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CD4⁺CD25⁺ Regulatory T Cells Control the Severity of Viral Immunoinflammatory Lesions

Susmit Suvas, Ahmet Kursat Azkur, Bum Seok Kim, Uday Kumaraguru, and Barry T. Rouse

CD4⁺CD25⁺ regulatory T cells (T_{reg}) can inhibit a variety of autoimmune and inflammatory diseases, but their involvement in regulating virus-induced immunopathology is not known. We have evaluated the role of T_{reg} in viral immunopathological lesion stromal keratitis. This frequent cause of human blindness results from a T cell-mediated immunoinflammatory response to HSV in the corneal stroma. The results show that lesions were significantly more severe if mice were depleted of T_{reg} before infection. The T_{reg} was also shown to modulate lesion expression induced by adoptive transfer of pathogenic CD4⁺ T cells in infected SCID recipients. The mechanism of T_{reg} control of stromal keratitis involved suppressed antiviral immunity and impaired expression of the molecule required for T cell migration to lesion sites. Interestingly, T_{reg} isolated from ocular lesions in nondepleted mice showed in vitro inhibitory effects involving IL-10, but were not very effective in established lesions. Our results decipher the in vivo role of T_{reg} in a virus-induced immunopathology and imply that manipulation of regulatory cell function represents a useful approach to control viral-induced immunoinflammatory disease.


Ocular infection with HSV can result in an immunoinflammatory lesion in the cornea which represents the most common infectious cause of blindness in the western world (1). Animal model studies have revealed that the blinding inflammatory lesions are orchestrated by CD4⁺ T cells that principally generate type 1 cytokines (2). However, the identity of target Ags which drive the immunopathology remains unclear (3). Lesions in some animals resolve without treatment and in such cases the cytokine IL-10 is up-regulated (4). In addition, expression of IL-10 or TGF-β early in the experimental disease may alleviate lesion severity (5, 6). Whether or not dedicated cell types that produce anti-inflammatory cytokines enter the cornea and regulate responses has not been explored. Thus, conceivably this viral-induced immunopathology, like several autoimmune lesions, might be subject to control by regulatory T cells (T_{reg}) such as the CD4⁺CD25⁺ T_{reg} population described initially by Sakaguchi et al. (7–9). To date, the role of CD4⁺CD25⁺ T_{reg} in the pathogenesis of viral immunopathological disease has not been reported.

Our results demonstrate a major role for CD4⁺CD25⁺ T_{reg} cells in the pathogenesis of murine herpetic stromal keratitis (HSK). We demonstrate that T_{reg} depletion before infection results in lesions of greater severity and the induction of disease with lower infecting doses of virus. However, the role of CD25⁺ T_{reg} in HSK pathogenesis was complex. One effect was to minimize the extent of immunopathogenic CD4⁺ T cell induction, whereas another was to limit the migration of pathogenic T cells to the tissue immunoinflammatory site. The CD25⁺ T_{reg} were present in the ocular immunoinflammatory sites, could be recovered from the site, and were shown to express IL-10-dependent inhibitory effects on activated CD4⁺ T cells in vitro. Our results indicate that CD25⁺ T_{reg} play a beneficial role to minimize viral immunological lesions and imply that procedures that enhance their function may prove therapeutically beneficial.

Materials and Methods

Mice and virus

Female 5–6-wk-old C57BL/6, Thy1.2⁺ BALB/c, and BALB/c SCID (H-2d) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and Thy1.1⁺ BALB/c mice were a kind gift from Dr. D. Woodland (Trudeau Institute, Saranac Lake, NY) and housed in the animal facilities of the University of Tennessee. SCID mice were kept in our special pathogen-free facility where all food, water, bedding, and instruments were autoclaved and all manipulations were done in a laminar flow hood. All experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE was propagated and titrated on Vero cells (ATCC CCL81) using standard protocols. The virus was stored in aliquots at −80°C until use.

Abs and reagents

PC61 (anti-CD25) hybridoma was purchased from American Type Culture Collection (Manassas, VA) and Ab was grown in tissue culture roller bottles. For all injections, an ammonium sulfate cut of PC61 mAb (1.0 mg/mouse) was used. Abs purchased from BD PharMingen (San Diego, CA) were FITC, PE, and biotin anti-CD4, FITC anti-CD25 (7D4), PE-anti-CD25 (PC61), CD62 ligand, CD44, CD69, CD49d, CTLA-4, streptavidin-PerCP-Cy5.5, and ELISA Abs such as anti-IFN-γ, anti-IL-2, anti-IL-10, and their biotinylated counterparts.

