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IL-10 and Natural Regulatory T Cells: Two Independent Anti-Inflammatory Mechanisms in Herpes Simplex Virus-Induced Ocular Immunopathology

Pranita P. Sarangi,* Sharvan Sehrawat,* Susmit Suvas,† and Barry T. Rouse2*

Two prominent anti-inflammatory mechanisms involved in controlling HSV-1-induced corneal immunopathology (stromal keratitis or SK) are the production of the cytokine IL-10 and the activity of natural regulatory T cells (nTregs). It is not known whether, under in vivo conditions, IL-10 and nTregs influence the corneal pathology independently or in concert. In the current study using wild-type and IL-10−/− animals, we have assessed the activity of nTregs in the absence of IL-10 both under in vitro and in vivo conditions. The IL-10−/− animals depleted of nTregs before ocular infection showed more severe SK lesions as compared with the undepleted IL-10−/− animals. In addition, nTregs purified from naive WT and IL-10−/− animals were equally able to suppress the proliferation and the cytokine production from anti-CD3-stimulated CD4+CD25− T cells in vitro. Furthermore, intracellular cytokine staining results indicated that nonregulatory cells expressing B220 and CD25 markers were the major IL-10-producing cell types in the lymphoid tissues of HSV-infected mice. In contrast, in the infected corneas, cells with the CD11b+Gr1− phenotype along with a minor population of Foxp3−CD4+ and a few F4/80+ cells produced IL-10. Our current investigations indicate that at least two independent anti-inflammatory mechanisms are involved in limiting the corneal lesions in SK, both of which may need to be modulated to control SK therapeutically. The Journal of Immunology, 2008, 180: 6297–6306.

Ocular infection with HSV results in a chronic immunoinflammatory reaction in the cornea that can result in blindness. Studies in animal models of stromal keratitis (SK)1 have revealed that the infection, possibly in part because of its TLR ligand activity, sets off a range of critical cellular and molecular events (1); the actual SK lesions, however, appear to be orchestrated mainly by CD4+ T cells that are primarily type 1 cytokine producers (2, 3). SK lesions may resolve spontaneously and, when this occurs in the mouse model, it may be associated with up-regulation of IL-10 production (4). Additionally, the severity of SK may also be affected by the activity of Foxp3+ regulatory T cells (Tregs) (5). Accordingly, the depletion of such cells may result in more severe lesions (5), and recently we have shown that the adoptive transfer of in vitro generated Foxp3+ Tregs may modulate the severity of SK lesions (6).

One potential mechanism by which the Tregs carry out their regulatory effects is by the production of inhibitory cytokines such as IL-10 and TGF-β (7). In fact some types of Tregs may be induced by IL-10 and mediate regulation principally by producing IL-10 (8). In some infectious disease models, these inducible Tregs (sometimes referred to as Tr1 cells) are considered to play a pivotal role in viral pathogenesis (9). That IL-10 is critical for the resolution of inflammatory lesions has been shown in many systems including SK (10). Thus, SK lesions may be more severe in IL-10−/− animals (11), but it is unclear whether this reflects the failed function of some type of Treg or is the consequence of the failure of IL-10 production by some innate inflammatory cell type. Thus, macrophages and some dendritic cell subtypes as well as highly activated CD4+ T cells may be major sources of IL-10 production (12, 13), and certain pathogens may encode ligands that can stimulate IL-10 production from some cells (14, 15).

In the present study, we have compared the pathogenesis of SK in wild-type (WT) and IL-10−/− mice in an attempt to further define whether Treg-mediated control of the inflammatory reaction is dependent on or independent of IL-10 production. Our results show that compared with WT controls, increased SK lesions occur in the absence of IL-10 as well as when natural Tregs (nTregs) are deleted. Interestingly, depletion of nTregs even in IL-10−/− animals resulted in even more enhanced SK lesion severity compared with IL-10−/− mice, indicating that the effect of Tregs mainly occurred independently of IL-10 production. In support of this finding, Tregs purified from IL-10−/− and WT mice appeared equally active at suppression and could inhibit the cytokine production from TCR-stimulated CD4+CD25− T cells isolated from naive and infected WT animals. Furthermore, in the infected corneas nonregulatory cells with the CD11b+Gr1− phenotype along with a minor population of Foxp3−CD4+ and a few F4/80+ cells, produced IL-10. Our results show that at least two independent anti-inflammatory mechanisms, namely IL-10 and nTregs, are involved in controlling corneal immunopathology in SK. We discuss the implication of our studies for future novel therapies for SK.

