

## Both CD4+ and CD8+ T cells are involved in protection against HSV-1 induced corneal scarring

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### Abstract

**Aim**—To determine the relative impact of CD4+ T cells and CD8+ T cells in protecting mice against ocular HSV-1 challenge.

**Methods**—CD4+ T cell knockout mice (CD4<sup>-/-</sup> mice), CD8+ T cell knockout mice (CD8<sup>-/-</sup> mice), and mice depleted for CD4+ or CD8+ T cells by antibody (CD4+ depleted and CD8+ depleted mice), were examined for their ability to withstand HSV-1 ocular challenge. The parental mice for both knockout mice were C57BL/6J.

**Results**—These results suggest that: (1) both CD4+ deficient mice (CD4<sup>-/-</sup> and CD4+ depleted mice) and CD8+ deficient mice (CD8<sup>-/-</sup>, and CD8+ depleted mice) developed significantly more corneal scarring than their C57BL/6J parental strain; (2) the duration of virus clearance from the eyes of the CD4+ deficient mice was 4 days longer than that of the CD8+ deficient mice; and (3) the severity of corneal scarring in the CD4+ deficient mice was approximately twice that of the CD8+ deficient mice.

**Conclusions**—It was reported here that: (1) CD4+ and CD8+ T cells were both involved in protection against lethal ocular HSV-1 infection; and (2) CD4+ and CD8+ T cells were both involved in protection against HSV-1 induced corneal scarring.

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Following ocular challenge of naive susceptible mice with HSV-1, the prevailing corneal disease is herpetic stromal keratitis (HSK).<sup>1</sup> HSK is also the main pathological response associated with ocular herpes simplex virus (HSV-1) infection in humans.<sup>2–4</sup> In mice, HSK first appears as geographic ulceration between days 3–5 after ocular challenge, proceeds to corneal ulceration between days 7–10, and finally to corneal scarring by day 21–28.<sup>5,6</sup> CD4+ and CD8+ T cell responses can influence the outcome of primary infection.<sup>1,6–8</sup> Depending on the infectious agent, T cells have been associated with protection or with progressive forms of infection.<sup>9–14</sup>

Studies in mice have alternatively suggested that the immune response leading to corneal scarring appears to be associated with CD4+ T cells alone,<sup>15–17</sup> CD8+ T cells alone,<sup>17</sup> or both together.<sup>18–20</sup> This discrepancy may be related to the use of different mouse or virus strains or the methods used to measure protection.

Most studies directed at examining the role of CD4+ or CD8+ T cells in corneal scarring have used mouse strains that are highly susceptible to HSV-1 infection and HSV-1 induced corneal scarring (that is, BALB/c, C3H, A/J, SCID, nude).<sup>6,20–27</sup> In these susceptible strains of mice, only 0–30% of the mice typically survive lethal ocular HSV-1 challenge.<sup>5,27–29</sup> Death usually occurs between days 7–10 after challenge. This greatly complicates the study of corneal scarring, which is usually measured after day 21. T cell depletion can further reduce survival rates, making studies of corneal scarring in these mice even more difficult.<sup>26–28</sup>

To help alleviate these difficulties, C57BL/6J mice were used in the current studies. These mice are highly resistant to lethal HSV-1 infection and develop little or no HSV-1 induced corneal scarring.<sup>30,31</sup> In addition, these studies were done using CD4+ KO mice, CD8+ KO mice, and in mice depleted for CD4+ or CD8+ T cells by antibody treatment. The rationale was to avoid any bias that might exist in any single model. In contrast with the results obtained in highly susceptible mice, in which lack of CD4+ T cells (or CD8+ T cells) appears to decrease corneal scarring, the results presented here for these highly resistant mice suggest that the lack of either CD4+ T cells or CD8+ T cells resulted in increased corneal scarring.

### Materials and methods

#### VIRUS AND CELLS

Plaque purified HSV-1 strain McKrae was grown in rabbit skin (RS) cell monolayers in minimal essential media (MEM) containing 5% fetal calf serum as described.<sup>5</sup>

#### MICE

Inbred C57BL/6J mice and homozygous C57BL/6J-CD4<sup>-/-</sup> and C57BL/6J-CD8<sup>-/-</sup> knockout mice (5–8 weeks old) were used in the study (The Jackson Laboratory). Animals were handled in accordance with the ARVO

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statement for the use of animals in ophthalmic and vision research.

