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Murine Herpetic Stromal Keratitis: Cellular and Molecular Mechanisms Involved in its Pathogenesis

Kaustuv Banerjee
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Kaustuv Banerjee entitled "Murine Herpetic Stromal Keratitis: Cellular and Molecular Mechanisms Involved in its Pathogenesis." I have examined the final electronic 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:

Robert Moore, Pamela Small, Albert T. Ichiki

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Vice Chancellor and
Dean of Graduate Studies

MURINE HERPETIC STROMAL KERATITIS: CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN ITS PATHOGENESIS

A

Dissertation Presented

For the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Kaustuv Banerjee

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ABSTRACT

Herpetic Stromal Keratitis (HSK) is a leading cause of infectious blindness resulting from corneal infection with herpes simplex virus (HSV-1). Extensive corticosteroid therapy is required to achieve remission and sometimes corneal transplantation is the only means of restoring vision. Murine model research has revealed that the immunological process that clears infection conversely leads to corneal tissue damage. This immuno-pathological reaction involves complex interactions of cellular and molecular events.

Current knowledge about human and murine HSK pathogenesis is summarized in Part I. Parts II, III and IV progresses this knowledge using knockout and transgenic mice. Results in Part II clarify mechanisms that operate shortly after corneal infection using a mouse with defective neutrophil migration. Impaired corneal neutrophil migration delayed corneal viral clearance causing heightened inflammation in the c ornea. The c ytokine IL-6, a component of this response, was shown to be capable of inducing VEGF, resulting in an increased angiogenic response and enhanced HSK lesions. These results provide novel insights into the link between viral infection, pro-inflammatory molecules, neutrophil migration, angiogenesis and HSK development. The role of HSV reactive and non-reactive $CD8^+$ T cells in HSK pathogenesis is discussed in Parts III and IV. It has been demonstrated that $CD4^+$ T cells, crucial to the development of HSK, do not require HSV antigen recognition to mediate HSK. Instead they are activated and recruited to the cornea by bystander mechanisms apparently through the action of pro-inflammatory molecules. Our recent

results indicate that bystander mechanisms also facilitate corneal recruitment of non-HSV specific $CD8^+$ T cells. However, HSV reactive $CD8^+$ cells were not involved directly in corneal lesions but instead modulated lesion severity and protecte mice from lethal HSV induced encephalitis. This latter effect appeared to be due to the ability of HSV specific $CD8^+$ T cells to control viral replication in the peripheral nervous system and thereby prevent spread to both the brain and the cornea.

This dissertation presents research aimed at elucidating both molecular and cellular events in HSK pathogenesis. These results will serve as guidelines for future development of more efficient prophylactic and therapeutic strategies.

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ABBREVIATIONS

CXCR2.....	CXC chemokine receptor 2
DC.....	Dendritic cell
HSV-1.....	Herpes simplex virus 1
ICAM-1.....	Intracellular cell adhesion molecule
IFNγ.....	interferon gamma
IL-1, 6, 8, 12, 18.....	Interleukins 1, 6, 8, 12, 18
IP-10.....	Interferon gamma inducible protein 10
MHC Class I, II.....	Major histocompatibility molecule Class I, II
MiG.....	Monokine induced by interferon gamma
MIP-1, 2.....	Macrophage inflammatory protein 1, 2
MMP-9.....	Matrix metalloproteinase 9
PAMP.....	Pathogen associated molecular patterns
PECAM-1.....	Platelet endothelial cell adhesion molecule 1
PFU.....	Plaque forming units
PMN.....	Polymorphonuclear leukocytes
RAG-/-.....	Recombinase activating gene knockout
SCID.....	Severe combined immunodeficiency
TCR.....	T cell receptor
TG.....	trigeminal ganglion
Tg.....	transgenic
TIMP-1.....	Tissue inhibitor of matrix metalloproteinase 1
TNF.....	Tumor necrosis factor
VEGF.....	Vascular endothelial growth factor

PART I

BACKGROUND AND OVERVIEW

Information provided in this chapter is a revised version of a review article by **Kaustuv Banerjee** and Barry T Rouse, submitted for publication as a chapter in the book:

Banerjee, K and Rouse, B. T. (2004). Immunopathological aspects of HSV infection. *In* Pathogenesis, Host response and Clinical Disease. Human Herpes Viruses: Biology, Therapy and Immunoprophylaxis. Eds: A. Arvin, G. Campadielli-Fiume, P. Moore, E. Mocarski, B. Roizman, R. Whitley and K. Yamanishi. Cambridge University Press.

My contributions in the review article include (1) literature survey (2) compilation of information (3) plan review outline (4) writing (5) preparation of figures and manuscript editing.

INTRODUCTION

Foreign material entering multicellular organisms triggers a range of defense reactions which, when successful, subjugates and removes the invaders. Invertebrates and plants suffice with natural defense systems, which recognize commonly shared patterns and usually react in a stereotypical manner. Long-lived animals such as vertebrates add to these natural defenses with adaptive systems that show discriminating recognition machinery, complex and varying effector mechanisms and an ability to learn from experience. Under ideal circumstances, immune defense proceeds with minimal or inapparent damage to the host itself. In other situations, the defense system is less successful and the host tissues become damaged by the

reaction. We usually consider the former situation as immunity and the latter as immunopathology. However, in both instances, mechanisms at play may be similar.

With microorganisms, the commonest circumstance that results in immunopathology is where the microbe persists and continues to cause an innate and adaptive response. These, however, prove ineffective to remove or neutralize the agent. Thus the reaction becomes chronic and host tissues become damaged as a consequence. This situation occurs in tuberculosis as well as hepatitis B and C virus infections. Over time many microbes with a long association with a host species find ways of persisting by evading responses that would either reject them or cause too much tissue damage. Human CMV infection in immuno-competent adults provides an example of this scenario (1). Other circumstances that result in immunopathology involve settings where one or more components of normal immune defense are compromised for genetic or other reasons. Prolonged severe genital herpes simplex virus (HSV) lesions in AIDS patients with very low $CD4^+$ T cells represent such an example (2). Atopics too often have problems clearing HSV and hence often develop skin and eye lesions (3). In addition, some microbes are considered as able to trigger immune reactions that target host components themselves (autoimmune disease) or cause infected cells to undergo neoplastic transformation. The herpesvirus EBV provides an example of the latter in genetically susceptible individuals (4). There are no undisputed examples wherein herpesviruses cause autoimmune diseases. However, circumstantial evidence exists for HHV-6 causing multiple sclerosis (5) and possibly HSV infection causing an autoimmune corneal inflammatory lesion (6). This later model is discussed in more detail subsequently.

Herpes simplex virus is a pathogen that only rarely appears as involved in immune mediated tissue damage. Characteristically, primary or recurrent infections at

superficial mucosal or dermal sites result in viral replication and destruction of most cells that support infection. This process induces an innate inflammatory reaction that contributes to infection control. Some cells, likely Langerhans dendritic cells, leave the site and carry viral antigens to draining lymph nodes where an adaptive response is induced or recalled. After a few days, effectors of adaptive immunity are recruited to the site, initially $CD4^+$ T cells followed by $CD8^+$ cells, and these T cells, probably assisted by antibody, complete the task of recovery (2). Virus is removed and the inflammatory reaction subsides usually without trace. These events can be judged to represent immunity. As already mentioned, when aspects of T cell function is impaired, as can happen in AIDS patients, HSV removal is impaired and the inflammatory reaction becomes unusually severe and prolonged. This situation can be taken to represent immunopathology.

Certain tissue sites are particularly vulnerable to damage by an inflammatory response. These are sites where virus is difficult to dislodge, so the inflammatory reaction becomes prolonged and destructive, or where tissue repair leaves a functionally damaged organ. The best site which exemplifies such circumstances is the eye. In this organ, where inflammation or scar tissue along the visual axis impairs function, HSV infection may permanently impair vision. The most frequent example is herpetic stromal keratitis (HSK). This chronic inflammatory reaction damages the stroma and can become sufficiently severe to merit corneal transplantation. About 20% of ocular HSV infections in humans result in stromal keratitis (7). In most instances, these are caused by HSV-1 and result from reactivation from latent infection in the trigeminal ganglion (7). Most of our understanding of the immunopathogenesis of HSK comes from studies in animal models, in former times

mostly the rabbit and now most usually the mouse. These studies are discussed subsequently.

HERPES INFECTIONS AND OCULAR DISEASE

At least four human herpesviruses have been implicated as causes of ocular disease. Two alphaherpesviruses, HSV and Varicella Zoster virus (VZV), the betaherpes virus CMV and the gammaherpesvirus EBV. CMV is a cause of retinitis, a lesion found only in immunosuppressed individuals, the majority of which were formerly AIDS patients, but now transplant recipients, especially recipients of bone marrow (8). With the widespread use of protease inhibitors to control HIV, CMV retinitis is now mainly a disease of transplant recipients. The lesion itself is likely a direct consequence of viral replication in retinal cells. EBV is an occasional cause of HSK lesions. These are characterized by an abundance of lymphoma like cells, in the stroma that are presumed to be mainly B cells. (9).

More commonly both HSV and VZV cause lesions in the anterior segment, principally the cornea. These lesions, many of which are immunopathological are discussed in the following sections.

KERATITIS IN HUMANS

Both HSV and VZV can infect multiple structures in the eye. Lesions caused by HSV are much more common. The incidence of HSV ocular disease ranges from 4.1-20.7 cases/100,000 patient years representing the commonest single infection cause of vision impairment in the western world (7). Of the three general types of HSV corneal disease, Infectious Epithelial Keratitis (IEK) is the most common lesion and this

appears to be a result of the direct effect of viral infection. Both disciform keratitis (HSV endotheliitis) and HSK are thought to be mainly the consequence of immune mediated mechanisms rather than by direct viral damage. Lesions seen with IEK are a result of viral replication and spread in the superficial epithelial layer of the cornea. This condition is usually self-limiting and no permanent corneal damage results. The quick remission seen with timely antiviral therapy suggests a simple viral cytolytic mechanism. However, virus invariably infects nervous ramifications in the cornea that have free ends within the epithelial layer, thus allowing retrograde transport and latency establishment (10). In addition, as a consequence of epithelial damage, virus can spread to the underlying stromal keratocytes and cause what is usually termed as necrotizing form of stromal keratitis (11). This terminology does not fit well with many ophthalmologists since necrosis also occurs in immune mediated HSK.

Disciform keratitis (DK) is a lesion in which the corneal endothelium is the primary site of damage. This form of ocular disease appears immunopathological based upon the fact that early intervention with corticosteroids leads to complete resolution (11). In DK, the inflammatory reaction of the endothelium sometimes results in secondary stromal and epithelial edema but there is usually no stromal infiltrate or neovascularization. One of the characteristic findings in patients is the demonstration of keratic precipitates or KP (11). The exact nature of the KP is unknown but they could be aggregates of macrophages or NK cells attracted by the immunoglobulins on the cell surface of infected cells (11). An alternative idea is that KP represents cytotoxic T cells recognizing viral epitopes on the endothelial cells (11). The role of live virus in disease development is supported by finding antigens, live virus and DNA in the anterior chamber and perhaps also corneal endothelial cells (12, 13). It has been postulated that productive infection of the endothelial cell elicits

a cellular and humoral immune response (14), but this evidence is only circumstantial. Alternative suggestions include a possible delayed type hypersensitivity reaction to persisting HSV antigens within the stroma or the endothelium (15). It is difficult to resolve the nature of DK pathogenesis since animal models to study it are less than ideal. Disciform disease is seen in rabbits with an intracorneal injection of soluble viral antigen (16). Using the rabbit model for DK some have suggested the lesions to involve immune complex formation and antibody-dependent cell-mediated cytotoxicity (17).

Inflammation of the corneal stroma (HSK) as a result of HSV-1 (rarely HSV-2) infection can lead to a blinding immuno-inflammatory lesion of the stroma. This only accounts for approximately 2% of initial episodes of ocular disease but approximately 50% of recurrent ocular HSV disease (18). A similar, but even more devastating lesion can be caused by VZV infection. Fortunately, this is quite rare and also usually occurs as a consequence of reactivation (zoster). They usually heal quickly unless the patient is immuno-suppressed (19). Recurrent lesions can be very severe and most difficult to treat and control. Frequently, corneal lesions are accompanied by conjunctivitis, anterior uveitis and lipid keratopathy (19). If the virus is not controlled, it spreads to involve the iris and the corneal stroma. Stromal lesions can become sclerotic and very persistent and is believed to be immune mediated, however, the mechanism is not known and is difficult to study. Patients often lose sensitivity of the cornea and involuntary physical damage can result in secondary bacterial infection.

Several observations suggest the operation of an immune etiology behind HSV induced HSK. These include the fact that the lesions are persistent and are manifest well beyond the time that virus or viral antigens can be demonstrated. Lesions often

need to be managed with indefinite corticosteroid treatment and reactivation lesions, except initially, do not benefit from acyclovir antiviral treatment. Also making a case for the pathogenesis of HSK involving immunopathology is the fact that the lesion is very uncommon in immuno-suppressed patients. Finally, clones of T lymphocytes reactive to viral epitopes and possessing cytotoxic activity can be cultured from corneas showing chronic HSK lesions (20, 21).

Approximately 90% of patients maintain good visual acuity despite prolonged disease. However, in many cases resolution of inflammation is associated with a permanent loss of vision resulting from corneal scarring and ulceration. This necessitates treatment by corneal transplantation, which in itself can sometimes be a high risk factor for recrudescent herpetic keratitis (also called newly acquired herpetic keratitis) (22) and super-infection with a different strain (23)

The corneal stroma may be affected by several mechanisms; this may be secondary to disease of the epithelium (IEK) or endothelium (DK) or as a stromal edema resulting from a damaged endothelium. In humans, HSK manifests itself in two primary forms that are perhaps mis-termed necrotizing HSK and immune HSK (11). While the former is thought to result from direct viral invasion of the stroma, chronic immune mechanisms, possibly of an autoimmune nature (yet unproven), are the suspected culprits in pathogenesis of the latter (7). These divisions are not mutually exclusive and necrosis can definitely occur in the immune form. Intact virions and antigens can be detected in corneal keratocytes, endothelial cells and foci of epithelial cells in specimens from patients with acute (necrotizing) stromal keratitis (24, 25). This suggests that replicating virus and the resulting host inflammatory response leads to stromal cell destruction. This acute necrotizing form of HSK eventually may become chronic, then considered as immune form of HSK, when viral

antigens are no longer present. The signs of HSK are generally quite variable but they involve the influx of a large number of different kinds of cells including polymorphonuclear leukocytes (PMN), macrophages, Langerhan's cells, natural killer (NK) cells, plasma cells and T lymphocytes (26-29). In chronic HSK in humans the predominant population are macrophages and T lymphocytes (27). Excess neovascularization also occurs in some patients.

The original mechanism proposed for the pathogenesis of the immune form of HSK focused on the role of anti-HSV antibodies. This was based on the finding that rings (Wessely rings) seen in the mid-stroma of the cornea in immune stromal keratitis were positive for IgM, IgG and IgA (30). Herpes virus particles have been demonstrated in these rings, many of them defective or incomplete (30). In addition, viral antigens have been found localized in the keratocytes of the corneal stroma in transplanted corneas (28, 31). Hence it has been speculated that viral antigens trapped in the stroma acted as a nidus for deposition of anti-HSV antibodies that fixes complement and leads to cellular damage (7). Viral antigens can also be presented to the infiltrating T lymphocytes. In clinical specimens, increased levels of class I and II HLA antigens have been noted in areas of the greatest infiltrate, suggesting active presentation of antigens (26). Both $CD4^+$ and $CD8^+$ T cells occur in chronic HSK with the former dominating the total T cell numbers (28). Most of these cells are reactive against HSV antigens with the $CD4^+$ subset reactive to peptide epitopes from UL21 and UL49 tegument proteins of HSV (20, 21). They do not apparently recognize antigens derived from corneal tissues which would provide evidence for an auto-immune mechanism (20). Corneal derived $CD4^+$ cells have been shown to possess cytotoxic activity suggesting the possible operation of this mechanism in stromal cell injury (20, 21).

ANIMAL MODELS FOR HSK

Understanding the pathogenesis of human HSK must rely only on clinical observations, transplant material and the occasional samples obtained at biopsy. Not surprisingly with such data it is difficult to construct a coherent understanding of HSK pathogenesis. Fortunately, convenient animal models exist wherein HSV infection of the eye reproducibly generates a stromal inflammatory response. Moreover, this appears to reflect human immune HSK at least prior to aggressive treatment. The usual animal models are the mouse and rabbit with the latter now rarely used except for studies on therapy. Understanding events involved in HSK pathogenesis comes mainly from studies of primary infection in various mouse strains. Since human HSK is most commonly a sequel to reactivated infection, a better animal model should be one where lesions follow reactivation. Such models have in fact, been described for both mice and rabbit (32, 33) but these are expensive and inconvenient and have contributed minimally to the understanding of pathogenesis. Rabbit reactivation can be achieved by ocular iontophoresis but this seldom gives rise to HSK lesions (32). The mouse reactivation model can be achieved by infecting mice under a cover of neutralizing antibody, then after some weeks asymptomatic animals are exposed to UV light. In usually a minority of animals, virus reactivates and generates an inflammatory reaction in the stroma (33). Few papers have employed the model, and the results of these usually support the basic findings of the primary infection model; namely that HSK is an immuno-inflammatory lesion mainly orchestrated by $CD4^+$ T cells (34).

The primary infection model usually uses strain HSV–1 RE and involves virus application to a lightly scratched cornea. Replication begins in epithelial cells of the cornea and usually the conjunctiva, but in immuno-competent mice rarely spreads to involve stromal cells or cells in uveal tissue. Characteristically, the viral replication events are over by 5-6 days and viral gene expression, as judged by protein detection or viral mRNA, are undetectable beyond a further 2-3 days (35). Viral DNA, however, can be detected for prolonged periods although copy numbers, detectable by real-time PCR, do not exceed 2000 – 5000 copies per cornea by 14 days p.i. (our unpublished results). When looked at with an ophthalmoscope, the initial viral replication events are accompanied by a barely detectable inflammatory reaction with new blood vessel growth from the limbus (the location of blood vessels at the edge of the vessel free normal cornea) the most obvious feature. This is often referred to as the pre-clinical phase, although in fact with appropriate tests is readily observable.

Innate immune response to infection in the mouse model

HSV infection of the corneal epithelium sets off a range of humoral and cellular events that taken together help contain infection. Unfortunately, some of these also set the stage for subsequent immunopathology. A prominent early cellular event is the influx of polymorphonuclear neutrophils (PMN). This occurs mainly into stromal tissues subjacent to the infected epithelium. Such PMN escape from blood vessels at the limbus presumably in response to signaling molecules generated from virus infected cells. The nature of such signaling molecules is unclear but several chemokines, including those known to be chemotactic to PMN, can be demonstrated within 12 hr p.i. (36, 37)

The PMN response is at its peak around 48 hrs and it seems that this response helps control viral replication. Thus depleting PMN with specific monoclonal antibodies results in more intense and prolonged virus infection in the cornea (37, 38). Moreover, PMN suppressed animals may succumb to encephalitis since virus now spreads to the brain. Such observations indicate that PMN are part of the anti-viral defense system although it is unclear how this function is performed. Accordingly, virus infected cells and PMN are usually not in direct contact implying that the protective function is indirect. Ideas for the mediation of such defenses include IFNγ and TNFα production as well as nitric oxide production by PMN (39). This topic has not been fully explored using, for example, knockout mice and other means of implicating potential anti-viral mechanisms.

The PMN response to virus is not only a defense reaction. Indeed products released from PMN have been proposed to contribute to corneal damage possibly unmasking auto-antigens subsequently involved in the immunopathology (37). In addition, PMN contribute to the process of neovascularization, a prominent feature of HSK and a necessary step in its pathogenesis (40, 41). It appears that PMN may be a source of angiogenesis factors such as VEGF as well as tissue degrading enzymes which breakdown the stromal matrix and facilitates the growth of new blood vessels. One such enzyme released by the granules of activated PMN is MMP9 (42). Since infected mice given the MMP-9 inhibitor TIMP-1 as well as MMP9-/- mice have reduced angiogenic and SK responses, MMP9 appears to be intricately involved in HSK pathogenesis (42).

Although PMN dominate the early inflammatory reaction to ocular HSV infection, other cell types can also be demonstrated. These include macrophages, dendritic cells (DC), NK cells but not B or αβ TCR T cells. The roles for these other

cell types have received minimal investigation. It is likely, however that the macrophage is a source of angiogenic factors such as VEGF and FGF as well as the angiogenic CXC chemokines. Some of these chemokines may also be involved in facilitating neutrophil influx. Thus, *in vivo* neutralization of MIP-2 in HSV infected mice reduces PMN migration (43). In other models such as in the *Onchocerca volvulus* ocular infection model, MIP-2 (CXCL8) appears important since infected mice lacking the receptor for MIP-2 (CXCR2-/- mice) have an impaired PMN response (44). Macrophages, along with DC, also act as a source of cytokines demonstrable early after infection. Most prominent of these are IL-1 and IL-6, both of which can also be produced by virus infected epithelial cells themselves (45, 46). Indeed it could be that these two cytokines are critical signaling molecules responsible for the many paracrine events set off by virus infected epithelial cells. The other early events described include the production of IL-12, VEGF and TNFα, but none of these are thought to be products of virus infected cells themselves (47, 48).

The cytokine IL-12 appears as a pivotal molecule in HSK pathogenesis. Knockout mice, for example, unable to produce IL-12 have only mild HSK lesions (49). The source of IL-12 following HSV infection remains to be clarified, since as mentioned it does not appear to be HSV-infected cells themselves (48). However conceivably viral DNA that has pathogen associated molecular pattern (PAMP) activity could represent such a stimulus (50). The most likely producer cell types are DC and macrophages. The DC initially involved would seem to be the resident cells only recently demonstrated as present in normal noninflamed corneas (51). A prominent feature of the injured cornea, including that caused by HSV, is the invasion of Langerhans DC, likely from the conjunctiva, into the cornea (52). However, this event takes several days to occur. Likely such cells also act as a source of cytokines

and chemokines but their major function in HSK pathogenesis is transport of viral antigens to lymphoid tissue where the adaptive immune response is initiated (discussed later).

The cytokine IL-12 has several downstream effects that impact on HSK pathogenesis. The primary effect is induction of IFNγ production by cells with IL-12 receptors. Although not proven in the eye, the most likely cells that respond and produce IFNγ are natural killer (NK) cells. Such cells in non-ocular systems have been shown to be important for resistance to HSV. In fact, removing them results in heightened susceptibility (53). An old study on HSK indicated that NK removal ameliorated HSK (54, 55), although this issue warrants further investigation. Whatever the source of IFNγ, this molecule appears intricately involved in antigen processing as well as other events critical for HSK pathogenesis. These include upregulation of the cell adhesion molecule PECAM-1 on vascular endothelial cells, at the limbus (56). This is a necessary step for normal PMN invasion as evidenced by the fact that neutralization of IFNγ or PECAM-1 results in diminished PMN ingress (56).

The importance of IFNγ in facilitating cell migration is further underscored by studies with human corneas. Stimulation of human corneal cells *in vitro* with IFNγ, and also IL-1 and TNFα, rapidly upregulates ICAM-1 expression (another cell adhesion molecule that participates in the adhesion and extravasation of cells) (57). IFNγ also upregulates MHC Class II expression on the antigen presenting cells involved in the induction of the initial antigen specific $CD4^+$ T cell response in local draining lymph nodes (58, 59). On the other hand, IFNγ could help modulate lesion development since it also induces angiostatic chemokines such as IP-10 and MIG

(41). Accordingly the IL-12 response to HSV infection indirectly impacts on both inflammatory and regulatory effects on HSK.

In Figure 1 (for all figures see appendix) several critical events are shown that are set into play by HSV during the first 6-7 days post infection. Interestingly, by the end of this often-called pre-clinical phase, the corneal tissues show little or no damage. The epithelium is fully intact, the stroma has few if any inflammatory cells and cytokine/chemokine levels have fallen significantly. The most obvious sign of change is a neovascular bed that continues to expand slowly beyond the limbal region. Nevertheless, in spite of the quiet appearance, notable changes begin to occur which constitute the true immunopathological events of HSK. Accordingly, the T cell orchestrators begin to invade via the new blood vessels and an intense inflammatory response ensues. This becomes obvious upon ophthalmoscopic examination and is frequently referred to as the clinical phase.