In vivo depletion, corneal HSV-1 infections, and clinical observations

BALB/c mice were given 1.0 mg PC61 mAb i.p. 3 days before corneal infection. Initial kinetics of depletion was studied in uninfected mice. Corneal infections of PC61-treated or nontreated BALB/c mice were conducted under deep anesthesia. Mice were sacrified on their corneas with a 27-gauge needle, and a 3-μl drop containing the required viral dose was applied to the eye. The eyes were examined on different days postinfection (p.i.) with a slit-lamp biomicroscope (Kowa, Nogoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The

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3 Abbreviations used in this paper: T_{reg}, T regulatory cell; SK, stromal keratitis; p.i., postinfection; HSK, herpetic stromal keratitis; DLN, draining lymph node; SPC, spot- forming cell; VLA, very late Ag.
scoring system was as follows: +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity, but iris visible; +4, opaque cornea, iris invisible; and +5, necrotizing SK.

**Immunofluorescence and immunohistochemical staining**

Eyes were frozen in OCT compound (Miles, Elkart, IN) at different days p.i. Six-micrometer-thick sections were cut, air dried, and fixed in cold acetone for 5 min. The sections were then blocked with 3% BSA and stained for the presence of CD4+ T cells and neutrophils with their respective mAbs. For immunohistochemistry, the biotinylated primary Abs were used followed by HRP-conjugated streptavidin, (1/1000; Jackson ImmunoResearch Laboratories, West Grove, PA) and 3,3’-diaminobenzidine (Vector Laboratories, Burlingame, CA). In the case of immunofluorescence, the frozen sections were fixed in 4% paraformaldehyde and the primary Ab was labeled with FITC. Finally, sections were washed with PBS and mounted with Vectashield mounting medium with propidium iodide (Vector Laboratories).

**Flow cytometric analysis**

**Cell preparation.** Single-cell suspensions were prepared from the cornea, draining lymph nodes (DLN), and spleen of mice at different time points p.i. Cornes were digested with Liberase (Roche Diagnostics). Briefly, a small incision was made at the junction of the limbus and the corneal cap was carefully removed. The pooled corneas were incubated with 60 U/ml Liberase for 60 min at 37°C in a humidified atmosphere of 5% CO2. After incubation, the corneas were disrupted by grinding with a syringe plunger on a stainless steel mesh and a single-cell suspension was made in complete RPMI 1640 medium. Cells were counted with trypan blue exclusion with high viability.

**Staining for flow cytometry.** The single-cell suspension obtained from DLN, and corneal samples were stained for different cell surface molecules for FACS. Briefly, a total of 1 × 10^6 cells were first blocked with an unconjugated anti-CD32/CD16 mAb for 30 min. In FACS buffer followed by addition of fluorochrome-labeled respective Abs for an additional 45 min. For intracellular staining, surface-labeled cells were permeabilized with permeabilization buffer (BD Biosciences, Mountain View, CA) and fluorochrome-labeled mAb was added. Finally, the cells were washed three times and samples were acquired on a FACScan (BD Biosciences). The data were analyzed using the CellQuest 3.1 software (BD Biosciences).

**Cytokine ELISA**

The culture supernatants from the bulk test cultures without addition of any exogenous cytokines were screened for the presence of IL-10 and IL-2 by addition of alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch) and nitroblue tetrazolium (Sigma-Aldrich) and 5-bromo-4-chloro-3-indolyl-phosphosphate (Sigma-Aldrich) as substrates. The absorbance was measured at 490 nm using a microtiter plate reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA) with the concentration was calculated with an automated ELISA reader (Dynatech). Data are expressed as mean ± SD.

**Quantification of IL-2- and IFN-γ-secreting cells by ELISPOT**

The ELISPOT assay was used for quantification of cytokine-producing cells as reported earlier (11). Briefly, ELISPOT plates (Millipore, Molsheim, France) were coated overnight at 4°C with anti-mouse IL-2 and IFN-γ Ab (1.0 M Na2HPO4, pH 9.0/0.1 M NaH2PO4, pH 6.0) for 1 h at room temperature. After washing, serially diluted samples and standards were added to the plate and incubated at 4°C overnight. The plates were washed following by the addition of cytokine-specific detection Abs for 2 h. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was added at 1/1000 dilutions. The color was developed by adding the substrate (ABTS) solution (Sigma-Aldrich, St. Louis, MO) and the concentration was calculated with an automated ELISA reader (SpectraMax 340; Molecular Devices, Sunnyvale, CA).

**Statistical analysis**

All analysis for statistically significant differences were performed with a Student’s paired r test. Values of p < 0.01 and p < 0.05 were considered to be statistically significant. Results are expressed as mean ± SD.