#### OCULAR CHALLENGE

Mice were challenged ocularly with HSV-1 strain McKrae using  $2 \times 10^6$  pfu (plaque forming units) of virus in 10  $\mu$ l of tissue culture media without corneal scarification as previously described.<sup>5</sup>

#### VIRAL CLEARANCE FROM THE EYE

Eyes were swabbed with a Dacron swab (Spectrum, Dallas, TX, USA), the swab was placed in tissue culture media, and the presence of infectious virus was assayed in RS cells as we previously described.<sup>27</sup>

#### CORNEAL SCARRING

Severity of corneal scarring in surviving mice was scored in a masked fashion by examination with slit lamp biomicroscope using 1% fluorescein and a 0 to 4 scale (0 = no disease, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% corneal staining or involvement).

#### DEPLETION OF CD4+ OR CD8+ T CELLS

Each mouse received an intraperitoneal injection of 100  $\mu$ g of purified GK1.5 (anti-L3T4 (CD4+)) or 2.43 (anti-Lyt-2 (CD8+)) monoclonal antibody (National Cell Culture Center, Minneapolis, MN, USA) in 100  $\mu$ l of phosphate buffered saline (PBS), 96 and 24 hours before ocular challenge. The injections were repeated 24 and 96 hours after ocular challenge. Efficiency of CD4+ and CD8+ T cell depletion was monitored by FACS analysis 24 hours after the second depletion and before ocular challenge.

#### FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS

Single cell suspensions of spleen cells from individual mice were prepared as described previously.<sup>32</sup> Staining of suspensions was done by incubating cells with monoclonal antibodies (FITC conjugated anti-L3T4 and PE conjugated anti-Lyt-2; Pharmingen, San Diego, CA, USA) as described by the manufacturer. Double colour flow cytometric analyses of total spleen cells were done using a FACScan (Beckton Dickinson, PA, USA). Controls included irrelevant antibody, no first antibody, or no second antibody. The percentage CD4+ and CD8+ T cells was calculated by forward scatter/side scatter gating of lymphocyte preparations. The results are presented as the percentage of CD4+ or CD8+ T cells compared with the number of CD4+ or CD8+ T cells in normal C57BL/6J mice.

#### STATISTICAL ANALYSIS

Survival and corneal scarring were analysed by Fisher's exact test and the Student's *t* test using InStat (GraphPad, San Diego, CA, USA). Results were considered statistically significant when the *p* value was  $< 0.05$ .

## Results

### SURVIVAL FOLLOWING HSV-1 CHALLENGE OF CD8+ DEFICIENT MICE

To determine if the absence of CD8+ T cells in C57BL/6J mice that are normally refractory to HSV-1 challenge would alter survival, mice were challenged ocularly with HSV-1 strain McKrae as described in Materials and methods. As expected, all C57BL/6J parental control mice survived ocular challenge (Fig 1A). Survival in the CD8<sup>-/-</sup> and CD8+ depleted mice appeared slightly reduced (80% survival) (Fig 1A). These differences were not statistically significant compared with the parental C57BL/6J mice ( $p > 0.05$ , Fisher's exact test).

### PROTECTION AGAINST CORNEAL SCARRING IN CD8+ DEFICIENT MICE

Corneal scarring was measured in the above surviving mice 28 days after ocular challenge. Both groups of CD8+ deficient mice developed corneal scarring (Fig 2A). There were no significant differences between the CD8<sup>-/-</sup> and the CD8+ depleted mice ( $p > 0.05$ , Student's *t* test). In contrast, compared with the C57BL/6J mice, both groups of mice with reduced CD8+ T cells had significantly more corneal scarring ( $p < 0.01$ , Student's *t* test). Thus, the absence of CD8+ T cells increased HSV-1 induced corneal scarring in C57BL/6J mice.

