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On the Role of Regulatory T Cells during Viral-Induced Inflammatory Lesions

Tamara Veiga-Parga,* Amol Suryawanshi,† Sachin Mulik,* Fernanda Giménez,* Shalini Sharma,‡ Tim Sparwasser,§ and Barry T. Rouse*

Ocular HSV-1 infection can result in stromal keratitis, a blinding immunoinflammatory lesion that represents an immunopathological response to the infection. CD4+ T cells are the main orchestrators, and lesions are more severe if the regulatory T cell (Treg) response is compromised from the onset of infection. Little is known about the role of Foxp3+CD4+ Tregs during ongoing inflammatory reactions, which is the topic of this article. We used DEREG mice and depleted Tregs at different times postinfection. We show that lesions became more severe even when depletion was begun in the clinical phase of the disease. This outcome was explained both by Tregs' influence on the activity of inflammatory effector T cells at the lesion site and by an effect in lymphoid tissues that led to reduced numbers of effectors and less trafficking of T cells and neutrophils to the eye. Our results demonstrate that Tregs can beneficially influence the impact of ongoing tissue-damaging responses to a viral infection and imply that therapies boosting Treg function in the clinical phase hold promise for controlling a lesion that is an important cause of human blindness. The Journal of Immunology, 2012, 189: 5924–5933.

In the present report, we have evaluated the lesion-modulating consequences of Treg removal after lesions have commenced in a system in which tissue damage is caused by an immunopathological response to infection. Our results show that Treg depletion resulted in increased lesion severity, with the effect less evident as depletion was delayed further from the time of infection. The consequences of Treg removal appeared to have two explanations. These were inhibitory effects at the lesion site, as well as effects in lymphoid tissues that reduced the migration of inflammatory cells to the tissue lesion. Our results indicate that Tregs have a beneficial role in minimizing and modulating the severity of viral-induced immunoinflammatory reactions. This may mean that expanding Treg function could represent a promising approach for therapy.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facilities. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Tennessee (Public Health Service Assurance number 83-R-0105). HSV-1 eye infection was performed under deep anesthesia (Avertin), and all efforts were made to minimize animal suffering.

Mice, virus, and cell lines

Breeder pairs of DEREG mice on C57BL/6 background were provided by Dr. Tim Sparwasser (Hannover, Germany), and additional mice were bred in the Walters Life Sciences animal facility at the University of Tennessee, Knoxville. For the experiments, females 5 to 6 wk old were used. 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, originally provided by the Robert N. Lausch laboratory (University of South Alabama, Mobile, AL), was used in all procedures. Virus was grown and titrated on Vero cells (ATCC no. CCL81) using standard protocols. The virus was stored in aliquots at −80˚C until use.

Abs

CD4-allophycocyanin (RM4.5), CD4-Percp (RM4.5), CD103-allophycocyanin (2E7), CTLA-4-PE (UC10-4F10-11), CD45-allophycocyanin (30-F11), CD11b-PerCP (M1/70), Ly6G-PE (1A8), CD49d-PE (MFR4.B), CD44-Percp (30-F11), CD69-PerCP (44C17.12), CD80-PerCP (20G2A12), CD86-PerCP (B7-15C16), and CD11c-PerCP (H11-22C9).