Adaptive immune response to infection in the mouse model

Migration of T cells that express appropriate homing molecules escaping from the newly established blood vessels represents a crucial step in HSK pathogenesis. Mice without T cells never develop typical HSK lesions (60, 61), but do if given T cell transfers (61, 62). Although debated early on, most investigators now agree that $CD4^+$ T cells, with the type 1 producing cytokine phenotype, are the main aggressors in HSK (61, 63). Such cells trigger the invasion of non-specific inflammatory cells, surprisingly once again dominated by PMN, giving rise to a peak response around 15 days after initial infection. The inflammatory response considerably thickens the stroma and neovascularization continues almost reaching the central cornea (see Fig 2). Severe lesions have areas of necrosis and epithelial ulcers and uveal tissues may

also be involved. The lesional T cells, which account for only a minority of the inflammatory cells present, are mainly $CD4^+$ T cells. Judging from a variety of approaches, the principal cytokine necessary for the lesion expression is IFNγ (56, 64). However, HSK can still be induced in animals lacking this cytokine (65). In cases where lesions do diminish in severity, the cytokine IL-10 is upregulated (66). Furthermore, the artificial expression of IL-10 or IL-4 early in the syndrome can markedly diminish lesions (67). Such observations indicate that $CD4^+$ Th1 are the principal aggressors but if a type 2 response can be induced, lesions will resolve. Whether such ideas can be applied usefully to the human system warrants investigation.

Very recently the severity of HSK lesions was shown to be modulated by a second species of $CD4^+$ T cells (68). These were $CD4^+CD25^+$ T regulatory cells (T_{reg}) found operative in autoimmune inflammatory lesions (69). Accordingly, in animals unable to generate T_{reg} responses, HSK lesions were more severe and animals more susceptible to a low dose of infection (68). In addition, there is evidence that the $CD8^+$ T cell response to HSV provides a protective function against HSK (61, 70). The mechanisms by which T_{reg} or $CD8^+$ T cells exert controlling effects on HSK expression are not currently understood.

A central issue in HSK pathogenesis is the nature of antigens recognized by the $CD4^+$ T cell orchestrators and if such recognition occurs in the extra-lymphoid or lymphoid sites (or both). This issue becomes of interest since at the time when T cells invade the cornea, replicating virus has usually disappeared (35). Moreover, certainly at the time of peak lesions (15 days), the presence of viral antigens in stromal tissues cannot be demonstrated (35). Conceivably, viral peptides expressed by DC could still be present in the cornea and draining lymph nodes, although usually T cell target

peptides turn over within 2-3 days after protein processing. Since new protein formation appears to have ceased by 6 days p.i., it is difficult to support the logical notion that peptides derived from viral proteins are the target antigens recognized by the $T_{aggressors}$.

An alternative concept is that viral specific T cells are initially responsible for the immunopathology but subsequently the chronic phase is maintained by an auto-reactive response (64). Here the idea is that the virus infection results in unmasking of the some corneal auto-antigen (see Fig 3), tolerance is broken and the auto-reactive T cells induced are responsible for orchestrating lesions. A modification of this idea favored by the Cantor group is that the auto-immune process is set off by some viral peptide sharing reactivity to the unmasked corneal auto-antigen (71). Thus the initial anti-viral response subsequently becomes sustained by auto-reactive $T_{aggresor}$ cells. This concept of molecular mimicry has aroused much interest and discussion (64). Its best support comes from studies on closely related inbred mice. Here it would seem that the UL6 protein of HSV possesses molecular mimicry with an auto-peptide that in fact represents a sequence also found on an immunoglobulin isotype (71). The molecular mimicry idea is not accepted by other groups for a number of reasons (Table 1, appendix). Most, especially the UL6 proteins of HSV appear not to induce T cell responses in animals following infection with HSV (72). In humans also the UL6 protein appears not to be recognized (20, 21, 73).

An alternative idea to explain how $CD4^+$ T cells become activated is that the inflammatory process could be initiated by viral antigen recognizing T cells, but subsequently is maintained by cells of the effector memory phenotype that escape into the cornea because of the highly permeable neovascular bed. Such cells in turn become activated by inflammatory molecules initially released by viral antigen

reactive cells. The responding cells, release inflammatory cytokines and so the process continues (see Fig 4). This idea is supported by the observation that abundant non-antigen T cells can be demonstrated and that it is possible to develop lesions identical to HSK in animals whose T cells are genetically incapable of recognizing viral antigens (74, 75). Such was shown in several T cell transgenic mice on SCID or RAG-/- backgrounds whose recognition repertoire did not include HSV antigen recognition (74, 75). In these models, the chronic source of activating cytokines were cells dying of HSV infection since in this instance virus persisted and spread to the stromal site of inflammation (74, 75).

Other ideas have also been advocated to explain which agonists drive HSK, especially in the chronic phase, but the issue remains unresolved. The candidate agonists include PAMP expressed by virus, superantigen expression and inflammatory reactions driven by stress proteins (76). Currently, a favored idea is that the HSV DNA could itself be pro-inflammatory because of its high content of bioactive CpG containing deoxynucleotide motifs (50). Such ideas await verification.

SPECIFIC AIMS AND RATIONALE

Studies with the murine model for HSK have revealed the involvement of complex mechanisms in its pathogenesis. Several areas, both in the pre-clinical and the clinical phase, require further elucidation. These include the molecular events that direct the neutrophil influx and promote viral clearance. In addition, it is not clear as to how the early pre-clinical events lead to the development of angiogenesis, which is important in the migration of the T lymphocytes. Furthermore, events that lead to the influx of T lymphocytes and the role of the different T lymphocyte subsets in the pathogenesis of

HSK is not entirely understood. Studies described here have been aimed at understanding the above mechanisms further.

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LIST OF REFERENCES

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APPENDIX

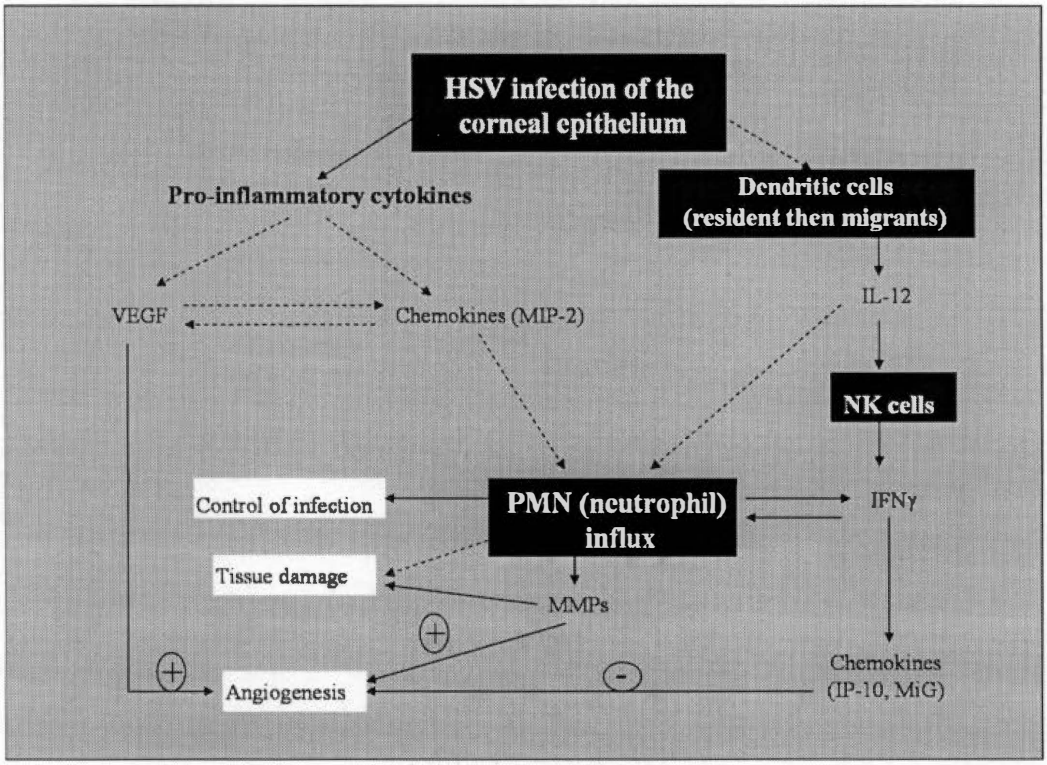

Figure 1. Critical events of the pre-clinical phase of murine HSK

HSV-1 infection of the corneal epithelium sets up a pro-inflammatory environment. Such an environment is responsible for directing subsequent immuno-pathology resulting in tissue damage. Shortly after infection there is a production of various cytokines and chemokines. These serve to activate and recruit cells of the innate immune system. Polymorphonuclear cells (PMN), mainly neutrophils, comprise a large number of the total cellular influx at this stage and are responsible for the clearance of virus. Pre-clinical events set the stage for (1) tissue damage by secreted products of neutrophils and other unidentified molecules (2) Angiogenic response (neovascularization) in the cornea brought about by the interplay between pro (+) (VEGF, MMP) and anti (-) (IP-10, MiG) angiogenic factors. Dashed lines represent those steps that require further investigation.

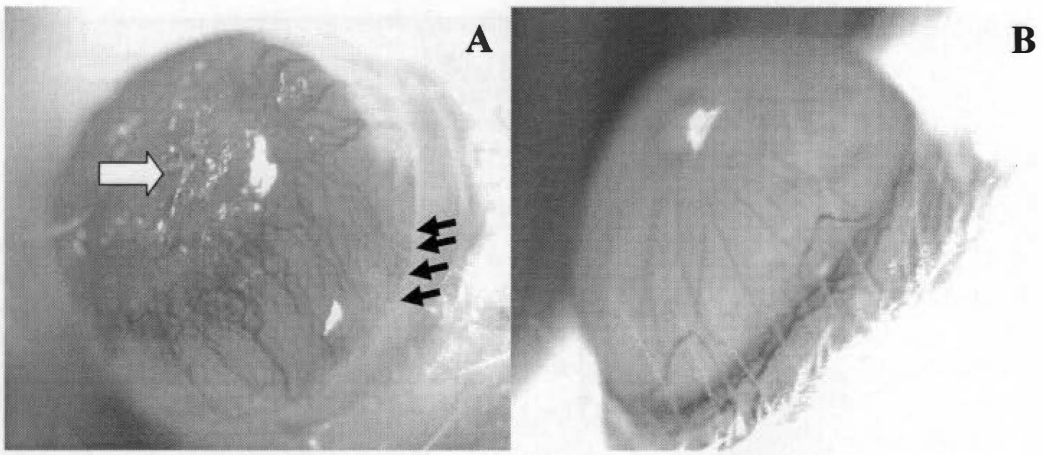

Figure 2. Typical features of an HSV-1 infected murine eye

The photograph shows a representative eye at day 15 post infection (A). At this stage eyes display characteristic growth of blood vessels from the corneal limbus (black arrows) and corneal opacity, necrosis and epithelial ulcers (white arrow). Shown for comparison is an uninfected eye (B).

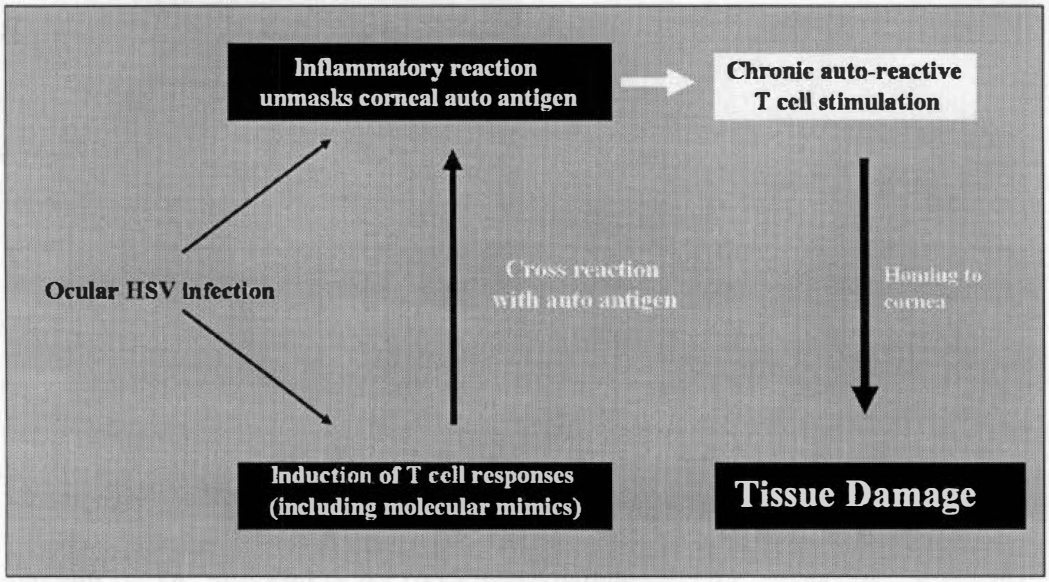

Figure 3. The molecular mimicry model to explain the pathogenesis of murine HSK

The figure summarizes the autoimmune events thought to explain HSK pathogenesis as proposed by the Cantor laboratory (Reference 71). $CD4^+$ T cells in the susceptible mouse strains are activated by a peptide of the HSV UL6 tegument protein. Such cells are also reactive to peptides from corneal antigens (yet undefined), released as a result of viral replication in the cornea, that mimic the HSV derived peptide. Corneal tissue damage is mediated by these auto-reactive cells.

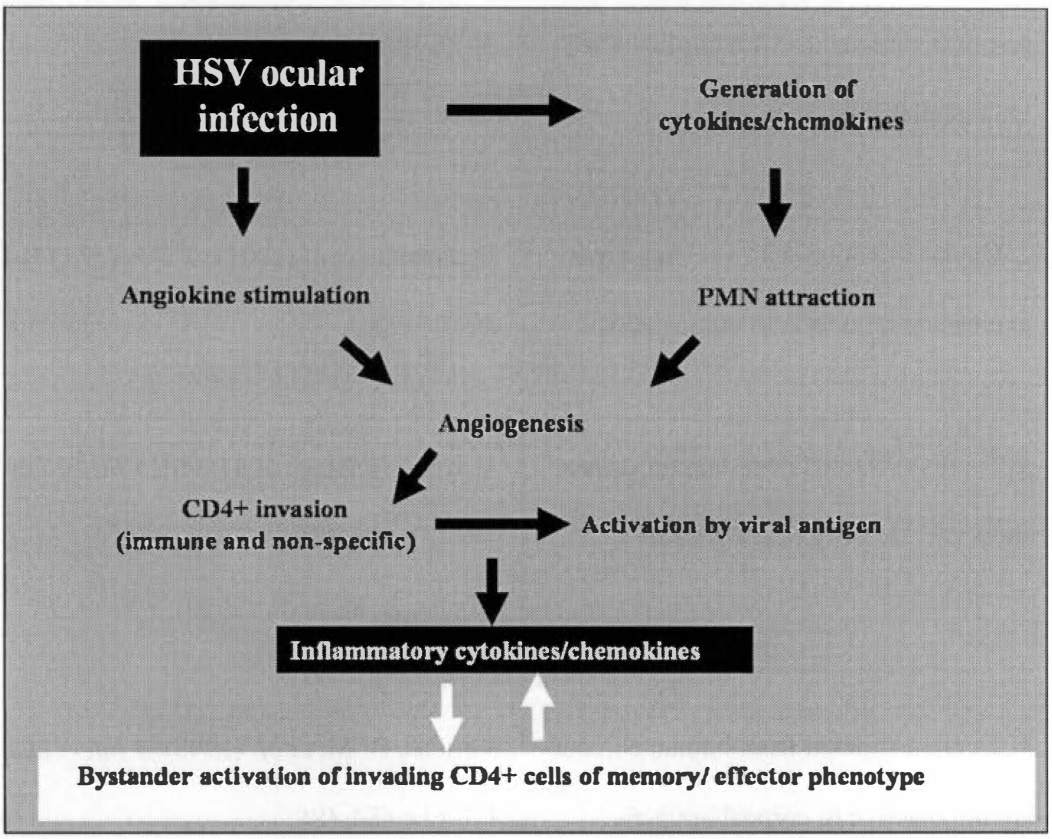

Figure 4. The bystander activation model to explain the pathogenesis of murine HSK

The bystander activation model proposes that viral antigen specific activation (non-requirement of the TCR-MHC interaction) is not a requirement for T cells to enter the cornea (References 74, 75). Such cells are activated by the pro-inflammatory mediators produced in the cornea after viral infection.

Table. 1. Evidence that challenges the molecular mimicry hypothesis

Observation	Reference
1. Failure to demonstrate an immune response directed to UL6 peptide after HSV ocular infection	Deshpande *et al* (2001). *J. Virol.* 75 (7): 3077-3088
2. Ocular infection with vaccinia virus expressing UL6 fails to induce HSK	Deshpande *et al* (2001). *J. Virol.* 75 (7): 3077-3088
3. T cells extracted from human corneas show no reactivity to HSV UL6	Verjans, G. M. *et al.* (1998). *J Infect Dis.* 177 (2): 484-488 Koelle, D. M. *et al.* (2000). *J Virol.* 74 (23). 10930-10938
4. T cells extracted from human corneas are not reactive to corneal antigens	Verjans, G. M. *et al.* (1998). *J Infect Dis.* 177 (2): 484-488,
5. Variation in clinical presentation of SK is not due to genetic variation in the UL6 epitope	Ellison, A. R. *et al* (2003). *Virology.* 310 (1): 24-28

PART II

EARLY EVENTS THAT DETERMINE SUSCEPTIBILITY TO HSK: THE ROLE OF IL-6 IN THE INDUCTION OF ANGIOGENESIS

Research described in this chapter is a slightly modified version of an article published in 2004 in *The Journal of Immunology* by Kaustuv Banerjee, Partha Sarathi Biswas, Bumseok Kim, Sujin Lee and Barry T Rouse:

Banerjee, K., Biswas, P. S., Kim, B., Lee, S. and Rouse, B. T. (2004). CXCR2-/- mice show enhanced susceptibility to herpetic stromal keratitis: a role for IL-6 induced neovascularization. *The Journal of Immunology*, 172: 1237-1245. *Copyright 2004. The American Association of Immunologists, Inc.*

In this chapter "we" and "our" refers to co-authors and me. My contributions in the paper include (1) selection of the topic (2) data analysis and interpretation (4) planning experiments (5) compiling and interpretation of the literature (6) understanding how results fit with the literature (7) compilation of contributions into one paper (8) providing structure to the paper (9) making graphs, figure and tables (10) writing and editing

ABSTRACT

Ocular infection with herpes simplex virus (HSV) results in a blinding immuno-inflammatory lesion known as Herpetic Stromal Keratitis (HSK). Early pre-clinical events include inflammatory cell, mainly neutrophils, infiltration of the stroma and neovascularization. To further evaluate the role of neutrophils in pathogenesis, HSV infection was compared in BALB/c and mice of the same background but lacking CXCR2, the receptor for chemokines involved in neutrophil recruitment.

Our results show clear differences in the outcome of ocular HSV infection in CXCR2-/- compared to control BALB/c mice. Thus CXCR2-/- animals had minimal PMN influx during the first 7 days post infection and this correlated with a longer duration of virus infection in the eye compared to BALB/c mice. The CXCR2-/- mice were also more susceptible to HSV induced lesions and developed HSK upon exposure to a dose of HSV that was minimally pathogenic to BALB/c mice.

The basis for the greater HSK lesion susceptibility of CXCR2-/- mice was associated with an elevated IL-6 response, which appeared in turn to induce the angiogenic factor VEGF. Our results serve to further demonstrate the critical role of angiogenesis in the pathogenesis of ocular lesions.

INTRODUCTION

Herpetic Stromal Keratitis (HSK) is an immunopathological reaction that results from ocular infection with herpes simplex virus (HSV) (1) . The lesion occurs naturally in humans and is a frequent cause of blindness (2). HSK is usually studied in the mouse and, as in humans, the pathogenesis involves immunopathology (1, 3). Multiple events are involved in the pathogenesis of HSK with $CD4^+$ T cells and neutrophils appearing as the pivotal cellular mediators (3-7). Neutrophil infiltration into the avascular cornea occurs promptly after HSV infection likely in response to signaling molecules produced either by infected cells or nearby cells stimulated by products released from infected cells (6, 8). The PMN influx may have several consequences. These include anti-viral effects, release of angiogenesis factors and possibly the unmasking of corneal autoantigens that help drive the ocular inflammatory response (6, 9, 10). Currently, the identity of the major signals responsible for the PMN influx into the HSV infected eye remain uncertain. However, several CXC chemokines that

cause PMN chemotaxis in other systems are present soon after HSV infection (11), and MIP-2 (CXCL1) was implicated indirectly as involved in HSV induced ocular neutrophil influx (12, 13). Although in humans the main chemokine attractant for PMN is IL-8, which engages the two high affinity receptors CXCR1 and CXCR2, the mouse lacks IL-8 and only CXCR2 acts as the receptor for PMN attracting CXC chemokines (14, 15). In consequence, animals lacking CXCR2 due to gene knockout (CXCR2-/-) are expected to show defects in neutrophil chemotaxis. Such was observed in at least 4 systems including ocular inflammatory responses induced by infection with the parasite *Onchocerca volvulus* (16-22). Since the PMN influx appears as a crucial event in HSK, the availability of the CXCR2-/- mice on the HSV susceptible BALB/c background should clarify the actual role of the PMN influx in the pathogenesis of lesions.

Our results show clear differences in the outcome of ocular HSV infection in CXCR2-/- compared to control BALB/c mice. Thus CXCR2-/- animals had minimal PMN influx during the first 7 days p. i. and this correlated with a longer duration of virus infection in the eye compared to BALB/c mice. The CXCR2-/- mice were also more susceptible to HSV induced lesions and developed HSK upon exposure to a dose of HSV that was minimally pathogenic to BALB/c mice. The basis for the greater HSK lesion susceptibility of CXCR2-/- mice was associated with an elevated IL-6 response, which appeared in turn to induce the angiogenic factor VEGF. Our results serve to further demonstrate the critical role of angiogenesis in the pathogenesis of ocular lesions.

MATERIALS AND METHODS

Animals

Female BALB/c-Cmkar2^{tm1Mwm} (CXCR2-/-) [Jackson Laboratory, Bar Harbour, Maine], BALB/c (Harlan Sprague Dawley, Indianapolis, IN), 6 to 8 week old, were used for the studies. Mice were housed in sterile micro-isolator cages and all food, bedding and instruments were autoclaved or disinfected. Manipulations were done under a laminar flow hood. To prevent bacterial super-infection, mice received prophylactic treatment of Sulfamethoxazole/Trimethoprim (Alpharma, Baltimore, MD) at the rate of 5ml per 200ml of drinking water. Antibiotic treatment was started 1 day before the beginning of experiments. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research.

Virus

HSV-1 RE strain (obtained from the laboratory of Dr. Robert Hendricks, University of Illinois at Chicago) was propagated and assayed on Vero cells for the measurement of PFU by standard protocols (23).

Corneal HSV infection, clinical observation and angiogenesis measurement

Corneas of mice, deeply anesthetized by Avertin (Pittman Moore, Mondelein, IL), were scarified with a 27-gauge needle. A 4µl drop containing the required dose of virus was applied to the scarified cornea and gently massaged with the eyelids. Animals were examined at different days post infection with a slit lamp biomicroscope (Kowa Co., Nagoya, Japan) and the severity of clinical keratitis of individually marked mice was recorded. Briefly, the clinical lesion score of HSK was

described as 0, normal cornea; 1, mild haze; 2, moderate haze, iris visible; 3, severe haze, iris not visible; 4, severe haze and corneal ulcer; 5, corneal rupture. Angiogenesis scoring was done as previously described (24). To quantify the degree of neovessel formation two primary parameters were used: 1) the circumferential extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle. The longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~0.4 mm (radius of the cornea is ~1.5 mm). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summated to derive the neovessel index (range, 0 to 16) for each eye at a given time point.

Virus recovery and titration

Eye swabs were taken from infected corneas (four animals/group) using sterile swabs soaked in DMEM containing 10 IU/ml penicillin and 100μg/ml streptomycin. Swabs were put in sterile tubes containing DMEM and stored at -80° C. For detection of virus, samples were thawed and vortexed. Duplicate 200μl aliquots of dilutions of each sample were plated on Vero cells grown to confluence in 24 well plates at 37° C in 5% CO_2 for 1hr 30 min. Medium was aspirated and 500μl of 2x DMEM containing 1% low-melting-point agarose was added to each well. Titers were calculated as $\log_{10}$ pfu/ml as per standard protocol (23).