**Results**

**CD4+CD25+ Treg depletion increases lesion severity**

BALB/c mice were treated with the PC61 mAb using conditions in which >90% of CD4+CD25+ T cells were depleted by day 3 and ~40–50% depleted at 18 days after depletion (data not depicted). Three days posttreatment, mice were infected on the ocular surface with different doses of HSV-1 and the severity of their stromal keratitis (SK) lesions were compared with age-matched control-infected mice treated with isotype control rat Ig. The results of a representative experiment are shown in Fig. 1a. As is evident, CD25-depleted animals developed more severe lesions, an effect most apparent in animals infected with a lower dose (10^4 PFU/eye) of virus. In this instance, 60% of eyes from depleted animals had lesion severity scores of 3.0 or greater on day 15 p.i. compared with none in the nondepleted animals (p < 0.01). A similar pattern in CD4+CD25+ Treg cells were purified as reported by others (36). Briefly, CD4 columns (R&D Systems, Minneapolis, MN) were used for the isolation of CD4+ T cells. CD4+CD25+ Treg were then purified by incubating the enriched CD4+ T cells obtained from the spleen and lymph node with biotin-conjugated anti-CD25 (15 μg/10^6 cells) mAb in PBS-2% FCS for 15 min at 4°C. Positive magnetic separation was then performed with LS columns (Miltenyi Biotec, Auburn, CA) according to the suggested protocol. The purity of cells ranged from 85–95%. The CD4+CD25+ T cells were adoptively transferred into SCID mice either alone or with CD25+ Treg 24 h before virus infection. Immune responses were analyzed 12 days p.i. Primed CD4+CD25+ T cells were also purified by the same procedure 4 wk after HSV (i.p) infection. In some experiments, corneal CD4+CD25+ T cells were purified by FACS sorting. Briefly, corneal samples were collagenase digested and MACS-purified CD4+ T cells were sorted into CD25+ and CD25- fractions. The purity of sorted fractions was >95%.

**Proliferation assay**

The purified CD4+CD25+ Treg or CD4+CD25neg T cells (5 × 10^5/well) were cultured with soluble anti-CD3 mAb (1.0 μg/ml) along with irradiated spot-forming cells (SPC) in the presence or absence of IL-2 (20 U/ml) for 72 h in 96-well round-bottom plates. To assess suppression by CD25+ Treg, a graded number of Treg were added into the anti-CD3 mAb-stimulated culture of CD4+CD25neg T cells (10^5 cells/well) in the presence of irradiated SPC. In one particular experiment, anti-IL-10R mAb (10 μg/ml) was added to a CD25+ Treg (10^5/well) and CD25neg T cell (10^5/well) co-cultured in the presence of anti-CD3 and irradiated SPC. Finally, [3H]thymidine (ICN Biochemicals, Irvine, CA) was added 1 μCi/well for the last 18 h. The plates were harvested and read using an scintillation counter (Trace 95; Inotech, Wohlen, Switzerland). The results were expressed as mean cpm ± SD.

**Real-time quantitative PCR**

The total cellular RNA was isolated from corneas by using a RNeasy protect mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instruction. All samples were treated with RNase-free DNase (Qiagen). The extracted RNA was reverse transcribed using oligo(dt) primers and reverse transcriptase enzyme (Promega, Madison, WI) according to standard protocol (4). The cDNA obtained was used as a template for real-time quantitative PCR and real-time PCR was performed using a QuantiTect SYBR green PCR kit (Qiagen). Initially, mRNA levels were normalized to the GAPDH mRNA level. PCR primer pairs used for GAPDH were 5'-GCC TGC TTC ACC ACC TTC TTG ATG-3' and 5'-CAT CCA CCA ACT GAG A-3' and 5'-GCT GAT CAT GGC TGG GTT GT-3'. Briefly, for real-time PCR, a standard curve was generated using the PCR product of a Foxp3 cloned into a plcX-RL-TOPO vector (Invitrogen, San Diego, CA). After spectrophotometric determination of the plasmid DNA concentration, the copy number was calculated using the following formula: (X g/μl DNA/planmid length in bp × 6600) / 6.022 × 10^23 = Y molecules/μl. Test samples were run in triplicate with a dilution series and the mean value of a particular dilution was used. Data are expressed as copy number per sample.
of results was noted when comparing lesion severity in the HSV-resistant C57BL/6 mouse strain (Fig. 1b). In these experiments, CD25⁺ Treg depletion resulted in approximately a 10-fold greater susceptibility to ocular HSV infection with an infecting dose of virus (10⁵ PFU/eye) producing lesions in depleted, but not in undepleted animals.

Lesions in Treg-depleted mice demonstrate enhanced CD4⁺ T cell infiltration and an altered phenotype

SK is well known to be orchestrated by CD4⁺ T cells (1). As shown in Table I, the percentage of CD4⁺ T cells recovered by collagenase digestion of corneas was greater in Treg⁻ depleted animals at all time points tested after ocular HSV infection. Sample animals evaluated by immunocytochemistry at the time of peak lesions (day 15) are shown in Fig. 2a. Increased T cell infiltration was notably more evident in sections from Treg⁻ depleted animals.

