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Regulatory T Cells Shape the Resident Memory T Cell Response to Virus Infection in the Tissues

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Regulatory T cells (Tregs) are well known for their role in dampening the immune responses to self-Ags and thereby, limiting autoimmunity. However, they also must permit immune responses to occur against foreign infectious agents. Using a mouse model of West Nile virus infection, we examined the role of Tregs in the generation of effector and memory T cell responses in the secondary lymphoid organs, as well as in the infected tissues. We found that Treg numbers and activation increased in both the secondary lymphoid organs and CNS postinfection. Using Foxp3ΔΔΔΔΔΔ knock-in mice, we found that Treg-deficient mice had increased Ag-driven production of IFN-γ from both CD4+ and CD8+ T cells in the spleen and CNS during the effector phase. In mice lacking Tregs, there were greater numbers of short-lived effector CD8+ T cells in the spleen during the peak of the immune response, but the memory CD8+ T cell response was impaired. Specifically, we demonstrate that Treg-dependent production of TGF-β results in increased expression of CD103 on CD8+ T cells, thereby allowing for a large pool of resident memory T cells to be maintained in the brain postinfection. The Journal of Immunology, 2014, 192: 683–690.

Regulatory T cells (Tregs) are well known for their suppressive properties, which can reduce immune responses to self-Ags and prevent autoimmunity (1, 2). Recent work also highlighted the role of Tregs in the immune response to microbial infection (3). Several groups reported that Tregs limit vigorous immune responses that would assist in pathogen clearance at the expense of damaging healthy tissue (4). In some cases, this leads to a severely diminished effector T cell response that is unable to clear the infection adequately (5). Additionally, Tregs were shown to facilitate early immune responses to viral infection by coordinating a timely trafficking of lymphocytes to the infection site in an HSV-2 model (6). Thus, because Tregs have demonstrated roles in the suppression of, as well as the generation of, antimicrobial immunity, we hypothesized that Tregs could have distinct roles in antiviral immunity depending on the time postinfection, as well as the tissue microenvironment.

Thus, in this study, we used a well-established mouse model of West Nile virus (WNV) infection to investigate a role for Tregs in T cell responses to neurotropic virus infection at various times postinfection, as well as in various tissues. WNV is an ssRNA virus that cycles between mosquitoes and birds, with humans and other mammals serving as incidental hosts. Approximately 20% of infected individuals experience a limited febrile illness, with 1% developing a more severe neuroinvasive disease characterized by encephalitis and meningitis (7). The immune response to WNV is known to involve both innate and adaptive responses, including humoral and cellular components. Upon infection in the skin following injection or mosquito bite, WNV replicates and is able to infect dendritic cells (DCs), including Langerhans cells, which can subsequently migrate to the draining lymph nodes (dLNs) where they initiate immune responses. DCs and other cells sense the presence of RNA virus infection through TLRs expressed within the endosomal compartment, as well as ubiquitously expressed cytoplasmic RNA sensors, such as RIG-I and MDA-5 (8). One key immune mediator downstream of this virus-sensing mechanism is type I IFN, an important antiviral molecule capable of eliciting multiple antiviral pathways. Both T and B lymphocytes are involved in protection against WNV, and it was demonstrated in mouse studies that humoral immunity is involved in peripheral clearance of WNV, whereas T cells are critical for viral clearance within the CNS. Specifically, the induction of virus-specific IgM early postinfection with WNV limits viremia and spread to the CNS, thus helping to protect against lethal infection (9, 10). CTLs are also known to mediate immunity to WNV infection, because adoptive transfer of WNV-specific CD8+ T cells results in a reduction of mortality and prolonged survival after WNV infection of recipient mice. CD8+ T cells were found to infiltrate the infected brain, suggesting that they could be involved in recovery from encephalitis (11). CD4+ T cell responses are also strongly induced and are required for the maturation of IgG responses, as well as sustaining CD8+ T cell responses, both in the periphery and the CNS. Nevertheless, an absence of CD4+ T cells did not cause a significant difference in viral titers in the periphery (12). WNV likely traffics from peripheral tissues to the CNS via axonal spread or by a hematogenous route across the blood–brain barrier (13). The generation of immune responses within the CNS is critical to clear the virus; however, at the same time, they must be regulated such that damage to nonrenewing populations of neurons is limited. Postinfection, CD8+ T cells migrate to the brain, and their presence correlates with viral clearance (14). Indeed, in the absence of CD8+ T cells, WNV persists in the brain of infected mice (15). Recruitment of T cells to the CNS is mediated by both CCL5 and CXCL10 (16–18). Although it was postulated that CD8+ T cells may have a pathologic role in terms of damaging infected neurons, in addition to their protective role during WNV infection, it is
certainly clear that CD8+ T cells are required for clearance of WNV from the CNS.