Corneal Intrastromal Injection assay

Corneal intrastromal injection was performed as described before (25). Under direct stereomicroscopic observation, a nick in the epithelium and anterior stroma of mouse cornea was made with a 1/2 –inch 30-gauge needle with a 30° bevel, in the mid-periphery. For each cytokine to be tested 8 eyes were injected. The needle was introduced into the corneal stroma and advanced 1.5 mm to the corneal center. Two microliters of solution containing the required concentration of cytokine was forcibly injected into the stroma to separate the corneal lamellae and disperse the solution. Recombinant murine IL-6 (Endotoxin level < 1.0 EU per 1μg of protein) and VEGF (Endotoxin level < 0.1ng per 1μg of protein) were purchased from R & D systems. PBS was used as a control. For VEGF neutralization experiments, anti-mVEGF neutralizing antibody (2 μg) (R & D Systems) was mixed with 200ng of IL-6 and injected intrastromally. The length of the neovessels generated from the limbal vessel ring toward the center of the cornea was measured on days 2, 4 and 7. The length and width of the neovessels was calculated in clock hours (each clock hour equal to 30° at the circumference). The angiogenic area was calculated according to the formula $A=\{\text{Clock hours} \times 0.4 \times \text{vessel length(mm)} \times \pi\} /2$ and expressed as mm^2.

Quantitative RT-PCR

Total RNA from 4 corneas/time point was extracted by using RNeasy protect mini kit (Qiagen, CA). Briefly tissues were lysed in RLT buffer and RNA was purified according to manufacturer's instructions. RNase free DNase set (Qiagen, CA) was used to remove any contaminating genomic DNA. To generate cDNA, 1μg total RNA

was reverse transcribed by MMLV reverse transcriptase (Promega, WI). All cDNA samples were aliquoted and stored at -20° C till further use.

Real time PCR was performed using a DNA Engine Opticon (MJ research Inc., MA). Quantitect SYBR green PCR kit (Qiagen, CA) was used according to manufacturer's protocol. PCR amplification of house keeping gene, murine GAPDH, was done for each sample as a control for sample loading and normalization between samples. A standard curve was constructed with PCR-II Topo cloning vector (Invitrogen, CA) with the inserted fragment amplified by the SYBR green I system. PCR reaction was carried out for three dilutions of each sample (in duplicate). To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. Copy number for the target gene was then normalized to 10^6 copies of GAPDH control and data represented as copy numbers /cornea. The primers used were murine IL-6 primer, Forward TTCCATCCAGTTGCCTTCTT and Reverse CAGAATTGCCATTGCACAAC and murine GAPDH primer, Forward CATCCTGCACCACCAACTGCTTAG and Reverse GCCTGCTTCACCACCTTCTTGATG

Histopathology, Immunohistochemistry and Immunoflourescence

For histopathological analysis eyes were extirpated and fixed in 10% buffered neutral formalin and embedded in paraffin. Sections (5μm thick) were cut, deparaffinized and stained with hematoxylin and eosin. PMN were identified based on their morphology under 1000x magnification. Cell counts were done on three eyes/group, two sections/eye scanning the limbus, paracentral and central areas of the cornea. The number of PMN per section was averaged and the SD between the three eyes was computed.

For immunohistochemistry and immunofluorescence analysis, eyes were enucleated at the indicated time points and snap frozen in OCT compound (Miles, Elkart, IN). Six micron thick sections were cut, air dried and fixed in acetone:methanol (1:1) at -20° C for 10 min. Endogenous peroxidase activity was blocked using a 50 % alcohol solution containing 0.3 % hydrogen peroxide for 15 minutes and sections were blocked with 3 % BSA-PBS. Antibody dilutions were made in 1% BSA-PBS. For detection of neutrophils, biotinylated anti-Grl mAb (Clone RB6-8C5, Pharmingen) was diluted 1/100 and incubated overnight at 4° C. For detection of HSV antigens, sections were treated with rabbit anti-HSV anti-serum (10min) (Dako, Carpentaria, CA) followed by biotinylated anti-rabbit Ab (20 min) (Biogenex, San Ramon, CA). Sections were then treated with HRP- conjugated streptavidin for 45 min (1:1000 dilution, Jackson Immunoresearch Laboratories) followed by 3, 3' – diaminobenzidine substrate (Biogenex, San Ramon, CA) and counterstained with hematoxylin (Richard Allen Scientific, Kalamazoo, MI). Irrelevant biotinylated rat antibodies and normal rabbit serum was used as negative controls. For immunoflourescence counting of neutrophils acetone:methanol fixed sections were blocked with 5% BSA-PBS-0.05% Tween 20 containing 1/200 dilution of Fc block (Clone 2.4G2, Pharmingen) for 2 hrs followed by overnight incubation with 1/500 dilution of FITC anti-Grl (Pharmingen) in 1% BSA-PBS-0.05% Tween 20. Slides were mounted with Vecta-Shield reagent (Vector Laboratories, Burlingame, CA). Gr-1 +ve cells were counted from two sections/eye and three-eyes/time point using a fluorescence microscope. For this purpose, the corneal section was divided into the limbal regions (roughly the part of the cornea marked by area of origin of the iris) and the central region (paracentral and central).

Cytokine ELISA of corneal lysates

For preparation of corneal lysates, 5-6 corneas/time point were pooled and minced. All procedures were done on an ice bath. Minced pieces were collected in 1 ml of DMEM without FCS and homogenized using a tissue homogenizer (PRO Scientific Inc., Monroe, Conn) four times, 15 seconds each with a gap of 1 min between homogenization to allow the sample to cool on ice. The lysate was then clarified by centrifugation at 14,000 rpm for 5 min at 4° C. The supernatant was collected and used immediately or stored at -80° C till further use. Lysates were assayed using a standard sandwich ELISA protocol. Anti-IL-6 capture and biotinylated detection antibodies were from Pharmingen (Clone MP5-20F3) and standard rmIL-6 was from R & D systems Inc, Minneapolis, MN. Anti-MIP2 and anti-VEGF capture and biotinylated detection antibodies and recombinant standards for MIP-2 and VEGF were from R & D systems. The color reaction was developed using ABTS (Sigma) and measured with an ELISA reader (Spectramax 340, Molecular Devices) at 405nm. The detection limit was 2 pg/ml. Quantification was performed with Spectramax ELISA reader software version 1.2.

Statistics

Statistical analysis was performed utilizing standard student's *t* test.

RESULTS

Neutrophil influx following HSV infection

A prompt consequence of HSV infection of the cornea in BALB/c mice is PMN influx (6). This, as shown in Fig.1 (all figures located in appendix), is evident as early

as 12hr p.i., peaks around 2 days p.i. and subsides greatly by day 5. This is the preclinical phase of HSK. Clinically evident lesions start around day 8 p.i. and are at their peak at 15-21 days p.i. (3). The pattern of PMN influx in CXCR2-/- mice differed from that in BALB/c animals both in terms of the level of response and location of the cells in the corneal stroma (Fig 2A). Thus, whereas in BALB/c mice PMN were abundantly present in the paracentral and central cornea, in CXCR2-/- mice PMN were mainly confined to the limbal area (the vascular part of the cornea) (Fig 2A). In terms of total PMN numbers in the cornea at the 2 day peak, the response of CXCR2-/- mice was around 30% that of BALB/c mice (Fig 1). However, PMN numbers in the limbal areas were approximately equal (Fig 2A). Thus as noted in some other systems, PMN appeared not to migrate beyond the vascular release site (16, 26). In addition, as revealed by H & E staining, the total number of inflammatory cells infiltrating the corneas of CXCR2-/- mice, at day 2 p.i., was lower compared to BALB/c mice (Fig 2B and Table 1, all tables located in appendix). Neutrophils comprise the majority of cells infiltrating the corneas of BALB/c mice at this time point, being almost 2 times that of monocytes (Table 1). In contrast, neutrophil counts were far lower in CXCR2-/- corneas and interestingly a majority of cells in such corneas were monocytes (Table 1). However there was no significant difference in the numbers of infiltrating monocytes in corneas of CXCR2-/- mice compared to BALB/c mice (Table 1). Neutrophil counts in BALB/c and CXCR2-/- mice were also compared at day 15 during the clinical phase of HSK. Surprisingly, PMN were well represented in the central cornea in CXCR2-/- mice, and accounted for approximately the same percentage of the inflammatory cells as was the case with BALB/c mice (Table 1).

Viral clearance in CXCR2-/- and BALB/c mice

Previous reports had noted that PMN depletion in BALB/c mice resulted in prolonged viral presence and animals often succumbed to HSV induced encephalitis (6, 9). Thus it was anticipated that viral clearance might be impaired in CXCR2-/- mice because of the diminished PMN response. To measure such an effect, groups of CXCR2-/- and BALB/c mice were infected at either a high ($5x10^5$ pfu) or low ($5x10^4$ pfu) dose of virus, and eye swabs were collected daily for viral titration While virus was detectable in swabs from CXCR2-/- mice, until at least day 7, even after infection with the low dose, BALB/c mice had cleared virus by this time point (Table 2). Conceivably, in the corneal tissues themselves virus could have been present several days beyond day 7. Thus upon analysis of frozen sections for the presence of viral antigens on day 7, abundant antigen was present in CXCR2-/- eyes (Fig 3) but antigen was absent by day 6 in BALB/c mice [data not shown, (6)]. Even more of interest, whereas in BALB/c mice viral antigen was evident only in the epithelium [data not shown and (6)], in CXCR2-/- mice antigen found after day 5 p.i., including the day 7 samples, was present in the stroma itself (Fig 3). Such a pattern of antigen expression was noted previously in B cell K/O (27) and transgenic SCID/RAG-/- mice (28, 29).

Taken together, our results indicate that CXCR2-/- mice clear virus less effectively than wild type mice and the virus spreads to a tissue location that is not found in the wild type mice.

Stromal Keratitis in CXCR2-/- and BALB/c mice

Groups of CXCR2-/- and BALB/c mice of the same age (6-8 weeks) were infected ocularly with either $5x10^5$ or $5x10^4$ pfu HSV-1 RE and animals were followed at intervals over a 20-day observation period to measure a) the extent of corneal

angiogenesis and b) the clinical severity of HSK. At the high dose level of infection, the pattern of events in the two strains was similar (Fig 4 and 5). Thus cumulative results from 2 separate experiments revealed that 17 of 20 eyes from CXCR2-/- mice develop significant lesions compared to 19 of 20 eyes from infected BALB/c mice. The average severity score and time of peak lesions did not differ significantly between the two groups (Fig 4 A, C, D). In addition, the extent of corneal neovascularization in both groups of mice was of similar magnitude (Fig 5 A and B). Representative eyes from CXCR2-/- and BALB/c mice showing equal clinical severity (score of 4) were examined histologically following H & E staining. Such eyes showed similar inflammatory changes and influx of a large number of inflammatory cells (Fig 6A), which included Gr-1+ve cells, as revealed by immunohistochemical analysis (Fig 6B).

Whereas a comparable outcome of events occurred after high dose infection, dramatic differences in the responsiveness of CXCR2-/- and BALB/c mice was observed upon infection with the lower dose of virus. Accordingly only 3 of 20 eyes in BALB/c mice developed notable clinical lesions (score of 3 or higher), significantly more (12 of 20 eyes) CXCR2-/- mice responded with positive lesions (Fig 4 C, D). In consequence, the average severity score at the peak time point was significantly higher in CXCR2-/- mice (Fig 4 B). Eyes of CXCR2-/- mice developing disease with the lower dose of infection were histologically similar to that seen with eyes from the higher dose infected mice (Fig 6A) with large number of Gr-1+ve cells (Fig 6B).

As with lesion incidence and severity, significant differences were apparent in the average extent of angiogenesis in CXCR2-/- and BALB/c mice following infection with low dose virus (Fig 5). By day 10 p.i., extensive angiogenesis was

evident in the CXCR2-/- group (Fig 5A) and at day 20 p.i., 12 of 20 eyes from CXCR2-/- mice had developed an angiogenesis score greater than 10 (Fig 5B). In marked contrast, only 4 of the 20 eyes of BALB/c mice developed equivalent scores (Fig 5B). When the overall angiogenesis was compared in the groups of CXCR2-/- and BALB/c mice the average angiogenesis score was 3 times (day 20 p.i.) that observed in BALB/c mice (Fig 5 A and B). Thus in spite of a diminished neutrophil migration response to HSV infection in CXCR2-/- mice, angiogenesis appeared to be enhanced.

Cytokines and chemokine production in infected corneas

So far our experiments indicate that CXCR2-/- mice show greater susceptibility to HSV infection than BALB/c animals and that this response reflects as more neovascularization in CXCR2-/- mice. Previous reports had demonstrated that IL-6 was one of the few cytokines produced by susceptible corneal cells following HSV infection (30). Moreover, IL-6 was implicated as a critically important molecule during pathogenesis of corneal disease following HSV-1 infection (31). Furthermore CXCR2-/- mice express high circulating levels of IL-6 (15). Finally IL-6 could be involved in angiogenesis since it was shown to induce the potent angiogenesis factor VEGF (32, 33). To determine if levels of corneal IL-6 were elevated in CXCR2-/- mice compared to BALB/c controls, groups of mice were infected with a low dose of HSV and sacrificed at intervals during the first week p.i. Corneal tissues were processed both to measure IL-6 mRNA and protein levels. As is evident in Fig 7, both mRNA and protein levels for IL-6 were significantly higher in the CXCR2-/- mice. The greatest differences were noted between day 2 and 5 where protein levels were

upto 50 fold and mRNA levels 100 fold increased in CXCR2-/- compared to BALB/c mice.

Since the IL-6 cytokine may influence expression of the chemokine MIP-2 and the angiokine VEGF (31, 32), levels of these proteins were also measured by ELISA. Both molecules were elevated in CXCR2-/- samples taken from mice infected with low dose of virus (Fig 8 B and D). In addition, groups of mice were also infected with the high dose so that similar numbers of animals would eventually develop lesions. Protein levels of both VEGF and MIP-2 were higher in CXCR2-/- mice compared to BALB/c mice at day 5 and 7 post infection (Fig 8 A and C). Thus our data are consistent with the hypothesis that CXCR2-/- mice develop heightened angiogenesis, and susceptibility to HSK, because their elevated IL-6 response caused production of the angiokine VEGF. Moreover even though CXCR2-/- mice have elevated MIP-2 levels the PMN response to it is minimal over that seen in BALB/c mice.

Verification that IL-6 induces VEGF production in mouse corneas in vivo

To avoid the complication of virus infection and to determine the potential for IL-6 to induce VEGF production in the eye, experiments were done in BALB/c mice in which the IL-6 was injected directly into the corneal stroma. Measurements were then made of VEGF production (in corneal extracts) and levels of angiogenesis. Comparisons were made with recombinant $VEGF_{164}$ protein injected into the stroma as well as with negative PBS control injections.

The results show that IL-6 injection led to both angiogenesis and VEGF production (Fig 9 and 10). With regard to angiogenesis, IL-6 induced significant responses evident at 48hr but these had declined by day 4 post injection (Fig 9A). The

response to VEGF positive control protein was greater than that of IL-6 and in this case the peak response was evident at 4 days post injection (Fig 9A). In addition, the quality of the IL-6 and VEGF responses appeared to differ. Thus the blood vessels evident after IL-6 injection were finer and less dense that those induced by VEGF (Fig 9B). This may mean that additional factors are involved in IL-6 induced angiogenesis. However the results shown in Fig 9 and 10, do indicate that VEGF was part of the IL-6 induced angiogenic response. Thus the angiogenesis induced by the injection of IL-6 could be blocked by administration of anti-murine VEGF (Fig 9). In addition corneal lysates at 48hr post IL-6 injection contained significant levels of VEGF as measured by ELISA (Fig 10). In contrast VEGF was not present in PBS injected corneas.

IL-6 induces VEGF production in vitro

To confirm that IL-6 directly induces cells to produce VEGF, the J774A.1 murine macrophage cell line was used which can be induced to express VEGF upon infection with virus (Zheng and Rouse, unpublished results). Such cells were reacted for 12 or 24 hr with different concentrations of IL-6, LPS or unstimulated. VEGF production was measured in the cell supernates by ELISA, and the cells themselves were harvested to quantify VEGF mRNA by RT-PCR. The results shown in Fig. 11 demonstrate that IL-6 induced VEGF production, measured by both assays.

DISCUSSION

This report deals with stromal keratitis, a blinding immunoinflammatory lesion of the corneal stroma that results from HSV infection of the eye. It is well established that

HSK lesions in humans as well as animal model systems are largely immunopathological and orchestrated by T cells, principally the $CD4^+$ subset (3-5, 7). However, in the murine model the most prominent initial cellular event in HSV infected eye is an abundant influx of mainly PMN into the corneal stroma. Several functions have been attributed to such infiltrates, but their precise role as well as the identity of molecules responsible for the influx remain poorly defined. In the present report, we have compared the outcome of ocular infection in wild type BALB/c mice with mice of the same background but lacking the expression of the chemokine receptor CXCR2. Thus, as shown in other systems, such mice exhibit defective PMN migration to inflammatory sites (15, 17, 19, 21, 22). Our results show that CXCR2-/- mice generated minimal PMN influx into the paracentral and central corneal stroma following HSV infection. The knockout mice were also less able to clear HSV from the eye and more susceptible to HSK development than were BALB/c mice. The increased susceptibility of CXCR2-/- mice appeared to be the consequence of a heightened IL-6 response that in turn stimulated VEGF induced corneal neovascularization. Thus these data serve to further emphasize the importance of angiogenesis in the pathogenesis of HSK.

A prominent early event after virus infection of the cornea is influx into the avascular stroma of inflammatory cells, primarily PMN (6). This influx occurs promptly after infection and likely serves several functions. These include antiviral effects (6, 9) and an involvement in neovascularization (10). The signals responsible for the PMN influx are likely multiple and non-viral derived. Several CXC chemokines are upregulated following HSV infection (11, 30) and one of them, MIP-2, was indicated to be a major mediator in both HSV and other pathogen induced PMN corneal infiltrates (12, 13, 34, 35). These reports await confirmation.

Moreover, with HSV, the source of MIP-2 remains undefined and would not seem to be the infected cells themselves (our unpublished results). Since in the mouse a single receptor, CXCR2, is used by all CXC PMN-attracting ligands (14, 15), the absence of this molecule should result in changes in PMN recruitment and help reveal the role of such cells in pathogenesis. Our results clearly showed that only minimal PMN invasion occurred into the stroma following HSV infection of CXCR2-/- mice. The effects were most evident in the avascular paracentral and central corneal locations. Thus at the limbal region, which is close to blood vessels, PMN were abundant in both CXCR2-/- and BALB/c mice indicating that vascular escape still occurred in CXCR2-/- mice but PMN migration was compromised.

A major consequence of the diminished PMN influx was that CXCR2-/- mice were more susceptible to infection. Thus virus persisted for longer periods in the eye. In addition, the virus gained access to the stromal tissue site, a situation noted previously only in immuno-compromised animals (27-29) . This circumstance likely reflects movement to the stroma via zosteriform spread from infected nerve ganglia, an event normally contained by the immune system (36, 37). How PMN contribute to such immune control remains unknown but IFNγ and TNFα have been suggested to be participants in the antiviral effect (38, 39).

Previous reports had indicated that PMN contributes to corneal angiogenesis following HSV infection (10). Thus we expected to observe that this process might be diminished in CXCR2 mice that have minimal PMN responses to HSV. In fact the contrary result was observed with the extent of angiogenesis being enhanced in CXCR2-/- mice compared to BALB/c mice. This was particularly noticeable when low virus doses were used for infection. The heightened angiogenesis noted in

CXCR2-/- mice correlated with the increased severity of HSK lesions, supporting the concept that angiogenesis plays a crucial role in the pathogenesis of such lesions (40). Curiously, in the clinical phase, once extensive angiogenesis had occurred, the PMN representation in the central cornea of CXCR2-/- mice was comparable to that of BALB/c animals. However, the PMN in CXCR2-/- vs. the BALB/c mice did appear to occupy different locations in the two strains. Thus many of the PMN counted were seen to be within blood vessels in the CXCR2-/- corneas, while in the BALB/c mice these were mostly extravascular. In addition, the fact that PMN numbers were approximately equal in both strains, late in disease, might reflect the operation of additional PMN chemotactic factors. MIP-1α represents a possible candidate based on previous reports (41). This issue is under investigation.

An explanation for the greater neovascular response to HSV of CXCR2-/- mice could lie with their differential IL-6 production. Thus for unknown reasons, CXCR2-/- mice have elevated serum IL-6 levels compared to BALB/c mice (15). In our studies too, we observed that levels of both mRNA and IL-6 protein levels in the cornea of HSV infected CXCR2-/- mice were higher (up to 100 fold) than those in control infected mice. The cellular source of the increased IL-6 response was not defined but it could include virus-infected cells themselves. Thus IL-6 is known to be produced by virus infected cells whereas most host proteins are rapidly switched off by HSV (41). It is known that HSV infection does result in the up-regulation of several host proteins but these mainly derive from infected cells. Examples of this phenomenon include IL-12 and VEGF (8, 24). Our results indicate that IL-6 was involved in anagiogenesis as a consequence of inducing VEGF. In support of this notion, VEGF protein levels were increased in the corneas of HSV infected CXCR2-/- mice over those comparably infected BALB/c animals. In addition, confirming

previous reports (32, 33), we showed with J774 A.1 cells exposed to IL-6 *in vitro* caused them to express VEGF. Finally, we demonstrated that injection of IL-6 into the corneal stroma of mice resulted in angiogenesis, an effect inhibited by anti-VEGF administration.

Taken together, our results support the hypothesis that PMN play an important role in HSV induced ocular lesions. Their normal function is required to help minimize the development of HSV induced lesions. This is in part the consequence of PMN mediated antiviral effects. Another effect may involve the regulation of angiogenesis. This idea was supported by the observation that when PMN influxes into the cornea were minimal because of faulty responses to chemokines, angiogenesis was increased and animals developed more severe HSK lesions. This regulation is indirectly linked to antiviral effects and the pro-inflammatory environment. An important component of this environment, defined in the current study, is the cytokine IL-6. Apart from it's role in the induction of angiogenesis, the recent observations that IL-6 inhibits regulatory T cells (42), may provide an additional explanation for the heightened HSK seen in CXCR2-/- mice. This issue is currently under investigation. It will be important to define if the scenario that emerges from studies of the CXCR2-/- mouse can be confirmed in other models. Studies on double knockout CXCR2 and IL-6 mice would be of particular interest.

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APPENDIX

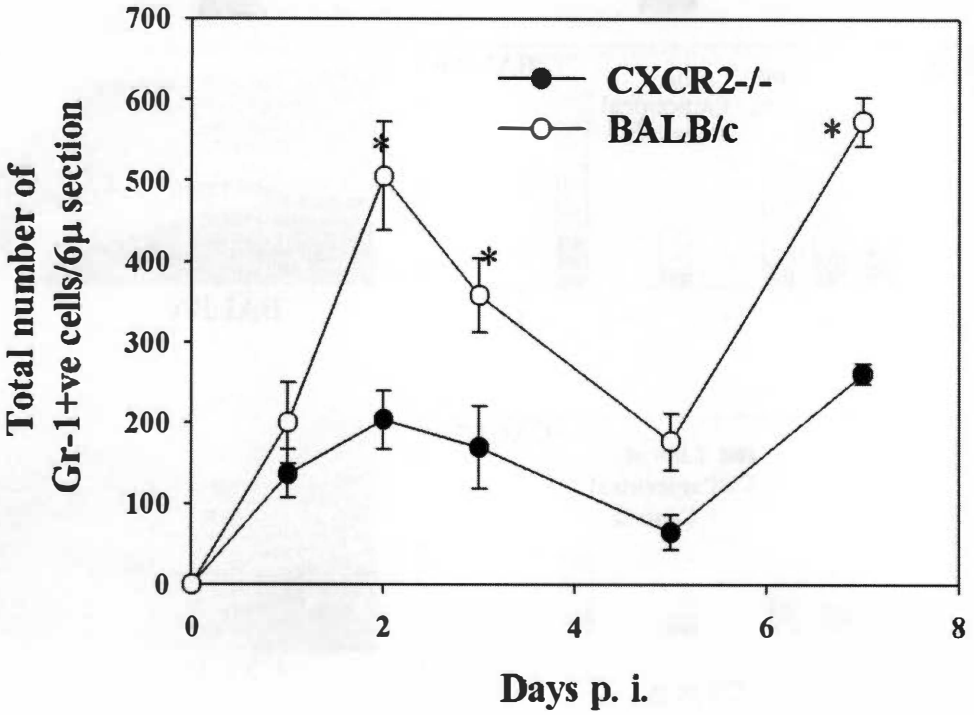


Figure 1. Kinetics of PMN migration into CXCR2-/- corneas after HSV-1 infection.

