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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 a chronic immunoinflammatory lesion in the corneal stroma, which is a significant cause of human vision loss (1, 2). Studies in animal models have revealed that stromal keratitis (SK) lesions are orchestrated mainly by IFN-γ-producing CD4+ T cells (Th1) (3, 4) and, to a lesser extent, by IL-17-producing CD4+ T cells (Th17) (5). Lesion severity is also subject to modulation by several host-derived factors, which include IL-10 (6), some galectins (7, 8), and microRNAs (9), as well as by regulatory T cells (Tregs) (10, 11). Thus, removing or expanding Tregs from the onset of lesion induction results in exacerbated or diminished lesions, respectively (10, 12). The role of Tregs in modulating lesion severity in multiple autoimmune diseases is well established (13, 14), and Tregs are suspected of playing a similar role in several chronic infections, which include hepatitis C and some of the lesions caused by HIV (15, 16). However, few, if any, studies have evaluated the role of Tregs in an infection-induced lesion by removing them during the ongoing disease process. Recently, Reuter et al. (17) did perform such a study, removing Tregs several days after intracerebral infection with measles virus. They showed that viral clearance was accelerated but did not evaluate any changes in tissue damage.

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 63-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

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.

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Corneal HSV-1 infection and clinical observations

Corneal infections of C57BL/6 mice were done with the mice under deep anesthesia induced by i.p. injection of trimbromothanol (Avertin), as previously described (18). Corneas were scarified with a 27-gauge needle, and a 3-μl drop containing the specific viral dose (2 × 10^6 PFU) was applied to the eye. Eyes were examined on different days postinfection (p.i.), with a slit-lamp biomicroscope (Kowa, Nagaoya, Japan) measuring the progression of SK lesion severity and angiogenesis of individual mice. 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; and +5, corneal rupture and necrotizing keratitis (19). The severity of angiogenesis was recorded as described previously (20). 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 scores 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.

Depletion of Tregs

To deplete Tregs, DEREG mice were injected with diphtheria toxin (DT) (from Corynebacterium diphtheriae; Sigma-Aldrich). Mice were injected i.p. with 1 μg DT every other day. Control mice were DEREG mice injected with the vehicle.

Histopathological examination

Eyes from control and DT-treated mice, starting on day 8 p.i., were excised, pooled according to group, and digested with 60 U/ml Liberase (Roche Diagnostics) for 35 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 cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium.

Staining for flow cytometry

The single-cell suspensions obtained from cornea, cervical draining lymph nodes (DNLs), and spleen of mice at different time points p.i. Corneas were excised, pooled according to group, and digested with 60 U/ml Liberase (Roche Diagnostics) for 35 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 cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium.

Flow cytometry

Cell preparation. Single-cell suspensions were prepared from cornea, cervical draining lymph nodes (DNLs), and spleen of mice at different time points p.i. Corneas were excised, pooled according to group, and digested with 60 U/ml Liberase (Roche Diagnostics) for 35 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 cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium.

Staining for flow cytometry. The single-cell suspensions obtained from cornea, DNLs, and spleen were stained for different cell surface molecules for FACs. All steps were performed at 4˚C. A total of 1 × 10⁶ cells were first blocked with an unconjugated anti-CD3/CD16 mAb for 30 min in FACs buffer. After washing with FACs buffer, fluorochrome-labeled respective Abs were added for 30 min on ice. Finally, the cells were washed three times and re-suspended in 1% paraformaldehyde. The stained samples were acquired with a FACsCalibur (BD Biosciences), and the data were analyzed using FlowJo software. For corneas, total cell numbers were calculated by acquiring the totality of the sample and taking into consideration the total number of corneas in the sample.

To determine the number of IFN-γ-producing CD4⁺ T cells, intracellular cytokine staining was performed as previously described (21). In brief, 10⁶ freshly isolated splenocytes, as well as lymph node and corneal cells, were cultured in U-bottom 96-well plates. For in vitro induced cultures, cells were left unstimulated or stimulated with PMA (50 ng) and ionomycin (500 ng) for 4 h in the presence of brefeldin A (10 μg/ml). Subsequently, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD Pharmingen) in accordance with the manufacturer’s recommendations. The Abs used were anti-IFN-γ allophycoerycin. The fixed cells were resuspended in 1% paraformaldehyde. The stained samples were acquired with a FACsCalibur (BD Biosciences), and the data were analyzed using FlowJo software. For intracellular staining of CTLA-4, we used the Foxp3 Staining Buffer Set (eBioscience).