In other experiments, the phenotype of CD4⁺ T cells isolated from the DLN as well as from corneal lesions after collagenase digestion was evaluated on both days 9 and 15 p.i. No significant difference in the average phenotype between Treg⁻ depleted and controls was noted for the following markers: CD62 ligand, CD69, and CD44 (data not depicted). However, a significant increase of DLN CD4⁺ T cells from Treg⁻ depleted mice expressed the integrin CD49d (very late Ag 4 (VLA-4)) at both time points (Fig. 2b). Moreover, on day 15, the ocular CD4⁺ T cell population of depleted animals also expressed an ~2-fold increase in CD49d⁺ CD4⁺ T cells. Additional experiments were conducted to compare the effector functions of infiltrated ocular CD4⁺ T cells in the nondepleted and Treg⁻ depleted mice. On day 15 p.i., cells isolated from collagenase-digested corneal tissue were stimulated with anti-CD3 mAb in the presence of IL-2 for 16 h. As shown in Fig. 2c, the percentage of ocular CD4⁺ T cells express more IFN-γ in the Treg⁻ depleted group compared with the nondepleted group. Taken together, we interpret these observations to mean that after viral infection, Treg may control the expression of molecules such as CD49d that are involved in migration of inflammatory cells to the ocular lesion site (10). Furthermore, once effector cells reach the cornea they function more effectively if not hampered by the presence of Treg⁻.

Treg⁻ depletion results in enhanced immune responsiveness to HSV

Another explanation for heightened lesion severity of Treg⁻ depleted animals could be that these animals developed enhanced HSV-specific immune responses. As demonstrated in Fig. 3, specific responses of both splenic and DLN CD4⁺ T cell were ~2-fold higher in Treg⁻ depleted animals compared with controls as measured by ELISA and ELISPOT analysis for both IL-2 and IFN-γ. Thus, as noted previously with the specific CD8⁺ T cell response to HSV (11), Treg⁻ depletion also results in elevated CD4⁺ T cell immunity to HSV.

Treg⁻ suppress pathology by CD4⁺ CD25⁺⁺⁺ T cells

Mice lacking T cells fail to express SK upon ocular infection with HSV, but readily do so if given adoptive transfers of CD4⁺ T cells.

Table 1. Effects of Treg depletion on the infiltration of CD4⁺ T cell in HSV-infected cornea

<table>
<thead>
<tr>
<th>% Age of CD4⁺ T Cells in Corneas on Different Days p.i.</th>
<th>Day 10</th>
<th>Day 15</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Ig</td>
<td>1.1 ± 0.04</td>
<td>1.8 ± 0.04</td>
<td>0.43 ± 0.02</td>
</tr>
<tr>
<td>Anti-CD25 mAb</td>
<td>1.9⁺ ± 0.2</td>
<td>5.07⁺⁺ ± 0.1</td>
<td>1.25⁺⁺ ± 0.07</td>
</tr>
</tbody>
</table>

* Four random corneal samples were pooled from each group and stained for CD4⁺ T cells by flow cytometry. Stained CD4⁺ T cells were gated and the percentage of gated population is depicted in the data.
  * p < 0.01
  ** p < 0.001
To assess the influence of T\textsubscript{reg} on this form of immunopathology, CD25\textsuperscript{+} T cells were isolated from naive BALB/c mice and transferred into SCID recipients along with a population of CD4\textsuperscript{+}CD25\textsuperscript{-} T cells from either naive or animals primed 5 wk previously by HSV infection. Mice were then infected ocularly with HSV. The results expressed in Fig. 4a indicate that the lesions induced by both naive and primed CD4\textsuperscript{+}CD25\textsuperscript{-} T cells were reduced in severity if animals additionally received T\textsubscript{reg}.

FIGURE 2. Removal of CD25\textsuperscript{+} T\textsubscript{reg} alters the CD4\textsuperscript{+} T cell phenotype and increases their influx into the cornea. a, Immunofluorescence staining of CD4\textsuperscript{+} T cells in HSV-infected corneas on day 15 p.i. in BALB/c mice. b, Cervical DLN and corneal samples were collected from HSV-infected rat Ig-treated and T\textsubscript{reg}-depleted BALB/c mice on days 9 and 15 p.i. Dot plots shown are representative of six individual mice from one such experiment, and values shown in each plot reflect the percentage of CD4\textsuperscript{+} T cells expressing a particular marker. c, Collagenase-digested ocular cells were polyclonally stimulated with anti-CD3 (2 \textmu g/ml) in the presence of IL-2 (10 U/ml) for 16 h. Cells were gated on CD4\textsuperscript{+} T cells and values shown in each plot represent the percentage of CD4\textsuperscript{+} T cells expressing IFN-\gamma. Data are representative of two similar experiments.
was greater against the naive cells than against the primed population, but in both instances the differences were statistically significant ($p < 0.01$). In these experiments, the transfer of $T_{reg}$ alone failed to cause SK and animals in this group died earlier from HSV encephalitis than those in the other groups. Curiously, the adoptive transfer recipients of both $T_{reg}$ and CD25$^{-}$ T cells also had less neutrophil infiltration in their inflammatory lesions (Fig. 4b). This might mean that the $T_{reg}$ additionally function in the eye to limit innate aspects of immunity.