Studies (19, 20) in mice showed that WNV can persist in the periphery and CNS, despite the presence of virus-specific immune cells and Abs, for ≥16 wk postinfection. It is hypothesized that the virus might persist in certain tissues, such as the brain, for longer periods, as a result of the slow turnover of neuronal tissue and the need to limit immunopathology within the brain (21). Given the persistence of WNV in the CNS and the role of Tregs in balancing an adequate, but not overly robust, immune response, we sought to investigate how Tregs might modulate the effector and memory T cell response to virus infection in both the secondary lymphoid organs (SLOs), as well as the infected neuronal tissues. Further, because depletion of Tregs is known to result in increased WNV disease, weight loss, and lethality (22), we hypothesized that there would be dramatic effects on the T cell response to virus infection upon Treg depletion.

Our results show that Tregs limit Ag-driven proliferation of effector T cells in the CNS, as well as the production of inflammatory cytokines. In mice depleted of Tregs, there is an expansion of short-lived effector cells (SLECs) in the spleen at the peak of the effector phase, but the retention of Ag-specific CD8+ T cells in the brain is impaired. Specifically, we found that Treg-dependent production of TGF-β leads to increased expression of CD103 on CD8+ T cells, allowing a greater number of these resident memory T cells to remain in the brain postinfection. In sum, our study demonstrates the two-sided nature of Tregs; they can suppress but also potentiate the immune response to microbes, depending on the time postinfection, as well as the location within the host.

Materials and Methods

Virus

WNV TX-2002-HC was kindly provided by Dr. Michael Gale, Jr. (University of Washington) and propagated as previously described (23, 24). Working stocks were generated from supernatants collected from infected Vero cell lines and were stored at −80°C.

Mice and infection

C57BL/6 mice, 6–8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in filtered cages. Foxp3GFP (25) and Foxp3SIPGFP (26) mice (kindly provided by Dr. Alexander Rudensky, Memorial Sloan-Kettering Cancer Center, New York, NY) were bred onsite at the animal facility at the Fred Hutchinson Cancer Research Center (FHCRC). Age-matched 6–12-wk-old mice were inoculated s.c. in the left rear footpad with 100 PFU WNV TX-2002-HC. To ablate Tregs, mice from C57BL/6 mice were also stained with Foxp3-APC (eBioscience), and stained with intracellular Abs were subsequently fixed, permeabilized (Foxp3 Fixation/Permeabilization Concentrate and Diluent; eBioscience), and stained with intracellular Abs from Integrated DNA Technologies (Coralville, Iowa). Cells were stained with intracellular Abs and all intracellular staining.

Intracellular cytokine staining

Splenocytes or CNS lymphocytes were resuspended at 1 × 106 cells/well in RPMI 1640 supplemented with 10% HI-FBS, 2.5 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 1 mM sodium pyruvate to be analyzed for intracellular cytokine production. Lymphocytes were stimulated with one of four different stimulation mixtures: media, the WNV NS4b peptide (SSVWNATTAI), heat-inactivated WNV (multiplicity of infection = 5) or polyclonal stimulation using anti-CD3/CD28, and incubated for 5 h at 37°C. Cells were washed and stained with fixable viability stain (Fixable Viability Dye eFluor 780; eBioscience) for 30 min on ice, followed by surface staining for CD4-Pe and CD8-PECy5 for 15 min on ice. The cells were then fixed, permeabilized, and stained with IFN-γ-PerCP/Cy5.5 and TNF-α–allophycocyanin, according to the manufacturer’s protocol (eBioscience). After staining, the cells were analyzed as described above.

RNA extraction

Five brains/group were collected after whole-body perfusion with 10 ml cold PBS, snap-frozen into pieces ∼1 mm in thickness, and immediately placed into preweighed tubes containing 7 ml RNA Later Stabilization Reagent (QIAGEN, Hilden, Germany). Samples were weighed to determine the weight of brain alone and placed in a −80°C freezer. Brains were thawed at 4°C and homogenized using a handheld homogenizer, and total RNA was extracted following protocol instructions included with the RNeasy Lipid Tissue Midi Kit (QIAGEN). RNA was eluted into PCR-grade nuclease-free water, and RNA concentration was measured using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific).