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2 This work was supported by National Institutes of Health Grants EY05093 and AI1063365.
3 Abbreviations used in this paper: SK, stromal keratitis; DLN, draining lymph node; p.i., postinfection; nTreg, natural regulatory T cell; TG, trigeminal ganglion; Treg, regulatory T cell; WT, wild type.
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Materials and Methods

Animals

IL-10-/- breeders were obtained from The Jackson Laboratory and were maintained in the animal facility of University of Tennessee (Knoxville, TN). C57BL/6 mice were obtained from Harlan Sprague Dawley. Foxp3-GFP mice (16) were a gift from Dr. M. Oukka (Harvard Medical School, Boston, MA). To prevent bacterial superinfection, all mice received prophylactic treatment with sulfamethoxazole/trimethoprim (Biocraft) at the rate of 5 ml per 200 ml of drinking water after virus infection. All experimental procedures followed the guidelines of the Association for Research in Vision and Ophthalmology (Rockville, MD) resolution on the use of animals in research. The animal facilities of the University of Tennessee are fully accredited by the American Association of Laboratory Animals in research. The animal facilities of the University of Tennessee (Knoxville, TN). C57BL/6 mice were obtained from Harlan Sprague Dawley. Foxp3-GFP mice (16) were a gift from Dr. M. Oukka (Harvard Medical School, Boston, MA). To prevent bacterial superinfection, all mice received prophylactic treatment with sulfamethoxazole/trimethoprim (Biocraft) at the rate of 5 ml per 200 ml of drinking water after virus infection. All experimental procedures followed the guidelines of the Association for Research in Vision and Ophthalmology (Rockville, MD) resolution on the use of animals in research. The animal facilities of the University of Tennessee are fully accredited by the American Association of Laboratory Animals in research. The animal facilities of the University of Tennessee (Knoxville, TN). C57BL/6 mice were obtained from Harlan Sprague Dawley. Foxp3-GFP mice (16) were a gift from Dr. M. Oukka (Harvard Medical School, Boston, MA). To prevent bacterial superinfection, all mice received prophylactic treatment with sulfamethoxazole/trimethoprim (Biocraft) at the rate of 5 ml per 200 ml of drinking water after virus infection. All experimental procedures followed the guidelines of the Association for Research in Vision and Ophthalmology (Rockville, MD) resolution on the use of animals in research. The animal facilities of the University of Tennessee are fully accredited by the American Association of Laboratory Animals in research.

**Clinical observation**

The eyes were examined on different days postinfection (p.i.) for the development of clinical lesions by slit lamp biomicroscopy (Kawa), and the clinical severity of keratitis lesions and the development of neovascularization of individually scored mice were recorded as described previously (18). In brief, the scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing stromal keratitis. Similarly, the angiogenic scoring system relied on quantifying the degree of neovessel formation based on three primary parameters: 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; and 3) 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 summed to derive the neovessel index (range, 0–16) for each eye at a given time point.

Histopathology

For histopathologic analysis, eyes from different groups of mice were excised at the indicated time point p.i. and snap frozen in OCT compound (Miles). Six-micrometer-thick sections were cut, air dried in a desiccation box, and stained with H&E (Richard Allen Scientific).
Flow cytometry

For flow cytometry measurement of the infiltrating cells, four corneas per point per group were collected at the indicated time points by dissecting the corneal buttons above the limbus by a scalpel. Similarly, two trigeminal ganglia (TGs) were collected per time point. Corneas or TGs were digested in Liberase (Roche Diagnostics) for 45 min at 37°C. Cervical draining lymph nodes (DLNs) and spleens were also collected from individual animals. Single cell suspension was prepared as described elsewhere (19). The Fc receptors were blocked with unconjugated anti-CD16/32 (BD Pharmingen) for 30 min. Samples were incubated with FITC-labeled anti-Gr-1, anti-CD11b, anti-CD11c, anti-NK1.1, anti-B220, anti-CD19, anti-CD25 (clone 7D4), anti-CD8 (BD Pharmingen), and anti-F4/80.

