ^c	Antigen specific proliferation (cpm) ^a					
			HSV stimulators			OVA stimulators		
			HSV infected stimulators	Uninfected stimulators	Stimulation index	OVA stimulators	Uninfected stimulators	Stimulation index
BALB/c	16050±1350	0.8	32877±5018 ^d	1763±694	19	1856±90	1430±596	1
DO11.10	12525±1878	0	13182±3256 ^e	2719±1452	5	27907±9175	1291±293	22
Tg-SCID	UD	2.5	76±2 ^f	110±49	1	8746±5842	122±66	72

^a On day 11 post challenge, mice splenocytes were individually assayed for antigen specific proliferation as described in materials and methods. The values {expressed as counts per minute (cpm)} represent the mean±SD.

^b On day 14 post immunization, serum IgG were measured by standard capture ELISA assay as described in Materials and Methods.

The values (expressed as ng/ml) represent the mean±SD. UD, undetectable i.e., below the sensitivity of the assay (100pg/ml).

^c Values represent mean clinical score of mice on day 11 post challenge with HSV-1RE

By Student's t -test: d versus f < 0.0003, e versus f < 0.002

Table 8: Quantitative comparison of leaky V α chain in Tg-SCID samples with BALB/c splenic T cells ^a

V α	% expression levels	
	Tg-SCID	BALB/c
1	2.5	98
2	6.3	76
5	4.5	53
11	3.2	93

^aEqual amount of cDNA samples were amplified with V α 1, V α 2, V α 5, and V α 11 primers and compared with V α 13.1 expression in Tg-SCID and BALB/c splenocytes. The V α 13.1 expression in each sample is taken as 100% and the results of other V α expression were compared. { V α 8 expression in both samples were also analyzed. The expression levels of V α 8 in BALB/c samples in relation to V α 13.1 is 94%. Since V α 8 is unproductively rearranged and expressed in Tg-SCID samples (Fig 8), it was omitted in quantitative comparison.

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**Part 5: Virus replication and dissemination in to the corneal stroma is
necessary for HSK in Tg-SCID mice.**

Chapter 1: Abstract

In murine model of HSK, the lesions in the corneal stroma are considered to be immunopathological and CD4+ T cells of the Th1 phenotype are shown to be essential. Recently we have shown that HSK could be induced in Tg-SCID mice (DO11.10 back crossed to SCID mice) that are incapable of eliciting HSV-specific immunity. Upon corneal scarification, Tg-SCID mice showed HSK prior to death due to encephalitis. In order to make Tg-SCIDs live longer and there by evaluate whether the lesions in these mice get severe or resolve, in the present study we transferred HSV immune CD8+ T cells or anti-HSV serum from BALB/c mice in to HSV infected Tg-SCID mice. Transfer of HSV-immune serum at 24h or 72h, or HSV-immune CD8+ T cells at 24h post infection protected mice from encephalitis and corneal disease. Interestingly enough, CD8+ T cells transferred at 72h post infection resulted in severe corneal pathology apart from protection from encephalitis. Analysis of cornea for the presence of virus by immunohistochemistry suggested that where as the cornea of Tg-SCID mice receiving immune serum showed virus only in the epithelium, CD8+ T cell recipients had virus even in the corneal stroma. These results indicate that a possible stimulus for the ocular lesion development in response to HSV-1 infection could be the virus replication driven proinflammatory milieu leading to bystander activation of CD4+ T cells in the cornea.

Chapter 2: Introduction

Ocular infection with herpes simplex virus-1 (HSV-1) results in blinding due to inflammatory reaction of corneal stroma termed as herpetic stromal keratitis(HSK). In humans, HSK is one of the common causes of nontraumatic corneal blindness (1). In mice, HSK as a result of primary infection with HSV-1 on scarified corneal surface is shown to be an immunopathological lesion mediated by CD4+ T cells that elaborate Th1 type of cytokines (2- 4). Although PMN, Langerhans cells and CD4+ T cells and their cytokine and chemokine pattern have been well documented, the precise mechanisms of disease expression remain to be resolved.

Although HSK is induced by HSV-1 infection, one of the characteristic feature of HSK is that the lesion occurs at the site and at a time when viral antigens are no longer detectable. Virus is cleared from the cornea several days before the lesions appear (5). Lesions do not occur in HSV-infected SCID mice but can be induced by transfer of either immune or naïve CD4+ T cells (6). This contrasts from other virus induced immunopathological conditions where virus persists in replicating form and T cells react against viral antigens (7-9). Recently Cantor and associates showed that two T cell clones reactive against HSV-infected mouse corneal lysate and IgG2ab peptide (298-314) also reacted to UL6(292-304), a HSV virion associated protein and that transferring any of these clones to HSV(KOS) infected nude mice resulted in corneal pathology. Recently, we have shown that HSK could be induced in mice that are incapable of eliciting HSV-specific immunity (10).

In this study we evaluated whether manifestation of HSK in Tg-SCID mice (DO11.10 mice back crossed to SCID mice) is indeed because of initial virus induced damage and could be mediated by CD4+ T cells of irrelevant specificity. HSV-infected Tg-SCID mice developed HSK before succumbing to encephalitis. In order to make these mice live longer which helps to evaluate whether the lesions get much severe to that of BALB/c mice or resolve, in the present study we transferred HSV immune CD8+ T cells or anti-HSV serum to HSV infected Tg-SCID mice. Transfer of CD8+ T cells or anti-HSV serum at 24h post infection protected mice from encephalitis and disease manifestation. Where as transfer of anti-HSV serum at 72h p.i., protected Tg-SCID from death and ocular pathology, CD8+ T cells transferred at 72h p.i., interestingly, resulted in severe corneal inflammation apart from protection from encephalitis. Analysis of the infected cornea for virus persistence revealed that anti-HSV serum and CD8+ T cells seem to regulate virus spread in different manner. These results indicate that a possible stimulus for the ocular lesion development in response to HSV-1 infection could be the virus replication driven cytokines and chemokine milieu leading to bystander activation of CD4+ T cells at an immune privileged site.

Chapter 3: Materials and methods

Mice

Four to five week old BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN), OVA-TCR transgenic mice (DO11.10 mice- kindly provided by Dr. Dennis Loh, Washington University School of Medicine, St. Louis, MO and Dr. Casey Weaver, University of Alabama, Birmingham, AL) were used. Transgenic mice were crossed to SCID mice for two generations and with brother - sister mating, it was possible to obtain Tg-SCID mice (as screened by PCR-tail DNA, and serum IgM-ELISA). Tg-SCID mice thus obtained had only CD4+ T cells and few or no CD8+T cells as tested by FACS analysis and were without any B cells as screened by serum IgM ELISA. Also almost all (>98%) of the CD4+ T cells were KJ1.26.1 (anti-OvaTCR antibody) positive T cells. Transgenic mice (DO11.10) and HSK susceptible BALB/c groups of mice were used as the control animals. All food, water, bedding, and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. To prevent bacterial superinfections, all Tg-SCID and SCID mice received prophylactic treatment of sulfatrim pediatric suspensions (Barre-National Inc., Baltimore, MD) at the rate of 5 ml per 200 ml of drinking water. Antibiotic treatment was started 1 day before the beginning of the experiments. All experiments were conducted in compliance with the *Guide for the Care and Use of Laboratory Animal Resources*, Commission Life Sciences, and National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All ocular

experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use and care of laboratory animals.

Virus and corneal infections

HSV-1 RE strain used was propagated on Vero cells and stored as infectious cell preparations at -70°C . The corneal surfaces of deeply anaesthetized mice (methoxy flurane; Pittman-Moore, Mondelein, IL) were scarified with a sterile 27-gauge needle and 5×10^5 TCID-50 HSV-1 RE strain was applied in a $4.0\ \mu\text{l}$ volume and gently massaged on to the eye lids.