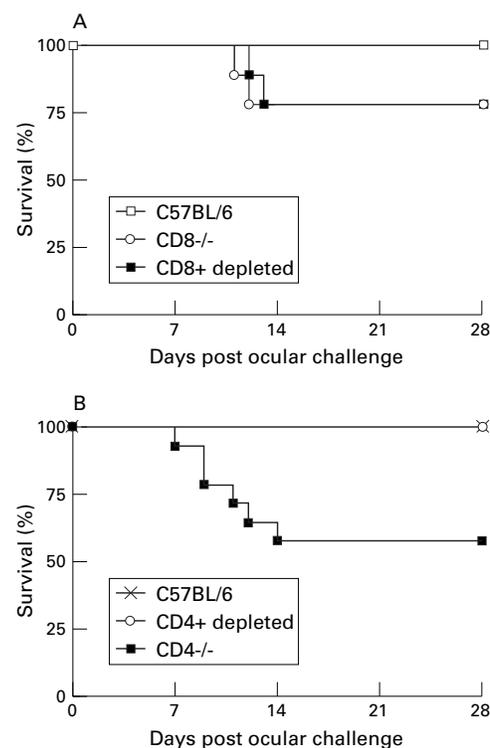
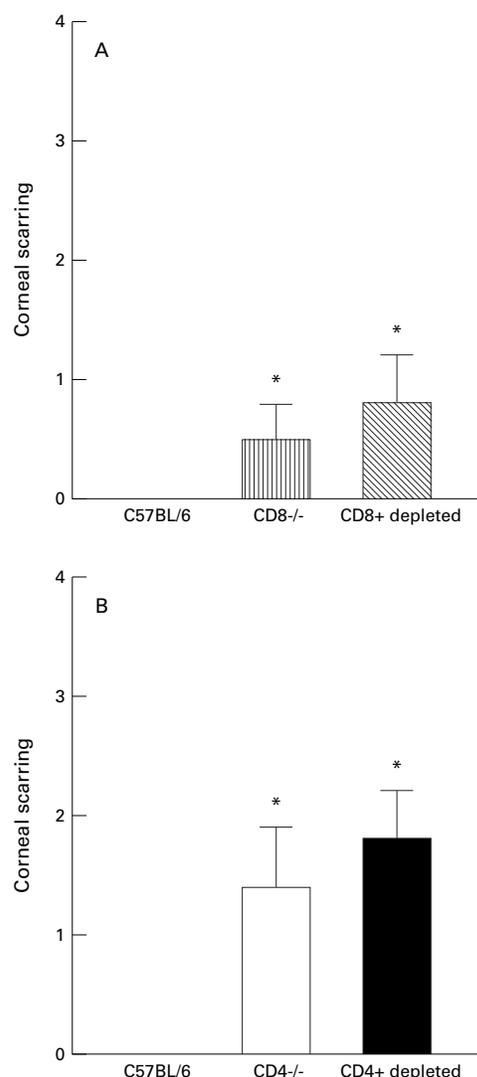


Figure 1 Survival of T cell deficient mice. Mice were inoculated ocularly with  $2 \times 10^6$  pfu of McKrae as described in Materials and method. Survival was measured 28 days after ocular challenge. (A) Survival of CD8<sup>-/-</sup> and CD8+ depleted mice compared with C57BL/6J control mice after ocular infection with HSV-1. CD8<sup>-/-</sup> mice ( $n=10$ ), CD8+ depleted ( $n=9$ ), and C57BL/6J ( $n=9$ ). (B) Survival of CD4<sup>-/-</sup> and CD4+ depleted mice compared with C57BL/6J control mice after ocular infection with HSV-1. CD4<sup>-/-</sup> mice ( $n=14$ ), CD4+ depleted ( $n=9$ ), and C57BL/6J ( $n=10$ ).