FIGURE 2. Increased inflammation in the corneas of mice deficient in IL-10 and nTregs. A, WT, Treg-depleted WT (DEP-WT), and IL-10−/− mice were infected with 5 × 10^3 PFU of HSV-1 RE. Mice were terminated at day 17 p.i. and eyes were processed for cryosection. H&E staining was conducted on 6-μm sections. The figure shows the pictures of the section taken at ×100 original magnification. B, Single-cell suspensions of the infected corneas were prepared from six corneas at day 17 p.i. for each group of mice. The cells were labeled for Gr1+CD11b+ (polymorphonuclear) cells. The dot plots represent the Gr1+CD11b+ cells in the WT, DEP-WT and IL-10−/− corneas. The number on the dot plots denotes the percentage of inflammatory cells expressing both Gr1+ and CD11b+ markers. C, The bars represent the total number of viable cells present per cornea of different groups of mice. D, The cells isolated from infected corneas at day 17 p.i. were stained for CD4 marker and the bars represent the total number of CD4+ T cells present per cornea from different groups of mice. E, Intranuclear staining for CD4+Foxp3+ nTregs was performed with the cells isolated from corneas. The bars represent the percentage of CD4+Foxp3+ cells present in the total CD4+ T cells per cornea of different groups of mice. F, Single-cell suspensions were prepared from four TGs at day 17 p.i. for each group of mice. The cells were labeled for Gr1+CD11b+ cells. The dot plots represent the Gr1+CD11b+ cells in the WT, DEP-WT, and IL-10−/− corneas. The number on the dot plots denotes the percentage of inflammatory cells expressing both Gr1+ and CD11b+ markers in TG. G, The cells from TGs were labeled for CD4+ and CD8+ cells. The dot plots represent the CD4+ and CD8+ cells in the WT, DEP-WT, and IL-10−/− corneas. The number on the dot plots denotes the percentage of inflammatory cells expressing CD4+ and CD8+ markers.

FIGURE 3. Increased number of CD4+IFN-γ+ T cells in the corneas of mice deficient in IL-10 and nTregs. Single-cell suspensions of corneal cells were prepared from six corneas at day 17 p.i. Cells were stimulated with either anti-CD3 and anti-CD28 Ab or UV-HSV. Intracellular IFN-γ staining along with CD4 surface staining was conducted as described in Materials and Methods. The results represent the data from one of two similar experiments. A, Dot plots represent the frequencies of CD4+IFN-γ+ T cells from WT, Treg-depleted WT, and IL-10−/− mice when gated on the lymphocytes. B, The bar diagram demonstrates the total number of CD4+IFN-γ+ T cells in the cornea in different groups. C, The bar diagram demonstrates the total number of CD4+IL-17+ T cells in the cornea in different groups.
(eBioscience) and PerCp-Cy5.5-labeled anti-CD4 (BD Pharmingen) Abs for 30 min. For intracellular cytokine staining, PE-labeled anti-IL-10, anti-IL-17, and anti-IFN-γ and FITC-labeled anti-IFN-γ and anti-TNF-α, Abs were used (BD Pharmingen). For Foxp3 IL-10−/− cell staining, mice expressing Foxp3-GFP were used and GFP−IL-10−/− cells were measured in such mice by using intracellular IL-10 staining. Intracellular staining for IL-10, TNF-α, and IFN-γ was performed using a BD Cytofix/CytoPerm fixation/permeabilization solution kit with BD GolgiPlug (BD Bioscience). For intracellular cytokine staining, cells were stimulated with either anti-CD3 and anti-CD28 Abs or UV-HSV as described in Materials and Methods. Intracellular cytokine staining along with CD4 surface staining was conducted as described in Materials and Methods. Dot plots represents the frequencies of CD4+ IFN-γ−/− (A) and CD4+ TNF-α−/− (B) T cells, respectively, in the lymphoid tissues of different groups of mice when gated on the lymphocytes. Bar diagrams demonstrate the total number of CD4+ IFN-γ−/− (A) and CD4+ TNF-α−/− (B) T cells per DLNs or spleens in different groups (n = 4 per group). *, p ≤ 0.05; statistically significant differences as compared with WT animals. Note: A similar pattern was also noticed in the number of CD4+ IL-17−/− T cells.