Adoptive transfer

To assess the role of virus replication in lesion development, mice were infected with virus on the cornea and 24h or 72h later were given i.v., $300\ \mu\text{l}$ of anti-HSV serum ($40\ \mu\text{g}/\text{ml}$, HSV-specific IgG as determined by ELISA) or HSV-immune CD8+ T cells (2.5×10^6 cells/mouse, i.v) from BALB/c mice. In such experiments controls were infected and given serum or CD8+ T cells from naïve BALB/c mice.

Clinical evaluation

Mice were scored according to their clinical severity by a person who was blinded to the experimental design using a slit lamp biomicroscope (Keeler Instruments, Broomall, PA) as follows; Score 0, normal cornea ; Score 1, Neovascularization at periphery, iris visible ; Score 2, partial opacity, iris not visible ; Score 3,

neovascularization at center, opacity ; Score 4, bleb formation ; Score 5, necrosis. The data were plotted as the mean daily clinical score for all animals in a particular treatment group.

Immunoglobulin ELISA

Serum collected was analyzed for HSV-specific total IgG, using standard ELISA as described previously (11). Basically, ELISA plates were coated with 100 μ l of HSV antigen in carbonate buffer (pH 9.8), and after overnight incubation at 40°C, the plates were washed three times in PBS containing 0.05% Tween 20, pH 7.2 (PBST) and then blocked using PBS (pH 7.2) with 3% dehydrated milk for 2 h at 37° C. A total of 200 μ l of serially diluted serum samples (prediluted in PBST) were added in duplicate, and washed wells coated with goat anti mouse IgG (0.25 μ g/ml) were treated with serially diluted standard mouse IgG. Plates were incubated for 2h at 37 C. After three washes, 100 μ l of goat anti-mouse IgG HRP was added. After three washes, 2, 2-azino-bis 3-ethyl benz-thiazoline- 6 -sulfonic acid substrate (Sigma, St. Louis, MO, no. A1888) was added. The concentration of the antibodies in the serum samples was determined from the standard curve.

Histopathology

Eleven days after infection, sections of eye were prepared for histopathology according to standard procedures. Briefly, at the termination of experiments whole eyes were fixed in 10% buffered neutral formalin and embedded in

paraffin. Tissue sections were stained with hematoxylin and eosin. Sections were observed for thickness of cornea, presence of inflammatory infiltrates, neovascularization epithelial erosions superficial or deep ulcers and corneal perforation.

Proliferation assay

Splenocytes (responders) at day 11 post infection were collected and restimulated in vitro with HSV-1 KOS (m.o.i.(multiplicity of infection) of 1.5 before UV irradiation) or OVA peptide (Research Genetics, Inc. Huntsville, AL). The HSV-1 specific lymphoproliferation was measured as described previously (12). Briefly, responders : stimulator ratios tested ranged from 10:1 to 0.625:1. Responders + stimulator mixtures were incubated at 37 C for 4 days. [3H] TdR (1.0 μ Ci/well) was added, harvested 18 h later, and read using a beta scintillation counter (Inotech, Trace 96). The results were expressed as mean+SD.

RNA isolation and RT-PCR

RNA extraction: On day 11 post infection, corneas from the mice were collected, transferred to Tri-reagent (Molecular Biology Inc., Cincinnati, OH). The corneas were then teased with sterile 21 gauge needle and titrated with a sterile 1 ml syringe plunger to expose the cells to lytic action of the Tri-reagent. The total cellular RNA was isolated from the Tri-reagent cellular lysate by adding chloroform and centrifugation followed by ethanol / isopropyl ethanol precipitation of the aqueous RNA solution according to the manufacturer's instructions. The RNA thus obtained was reverse transcribed using

oligodT primers and super script (Gibco BRL-Life Technologies- Bethesda, MD) according to standard protocol (13). The cDNA thus obtained was used as a template in subsequent qualitative and quantitative polymerase chain reactions for IFN- γ and TNF- α cytokine mRNA.

Flow cytometric analysis.

To analyze purity of CD8+ T cells in donor cell population, isolated cervical lymph node cells (viable cells) from BALB/c mice were blocked with heat inactivated FBS, washed three times with FACS buffer (1 X PBS with 1% BSA and 0.05% NaN₃). Cells were then stained with anti-CD4 FITC and anti-CD8 PE antibodies. As an isotype control, rat IgG2a FITC and rat IgG2a PE antibodies were used. For analyzing OVA-specific T cells in spleen and lymph node, KJ1-26.1 antibody (anti-OVA TCR Ab, a kind gift from Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO) was used and detected by adding goat-anti-mouse IgG2a-FITC antibody. Events were collected and analyzed using a Becton-Dickinson FACScan analyzer.

Immunohistochemical staining

At the termination of experiments, eyes were enucleated and snap frozen in OCT compound (Miles, Elkhart, IN). Six-micron- thick sections were cut, air dried, and fixed in cold acetone for 5 min. The sections were then blocked with heat inactivated goat serum and stained with biotinylated anti-CD4 or anti-CD8 antibody (PharMingen, San Diego, CA). Frozen sections were also stained for the presence of HSV antigens by the

use of rabbit anti-HSV anti-serum (Dako Corp, Carpinteria, CA) which was followed with biotinylated anti-rabbit Ig (1/20 dilution; Biogenex, San Ramon, CA). Sections were then treated with HRP-conjugated streptavidin (1:1000) and 3,3'-diaminobenzidine (Vector, Burlingame, CA) and counterstained with hematoxylin.

Delayed-type hypersensitivity (DTH) assays

On the day of testing, each mouse was injected with 20 μ l of UV-inactivated HSV-1 KOS (10^6 PFUs before inactivation) in the right ear and the same volume of vero cell extract in the left ear. The right and left ear thickness of each mouse were measured with a screw gauge Odimeter (Oditest, H.C. Kroeplin GMBH, Germany) and recorded individually. The thickness was measured at 1 h before ear injection, then at 24, 48, and 72 h after injections and the values were represented as $n \times \text{mm}^{-2}$. The mean ear thickness of each ear from each group of animals was calculated and the mean increase between before and 48 h after injections were compared between DO11.10 mice and Tg-SCID mice.

Virus recovery

At various time points p.i., eye swabs were taken using sterile swabs soaked in McCoy medium containing 100 IU/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY). The swabs were then placed in tubes containing 500 μ l of the above mentioned transport medium and stored at -80°C . For detection of HSV in swabs, the samples were thawed and vortexed, and 100 μ l of each sample from

individually marked mice was used for quantification of virus recovery by standard PFU assay on vero cell cultures as described elsewhere (14).

Statistical analysis

Wherever specified, data obtained were analyzed for statistical significance by Student's t- test.

Chapter 4: Results

Adoptive transfer of HSV immune CD8+ T cells or antiserum to Tg-SCID mice at 24h.p.i protects from encephalitis and ocular pathology:

Upon corneal scarification with HSV, the Tg-SCID mice succumbed to encephalitis by day 11 post infection and showed lesions comparable to BALB/c mice and DO11.10 mice (clinical score of 3) (16). However, the control BALB/c mice and the DO11.10 mice developed fulminant ocular lesions by day 18 post infection. In order to make the Tg-SCID mice survive for longer periods which would answer the question of whether the lesion of clinical score 3 in Tg-SCID mice resolves or becomes severe, CD8+ T cells were column purified from HSV-immune BALB/c mice and transferred to Tg-SCID mice at 24 hours post infection. As another means of protecting the mice from encephalitis, anti-HSV serum from the donor mice was also used on another group of HSV infected Tg-SCID mice. Control mice received naïve serum. This experiment was designed also to evaluate whether the lesions in Tg-SCID was the result of viral infection or bacterial superinfection that occasionally occurs even in immunocompetant mice. The Tg-SCID mice that received control antiserum (naïve BALB/c serum) showed ocular lesions as assessed by slit lamp biomicroscopy and histopathology (Figs. 1 and 2). However, such mice succumbed to encephalitis by day 11 post infection and hence were terminated and checked for HSV and OVA-specific T cell responses (Table 1). All the recipients of CD8+ T cells and anti-HSV serum were protected from encephalitis, and lived longer, but were also protected from corneal pathology (Figs 1 and 2). Transfer of column purified CD4+ T cells and CD8+ T cells together at 24h p.i. resulted in severe

lesions and protection from encephalitis (data not shown). The CD8+ T cells and anti-HSV serum recipient Tg-SCID mice were terminated on day 25 post infection and analyzed for reactivity to HSV and OVA peptide. The anti-HSV serum recipient Tg-SCID mice and the HSV-specific CD8+ T cell recipient Tg-SCID mice showed no proliferative response to HSV unlike HSV infected DO11.10 control mice. However all the Tg-SCID mice (the control serum recipient Tg-SCIDs, CD8+ T cell recipients and anti-HSV serum recipient) showed proliferative response to OVA peptide (Table1).

