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Coordination of Early Protective Immunity to Viral Infection by Regulatory T Cells

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Abstract

Suppression of immune responses by regulatory T cells (Tregs) is thought to limit late stages of pathogen-specific immunity as a means of minimizing associated tissue damage. We examined a role for Tregs during mucosal herpes simplex virus infection in mice, and observed an accelerated fatal infection with increased viral loads in the mucosa and central nervous system after ablation of Tregs. Although augmented interferon production was detected in the draining lymph nodes (dLNs) in Treg-deprived mice, it was profoundly reduced at the infection site. This was associated with a delay in the arrival of natural killer cells, dendritic cells, and T cells to the site of infection and a sharp increase in proinflammatory chemokine levels in the dLNs. Our results suggest that Tregs facilitate early protective responses to local viral infection by allowing a timely entry of immune cells into infected tissue.

Regulatory T cells (Tregs) expressing the transcription factor Foxp3 play an essential role in controlling immune response-mediated inflammation. Their importance is emphasized by the fact that deficiency in Tregs results in a fatal autoimmune syndrome affecting multiple organs (1, 2). Theoretically, the potent suppressor function of Tregs might present a serious obstacle to establishing robust protective immunity toward pathogens, and recent studies addressing a role for Tregs during infection have suggested several mutually exclusive scenarios. Some studies have suggested that by limiting late immune responses to an infectious agent, Tregs minimize associated tissue damage while at the same time preventing or diminishing pathogen clearance (3-5). Alternatively, it has been proposed that during viral infection, Tregs lose their suppressor capacity in response to engagement of virus-sensing mechanisms such as Toll-like receptor (TLR) signaling (6). Another study suggests that effector T cells responding to infection might become resistant to Treg-mediated suppression as a result of exposure to proinflammatory cytokines and increased costimulatory signals (7). Thus, with several scenarios proposed, the role for Tregs during infection remains unclear.

We examined a role for Tregs in mucosal herpes simplex virus (HSV) infection by taking advantage of *Foxp3^{gfp}* and *Foxp3^{DTR}* knock-in mice harboring Treg subsets tagged with green fluorescent protein (GFP) or a human diphtheria toxin receptor (DTR), respectively. This allowed us to track and isolate Tregs and to efficiently ablate these cells upon in vivo DT treatment (8, 9). In these studies we used a well-established model of genital HSV-2 infection via a natural route (10). In HSV-2-infected mice, initial viral replication is limited to the vaginal mucosa (11), followed by spread into the central nervous system (CNS) upon retrograde transport of virions into the sacral ganglia, resulting in a fatal paralysis. The adaptive immune response to HSV-2 is dominated by virus-specific T helper 1 (T_H1) cells essential for limiting HSV-2 replication (12, 13).

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We first examined whether Tregs respond to viral infection by monitoring the dynamics of the GFP-tagged Treg subset relative to “effector” T cells, as defined by the absence of Foxp3, in infected *Foxp3^{gfp}* mice. After genital infection with HSV, the total numbers of both non-Tregs and Tregs drastically increased in the draining lymph nodes (dLNs) and at the site of infection with essentially identical kinetics, and both subsets displayed an increased proportion of cells expressing activation markers (Fig. 1, A to D). Furthermore, using continuous in vivo 5-bromo-2'-deoxyuridine (BrdU) labeling, we observed that CD4⁺Foxp3⁻ and Foxp3⁺ Tregs that had undergone cell division could be found in both the dLNs and at the infection site within 4 days of viral challenge (Fig. 1, E and F). Finally, Tregs isolated from the dLNs of infected mice had a measurably greater potency in suppressing the virus-specific proliferative response of CD4 T cells relative to Tregs isolated from uninfected mice (Fig. 1G). Taken together, these results show that Tregs, rather than remaining refractory, can become activated upon local viral infection.

