Strongyloides ratti Infection Induces Expansion of Foxp3+ Regulatory T Cells That Interfere with Immune Response and Parasite Clearance in BALB/c Mice

Birte Blankenhaus, Ulrike Klemm, Marie-Luise Eschbach, Tim Sparwasser, Jochen Huehn, Anja A. Kühl, Christoph Loddenkemper, Thomas Jacobs and Minka Breloer

J Immunol 2011; 186:4295-4305; Prepublished online 18 February 2011;
doi: 10.4049/jimmunol.1001920
http://www.jimmunol.org/content/186/7/4295
Strongyloides ratti Infection Induces Expansion of Foxp3+ Regulatory T Cells That Interfere with Immune Response and Parasite Clearance in BALB/c Mice

Birte Blankenhaus,*† Ulrike Klemm,*‡ Marie-Luise Eischbach,* Tim Sparwasser,‡ Jochen Huehn,‡ Anja A. Kühl,§ Christoph Loddenkemper,§ Thomas Jacobs,* and Minka Breloer*

To escape expulsion by their host’s immune system, pathogenic nematodes exploit regulatory pathways that are intrinsic parts of the mammalian immune system, such as regulatory T cells (Tregs). Using depletion of Treg mice, we showed that Foxp3+ Treg numbers increased rapidly during infection with the nematode Strongyloides ratti. Transient depletion of Tregs during the first days of infection led to dramatically reduced worm burden and larval output, without aggravation of immune pathology. The transient absence of Tregs during primary infection did not interfere with the generation of protective memory. Depletion of Tregs at later time points of infection (i.e., day 4) did not improve resistance, suggesting that Tregs exert their counterregulatory function during the priming of S. ratti-specific immune responses. Improved resistance upon early Treg depletion was accompanied by accelerated and prolonged mast cell activation and increased production of types 1 and 2 cytokines. In contrast, the blockade of the regulatory receptor CTLA-4 specifically increased nematode-specific type 2 cytokine production. Despite this improved immune response, resistance to the infection was only marginally improved. Taken together, we provide evidence that Treg expansion during S. ratti infection suppresses the protective immune response to this pathogenic nematode and, thus, represents a mechanism of immune evasion.

The Journal of Immunology, 2011, 186: 4295–4305.

Pathogenic nematodes are large multicellular organisms that often reside for years within their mammalian hosts. To escape expulsion by the host’s immune system, they must avoid the induction of immune pathology and, thus, to avoid injuring their hosts, helminths need to dampen the host’s immune response. Therefore, helminths have used regulatory pathways that are intrinsic parts of the mammalian immune system for their purposes. By secreting analogs of regulatory cytokines, by inducing the upregulation of regulatory receptors, and by inducing the expansion of regulatory cell populations, these parasites actively interfere with the initiation of successful immune responses to themselves (1, 2). Regulatory T cells (Tregs) are the most prominent lymphocyte population involved in the counterregulation of adaptive immune responses (3). Tregs are characterized by the constitutive expression of the IL-2R α-chain, CD25, CTLA-4, and the transcription factor Foxp3. Although CD25 and CTLA-4 are also upregulated by effector T cells (Teffs) during activation, Foxp3 expression is still thought to be specific for Tregs in the murine system (4).

Several murine (5–12) and human (13–15) studies showed that ongoing helminth infections induce the expansion of Foxp3+ Treg numbers. Depletion or functional inactivation of Tregs resulted in a reduced worm burden in some studies, suggesting that Treg induction promotes helminth survival by interference with the generation of a protective immune response (6, 16–18). Because the majority of studies reported the occurrence of increased inflammation and immune pathology in the absence of Tregs (7, 19–21), their induction apparently also contributes to host survival. In most studies, the depletion or functional inactivation of Tregs was achieved by application of mAb specific for CD25 or CTLA-4, respectively. Because these receptors are also expressed by activated Teffs, the biologic effects observed could be due to interference with Treg and Teff function.