Virus-specific CD8+ IFN-γ+ staining

To determine the number of IFN-γ-producing CD8+ T cells in the infected DLN and spleen, intracellular cytokine staining was performed as previously described (20). Single cell suspension of infected DLN and

**FIGURE 4.** Increased number of IFN-γ−/− and TNF-α−/− CD4 T cells in the secondary lymphoid tissues of IL-10−/− mice as compared with nTreg-depleted WT (DEP-WT) and WT mice. Single cell suspensions of the individual spleen and the cervical DLNs were prepared from four mice of each group at day 17 p.i. Cells were stimulated with either anti-CD3 and anti-CD28 Abs or UV-HSV as described in Materials and Methods. Intracellular cytokine staining along with CD4 surface staining was conducted as described in Materials and Methods. Dot plots represents the frequencies of CD4+ IFN-γ−/− (A) and CD4+ TNF-α−/− (B) T cells, respectively, in the lymphoid tissues of different groups of mice when gated on the lymphocytes. Bar diagrams demonstrate the total number of CD4+ IFN-γ−/− (A) and CD4+ TNF-α−/− (B) T cells per DLNs or spleens in different groups (n = 4 per group). *, p ≤ 0.05; statistically significant differences as compared with WT animals. Note: A similar pattern was also noticed in the number of CD4+ IL-17−/− T cells.

(eBioscience) and PerCp-Cy5.5-labeled anti-CD4 (BD Pharmingen) Abs for 30 min. For intracellular cytokine staining, PE-labeled anti-IL-10, anti-IL-17, and anti-IFN-γ and FITC-labeled anti-IFN-γ and anti-TNF-α, Abs were used (BD Pharmingen). For Foxp3 IL-10−/− cell staining, mice expressing Foxp3-GFP were used and GFP−IL-10−/− cells were measured in such mice by using intracellular IL-10 staining. Intracellular staining for IL-10, TNF-α, and IFN-γ was performed using a BD Cytofix/CytoPerm fixation/permeabilization solution kit with BD GolgiPlug (BD Bioscience). For intracellular cytokine staining, cells were stimulated with either anti-CD3 and anti-CD28 (1 μg/ml) or PMA/ionomycin or UV-inactivated HSV (multiplicity of infection of 3). Foxp3 staining was performed using a mouse regulatory T cell staining kit (eBioscience). All samples were collected on a FACSscan (BD Biosciences) and data were analyzed by using CellQuest 3.1 software (BD Biosciences).
spleen was prepared, and 10^6 cells/well were cultured in 96-well U-bottom plates. Cells were left untreated or stimulated with SSIEFARL peptide (HSVgB(554-560) synthesized at Genemed Synthesis) (1 μg/ml) for 5 h at 37°C in 5% CO2. Brefeldin A (10 μg/ml) was added to the culture for the intracellular cytokine accumulation. Cell surface marker and intracellular cytokine staining for IFN-γ was performed using a Cytofix/Cytoperm kit (BD Pharmingen). All samples were collected with a FACScan and were analyzed with CellQuest 3.1 software.