**Mechanism of lesion suppression by $T_{reg}$**

The mechanism by which the adoptive transfer of CD25$^{+}$ $T_{reg}$ suppressed lesion induction by CD25$^{-}$ primed and naive CD4$^{+}$ T cells was further investigated and seen to have at least two explanations. First, as shown in Fig. 5a, cotransfer of $T_{reg}$ with CD25$^{-}$ T cells resulted in significantly diminished Th1-type immune responses in viral Ag-stimulated splenocytes. This effect was more evident against the response of naive CD25$^{neg}$ cells ($\sim$4-fold reduction) than against primed cells ($\sim$2-fold reduction). In neither group could Ag-induced IL-10 production be demonstrated in splenocytes (data not depicted). Such results indicate that the $T_{reg}$ served to inhibit the induction of HSV immunopathogenic CD4$^{+}$ T cells involved in lesion expression.

A second effect observed in the recipients of CD25$^{+}$ $T_{reg}$ with suppressed lesions was a change in the average phenotype of the CD4$^{+}$CD25$^{neg}$ population in lymphoid tissues compared with controls not receiving $T_{reg}$. In such experiments, recipient SCID mice were given adoptive transfers of Thy1.1 $T_{reg}$ along with Thy1.2 HSV-primed CD4$^{+}$CD25$^{neg}$ T cells isolated 5 wk p.i. Animals were ocularly HSV infected 1 day later and animals were sacrificed after an additional 12 days to measure the phenotype of CD4$^{+}$ T cells in spleens.

In these experiments, lesion severity was greater in control animals given only CD25$^{-}$ T cells compared with those that additionally received $T_{reg}$. In these experiments, recipient SCID mice were given adoptive transfers of Thy1.1 $T_{reg}$ along with Thy1.2 HSV-primed CD4$^{+}$CD25$^{neg}$ T cells isolated 5 wk p.i. Animals were ocularly HSV infected 1 day later and animals were sacrificed after an additional 12 days to measure the phenotype of CD4$^{+}$ T cells in spleens.

FIGURE 3. HSV-specific Th1-type response is enhanced in CD25-depleted BALB/c mice after virus infection. a, CD4$^{+}$ T cells obtained from cervical DLN and spleen on day 15 p.i from mice infected with $10^5$ PFU of virus were restimulated with UV-inactivated HSV-1 RE-pulsed irradiated SPC as described in Materials and Methods and supernatant was analyzed by ELISA for cytokine production. Without virus stimulation, there was no significant difference in the concentration of IL-2 and IFN-$\gamma$ secretion in nondepleted and CD25-depleted HSV-infected mice, and maximum concentration measured was always $<1000$ pg/ml for IL-2 and IFN-$\gamma$ both in DLN and spleen. b, Purified CD4$^{+}$ T cells from the DLN and spleen (10$^3$/well) were restimulated in vitro with UV-inactivated HSV-pulsed irradiated SPC at a stimulator:responder ratio of 1:5, 1:2.5, and 1:1.25, respectively. The frequency of SFC was determined by ELISPOT as detailed in Materials and Methods. In the absence of viral stimulation, no significant difference in the number of IL-2- and IFN-$\gamma$-producing CD4$^{+}$ T cells were observed in nondepleted and CD25-depleted HSV-infected mice, and the maximum number obtained either in DLN or in spleen was always $<25$ SFC/10$^6$ cells. * $p < 0.01$ when compared with the rat Ig-treated HSV-infected group. Each group consisted of six mice and results shown are representative of two similar experiments.
observed when naive CD4\(^+\)CD25\(^{neg}\) T cells were adoptively transferred with T\(_{reg}\) into SCID mice (data not depicted). Since before corneal HSV infection.