In this study, we used depletion of Treg (DEREG) mice to specifically investigate the role of Tregs during nematode infection. DEREG mice are transgenic for a bacterial artificial chromosome expressing a diphtheria toxin (DT) receptor-enhanced GFP (eGFP) fusion protein under the control of the foxp3 gene locus (22). Thus, they allow the detection of Foxp3+ Tregs due to eGFP expression and their depletion by application of DT without affecting Teffs. We used S. ratti as a model organism for pathogenic nematodes because it displays tissue-migrating and gut-dwelling life stages (23, 24). S. ratti infective third-stage larvae (iL3) actively penetrate the skin of their rodent hosts. They migrate via the head to the pharynx and are subsequently swallowed to reach the gut. The parasitic adults live embedded in the mucosa of the small intestine and reproduce by parthenogenesis. Eggs and hatched first-stage larvae (L1) are released with the feces to the free world...
They may develop directly into IL3 or grow up to free-living male and female adults that undergo sexual reproduction (26). Experimental infection of mice induces patent, but transient, infections that are resolved spontaneously within 2–4 wk and render the mice semiresistant to subsequent infections. Although mice, not being the natural host, display lower worm burden compared with the natural host, the rat, the overall kinetics of infection and immune response are comparable (27–29). S. ratti infection provokes a classical type 2 response in both species that is characterized by the induction of IL-13, IL-5, IL-3, and IL-10, as well as high titers of S. ratti-specific IgM and IgG1 (30, 31). Final expulsion of parasitic adults from the gut depends on the presence of mast cells that need to be activated in the context of this type 2 response (32–36).

In this article, we show that Foxp3+ Treg numbers increased rapidly during S. ratti infection. Transient depletion of Tregs during the first days of infection resulted in increased and accelerated immune responses and drastically reduced worm burden, without affecting the generation of protective memory or aggravating immune pathology. Selective CTLA-4 blockade also led to increased nematode-specific immune response, but resistance was only marginally improved. Taken together, we provide evidence that the expansion of Tregs during S. ratti infection suppresses the protective immune response to this pathogenic nematode.

Materials and Methods

Mice and parasites

All in vivo experiments were carried out at the animal facility of the Bernhard Nocht Institute for Tropical Medicine with permission of the Federal Health Authorities of the State of Hamburg, Germany. BALB/c mice were obtained from the animal facility of the University Hospital Eppendorf (Hamburg, Germany), BALB/c-DEREG mice were bred in the animal facilities of the Bernhard Nocht Institute for Tropical Medicine, and Wistar rats were purchased from Charles River (Sulzfeld, Germany). Animals were kept in individually ventilated cages under specific pathogen-free conditions and used at the age of 8–12 wk (mice) or 4–8 wk (rats). The S. ratti cycle was kindly provided by Dr. Uzinger (Swiss Tropical Institute, Basel, Switzerland) and maintained by serial passage of S. ratti through rats. IL3 of S. ratti were purified from charcoal feces cultures. Prior to infection, IL3 were stored overnight in PBS supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml).

Murine S. ratti infections

BALB/c-DEREG and wild-type littermates were infected by s.c. injections of 2000 purified IL3 in 30 µl PBS into the footpad. Groups of mice received 1 µg DT (Merck, Darmstadt, Germany) dissolved in PBS (pH 7.4) i.p. on three consecutive days, starting 1 d prior to S. ratti infection (early depletion), or at day 4 of S. ratti infection (late depletion). The depletion of Tregs was surveyed by analysis of peripheral blood samples at indicated time points for GFP, Foxp3, and CD4 expression. For the neutralization of CTLA-4 signaling, BALB/c mice received 300 µg anti-CTLA-4 mAb (clone 4F10). Hamster IgG was used for control groups. Mice were infected 3 h later with 1000 S. ratti IL3. Stool samples were collected for 24-h periods to detect eggs and L1 by quantitative PCR (qPCR). For analysis of worm burden, mice were sacrificed at the indicated time points and spleen, mesenteric lymph nodes (MLNs), and small intestine were prepared.