**Purification of CD4^+ CD25^+ and CD4^-CD25^- T cells**

CD4^+CD25^+ and CD4^-CD25^- Tregs were purified from the lymph nodes of WT and IL-10^-/- mice using a Treg isolation kit (Miltenyi Biotec) according to the suggested protocol. The purity of cells ranged from 89 to 92% (see Fig. 8A). Accessory cells were isolated from the spleens of WT mice by depleting Thy1.2^+ cells using Thy1.2 magnetic beads (Miltenyi Biotec). Thy1.2-depleted cells were irradiated before being added to the cultures. CD4^-CD25^- T cells from Thy1.1.C57BL/6 animals were used for in vitro cultures.

**Cytokine inhibition and in vitro suppression assay**

Purified Tregs (5 × 10^5/well) from IL-10^-/- and WT animals were cultured with CD4^-CD25^- T cells (5 × 10^5/well) isolated from naive and infected WT animals along with irradiated splenocytes (1 × 10^6/well). Anti-CD3 Ab was added to the wells (1 μg/ml). Cells were cultured in a 48-well plate, the culture supernatants were collected after 48 h, and the levels of IFN-γ and IL-2 were measured by sandwich ELISA. For in vitro suppression assays, in one of the experiments CD4^-CD25^- T cells from Thy1.1 mice (1 × 10^5/well) were labeled with CFSE (0.5 μM) and cultured in a 96-well plate with different dilutions of WT or IL-10^-/-/CD4^-CD25^- T cells (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32) in the presence of anti-CD3 Ab (1 μg/ml) and APCs (2 × 10^5/well).

**Cytokine ELISA**

Cervical DLNs from individual mice and six corneas per group were collected at the indicated time points. The DLNs and corneas were sonicated and the levels of IL-6, IL-12, and IL-10 were measured in the supernatants. Similarly, culture supernatants from the in vitro suppression assay were collected and the levels of IL-2 and IFN-γ were determined by a standard sandwich ELISA protocol. Anti IL-6, IL-12, IL-2, IL-10, and IFN-γ capture and detection Abs were purchased from BD Pharmingen and standard recombinant murine IL-6, IL-12, IL-2, IL-10 and IFN-γ were obtained from R&D Systems. Biotinylated detection Abs were purchased from BD Pharmingen. The color reaction was developed using ABTS (Sigma-Aldrich) and measured with an ELISA reader (SpectraMax 340; Molecular Devices) at 405 nm. Quantification was performed with SpectraMax ELISA reader software version 1.2.

**Statistical analysis**

Statistical significance was determined by Student’s t test. *p < 0.05 was regarded as a significant difference between the groups.

**Results**

**The absence of IL-10 and depletion of nTreg results in increased SK severity**

The outcome of HSV infection was followed and compared in three groups of mice that were age and sex matched and ocularly infected with the same dose of HSV-1 RE. The pattern of disease that developed differed significantly in the three groups. Thus, both IL-10^-/-^ and Treg-depleted animals showed heightened susceptibility compared with WT animals (Fig. 1, B and C). Clinical lesions were evident in IL-10^-/-^ animals before those in other groups. At 8 days p.i., the incidence of SK (lesion score, ≥3) was 0 and 30% in WT and IL-10^-/-^ animals, respectively (not shown). The majority of IL-10^-/-^ animals had developed moderately severe lesions (≥3) by 16 days p.i. (90% in the experiment shown in Fig. 1B). In contrast, <40% of WT animals in the same experiment developed ≥3 lesions and almost two-fold more than WT in the nTreg-depleted group (Fig. 1B). The pattern of more severe responses in IL-10^-/-^ and Treg-depleted animals was also evident when the extent of angiogenesis was quantified in the different groups (Fig. 1, E and F). Eyes showing median responses in each group were selected for histological analysis. As is evident in Fig. 2A, inflammatory reactions were more severe than in WT mice in both the IL-10^-/-^ and Treg-depleted animals. In other experiments, corneal samples were collected from eight randomly selected eyes taken from animals at 17 days p.i. to prepare single cell suspensions following collagen digestion for phenotypic analysis by flow cytometry. First, total viable cell numbers recovered from four mice of each group at day 17 p.i. SSIEFARL-specific CD8^-IFN-γ^- staining was conducted as described in Materials and Methods. A. The dot plots represent the frequencies of CD8^-IFN-γ^- cells (CD8^- on the x-axis and IFN-γ^- on the y-axis). The numbers on the upper right quadrants represent the percentage of CD8^-IFN-γ^- T cells in the gated population of lymphocytes in the spleens. The plots are representative of four animals analyzed per each group (n = 4). B, The bar diagram demonstrates the total number of double positive cells per DLN or spleen in different groups. *, p ≤ 0.05; **, p ≤ 0.01; statistically significant differences as compared with WT animals.
of cytokine producing cells were present in the corneas of IL-10−/− mice with Treg-depleted animals showing less and WT mice the least amount (Fig. 3). By intracellular cytokine staining, we also looked for the cytokine producing CD4+ T cells in the cervical lymph node and the spleen of these mice. As shown in Fig. 4, 3- and 8-fold increases in the numbers of CD4+ T cells producing IFN-γ and TNF-α were found in the DLN and spleens in the absence of nTregs and IL-10, respectively. This increase in proinflammatory mediators in the cornea and secondary lymphoid tissues along with higher Treg numbers in IL-10−/− animals may mean an inability of nTregs to suppress the disease pathology in the proinflammatory milieu (21, 22), or that the pathogenic Th1-type CD4+ T cells present at the site are resistant to nTreg-mediated suppression (23, 24).