CXCR2-/- and BALB/c mice were infected with $5x10^5$ pfu/eye with HSV-1 RE. Ocular sections from eyes of mice sacrificed at the indicated time points were stained with Gr-1 FITC and PMN were counted under 400x magnification using a fluorescence microscope (2 sections/eye and 3 eyes/time point). Results are expressed as mean counts for each time point ± SD. *$p<0.05$*

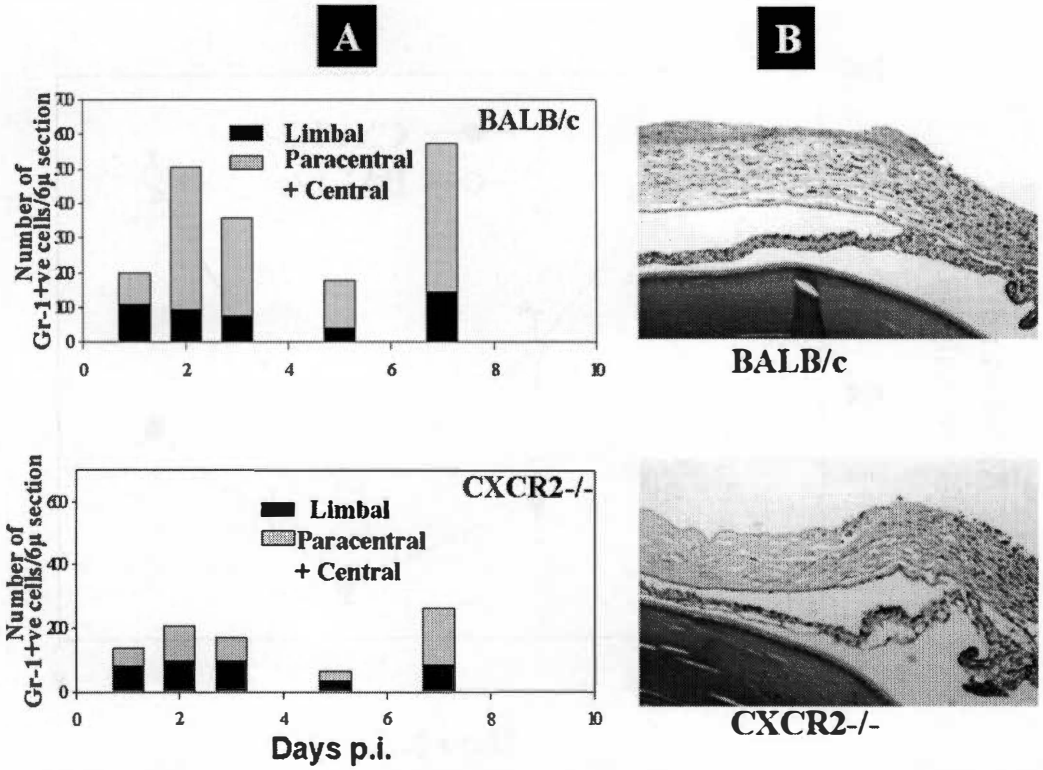


Figure 2. A majority of PMN in the HSV infected CXCR2-/- corneas are found at the limbus

A. Mice were infected with $5x10^5$ pfu/eye with HSV-1 RE. Ocular sections were made from eyes of mice sacrificed at indicated time points and stained with FITC labeled Gr-1 and PMN were counted under 400x magnification using a fluorescence microscope (2 sections/eye and 3 eyes/time point). Cells were counted in the limbal region (roughly the area marked by the point of origin of the iris) and the paracentral and central corneas. Results are expressed as mean counts for each region for each time point.

B. Mice were sacrificed at day 2 p.i. and eyes processed for paraffin embedding and H & E staining. Photographs show reduced number of inflammatory cells in the paracentral area of the cornea of a CXCR2-/- mouse compared to BALB/c mouse.

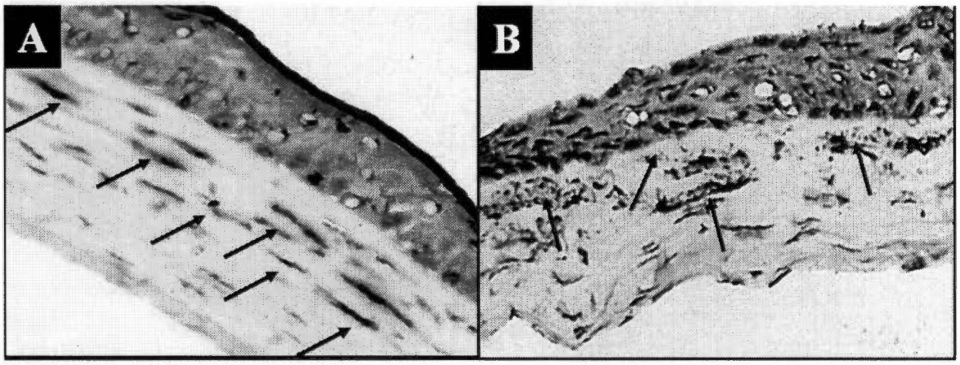


Figure 3. Immunohistochemistry for viral antigens in corneas of CXCR2-/- mice

A & B. Viral antigens (arrows) are detectable in corneal stroma of CXCR2-/- mice infected with high; $5x10^5$ pfu (**A**) and low; $5x10^4$ pfu (**B**) HSV-1 RE at day 7 p.i. DAB was used as substrate and sections were counterstained with haematoxylin. Original magnification x200.

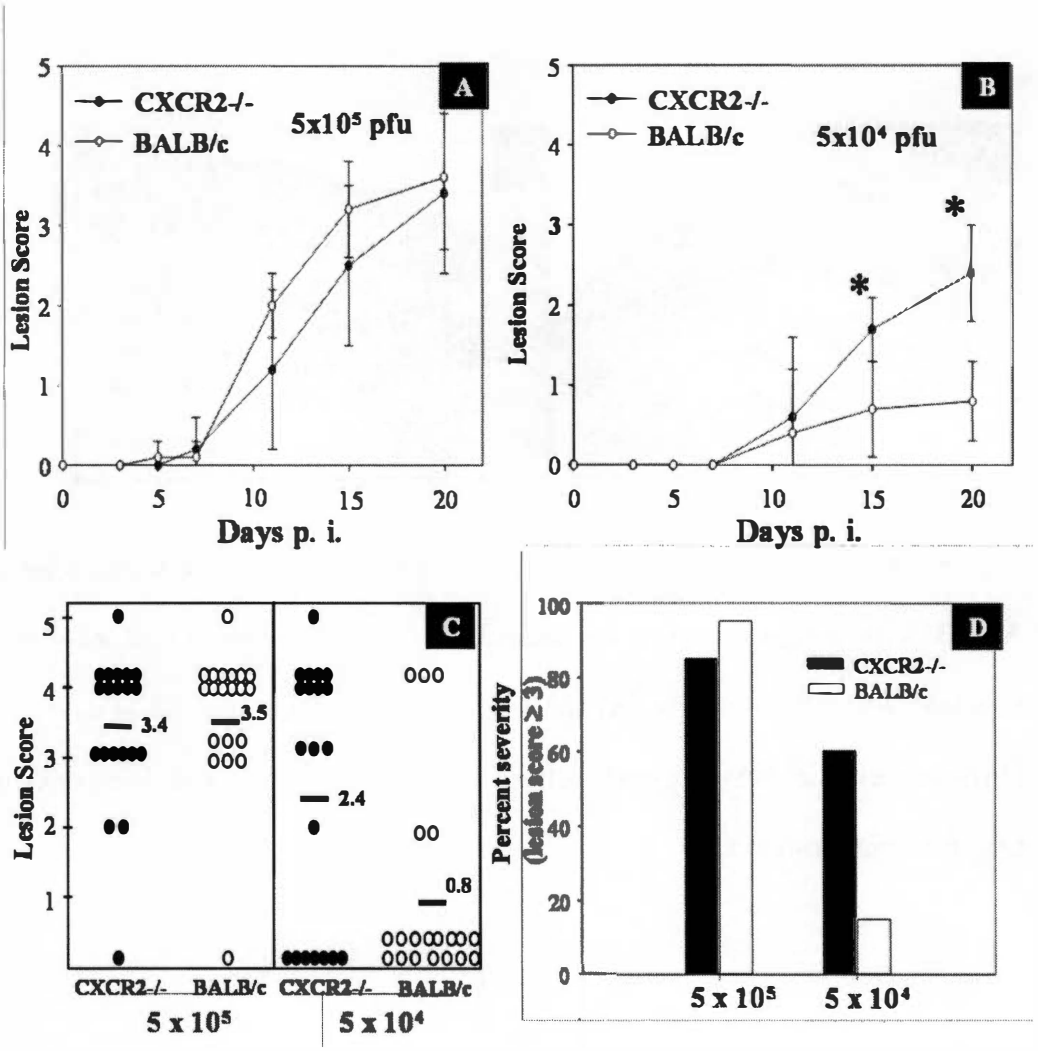


Figure 4. Enhanced HSK severity in CXCR2-/- mice

A & B. Results show the kinetics of the development of HSK lesions in CXCR2-/- and BALB/c mice infected with $5x10^5$ pfu (A) and $5x10^4$ pfu (B) HSV-1 RE.

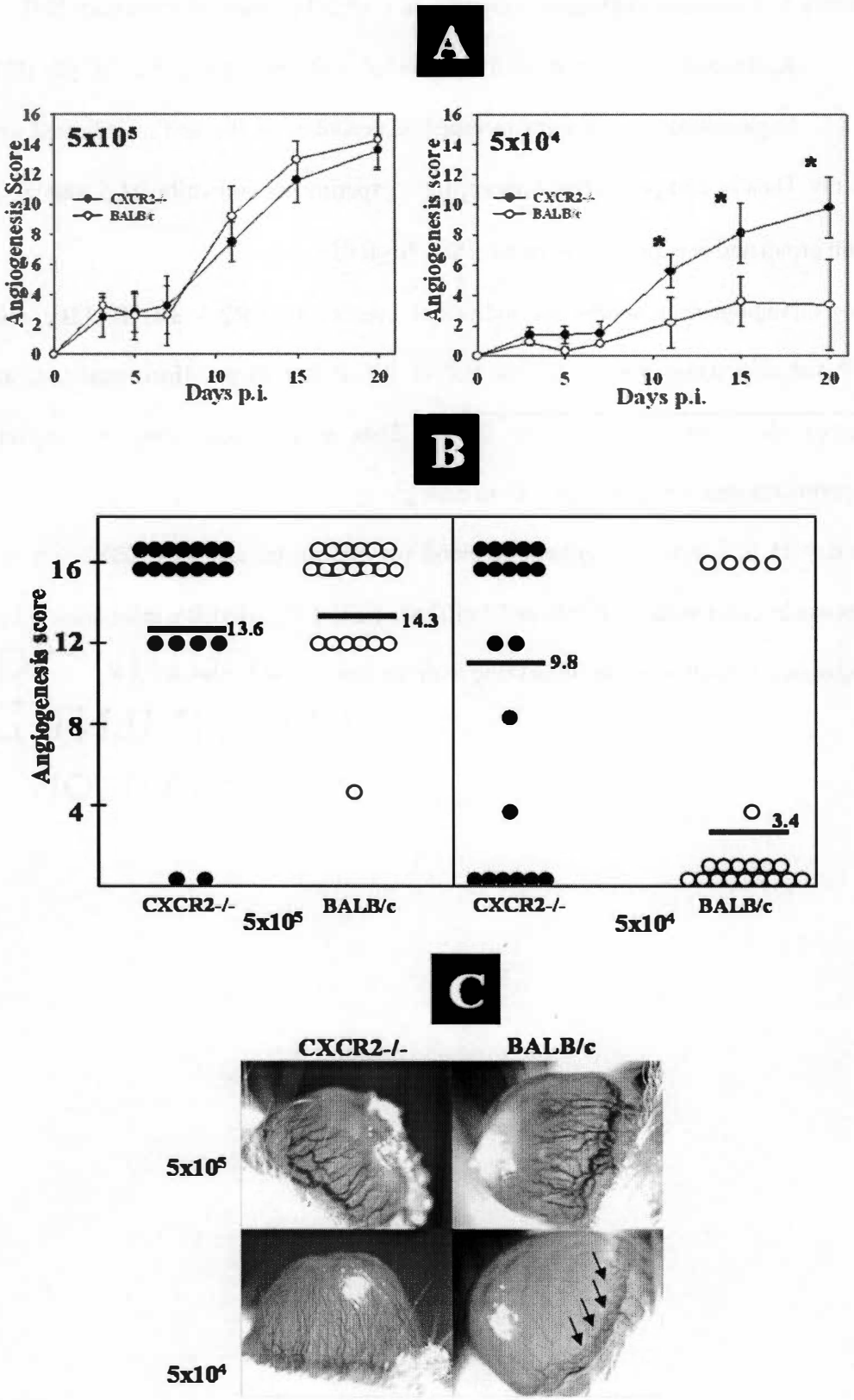
C & D. Severity of HSK lesions in mice infected with $5x10^5$ pfu and $5x10^4$ pfu HSV-1 RE at day 20 p.i. Each dot represents the HSK score from one eye. Horizontal bars and figures show the mean for the groups. Data is compiled from two separate experiments consisting of 5 animals in each group.

Figure 5. Enhanced angiogenic response in CXCR2-/- mice infected with HSV-1

A. Kinetics of angiogenesis in mice infected with $5x10^5$ pfu and $5x10^4$ pfu HSV-1 RE. Angiogenesis scores were recorded as described in the text at indicated time points. Data is compiled from two separate experiments consisting of 5 animals in each group and is expressed as mean ± SD. $*p<0.05$

B. Angiogenesis scores for individual eyes of CXCR2-/- and BALB/c mice infected with $5x10^5$ and $5x10^4$ pfu HSV-1 RE at day 20 p.i. Horizontal bars and figures show the mean for the groups. Data is compiled from two separate experiments consisting of 5 animals in each group.

At day 15 p. i. extensive growth of blood vessels can be seen in CXCR2-/- mouse corneas infected with $5x10^5$ pfu and $5x10^4$ pfu HSV-1 RE. BALB/c mice show minor angiogenic sprouts near the limbal ring with the lower dose (arrows)


A
5x10⁵
CXCR2-/-
BALB/c
Angiogenesis Score
Days p.i.
5x10⁴
Angiogenesis score
Days p.i.
B
Angiogenesis score
13.6
14.3
9.8
3.4
CXCR2-/-
BALB/c
5x10⁵
CXCR2-/-
BALB/c
5x10⁴
C
CXCR2-/-
BALB/c
5x10⁵
5x10⁴

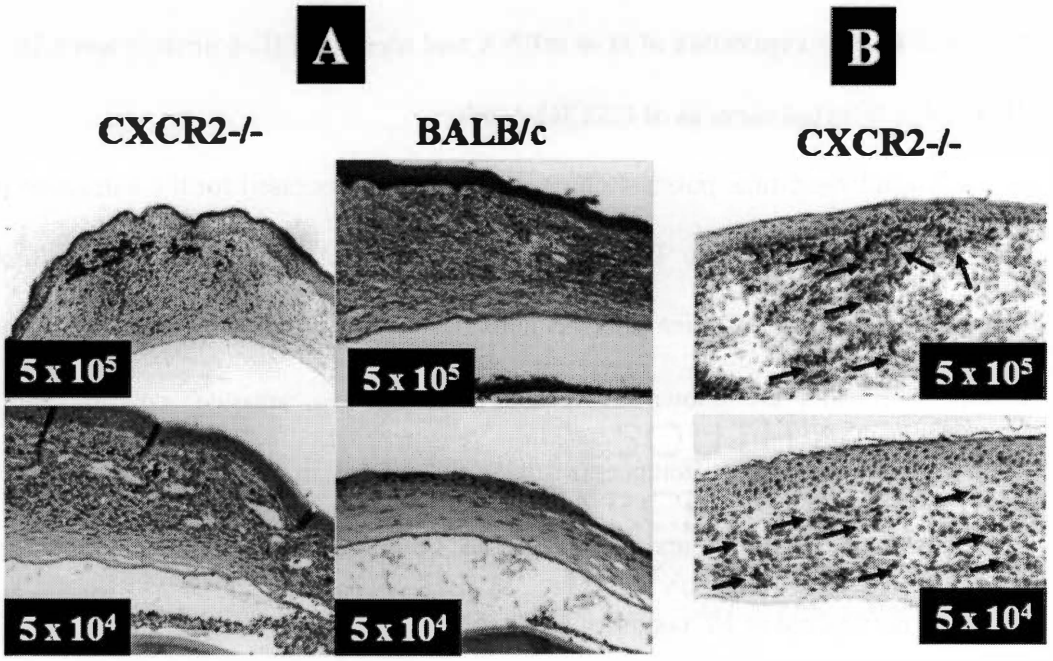


Figure 6. Ocular lesions seen in CXCR2-/- animals show histological characteristics similar to that seen in BALB/c

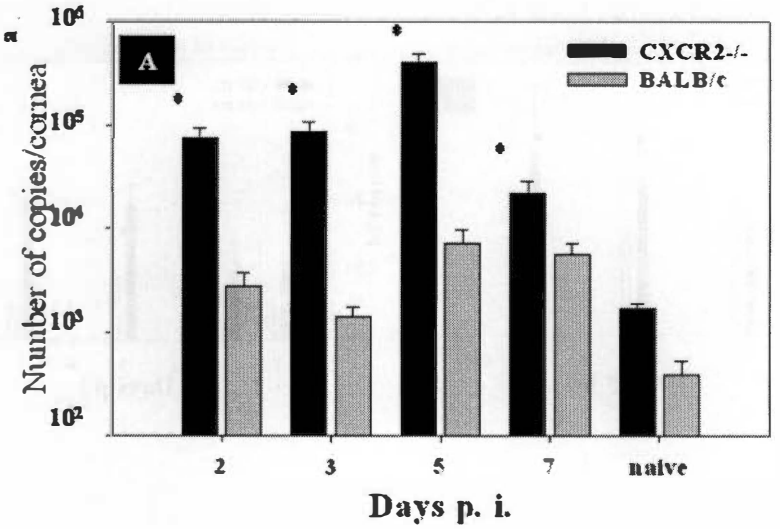
A. Mice were terminated on day 20 p.i. and eyes processed for paraffin embedding. Haematoxylin & Eosin staining was carried out on 6μ sections. Magnification x200.

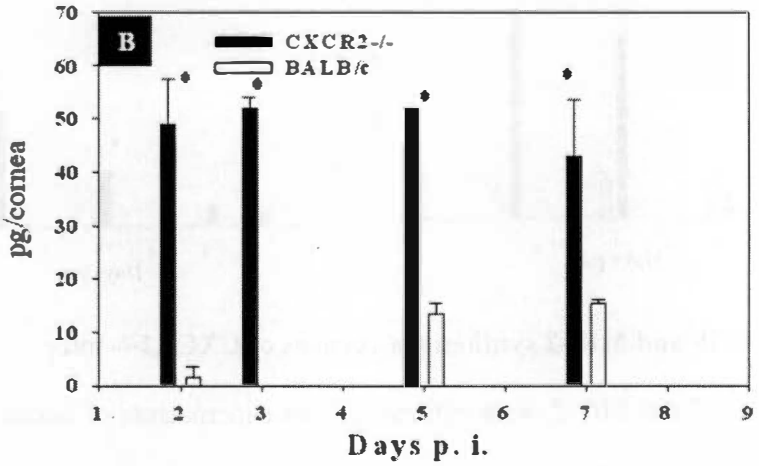
B. Large number of neutrophils can be detected in eyes from CXCR2-/- mice (day 20 p.i.) showing HSK with both $5x10^5$ pfu and $5x10^4$ pfu virus doses (arrows). DAB was used as the substrate and sections were counterstained with Haematoxylin. Original magnification x200.

Figure 7. Higher expression of IL-6 mRNA and increased IL-6 protein levels in HSV-1 RE infected corneas of CXCR2-/- mice.

A. At indicated time points 4 corneas/group were processed for the extraction of cellular mRNA. Real time PCR analysis was conducted to detect IL-6 mRNA expression in corneas of mice infected with $5x10^4$ pfu of HSV-1 (see materials and methods). Results are shown as mean ± SD of two separate experiments. * Statistically significant difference ($p<0.05$) was noted in the expression of IL-6 mRNA between CXCR2-/- and wild type BALB/c mice. [a] Copy number of the target gene was normalized to 10^6 copies of GAPDH.

B. Levels of IL-6 protein was estimated from supernatants of corneal lysates of mice infected with $5x10^5$ pfu HSV-1 RE by an antibody capture ELISA as outlined in materials and methods. Results are expressed as mean ± SD of two separate experiments (6 corneas/time point). *$p<0.05$


A
CXCR2-/-
BALB/c
Number of copies/cornea
Days p. i.
naive


B
CXCR2-/-
BALB/c
pg/cornea
Days p. i.