Quantitative RT-PCR for WNV RNA

Competent Escherichia coli cells, transformed with kanamycin-resistant plasmids containing the WNV PCR target region, were kindly provided by Dr. Michael Gale, Jr. (University of Washington). Cells were grown in kanamycin-containing media, and plasmids were isolated using the QIAGEN Plasmid Mini kit (QIAGEN). The plasmid concentration was determined using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific), and a 10-fold standard dilution series was generated spanning a concentration of 1 × 105 to 1 × 1010 plasmids/μl.

WNV primers and probe were used as previously described (27). The fluorescent probe was synthesized with a 5’ reporter dye FAM and a 3’ quencher dye TAMRA. Primers and probe were generated as custom assays from Integrated DNA Technologies (Coralville, Iowa). Quantitative RT-PCR assays were performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Life Technologies, Grand Island, NY). Reactions were carried out in a total volume of 20 μl, containing 5 μl template RNA, 1× reaction mix, 500 mM final concentration for forward and reverse primers and 250 mM final concentration for probe, 0.4 μM ROX dye, and 0.4 μl RT/Taq polymerase enzyme mix and brought up with nuclease-free water. After adding the reaction mixture and template RNA to MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems, Foster City CA), reverse transcription and amplification were carried out on the ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems) in standard mode. Cycling conditions were as follows: 50°C for 15-min hold (cDNA synthesis step), 95°C for 2-min hold, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min.
Whole-brain homogenates or purified samples of Tregs or conventional CD4+ T cells were prepared and assayed for TGF-β by ELISA. Cell populations were isolated using the CD4+ CD25+ Regulatory T Cell Isolation kit (Miltenyi Biotec, Auburn, CA). Non-CD4+ cells were indirectly magnetically labeled and depleted over a MACS column, and the flow-through fraction of pre-enriched CD4+ T cells was labeled with CD25 MicroBeads for subsequent positive selection of CD4+CD25+ Tregs. Brain homogenates and purified cell populations were prepared and lysed using Nonidet P-40 lysis buffer containing complete protease inhibitor mixture tablets (Roche, Indianapolis, IN) for protein quantification. Lysates were centrifuged, and supernatants were stored at −80°C until the ELISA was performed according to the manufacturer’s protocol (Mouse TGF-β ELISA kit; eBioscience). Briefly, samples were treated with acid and then neutralized to activate the latent TGF-β1 to the immunoreactive form. Standards and samples were incubated for 2 h at room temperature, followed by incubation with biotin and streptavidin-HRP. TMB substrate was used, and the plate was read by a spectrophotometer at 450 nm.

Statistical analysis

When comparing groups, two-tailed unpaired Student t tests were conducted, with p values < 0.05 considered significant. Error bars show ± SEM.

Results

Treg numbers increase and become activated in the dLNs and CNS after WNV infection

To determine whether Tregs respond to WNV infection, we first examined the kinetics and activation status of the Treg population following infection in both the footpad-draining popliteal lymph node and the brain. WNV infection was performed by s.c. injection in the rear footpad to mimic a mosquito bite, because WNV is a vector-borne viral infection. Importantly, Foxp3+ Tregs were detectable in the brain postinfection, although they were largely absent in the naive brain (Fig. 1A), thereby demonstrating that Tregs are present and could play an important role in the immune response to WNV within the infected tissues. In the dLNs, Tregs expanded rapidly, beginning by day 4 postinfection and peaking around day 15, with total Treg numbers then decreasing through day 20. In the brain, Treg expansion occurred more gradually, with high numbers maintained out to a memory time point: day 60 postinfection (Fig. 1B). Treg expansion kinetics in the dLNs and brain were similar to those observed for the effector CD4+ T cell population (data not shown). Likewise, activated effector T cell and Treg populations increased postinfection in both the dLN and brain, as indicated by increased expression of ICOS (Fig. 1C, data not shown). The frequency of CTLA-4+ and CXCR3+ Tregs also was increased in both the dLNs and brain (data not shown).