We also determined the magnitude of SSIEFARL (immunodominant epitope in C57BL/6 mice)-specific CD8+ T cell responses and cytokine production by CD8+ T cells in these animals. As shown in the Fig. 5, a higher magnitude of immune responses was noted in the absence of either Tregs (3-fold) or IL-10 (2-fold). Interestingly, in contrast to CD4+ T cells, the increase in total CD8+ IFN-γ+ T cells was greater in the absence of nTregs as compared with IL-10, which could mean that different pathways of immune response generation may be influenced by IL-10 and nTregs.

**Treg effects are still evident in IL-10−/− mice**

One explanation for the greater susceptibility of IL-10−/− animals could relate to the function of adaptive or nTregs. The former cell type is usually dependent on IL-10 and thus would not be expected to be present in IL-10−/− animals. Studies in some cases have indicated that Foxp3+ natural Tregs may also function at least in part by their production of IL-10 (25). Another explanation could be the dysfunction of nTregs in the presence of a high concentration of proinflammatory mediators such as IL-6 and IL-12 at the site (22, 23). In this regard, higher levels of IL-6 and IL-12 were detected in the corneas of the IL-10−/− mice.
found in the DLN and corneal lysates of IL-10−/− animals as compared with WT mice (Fig. 6).

Experiments were performed in older IL-10−/− male mice, which are more resistant to HSV-induced lesions than the 6- to 8-wk-old female mice used in previous experiments. To evaluate the role of nTregs in controlling SK lesion development, Treg-depleted IL-10−/− mice were compared with nondepleted IL-10−/− animals. As shown in Fig. 7, A–D, depletion of CD25+ cells from IL-10−/− animals resulted in increased SK lesions (Fig. 7, A and D) as well as angiogenic responses (Fig. 7, B and D). At day 20 p.i., 55% of the eyes in Treg-depleted IL-10−/− animals had SK scores of ≥3 compared with 25% in undepleted IL-10−/− mice (Fig. 7C). At this time point, the SK scores were 2.3 ± 0.3 (p = 0.05) in Treg-depleted IL-10−/− mice as compared with 1 ± 0.2 in normal IL-10−/− animals. A similar pattern was also evident in the development of neovascularization in the Treg-depleted IL-10−/− animals. At day 20 p.i., Treg-depleted IL-10−/− animals had neovascularization scores of 10.8 ± 1.1 as compared with 7.4 ± 1 in control IL-10−/− animals. Additionally, there was an earlier onset of SK lesions in the Treg-depleted IL-10−/− mice as compared with normal IL-10−/− animals. The incidence of SK at day 12 p.i. was 30 and 58% in the IL-10−/− and Treg-depleted IL-10−/− groups, respectively (Fig. 7C). Increased numbers of IFN-γ+CD4+ T cells (Fig. 7E) and other inflammatory cell infiltrates were also noted in the corneas of such animals as compared with the controls (not shown). Similarly, as shown in Fig. 7F, the virus-specific CTL response, as assessed by the frequency and the absolute number of virus-specific CD8+ IFN-γ+ T cells in the secondary lymphoid tissues, was also significantly increased in the absence of nTregs in the IL-10−/− animals. These results indicate that even in IL-10−/− animals, nTregs can exert their suppressive activity and that their function could be independent of IL-10 in HSV-induced corneal immunopathology.