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Abbreviations used in this article: DLN, draining lymph node; DT, diphtheria toxin; p.i., postinfection; Q-RT-PCR, quantitative RT-PCR; SK, stromal keratitis; Treg, regulatory T cell.
the mock-infected samples and control or day 8 DT-treated samples from day 15 p.i. was calculated using the $2^{-\Delta\Delta C_t}$ formula: $\Delta C_t = \Delta C_{t\text{sample}} - \Delta C_{t\text{reference}}$. In this equation, $\Delta C_t$ is the change in cycling threshold between the gene of interest and the “housekeeping” gene β-actin, where $\Delta C_{t\text{sample}}$ was the Ct value for any day 8 DT-treated or control samples from day 15 p.i. normalized to the β-actin gene, and $\Delta C_{t\text{reference}}$ was the Ct value for the mock-infected samples (scratched and infected only with PBS), also normalized to β-actin. Each of the samples was run in duplicate to determine sample reproducibility, and a mean Ct value for each duplicate measurement was calculated. The PCR primers used were the following: β-actin, F 5'-CTCTTTGGTATGGAATCTG-3' and R 5'-GCCAGCACATTGGTCTC-3'; β2-microglobulin, F 5'-GCTTCCTGAGGCTGGATTC-3' and R 5'-CAAGGCCAATTGGCAAAA-3'; R 5'-TCCGGATCAGGGCTAGTGA-3'; CXCL-10, F 5'-TGTGCGGCTTGAGGAATGGG-3' and R 5'-AAGCTTCCCATTGCTCCCTCA-3'; MCP-1, F 5'-TGGCGAGTCGAACCTGGTG-3' and R 5'-TGGGATACTATCCTCGGTG-3'; KCXCXL1, F 5'-GGTGGGCTCCTCGAGGCC-3' and R 5'-GGCTTGCGACACATCTCTTG-3'.

**ELISA**

DNL single-cell suspensions from individual mice were collected at day 15 p.i. Cells were stimulated in vitro with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 48 h at 37°C. In addition, corneal samples were pooled according to group (five corneas per sample) and homogenized using a tissue homogenizer (Pellet Pestle mortar; Kontes). For lymph node samples, four cervical DNLs were collected for each mouse sample, and ELISA was performed on the homogenized sample. The concentrations of IFN-γ were measured by sandwich ELISA kits from eBioscience per the manufacturer’s instructions.

**Statistical analysis**

Most analyses for determining the level of significance were performed using unpaired two-tailed Student t tests. Values of $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$ were considered significant. Results are expressed as means ± SEM. For some experiments, as mentioned in the figure legends, a one-way ANOVA test was applied.

**Results**

Lymphoid Treg and Th1 response changes after HSV-1 infection

Although previous studies reported that local infection with HSV results in expansion of the Treg population in infected tissues and the lymphoid sites (22), the kinetic nature of the response was not determined after corneal infection. As shown in Fig. 1A, corneal infection of Foxp3-GFP reporter mice, which express GFP knocked into the locus encoding the transcription factor Foxp3 uniquely expressed by Tregs (23), caused expansion of Tregs in the DNL as soon as day 4 p.i. Peak numbers were evident by day 8 p.i., when the expansion was ~4-fold. Numbers of Th1 cells also peaked on day 8 p.i. (Fig. 1B). The phenotype of Tregs was also measured and compared with uninfected controls in the DNL and spleen samples at different time points p.i. (days 0, 4, 8, 12, and 15). As is evident, a greater proportion of Tregs from infected animals showed increased expression of activation markers, and more were CD103+ than uninected controls (Fig. 2A). The expression of other activation markers and costimulatory molecules was also altered p.i. (Fig. 2B). The frequency of Tregs expressing CTLA-4 and the ICOS significantly increased in DNL and spleen, peaking on day 15 and day 12 p.i., respectively. The expression of the inhibitory receptor PD-1 also changed, being downregulated until day 8 p.i. and then increasing at later time points (days 12 and 15 p.i.) in both organs. Thus, Tregs after HSV-1 infection increase in numbers in lymphoid tissues and change their surface phenotype to a more increased activation phenotype.
depletion of Tregs owing to expression of DT receptor-enhanced GFP under the control of the Foxp3 promoter (24) were infected ocularly with HSV-1, and flow cytometry was used to quantify Treg and Th1 cells in DLN and spleen of naïve and infected mice at different times after corneal infection with HSV-1. (A) Representative frequencies and numbers of Tregs in DLN and spleen of infected mice at different time points. (B) Representative frequencies and numbers of Th1 cells in DLN and spleen of infected mice at different times. Data are representative of three independent experiments and show mean values ± SEM (n = 6 mice at each indicated time point p.i.). Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett’s post hoc test settings. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 1.** Lymphoid Treg and Th1 response changes after HSV-1 infection. Foxp3-GFP mice corneas were scarified and infected with HSV-1, and flow cytometry was used to quantify Treg and Th1 cells in DLN and spleen of naïve and infected mice at different times after corneal infection with HSV-1. (A) Representative frequencies and numbers of Tregs in DLN and spleen of infected mice at different time points. (B) Representative frequencies and numbers of Th1 cells in DLN and spleen of infected mice at different times. Data are representative of three independent experiments and show mean values ± SEM (n = 6 mice at each indicated time point p.i.). Statistical levels of significance were analyzed by one-way ANOVA test with Dunnett’s post hoc test settings. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