To investigate the mechanism of protection from encephalitis and ocular lesions by transfer of antiserum or CD8+ T cells, virus clearance was analyzed from the site of infection. Analysis of eye swabs for infectious virus showed that anti-HSV serum and CD8+ T cells cleared the virus from the cornea by day 3 and day 5 post transfer respectively (Table 2). These results indicate that both anti-HSV serum and HSV specific CD8+ T cells given at 24h p.i conferred protection from HSV and encephalitis by clearing the virus from the site of infection.

As the virus particles that are spread beyond the epithelium, to the stroma of the cornea can not be detected by the eye swabs, in order to confirm duration and extent of virus spread at the site of infection, in a separate experiment, adoptive transfer at 24h p.i was repeated and the eyes were collected for immunohistochemical analysis for the virus. As shown in Fig 3, where as the HSV infected BALB/c mice were positive for the virus till day 5, Tg-SCID mice that received HSV antiserum also showed virus till day 5 and in both cases the virus was restricted to epithelium. In those mice that received HSV-specific CD8+ T cells, virus was demonstrable in the stroma up to day 9 post

transfer although not as much as in the control Tg-SCID mice. This indicated that although both anti-HSV serum and HSV immune CD8+ T cells protected mice from encephalitis and HSK, unlike anti-HSV serum that restricts virus spread to the stroma, the HSV immune CD8+ T cells act in preventing the spread of virus distant from the site of infection (15). However, the control Tg-SCID mice (naïve serum recipients) showed presence of virus in the corneal stroma till they succumbed to encephalitis (day 11).

Transfer of CD8+ T cells at 72h p.i results in exacerbated lesions where as antiserum still confers protection from ocular pathology

In the above experiment, the recipient Tg-SCID mice could have failed to manifest lesions may be because the transfer of HSV specific CD8+ T cells or anti HSV serum at 24 hours post infection resulted in clearance of the virus from the site of infection. In order to check whether the OVA-specific CD4+ T cells would still mediate the disease if the virus is allowed to replicate for longer period of time, adoptive transfer was done at 72 hours post infection. Where as the control Tg-SCID mice showed lesions and succumbed to encephalitis by day 12 p.i, all the antiserum recipients were protected from encephalitis and ocular disease (Figs. 4 and 5). Interestingly, 60% (3 of the 5 mice in two separate experiments) of the HSV immune CD8+ T cell recipient Tg-SCIDs were protected from encephalitis and went on to show severe ocular lesions (Fig. 4). In a separate experiment adoptive transfer of HSV-immune CD8+ T cells in to HSV-infected SCID mice at 72h p.i. failed to show HSK in surviving mice (data not shown). Histopathological section of such eyes showed severe inflammatory infiltrates

comparable to HSV infected DO11.10(wilt type transgenics) mice (Fig.5). By immunohistochemical analysis, the corneal stroma stained positive for CD4+ T cells (Fig.7). Further, such inflamed cornea at day 20 post transfer showed no viral antigens (Fig.6) and CD8+ T cells (Fig.7) in the cornea. However, the spleen and draining lymphnodes still possessed the transferred CD8+ T cells at day 22 (Fig 8). Also, analysis of CD8+ T cell recipient Tg-SCID spleen and cervical lymphnodes revealed that all the CD4+ T cells were OVA-TCR (KJ1-26) positive(Fig.8). To rule out the possibility of contaminating HSV-specific CD4+ T cells in the donor population, before cell transfer, the donor cells were purified on T cell enrichment columns and the purified cells were devoid of any CD4+ T cells by FACS analysis (Fig.9). Analysis of eye swabs showed presence of virus up to day 6 post transfer (day 9 post infection) (Table 2). Analysis of the immune responses in splenocytes at day 20 post transfer revealed that the HSV immune CD8+ T cell recipient Tg-SCID mice showed marginal DTH response to HSV however no detectable proliferative response to HSV as measured by HSV-specific DTH and proliferation assays (Table 3). Once again, all the recipient Tg-SCID mice proliferated to OVA-stimulators. The finding that the lymphoproliferation assay did not show any cross reactive recognition for HSV antigens with OVA-specific T cells was further confirmed by computer based sequence analysis. No significant amino acid sequence homology was found between the known HSV proteins(77 proteins) and OVA peptide (17amino acids). RT-PCR analysis of inflamed cornea of the CD8+ T cell transferred mice revealed enhanced mRNA for cytokines IFN- γ and TNF- α comparable to DO11.10 mice (Fig.10). These results indicate that the virus replication and its spread

to corneal stroma and hence the proinflammatory milieu is necessary in Tg-SCID mice for making the cornea permeable to inflammatory cells that may lead to bystander activation of CD4+ T cells that culminates in virus induced ocular immunopathology.

Chapter 4: Discussion

Previous experiments using Tg-SCID mice showed that HSK occurred in HSV infected Tg-SCID mice as a result of activated CD4+ T cells was with out any detectable reactivity towards HSV antigens (16). In that study HSV infected Tg-SCID mice succumbed to encephalitis by 12 days p.i.. In our attempts to render these mice live longer by transfer of antiHSV serum or CD8+ T cells from HSV immune BALB/c mice, the results of this study indicates that HSK in Tg-SCID mice is virus replication and persistence dependent. Additionally, transfer of anti-HSV serum and CD8+ T cells seem to differentially regulate the virus spread in the host.

Although transfer of both anti-HSV serum and CD8+ T cells was effective in rendering the mice resistance to death due to encephalitis, time of transfer yielded interesting results with respect to corneal pathology. Transfer of anti-HSV serum at either 24h or 72h p.i. protected Tg-SCID mice from encephalitis and corneal pathology. But where as transfer of CD8+ T cells at 24h protected mice from encephaltis and ocular disease, transfer at 72h p.i. inaddition to protection from encephaltis in 60% of mice, also resulted in severe corneal pathology. Analysis of viral antigen upon transfer revealed that where as anti-HSV serum restricted virus to corneal epithelium, Tg-SCID mice that received CD8+ T cells showed virus all over the corneal stroma.