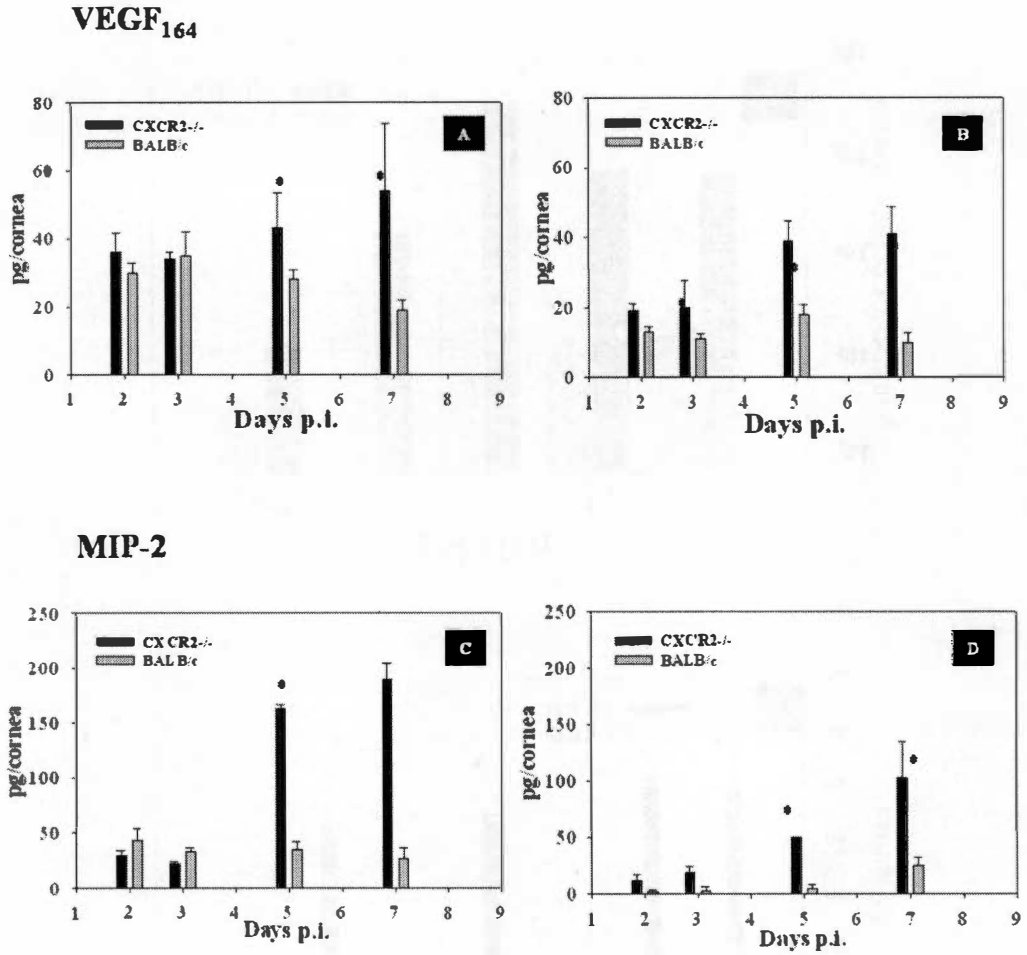


Figure 8. VEGF and MIP-2 synthesis in corneas of CXCR2-/- mice

Levels of VEGF and MIP-2 were estimated from supernatants of corneal lysates of mice infected with $5x10^5$ pfu (A, C) and 5×10^4 pfu (B, D) HSV-1 RE by an antibody capture ELISA as outlined in materials and methods. Results are expressed as mean ± SD of two separate experiments (6 corneas/time point). *$p<0.05$

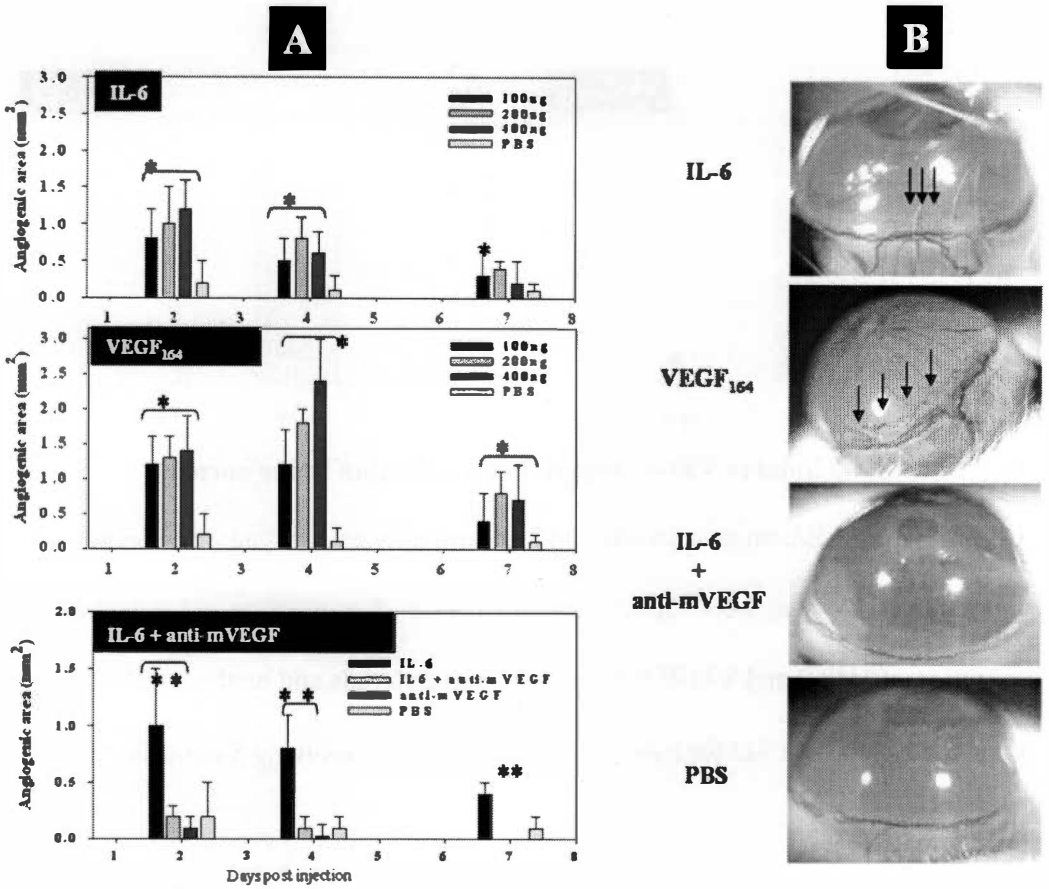


Figure 9. IL-6 induces angiogenesis in a corneal intrastromal injection assay

A. Different concentrations of recombinant murine IL-6 was injected intrastromally into BALB/c corneas (*n=8*) and angiogenesis scoring was carried out on days 2, 4 and 7 after injection. VEGF was used as a positive and PBS as a negative control. For neutralization experiments a mix of 200ng IL-6 and 2ug anti-mVEGF were injected in a 2µl volume. Results expressed as mean ± SD. * Significant difference ($p<0.05$) compared to PBS treatment. ** Significant difference ($p<0.05$) compared to administration with anti-VEGF neutralizing antibody

B. Representative photographs of eyes at day 2 injected with 200ng of IL-6 showing finer and less dense blood vessel development than that seen with the same dose of VEGF (arrows). Eyes that received IL-6 mixed with VEGF neutralizing antibody or PBS show negligible blood vessel development at this time point.

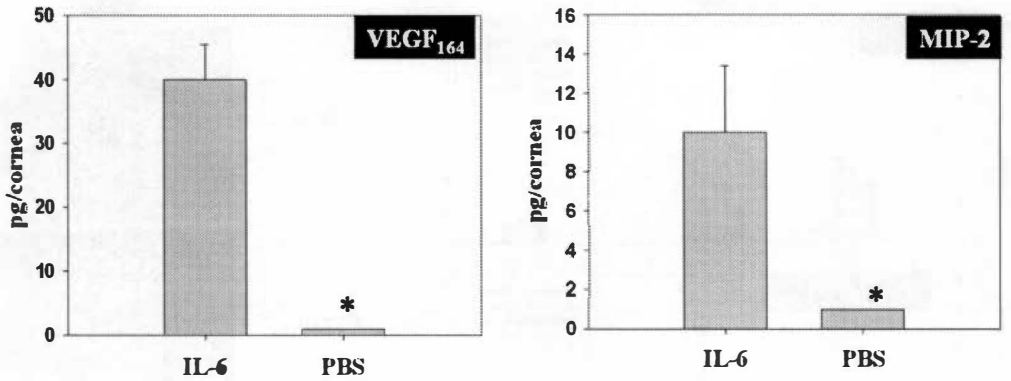


Figure 10. IL-6 induces VEGF and MIP-2 production in the cornea

Corneas of BALB/c mice were injected intrastromally with 200ng of recombinant murine IL-6. Corneas were excised from mice 48hr after injection and processed for detection of MIP-2 and VEGF from lysates (see materials and methods). Results are expressed as mean ± SD for two separate experiments involving 5 corneas. * Below detection limit

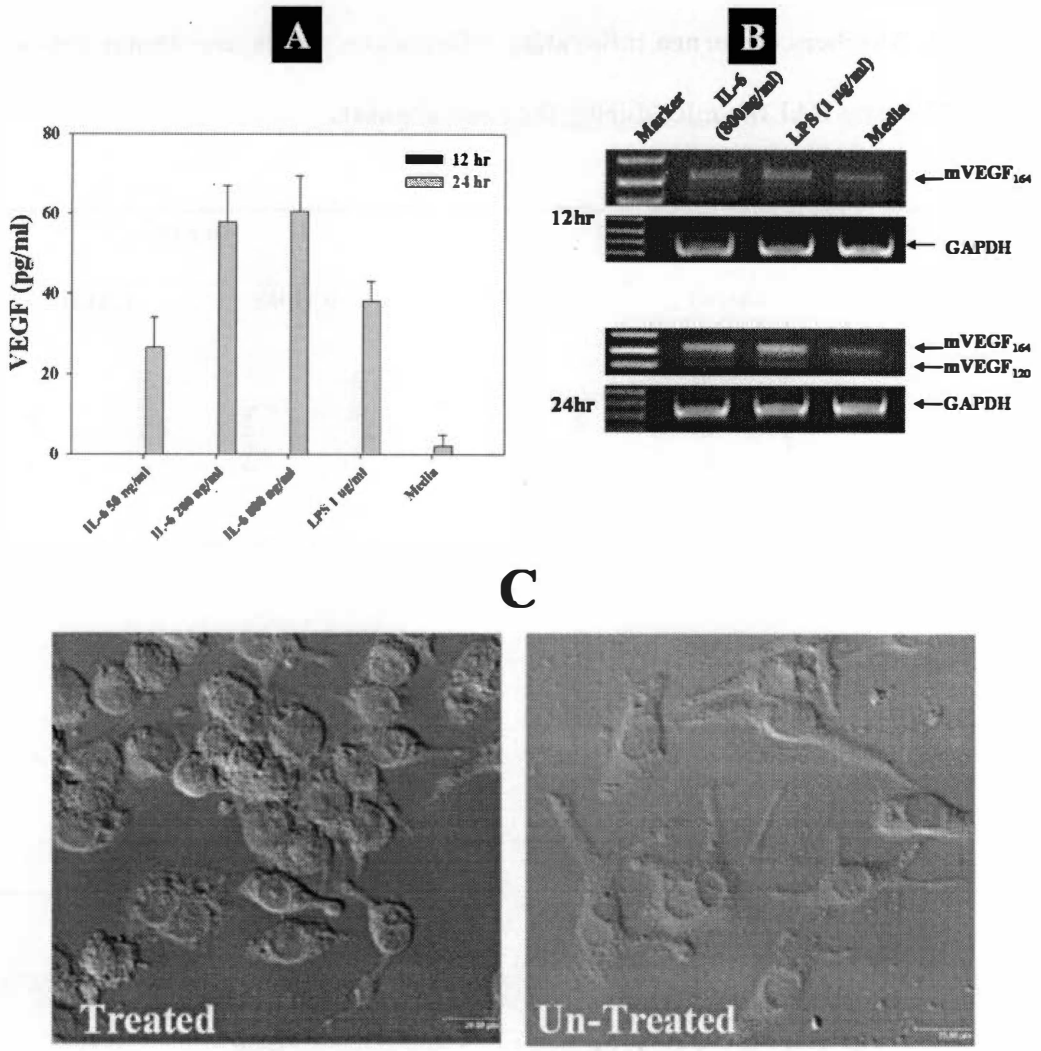


Figure 11. IL-6 induces VEGF production in J774A.1 cells

J774A.1 cells were incubated for 12-24 hrs with recombinant IL-6 and LPS. Supernatants were used in a sandwich ELISA to detect secreted VEGF (A) and total RNA (from cell pellet) was used to detect VEGF mRNA expression by PCR (B). GAPDH served as a standard. In other experiments J774A.1 cells were treated with 200ng of IL-6 and VEGF production was detected by immuno-cytochemistry using biotinylated anti-VEGF MAb (R & D systems) and Streptavidin-Alexa Fluor 546 after 24 hrs of incubation (C). Images were captured using a Leica SP2 laser scanning confocal microscope.

Table 1. Numbers of cornea infiltrating inflammatory cells are similar between CXCR2-/- and BALB/c mice during the clinical phase.

	Day 2[A]				Day 15[A]			
	BALB/c		CXCR2-/-		BALB/c		CXCR2-/-	
	Limbal	Paracentral + Central	Limbal	Paracentral +Central	Paracentral	Central	Paracentral	Central
Neutrophils	161 ± 27 (64%)[B]	337 ± 38 (72%)	58 ± 12 (31%)	36 ± 16 (32%)	383 ± 128 (62%)	343 ± 97 (74%)	495 ± 139 (57%)	271 ± 90 (62%)
Monocytes	91 ± 15 (36%)	131 ± 41 (28%)	126 ± 21 (68%)	76 ± 20 (68%)	237 ± 129 (38%)	118 ± 61 (25%)	368 ± 95 (42%)	164 ± 77 (38%)
Total	251 ± 24	467 ± 61	184 ± 31	111 ± 30	620 ± 92	462 ± 129	863 ± 227	435 ± 154

[A] Eyes infected with 5 x 10^5 pfu HSV-1 were enucleated and processed for paraffin embedding and H & E staining, on the day 2 and day 15 (HSK score of 3) p. i. A differential count was carried out under 1000x magnification based on cellular morphology. Two sections per eye were assayed and a total of three eyes were studied. Results are shown as Mean ± SD for the six sections.

[B] Figures in parentheses denote the percentage of each cell type of the total number counted.

Table 2. Viral titration shows a defect in viral clearance in CXCR2-/- mice.

Days p.i.	Virus titer (log_{10} pfu)[A]			
	$5x10^4$ pfu		$5x10^5$ pfu	
	CXCR2-/-	BALB/c	CXCR2-/-	BALB/c
1	5.3 ± 0.2	5.5 ± 0.3	5.7 ± 0.2	5.9 ± 0.1
3	2.8 ± 0.8	3 ± 0.1	3.3 ± 0.5	2.9 ± 0.6
5	3.1 ± 0.6	2.9 ± 0.5	3.3 ± 0.4	2.9 ± 0.8
7	2.7 ± 0.7	UD	3.7 ± 0.2	UD[B]
10	UD	UD	UD	UD

[A] Mice were infected with $5x10^5$ pfu and $5x10^4$ pfu of HSV-1 RE. Virus titer was estimated by standard plaque assay from swabs taken from infected corneas of mice (*n=4*) at the indicated time points.

[B] UD, Undetected, i.e. below the sensitivity of the assay (<10 pfu/ml)

PART III

$CD8^+$ T CELL TRANSGENIC MOUSE MODELS FOR HSK

Research described in this chapter is a slightly modified version of an article published in 2002 by Kaustuv Banerjee, Shilpa Deshpande, Mei Zheng, Udayasankar Kumaraguru, Stephen P. Schoenberger and Barry T Rouse:

Banerjee, K., Deshpande, S., Zheng, M., Kumaraguru, U., Schoenberger, S. P. and Rouse, B. T. (2002). Herpetic Stromal Keratitis in the absence of viral antigen recognition. *Cellular Immunology*, 219: 108-118

In this chapter "we" and "our" refers to co-authors and me. My contributions in the paper include (1) selection of the topic (2) data analysis and interpretation (4) planning experiments (5) compiling and interpretation of the literature (6) understanding how results fit with the literature (7) compilation of contributions into one paper (8) providing structure to the paper (9) making graphs, figure and tables (10) writing and editing.

ABSTRACT

Herpetic Stromal Keratitis (HSK), resulting from ocular infection with Herpes Simplex Virus (HSV), is thought to represent a T cell mediated immunopathologic lesion. Antigens recognized by the inflammatory T cells remain unresolved and non-TCR mediated activation of T cells (bystander activation) is considered as also

involved. This report documents further evidence for the bystander activation mechanisms using two $CD8^+$ T cell transgenic RAG-/- mouse strains. Accordingly HSK occurred in P14 RAG-/- and OT-1 RAG-/- mice. In none of the models could HSV specific T cell reactivity be demonstrated and animals were unprotected from lesion development by immunization prior to HSV ocular infection. The results support the role of bystander activation as a mechanism of T cell mediated immunopathology and show that $CD8^+$ T cells can participate in HSK lesion development.

INTRODUCTION

Viruses are incriminated but difficult to convict as causes of autoimmune disease(1). Lesions have been advocated to result from molecular mimicry between viral encoded antigens and host autoantigens involved in autoimmunity(1, 2). Such a scenario receives strong support in a model where ocular infection with herpes simplex virus (HSV) results in a progressive $CD4^+$ T cell mediated immunoinflammatory reaction in the cornea termed herpetic stromal keratitis (HSK)(3). In situations where molecular mimicry operates, the cross-reacting antigen involved is expected to be a notable immunogen eliciting both a viral and host reactive immune response. Molecular mimicry is considered an explanation for HSK lesions with a peptide derived from the UL6 protein of HSV as the apparent cross-reacting antigen (4). This provocative idea awaits confirmation and has in fact been disputed (5, 6). Moreover, in fact it has also been observed that HSK can be induced by virus infection in situations where CD4+ T cells fail to recognize any viral antigens including candidate cross reacting molecular mimics (7-9). The pathogenesis in such a model was assumed to involve a

bystander activation mechanism with the virus inducing an inflammatory environment in the cornea that serves to activate ingressing $CD4^+$ T cells to organize a progressive inflammatory reaction. The T cells were assumed to be activated by non-TCR mediated events(7-9). Bystander activation occurs in DO11.10 mice backcrossed to RAG-/- or SCID, in which the great majority (>95%) of their T cells reacted with the $OVA_{323-339}$ peptide(7-9). Although cross reactivity between the peptides and HSV could not be demonstrated and a search of the gene bank revealed no detectable homology, given the fact that T cells may show considerable redundancy in epitope recognition(10), the observation of bystander activation in the DO11.10 SCID/RAG-/- might in fact represent undemonstrable cross reactivity.

In the present report, we have studied the pathogenesis of HSK in two $CD8^+$ TCR transgenic mice strains backcrossed to RAG-/- that expressed almost monoclonal T cell reactivity. One mouse strain possessed $CD8^+$ T cells, the majority of which recognized the GP_{33-41} peptide of lymphocytic choriomeningitis virus (LCMV) P14 protein and the other possessed transgenic $CD8^+$ T cells that recognized the $OVA_{257-264}$ peptide of OVA. In both these TCR Tg RAG-/- mouse strains animals developed HSK following ocular HSV infection and none developed detectable T cell responses to HSV after either infection or immunization. These results further indicate that the ocular immunopathology resulting from HSV infection likely proceeds via a bystander activation mechanism of T cell activation and that $CD8^+$ T cells can participate in such reactions.

MATERIALS AND METHODS

Mice

C57BL/6 x C57BL/10SgSnAi-[Tg]TCR LCMV P14-[KO]rag2 (P14 RAG-/-) mice were obtained from the National Institute of Allergy and Infectious Disease (NIAID) contract facility at Taconic Farms, Inc. (Germantown, N.Y.). OT-1 RAG-/- mice produced in the laboratory of Dr. Stephen Schoenberger (La Jolla Institute for Allergy and Immunology, San Diego, CA). C57BL/6 (B6) and BALB/c mice (5-6 wk) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were sex and age matched for all experiments. All manipulations involving the immunocompromised mice were performed in laminar flow hood. To prevent bacterial super infections, all mice received prophylactic treatment with sulfatrim pediatric suspension (Barre-National, Baltimore, MD) at the rate of 5 ml per 200 ml of drinking water. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research.

Virus and reagents

HSV-1 RE (obtained from the laboratory of Dr. Robert Hendricks, University of Illinois, Chicago) and HSV-1 KOS strains were propagated and titrated on monolayers of Vero cells (ATCC CCL81) using standard protocols(11). HSV-1 RE was used for corneal infections and HSV-1 KOS was used to elicit cutaneous delayed type hypersensitivity reactions, in vitro lymphoproliferation assays and immunization of mice. Cyclosporin A (CsA) (Sandimmune, Novartis) was administered intraperitoneally daily beginning from the day of ocular infection in 100µl volume at a dose of 50mg/kg (12, 13).

Corneal HSV Infections and Clinical Observations.

Corneal infections of all mice groups were conducted under deep anesthesia induced by avertin (Sigma). Mice were scarified on their corneas with a 27- gauge needle, and a 4 μl drop containing required viral dose was applied to the eye and gently massaged with the eyelids. The eyes were examined on different days post infection with a slit lamp biomicroscope (Kowa Co., Nagoya, Japan) and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity, but iris invisible; +4, opaque cornea; and +5, necrotizing stromal keratitis.

Quantification of IFNγ production by intracellular staining

To enumerate the IFNγ producing $CD8^+$ T cells, intracellular cytokine staining was performed as previously described (14). In brief, 10^6 freshly explanted splenocytes and/or draining lymph node cells were cultured in flat bottom 96-well plates. Cells were left untreated, stimulated with HSV $gB_{(498-505)}$ peptide (SSIEFARL), $OVA_{(257-264)}$ peptide (SIINFEKL), $P14_{(gp33-41)}$ peptide (1 μg/10^6 cells), or treated with PMA (10 ng/ml) and ionomycin (500 ng/ml), and incubated for 6hr at 37° C in 5% CO_2. Brefeldin A (10 μg/ml) and IL-2 (50 U/ml) was added for the duration of the culture period. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a cytofix/cytoperm kit (Pharmingen, San Diego, CA) in accordance with the manufacturer's recommendations. PE labeled anti-IFNγ antibodies were used for intracellular cytokine staining (Clone XMG1.2, Pharmingen).

Flow cytometry and tetramer staining

Single cell suspensions were prepared fromDLN cells and spleens of mice. For analysis of HSV reactivity of the P14 RAG-/- and OT-1 RAG-/- mice, splenocytes obtained from ocularly infected mice were expanded in vitro for 5 days in the presence of stimulating peptide (2 $\mu g/10^6$ cells) and IL-2 (50 U/ml). MHC class I (H-2b) tetramers were used to measure SSIEFARL-specific, SIINFEKL specific and P14 specific T cells (Provided by NIAID Tetramer Facility and Dr. Rafi Ahmed, Emory University, respectively). Cells (10^6) were suspended in FACS buffer and stained with CD8-FITC (Pharmingen) and tetramers. They were incubated for 30 min washed and analyzed using FACScan and Cell Quest software (Becton Dickenson).

Histopathology and Immunohistochemical staining

Eyes were enucleated and fixed in 10% buffered neutral formalin and embedded in paraffin. Sections (five-micron-thick) were cut and stained with hematoxylin and eosin. For immunohistochemistry, eyes were frozen in optimum cutting temperature (OCT) compound (Miles, Elkart, IN). Six-micron-thick sections were cut, air dried, and fixed in cold acetone for 5 minutes. For detection of viral antigens sections were blocked with heat inactivated goat serum (Sigma) and incubated with rabbit anti-HSV anti-serum (Dako Corp, Carpentaria, CA) followed by biotinylated goat anti-rabbit Ab (Biogenex, San Ramon, CA). For detection of T cells, sections were blocked with 3% BSA and then incubated with hamster anti-mouse TCR β monoclonal antibody (Pharmingen) followed by biotinylated anti-hamster IgG (Vector Labs, Burlingame, CA). Sections were then treated with HRP-conjugated streptavidin (1/1000 dilution,

Jackson Immunoresearch Laboratories Inc.) and 3,3'-diaminobenzidine substrate (Biogenex, San Ramon, CA), and counter stained with hematoxylin (Sigma).

Statistical Analysis

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

RESULTS

Herpetic Stromal Keratitis in $CD8^+$ TCR transgenic mice

Ocular infection of P14 RAG-/- mice, whose peripheral T cells were $CD8^+$ T cells and recognized LCMV gp33-41 (Dr. B. J. Fowlkes, NIAID-personal communication), resulted in c linical HSK evident around 7-8 days post infection (Fig 1, all figures located in appendix). However, animals usually died of herpetic encephalitis by 11-12 days post infection. In such experiments, around 40% of control B6 animals developed clinical HSK and none died of herpetic encephalitis. In addition, lesions in the control immunocompetant mice developed more slowly than in the P14 RAG-/- animals (Fig 1). Both $CD8^+$ T cells and viral antigens were present in the stroma of corneal lesions from P14 RAG-/- mice tested histologically at 10 days post infection (Fig 2A and B respectively). In contrast detectable antigen had disappeared from sections of control B6 animals after the first 2-3 days of infection and was undetectable in sections showing HSK lesions (data not shown).

Sample animals killed at 10 days post infection, were also tested for HSV gB and LCMV gp33-41 peptide specific immune reactivity, measured by both intracellular IFNγ production as well as with specific tetramers following in vitro expansion. Cells from P14 RAG-/- animals failed to show significant reactivity to the

gB peptide, unlike control B6 mice which showed robust responses (Table 1 A, all tables located in appendix). Such data indicate that the P14 RAG-/- mice failed to respond immunologically to HSV antigens. Mice immunized with UV inactivated HSV also failed to generate gB peptide specific responses (data not shown).

In a separate experiment, a group of P14 RAG-/- and control B6 mice were immunized with UV inactivated virus 10 days prior to ocular infection with HSV-RE. Animals were followed for clinical signs of HSK and encephalitis. Immunized P14 RAG-/- mice all developed HSK of approximately equal severity and time course as in the non-immune infected P14 RAG-/- mice (Fig 1). In addition, immunized animals all died of encephalitis by 11 days p.i showing that vaccination failed to induce immunity in the P14 RAG-/- mice. In contrast, detectable clinical lesions did not occur in immunized B6 animals following HSV ocular infection (Fig 1).

Limited experiments were also performed with OT-1 RAG-/- animals whose $CD8^+$ T cells recognize the $OVA_{257-264}$ peptide. Infected OT-1 RAG-/- animals developed HSK but with slower kinetics than observed in other strains studied (Fig 1). Moreover, animals lived longer than the usual 11-12 days. Evaluation of lesions histologically and immune reactivity of splenocytes was tested 13 days post infection. Typical HSK lesions, with both $CD8^+$ T cells and viral antigen present in the stroma, were evident in corneal sections of OT-1 RAG-/- infected mice (Fig 2 C and D respectively). Moreover lymphocytes from HSV infected OT-1 RAG-/- animals stimulated with HSV gB peptide failed to develop significant specific responses as tested both by intracellular cytokine expression (for IFNγ) or tetramer analysis (Table 1B). However lymphocytes from these mice responded positively to the $OVA_{257-264}$ peptide (Table 1B).