a, CD4\(^+\)/H11001 observed that in the SK model, T\(_{reg}\) were present in tissue lesions. The first approach took advantage of the observation that CD25\(^+\) T\(_{reg}\) express higher levels of intracellular CTLA-4 and lower cell surface expression of CD45RB (15). At 15 days p.i., corneal inflammatory cells were recovered by collagenase digestion and analyzed by FACS for their CD4, CD25, CD45RB, and intracellular Foxp3 expression in the DLN and corneal tissue from infected undepleted animals. This molecule was recently shown to be present in high levels in CD25\(^+\) T\(_{reg}\) (16, 17). Foxp3 expression was readily detectable in samples from virus-infected undepleted mice, but were significantly less in material from depleted mice even though such samples were obtained from mice with significantly more severe lesions (Fig. 6b). Furthermore, a comparative analysis of Foxp3 expression in the DLN and corneal tissue from the same HSV-infected mouse exhibited higher copy number of Foxp3 at the site of inflammation (Fig. 6b). Such results add further evidence that T\(_{reg}\) are present in lesions of undepleted mice.

b, CD4\(^+\)/H11001 Role of lesion CD4\(^+\)CD25\(^+\) T\(_{reg}\) in controlling SK

To seek evidence that the lesion T\(_{reg}\) in undepleted animals expressed immunomodulatory activity, cells were collected and fractionated into CD4\(^+\)CD25\(^{neg}\) and CD4\(^+\)CD25\(^+\) T cells. The cell fractions were then tested for their ability to proliferate in response to a polyclonal stimulant, soluble anti-CD3 mAb. The CD25\(^{neg}\) population underwent extensive proliferation but the CD25\(^+\) cells proliferated only when exogenous IL-2 was added (Fig. 7a). Furthermore, the addition of graded numbers of ocular CD25\(^+\) cells to a fixed number of ocular CD25\(^{neg}\) responders showed a dose-dependent inhibition of anti-CD3-driven proliferation of the CD25\(^{neg}\) cells (Fig. 7b).

A second approach to detect T\(_{reg}\) in lesions involved isolation of RNA from the corneas of infected depleted and nondepleted mice and to quantify by real-time PCR the levels of mRNA of transcription factor Foxp3. This molecule was recently shown to be present in high levels in CD25\(^+\) T\(_{reg}\) (16, 17). Foxp3 expression was readily detectable in samples from virus-infected undepleted mice, but were significantly less in material from depleted mice even though such samples were obtained from mice with significantly more severe lesions (Fig. 6b). Furthermore, a comparative analysis of Foxp3 expression in the DLN and corneal tissue from the same HSV-infected mouse exhibited higher copy number of Foxp3 at the site of inflammation (Fig. 6b). Such results add further evidence that T\(_{reg}\) are present in lesions of undepleted mice.

**FIGURE 4.** Adoptive transfer of CD25\(^+\) T\(_{reg}\) diminishes the SK lesion severity induced either by naive or primed CD4\(^+\)CD25\(^{neg}\) T cells in BALB/c SCID. BALB/c SCID mice were either adoptively transferred with CD25\(^+\) T\(_{reg}\) or CD4\(^+\)CD25\(^{neg}\) T cells or a cotransfer of T\(_{reg}\) and CD25\(^{neg}\) T cells 24 h before corneal HSV infection. a, Lesion severity was scored clinically for SK on a score of 0–5 as mentioned in Materials and Methods at different time intervals. ○, Mice receiving only CD4\(^+\)CD25\(^{neg}\) T cells; ●, receives both T\(_{reg}\) and CD25\(^{neg}\) T cells; and ▼, mice given T\(_{reg}\) only. Results are expressed as mean clinical score ± SD. Data shown are representative of two similar experiments. *, p < 0.01 when compared with group adoptively transferred with only CD4\(^+\)CD25\(^{neg}\) T cells. b, Histopathological sections of a BALB/c SCID cornea at day 12 p.i. were stained with anti-Gr1 mAb (for neutrophil staining) as mentioned in Materials and Methods. Immunoperoxidase staining demonstrated infiltration of neutrophils in different groups. Magnification, ×100.
of mice was comprised of six animals.

Inflammation (VLA-4), an integrin likely involved in T cell migration to the ocular surface of CD4+ cells was associated with a greater infiltration into the cornea at day 12 p.i. Arrows denote infiltrating CD4+ T cells. Magnification, ×100. d, Collagenase-digested corneal samples (four corneas per set) as described in Materials and Methods were stained for CD4+ T cells by flow cytometry, and the percentage of infiltrating CD4+ T cells was determined in HSV-infected SCID mice groups receiving CD25neg T cells alone or Treg plus CD25neg together. Each group of mice was comprised of six animals. *, p < 0.01 when compared with control group.