Inflammatory consequences of Treg depletion in the clinical phase of SK

In experiments in which depletion was commenced on day 8 p.i., pools of corneas were collected on day 15 p.i. from DT-treated and control animals either to recover inflammatory cells by collagenase digestion or to prepare samples for analysis by Q-RT-PCR or protein measurement by ELISA. The numbers of CD45+ cells on day 15 p.i. were ~3-fold greater in Treg-depleted compared with control animals. In conclusion, depletion of Tregs during the clinical stages of SK resulted in significantly increased lesion severity, indicating that Tregs play a modulating role during the immunopathological process.

Corneas showed more severe inflammatory reactions in animals depleted from day 8 p.i. compared with vehicle-treated controls (Fig. 3D). In conclusion, depletion of Tregs during the clinical stages of SK resulted in significantly increased lesion severity, indicating that Tregs play a modulating role during the immunopathological process.
(CD45^+CD11b^+F4/80^+) were also significantly greater in Treg-depleted animals than in control mice (Fig. 4C, 4D).

Experiments of the same design were conducted to compare the effector functions of infiltrated ocular CD4^+ T cells in the nondepleted and Treg-depleted DEREG mice. Cells isolated from corneal tissues were stimulated with PMA/ionomycin in the presence of Golgi-Plug for 4 h. The numbers of Th1 cells were increased ~2-fold in the Treg-depleted group compared with control animals (Fig. 4E). The effect of Treg depletion on levels of chemokines present in corneas was also measured either by Q-RT-PCR or by protein measurement with ELISA. As indicated in Fig. 5A, samples from depleted animals showed increased levels of molecules known from previous studies to participate in the pathogenesis of SK (25). These included IL-6 and IL-1β, which were increased 5- and 13-fold, respectively. In addition, significantly increased levels of chemokines involved in neutrophil (KC) and monocyte (MCP-1) migration, as well as lymphocyte migration (CXCL-9 and CXCL-10), were noted (Fig. 5B). IFN-γ protein levels were also increased ~2-fold in Treg-depleted mice (Fig. 5C).

The increase in CD4^+ T effector cells in Treg-depleted corneas was attributed to an increase in proinflammatory chemokines in the corneas that recruited the inflammatory cells. Another possible explanation could be that Tregs control the number of effector T cells generated in lymphoid tissues, making fewer available to enter the inflamed tissues. To assess this possibility, DLN and spleen cells from day 8 p.i. depleted and control animals were...
compared for the number of Th1 cells on day 15 p.i. The results shown in Fig. 6A and 6B demonstrate that Th1 cell total numbers in both DLN and spleen were significantly increased in DT recipients when compared with controls. In another set of experiments, DLN extracts were collected on day 15 from control and Treg-depleted mice, and samples were analyzed by ELISA for IFN-γ protein levels. The data show that Treg depletion increased the production of IFN-γ by almost 2-fold in the depleted mice (Fig. 6C).

Taken together, our results show that Treg depletion during the clinical phase of the disease caused a significant increase in the total cellular infiltration of Foxp3+ CD4+ T cells, neutrophils, and macrophages, as well as the amount of proinflammatory cytokines and chemokines in the corneas of HSV-1 infected mice. In addition, effector cell numbers and products in lymphoid tissues were increased in the absence of Tregs. We interpret these data to mean that Tregs normally act to modulate the extent of inflammatory cell molecule production at the tissue site. Tregs also influence Th1 cell numbers and responses in secondary lymphoid tissues, which would reduce the number of cells available to infiltrate the inflamed cornea. Moreover, Tregs might act to inhibit the recruitment of inflammatory cells to the tissue site. Some evidence for this effect is described in the next section.