Tregs limit CD4+ and CD8+ T cell activation and infection-induced cytokine production

Because we observed Treg expansion and activation during WNV infection, we next examined the immune response in the infected tissues. In the context of infection. Twelve days postinfection, we performed intracellular cytokine staining to evaluate the number of CD4+ and CD8+ T cells that were producing IFN-γ, TNF-α, or both cytokines. The frequency of both CD4+ and CD8+ T cells producing IFN-γ directly in response to virus was increased in both the spleen and the brain of Treg-deficient mice, reaching statistical significance in the peripheral site of virus infection, the brain (Fig. 2C). Additionally, of the WNV-specific CD4+ and CD8+ T cells in both the SLOs and brain, Treg-ablated mice had an increased proportion of cells that were polyfunctional in their ability to produce both IFN-γ and TNF-α (Fig. 2D), likely leading to a more proinflammatory environment. In contrast, Treg-sufficient mice had more CD8 T cells producing IFN-γ or TNF-α alone,
was demonstrated by other investigators examining CD8 T cell functionality after WNV infection (Fig. 2D) (28–30). Finally, we quantified the levels of WNV present in the brain and spleen in Treg-sufficient or Treg-deficient mice. At day eight postinfection, there was no difference in WNV RNA in the spleen, regardless of the presence or absence of Tregs; further, there was no statistically significant difference at day 8 or 11 postinfection in the brain (Fig. 3), suggesting that the increased proinflammatory cytokine production observed in the brain upon Treg depletion is not due simply to enhanced virus replication upon Treg ablation.

Tregs modulate the fate of WNV-specific effector CD8 T cells in distinct tissue compartments

Because we observed differences in effector T cell activation and cytokine production during WNV infection in the absence of Tregs, we next considered how Tregs could affect the fate of WNV-specific T cells. To evaluate the effect of Treg ablation on the effector-to-memory cell transition in the context of WNV infection, we examined the frequency of WNV-specific CD8+ T cells expressing the cell surface markers CD127 or KLRG-1 in Treg+ or Treg− infected mice (31). Similar to results published previously using a vaccinia virus vector (32), ablation of Tregs resulted in increased numbers of SLECs, defined as CD127− KLRG-1+ tetramer+ CD8+ T cells, in the spleen and CD8+ T cells in the brain at day 12 postinfection.
a stable memory pool at day 60 postinfection. The pattern of CD8 T cell dynamics was similar, regardless of the presence or absence of Tregs, but Treg ablation resulted in an increase in the total number of Ag-specific CD8+ T cells in the spleen at the peak of the effector phase (Fig. 4C). Strikingly, in the brain we observed that, in Treg-sufficient animals, there was a steady increase in WNV-specific CD8+ T cells following infection out to a memory time point. In the absence of Tregs, there were similar numbers of Ag-specific CD8+ T cells in the brain at day 60 in the absence of Tregs compared with in the presence of Tregs (\(p = 0.0284\), Fig. 5), suggesting that Tregs are vital in the establishment of a TRM population in the brain following WNV infection, as well as hinting at a mechanism by which Tregs could control recruitment and retention of these cells. Of note, WNV-infected Treg-deficient mice have a sizable population of tetramer- CD8 T cells in the brain that express CD103; however, because this population is similar in frequency to that observed in the brains of naive mice, we hypothesize that these are not WNV-specific T cells but rather are cells that are resident in the brain prior to infection with WNV (Fig. 5A).

Because the expression of CD103 is known to be positively regulated by TGF-\(\beta\) (33, 35), we next examined the levels of TGF-\(\beta\) protein in the brain at various times post-WNV infection. TGF-\(\beta\) levels were significantly lower in Treg-deficient mice 7 d postinfection compared with Treg-sufficient mice (\(p = 0.0423\)). Additionally, there was a trend for decreased TGF-\(\beta\) levels in Treg-deficient mice at days 9 and 12 post-WNV infection (Fig. 6A). Further, Tregs are a key cellular source of TGF-\(\beta\) in the brain after WNV infection because they contain significantly higher levels of TGF-\(\beta\) protein compared with conventional CD4 T cells (\(p = 0.0003\), Fig. 6B). Therefore, we conclude that Tregs directly modulate the TRM response to WNV in the brain via production of TGF-\(\beta\).
Discussion

The generation of a robust antiviral immune response involves a rapid and dramatic clonal expansion of Ag-specific effector CD4+ and CD8+ T cells, which then contract to form a small, but potent, pool of stable memory T cells. In non-SLO tissues, it was shown that effector memory T cells are particularly poised for instant response to infection, because memory CD8+ T cells from non-lymphoid tissues display effector levels of lytic activity directly ex vivo (36). Because it is known that circulating memory T cells do not access all tissues during immune surveillance, particularly the brain and intestinal lamina propria (37), the generation of TRM populations may be of particular importance in these tissues. TRMs are characterized as being tissue resident and self-renewing, as well as extremely protective against repeat infections. An elegant study of TRMs in the brain by Wakim et al. (34) demonstrated that these cells express CD103, which was shown to be a phenotypic marker, as well as critical for their generation and accumulation. Further, Wakim et al. (34) demonstrated that brain TRMs can persist in the absence of persistent antigenic stimulation, although they require local DC-dependent Ag presentation to upregulate CD103 on the incoming T cells.