Discussion

This investigation demonstrates that CD25+CD4+ Treg cells notably influence the expression of a viral-induced immunopathological disease. Using a mouse model of the blinding immunoinflammatory disease, SK, our results show that lesions are significantly more severe and animals become more susceptible to infection if depleted of CD25+ Treg before infection. Increased lesion severity was associated with a greater influx of CD4+ T cells into the cornea. In an adoptive transfer model of disease, the cotransfer of Treg with primed or naive pathology inducing CD4+CD25neg T cells reduced lesion severity and diminished the Ag-specific cytokine response of splenic CD4+ T cells. In addition, Treg recipients had significantly fewer splenic CD4+ T cells that expressed CD49d (VLA-4), an integrin likely involved in T cell migration to the ocular inflammatory site. Interestingly, in undepleted animals, Treg could be demonstrated in the inflamed corneas and CD4+CD25neg cells isolated from such corneas inhibited the proliferation of polyclonal stimulated CD25neg T cells, an effect impaired by anti-IL-10R mAb. These results are the first to indicate a role for Treg in viral-induced immunopathology and imply that manipulation of regulatory cell function might represent a useful approach to control viral-induced immunoinflammatory disease.

The surge of recent interest in regulatory cells has primarily focused on disease models that involve autoreactivity (18, 19). However, increasing reports document that Treg also influence immunity to several pathogens (11, 20, 21). Thus, we and others have shown that the absence of a Treg response may result in heightened immunity which includes a more durable memory response (11, 22). However, a downside of heightened immune reactivity might be immunopathogenesis. Indeed, the initial description of CD25+ Treg cell function emerged from studies showing that their absence resulted in organ-specific autoimmune diseases in an adoptive transfer system (7). The SK model represents a situation where normally immunoprotective CD4+ T cells exert an immunopathological function in the special environment of the eye’s cornea (23). Thus, for reasons still unclear, corneal infection with HSV sets off an inflammatory reaction that usually fails to fully resolve, even long after viral replication has ceased and viral Ags are no longer detectable (24). Such observations have led to the suggestion that SK ultimately becomes an autoimmune lesion (25), but this attractive hypothesis remains to be substantiated.

Our observation that the severity of SK lesions was influenced by Treg was supported by the observation of more severe lesions in Treg-depleted animals, as well as by noting a modulatory effect of Treg on lesions induced by pathogenic CD4+ T cells in an adoptive transfer system. Our results suggest that Treg may play a pivotal role in microbial pathogenesis, especially in situations that involve...
chronic inflammation and persistent infection. Other examples have also been recently reported, especially with parasitic infections (26, 27). Thus, with Leishmania infection in the susceptible BALB/c mouse strain, T reg appeared to limit the extent of T cell-mediated inflammatory disease (28). This is similar to the circumstances described for SK as well as for Pneumocystis carinii-induced lung pathology in mice (21). However, in resistant mice the Treg plays a crucial role in maintaining concomitant immunity to Leishmania (20). Accordingly, T reg limit the protective effect of CD4\(^+\) Th1 cells, resulting in a balance that permits parasite persistence, immunity to reinfection, and an absence of lesions. Removal of Treg results in parasite clearance but susceptibility to infection. Clearly, manipulating Treg function in chronic microbial infections could represent a valuable approach to disease management.

Although our results substantiate that Treg modulate the severity of SK lesions, we can only speculate how this process is accomplished. We anticipate that the effects are multiple. They include inhibition of virus-specific CD4\(^+\) T cell induction, the cells that help orchestrate SK lesions (3). Accordingly, the presence of Treg limited the extent of CD4\(^+\) T cell immunity, as was shown previously to occur with CD8\(^+\) T cell responses (29). In immunocompetent mice, these inhibitory effects were modest. Greater effects were evident in an adoptive transfer model in which Treg were transferred along with naive or primed CD4\(^+\) T cells and HSV-specific immunity was compared with recipients that received responder cells only. In such experiments, the HSV-specific CD4\(^+\) T cell response was inhibited by up to 4-fold in animals that received Treg. Thus, one explanation for the Treg

**FIGURE 6.** CD4\(^+\)CD25\(^+\) T cells isolated from HSV-infected corneas express higher levels of intracellular CTLA-4 and Foxp3. Collagenase-digested corneal samples obtained from HSV-infected BALB/c mice were stained for CD4\(^+\)CD25\(^+\) cells on day 15 p.i. a, CD4\(^+\)CD25\(^+\) T cells from nondepleted animals expressed higher levels of intracellular CTLA-4 and low cell surface CD45RB when compared with CD25\(^-\) T cells from Treg-depleted animals. b, RNA was isolated from rat Ig and anti-CD25 mAb-treated HSV-infected corneas (two corneas), DLN on day 15 p.i., and reverse transcribed as described in Materials and Methods. Foxp3 expression was determined in cDNA samples by a real-time RT-PCR using the CYBR green method. Data shown are the Foxp3 copy number in four pooled corneas obtained from naive, rat Ig-treated, and CD25-depleted HSV-infected BALB/c mice. Data are representative of three independent experiments. *, p < 0.001 when compared with the rat Ig-treated group.
The effect on SK lesion expression was immunosuppression, an effect likely mediated at the lymphoid sites of immune induction.