In humans as well as in primary infection of mice with HSV, HSK is considered to be an immunopathological lesion(17). In immunocompetant mice, topical corneal infection with HSV results in HSK mediated primarily by CD4+ T cells (2,3,6). Previously it has been shown that replicating virus as well as viral mRNA is absent from

the cornea at the time when lesions are clinically apparent, and that an initial period of virus replication is necessary to induce HSK (18). Present report is in agreement with such findings that when virus dissemination occurred in to the corneal stroma in case of CD8+ T cell transfer at 72h p.i., severe lesions were observed in the surviving mice. Most virus induced immunopathological disorders result in response to active persistent virus infections where inflammation occurs in response to recognition of viral antigens (7-9). The scenario in HSK was shown to be quite different i.e the virus is usually eliminated from the immunocompetant host atleast from the site of infection before the lesions become apparent (5). In the present study virus was found to be present until the time of death in ocular lesion positive unreconstituted Tg-SCID mice. Failure of anti-HSV serum recipient Tg-SCID mice to show HSK could be that the virus was cleared from the site of infection without spread in to the stroma. Also the reason for lesion development in CD8+ T cell recipient Tg-SCID mice could be that virus disseminated from corneal epithelium to stroma. This is unlikely because immunoperoxidase staining of the inflamed corneas of CD8+ T cell recipient Tg-SCIDs were negative for viral antigens. Another possibility is that CD8+ T cells could themselves be orchestrating the disease along with OVA-specific CD4+ T cells. The latter could be unlikely because we were not able to find CD8+ T cells in lesion manifesting sections by immunochemical staining. Yet another possibility that is gaining importance by other investigators (19) is that virus replication could unmask host-derived determinants or initiate a proinflammatory milieu leading to activation of CD4+ T cells that mediate the inflammatory events. In that study the investigators used a similar approach described in

the present study to address Coxsackie B virus induced IDDM. Their results suggested that islet antigen-specific TCR transgenic mice that were incapable of reactivity to Coxsackie virus protein and well established GAD epitope developed IDDM when infected with Coxsackie B virus. Another possibility worth exploring is that the generation of abundant virus particles in the stroma of the Tg-SCID mice may result in up take by the inflammatory cells, in particular by neutrophils found abundant in early phase of infection and that the apoptotic remnants of inflammatory cells could possibly initiate an autoreactive response.

Figure 1: Clinical score from HSV RE-infected Tg-SCID mice transferred with naïve BALB/c serum, anti-HSV BALB/c serum or HSV-immune CD8+ T cells at 24h p.i..

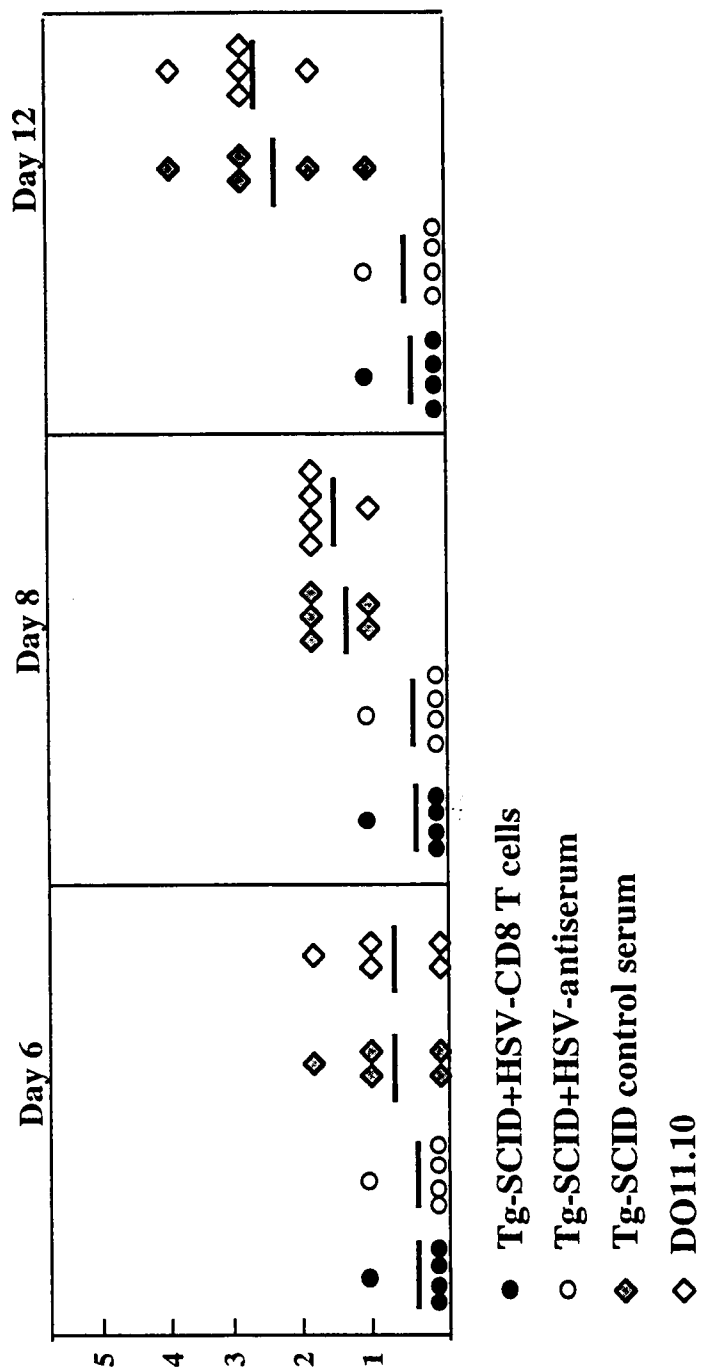


Figure 2: Histopathologic sections (at day 11 p.i.) of cornea of HSV-infected Tg-SCID mice transferred with naïve BALB/c serum(A), anti-HSV serum (B) or HSV-immune CD8+ T cells (C) at 24h p.i..

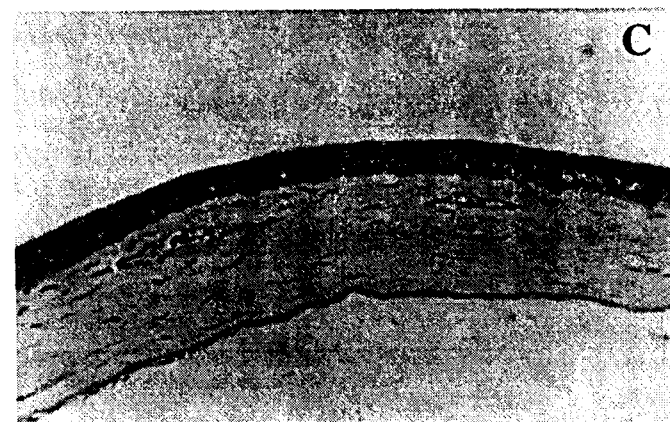
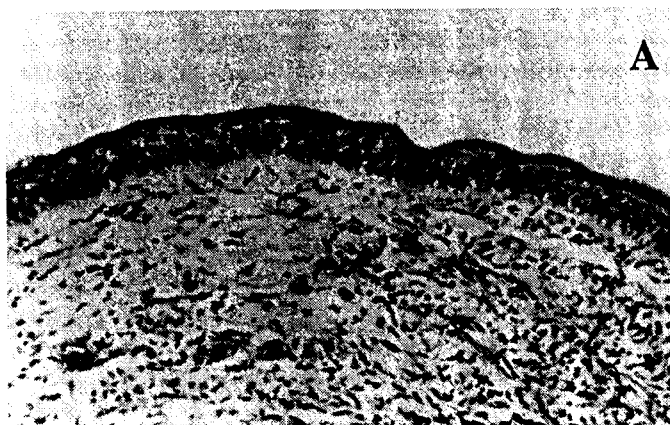


Figure 3: Immunoperoxidase detection for viral antigens (at day 10 p.i.) in corneas of mice infected with HSV and transferred with naïve BALB/c serum (A), anti-HSV serum (B) or HSV-immune CD8⁺ T cells (C) at 24h p.i..



Figure 4: Clinical score from HSV infected Tg-SCID mice transferred with naïve BALB/c serum, anti-HSV serum or HSV-immune CD8+ T cells at 72h p.i..