Analysis of worm burden

In experimental infections with S. ratti, egg and L1 output was analyzed by collecting feces over 24-h periods. DNA was extracted from 200 mg representative stool samples using the QIAamp DNA stool kit (Qiagen, Hilden, Germany), according to the manufacturer’s recommendations. DNA was eluted in 200 µl water and, finally, 2 µl a 1:10 dilution (in water) was used as template for the qPCR. S. ratti 28S RNA gene was quantified by qPCR, as described (31). Briefly, a 180-bp fragment of the S. ratti 28S RNA gene was amplified by qPCR in duplicates. For each run, a melting-curve analysis was performed to guarantee the specificity in each reaction tube. Comparative quantification (efficiency-corrected C T method) was used to transform the difference in C T values between the test samples and the calibrator sample into a copy number ratio. To count the number of adult nematodes in the gut, mice were sacrificed at the indicated time points postinfection (p.i.). The small intestine was sliced open longitudinally and incubated at 37°C for 3 h in a petri dish with tap water. The released adult females were collected, centrifuged for 5 min at 1200 rpm at RT, and counted. Microscopic analysis of the small intestine revealed that no significant numbers of adults remained. To count the number of migrating larvae in the tissue, total heads and lungs were prepared, minced, and incubated in petri dishes with tap water at 37°C for 3 h. Released larvae were collected by centrifugation for 10 min at 4000 rpm (no brake) and counted.

Cytokine production and proliferation

A total of 2 × 105 spleen or MLN cells was cultured in 96-well round-bottom plates in RPMI 1640 medium supplemented with 10% FCS, 20 mM HEPES, l-glutamine (2 mM), and gentamicin (50 µg/ml) at 37°C and 5% CO2. The cells were stimulated for 72 h with medium, anti-mouse CD3 (145-2C11, 1 µg/ml), or S. ratti IL3 lysate (20 µg/ml) that was prepared as described (31). The supernatant was harvested for analysis of cytokine production by ELISA. Cell proliferation was measured by uptake of [3H] thymidine for an additional 18 h of culture. All cultures were performed quadruplicate.

ELISA

Strongyloides-specific Ig in the serum was quantified by ELISA, as described (31). Briefly, 50 µl/well S. ratti Ag lysate (2.5 µg/ml) in PBS was coated overnight at 4°C on Microlon ELISA plates (Greiner, Frickenhause, Germany). Plates were washed four times with PBS 0.05% Tween 20 and blocked by incubation with PBS 1% BSA for 2 h at RT. Serial dilutions of sera in PBS 0.1% BSA were incubated in duplicate, adding 50 µl well overnight at 4°C. Plates were washed five times, and Strongyloides-specific Ig was detected by incubation with 50 µl/well HRP-conjugated anti-mouse IgG1 and IgM (Zymed Karlsruhe, Germany) for 1 h at RT. Plates were washed five times and developed by incubation with 100 µl/well tetramethylbenzidine 0.1 mg/ml, 0.003% H2O2 in 100 mM NaH2PO4 (pH 5.5) for 2.5 min. Reaction was stopped by addition of 25 µl well 2 M H2SO4, and OD at 450 nm (OD450) was measured. The titer was defined as the highest dilution of serum that led to an OD450 above the doubled background. Background was always <0.15 OD450. Murine cytokines (IL-3, IL-5, IL-10, IL-13, and IFN-γ) were measured in the culture supernatant of in vitro-stimulated spleen and MLN cells using DuoSet ELISA development kits (R&D Systems, Wiesbaden, Germany), according to the manufacturer’s instructions. MMPC-I in serum was detected using the MMPC-I ELISA assay kit (Moreduin Scientific limited, Penicuik, Scotland).

Flow cytometry

The expression of the surface molecule CD4 and the intracellular expression of Foxp3 and GFP in PBLs, spleen, and MLN cells were analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences). To prevent specific binding of mAbs, all samples were preincubated with 10% mouse serum at 4°C for 10 min. Surface staining was carried out for 20 min at 4°C using allophycocyanin-labeled anti-CD4 (clone RM4-5; BD Biosciences). Cells were permeabilized with 250 µl fixation/permeabilization buffer (eBioscience) for 30 min at 4°C, washed with permeabilization buffer (eBioscience), and stained with PE-labeled anti-Foxp3 (staining kit, clone FJK-16s; eBioscience) and FITC-labeled anti-GFP (Novus Biologicals) to enhance the GFP signal that is bleached during permeabilization. For intracellular staining of CD3, cells were surface stained with FITC-labeled anti-CD4 (clone RM4-5; BD Biosciences), permeobilized with Cytofix/ Cytoperm (BD Biosciences) for 20 min at 4°C, blocked with 10% mouse serum in Perm/Wash (BD Biosciences) for 15 min at 4°C, and stained by incubation with PE-labeled anti-CTLA-4 mAb (clone UC10-4B9; eBioscience) and allophycocyanin-labeled anti-Foxp3 (clone FJK 16s; eBioscience). Data were analyzed via CellQuest software (BD Biosciences).