In light of this activation, we next examined whether Tregs might fulfill a specific role during the immune response to HSV-2. Previous studies have shown enhanced CD8⁺ T cell responses in mice treated with antibody to CD25 that were infected with HSV-1 via footpad injection, whereas depletion of CD25⁺ Tregs in a corneal HSV-1 infection model has been reported to increase severity of T cell-mediated tissue lesions (14, 15). On the basis of these studies, we anticipated that Treg depletion during genital HSV infection might enhance the immune response to the virus. However, *Foxp3^{DTR}* mice succumbed more rapidly to genital HSV infection after DT-induced depletion of Foxp3⁺ Tregs (Fig. 2A), developing severe lesions and hindlimb paralysis 4 to 5 days earlier than Treg-sufficient mice (Fig. 2B).

The uninfected *Foxp3^{DTR}* mice did not develop any signs of CNS and genital tract pathology resulting from Treg ablation. Unexpectedly, infected, DT-treated *Foxp3^{DTR}* mice carried significantly elevated viral loads at the site of infection, relative to *Foxp3^{WT}* mice, by day 3 after infection (Fig. 2C). High titers of virus were also present in the spinal cord of *Foxp3^{DTR}* mice 4 days after infection, a time at which *Foxp3^{WT}* spinal cords remained virus-free (Fig. 2D). Note that viral titers from infected *Foxp3^{DTR}* mice reconstituted with DTR-negative Foxp3⁺ Tregs were similar to those detected in *Foxp3^{WT}* mice (fig. S1); this result indicates that ablation of Tregs was solely responsible for the increased viral titers and that Tregs facilitate protective immunity to HSV infection.

Type I interferons, produced primarily by plasmacytoid dendritic cells (pDCs) in response to TLR9 stimulation, and interferon- γ (IFN- γ), produced by CD4⁺ T cells and natural killer (NK) cells, are indispensable for the protective immune response to HSV (12, 16). This led us to hypothesize that ablation of Tregs might in some way impair the production of these essential antiviral cytokines. However, we found sharply augmented IFN- γ production by virus-specific CD4 T cells (Fig. 3A) as well as enhanced levels of IFN- α in the dLNs of infected Treg-ablated mice (Fig. 3B). Furthermore, a comparable increase in the number of activated T cells and in the production of IFN- γ , tumor necrosis factor- α , and interleukin-2 (IL-2), potentially produced by “self”-reactive CD4⁺ T cells, was observed both in uninfected and infected *Foxp3^{DTR}* mice after Treg ablation (figs. S2 and S3). These results suggest that Tregs limit proinflammatory responses regardless of infection status.

Although interferon levels in the dLNs were increased, we reasoned that local rather than systemic interferon production might restrain the infection. To test this idea more directly, we examined type I and II interferon levels at the site of viral replication, the vaginal mucosa. Indeed, in contrast to augmented IFN- γ production in the dLNs, vaginal IFN- γ as well as IFN- α levels remained low in the absence of Tregs (Fig. 3, C and D). This discrepancy between interferon production in the dLNs and in infected tissue suggested that ablation of Tregs might impair immune cell trafficking to the infected tissue.

To test this, we examined the proportion of effector immune cell subsets including NK cells, CD4⁺ T cells, pDCs, and the dominant antigen-presenting cells (APCs), DCs, in the dLNs and vaginal mucosa early during infection. On day 2 after infection, we found an increased presence of these cells in the dLNs of infected, Treg-ablated mice. Comparable increases in these cell subsets were found in the LNs of DT-treated uninfected *Foxp3^{DTR}* mice (Fig. 3E). In contrast, the site of infection showed a marked decrease in the number of these immune cells at 2 days after infection when Tregs were absent (Fig. 3E). Because the peak of IFN- α produced by pDCs and IFN γ produced by NK cells occurs at this early time point, this result was in full agreement with the negligible local interferon production in Treg-depleted mice.

At day 4 after infection, the numbers of NK cells and pDCs were low irrespective of the presence of Tregs. Thus, it appeared that ablation of Tregs impaired the migration of these critical antiviral effector cells to the site of infection. At this time, however, CD4⁺ T cell numbers at the site of infection were greater in the absence of Tregs than in their presence, whereas CD11b⁺ DC numbers were similar in both groups, indicating that arrival of these two cell subsets had been delayed by about 2 days. Together, these results suggest that early during infection, Tregs provide an important means of balancing the trafficking of effector cells between the sites of immune induction and sites of infection.