To support the apparent IL-10-independent in vivo suppressive activity of nTregs, we isolated CD4+CD25+ nTregs from uninfected WT and IL-10−/− animals and those were cultured with CFSE-labeled CD4+CD25+ cells isolated from naive and 12-day p.i. WT animals. Cells were stimulated with anti-CD3 and intracellular staining for IL-10 was performed as outlined in the Materials and Methods. As shown in the dot plots, no IL-10+ or GFP+ cells were detected in the lymphoid tissues after infection.

### Table I. Phenotype of IL-10 producing cells in various tissues

<table>
<thead>
<tr>
<th>Day (p.i.)</th>
<th>Surface Markers</th>
<th>Percentage of Total IL-10+ Cells</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Cornea</td>
<td>Spleen</td>
</tr>
<tr>
<td>5</td>
<td>CD4+</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td>Foxp3+</td>
<td>UD</td>
</tr>
<tr>
<td></td>
<td>Gr1 CD11b+</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>F4/80+</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>B220+</td>
<td>62 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>CD25+</td>
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</tr>
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<td>CD4+</td>
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<td>F4/80+</td>
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<td></td>
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<td>83 ± 3</td>
</tr>
<tr>
<td></td>
<td>CD25+</td>
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</tr>
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*The number of Foxp3+ IL-10+ cells was determined by using the GFP+IL-10+ cell number. After day 20 p.i. an increase in the number of these double positive cells was noticed in the infected corneas but not in the lymphoid tissues. UD. Undetected.*
IL-10$^{-}$Foxp3$^{+}$CD4$^{+}$ T cells were not evident in either cornea or lymphoid tissues (not shown). These data suggest that nonregulatory cells could be the major cellular source of IL-10 p.i.

To further study the phenotype of the IL-10-producing cells in the infected corneas and secondary lymphoid tissues, intracellular cytokine staining was performed with cells taken from infected WT corneas, spleens, and DLNs at days 5, 11, and 20 p.i. The cells were separated using antibodies against markers such as c-Kit, Gr-1, CD11c, CD11b, B220, CD19, CD25, CD3, NK1.1, and CD4 along with intracellular IL-10. As shown in Table I, the majority of the IL-10-producing cells in the spleens and DLNs also expressed B220, CD25, and F4/80 markers. More than 50% of the cells also expressed c-Kit and Gr1 markers (data not shown). Interestingly, as shown in Table I, in the infected corneas nonregulatory cells with the CD11b$^{+}$Gr1$^{+}$ phenotype along with a minor population of Foxp3$^{+}$CD4$^{+}$ and a few F4/80$^{+}$ cells produced IL-10.

**Influence of nTreg on IL-10 production**

Because our results indicated that Tregs could have IL-10-independent suppression in SK and that these cell types did not constitute a significant part of the IL-10-producing cell types, we determined whether the activity of nTregs could influence the production of IL-10 p.i. In many reports, Tregs were shown to suppress immune responses via IL-10 production (8, 28). Interestingly, as measured by intracellular cytokine staining and cytokine ELISA an increase in the absolute number and frequencies of IL-10-producing cells as well as IL-10 proteins was noted in the lymphoid tissues in the absence of Tregs in the WT animals as compared with undepleted WT animals (Fig. 9, A and B). But the mean fluorescence intensity of IL-10$^{+}$ cells remained comparable among Treg-depleted and undepleted WT animals, and the case of the phenotype of IL-10-producing cells was similar (data not shown). This could be due to the generalized suppressive activity of Tregs on the IL-10-producing cell types such as macrophages or dendritic cells or the activated CD4$^{+}$ T cells. In contrast, as is evident in Fig. 9C, the level of IL-10 in the corneas of Treg-depleted WT mice was lower as compared with that in undepleted WT animals at days 4 and 9 p.i.