T cell activation requirements for HSK development in P14 RAG-/- mice

TCR activation of cells is subject to inhibition by the drug Cyclosporin A (CsA) (15). To determine if HSK lesions in the transgenic system were mediated by TCR stimulation of the transgenic T cells, the effects of CsA on lesion expression were measured. As recorded in Fig 3A, CsA treatment had no significant inhibitory effect on the expression of lesions in the HSV infected P14 RAG-/- mice. In contrast, in control immunocompetent BALB/c mice, in which the pathogenesis of HSK likely involves TCR mediated stimulation of ingressing T cells (16), CsA was markedly inhibitory on lesion progression and severity (Fig 3B). These results are consistent with the notion that the mechanism by which transgenic T cells become activated to participate in HSK lesions may differ from that in immunocompetent animals.

DISCUSSION

Herpes simplex virus induced stromal keratitis is an immunoinflammatory reaction in the cornea organized primarily by $CD4^+$ T cells (3). Currently the identity of antigens recognized by the T cells involved in lesion induction remains unresolved. The logical idea that viral proteins provide the nominal antigens recognized has been questioned by the observation that HSK can be induced in mice whose $CD4^+$ T cells lacked detectable reactivity to viral antigens (7-9). Initial reports came from studies in TCR transgenic (TCR Tg) SCID or RAG-/- mice where the bulk of T cells recognized the $OVA_{323-339}$ peptide (7-9). The present report demonstrates that HSK can be induced in additional TCR Tg RAG-/- mice which react with different peptides but which also fail to show detectable reactivity to HSV. The models were two TCR Tg RAG-/- mice that possessed only $CD8^+$ T cells. Such cells reacted with the Lymphocytic

choriomeningitis virus $P14_{(gp33-41)}$ peptide and the $OVA_{(257-264)}$ peptide respectively. These TCR Tg RAG-/- strains showed no detectable cross reactivity to HSV antigens and animals failed to develop HSV specific immunity upon infection or immunization with viral antigens. Our results further support the notion that T cell dependent ocular inflammatory lesions initiated by HSV infection can be mediated by $CD8^+$ T cells that do not recognize nominal antigens derived from viral proteins. This mechanism of immunopathology has been referred to as bystander activation (7-9) and may be the consequence of non-TCR mediated activation of T cells.

Initial reports of bystander activation HSK in the DO11.10 SCID and RAG-/- mice could not exclude the possibility that some low level cross reactivity existed between the OVA peptide and peptides derived from one or more of the approximately 80 proteins encoded by HSV. Thus the lesions could result from viral epitope peptide activation of the OVA specific $CD4^+$ T cells. Aside from the fact that no cross reactivity was demonstrable, a search of the gene bank revealed no molecular mimics of the sequence that encoded the OVA peptide(14). Our model strains had transgenic $CD8^+$ T cells and $CD8^+$ T cells, in recognizing shorter peptides in a closed MHC cleft, may be expected to show less cross-reactivity than do $CD4^+$ T cells. We contend that the observation that $CD8^+$ TCR Tg RAG-/- animals whose T cells recognize different nominal antigens, but not HSV, makes it even less likely that the lesion orchestrating T cells are reacting with peptide epitopes derived from HSV. Also arguing against cross reactivity was the observation that all TCR Tg RAG-/- animal models studied were unable to control HSV infection and that the pattern of immunological and clinical events were unchanged following preimmunization with HSV. This stands in contrast to the situation in immunocompetant mice, as

demonstrated in this report, where preimmunization protects against the development of HSK following subsequent ocular infection with virulent HSV(17, 18).

We and others have demonstrated that lesions resulting from HSV infection of the cornea are T cell mediated (19-22). In immunocompetant animals, as well as in previously studied TCR Tg models, the T cells involved in HSK have been predominantly of the $CD4^+$ phenotype. In fact few $CD8^+$ T cells infiltrate ocular lesions at least in the BALB/c mice(23). Moreover with adoptive transfer studies with immune T cell subsets, lesions can only be induced with $CD4^+$ T cells, with $CD8^+$ cells actually serving to modulate rather than mediate HSK(8, 21). The finding that transgenic $CD8^+$ T cells could mediate HSK in the bystander activation model was in consequence unexpected. Furthermore at present it is not clear how either the transgenic $CD4^+$ or $CD8^+$ T cells are activated to mediate lesions if recognition of a specific viral derived epitope is not involved. Previous reports, however, documented situations in which either naïve or memory cells may be activated without exogenous application of cognate antigen. One now well studied example is the homeostatic expansion and activation of naïve cells into pseudo-memory cells that occurs upon transfer of naïve T cells into lymphopenic animals(24, 25). The expanded cells take on an activation phenotype and may participate in inflammatory reactions. Thus previously we have shown that naïve transgenic $CD4^+$ T cells transferred into SCID recipients underwent homeostatic activation in 2-3 weeks and animals subsequently infected with HSV were significantly more susceptible to HSK than were recipients infected soon after cell transfer (14).

Other reports have documented that cells with a memory phenotype may be activated by exposure to cytokines without the addition of exogenous nominal antigen

(26). In the HSV system described in the report, we suggest that the T cells which enter the cornea in response to the inflammatory reaction set off by HSV, are exposed to multiple signaling molecules that may activate them directly, rather than by TCR mediated signaling, to participate in the inflammatory response. Most likely the initial source of some of the proinflammatory cytokines are the infected cells themselves. There are several likely candidates. IL-6, known to play a role in amplifying leukocyte accumulation at sites of inflammation, by augmenting the local production of chemokines (27) has been previously shown to be produced in HSV infected corneas (28). Recent observations also point to the fact that this cytokine is an important participant in the cytokine cascade triggered by HSV corneal infection(29). Subsequently the main source may be accessory cells such as dendritic cells that respond to factors released from viral infected cells, such as stress proteins and viral DNA. Accordingly, HSV DNA contains numerous bioactive CpG motifs, that could cause dendritic cells to produce proinflammatory cytokines IL-12 and IL-18 known to directly activate T cells(15).

Our results also provide evidence that events involved in activating transgenic T cells to mediate HSK differ from those occurring in immunocompetent animals. Accordingly, we observed that HSK in the P14 RAG-/- mice was unaffected by treatment with the drug cyclosropin A. In contrast, HSK in immunocompetent control animals, where early events at least are considered as mediated by TCR activation of viral antigen specific T cells(16), was markedly diminished by cyclosporin A treatment. We interpret such results to mean that mechanisms of activation of transgenic T cells in HSK lesions occurs other than by TCR activation. Presumably one or more cytokines or chemokines are involved with cytokine activation of T cells

unaffected by cyclosporin A. Thus as reported by others, direct activation of T cells by IL-12/IL-18 cannot be inhibited by cyclosporin A treatment (15). It will be important to understand how activation of T cells resulting in HSK, is mediated at the molecular level. Such investigation is currently underway in our laboratory.

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APPENDIX

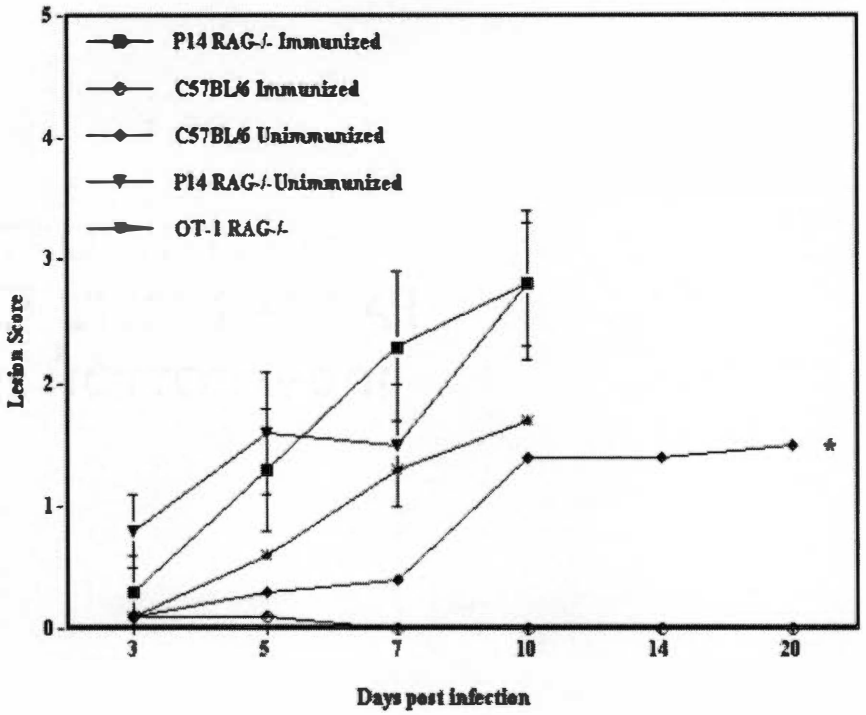

Figure 1. CD8$^+$ TCR transgenic mice develop HSK and die of encephalitis despite UV-HSV immunization.

P14 RAG-/- mice and C57BL/6 mice (n=5) were immunized with UV-HSV i.p (10^7 pfu prior to UV irradation) 10 days before infection on scarified corneas (5x 10^7 pfu HSV-1 RE). Similar dose was used for the OT-1 RAG-/- mice. Unimmunized P14 RAG-/- and C57BL/6 mice (n=5) were used as controls. Mice were scored clinically for HSK on a score of 0-5 as described in materials and methods. Data plotted for P14 RAG-/- and OT-1 RAG-/- mice are for days 3, 5, 7 and 10 post infection. These animals succumbed to encephalitis by day 10-11 post infection. Data plotted for C57BL/6 mice are for days 3, 5, 7, 10, 14 and 20 post infection. Results are expressed as mean clinical score ± SD. * 40% of mice developed disease.

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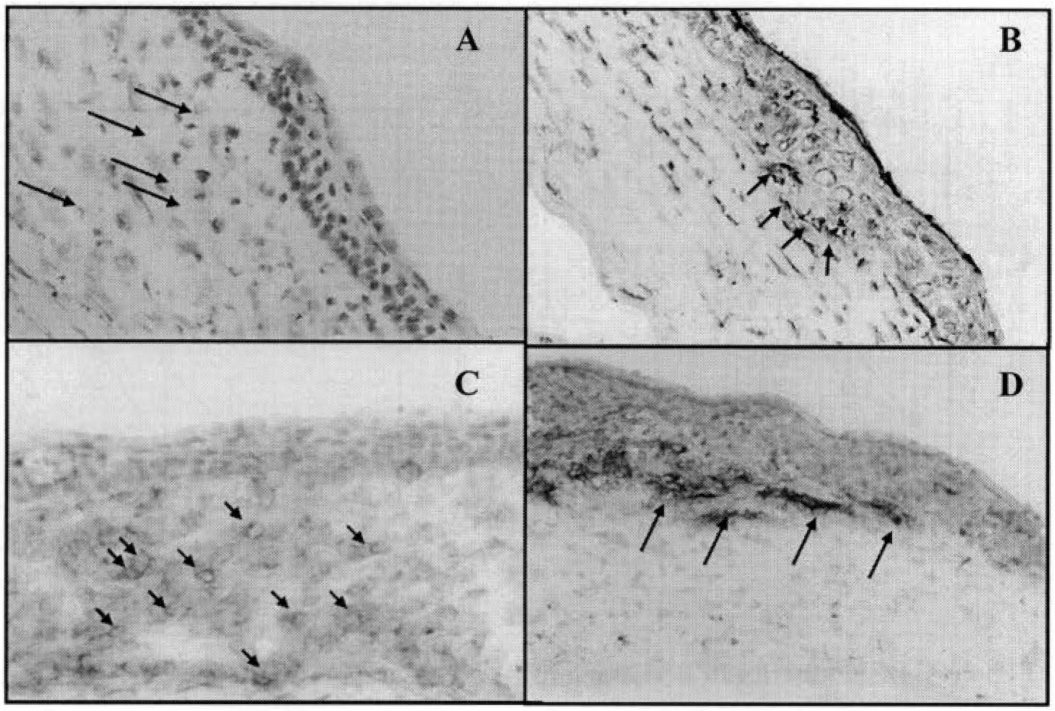

Figure 2. Imunohistochemistry of infected $CD8^+$ TCR transgenic mouse corneas shows infiltrating T cells and persisting viral antigens.

(A & C). P14 RAG-/- (A) and OT-1 RAG-/- (C) eyes were enucleated on day 10 and day 13 respectively post infection and were sectioned and processed for immunohistochemistry for T cell detection (hamster anti mouse TCR β antibody-Pharmingen) as described in materials and methods. Arrows show the presence of infiltrating T cells in the stroma (as detected by brown staining). Sections were counterstained with haematoxylin.

(B & D). Arrows show viral antigen staining in the corneal stroma of P14 RAG-/- (B) and OT-1 RAG-/- (D) mice.

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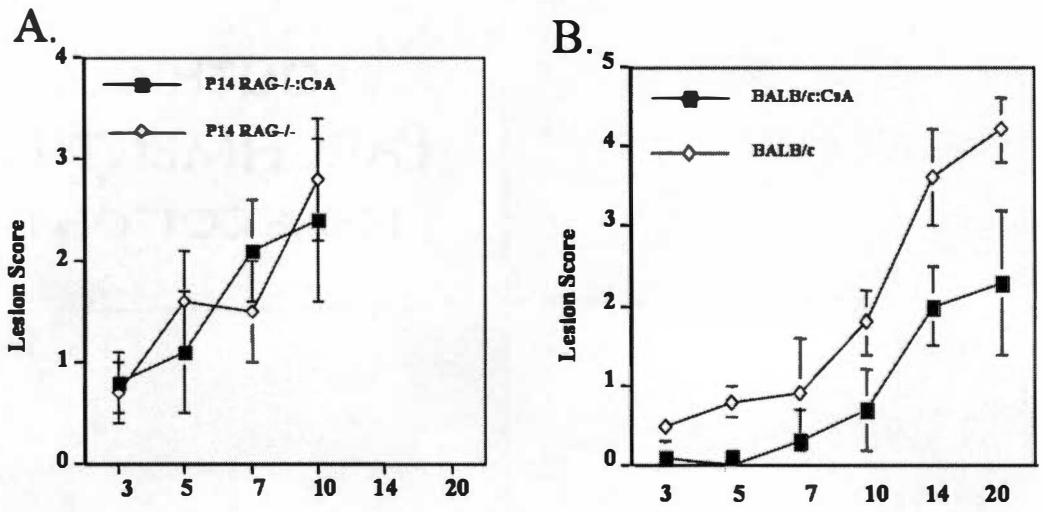

Figure 3. HSK kinetics in BALB/c and P14 RAG-/- transgenic mice treated with cyclosporin A (CsA).

Groups of P14 RAG-/- and BALB/c (n=4) infected with HSV-1 RE ($5x10^7$ pfu for P14 RAG-/- and $5x10^5$ pfu for BALB/c) on scarified corneas were treated with cyclosporin A (50 mg/kg) intraperitoneally daily. P14 RAG-/- mice (A) and BALB/c mice (B) were scored clinically for HSK on a score of 0-5 as described in materials and methods. Data plotted for P14 RAG-/- mice are for days 3, 5, 7 and 10 post infection. These animals succumbed to encephalitis by day 10-12 post infection. The data plotted for BALB/c mice are for days 3, 5, 7, 10, 14 and 21 post infection. Results are expressed as mean clinical score ± SD.

Table 1. P14 RAG-/- and OT-1 RAG-/- mice show no reactivity to HSV

Mice	Stimulating peptide	Frequency of CD8 response		
		% intracellular cytokine positive[a]	% Tetramer positive[b]	
			$P14_{(gp33-41)}$	$gB_{(498-505)}$
A.				
P14 RAG-/-	$gB_{(498-505)}$	0	-	0.3 ± 0.2
	$P14_{(gp33-41)}$	17 ± 2.3	91 ± 3.5	0
C57BL/6	$gB_{(498-505)}$	6.1 ± 1.4	-	7.5 ± 2
	$P14_{(gp33-41)}$	0.8 ± 0.2	1 ± 0.6	-
B.				
OT-1 RAG-/-	$gB_{(498-505)}$	0	-	0
	$OVA_{(257-264)}$	11.6 ± 2.1	89 ± 3	0
C57BL/6	$gB_{(498-505)}$	5.8 ± 1.1	0	8.7 ± 1.3
	$OVA_{(257-264)}$	0	0	-

Mice ocularly infected with HSV-RE were terminated on day 10 (A) and on day 13 (B) post infection and their splenocytes used for intracellular cytokine assay and for tetramer analysis.

(-) Not done

[a] *In vitro* stimulation of 10^6 splenocytes for 6 hours in the presence of stimulating peptide, IL-2 (50 U/ml) and brefeldin A (10 µg/ml)

[b] *In vitro* expansion for 5 days in the presence of stimulating peptide and IL-2 (50 U/ml)

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PART IV

EVALUATION OF THE ROLES OF HSV REACTIVE AND UNREACTIVE $CD8^+$ T CELLS IN HSK PATHOGENESIS

ABSTRACT

Herpetic stromal keratitis (HSK), resulting from corneal HSV-1 infection, represents a T cell mediated immuno-pathologic lesion. The identity of the antigens recognized by the T cells remains unresolved. Transgenic mice with T lymphocytes unresponsive to the viral antigens develop corneal pathology and both $CD8^+$ and $CD4^+$ T cells can enter the cornea without the need for virus recognition (bystander activation). Using a $CD8^+$ T cell transgenic mouse model we investigated whether recruitment also extends to virus reactive $CD8^+$ T cells. Adoptively transferred virus reactive $CD8^+$ T cells protected ocularly infected mice from encephalitis. In animals that developed severe HSK lesions, only HSV non-reactive $CD8^+$ T cells infiltrated the cornea. HSV reactive $CD8^+$ T cells, on the other hand, were detectable in the secondary lymphoid organs, the trigeminal ganglion and the brain. Our results indicate that after corneal HSV infection, bystander mechanisms activate and recruit non-HSV reactive, but not virus reactive, pathogenic $CD8^+$ T cells to corneas. Virus reactive $CD8^+$ T cells are recruited to both the central and peripheral nervous system where they function to clear virus or presumably prevent virus reactivation and achieve both protection and HSK modulation.

INTRODUCTION

Herpetic Stromal Keratitis (HSK), resulting from infection of the cornea with Herpes Simplex virus type 1 (HSV-1), represents a leading cause of infectious blindness in the western world (1). Lesions are considered to be immuno-pathological and once a clinical episode occurs patients frequently are placed on long term

corticosteroid therapy (2), which helps prevent clinical recurrences. Whereas human HSK is considered as a T cell mediated immuno-inflammatory lesion, definite evidence for such a mechanism comes from animal studies. Most studies in the mouse model indicate a primary role for $CD4^+$ T cells of the Th1 phenotype (3-8). The identity of target antigens recognized by the lesion orchestrating $CD4^+$ T cells remains unclear. Thus only a minority of T cells in ocular lesions react with viral antigens, leading to the suggestion that most inflammatory T cells in lesions represent bystanders (9-14). In some models it is possible to induce HSK lesions with either $CD8^+$ or $CD4^+$ T cells that are judged to be bystanders alone(9, 12-14). Thus in these TCR transgenic models, the T cells lacked demonstrable reactivity with any antigens. Nevertheless T cells were needed since animals lacking such cells fail to express HSK lesions (5, 6, 15).

In this report, using the $CD8^+$ T cell bystander model, we have attempted to define the role of the HSV specific $CD8^+$ T cells in HSK pathogenesis. Previous reports indicate a protective role for HSV immune $CD8^+$ T cells with respect to ocular HSV infection (3, 5, 6, 13). This interpretation emerged from the finding that SCID mice given an adoptive transfer of HSV immune $CD8^+$ T cells from immunocompetent mice protected ocularly infected mice from encephalitis and extended their life span dramatically, but did not lead to HSK lesions (3, 5, 6, 13). In light of our recent findings with the recruitment of non-HSV-specific $CD8^+$ T cells into corneas we investigated whether such recruitment extends to HSV immune $CD8^+$ T cells using the $CD8^+$ T cell transgenic mouse model (OT-1 RAG-/- mice). Adoptively transferred, purified, immune $CD8^+$ T cells protected ocularly infected mice from encephalitis that was presumably via a combination of the ability of these

cells to clear virus from the CNS and prevent virus reactivation from the peripheral nervous system (trigeminal ganglion, TG). In mice that received cells 3 days after infection, survived encephalitis and developed severe HSK lesions, $CD8^+$ T cells infiltrating the cornea were identified to be HSV non-reactive. HSV reactive $CD8^+$ T cells, on the other hand, were detectable in the secondary lymphoid organs, the trigeminal ganglion and the brain. Thus, after corneal HSV infection, bystander mechanisms activate and recruit non-HSV reactive, but not virus reactive, $CD8^+$ T cells to corneas and these participate in HSK pathogenesis. Virus reactive $CD8^+$ T cells are recruited to both the central and peripheral nervous system where they function to clear virus or presumably prevent virus reactivation and achieve both protection and HSK modulation.

MATERIALS AND METHODS

Mice

Five to six week old female mice were used for the experiments. OT-1 RAG-/- CD45.1 mice were produced in the laboratory of Dr. Stephen Schoenberger (La Jolla Institute of Allergy and Immunology, San Diego, CA). These mice are referred to in the text as OT-1 RAG-/- mice. HSV specific TCR transgenic mice (gBT-1.1) were produced in the laboratory of Dr. Francis Carbone (University of Melbourne, Melbourne, Australia) (16). C57BL/6 (B6) mice ($CD45.2^+$) were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Animals were age and sex matched for all experiments. All manipulations involving immunocompromised mice were performed in laminar flow hood. To prevent bacterial super infections, mice received prophylactic treatment, starting one day prior to corneal infection, with sulfatrim

pediatric suspension (Barre National, Baltimore, MD) at the rate of 5ml per 200ml of drinking water. All experimental procedures were in complete agreement with the Association of Research in Vision and Ophthalmology (ARVO) resolution on the use of animals in research.

Virus

HSV-1 RE (obtained from the laboratory of Dr. Robert Hendricks, University of Illinois, Chicago) and KOS strains were propagated and titrated on monolayers of Vero cells (ATCC CCL81) using standard protocols (17). HSV-1 RE was used for corneal infections and HSV-1 KOS was used to immunize mice and elicit cutaneous delayed type hypersensitivity reactions.

Corneal HSV infections and clinical observation

Corneal infections of all mice groups were conducted under deep anesthesia induced by avertin (Sigma). Mice were scarified on their corneas with a 27 gauge needle and a 4μl drop containing 5 x 10^6 pfu of HSV-1 RE was applied to the eye and gently massaged with the eyelids. The eyes were examined on different days post infection with a slit lamp biomicroscope (Kowa, Nagoya, Japan) and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity, but iris invisible; +4, opaque cornea; and +5, necrotizing stromal keratitis.

Virus recovery and titration

Eye swabs were taken from infected corneas (four eyes/group) using sterile swabs soaked in DMEM containing with 10IU/ml penicillin and 100μg/ml streptomycin. Swabs were put in sterile tubes containing DMEM and stored at -80° C. For detection of virus, samples were thawed and vortexed. Duplicate 200μl aliquots of dilutions of each sample were plated on vero cells grown to confluence in 24 well plates at 37° C in 5% CO_2 for 1hr 30 min. Medium was aspirated and 500μl of 2x DMEM containing 1% low-melting point agarose was added to each well. Titers were calculated as $\log_{10}$ pfu/ml as per standard protocol (17).

Adoptive transfer of HSV immune $CD8^+$ T cells

To generate HSV immune $CD8^+$ T cells, B6 and gBTI.1 mice were injected with 5 x 10^6 pfu of HSV-1 KOS into the footpad. Single cell suspensions of pooled spleens and popliteal lymph nodes were prepared from immunized mice 7-8 days later and $CD8^+$ T cells were purified using a mouse CD8 subset column (R & D systems, Minneapolis, MN). By flow cytometry analysis, the purified population consisted of 80% $CD8^+$ T cells with no detectable $CD4^+$ T cells. Approximately 9% (B6) and 50% (gBT-I.1) of the $CD8^+$ T cells produced IFNγ upon re-stimulation to the $gB_{498-505}$ immunodominant epitope of HSV, measured by intracellular IFNγ production (measured prior to purification from cell samples taken from pooled splenocyte and DLN single cell suspensions. See below for details). Ocularly infected OT-1 RAG-/- animals received an intravenous injection of 5 x 10^6 purified cells at 24 (referred to in the figures as OT-1 RAG-/-: CD8 24hr) and 72 hr (referred to in the figures as OT-1

RAG-/-: CD8 72hr. For cells from gBT-I.1 mice referred to as OT-1 RAG-/-: gBT-I.1 CD8 72hr) post infection.