To directly address this question, we adoptively transferred carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cell populations into HSV-infected Treg-sufficient or Treg-deficient hosts. In the absence of Tregs, the proportion of CFSE⁺ DCs, pDCs, and CD4⁺ T cells increased in the LNs 24 hours after transfer relative to Treg-intact mice (Fig. 4A). In contrast, there was a striking absence of the CFSE⁺ NK, DC, and CD4 T cells and a major reduction in pDCs in the vaginal tract of the infected hosts (Fig. 4A). Thus, the absence of Tregs resulted in enhanced entry and retention of effector cells in the dLNs, coupled with a lack of migration to the site of infection. One likely scenario for this would be an alteration in essential chemotactic cues for migrating effector cells. Consistent with this idea, global disruption of cellular trafficking in HSV-infected mice treated with pertussis toxin resulted in an increase in viral titers similar to that seen upon ablation of Tregs (fig. S4). In parallel, we also observed that levels of chemokines CXCL10, CCL2, CXCL9, and lymphoid chemokine CXCL13 were greatly increased in the dLNs upon Treg ablation (Fig. 4B and fig. S5). Upon infection of Treg-intact mice, CCL21 and CXCL13 were decreased, in agreement with a recent report (fig. S5) (17).

The data thus far suggest that by exerting control over inflammatory chemokine cues, Tregs might achieve immune control indirectly by limiting CD4 T cell activation or by directly affecting chemokine-producing cell types. To distinguish between these alternatives, we measured chemokine levels in the LNs of HSV-infected *Foxp3^{DTR}* mice subjected to DT-mediated ablation of Foxp3⁺ Tregs alone or in combination with antibody-mediated CD4⁺ T cell depletion. Simultaneous depletion of CD4⁺ T cells and Tregs prevented the lymphadenopathy characteristic of Treg depletion alone (fig. S6A), yet resulted in a similar increase in inflammatory chemokines in the LNs (fig. S6B). Thus, it is likely that Tregs suppress inflammatory chemokine production in the LNs directly. To identify cellular sources of LN chemokines, we measured chemokine mRNA levels in fluorescence-activated cell sorter-purified cell subsets from *Foxp3^{WT}* or *Foxp3^{DTR}* mice. We found that CXCL9 and CCL2 mRNA levels strongly increased in DCs and that CXCL10 increased in both DCs and CD45⁻ stromal cells in the absence of Tregs (Fig. 4C). Additionally, CXCL10, CCL2, and CCL5 were elevated in LN NK cells, whereas pDCs did not show differences in the absence of Tregs (fig. S7). In contrast to increased chemokine production, CD4⁺ T cell levels of sphingosine 1-phosphate receptor-1 (S1P₁) were unchanged in the absence of Tregs (fig. S8), excluding potential modulation of S1P₁ as a cause of delayed egress of CD4⁺ T

cells from the dLNs. Examination of chemokines in the vaginal tract of infected Treg-deficient mice revealed increased expression of CXCL10 and CCL2 relative to Treg-sufficient mice, albeit at levels considerably lower than those found in the dLNs (Fig. 4B). Unlike CXCL10 and CCL2, CCL5 was largely lacking in the vaginal tissue of Treg-depleted mice, in contrast to a markedly augmented CCL5 expression found in Treg-replete HSV infected mice (Fig. 4B). This could account for a failure of recruitment to the infected tissue of CCR5-expressing cells, including pDCs. In agreement with this notion was a previous finding that protection against lethal HSV-2 challenge afforded by administration of CpG was coincident with a wave of CCL5 as well as IFN- γ , IL-12, and IL-18 production in the genital mucosa (18).

It is likely that Treg-dependent suppression of proinflammatory chemokines in the lymph nodes plays an important role in restraining viral replication in nonlymphoid tissues other than mucosal epithelium. To test this possibility, we examined liver viral titers and LN chemokine levels during lymphocytic choriomeningitis virus (LCMV) Armstrong infection of Treg-replete or Treg-depleted mice. In agreement with the HSV data, in the absence of Tregs we observed an increase in LCMV liver titers on day 5 after infection, corresponding to the peak viral load (19), as well as increases in CXCL10 and CCL2 in the LNs (Fig. 4D). Thus, the observation of increased pathogen replication and altered chemokine milieu in secondary lymphoid organs in the absence of Tregs in mucosal HSV infection can be extended to a systemic LCMV infection.

