Ocular infection with herpes simplex virus (HSV-1) may cause a vision impairing inflammatory reaction in the corneal stroma called herpetic stromal keratitis (HSK). The lesion in humans is one of the common causes of nontraumatic corneal blindness. In murine models, HSK lesions appear to be immunopathologic mediated by CD4+ T cells of the Th1 phenotype. Nevertheless, the pathogenesis of HSK remains to be resolved, particularly the identity of agonists that drive CD4+ T cells to orchestrate the inflammatory reaction. The most logical idea, that virus-derived proteins or peptides expressed on invading Langerhans cells are recognized by CD4+ T cells, has been difficult to prove. Thus, usually in immunocompetent mice viral antigens are not evident when HSV can be induced in athymic or SCID mice by adoptive transfer of CD4+ T cells that could become activated by an inflammatory milieu consisting of enhanced accessory molecules and proinflammatory cytokines in the cornea.

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Bystander Activation of CD4+ T Cells Accounts for Herpetic Ocular Lesions

Shivaprakash Gangappa,1 Shilpa P. Deshpande, and Barry T. Rouse

Purpose. Stromal keratitis is an immunopathologic consequence of herpes simplex virus (HSV) infection of the cornea. The lesion is immunopathologic, but the identities of molecules that drive the reaction remain unresolved. To exclude viral antigen recognition as a necessary step in the disease process, ocular HSV infection was followed in Tg-RAG mice (OVA-TCR transgenic mice crossed to RAG2-deficient mice) whose limited T-cell repertoire did not include immune responsiveness to HSV.

Methods. Mice with T-cell specificity to OVA peptide (Tg-RAG mice) as well as control DO11.10 and BALB/c mice were infected with HSV on the scarified cornea and subjected to clinical, histologic, and immunologic analysis. To evaluate involvement of OVA-specific CD4+ T cells in lesion development in Tg-RAG mice, monoclonal antibody to CD4+ T cells was used for in vivo CD4+ T-cell depletion.

Results. Tg-RAG mice were capable of eliciting ocular lesions in the absence of detectable reactivity to viral antigens. Lesion manifestation in Tg-RAG mice was CD4+ T-cell dependent and the cellular infiltrates and their inflammatory products in the HSV-infected cornea were comparable to similarly infected BALB/c and DO11.10 mice.

Conclusions. The authors conclude that mechanisms other than viral antigen recognition, and hence molecular mimicry, are at play and are sufficient to cause HSV-induced stromal keratitis. The data imply a significant role for non–virus-specific CD4+ T cells that could become activated by an inflammatory milieu consisting of enhanced accessory molecules and proinflammatory cytokines in the cornea.

Materials and Methods

Mice

Four- to five-week-old BALB/c mice (H-2d) (Harlan Sprague-Dawley, Indianapolis, IN), OVA-TCR transgenic mice...
(DO11.10 mice, H-2d; kindly provided by Dennis Loh, Washington University School of Medicine, St. Louis, MO and Casey T. Weaver, University of Alabama, Birmingham, AL) were used. Tg-RAG2−/− (Tg-RAG, H-2d)10 mice were kindly provided by Osami Kanegawa (Washington University School of Medicine). Transgenic mice (DO11.10) and HSK-susceptible BALB/c groups of mice were used as control animals. DO11.10 mice were housed in a barrier facility. All food, water, bedding, and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. To prevent bacterial super infections, DO11.10 and Tg-RAG mice received prophylactic treatment of sulfatrim pediatric suspensions (Barre-National, Baltimore, MD) at the rate of 5 ml/200 ml drinking water. Antibiotic treatment was started 1 day before the beginning of experiments. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animal Resources, Commission Life Sciences, and National Research Council. The facilities used were accredited by the American Association for Accreditation of Laboratory Animal Care. All ocular experimental procedures were conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Virus and Corneal Infections**

The HSV-1 RE strain used was propagated on Vero cells and stored as infectious cell preparations at −70°C. The corneal surfaces were deeply anesthetized (methoxyflurane; Pittman Moore, Mondelein, IL) and were scarified with a sterile 27-gauge needle and 5 × 105 pfu. The HSV-1 RE strain was applied in a 4.0-μl volume and was massaged gently on the eye lids.