During infection with WNV, a balance must exist between a robust immune response that clears the pathogen but limits destruction of nonrenewing populations of neurons in the brain. Given the role of Tregs in regulating and maintaining a delicate balance in immune responses to viral infections, we investigated the role of Tregs in the generation of effector and memory T cells both systemically and in the infected CNS. Using a mouse model in which Tregs could be ablated prior to infection, we showed that Treg-deficient mice had greater numbers of short-lived effector CD8+ T cells in the spleen during the peak of the immune response, but the memory CD8+ T cell response in the brain was impaired. Specifically, we demonstrate that Treg production of TGF-β results in increased expression of CD103 on CD8+ T cells, thereby allowing for a large pool of resident memory T cells to be maintained in the brain postinfection. In our model of WNV infection in mice, as opposed to the work done by Wakim et al. (34) using intranasal infection with vesicular stomatitis virus, it is possible that there is prolonged Ag presentation in the brain, even after viral clearance, because Appler et al. (19) showed that WNV RNA persists in the CNS up to 3 mo postinfection. This prolonged Ag presentation could drive CD103 expression on TRMs, perhaps through induction of Treg-dependent TGF-β production.

In the absence of Treg regulation during the immune response to viral infection, it is possible that bulk expansion of nonspecific CD4+ and CD8 effector T cells contributes to the net increase in effector T cells observed in the CNS. However, we showed that Treg-deficient mice had increases in CD4 and CD8 cells specifically producing cytokine in response to WNV stimulation. Although these Treg-deficient mice had increased numbers of effector T cells and more robust cytokine production during the peak of infection, the number of memory T cells in the brain was significantly decreased compared with wild-type–infected mice at day 60 postinfection. This demonstrates that Tregs are vital for the creation of...
memory T cells in the brain and that certain mechanisms and instructional cues must be in place to allow T cells to remain in such a tightly regulated area. Alternatively, it is possible that elimination of Tregs could impact the trafficking of CD8 T cells into the brain, which may explain the resultant differences that we observed in memory CD8 T cell numbers in the brain. Although this is possible, we favor the hypothesis that a lack of retention of CD8 T cells in the brain when Tregs are absent at the time of infection results in the deficit of TRM, because we note a decreased expression of CD103 on WNV-specific CD8 T cells in the brain upon Treg ablation (Fig. 5). Additionally, we did not observe a lack of WNV-specific CD8 T cells in the brain at early time points (Fig. 4A, 4C), suggesting that these cells are able to traffic to the CNS but fail to persist.

Our finding that Tregs control the generation of SLECs in the context of virus infection is similar to the findings by Kastenmüller et al. (32), who demonstrated that this was mechanistically due to a limited availability of IL-2, which is required for optimal SLEC generation. However, our results differ in that we uncovered a role for Tregs in the generation of immunological memory, likely due to the fact that we used a neurotropic virus capable of inducing CD8+ T cell memory responses in the brain, pointing to the different roles that Tregs can play in immunity, depending on tissue location. Indeed, we speculate that the presence of Tregs during neurotropic virus infection can restrain CD4+ and CD8+ T cell production of proinflammatory and antiviral cytokines in the brain, thereby resulting in the incomplete viral clearance demonstrated by other investigators (19). This continued antigenic presence, in turn, could be important for sustained TRM population.

In sum, our results suggest that Tregs are necessary to generate a pool of resident memory T cells, again highlighting the important role that these regulatory cells play in potentiating antiviral immunity. Although a significant population of T cells persisting in the brain could present a dangerous situation to the host, it is necessary to defend such critical tissues from neurotropic infection. Although we demonstrate that Tregs can negatively regulate T cell cytokine function in response to virus infection in the brain, they also play a pivotal role in establishing an appropriate TRM population with which to protect the host from reinfection. Finally, the differing roles of Tregs in the SLOs compared with neuronal tissues suggest that the tissue microenvironment provides vital cues that play a significant role in defining T cell fates and balancing the host immune response to microbial infection.

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Disclosures
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