Delayed type hypersensitivity (DTH)

At indicated time points after corneal HSV-1 infection, UV inactivated HSV (10^5 pfu prior to UV inactivation) was injected in 20µl of PBS in the right ear pinna of anesthetized mice. As a control vero cell extract was injected in the left ear pinna. Ear thickness was measured 48 hr post injection with a screw gauge meter (Oditest; H. C. Kropelin GhBH, Schluechtern, Germany) as described elsewhere (9). The mean increase in ear thickness before and after injection was calculated and expressed as 10^{-2} mm.

Cellular analysis and flow cytometry

a. *Surface staining for the detection of adoptively transferred $CD8^+$ T cells.* Single cell suspensions were prepared from spleen and draining lymph nodes (DLN) of mice. The draining lymph nodes that were used for cellular analysis were the mandibular and the superficial cervical lymph nodes. Whole brains were minced and single cell suspensions were made by passing minced tissue through a 70µm nylon cell strainer. Isolated TGs were pooled, minced and digested in 1mg/ml collagenase/dispase (Roche Diagnostics, Mannheim, Germany) for 1hr 30 mins followed by two washes in PBS. Surface staining of cells was carried out in flow cytometry buffer (1x PBS with 3% FCS and 0.1% Sodium Azide). Viable cells (10^6) were blocked with Fc Block (Clone 2.4 G2, Pharmingen). Cells were double stained with FITC labeled anti-CD8α (Clone 53-6.7, Pharmingen) and biotin labeled anti-CD45.1 (clone A20, Pharmingen) (host cells) or anti-CD45.2 (clone 104, Pharmingen) (Donor cells).

Biotin labeled antibodies was detected by further incubation of cells with a 1/200 dilution of streptavidin-PerCP (Pharmingen). Events were collected on FACScan (Becton-Dickinson, San Jose, CA) and analysed using Cellquest version 3.0 (Becton-Dickinson, San Jose, CA).

b. *Quantification of IFNγ production by intracellular staining:* To enumerate IFNγ producing $CD8^+$ T cells, intracellular cytokine staining was performed as previously described (12). In brief, 10^6 splenocytes or DLN cells were cultured in flat bottom 96-well plates. Cells were left untreated, stimulated with HSV $gB_{498-505}$ peptide (SSIEFARL), $OVA_{257-264}$ peptide (SIINFEKL) (1μg/10^6 cells), or treated with PMA (10ng/ml) and ionomycin (500ng/ml) and incubated for 6hrs at 37° C in 5% CO_2. Brefeldin A (10μg/ml) and IL-2 (50 U/ml) was added for the duration of the culture period. After this period, cell surface staining was performed as described above. This was followed by intracellular cytokine staining using a cytofix/cytoperm kit (Pharmingen, San Diego, CA) in accordance with the manufacturer's recommendations. PE labeled anti-IFNγ antibody was used for intracellular cytokine staining (clone XMG1.2, Pharmingen). Events were collected on FACScan (Becton-Dickinson, San Jose, CA) and analysed using Cellquest version 3.0 (Becton-Dickinson, San Jose, CA).

Histopathology, Immunohistochemistry and Immunofluorescence

Eyes were enucleated and fixed in 10% buffered neutral formalin and embedded in paraffin. Sections (5μm thick) were cut, deparaffinized and stained with haematoxylin and eosin. For immunohistochemistry and immunofluorescence analysis, eyes were

enucleated at indicated time points, frozen in optimum cutting temperature (OCT) compound (Miles, Elkart, IN). Six micron thick sections were cut, air dried and fixed in acetone:methanol (1:1) at -20° C for 10 min. For detection of viral antigens sections were blocked with 5% BSA in PBS containing 1:200 dilution of Fc block (2 hrs) and incubated with rabbit anti-HSV serum (10 min) (Dako, Carpentaria, CA) followed by biotinylated goat anti-rabbit Ab (20 min) (Biogenex, San Ramon, CA). Sections were then treated with HRP-conjugated streptavidin for 45 min (1/1000 dilution, Jackson Immunoresearch Laboratories) followed by 3, 3' – diaminobenzidine substrate (Biogenex, San Ramon, CA) and counterstained with haematoxylin (Richard Allen Scientific, Kalamazoo, MI).

For immunofluorescence endogenous biotin was blocked in acetone:methanol fixed frozen sections with endogenous biotin-blocking kit (Molecular probes, Eugene, OR). This was followed by blocking with 5% BSA-PBS-0.05% Tween 20 containing 1/200 dilution of Fc Block (clone 2.4G2, Pharmingen) for 2 hrs. Antibodies used for staining were anti-CD8-FITC (5μg/ml) (Clone 53-6.7, Pharmingen) and biotin labeled anti-CD45.2 (10μg/ml) (Clone 104, Pharmingen). Antibody dilutions were made in 1% BSA-PBS. After incubating overnight at 4° C slides were washed thoroughly in PBS and Streptavidin conjugated Alexa Fluor 546 (1μg/ml) (Molecular Probes, Eugene, OR) in 1% BSA-PBS was added for 1hr at room temperature. Slides were mounted with Vectashield without propidium iodide (Vector Laboratories, Burlingame, CA). For slides single stained with FITC labeled antibodies, propidium iodide was used a counterstain (Vectashield with propidium iodide, Vector laboratories, Burlingame, CA). Images were captured with a Leica SP2 Laser scanning confocal microscope.

Statistical analysis

Wherever specified, data obtained were analysed for statistical significance by a standard students *t* test.

RESULTS

OT-1 RAG-/- mice are protected from lethal HSV induced encephalitis by HSV immune CD8$^+$ T cells

We have previously shown that CD8$^+$ T cells transgenic mice develop typical HSK lesions upon ocular infection with HSV-1 and transgenic CD8$^+$ T cells (specific to either the LCMV P14 or the $OVA_{257-264}$ epitope) can be found in such lesions (12). However, in the absence of the full repertoire of immune competent cells, HSV-1 induced encephalitis results in death of such mice within 12 days p. i. These results were confirmed in the present study with the OT-1 RAG-/- CD45.1 transgenic mice. Indeed, all mice succumbed to encephalitis with an ocular infection dose $5x10^6$ pfu of HSV-1 RE strain by day 12 p. i., a dose that failed to have any impact on the survival of control C57BL/6 (B6) mice (Fig. 1, for all figures see appendix). Such mice also developed characteristic HSK lesions at the time of their death (see below).

Utilizing this characteristic of the transgenic non-HSV specific CD8 system we sought to assess the contribution of HSV immune CD8$^+$ T cells to both survival and HSK development in such mice. Purified CD8$^+$ T cells from HSV infected B6 mice (CD45.2) were adoptively transferred into OT-1 RAG-/- animals at two time

points post ocular infection and survival of animals was monitored over a 25 day period post ocular infection (the last time point analyzed).

Interestingly, adoptive transfers at both 24 and 72 hr p. i. protected mice from encephalitis (Fig 1). In mice that survived well beyond 12 days p. i. no visible clinical sign of encephalitis was evident. From two separate experiments, 6 of 8 (75%) and 7 of 8 (88%) mice from the 24 and 72 hr adoptively transferred groups survived encephalitis. However, though control B6 animals showed normal DTH responses to viral antigens, no such response was demonstrable in transgenic animals receiving immune cells and surviving lethal encephalitis (Fig 2), and re-ascertains the inability of $CD8^+$, but not $CD4^+$ T cells to participate in DTH reactions (18).

Thus HSV immune $CD8^+$ T cells are protective and prevent the development of lethal HSV induced encephalitis.

Ocular virus clearance in mice receiving immune $CD8^+$ T cells

Two separate assays were used to determine the clearance of HSV from infected eyes of different groups of transgenic animals. As evident from Fig. 3 clearance of both infectious virus and viral antigens was impaired in mice not receiving immune cells, but succumbing to encephalitis. As measured on day 8 p. i. replicating virus was still detectable in eye swabs from OT-1 RAG-/- animals that did not receive any immune cells (Fig 3A). In control B6 mice infectious virus was no longer detectable in eye swabs from day 6 p. i. (data not shown). When ocular sections of such mice were processed at day 12 p. i., for the detection of viral antigens, antigens were clearly detectable both in the epithelium and in the stromal layers of the cornea (Fig 3B). In

control B6 mice, antigens were only detectable in the epithelium till day 5 p. i. (data not shown).

In marked contrast, infectious virus was undetectable from corneas of most of the ocularly infected OT-1 RAG-/- mice that received the immune $CD8^+$ T cells. At day 8 p. i., while infectious virus was readily isolated from the mice not receiving cells, eye swabs from a majority of the eyes of OT-1 RAG-/- mice receiving cells at both 24 and 72 hr p. i., and eventually surviving lethal encephalitis, failed to show any evidence of replicating virus (Fig 3A). In addition, eyes from mice that were protected from encephalitis by the adoptively transferred cells (day 12 p. i.), did not reveal the presence of viral antigens (Fig 3 C & D). However, a dense infiltrate of inflammatory cells was clearly evident in corneas of mice receiving cells at 72 hrs (Fig 3D) but mostly absent from corneas of mice receiving them at 24 hrs (Fig 3C).

These results indicate that the HSV immune $CD8^+$ T cells facilitate virus clearance from infected corneas.

HSV immune $CD8^+$ T cells modulate HSK development

The severity of HSK lesions depends on clearance of virus/viral antigens from infected corneas (9). Since, adoptive transfer of the immune $CD8^+$ T cells had an effect on the clearance of ocular HSV from infected corneas of OT-1 RAG-/- mice, it was interesting to see how HSK lesions might be affected in such mice. Indeed, a difference was noted in the development of HSK lesions in mice that received immune $CD8^+$ T cells at different times points post infection.

Firstly, we confirmed the development of typical HSK lesions in OT-1 RAG-/- mice. By the time all of these animals had succumbed to encephalitis (day 12 p. i.), the incidence of HSK lesions was approximately 80% (Fig 4 A & B). When visualized with a slit lamp microscope, typical corneal opacification and growth of blood vessels was seen from the limbus (Fig 4C). Histopathologically, the lesions showed inflammatory cell infiltrates and pathology consistent with typical HSK lesions (Fig 5A). Interestingly, very similar disease kinetics was seen in mice that received cells at 72 hr p. i. (Fig 4 A). By day 12, 45% of eyes from such animals showed clinically evident HSK lesions, and average disease scores were similar to that in mice that did not receive cells (Fig 4B). However, in mice that received cells at 24 hrs p. i. lesion development was slower and average scores were 2 fold lower than in mice that did not receive cells or received them at 72 hrs p. i. (Fig 4 A and B).

HSK lesions were then monitored in mice that survived encephalitis. In mice that received the cells at 72 hrs p. i., and showed clinically evident signs of HSK development at day 12 p. i., lesions developed further or persisted throughout the course of the study (Fig 4A) and at day 25, which was the last time point analyzed, the HSK incidence was 64% (Fig 4B). The corneas of many of such eyes had severe pathology, with necrosis and scarification and the development of blood vessels reaching the centre of the cornea and massive infiltration of inflammatory cells (Fig 4C and 5B). In contrast to these findings, lesion development remained slow, or regressed, in mice that received cells at 24 hrs (Fig 4A) and at the end of the study period the incidence was greater than 3 fold lower (Fig 4B). Eyes from this group showed very mild pathological changes at day 25 p. i. (Fig 5C). Thus $CD8^+$ T cells regulate HSK lesion development. This depends on the time of adoptive transfer post

infection and is related with the ability of such cells to facilitate virus clearance from infected corneas.

Bystander $CD8^+$T cells mediate HSK lesions

As evident from the above mentioned HSK studies, OT-1 RAG-/- mice that did not receive immune cells succumbed to encephalitis by day 12 p. i., at which time a majority of the infected eyes showed clinical signs of HSK lesions. T cells infiltrating corneas in such mice were $CD8^+$ T cells, as judged by immunohistochemical analysis of frozen ocular sections (Fig 6A). Since greater than 98% of the OT-1 RAG-/- T cell population is dominated by $CD8^+$ T cells that recognize the $OVA_{257-264}$ epitope and do not show any cross reactivity to HSV antigens (12), it was obvious that the lesion infiltrating T cells were non-HSV specific. Thus HSK lesions were orchestrated by bystander activated $CD8^+$ T cells, since RAG-/- mice succumb to encephalitis at around the same time point, but do not show evidence of HSK lesions (data not shown, (14)).

The presence of $CD8^+$ T cells in lesions from mice that had received immune $CD8^+$ T cells at 72 hrs p. i., survived encephalitis, and developed severe HSK lesions by day 25 p. i. was determined by a similar procedure. Indeed, as evident from Fig. 6B, large numbers of $CD8^+$ T cells were demonstrable at this time point. Two color immunofluorescence and confocal microscopy was used to define the host and the donor $CD8^+$ T cells in corneal lesions. Serial ocular sections were stained with anti-CD8 and anti-CD45.2 (donor cells). As revealed by this assay and evident from Fig 6C, visually, a majority of the $CD8^+$ T cells were of the host origin (i. e. non-HSV

specific). A few, very rare, scattered $CD8^+$ cells were found to be of donor origin. At this time very few of the $CD8^+$ T cells in the DLN and the spleen of such mice were of donor origin (Fig. 7). In addition, upon re-stimulation with the HSV immunodominant $gB_{498-505}$ peptide none (in the DLN) or a negligible (in the spleen) number of such surviving transferees were capable of IFN□ production revealing the absence of functional $CD8^+$ T cells reactive to HSV (Fig 7). Thus, by extrapolation, the few rare donor cells in the cornea at this time point were likely to be non-HSV specific as well.

Further investigations were carried out to determine whether in the presence of large number of HSV reactive $CD8^+$ T cells in the periphery, such cells could be found in the cornea. For this we utilized HSV immune $CD8^+$ T cells from the gBT-I.1 mice. Greater than 98% of $CD8^+$ T cells in such mice have TCRs recognizing the immunodominant HSV-1 $gB_{498-505}$ epitope (16). OT-1 RAG-/- mice received an adoptive transfer of HSV immune gBT-1.1 $CD8^+$ T cells 72 hours after ocular infection. Adoptively transferred $CD8^+$ T cells were readily demonstrable 12 days p. i., in both spleens and DLN (total number of recovered $CD8^+$ T cells in DLN approximately double that seen in the spleen, data not shown) and with greater than 50% of the transferees capable of IFNγ production upon re-stimulation with the immunodominant peptide (Fig 7). Interestingly, even at this time point no HSV reactive donor cells were detectable in corneas, even though as expected there were host (HSV un-reactive) $CD8^+$ T cells (Fig 6D).

Taken together, our results imply the ability of bystander activated $CD8^+$ T cells, but not HSV reactive $CD8^+$ T cells, to migrate to the cornea and orchestrate lesion development

HSV specific $CD8^+$ T cells migrate to the brain and trigeminal ganglion

Additional experiments were carried out to understand the protection from encephalitis and the modulation of HSK afforded by the HSV immune $CD8^+$ T cells. Experiments involved analysis of whether such cells could be detectable in the brain and trigeminal ganglion (TG), based upon the understanding that $CD8^+$ T cells are (1) involved in CNS viral clearance (19, 20) and (2) prevent virus reactivation from the peripheral nervous system after ocular infection (21)

In our experiments, we used the ocularly infected OT-1 RAG-/- animals that had received an adoptive transfer of gBT-I.1 $CD8^+$ T cells 72 hrs after infection (such mice are protected from HSV induced encephalitis but not from HSK development). The trigeminal ganglia and brain from such mice were processed on day 12 post infection (9 days after adoptive transfer). Donor HSV reactive cells were evident in the trigeminal ganglion both by microscopic (Fig 6E) and flow cytometry analysis (Fig 7). Interestingly, the TG also contained virus non-reactive host $CD8^+$ T cells, though outnumbered by the HSV reactive species almost twofold (Fig 7). In addition, a similar presence of $CD8^+$ T cells was noted in the brains by flow cytometry (Fig 7). However, the ratio of virus reactive to non-reactive cells in the brain was almost 6 to 1 (Fig 7).

Thus after an ocular HSV infection, virus specific CD8's migrate to the trigeminal ganglion and the brain, but not to the cornea. Their presence in the brain accounts for the protection from encephalitis, presumably through virus clearance. Further, finding virus specific cells in the TG implies their possible role in controlling

virus reactivation from this site and anterograde transport to the cornea, since in mice that received cells very early (24 hrs) corneal virus was cleared and HSK failed to occur.

DISCUSSION

It is established that T lymphocytes are the crucial mediators in the pathogenesis of Herpetic Stromal Keratitis (HSK). So far the identity of agonists responsible for the influx of these critical cells is unclear. Viral proteins, processed and presented by professional antigen presenting cells (APCs) or even by corneal cells themselves (22), are unlikely involved in T cell activation since T cells lacking reactivity to viral antigens can induce HSK (9, 11, 12). This mechanism of immunopathology, now well studied in several murine models, is referred to as bystander activation (23). Using these models, it has been shown that HSK can be mediated by both $CD8^+$ (the LCMV P14 RAG-/- and OT-1 RAG-/- mouse models for $CD8^+$ T cell activation) and $CD4^+$ T cells (the DO11.10 SCID and PCC RAG-/- mouse models for $CD4^+$ T cell activation) (9, 11-13). In the present report we have used the $CD8^+$ T cell bystander activation HSK model to evaluate whether signals responsible for bystander activation influence HSV specific $CD8^+$ T cells. Our results indicate that HSV reactive $CD8^+$ T cells do not to migrate to the corneal site of inflammation, a capability possessed mainly by the non-virus reactive $CD8^+$ T cells. However, the former cells appear to facilitate the control of viral replication in the cornea presumably by preventing virus reactivation from sites of latency and anterograde transport to the original site of infection. These results further strengthen the concept that murine HSK is mainly mediated by non-

HSV specific T cells that are activated by bystander mechanisms and confirm the protective and HSK immunomodulatory role of virus specific $CD8^+$ T cells.

It is evident that in the HSV infected RAG-/- or the SCID there exists a pro-inflammatory environment that overrides regulatory controls. Quiescent cells are activated, by bystander mechanisms into aggressive effector cells ($T_{aggressors}$) that induce HSK lesions. This scenario is reminiscent of the activation of naïve T cells into pseudo-memory cells, when transferred into lymphopenic animals (24). Thus we have previously shown that if T cells are allowed homeostatic expansion animals became more susceptible to HSK (4). In addition, other factors such as the lack of regulatory $CD4^+CD25^+$ (T_{regs}) cells have a likely influence. As shown recently, with the RAG-/- mouse model, in which animals develop HSK only when reconstituted with aggressor $CD4^+$ T cells (25), this is indeed the case. These studies demonstrate that animals fail to develop HSK lesions when reconstituted with a mixture of T_{regs} and $T_{aggresors}$ (25). Another contributing factor is virus persistence and dissemination into the corneal stroma, as shown in the present report and previously (9, 12, 13, 26). Virus derived molecules (proteins, DNA) in the stroma set up a chronic inflammatory milieu that provides a constant supply of pro-inflammatory molecules (4). If viral replication induced events occur favorably within the first few days after infection the stage is set for the successful development of HSK lesions. Thus upon transfer of HSV immune $CD8^+$ T cells, 24 hrs after infection, virus replication was controlled shortly after, preventing the induction of pro-inflammatory events. This pro-inflammatory milieu was however induced optimally in mice that received cells 3 days later and by extrapolation from our results it appears that optimal induction in the bystander model requires a replication of 6-8 days p.i. Thus the latter and not the former mice developed HSK lesions.

Most likely the sources of the initial pro-inflammatory molecules are the virus infected corneal cells themselves. Of the several likely candidates, IL-1 and IL-6, both of which have been shown to be involved in cytokine cascades and inflammatory cell influx (27-29), maybe the initial molecules. Subsequently, after virus replication events have ceased, as in the case of mice that received cells at 72 hrs, the main producers of T lymphocyte activating cytokines are infiltrating non-resident DCs or macrophages that respond to molecules released from virus infected cells. Cytokines that are thought to be involved in this process are IL-12 (30) and IFNγ (31). These molecules are currently under investigation. Another potential candidate that is thought to sustain the inflammatory process, possibly through its action on macrophages and dendritic cells, is HSV DNA. This molecule persists in corneas throughout the course of murine HSK (32) and contains abundant CpG motifs (33). Recent evidence points to the fact that, indeed, CpG motifs possess immunostimulatory capabilities required to drive Th1 type responses after HSV infection (33) and cornea infiltrating T cells show the same phenotype (7, 8). Supporting the role of HSV DNA derived CpG motifs in the bystander phenomenon is the observation of aggravated HSK lesions in DO11-10 SCID mice previously exposed to CpG (9). In addition, CpG has also been shown to be involved in the formation of new blood vessels (angiogenesis) in the cornea (34) that serve as conduits for T lymphocytes.

The results from the present study provide insights about the functions of the HSV specific $CD8^+$ T cells after an ocular HSV-1 infection. First and the most obvious is presence of HSV reactive $CD8^+$ T cells in the brains and their ability to protect from lethal encephalitis. As also observed in other HSV-1 models of encephalitis, indeed, when such cells are present early, but not late in infection, mice

are protected (20). Several studies, both *in vitro* and *in vivo*, have investigated mechanisms operational in the protection from uncontrolled viral replication in the nervous system and have implied non-cytolytic mechanisms involving IFNγ (21, 35, 36) and Granzyme A (37). Secondly, these cells are by themselves unable to mediate HSK, a capability possessed by bystander activated $CD8^+$ T cells. This is probably due to the inability of such cells to migrate to and reach sufficient numbers in the cornea as evident both from previous studies (3, 6, 13) and results presented here.

There could be two possible explanations for the inability of HSV CD8s to enter the cornea. The simplest would be to assume that one or more corneal homing molecules are different between the two types of $CD8^+$ T cells. However, the most probable scenario is that HSV specific cells only migrate to sites that are virus infected. Thus, shortly after HSV infection, the focus of migration of HSV immune CD8s is likely to be the draining lymph nodes associated with the cornea and trigeminal ganglion (TG) which is also a site of active viral replication and the induction of inflammatory cytokines (38). Extensive corneal angiogenesis is lacking at this stage and being a determining factor in T cell migration to corneas (39), even though the cornea is the site of viral replication, HSV specific $CD8^+$ T cells are unable to enter. Eventually during the clinical phase, when conditions become favorable, by corneal neovascularization, replicating virus or viral antigens are no longer detectable in the cornea making it unnecessary for these cells to extravasate into corneal tissues. However, in our experiments, in some instances few rare donor cells were indeed noted in the corneas at this stage and we contend that these are probably donor bystander CD8s that comprised 90% of the input population and survived within the host. Such cells were likely influenced by the bystander signals that effectively

recruited the OVA specific CD8s into the cornea. Further investigations are ongoing to substantiate these speculations.

Finally, and most importantly, though unable to migrate to the cornea, $CD8^+$ T cells facilitate corneal viral clearance. This indirect, remote, mechanism of viral clearance can be explained by a possible effect that the HSV immune $CD8^+$ T cells have on neutrophils. The latter cells migrate into corneas shortly after infection and are involved in viral clearance (40). Previous studies have implied that the cytokine IFNγ is involved in neutrophil extravasation from corneal blood vessels by upregulating PECAM-1 expression on vascular endothelial cells (41). One likely scenario is that the immune CD8s were a source of vast amounts of this critical cytokine, otherwise lacking in the un-reconstituted mice, allowing neutrophils to enter the cornea through similar integrin up-regulation associated events. This needs further evaluation. The other possibility that HSV reactive $CD8^+$ T cells might have stopped reactivation events in the TG is supported by recent observations by the Hendricks laboratory (21, 36, 42) and in this study. In their ocular infection model, HSV specific $CD8^+$ T cells that infiltrate the TG are the dominant population amongst the $CD8^+$ T cells, persist for months at this site and appear to prevent virus reactivation by monitoring latently infected neurons by mechanisms involving IFNγ (21, 36, 42). In the immune-compromised mice that we have used, presumably in the absence of a monitoring system in the TG, the virus repeatedly finds its way back to the original site of infection and is responsible for a continuous source of inflammatory molecules. Our results show that indeed these cells can find their way to the TG and interestingly when given early (24hrs p. i.) prevent HSK. Thus HSK lesions in the immune-compromised bystander model maybe a result of virus reactivation. This

speculation is supported by studies that show the inability to induce HSK, in DO11.10 SCID mice, if infected with a virus that is unable to reactivate from the TG (9).