Histology

Sections of formalin-fixed paraffin-embedded tissue (Swiss rolls of small intestine) were subjected to H&E staining for the evaluation of nematode-induced enteritis. Histological signs of inflammation were assessed in
a blinded fashion using a combined score of intestinal architecture (0–4) and inflammatory cell infiltration (0–4). The resulting score ranged from 0 to 8, with 0 representing no signs of inflammation and 8 representing strong villus atrophy and crypt hyperplasia with transmural infiltration, ulcerations, and mucin depletion associated with strong bowel wall thickening.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism software (San Diego) using the unpaired two-tailed t test or ANOVA followed by the Bonferroni posttest to calculate the significance of differences among multiple groups. The data are presented as mean ± SEM; p ≤ 0.05 was considered statistically significant.

**Results**

**Expansion of Tregs during S. ratti infection**

To monitor the Treg compartment during nematode infection, BALB/c-DEREG mice were infected with S. ratti by s.c. injection of 2000 iL3 into the footpad. Using this infection regimen, we previously showed that migrating larvae are present in the lung and in the head by day 1, with a maximum at day 2 p.i. Larvae are swallowed and reach the small intestine where they molt via the fourth larval stage to become parasitic adults that are detectable by day 5 p.i. (31). Tregs in the lymphatic organs were identified at different points in time p.i. by eGFP/CD4 expression. Fig. 1A shows

**FIGURE 1.** Treg expansion during S. ratti infection in BALB/c-DEREG mice. Eight- to ten-week-old BALB/c-DEREG mice were infected s.c. with 2000 S. ratti iL3. Mice were sacrificed at the indicated times, and spleen and LNs were stained for CD4 (surface) and Foxp3 and GFP (intracellular). A total of 1 × 10^5 lymphocytes was analyzed on a FACSCalibur using Cellquest software. A, Representative stainings of MLNs derived from naive mice (upper panel) and S. ratti-infected mice (lower panel) gated on CD4+ lymphocytes. B, Absolute numbers of CD4+/GFP+ cells (top row) and CD4+/GFP− cells (middle row) and the frequency of CD4+/GFP+ cells in the CD4+ T cell population (bottom row) in naive mice or mice that had been infected for 2, 7, or 14 d. The combined results of two independent experiments are shown (mean ± SEM; n = 9). *p < 0.05, **p < 0.01 compared with the naive control group; ANOVA with Bonferroni correction.
representative intracellular Foxp3 staining of the CD4+ cell population in MLNs derived from naive and S. ratti-infected BALB/c-DEREG mice. This GFP/Foxp3 double staining verified that the CD4/eGFP+ cell population represented the CD4/Foxp3+ Tregs, as was shown in naive (22) and nematode-infected mice (19). S. ratti infection induced a rapid increase in Treg numbers that was visible as early as day 2 p.i. Treg numbers in the popliteal LN that drain the site of infection, as well as in the inguinal LN that drain the site of migrating iL3, were maximal by day 2 p.i. and decreased nearly to naive levels by day 7, correlating with the transient presence of S. ratti in these tissues (Fig. 1B, middle panel). In contrast, expansion of Tregs in the MLN was already detectable by day 2 p.i., but it increased even further from days 2–14 p.i., reflecting the sustained presence of S. ratti parasitic adults in the small intestines. Because Foxp3+ Teffs expanded during infection with similar kinetics, the frequency of Tregs within the CD4+ T cell compartment did not increase to the same extent (Fig. 1B, upper and lower panels). In addition to this local Treg expansion, we observed systemic expansion of Treg numbers in the spleen; however, it was not statistically significant. Infection-induced expansion was also observed for total lymphocytes and total CD4+ T cells (Supplemental Fig. 1).