Our results show that Tregs facilitate early immune responses to local viral infection at least in part by orchestrating a timely homing of immune effector cells to the site of infection. This unexpected finding of an immune response-promoting role for Tregs seems to be applicable to other infections resulting in pathogen replication in nonlymphoid tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Tregs respond to local HSV-2 infection with kinetics similar to those of effector CD4⁺ T cells. (A to F) *Foxp3^{gfp}* mice were infected with HSV-2, and vaginas and dLNs were prepared for analysis by flow cytometry. CD4⁺ T cells are CD4⁺Foxp3⁻ and Tregs are CD4⁺Foxp3⁺. CD4⁺ T cells (C) and Tregs (D) in the dLNs were stained for inducible costimulatory molecule expression. Mice were administered BrdU in the drinking water throughout the course of infection (E and F), and BrdU incorporation by CD4⁺ T cells and Tregs was detected by flow cytometry. (G) Effector T cells and antigen-pulsed APCs were cocultured with Foxp3⁺ suppressor cells as indicated, ³H was added, and cells were cultured for an additional 20 hours before harvest and measurement of proliferation with a beta-counter.

Fig. 2.

Tregs are required to prevent early death from HSV-2 infection. *Foxp3*^{WT} or *Foxp3*^{DTR} mice were infected with HSV-2 and treated with DT. Survival (A) and disease score (B) were monitored daily and vaginal washes were collected daily to assess vaginal viral titer (C) by plaque assay on Vero cells. (D) Spinal cords were collected 4 days after infection and plaque assays were performed on homogenates.

Fig. 3.

Tregs are required to mount a protective immune response at the site of infection subsequent to genital HSV-2 infection. *Fcpx3^{WT}* or *Fcpx3^{DTR}* mice were infected with HSV-2 and treated with DT. (A) For ex vivo cultures, CD4⁺ T cells were isolated from the dLNs of naive or infected *Fcpx3^{WT}* or *Fcpx3^{DTR}* mice and cultured for 3 days with irradiated, heat-inactivated HSV-2-pulsed APCs. IFN- γ was detected by enzyme-linked immunosorbent assay (ELISA). (B) Mice were infected and treated with DT as in Fig. 2, dLNs were collected 2 days after infection, and extracts were prepared for IFN- α detection by ELISA. (C and D) IFN- γ present in vaginal washes collected at various times after infection (C) and IFN- α from vaginal washes collected 2 days after infection (D) were measured by ELISA. (E) At the indicated times after infection, dLNs and vaginal tracts were subjected to flow cytometric analysis. Naive mice received DT according to the same schedule as infected mice.

Fig. 4.

Tregs modulate the chemokine gradient that controls proper effector cell homing to the dLNs and site of infection. (A) NK cells, CD11b⁺ DCs, and pDCs from naïve donors or CD4⁺ T cells isolated from the dLNs of *Foxp3*^{gfp} donor mice infected with HSV-2 for 6 days were labeled with CFSE and injected into recipient mice. Recipients were *Foxp3*^{WT} or *Foxp3*^{DTR} mice treated with DT and infected with HSV-2, and mice received CFSE-labeled cells 2 days after infection. Twenty-four hours after transfer, dLNs and vaginal tracts from recipient mice were examined for percentage of the indicated population that was CFSE⁺ by flow cytometry. (B) *Foxp3*^{WT} or *Foxp3*^{DTR} mice were infected with HSV-2 and treated with DT. Two days after infection, dLNs and vaginal tract extracts were prepared for chemokine detection by Luminex bead assay. (C) Chemokine mRNA amounts in DCs and CD45⁺ stromal cells were measured by real-time PCR and are shown as the mean of three independent experiments relative to hypoxanthine-guanine phosphoribosyl transferase

expression. **(D)** Foxp3^{DTR} mice were treated with DT and infected with LCMV. Five days after infection, livers were collected and assayed for viral titer, and LNs were analyzed for chemokines as in (B).