**Figure 2** Corneal scarring in T cell deficient mice. Corneal scarring was measured 28 days after ocular challenge as described in Materials and method in the mice shown in Figure 1 that survived ocular challenge. (A) Corneal scarring in CD8<sup>+</sup> deficient mice; (B) corneal scarring in CD4<sup>+</sup> deficient mice. For each bar, the corneal scarring (y axis) represents the average from 16 eyes (CD8<sup>-/-</sup> mice), 14 eyes (CD8<sup>+</sup> depleted mice), 20 eyes (C57BL/6 mice), 16 eyes (CD4<sup>-/-</sup> mice), 18 eyes (CD4<sup>+</sup> depleted mice), and 20 eyes (C57BL/6 mice) (SEM). \*Similar to each other and significantly different from other groups ( $p < 0.001$ , Student's *t* test).

**Table 1** HSV-1 in tears following ocular challenge\*†

| Days post challenge | CD8 <sup>+</sup> deficient |          | CD4 <sup>+</sup> deficient |          | C57BL/6J |
|---------------------|----------------------------|----------|----------------------------|----------|----------|
|                     | CD8 <sup>-/-</sup>         | Depleted | CD4 <sup>-/-</sup>         | Depleted |          |
| Day 1               | 100%                       | 100%     | 100%                       | 100%     | 100%     |
| Day 2               | 100%                       | 100%     | 100%                       | 100%     | 100%     |
| Day 3               | 100%                       | 100%     | 100%                       | 100%     | 100%     |
| Day 4               | 100%                       | 100%     | 100%                       | 100%     | 100%     |
| Day 5               | 100%                       | 100%     | 100%                       | 100%     | 100%     |
| Day 6               | 60%                        | 50%      | 80%                        | 80%      | 60%      |
| Day 7               | 50%                        | 50%      | 80%                        | 80%      | 50%      |
| Day 8               | 0%                         | 0%       | 60%                        | 50%      | 0%       |
| Day 9               | 0%                         | 0%       | 20%                        | 30%      | 0%       |
| Day 10              | 0%                         | 0%       | 10%                        | 20%      | 0%       |
| Day 11              | 0%                         | 0%       | 0%                         | 0%       | 0%       |

\*Mice were challenged ocularly with  $2 \times 10^6$  pfu/eye of HSV-1 McKrae as described in Materials and methods. Tears were collected from five mice (10 eyes) on the days indicated using a Dacron swab and plated on RS indicator cells to determine the presence of infectious virus. †Percentage eyes with detectable virus.

#### SURVIVAL FOLLOWING HSV-1 CHALLENGE OF CD4<sup>+</sup> DEFICIENT MICE

To determine if the absence of CD4<sup>+</sup> T cells in C57BL/6J mice would alter survival, mice were challenged ocularly with HSV-1 strain McKrae as above. As expected, all 10 of the parental C57BL/6J mice survived (Fig 1B). All CD4<sup>+</sup> depleted mice survived ocular HSV-1 challenge. In contrast, only 57% (eight out of 14) of the CD4<sup>-/-</sup> mice survived ocular challenge (Fig 1B). This was significantly different from the C57BL/6J mice ( $p = 0.006$ , Fisher's exact test). Thus, survival in the CD4<sup>-/-</sup> mice was reduced, while survival in the CD4<sup>+</sup> depleted mice was not.

#### PROTECTION AGAINST CORNEAL SCARRING IN CD4<sup>+</sup> DEFICIENT MICE

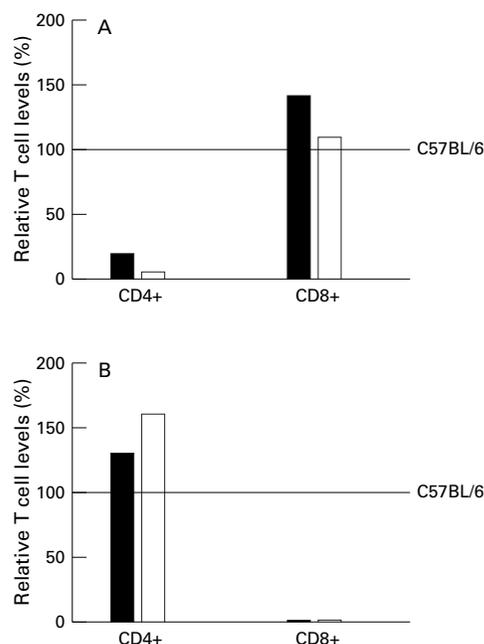
Corneal scarring in the surviving mice with reduced CD4<sup>+</sup> T cells was determined as above. In contrast with the parental C57BL/6J mice, both the CD4<sup>-/-</sup> mice and the CD4<sup>+</sup> T cell depleted mice developed significantly more corneal scarring (Fig 2B,  $p < 0.01$ , Student's *t* test). The amount of corneal scarring in the CD4<sup>-/-</sup> and the CD4<sup>+</sup> T cell depleted mice was similar ( $p > 0.05$ ).