Consequences of Treg depletion on phenotype of effectors in secondary lymphoid tissue

To measure the putative effects of Tregs on inflammatory cell recruitment in DLN cell populations from day 8 p.i., depleted and control animals were compared for surface expression of molecules considered to be involved in tissue migration. Mice were sacrificed on day 15 p.i., compared with vehicle-treated animals. Data are representative of three independent experiments and show mean values ± SEM (n = 12–15 mice per group). Statistical levels of significance were analyzed by the Student t test (unpaired). **p ≤ 0.01, ***p ≤ 0.001.

FIGURE 3. Depletion of Tregs during clinical stages of disease increases SK lesion severity. DEREG mice infected with HSV-1 were given either DT or vehicle IP, starting on day 5 or 8 or 12 p.i., and treated every other day until the termination day (day 15 p.i.). (A) Starting on day 8 p.i., mice were treated with either DT (1 μg/d) or vehicle every other day, mice were sacrificed on day 15 p.i., and lymphoid organs were removed. Flow cytometry analysis of lymphoid cells from cornea, DLN, and spleen shows efficient depletion of Tregs in DT-treated DEREG mice compared with DT-PBS–treated DEREG mice. (B) The progression of SK lesion severity was significantly increased in the group of mice treated with DT compared with control mice. Kinetics of SK lesion severity are shown. Individual eye scores of SK lesion severity on day 15 p.i. are shown. SK lesion severity was significantly increased in the groups of mice treated with DT, starting on day 5 and 8 p.i., compared with control mice. (C) Representative eye photos show increased SK lesion severity, as well as angiogenesis, from DT-treated mice, starting on day 8 p.i., compared with vehicle-treated mice. (D) Eyes were processed for cryosections on day 15 p.i. H&E staining was carried out on 6-μm sections, and pictures were taken at different microscope augmentations (original magnification ×20 and ×40). Representative eye sections show increased cellular infiltration in DT-treated mice, starting on day 8 p.i., when compared with vehicle-treated animals. Data are representative of three independent experiments and show mean values ± SEM (n = 12–15 mice per group). Statistical levels of significance were analyzed by the Student t test (unpaired). **p ≤ 0.01, ***p ≤ 0.001.
FIGURE 4. Depletion of Tregs increases cellular infiltration in corneas of HSV-1–infected animals. DEREG mice infected with HSV-1 were given either DT or vehicle i.p., starting on day 8 p.i., every other day until the termination day (day 15 p.i.). (A) Representative FACS plots, and frequencies and numbers of CD45+ cells infiltrated in the corneas of control and DT-treated mice, are shown. (B) Representative FACS plots, and frequencies and numbers of CD4+ T cells infiltrated in the corneas of control and DT-treated mice. (C) Representative FACS plots, and frequencies and numbers of CD11b+Ly6G+ polymorphonuclear neutrophils gated on total CD45+ cells infiltrated in the corneas of control and DT-treated mice, are shown. (D) Representative FACS plots, and frequencies and numbers of CD11b+F4/80+ macrophages gated on total CD45+ cells infiltrated in the corneas of control and DT-treated mice, are shown. (E) Representative FACS plots, and frequencies and numbers of IFN-γ–secreting cells gated on CD4+ T cells infiltrated in the cornea on day 15 p.i. and stimulated with PMA/ionomycin for 4 h, from control and DT-treated mice. Data are representative of three independent experiments and show mean values ± SEM (n = 10–12 mice per group; each sample is representative of two corneas). Statistical levels of significance were analyzed by the Student t test (unpaired). *p ≤ 0.05, **p ≤ 0.01.
Depletion of Tregs increases cytokine and chemokine levels in the corneas of HSV-1–infected animals. DEREG mice infected with HSV-1 were given either DT or vehicle i.p., starting on day 8 p.i., every other day until the termination day (day 15 p.i.). Mice were sacrificed on day 15 p.i., and corneal extracts were collected for measuring inflammatory factors, using either Q-RT-PCR or sandwich ELISA. (A) Relative fold change in mRNA expression of the proinflammatory cytokines IL-1β and IL-6 was examined and compared between control and DT-treated mice on day 15 p.i. by Q-RT-PCR. (B) Relative fold change in mRNA expression of the chemokines KC, MCP-1, CXCL9, and CXCL10 was examined and compared between control and DT-treated mice on day 15 p.i. by Q-RT-PCR. (C) IFN-γ protein levels from pooled corneal samples from control and DT-treated animals were quantified using ELISA. Data are representative of three independent experiments and show mean values ± SEM (n = 12 mice per group; each sample is representative of four corneas). Statistical levels of significance were analyzed by the Student t test (unpaired). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