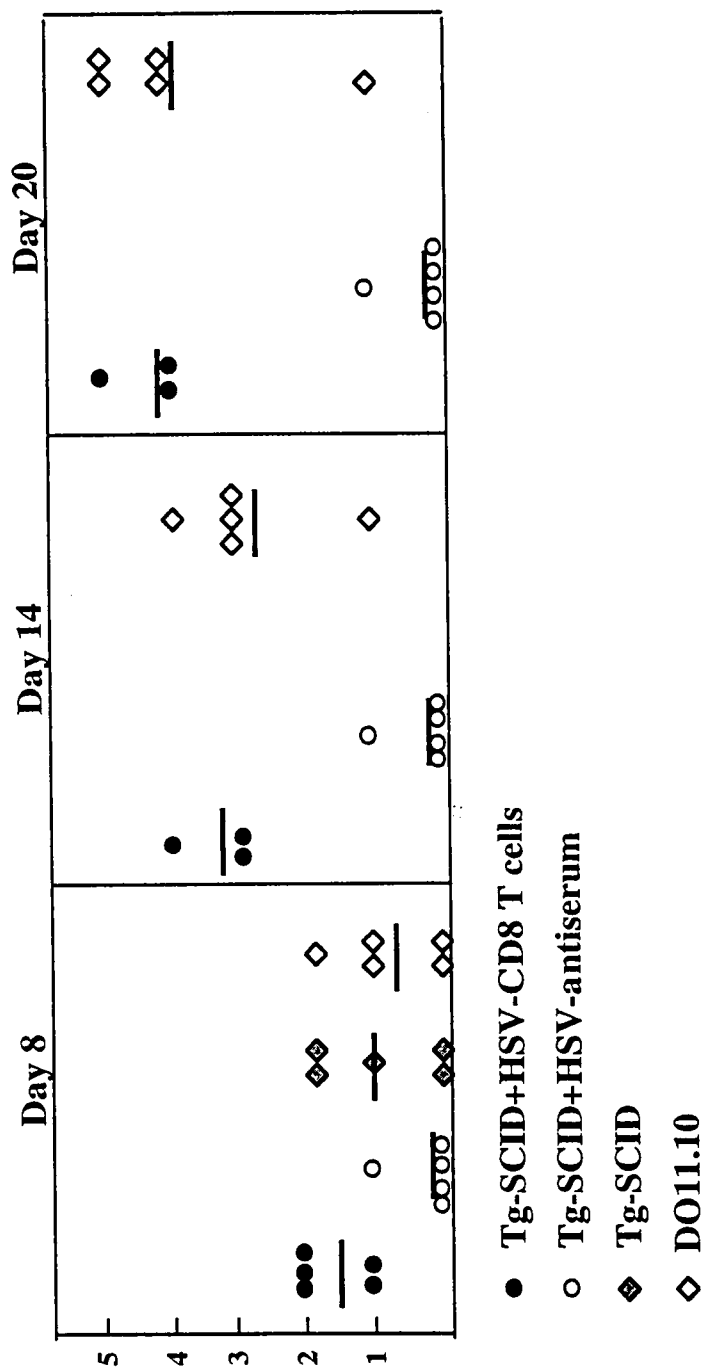


Figure 5: Histopathological sections of HSV infected Tg-SCID mice (A and B) and BALB/c mice (C) cornea at day 22 p.i.. The Tg-SCID mice were given either HSV-immune CD8+ T cells (A) or anti-HSV serum (B) at 72h p.i..



Figure 6: Immunoperoxidase detection of viral antigens in HSV- infected Tg-SCID mice (A and B) and BALB/c mice at day22 p.i.. The Tg-SCID mice were given either HSVimmune CD8+ T cells(A) or anti-HSV serum(B) at 72 h. p.i..

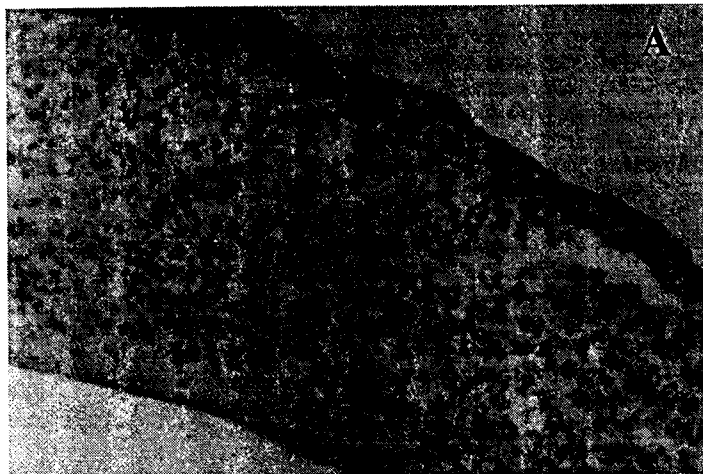


Figure 7: Immunoperoxidase detection of CD4+ T cells (A, C) and CD8+ T cells (B, D) in HSV-infected BALB/c mice (A, B) and Tg-SCID mice (C, D) at day 22 p.i.. The Tg-SCID mice were given HSV-immune CD8+ T cells at 72h.pi..

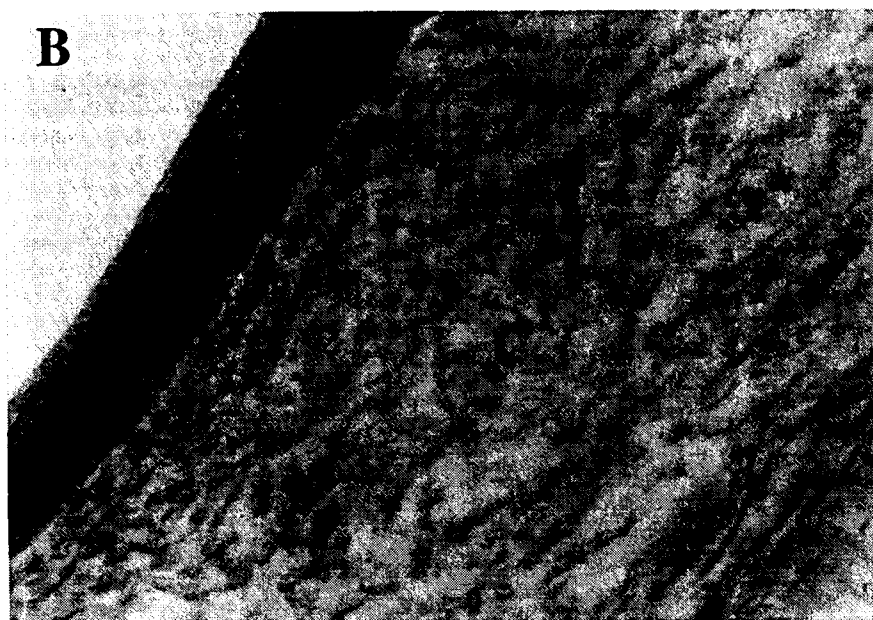
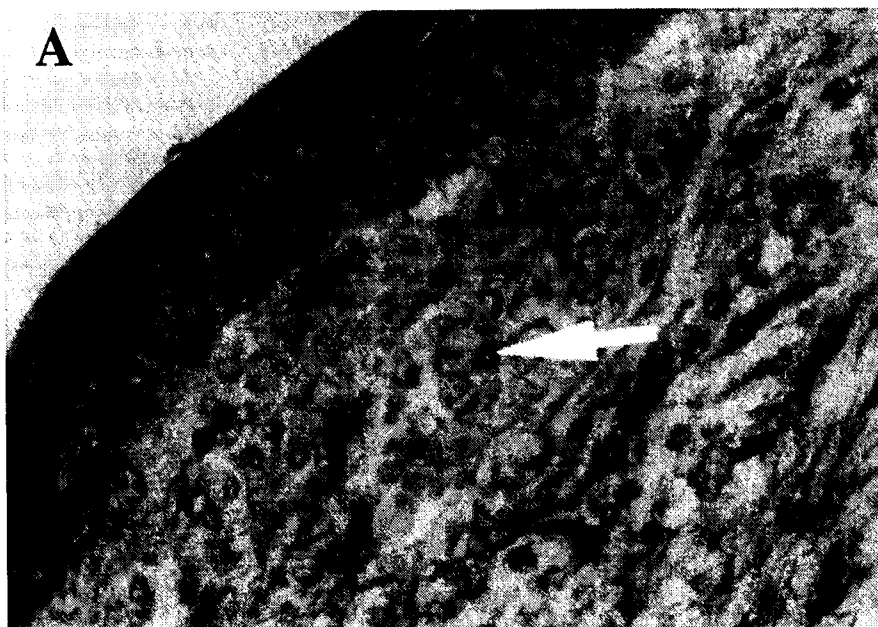




Figure 8: Flow cytometric analysis of spleen and cervical lymphnode cells from CD8+ T cell recipient Tg-SCID mice at day 22 p.i..