**In Vivo CD4+ T-Cell Depletion**

For CD4+ T-cell depletion, ascitic fluid containing anti-CD4 monoclonal antibody (Ab) (GK 1.5 hybridoma; ATCC TIB207, Rockville, MD) was used. Ascitic fluid containing rat IgG2b antibody was used as isotype control. The determination of depletion dose (0.5 mg/mouse on days −2, 0, +2, and +5) was followed by the method described previously.11 The ascitic fluids were titrated for the Ab content using an indirect enzyme-linked immunosorbent assay (ELISA) as described previously.12

**Clinical Evaluation**

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

**Immunoglobulin ELISA**

Serum collected was analyzed for HSV-specific total IgG, using a standard ELISA as described previously.13 Basically, ELISA plates were coated with 100 μl HSV antigen in carbonated buffer (pH 9.8), and after overnight incubation at 4°C, the plates were washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 20, pH 7.2 (PBST), and then were blocked using PBS (pH 7.2) with 3% dehydrated milk for 2 hours at 37°C.

A total of 200 μl serially diluted serum samples (prediluted in PBST) was added in duplicate and washed. Wells coated with goat anti-mouse IgG (0.025 mg/ml) were treated with serially diluted standard mouse IgG. Plates were incubated for 2 hours at 37°C. After three washes, 100 μl goat anti-mouse IgG horseradish peroxidase was added. After three washes, 2,2-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid substrate (no. A1888; Sigma, St. Louis, MO) was added. The concentration of the antibodies in the serum samples was determined from the standard curve.

**Cytokine Assay**

For cytokine [interferon gamma (IFN-γ)] assay, splenocytes from mice were suspended in 10% RPMI-1640, and 106 cells in 1 ml were stimulated in vitro with 1.5 MOI (multiplicity of infection, before inactivation) of UV-inactivated HSV-1 (KOS strain). A similar number of cells were concanavalin A–stimulated (5 μg/106 cells/ml) in 12-well culture plates. Plates were incubated at 37°C for 72 hours. The supernatant fluid was collected and stored at −20°C until use. These supernatants were screened for the presence of IFN-γ by ELISA as described previously.14

**Histopathology**

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

**Immunohistochemical Staining**

At the termination of experiments, eyes were removed and snap-frozen in OCT compound (Miles, Elkhart, IN). Six-micron-thick sections were cut, air-dried, and fixed in cold acetone for 5 minutes. The sections were then blocked with heat-inactivated goat serum and stained with biotinylated anti-CD4 (Pharmingen, San Diego, CA). Frozen sections also were stained for the presence of HSV antigens by the use of rabbit anti-HSV antiserum (Dako Corp., Carpinteria, CA), which was followed with biotinylated anti-rabbit Ig (1/20 dilution; Biogenex, San Ramon, CA). Sections were then treated with horseradish peroxidase–conjugated streptavidin (1:1000) and 3,3′-diaminobenzidine (Vector, Burlingame, CA), and counterstained with hematoxylin.

**Proliferation Assay**

Splenocytes (responders) at day 11 p.i. were collected and restimulated in vitro with HSV-1 KOS (MOI of 1.5 before UV irradiation) or OVA peptide (Research Genetics Inc., Huntsville, AL). The HSV-1–specific lymphoproliferation was measured as described previously.14 Briefly, responders: stimulator ratios tested ranged from 10:1 to 0.625:1. Responders + stimulator mixtures were incubated at 37°C for 4 days. [3H]TdR (1.0 μCi/well) was added, harvested 1 hour later, and results were read as counts per minute.
later, and read using a beta scintillation counter (Trace 96; Inotech, Lansing, MI). The results were expressed as means ± SD.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction

On day 11 p.i., splenocytes were collected, transferred to Tri-reagent (Molecular Biology Inc., Cincinnati, OH). The total cellular RNA was isolated from the Tri-reagent cellular lysate by adding chloroform and with centrifugation, followed by etha-nol/isopropyl ethanol precipitation of the aqueous RNA solution according to the manufacturer's instructions. The RNA thus obtained was reverse transcribed using oligo(dT) primers and superscript (Gibco BRL-Life Technologies, Bethesda, MD) according to standard protocol. The cDNA thus obtained was used as a template in subsequent qualitative for viral gD mRNA expression and semi-quantitative polymerase chain reactions (PCRs) for TNF-α and IFN-γ mRNA expression as described previously. Preliminary testing established that the number of cycle runs and cDNA dilutions were within the linear range of amplification for the sets of primers used. Sequences of primers (5'-3') used were as follows: TNF-α, ATG AGC ACT GAA AGC ATG ATC (sense) and AAA GCC TAG TAA CGG GAC ACT (antisense); IFN-γ, 5'-GCA GCG ACT CCT TTT CCG CTT (sense) and GCA GCG ACT CCT TTT CCG CTT (antisense); β actin, 5'-GTG GGG CGC CCC AGG CAC CAG (sense) and TAG