#### CLEARANCE OF VIRUS FROM EYES

To determine if the absence of CD4<sup>+</sup> or CD8<sup>+</sup> T cell subtypes affected the amount of time it took for infectious virus to be cleared from HSV-1 challenged eyes, CD4<sup>+</sup> deficient, CD8<sup>+</sup> deficient, and parental control mice were ocularly challenged as described in Materials and methods. Tear films were collected from 10 eyes/group on days 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 after challenge and cultured for the presence of infectious virus (Table 1). In the parental C57BL/6J wild type mice, virus was completely cleared by day 8. The results for the CD8<sup>+</sup> deficient mice were similar, indicating that viral clearance was not impeded by the absence of a strong CD8<sup>+</sup> T cell mediated immune response. In contrast, viral clearance appeared impaired in the CD4<sup>+</sup> deficient mice. Virus was partially cleared by day 8 and completely cleared by day 11. Thus, the decreased CD4<sup>+</sup> T cell response in CD4<sup>+</sup> deficient mice appeared to decrease the ability of these mice to clear HSV-1 from the eye.

#### T CELLS IN THE SPLEENS OF MICE WITH REDUCED CD4<sup>+</sup> T CELLS

Because of the differences in survival seen above between the two groups of mice with reduced levels of CD4<sup>+</sup> T cells, it was important to determine the levels of T cells in these mice. The amount of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mouse spleens were compared using two colour flow cytometry as described in Materials and methods (Fig 3A and B). The results are presented as the percentage of CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells compared with the number of CD4<sup>+</sup> T cells or CD8<sup>+</sup> T cells in normal C57BL/6J mice. CD4<sup>-/-</sup> mice retained approximately 21% of the normal level of CD4<sup>+</sup> T cells in their spleens (Fig 3A, left side, solid bar). This unexpectedly high level of CD4<sup>+</sup> T cells is consistent with previ-



**Figure 3** Cytofluorimetric analysis of spleen cells from T cell deficient mice. (A) Splens from two  $CD4^{-/-}$  mice (solid bars) or two C57BL/6 mice depleted for  $CD4^{+}$  T cells with antibody (open bars) were harvested. Single cell suspensions of splenocytes were prepared, reacted with mAbs to  $CD4^{+}$  T cells (L3T4 mAb) and  $CD8^{+}$  T cells (Lyt-2 mAb) and FACS analysis was performed as described in Materials and methods. The level of  $CD4^{+}CD8^{-}$  T cells ( $CD4^{+}$ ) and the level of  $CD4^{-}CD8^{+}$  T cells ( $CD8^{+}$ ) are shown relative to the level of these cells in untreated control C57BL/6 mice (horizontal line at 100%). Each bar represents the mean from two to four FACS analyses. (B) Spleen cells from two  $CD8^{-/-}$  mice (solid bars) or two C57BL/6 mice depleted for  $CD8^{+}$  T cells with antibody (open bars) were prepared and FACS analysis performed as in (A).

ous reports showing that  $CD4^{+}$  T cells can be detected in these  $CD4^{-/-}$  mice by both FACS and functional assays.<sup>33–36</sup> Depletion of  $CD4^{+}$  T cells with GK1.5 monoclonal antibody resulted in a residual of only 7% of the normal  $CD4^{+}$  T cell level (Fig 3A, left side, open bar). Thus, the  $CD4^{-/-}$  mice appeared to retain more  $CD4^{+}$  T cells than did the antibody depleted C57BL/6J mice. The number of  $CD8^{+}$  T cells in both groups with reduced  $CD4^{+}$  T cell numbers appeared slightly higher than in control C57BL/6 mice (Fig 3A, right side).