enhanced the expression of activation markers on Foxp3− CD4+ T cells in DLN and spleen. As shown in Fig. 7B, the frequencies of CD4+ T cells expressing CD44hi in the DLN and spleen significantly increased in the Treg-depleted group compared with controls. Furthermore, ∼35% of cells in the DLN and 55% in the spleen were CD62Llow in the Treg-depleted group compared with 28% and 35%, respectively, in control mice (Fig. 7C). The up-regulation of CD49d on CD4+ T cells is likely relevant, because previous studies indicate that blocking CD49d reduces SK (26, 27). Our results are consistent with the possibility that Tregs normally act within lymphoid tissues to reduce the expression of surface molecules such as CD49d on activated cells that permit them to migrate to the tissue site. This observation may mean that effector cells could traffic more effectively to tissue sites when not controlled by Tregs during clinical stages of the disease.

Discussion

Many studies have demonstrated that the extent of inflammatory responses is affected by the balance between proinflammatory effectors and events that regulate them, such as the activity of Foxp3 Treg cells (11, 13, 28). The conclusion that Tregs play a modulatory role comes largely from studies that compare the outcome in normal animals to that in which the Treg composition is manipulated from before or early in the disease process. In this investigation, we asked if Tregs play an active modulatory role during the clinical expression of lesions. Results were accomplished by taking advantage of DEREG mice, in which Tregs can be successfully and rapidly removed by exposure to DT, which depletes the Foxp3+ cells because they express the DT receptor (24, 29). We show, using a model in which an inflammatory lesion occurs in the cornea after HSV infection, that lesions became more severe even when depletion was begun in the clinical phase of the disease. This outcome was explained both by Tregs’ influence on the activity of inflammatory effector T cells at the lesion site and by an effect in lymphoid tissues that led to reduced numbers of effectors and less trafficking of T cells and neutrophils to the eye. Our results demonstrate that Tregs can beneficially influence the impact of ongoing tissue-damaging responses to a virus infection and imply that therapies boosting Treg function in the clinical phase hold promise for controlling a lesion that is an important cause of human blindness.

Since the milestone report from Belkaid and colleagues (30), we have known that Tregs are induced by infections and that they have a vital part in shaping the pattern of disease responses. Tregs, for instance, are considered to influence the outcome of persistent infections, just as they do in autoimmune diseases (13, 15). However, the majority of studies that implicate Tregs during an infectious disease either compared the outcome of disease in animals depleted of Tregs from the onset with that in controls, or observed the consequence of boosting Treg responses early or before infection (11, 31, 32). In our study, we attempted to evaluate the function of Tregs during an ongoing inflammatory process by removing them when clinical lesions were already present. In so doing, we exploited the transgenic mouse system developed by Sparwasser and colleagues (29), which permits rapid and almost total selective elimination of Foxp3+ Tregs at any stage in the disease process. Our findings clearly show that removing Tregs in the clinical phase of the disease resulted in lesions becoming more severe. This increased severity could be demonstrated not only clinically but also by comparing the number of inflammatory cells and their products in the corneas of Treg-depleted animals with that in control animals. In the absence of Tregs, greater numbers of the effector T cells that orchestrate lesions were present, as well as nonlymphoid inflammatory cells such as neutrophils, the latter mainly responsible for damage to the corneal stroma (33, 34). Although our results demonstrate that without Tregs, lesions became more severe, we provide no direct evidence that Tregs subserve their function only at the tissue site.