A second effect accounting for reduced lesion severity could be that Treg, perhaps as a consequence of activation by HSV as was shown to occur previously (11), could serve to modulate the expression of one or more homing molecules involved in T cell migration to the ocular inflammatory site. Although the signature of homing molecules involved in ocular migration remains unknown, there is evidence that the integrin VLA-4 is involved in the process (10). In SK 70–80% of corneal T cells in disease express VLA-4 early in lesion development. Thus, it was intriguing to observe that the VLA-4 integrin was expressed on a significantly lower percentage of lymphoid CD4+ T cells when these were activated in the presence of Treg. Our observations could mean that Treg also act to control lesion development by limiting the migration of pathogenic T cells to the extralymphoid inflammatory site. Such concepts are now under further investigation.

A final effect of Treg on SK may be that such cells also function at tissue sites to limit inflammatory events. Whether or not Treg act at nonlymphoid or lymphoid sites (or both) remains an unresolved issue. In some systems where Treg effects have been noted, the regulatory cells cannot be demonstrated in lesions (14). In others they are present, but it was not clear whether this was the exclusive site for their activity. For example, in the inflammatory bowel disease model, Treg could be demonstrated both in colon lesions as well as in the mesenteric lymph nodes, but whether they act at both

FIGURE 7. CD4+CD25+ T cells isolated form SK lesions are suppressive and secrete IL-10 in response to polyclonal stimulation. CD4+ T cells purified from SK-developed corneas (32 corneas) on day 15 p.i. were sorted into CD25+ and CD25neg subpopulations as described in Materials and Methods. a. Sorted fractions of CD4+CD25+ (5 × 10⁴ cells/well) and CD4+CD25neg (5 × 10⁴ cells/well) T cells were stimulated with anti-CD3 (1.0 µg/ml) and irradiated SPC (10⁴ cells/well) in the presence or absence of IL-2. Proliferation assay was performed as described in Materials and Methods. b. CD4+CD25neg T cells (10⁴/well) isolated from SK lesions were stimulated with anti-CD3 mAb and irradiated SPC in the absence or presence of different numbers of CD4+CD25+ T cells from SK lesion, and 72 h later proliferation was assayed as described in Materials and Methods. c, CD25+ and CD25neg CD4+ T cells were stimulated with anti-CD3 (1.0 µg/ml) for 48 h and the supernatant was used to measure the IL-10 production by ELISA. nd, Not done. d. Anti-IL-10R mAb was added in the coculture of CD4+CD25+ (10⁴ cells/well) and CD4+CD25neg (10⁴ cells/well) T cells and proliferation was measured by ³H-thymidine incorporation in the last 18 h of culture. Values shown are mean ± SD of triplicate wells. *, p < 0.01 when compared with control group.
sites was not evaluated (13). In the SK model, two lines of evidence indicated that Treg were present in tissues. First, a high percentage of CD4+ CD25+ T cells isolated from lesions of undepleted mice also expressed high intracellular CTLA-4 and low cell surface expression of CD45RB, accepted markers for Treg (15). Additionally, taking advantage of the recent observation that the transcription factor Foxp3 is a marker product for Treg (16), we showed by real-time PCR abundant Foxp3 message only in the samples from nondepleted mouse lesions. Together, these approaches indirectly establish the presence of Treg in lesions, but do not indicate that they act to control immunopathology at the site.

Two lines of evidence indicated that such lesional Treg were functioning in the SK system. First, the homing/activation marker VLA-4 was expressed on a significantly lower percentage of lesional CD4+ T cells late after infection (15 days p.i.) in control compared with undepleted animals. This could mean that the Treg were responsible for down-regulation. Second, CD25+ T cells isolated from lesions at this late stage were shown to inhibit in vitro proliferation of polyclonal stimulated CD4+ CD25neg T cells isolated from the same lesions. This modulatory effect was partially inhibited by anti-IL-10 mAb, indicating that the Treg acted via IL-10 secretion as noted in some other systems (30, 31).

Further studies are underway to directly demonstrate Treg in SK lesions and to determine whether and how they control the immunopathology. We anticipate that the efficacy of such a function might vary as lesions progress. Conceivably, regulation may not occur in the early lesions when virus is still present because HSV infection is a potent stimulus for IL-6 production (32, 33), and this cytokine was elegantly shown to render pathogenic T cells refractory to the effects of Treg (34). Furthermore, the corneal IL-6 concentrations have also been reported to decline during the later phase of HSK (35), the time when Treg might help to resolve the inflammatory events. The kinetics of Treg ingress and their control of SK lesions are under further investigations.

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References