#### T CELLS IN THE SPLEENS OF MICE WITH REDUCED $CD8^{+}$ T CELLS

As expected, both  $CD8^{-/-}$  mice and  $CD8^{+}$  T cell depleted mice retained almost none of their  $CD8^{+}$  T cell populations (1%) (Fig 3B, right side). Interestingly, both groups of mice with reduced  $CD8^{+}$  T cells had increased levels of  $CD4^{+}$  T cells in their spleens (Fig 3B, left side, solid and open bars) compared with control C57BL/6 mice (horizontal line). These results suggest that both types of  $CD8^{+}$  T cell deficient mice had similar  $CD8^{+}$  and  $CD4^{+}$  T cell profiles.

#### Discussion

The contribution of  $CD4^{+}$  and  $CD8^{+}$  T cells in protection against HSV-1 induced corneal scarring in naive mice was investigated using

knockout mice and mice depleted for either  $CD4^{+}$  T cells or  $CD8^{+}$  T cells using monoclonal antibodies. The two strains of KO mice used here each differ from their parental C57BL/6J mouse strain by the absence of a single gene.<sup>33–37</sup> Therefore, these mice were expected to form a useful tool to look at the comparative involvement of  $CD4^{+}$  T cell and  $CD8^{+}$  T cell immune responses in the development of HSV-1 induced corneal scarring.

Both groups of mice with reduced  $CD8^{+}$  T cells were more susceptible to ocular HSV-1 challenge as measured by survival. This suggests that in C57BL/6J mice,  $CD8^{+}$  T cell responses play a part in protecting against primary HSV-1 ocular challenge. In contrast, only  $CD4^{-/-}$  mice appeared to be more susceptible to ocular HSV-1 challenge as measured by survival.

Mice with reduced  $CD8^{+}$  T cells or reduced  $CD4^{+}$  T cells developed significantly more corneal scarring than the C57BL/6J mice. This suggests that in naive C57BL/6 mice  $CD8^{+}$  T cells and  $CD4^{+}$  T cells are both normally involved in protection against HSV-1 induced corneal scarring. The amount of corneal scarring observed in  $CD4^{+}$  T cell deficient mice was twofold higher than the level of corneal scarring in  $CD8^{+}$  T cell deficient mice. The higher corneal scarring detected in  $CD4^{+}$  deficient mice may be due to the 4 day longer duration of virus in these mice compared with  $CD8^{+}$  deficient mice.

Previous studies directed at the role of  $CD4^{+}$  and  $CD8^{+}$  T cells in HSV-1 induced eye disease and corneal scarring were done with mouse strains that are susceptible to HSV-1 induced corneal scarring. In those studies, the consensus is that reduced levels of  $CD4^{+}$  T cells result in reduced corneal scarring.<sup>1–15, 38</sup> In contrast, in this report, the role of  $CD4^{+}$  and  $CD8^{+}$  T cells in HSV-1 induced eye disease was examined in a mouse strain that is highly resistant to HSV-1 eye disease and corneal scarring.<sup>30</sup> In this system, reduced levels of either  $CD4^{+}$  or  $CD8^{+}$  T cells appeared to increase, rather than decrease, corneal scarring. The reason for the apparent discrepancy between these models is probably due to the fact that in the resistant C57BL/6J mice used here, viral replication in the eye is dramatically less than in susceptible mice such as BALB/c.<sup>30</sup> Thus, in the C57BL/6J mice, the more protective immune response to HSV-1 probably overwhelms any deleterious immune response. Altering the protective immune response, such as by reducing either  $CD4^{+}$  or  $CD8^{+}$  T cell levels, may then allow deleterious immune responses to produce corneal scarring. This suggests that both  $CD4^{+}$  and  $CD8^{+}$  T cell responses are involved in the high resistance of C57BL/6J mice to ocular HSV-1 challenge. This in turn would suggest that even in susceptible mice, such as BALB/c,  $CD4^{+}$  T cells might be both protective and deleterious for ocular HSV-1 infection. This has important implications for HSV-1 vaccine development, suggesting that highly efficacious vaccines will

have to induce a fine balance between protective and harmful CD4+ T cell responses.

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