The problem with attempts to conclude that Tregs exert functional effects solely within inflammatory reactions is that the
The depletion procedure also had proinflammatory consequences in lymphoid tissues. Accordingly, effector cell numbers were increased by Treg ablation, and the average phenotype of CD4+ T cells involved in orchestrating SK lesions showed changes. These included increased numbers of cells with an activation phenotype and increased cell numbers that expressed molecules shown in previous studies to be required for migration to the corneal tissue site (26). Consequently, it remains possible that the major influence of Tregs during infection occurs by the DLN acting to limit the number of activated T effectors and the generation of inflammatory cells that express receptors permitting their passage to tissue sites. Further experiments are needed to define the relative importance of tissue-acting versus lymphoid organ effects of Tregs on the inflammatory response. However, as reviewed by Shevach (35), Tregs suppress immune responses at multiple levels with different mechanisms of action in different experimental models.

Our observation adds further evidence to the idea that Tregs can play a critical role during responses to infections and implies that amplifying their function in the phase of active lesions would likely prove beneficial to the outcome. This beneficial effect may be especially relevant in situations such as SK, in which replicating virus is minimal or absent at the phase of the inflammatory response (25). However, it remains to be shown how the latter effect can be most effectively achieved, particularly if, as has been advocated (36), the Tregs that function most effectively need to be Ag specific. In the case of the virus-induced immunopathological disorders caused by HSV ocular infection, Tregs may not need to be virus specific as long as they express the activation and migration phenotypes that permit them to access the site of inflammation (12). Whereas Ag specificity may facilitate the regulatory activity of Tregs, it is also conceivable that as long as Tregs are activated and can successfully gain entrance into inflammatory sites, Tregs of multiple specificities could still subserve a useful function. This result is to be expected, especially when Treg function by producing soluble anti-inflammatory mediators such as IL-10 and TGF-β, as well as IL-35 (35–37). In addition, in past studies we have shown that the adoptive transfer of Foxp3+ Tregs reactive with OVA peptide can readily enter the inflamed cornea (12). Moreover, some evidence exists that SK lesions may be, in part, autoreactive (38), meaning that self-reactive Tregs, a major fraction of the Foxp3+ Treg population (13, 36), could participate in regulating lesion severity.

Our study shows that Treg function is needed to limit the extent of virus-induced inflammatory lesions, and implies that expanding and activating Tregs could be therapeutically valuable. Moreover, if, as we advocate, Tregs do not need to share Ag reactivity with the proinflammatory effectors they regulate, the therapeutic options...
increase, as approaches that expand Tregs polyclonally are more available than are maneuvers to expand Ag-specific Tregs. Of the polyclonal approaches reported, the ones described by Sprent et al. (39, 40) and Podack and colleagues (41, 42) have particular appeal. Podack’s approach exploits the fact that among naive and resting T cells, the mAb for the TNF receptor 25 (mAbT25) is expressed predominantly on Foxp3+ Tregs (41). Moreover, if the receptor is engaged with an agonistic mAb, the Treg population is preferentially expanded and activated. We have found this approach to be effective in expanding Tregs and diminishing SK lesions (42). However, the downside of using mAbT25 is that activated effectors also express TNF receptor 25 (mAbT25) and may also be expanded, which could exacerbate tissue damage. Fortunately, this problem can be overcome by the coadministration of additional therapies that selectively cause apoptosis of effectors (42).

In conclusion, our results indicate that Tregs help minimize and modulate the severity of SK lesions during the clinical phase. Our results also suggest that therapies boosting Treg function in the clinical phase help control a lesion that is an important cause of human blindness.

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Disclosures
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