### Table 1. Immune Responses in HSV-RE–Infected BALB/c, DO11.10, and Tg-RAG Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>HSV Stimulators</th>
<th>OVA Stimulators</th>
<th>ConA Stimulation (cpm)</th>
<th>HSV-Specific IgG (ng/ml)</th>
<th>IFN-γ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>41</td>
<td>3</td>
<td>26821 ± 2102</td>
<td>3822 ± 704</td>
<td>27 ± 11</td>
</tr>
<tr>
<td>DO11.10</td>
<td>29</td>
<td>76</td>
<td>19122 ± 1681</td>
<td>2178 ± 191</td>
<td>19 ± 09</td>
</tr>
<tr>
<td>Tg-RAG</td>
<td>1</td>
<td>142</td>
<td>18617 ± 3228</td>
<td>UD</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Antigen-specific proliferation in HSV-1 infected BALB/c, DO11.10, and Tg-RAG mice. Five mice per group were individually assayed for proliferation in response to UV-HSV and OVA peptide (323–339) by incorporation of [3H]thymidine.
† The values represent stimulation index calculated based on response to antigen pulsed and naive stimulators.
‡ Splenocytes from individual mice were restimulated in vitro with UV-HSV or ConA and the supernatants were assayed by ELISA for IFN-γ as described in the Materials and Methods section. The values represent the means ± SD.
§ Serum IgG was measured by standard capture ELISA assay with five mice per group.
CAG GCA CTG TAA TTC CTC (antisense). Values are expressed as ratios of cytokine to β-actin to account for RNA isolation and reverse transcription efficiencies. Viral gD primers used were as follows: GC CCG AGA CCC CCA ACG CCA (sense) and TG CGC GTG GAC AAG GCG GAC (antisense).

Flow Cytometric Analysis
Isolated splenocytes or cervical lymph node cell populations were counted by the trypan blue exclusion method and analyzed for cell surface markers by flow cytometry. Viable cells were blocked with heat-inactivated fetal bovine serum, washed three times with fluorescence activated cell sorter buffer (1× PBS with 1% bovine serum albumin and 0.05% NaN₃). For Tg-RAG mice characterization, cells from cervical lymph nodes were stained with anti-CD4 fluorescein isothiocyanate (FITC), anti-CD8 PE. For detection of Tg-RAG mice, cells from cervical lymph nodes were stained with anti–CD4-fluorescein isothiocyanate (FITC), anti–CD8-PE. For detection of transgenic, TCR-positive T cells, KJ1-26.1 (anti-OVA TCR Ab, a kind gift from Philippa Marrack, National Jewish Hospital, Denver, CO) antibody was used and was detected by adding goat anti-mouse IgG2a-FITC antibody. Events were collected and analyzed using a Becton-Dickinson FACS analyzer.

Virus Recovery
At various time points after infection, swabs of the corneal surface were taken using sterile swabs soaked in McCoy me-

### Table 2: Proliferative Response in CD4⁺ T-Cell-Depleted BALB/c and Tg-RAG Mice Ocularly Infected with HSV-1

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment</th>
<th>HSV Stimulators</th>
<th>OVA Stimulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg-RAG</td>
<td>Rat IgG control antibody</td>
<td>1</td>
<td>181</td>
</tr>
<tr>
<td>Tg-RAG</td>
<td>Anti-CD4 antibody</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Rat IgG control antibody</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Anti-CD4 antibody</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

On day 12 p.i., splenocytes from five mice/group were individually assayed for antigen-specific proliferation in response to UV-HSV and OVA peptide (323–339) by incorporation of [³H]thymidine. *The values represent stimulation index calculated based on the response to antigen pulsed and naïve stimulators.

In the next section, we present data on the absence of immunity in Tg-RAG mice following corneal scarification and infection with HSV. We show that Tg-RAG mice do not generate T-cell immunity against HSV, as measured by T-cell proliferative and cytokine responses.

#### Statistical Analysis
Wherever specified, data obtained were analyzed for statistical significance by Student’s t-test.