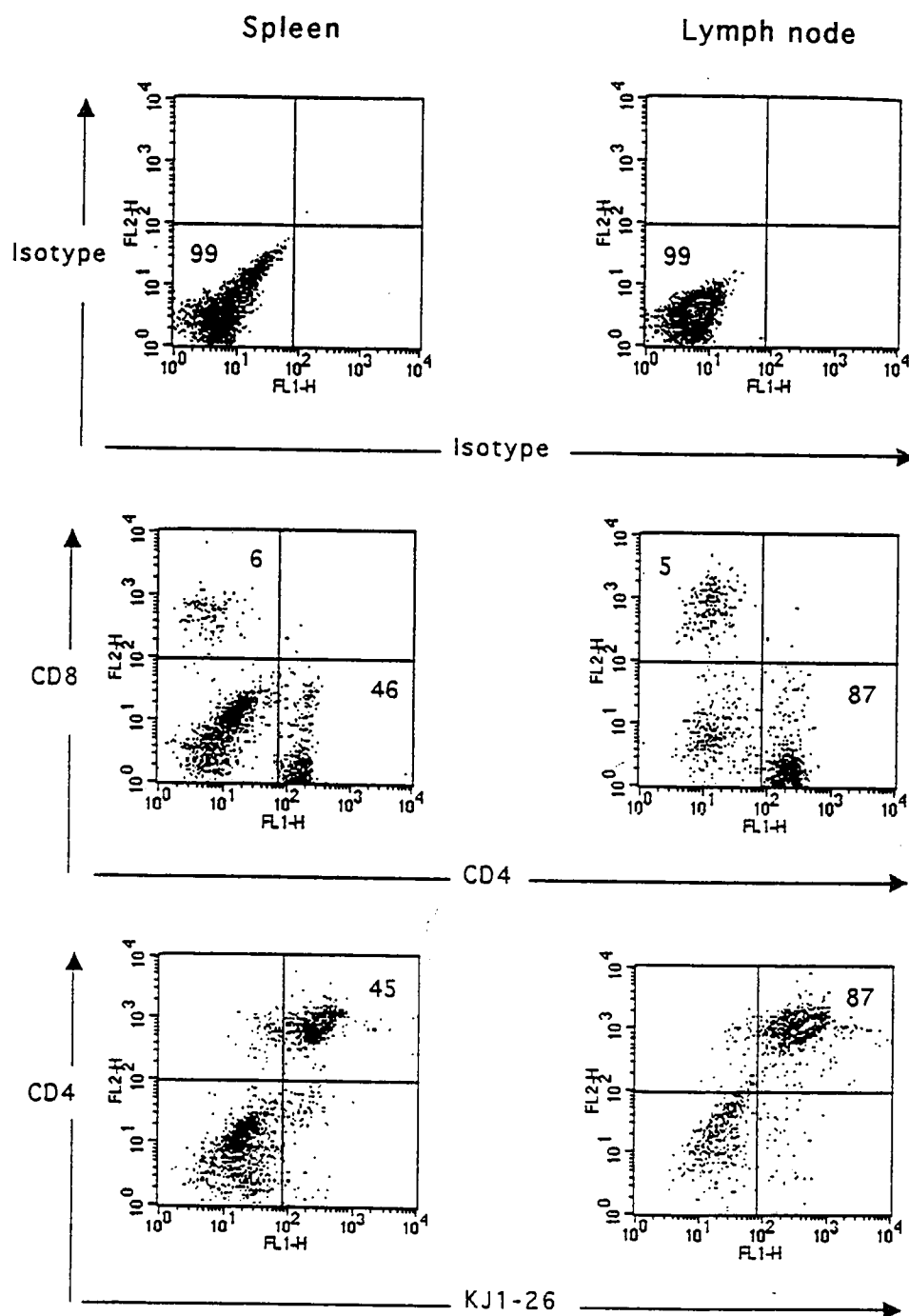


Figure 9: Flow cytometric analysis of CD8+ T cells in donor cells that were transferred to Tg-SCID mice at 72h p.i.

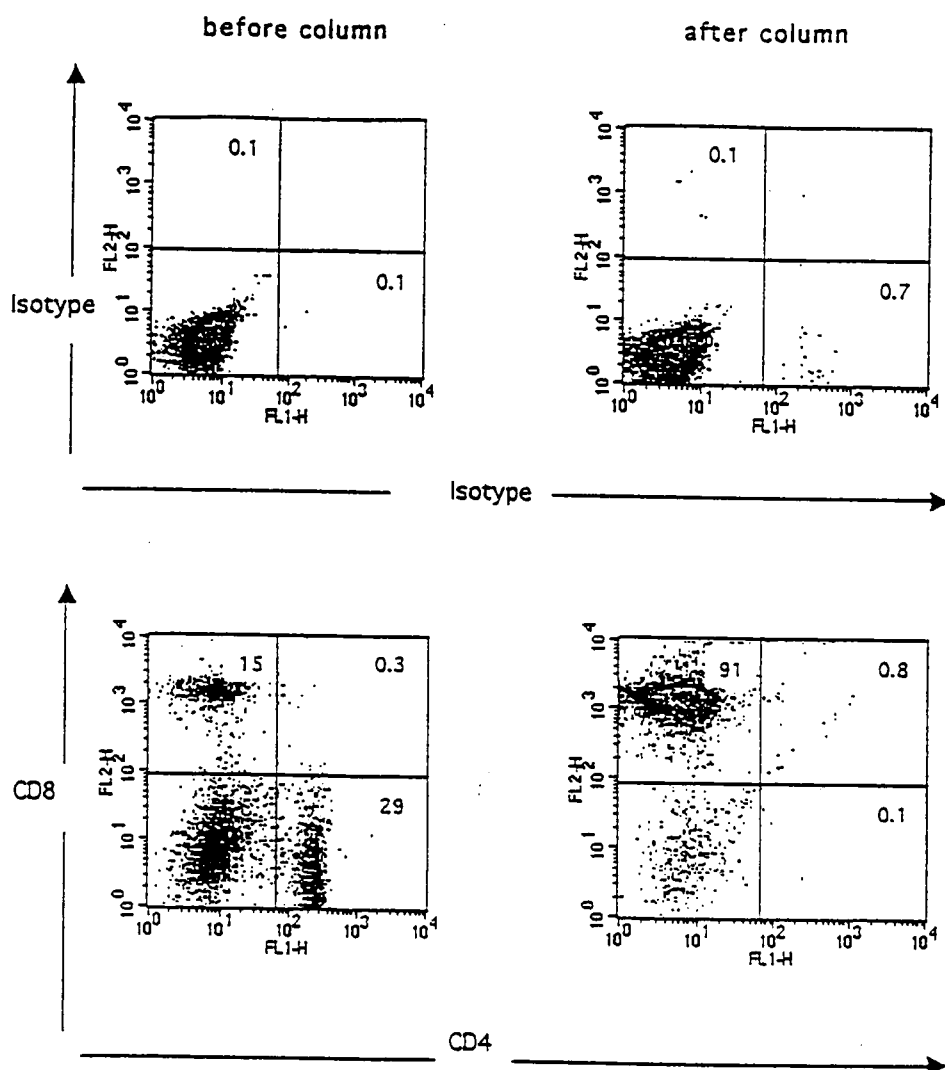


Figure 10: RT-PCR analysis of the inflammed cornea of Tg-SCID mice and BALB/c mice for IFN- γ and TNF- α mRNA. Four corneas from HSV infected Tg-SCID mice (transferred with HSV-immune CD8+ T cells) and BALB/c mice were analyzed. Upper panel, A: Lane1, marker; lane2, naïve BALB/c; lane3, HSV-infected BALB/c; lane4, naïve Tg-RAG; lane5, HSV-infected Tg-RAG; lane6, naïve Tg-SCID. Middle panel, B: lane1, marker; lane2, naïve BALB/c; lane3, HSV-infected BALB/c; lane4, HSV-infected Tg-RAG, lane5, HSV-infected Tg-SCID. Lower panel: lane1, marker; lane2, naïve BALB/c; lane3, HSV-infected BALB/c, lane4, naïve Tg-RAG; lane5, HSV-infected Tg-RAG; lane6, naïve Tg-SCID; lane7, HSV-infected Tg-SCID.