Real-time PCR

RNA was extracted from cells and tissue with TRIzol LS reagent (Invitrogen). Total cDNA was made with 500 ng RNA using oligo(dt) primer. Quantitative RT-PCR (Q-RT-PCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with an iQ5 RT-PCR detection system (BioRad, Hercules, CA) using 5 μl cDNA for 40 cycles. The expression levels of different molecules were normalized to β-actin with the Δ threshold cycle method calculation. Relative expression between mock-infected samples and control or day 8 DT-treated samples from day 15 p.i. was calculated using the 2−ΔΔCt formula: ΔΔCt = ΔCt sample − ΔCtreference. In this equation, ΔCt is the change in cycling threshold between the gene of interest and the “housekeeping” gene β-actin, where ΔCt Sample was the Ct value for any 8 DT-treated or control samples from day 15 p.i. normalized to the β-actin gene, and ΔCt 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'-CCTCTTCGGGTATGATGCCTG-3' and R 5'-GGCCATTAGGAGGTTACCGATG-3'; IL-6, F 5'-GGATGACCCAATTTGACAC-3' and R 5'-GGTCCCCGGCCGTTGGT-3'; IL-1β, F 5'-GAAATGCCACTTTCGAAGAC-3' and R 5'-CAGGCCACAGGTCCTTTGATC-3'; CXCL-10, F 5'-GGGAGCCTTCGACCACTTGGA-3' and R 5'-CTGGGTCATCCAAGGCTGTA-3'; IP-10, F 5'-GTGGGATGCTCCAAGGATCT-3' and R 5'-AGCTGTGGATGCTCTATGC-3'; and R 5'-GGCTCAGGACCTATCTTC-3'.
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 naive and infected mice at different times after cornea 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 animals than in controls (Fig. 4A). CD4+ T cell numbers, too, were ∼3-fold greater in Treg-depleted mice than in controls (Fig. 4B). In addition, the numbers of neutrophils (CD45+CD11b+Ly6G+) and macrophages...
(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 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

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**FIGURE 2.** HSV-1 infection changes Treg cell surface phenotype. Foxp3-GFP mice corneas were scarified and infected with HSV-1. (A) Flow cytometry was used to detect CD103 expression on Tregs. Cells from DLN and spleen of naive (day 0) and HSV-1–infected mice at different time points were stained for CD4 and CD103. Histograms were gated on CD4+Foxp3+ GFP+ cells. The frequencies of Tregs expressing CD103 are shown. (B) Flow cytometry was used to detect different surface markers on Tregs: CTLA-4, ICOS, and PD-1. Cells from DLN and spleen of naive (day 0) and HSV-1–infected mice at different time points were stained for CD4, CTLA-4, ICOS, and PD-1. Histograms are gated on CD4+Foxp3+ GFP+ cells. The frequencies of Tregs expressing CTLA-4, ICOS, and PD-1 are shown. 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.
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 2 d after depletion (day 10), and the phenotype of CD4+ T cells isolated from DLN and spleen was evaluated. Representative eye sections show increased cellular infiltration in DT-treated mice, starting on day 8 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 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.
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 CD49d^hi 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 CD62L<low> 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 effector T cells and neutrophils trafficked 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.

The problem with attempts to conclude that Tregs exert functional effects solely within inflammatory reactions is that 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 Tregs 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

![FIGURE 6. Treg depletion increases Th1 responses and cell numbers in response to HSV-1 infection. 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 IFN-γ-secreting cells gated on CD4+ T cells infiltrated in DLN on day 15 p.i. and stimulated with PMA/ionomycin for 4 h, from control and DT-treated mice. (B) Representative FACS plots, and frequencies and numbers of IFN-γ-secreting CD4+ T cells from spleen on day 15 p.i., from control and DT-treated mice. (C) IFN-γ protein levels analyzed by ELISA from control and DT-treated DLN and spleen on day 15 p.i. Data are representative of three independent experiments and show mean values ± SEM (n = 10–12 mice per group). Statistical levels of significance were analyzed by the Student t test (unpaired). **p ≤ 0.01, ***p ≤ 0.001.](http://www.jimmunol.org/)}
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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