Increased resistance to S. ratti infection in the absence of Tregs

To analyze this nematode-induced Treg expansion in host defense, we compared the course of infection in the presence and absence of Tregs. To this end, Tregs were depleted by injection of DT for three consecutive days starting 1 d before infection. This treatment reduced the proportion of GFP+ Tregs within the CD4+ T cell compartment in the peripheral blood to almost 0 at days 1–3 p.i. (Fig. 2A). A representative staining of PBLs for Foxp3 expression verified that Foxp3+ Tregs were absent in the PBLs of the DT-treated DEREG mice, but not the untreated DEREG mice, at day 2 p.i. (Fig. 2B). A similar reduction in Foxp3+/GFP+ Tregs was observed in the MLNs and spleen of DT-treated DEREG mice at day 2 p.i. (Supplemental Fig. 2, upper panel). Tregs rapidly reappeared in the peripheral blood (Fig. 2A), as well as in MLNs and spleen (Supplemental Fig. 2, lower panel), at later time points. Thereby, Foxp3 expression preceded GFP expression as described previously (19). In summary, our protocol led to a complete, but transient, depletion of Tregs during the first days of S. ratti infection.

Using this transient depletion protocol, we compared the numbers of migrating viable S. ratti larvae in DT-treated DEREG mice and DT-treated wild-type littermates. Despite the absence of Tregs at days 1 and 2 of S. ratti infection, the numbers of migrating larvae were not reduced at these time points (Fig. 3A). In contrast, numbers of iL3 detected in the head were slightly, but not significantly (p = 0.14), increased in the absence of Tregs. Next, we assessed the worm burden by counting the numbers of parasitic female adults in the small intestine. At day 7 p.i., worm burden was dramatically reduced in the absence of Tregs compared with untreated DEREG mice or wild-type littermates that had been treated with DT (Fig. 3B). It should be noted that the DT-treated wild-type littermate group controlled for any possible side effect of the DT treatment itself on the susceptibility of wild-type mice to S. ratti. In contrast, the comparison of DT-treated and untreated DEREG mice verified that the different susceptibility was not due to genetic differences between DEREG mice and their littermate controls. These combined controls clearly showed that the reduced worm burden observed in the DT-treated DEREG mice was due to the absence of Tregs.

To monitor the course of nematode infection, we quantified the output of S. ratti eggs and hatched larvae in the feces by qPCR for the S. ratti 28S RNA gene locus, as described (31). S. ratti-derived DNA in the feces of infected mice was detectable at day 6 p.i., reached a maximum output between days 7 and 8, and rapidly declined until day 22 p.i. The transient depletion of Tregs led to a clear reduction in the S. ratti larval output at every time point analyzed compared with both control groups containing normal Treg numbers (Fig. 3C). In summary, these results strongly suggested that Treg depletion improved resolution of S. ratti infection at the stage of parasitic adult expulsion and not by improved eradication of migrating larvae.

Irrespective of the presence or absence of Tregs, all mice finally cleared the infection spontaneously within a month; by day 49 p.i., no S. ratti-derived DNA was detectable in the feces in all groups tested (Fig. 3C). To analyze the impact of Tregs on the establishment of protective immune memory, all mice were reinected, and output of eggs and larvae was again monitored by qPCR. The resolved primary S. ratti infection rendered the mice semiresistant to secondary infection, as was shown previously (28, 31). This resistance was reflected by reduced larval output upon infection with the same number of iL3 compared with the first infection (Fig. 3C) and a dramatically reduced number of parasitic adults in the gut to less than five adults per gut (data not shown). Resistance to second infection is thought to be mediated by T and B cell memory responses (37–40). The encounter of a primary infection in the absence of Tregs also resulted in lower S. ratti output in the second infection (Fig. 3A, inset), although the Treg compartment started to repopulate by day 6 p.i. (Fig. 2A; Supplemental Fig. 2).
This result clearly showed that encounter of a primary infection in the absence of Tregs did not interfere with the generation of a protective memory response.