**Discussion**

Many chronic immunoinflammatory diseases are difficult to control therapeutically. One such example is SK, which is caused by HSV infection of the eye. It is anticipated that control measures will improve once all of the mechanisms involved in lesion resolution are identified and their respective roles are fully understood. With regard to SK, few studies have been done but reports have demonstrated that up-regulation of certain cytokines such as IL-10 and TGF-β help resolve lesions (29, 30). Recently, observations in a variety of inflammatory diseases indicate that several types of regulatory cells control and resolve inflammatory lesions, and some of these appear to function by producing either or both IL-10 and TGF-β (31). With SK, natural regulatory T cells were shown previously to influence lesions, but the mechanisms by which this is achieved were not identified (5). In some systems nTregs appear to function independently of any cytokine production (32), whereas in others IL-10 production is a necessary event (25, 28).

In this report, we show that both nTreg and IL-10 participate in the control of SK lesion severity, but these two mechanisms proceed largely independently of each other. Our results show that the severity of SK lesions becomes enhanced if animals are unable to produce IL-10 because of gene knockout or have been depleted of nTreg. Furthermore, depletion of nTreg in IL-10$^{-/-}$ mice led to even higher levels of disease severity than in undepleted IL-10$^{-/-}$ mice. In addition, nTreg purified from WT and IL-10$^{-/-}$ animals appeared to be equally suppressive in vitro to the activity of nonregulatory cells. Few if any Foxp3$^{+}$CD4$^{+}$ Tregs in either the ocular lesions or DLNs were IL-10 producers. In fact, the major source of IL-10 appeared to be non-T cells such as granulocytes and mononuclear inflammatory cells. Accordingly, these HSV-induced immunoinflammatory lesions can be modulated independently by both IL-10 and nTreg activity, which could mean that future novel therapies will need to manipulate both mechanisms to achieve maximal effects.

The means by which inflammatory lesions are influenced by regulatory T cells remain poorly understood, especially in responses caused by pathogens (33). In some virus-induced immunoinflammatory lesions, some advocate a major role for adaptive Tregs induced by Ag in the presence of IL-10 (9, 34). These so-called Tr1 cells regulate responses by producing an abundance of IL-10 (9, 34). Their role in SK remains unexplored. In other systems, such as *Leishmania* infections, cells of the nTreg phenotype control lesions, but these appear to act by producing IL-10 (35, 36). The same may also be true in other parasite-mediated lesions as well as in bacteria-induced colitis (36, 37). Our results showing that a significant nTreg effect could be demonstrated in mice genetically unable to produce IL-10 strongly argues against IL-10 being a dominant mechanism by which nTregs act in vivo. Similarly, such cells isolated from IL-10$^{-/-}$ mice acted equally as those from WT animals in vitro, indicating no role for IL-10 to mediate their function as others have argued to be the case using different approaches (38).

It is conceivable that several functional subsets of the Foxp3$^{+}$ regulatory phenotype exist. Those thymus-derived autotigent-specific cells that are primarily depleted by pc61 mAb in naive
mice may function in a different way as those converted in vivo from CD4+ Foxp3+ foreign Ag-specific precursors. This conversion process was shown recently to occur by several groups (39–41) including ourselves (6). We suspect that the nTregs preexisting at the time of infection are signaled in some way by the HSV infection and that these control SK in a manner that does not depend on IL-10 production. In contrast, the Foxp3+ Tregs that act in more chronic infections may largely represent Foxp3+ IFN-γ+ T cells by inhibiting the production of proinflammatory cytokines such as IL-6 and IL-12 in the lymph nodes and ocular tissues during herpetic stromal keratitis. J. Immunol. 149: 3035–3039.


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Disclosures

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References