In conclusion, our results serve to demonstrate the distinct roles of HSV specific $CD8^+$ T cells and $CD8^+$ T cells of other specificities with respect to HSK pathogenesis. The picture that emerges from these studies is the importance of the immune $CD8^+$ T cell as a modulator of HSK development. Other studies have demonstrated the increase in HSK severity and the enhanced susceptibility to encephalitis (19, 31, 35), in the absence or dysfunction of these cells. Further investigations will focus on harnessing the functions of these cells and possibly utilizing their potential to develop prophylactic and therapeutic strategies.

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APPENDIX

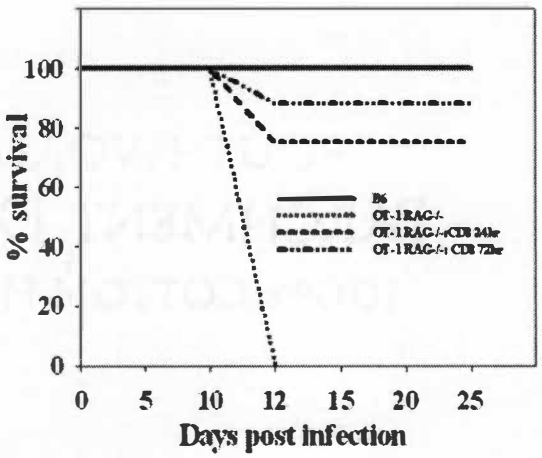


Figure 1. Ocularly infected OT-1 RAG-/- mice receiving HSV immune $CD8^+$ T cells are protected from death due to herpetic encephalitis.

Mice were infected on scarified corneas with $5x10^6$ pfu of HSV-1 RE. Adoptive transfer of HSV immune $CD8^+$ T cells was carried out via tail vein at 24 hrs and 72 hrs p. i. Data compiled from two separate experiments consisting of 4 animals in each group.

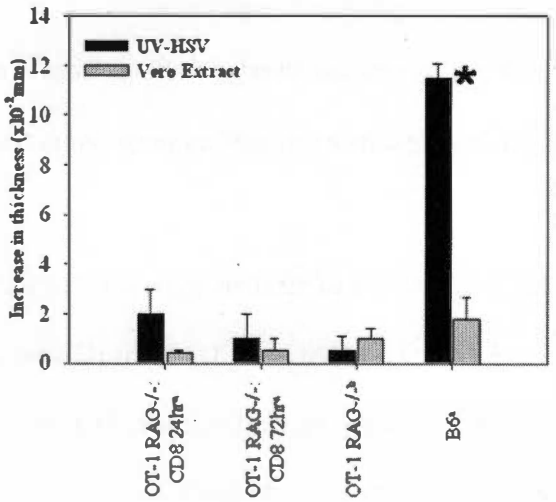


Figure 2. OT-1 RAG-/- mice receiving immune cells fail to mount a DTH response to HSV.

Ocularly infected mice from different groups (n=4) were injected with UV-HSV and vero cell extract on the left and the right ear pinna respectively. Increase in ear thickness was measured after 48hrs. Results expressed as mean ± SD. $*p < 0.05$

[a] Antigen was injected on day 23 p. i. and the DTH response measured on day 25 p. i.

[b] For OT-1 RAG-/- mice not receiving cells antigens were injected on day 8 p. i. and DTH response was measured on day 10 p. i.

Figure 3. Early clearance of virus from corneas of mice receiving immune $CD8^+$ T cells.

A. Viral titers were determined on day 8 p.i. by agarose overlay method from 4 eyes for each group and expressed as $\log_{10}$pfu/ml. Results expressed as mean virus titre ± SD. $*p < 0.05$

B, C and D. Immunohistochemistry for the detection of viral antigens (day 12 p.i.) in frozen sections of eyes from OT-1 RAG-/- (B), OT-1 RAG-/-: CD8 24 hr (C) and OT-1 RAG-/-: CD8 72 hr (D). Arrows show the presence of viral antigen. The substrate was DAB and counterstaining was done with Hematoxylin. Magnification x200.

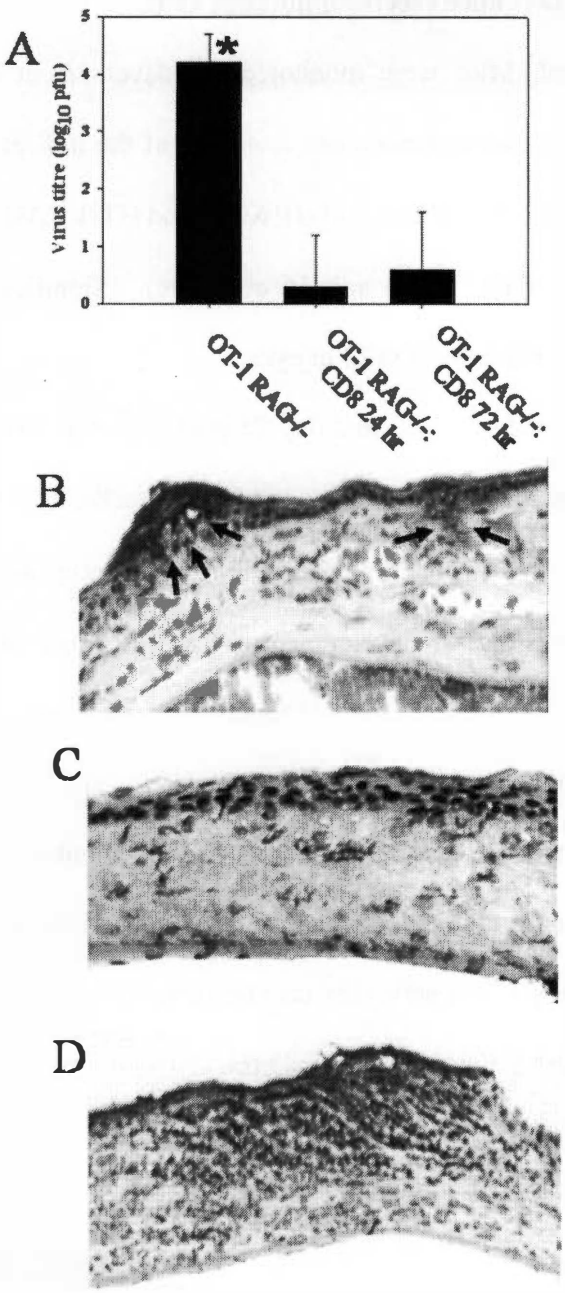
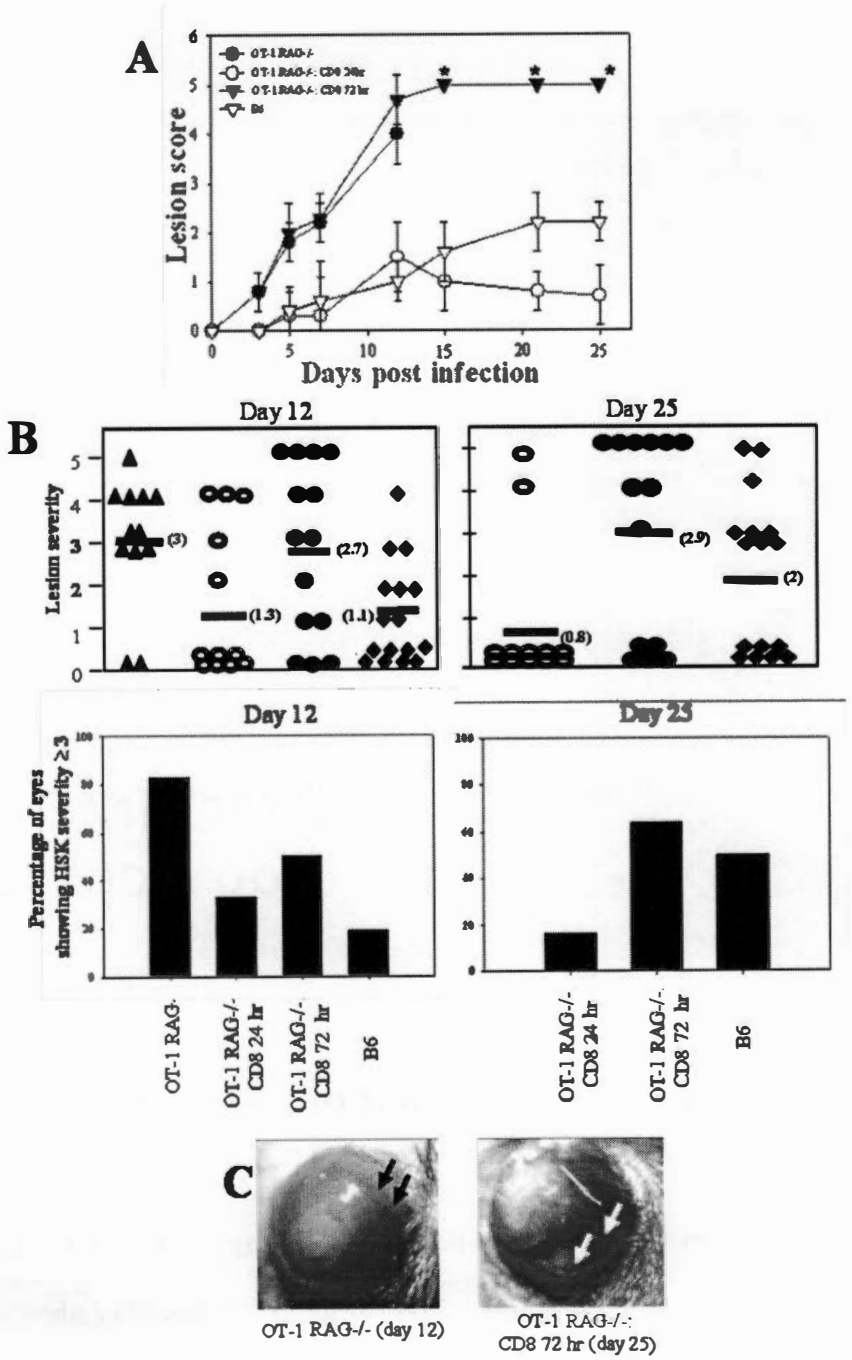

A
Virus titre (log_{10} pfu)
5
4
3
2
1
0
*
OT-1 RAG-/-
OT-1 RAG-/-: CD8 24 hr
OT-1 RAG-/-: CD8 72 hr
B
C
D

Figure 4. HSK severity in OT-1 RAG-/- mice receiving immune cells.

A. Kinetics of HSK development. Mice were monitored for development of lesions by a slit lamp ophthalmoscope (see materials and methods) at the indicated time points. Results show the mean ± SD for 12 eyes (OT-1RAG-/- and OT-1 RAG-/: CD8 24 hr), 14 eyes (OT-1 RAG-/-: CD8 72 hr) and 16 eyes (B6). *Significant difference ($p<0.05$) compared to OT-1 RAG-/-: CD8 24 hr eyes.

B. Results show HSK lesion scores for day 12 and day 25 post infection. OT-1 RAG-/- mice received an adoptive transfer of 5 x 10^6 immune $CD8^+$ T cells at 24 hr (o) and 72 hr (•) p.i. Control OT-1 RAG-/- mice (▲) succumb to viral encephalitis on day 12 p.i. Shown for comparison is disease development in C57BL/6 mice (◆). Each dot represents the HSK score from one eye. The horizontal bars and figures in parentheses show the mean for the groups.

C. Eyes of OT-1 RAG-/- mice, not receiving immune CD8s and succumbing to encephalitis by day 12 p.i., show evidence of opacification and angiogenesis (arrows). Mice receiving immune cells at 72 hr p.i. and surviving encephalitis, show necrotic lesions of the central cornea along with angiogenesis at day 25 p.i.


A
Lesion score
Days post infection
B
Day 12
Day 25
Lesion severity
Percentage of eyes showing HSK severity ≥ 3
OT-1 RAG-
OT-1 RAG-/- CD8 24 hr
OT-1 RAG-/- CD8 72 hr
B6
C
OT-1 RAG-/- (day 12)
OT-1 RAG-/-: CD8 72 hr (day 25)

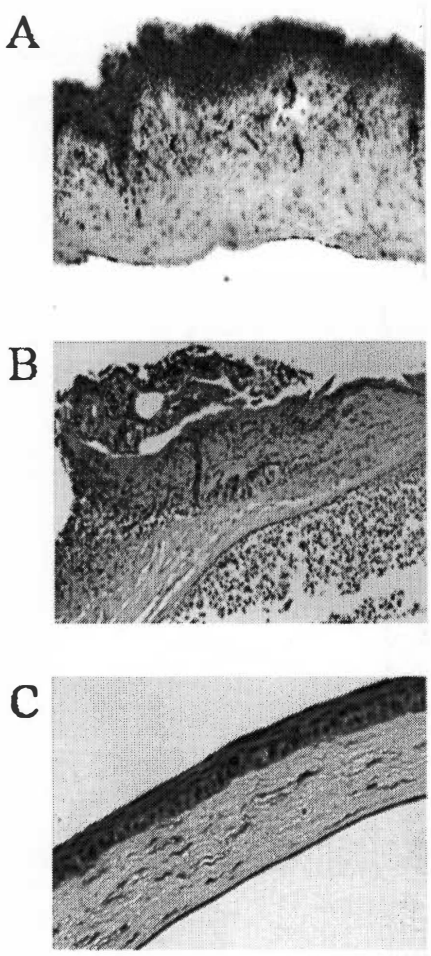


Figure 5. Inflammatory cells infiltrate corneas of OT-1 RAG-/- mice showing HSK lesions.

Photographs of representative corneas of OT-RAG-/- mice at day 12 (A), OT-1 RAG-/-: CD8 72 hr at day 25 (B) and OT-1 RAG-/-: 24 hr at day 25 (C). Paraffin embedded eyes were sectioned and hematoxylin and eosin staining was carried out on 6μ sections. Magnification x200.

Figure 6. $CD8^+$ T cells in corneas and trigeminal ganglia.

Frozen sections (6μ) of representative eyes of OT-1 RAG-/- mice day 12 (A) and OT-1 RAG-/-: CD8 72 hr day 25 (B) was stained with FITC labeled anti-CD8α MAb. Propidium iodide was used as a counterstain. Scale Bars: 80μm

Frozen sections (6μ) of eyes of OT-1 RAG-/-: CD8 72 hr at day 25 p. i. (C) and of eyes (D) and trigeminal ganglia (E) of OT-1 RAG-/-: gBT-I.1 CD8 72 hr at day 12 p. i. were incubated with FITC labeled anti-CD8α (green) and biotin labeled anti-CD45.2. After incubation with streptavidin-Alexa fluor 546 (red), images were captured using a confocal microscope. The differential interference contrast (DIC) image shows the location of the epithelium (epi) and stroma (str) of the cornea. Arrows show $CD8^+CD45.2^+$ cells. Scale Bars: 80μm

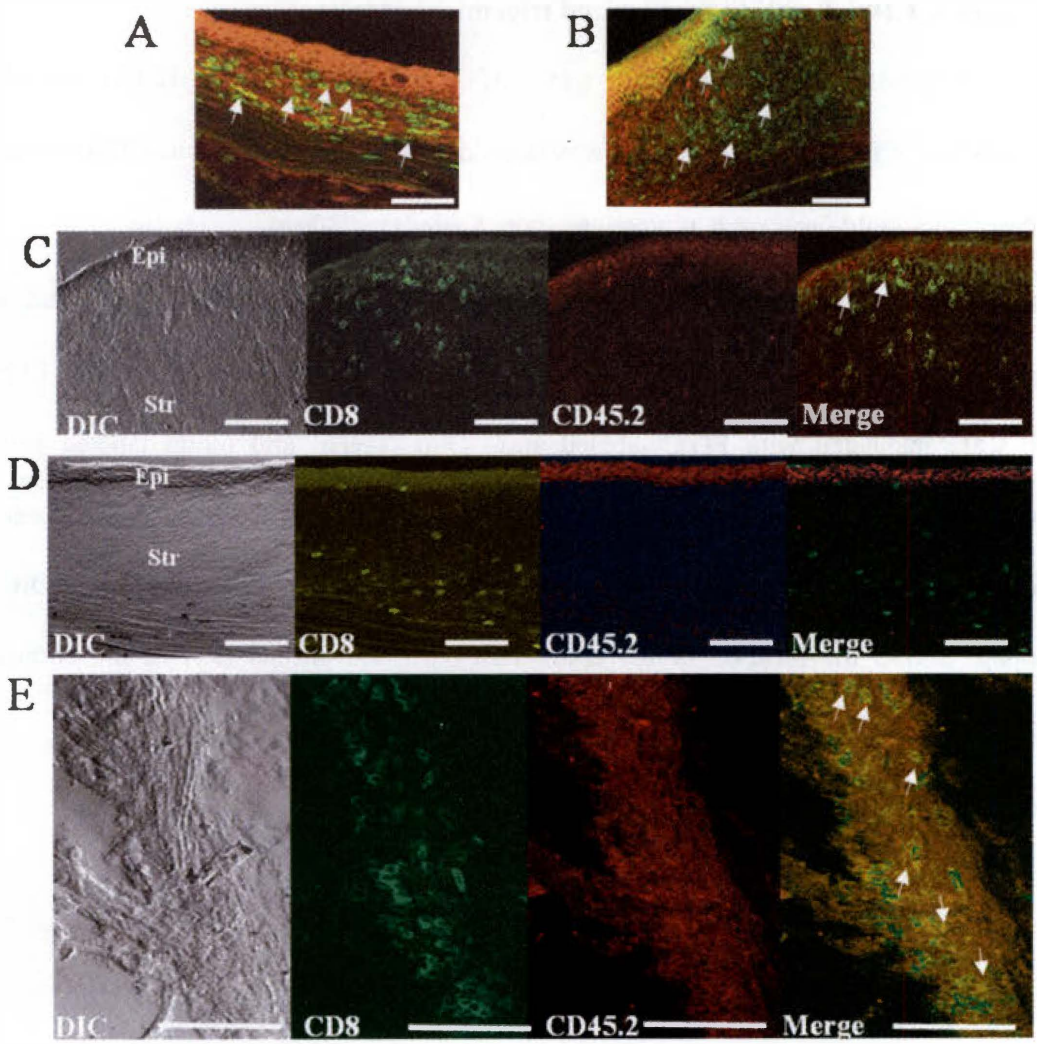
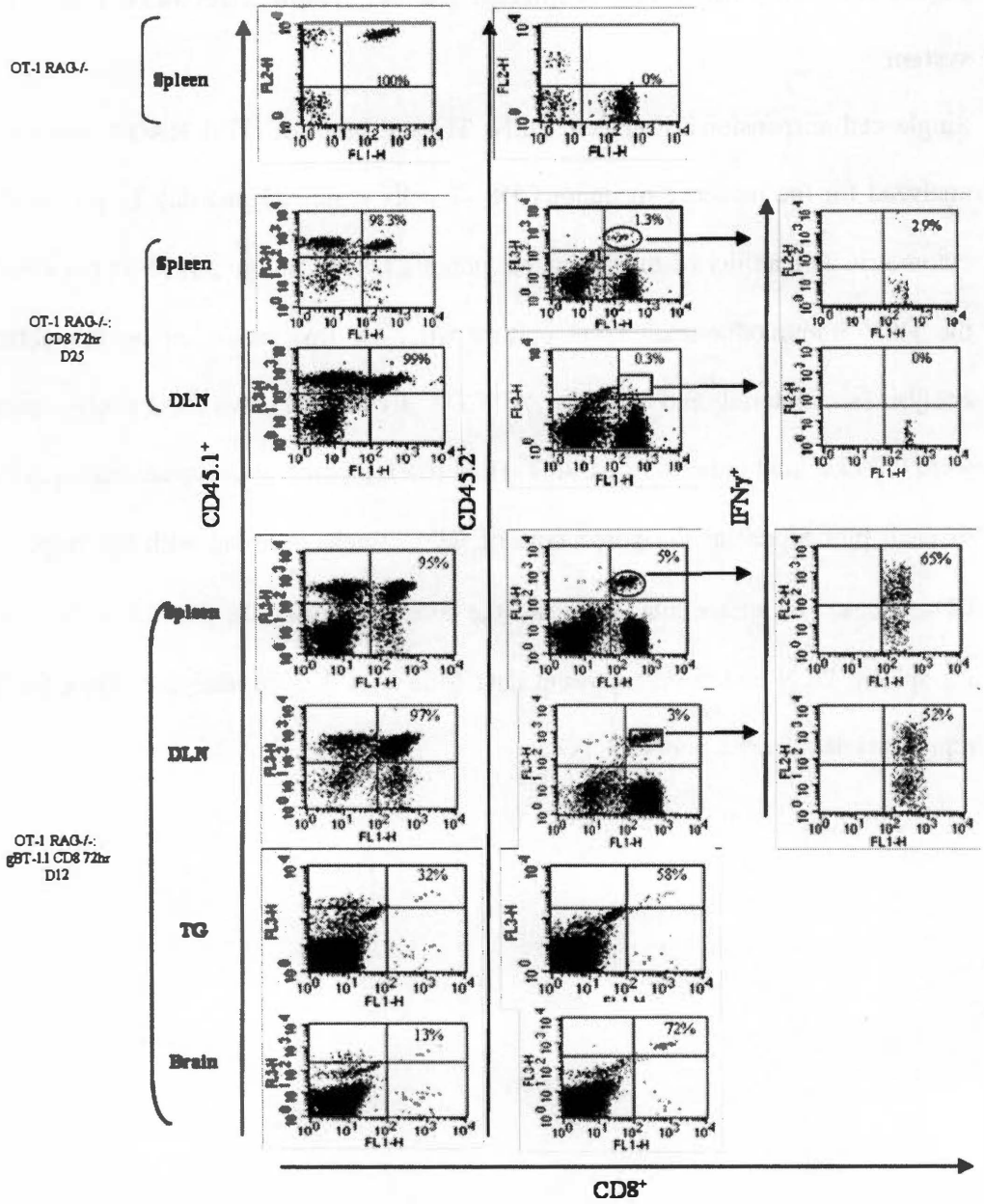

A
B
C
Epi
Str
DIC
CD8
CD45.2
Merge
D
Epi
Str
DIC
CD8
CD45.2
Merge
E
DIC
CD8
CD45.2
Merge

Figure 7. Donor $CD8^+$ T cells in spleens, draining lymph nodes and the nervous system

Single cell suspensions of spleen, DLN, TG and brain of OT-1 RAG-/- mice were analyzed for the presence of donor $CD8^+$ T cells at day 25 and day 12 p.i. by flow cytometry. The ability of the detectable donor CD8s to produce IFNγ in response to the HSV immunodominant CD8 epitope $gB_{498\text{-}505}$ was measured by intracellular analysis (see materials and methods). All $CD8^+$ T cells in a naïve OT-1 RAG-/- mouse were $CD45.1^+$ and none were $CD45.2^+$ (first row). Figures in the upper right quadrant of each plot represent the percentage of $CD8^+$ T cells staining with the respective CD45 marker or intracellular IFNγ (for the latter gated on $CD8^+CD45.2^+$ cells). Plots for spleen, DLN and brain represent data from 1 of 3 mice analyzed. Plots for TG represents data from a pool of 3 TGs.


OT-1 RAG-/-
Spleen
100%
0%
OT-1 RAG-/-:
CD8 72hr
D25
Spleen
98.3%
1.3%
2.9%
DLN
99%
0.3%
0%
CD45.1+
CD45.2+
IFNγ+
Spleen
95%
5%
65%
DLN
97%
3%
52%
OT-1 RAG-/-:
gBT-1.1 CD8 72hr
D12
TG
32%
58%
Brain
13%
72%
FL1-H
FL2-H
FL3-H
CD8+

Vita

Kaustuv Banerjee was born in Calcutta (now Kolkatta), India on August 12th, 1975. He attended schools at Bangalore, India and graduated from the Bishop Cotton Boys School in 1991 after completing his Indian Council of Secondary Examination. He completed his Indian School Certificate examination in the year 1993 again from Bishop Cotton Boys School, Bangalore.

In October of 1993 he started his Veterinary education at the West Bengal University of Animal and Fishery Sciences, Calcutta and received the Bachelor of Veterinary Sciences and Animal Husbandry (B.V.Sc and A.H) degree in the year 1998.

Kaustuv joined the Indian Veterinary Research Institute, Uttar Pradesh in September of 1998 and obtained his Master of Veterinary Sciences (M.V.Sc) degree from the department of Veterinary Virology in the year 2000. This included a year of research at the Indian Veterinary Research Institute campus at Bangalore. He joined the University of Tennessee, Knoxville in August 2000 and received the Doctor of Philosophy degree from the Department of Comparative and Experimental Medicine, College of Veterinary Medicine in August 2004. He plans to further pursue post doctoral training.