#### Results

**Absence of Immunity in Tg-RAG Mice**

Corneal scarification and infection with HSV results in an inflammatory reaction in the corneal stroma of both BALB/c and DO11.10 mice, but not in mice lacking T cells. Lesions of HSK appear to be mediated by CD4⁺ T cells. To study the outcome of ocular infection in mice with CD4⁺ T cells yet unable to recognize HSV immunologically, Tg-RAG mice were used. Such mice possess only CD4⁺ T cells, but these recognize an OVA peptide. As shown in Figure 1, all T cells in Tg-RAG mice were CD4⁺ and more than 98% of them additionally reacted with the monoclonal antibody, KJ1-26.1, indicating that they are OVA-specific. That Tg-RAG mice failed to generate T-cell immunity is documented in Table 1. Thus, analysis of T-cell-proliferative and antigen-induced IFN-γ responses in splenocytes 10 to 12 days p.i. failed to detect HSV-specific reactivity. Animals also failed to manifest delayed-type hypersensitivity reactions to HSV (data not shown). In contrast, animals showed strong proliferative and cytokine responses to stimulation by OVA peptide. Depletion of CD4⁺ T cells from either Tg-RAG mice or BALB/c mice markedly reduced or abrogated the proliferative responses (Table 2). Whereas BALB/c mice, and in addition DO11.10 mice, infected via the cornea with HSV RE survived infection, similarly infected Tg-RAG mice were all dead from encephalitis by 11 to 13 days p.i.

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HSK Lesions in Tg-RAG Mice

Mice of three types (Tg-RAG, BALB/c, and DO11.10 mice) were infected on the scarified cornea with 5 × 10⁵ pfu of HSV-1 RE and followed clinically for the expression of HSK lesions. Sample mice in some experiments also were analyzed at different times after infection to observe the histologic nature of lesions. As is shown in Figure 2, in sample Tg-RAG mice with clinical disease examined histologically, lesions typical of HSK were present. Unfortunately, Tg-RAG mice could not be studied beyond 11 to 13 days p.i., because death from herpetic encephalitis occurred at that time. The lesions evident in Tg-RAG mice were mediated by CD4⁺ T cells, because if such mice were CD4⁺ T-cell depleted, clinical and histologic lesions failed to occur (Fig. 3). In addition, in the lesions of Tg-RAG mice CD4⁺ T cells could be demonstrated by immunocytochemistry (Fig. 2B). Although only few CD4⁺ T-cell–depleted animals were followed, death in such animals occurred no more rapidly than in nondepleted Tg-RAG mice.

Why Do HSV-Infected Tg-RAG Mice Develop HSK?

The above data demonstrate that Tg-RAG mice develop a CD4⁺-dependent HSK lesion, yet their CD4⁺ T cells fail to recognize HSV antigens. As shown in Table 3, one major difference between BALB/c and Tg-RAG mice after HSV infection is that infectious virus was recoverable from eye swabs until the time of death. Furthermore, viral antigens appeared present deep within the stroma at least by 10 days p.i. (Fig. 4). In contrast, BALB/c mice usually cleared virus by 6 days p.i. (Fig. 4), and viral antigen expression was confined mostly to the corneal epithelium. Sample infected Tg-RAG mice also were analyzed for the presence of mRNA for TNF-α, IFN-γ, and the viral protein (gD) at 10 days p.i. All three species of mRNA were present in RNA samples from Tg-RAG mice, but only the cytokine mRNA species were present in BALB/c mice (Figs. 5, 6). We interpret our experiments to mean that HSK lesions were orchestrated by CD4⁺ T cells being activated in the cornea rich in cytokines as a result of continuous HSV replication.

DISCUSSION

Herpetic stromal keratitis is usually considered to represent an immunoinflammatory response to ocular HSV infection organized by CD4⁺ T cells. The present data using a transgenic mouse model with a highly restricted T-cell repertoire demonstrate that CD4⁺ T cells are required for the expression of HSK, but such T cells need not be capable of recognizing HSV antigens. We interpret our observations to mean that CD4⁺ T-cell–mediated immunopathology can proceed by bystander activation of T cells likely by one or more proinflammatory molecules generated by persistent HSV infection of the cornea.

The mice chosen for study were transgenic for the Va and Vβ T-cell receptor, which recognizes a class II–restricted OVA peptide. The animals were repeatedly back-crossed to the RAG-2–deficient mouse to produce Tg-RAG mice that possessed only CD4⁺ T cells, 98% of which expressed the KJ1-26.1 idiotype characteristic of the TCR reactive with the OVA peptide (323–339). Such mice were highly susceptible to