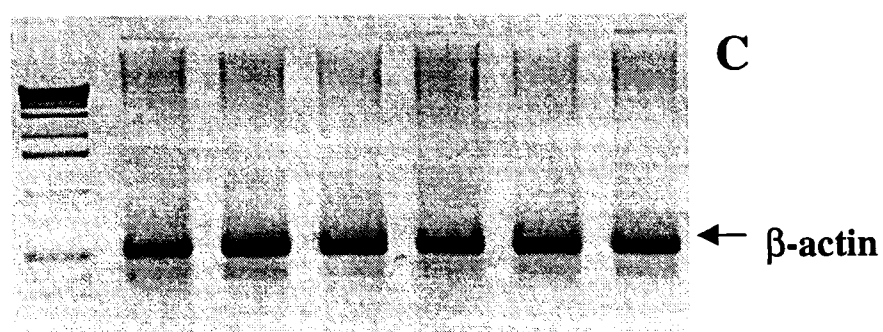
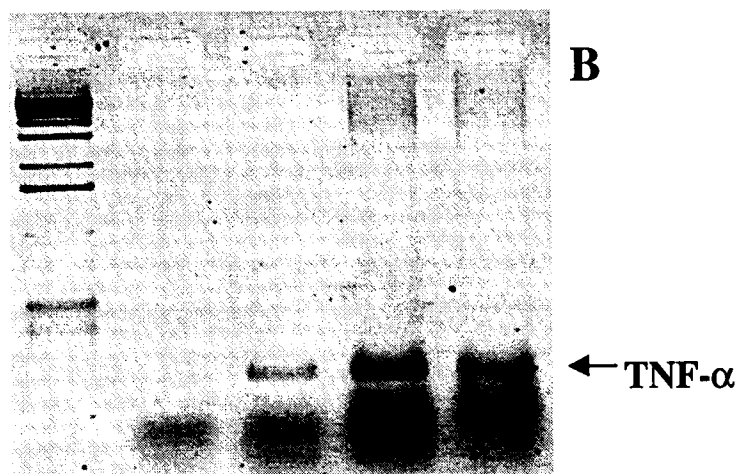
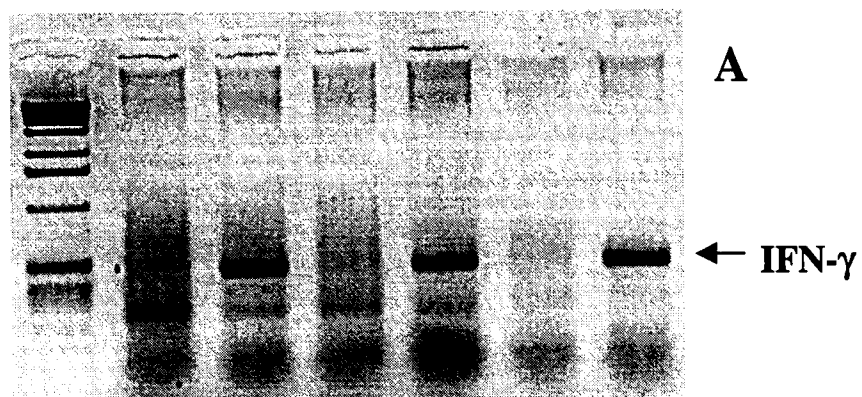


Table 1: In vitro proliferative responses in HSV-RE infected Tg-SCID and DO11.10 mice.

Mice	Antigen-specific proliferation (cpm) *							
	HSV stimulators				OVA stimulators			
	Responders	Responders+ uninfected stimulators	Reponders+ HSV stimulators	SI ^b	Responders	Responders+ uninfected stimulators	Responders+ OVA stimulators	SI
Tg-SCID HSV-CD8 T cells	224±109	213±76	445±49 ^c	2	109±44	176±37	14180±486	81
Tg-SCID HSV-antisera	115±68	189±37	232±28 ^d	1	77±41	128±53	19909±1973	155
Tg-SCID naive serum	88±23	126±31	108±43 ^e	1	105±51	193±102	24482±3128	127
DO11.10	301±141	130±29	6670±1149 ^f	51	224±103	344±115	14248±4449	41

* Antigen-specific proliferation in HSV-1 RE infected DO11.10 and CD8 T cells or HSV-antisera recipient Tg-SCID mice. Five mice/group were individually assayed for proliferation in response to UV-HSV and OVA- peptide(323-339) by incorporation of ³H. The values {expressed as counts per minute (cpm)} represent the mean ± SD. ^b SI, Stimulation Index. By Student's t-test: ^c Vs ^f, p<0.00004; ^d Vs ^f, p<0.0002; ^e Vs ^f, p<0.00003.

Table 2.: Duration of the presence of infectious virus in the eyeswabs following ocular HSV-1 RE infection^a

Mice	Days postinfection											
	1	2	3	4	5	6	7	9	11	14	16	
BALB/c	10x10 ^{3b}	9x10 ³	7x10 ³	5x10 ³	2x10 ³	0.6x10 ³	UD	UD	UD	UD	UD	
Tg-SCID- naive serum	24h. 9x10 ³	8x10 ³	9x10 ³	10x10 ³	12x10 ³	15x10 ³	12x10 ³	20x10 ³	10x10 ³	NS ^d	NS	
Tg-SCID HSV-antiserum	24h. 8x10 ³	4x10 ³	1x10 ³	0.2x10 ³	UD ^c	UD	UD	UD	UD	UD	UD	
	72h. 7x10 ³	7x10 ³	8x10 ³	5x10 ³	2x10 ³	0.4x10 ³	UD	UD	UD	UD	UD	
Tg-SCID- HSV-CD8	24h. 9x10 ³	5x10 ³	3x10 ³	2x10 ³	1x10 ³	0.3x10 ³	UD	UD	UD	UD	UD	
	72h. 10x10 ³	6x10 ³	8x10 ³	8x10 ³	5x10 ³	3x10 ³	1x10 ³	0.4x10 ³	UD	UD	UD	

^a HSV-1 RE (5x10⁵ pfu) was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in eye swabs was determined by the agarose overlay method, the number of pfu/ml were estimated. At each time point eye swabs were collected from four mice (n=4) and viral titers determined.^b pfu/ml, plaque-forming unit per ml, ^c UD, undetectable i.e., below the sensitivity of the assay (<10 pfu), ^d NS, no survivors.

Table 3: HSV-specific *in vitro* proliferative responses of splenocytes and *in vivo* DTH responses in Tg-SCID and DO11.10 mice ocularly infected with HSV-1.

Mice	Antigen specific proliferation (cpm) ^a						DTH response (mm ⁻²) ^b	
	HSV stimulators			OVA stimulators			Challenged with	
	HSV infected stimulators	Uninfected stimulators	Stimulation index	OVA stimulators	Uninfected stimulators	Stimulation index	left ear	right ear
Tg-SCID* HSV-CD8 T cells	269±91 ^d	175±44	1	13991±3067	132±23	106	1.7±0.5	3.3±1.1 ⁱ
Tg-SCID** naive-CD8 T cells	124±46 ^e	107±26	1	22681±4622	206±81	110	ND	ND
Tg-SCID HSV-anti sera	189±69 ^f	135±14	1	24467±519	183±58	134	2.1±1.3	1.8±0.6 ⁱ
Tg-SCID** naive anti serum	72±26 ^g	87±39	1	27034±2086	144±76	188	ND ^c	ND
DO11.10	7806±1775 ^h	163±62	48	14016±7132	178±146	79	2.5±1.0	12.0±1.6 ^k

On day 30 p.i.(**day12 p.i) splenocytes from five mice/group (*3 mice) were individually assayed for proliferation in response to UV-HSV and OVA-peptide(323-339) by incorporation of ³H. The values (expressed as counts per minute (cpm)) represent the mean ± SD.