Increased immune response in the absence of Tregs

The expulsion of parasitic *S. ratti* adults from the small intestine depends on degranulating mast cells that are activated in the context of a Th2-like response (32, 33). We monitored mast cell degranulation by measuring the concentration of MMCP-I in the serum. Maximal mast cell activation occurred at day 14 p.i. in the control groups and remained detectable until day 21, but it was not visible at later time points (e.g., day 45 p.i.) (Fig. 4A). In contrast, MMCP-I serum concentrations in Treg-depleted mice reached the maximum 7 d earlier. MMCP-I remained at maximal concentrations through day 14 p.i. and was still measurable at late time points, such as day 45 p.i., when MMCP-I in the serum of the control groups had returned to background concentration. The humoral response, assessed as serum titer of *S. ratti*-specific IgM (Fig. 4B) and IgG1 (Fig. 4C), was only slightly increased upon Treg depletion. In summary, these results showed that Treg depletion led to accelerated and prolonged mast cell activation while not inducing significant changes in other early B cell responses.

Efficient mast cell activation during *S. ratti* infection depends on a Th2 response, specifically the production of IL-5 and IL-3 (31, 34, 41–44). To compare *S. ratti*-specific T cell responses in the presence and absence of Tregs, we stimulated MLN cells at day 7 p.i. with *S. ratti* Ag and measured proliferation and cytokine response. *S. ratti*-specific proliferation was visible in the presence and absence of Tregs in infected mice but not in naive mice (Fig. 5A). However, depletion of Tregs in DT-treated DEREG mice resulted in a clear increase in Ag-specific proliferation compared with untreated DEREG and DT-treated wild-type mice. Treg depletion did not induce an unspecific proliferation, because MLN cells from DT-treated naive mice did not respond to *S. ratti* lysate. Analysis of the produced Ag-specific cytokines revealed that Th2-associated cytokines, such as IL-13, IL-5, and IL-3, were barely detectable in the presence of Tregs (Fig. 5B). In vivo depletion of Tregs resulted in increased production of all of these cytokines upon in vitro stimulation of MLNs with *S. ratti* Ag. Interestingly, the production of IL-10 was also increased upon Treg depletion. The production of proinflammatory cytokines, such as IFN-γ, in *S. ratti* Ag-stimulated MLN cells did not increase above background levels in the presence of Tregs, and it was only slightly increased upon Treg depletion (Fig. 5B). Analysis of spleen cell cultures led to similar results (data not shown).

**FIGURE 3.** Increased resistance to *S. ratti* infection upon Treg depletion. BALB/c-DEREG mice that received DT (black squares/bars) or PBS (gray squares/bars) or wild-type littermates that received DT (open squares/bars) were infected with 2000 *S. ratti* iL3 at day 0 and reinfected at day 49 of infection. A, Mice were sacrificed 24 or 48 h p.i., and migrating larvae in lung and head were counted. Combined results of three independent experiments are shown (mean ± SEM; *n* = 8). B, Five mice per group were sacrificed at day 7 p.i., and the number of adult *S. ratti* in the small intestine was counted. One representative experiment of four (mean ± SEM). C, Release of *S. ratti*-derived DNA (gene coding for 28S RNA) in the feces over 24 h was quantified at the indicated time points. The inset shows the reinfecition with 0–450 copies/μL. Combined results of three independent experiments (mean ± SEM; *n* = 8). ***p < 0.001 compared with controls; ANOVA with Bonferroni correction.

**FIGURE 4.** Accelerated mast cell activation in the absence of Tregs. BALB/c-DEREG mice that received DT (black bars) or PBS (gray bars) or wild-type littermates that received DT (open bars) were infected with 2000 *S. ratti* iL3. MMCP-I (A), *S. ratti*-specific IgM (B), and *S. ratti*-specific IgG1 (C) in the serum were quantified at the indicated time points p.i. Combined results of three (MMCP-I) or two (Ig) independent experiments [mean ± SEM, *n* = 9 (A); *n* = 5 (B, C)]. *p < 0.05, ***p < 0.001 compared with both control groups; ANOVA with Bonferroni correction.
We showed previously that *S. ratti* infection induced the production of type 2 cytokines to polyclonal stimulation of T cells by CD3 engagement in C57BL/6 mice, whereas T cells derived from naive mice did not produce significant amounts of IL-3, IL-5, IL-13, or IL-10 (31). Depletion of Tregs also led to an increased production of type 2-associated cytokines upon CD3 engagement in BALB/c-DEREG mice (Fig. 5C). Strikingly, these increased polyclonal T cell responses were not restricted to the ongoing type 2 response during *S. ratti* infection because the IFN-γ response to anti-CD3 was also increased in Treg-depleted mice. These results suggested that Treg depletion during *S. ratti* infection increased the magnitude of all immune reactions, regardless of their quality.