<table>
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<th>Mice</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>8 × 10⁴</td>
<td>3 × 10⁵</td>
<td>1 × 10⁵</td>
<td>UD</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>DO11.10</td>
<td>10 × 10⁵</td>
<td>7 × 10⁵</td>
<td>5 × 10⁵</td>
<td>0.6 × 10³</td>
<td>UD</td>
<td>UD</td>
</tr>
<tr>
<td>Tg-RAG</td>
<td>9 × 10⁶</td>
<td>8 × 10⁵</td>
<td>10 × 10⁵</td>
<td>12 × 10³</td>
<td>15 × 10³</td>
<td>12 × 10³</td>
</tr>
</tbody>
</table>

HSV-1 RE (5 × 10⁵ pfu) was inoculated on the scarified cornea, and at the indicated times the presence of infectious virus in eye swabs was determined by the agarose overlay method and the number of plaque forming units (pfu) per milliliter were estimated. At each time point, eye swabs were collected from four mice (n = 4), and viral titers were determined.

UD, undetectable, i.e., below the sensitivity of the assay (<10 pfu).

**TABLE 3.** Presence of Infectious Virus in the Eye Swabs after Ocular HSV-1 RE Infection

**FIGURE 4.** Immunoperoxidase staining for viral antigen (indicated by arrow) in the cornea of Tg-RAG mice (A; day 10 p.i.) and BALB/c mice (B; day 6 p.i.). Magnification, ×200.
HSV infection and, as far as could be determined, failed to generate HSV-specific T-cell responses after infection or immunization (Gangappa S, Rouse BT, unpublished observations). The mice also lacked B cells or CD8$^+$ T cells. Unexpectedly, however, the Tg-RAG mice still expressed lesions typical, both clinically and histologically, of HSK. Such lesions did not occur if the HSV-infected, Tg-RAG mice were depleted of CD4$^+$ T cells. These observations indicate that the CD4$^+$ T cells are necessary to orchestrate the immunoinflammatory lesions but fail to function by specifically recognizing HSV-encoded antigens. They also serve to argue against the molecular mimicry hypothesis for HSK pathogenesis. Some have advocated that autoantigens rather than viral antigens provide the targets for immunoinflammatory CD4$^+$ T cells in the cornea. Our data cannot formally exclude such a hypothesis, but with the highly restricted T-cell repertoire expressed by Tg-RAG mice, the notion is unlikely.

Currently, a mechanistic explanation for the immunopathology observed in Tg-RAG mice is lacking. However, our working hypothesis is that CD4$^+$ T cells enter a cornea rich in proinflammatory molecules, present as a consequence of continuous HSV replication in Tg-RAG mice. Thus, in Tg-RAG mice, virus remained present in the cornea until death and disseminated beyond the epithelial location, typical of the situation in immunocompetent mice. Accordingly, by immunocytochemistry, viral antigen was abundantly evident in the stroma itself. We assume that CD4$^+$ T cells entering the cornea, perhaps after effective angiogenesis has occurred, become activated by a non-TCR-mediated process and respond by themselves, producing proinflammatory molecules, and orchestrate the inflammatory lesion. A similar idea recently was proposed to explain the pathogenesis of Coxsackie virus-induced, insulin-dependent diabetes mellitus (IDDM) in transgenic mice incapable of recognizing Coxsackie viral antigen or GAD, an antigen often involved in IDDM. We are currently attempting to identify the nature of activators that participate in this bystander activation mechanism of immunopathology. It also will be important to establish what part if any bystander activation plays in HSK in immunocompetent mice or in the natural human disease.

**FIGURE 5.** Semiquantitative analysis of IFN-γ (A) and TNF-α (B) mRNA expression in the cornea of BALB/c and Tg-RAG mice at day 11 p.i. Total RNA was isolated from corneal lysate and used for RT-PCR as described in the Materials and Methods section. Lane 1, trauma-control BALB/c cornea; lane 2, HSV-1–infected BALB/c cornea; lane 3, trauma-control Tg-RAG cornea; lane 4, HSV-1–infected Tg-RAG cornea; lane 5, Marker (1-kb DNA ladder). (C) β-actin expression in the respective samples.

**FIGURE 6.** Qualitative analysis for viral gD (A) and β-actin (B) mRNA expression in the cornea of BALB/c and Tg-RAG mice at day 10 p.i. Total RNA was isolated from corneal lysate and used for RT-PCR as described in the Materials and Methods section. Lane 1, Marker (1Kb DNA ladder); lane 2, trauma-control BALB/c cornea; lane 3, HSV-1–infected BALB/c cornea; lane 4, trauma-control Tg-RAG cornea; lane 5, HSV-1–infected Tg-RAG cornea; lane 6, HSV-1–infected BALB/c cornea at day 2 p.i.
Acknowledgments

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References