The values (expressed as counts per minute (cpm)) represent the mean±SD.

^b Forty eight hours before the termination of the experiment, mice were checked for DTH responses as described in materials and methods.

Results shown above represent the mean increase in ear thickness after challenge. Values (expressed as nxmm⁻²) represent the mean±SD.

^c ND, not done. By Student's t-test: ^d Vs ^h, p<0.0002; ^e Vs ^h, p<0.0001; ^f Vs ^h, p<0.0001; ^g Vs ^h, p<0.0001; ^h Vs ^k, p<0.0004; ^j Vs ^k, p<0.00002

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Part 6: Concluding remarks

This study was initiated with the aim of understanding mechanisms by which infection with HSV-1 culminates in an immunoinflammatory reaction in the cornea. The initial experiments addressed requirement of CD4+ T cells and their activation in the disease process. The results of the subsequent experiments propose a novel mechanism by which CD4+ T cells get activated and orchestrate virus-initiated stromal pathology in the absence of viral antigen recognition. Previous studies have given considerable insight into the critical events involved in the pathogenesis. Those studies have dissected the type of cells and the inflammatory molecules involved in driving the disease process. Some of the recent reports suggested that T cells reactive to corneal autoantigens determine the outcome of HSV infection in mice. Apart from suggesting that virus-specific responses are not the only factors in deciding the outcome of HSK, data presented here also proposes 'bystander activation' of CD4+ T cells as a novel mechanism in the pathogenesis of murine HSK. The data presented here demonstrate that HSK is induced in the absence of detectable virus specific responses. These findings surface a new dimension to the complexity of HSV-induced ocular immunopathology and adds to the perplexing multitude of mechanisms.

Initial experiments evaluated the requirement of activation of CD4+ T cells as a necessary event in disease expression. This issue was approached taking advantage of the signals required for T cell activation. T cell activation involves recognition of antigen through TCR-MHC interaction (signal 1) and costimulation via accessory molecules (signal 2). In the absence of second signal (costimulation through CD28-B7 molecule interaction) T cells have been shown to attain the state of anergy.

Administration of CTLA4Ig was effective in blocking T cell activation by binding to B7 molecule on antigen presenting cells and hence prevented the disease on set. However whether the antigen recognition is a continuous process where by T cells of different specificity (viral and host antigens) continue to react to determinants in the inflamed cornea was not studied and hence remains to be defined by local expression of CTLA4Ig in the virus infected cornea.

Significant finding of the subsequent experiment using mice with handicapped T cell repertoire was that virus induced lesions were demonstrable in these mice in the absence of reactivity to viral antigens. The Tg-SCID mice generated in the present study was unique in that it possessed a skewed T cell repertoire and hence lacked reactivity to any antigens other than OVA-peptide(323-339). Previous reports have indicated that viral antigens are not detectable by the time lesions become apparent in the cornea. Therefore, Tg-SCID mice with their ability to recognize only OVA-peptide served as a suitable model to evaluate whether HSK could be induced in the absence of T cell reactivity to viral antigens.

This study was extended to understanding the mechanisms by which virus spread is restricted in the immunocompetent and TCR repertoire-restricted mice. HSV-infected Tg-SCID mice were given either HSV-immune CD8+ T cells or anti-HSV serum to protect from death due to encephalitis. In the adoptive transfer studies, delayed transfer (day 3 p.i.) of CD8+ T cells to HSV infected Tg-SCID mice resulted in severe stromal disease. In the cornea of such mice no viral antigens and CD8+ T cells were demonstrable at the peak of the disease process. However CD4+ T cells were readily

demonstrable in such inflamed cornea. Although the transferred CD8+ T cells were detectable in the cervical lymph node and spleen, it may be possible that very few CD8+ T cells that escaped in our detection system could infiltrate the cornea and contribute to lesion development. Further studies are warranted to titrate CD8+ T cell transfer in SCID mice at different time points after HSV infection. In the present study transfer of anti-HSV serum protected mice from encephalitis and corneal pathology. Analysis of viral antigens in the cornea revealed that, whereas anti-serum restricted the virus to corneal epithelium, the CD8+ T cells recipients showed virus dissemination even in the corneal stroma.

Another interesting finding of this study was that all of the Tg-SCID mice generated for studying antigen specificity in HSK had the same pattern of endogenous V α chain rearrangement even after several back crossing with SCID mice. All of these mice had only the rearranged V β 8.2 chain but possessed V α 2, V α 5, V α 8, V α 11 in addition to the transgene V α 13.1. Additionally, even Tg-RAG2^{-/-} mice showed endogenous V α 2, V α 5, V α 8, and V α 11 TCR chains in addition to the transgene V α 13.1. It will be of interest to determine why the transgenic mice rearrange the same segment of the TCR-V α chain when back crossed to SCID or RAG-2^{-/-} mice.

Future prospects of this study involves evaluating the bystander activation phenomenon in another transgenic setting. TCR-HA mice which recognize hemagglutinin peptide of influenza virus could serve as a good model. Assuming that any form of bystander activation in vivo, occur in connection with a T cell response, one could consider at least three possible mechanisms: 1. It could simply be that cytokines

produced by antigen specific T cells and antigen presenting cells can stimulate the growth of T cells to get activated and then migrate to inflammation sites as already activated T cells: 2. It is possible that, because an inflammatory milieu can lower the threshold of TCR-mediated activation via increasing costimulatory signals, bystander cells are activated through low-affinity interactions of their TCR with self proteins or cross-reactive environmental antigens; 3. It is also possible that resting T cells are activated in the absence of TCR occupancy. In principle, this last possibility may occur via cell-to-cell contacts, cell-tissue interactions, or soluble factors. With regard to cell-to-cell contacts, it has been demonstrated that agonistic antibodies to several T-cell surface molecules (CD2, CD5, CD28) used alone or in combinations can activate T cells. However it is not clear whether the natural ligands of these molecules can stimulate T cells in vivo. Therefore, evaluating the possible proinflammatory cytokines and costimulatory molecules (LFA-3, CD59) for non specific activation of CD4+ T cells in the inflamed cornea would lend support to the emerging concept of 'bystander activation'. Another important aspect of this study is to determine if such nonspecifically activated CD4+ T cells invitro would mediate the disease in HSV-infected immunocompromised mice. A challenging aspect of such study would be dissecting the contributions of virus-specific, virus-induced corneal antigen-specific and nonspecific CD4+ T cells in the corneal microenvironment.

Vitae

Shivaprakash Gangappa was born in Bangalore, India on May 17, 1964. He attended private schools at Bangalore and graduated from Vidya Vardhaka High School May 1978 after completing his Secondary School Certificate Exam. The following August he joined Malleswaram Education Society college, affiliated to Bangalore University and finished his Pre-University Exam in May 1981. He did his Bachelor of Veterinary Sciences (B.V.Sc.), Veterinary College, University of Agricultural Sciences and received his degree in Aug 1986. He worked as a Veterinarian at Karnataka State Government Hospital for 2 years (1986-1988), and continued his education and graduated with a Master of Veterinary Science in Pharmacology from Veterinary College, University of Agricultural Sciences in December 1991. He worked for 3 years in Haematology and Clinical Biochemistry division, Department of Toxicology at Rallis Research Center, Bangalore.

Shivaprakash joined the University of Tennessee, Knoxville in August 1994. He received the Doctor of Philosophy degree from the Department of Comparative and Experimental Medicine in May 1999. Shivaprakash plans to pursue postdoctoral training at Harvard University.