**Tregs modulate the immune response to *S. ratti* infection during priming**

With regard to the kinetics of *S. ratti* infection, worm burden and the nematode-specific Th2 response were maximal through days 6–10 p.i., as we showed previously (31). Strikingly, transient Treg depletion during the first days of *S. ratti* infection resulted in reduced worm burden, improved mast cell activation, and nematode-specific T cell response at later time points when the Tregs had already started to repopulate the lymphatic organs. To analyze the impact of Tregs during the peak of infection, we compared the effect of Treg depletion during the first days of infection (“early depletion”) with the effect of Treg absence selectively during the peak of infection (“late depletion”). Early depletion was achieved by our usual DT treatment starting 1 d before infection. We achieved late depletion by starting the DT treatment at day 4 p.i., resulting in Treg absence during days 5–7 p.i. (Fig. 6A). While the early Treg depletion reproducibly resulted in reduced worm burden and reciprocally increased mast cell degranulation at day 7 p.i., we did not observe improved host defense upon late Treg depletion (Fig. 6B, 6C). Worm burden in the small intestine and serum concentration of MMCP-I were comparable between day 4 DT-treated DEREG mice and wild-type littermate control mice. This result strongly suggested that Tregs exert their counter-regulatory function during the first days of murine *S. ratti* infection (i.e., in the priming phase of the immune response).

**Transient Treg depletion does not aggravate parasite-induced pathology**

Because Treg depletion during the first days of *S. ratti* infection clearly accelerated parasite expulsion in the context of an increased mast cell and T cell response, we asked whether this would also result in increased inflammation and immune pathology in the intestine. Therefore, we performed histological analyses of the small intestines of naive or infected DT-treated DEREG mice, DT-treated wild-type littermates, and untreated DEREG mice. Infection with *S. ratti* induced only moderate changes in the small intestine, such as slight villous blunting, crypt hyperplasia, and cellular infiltrates into the lamina propria, that were most prominent at day 7 p.i. (Fig. 7A). However, quantification of this *S. ratti*-induced pathology by scoring changes in intestinal architecture and signs of inflammation revealed that pathology was comparable in all groups; thus, it was not aggravated by the transient absence of Tregs (Fig. 7B).
Increased nematode-specific type 2 immune response upon CTLA-4 blockade

Tregs counterregulate adaptive immune responses by many mechanisms that have not been completely analyzed (3, 45). Suppression of leukocyte function is among other things mediated by CTLA-4, which is constitutively expressed by Tregs (46, 47). Analysis of CTLA-4 expression revealed that Foxp3+ Tregs represented the dominant CTLA-4+ T cell population, but this CTLA-4 expression on Tregs was not clearly modulated during S. ratti infection in MLNs or spleen (Fig. 8, data not shown). However, Foxp3+ Teffs displayed only a very low frequency of CTLA-4+ cells that expanded during S. ratti infection selectively in MLNs.

Having shown that Tregs suppressed the protective immune response to S. ratti infection, we intended to analyze the role of CTLA-4 on Tregs and Teffs in the transduction of this suppression. To this end, we blocked CTLA-4 during S. ratti infection by application of an antagonistic mAb. Application of 300 μg anti–CTLA-4 (4F10) at the day of S. ratti infection led to detectable serum concentrations of the mAb until day 13 p.i. (data not shown). Blockade of CTLA-4 in vivo did not change the overall output of S. ratti eggs and larvae in the feces (Fig. 9A). Nevertheless, comparison of parasitic adults in the small intestine revealed a slight reduction in parasite numbers upon CTLA-4 blockade that was significant at day 7 p.i. (Fig. 9B). Neither mast cell degranulation (Fig. 9C) nor Ag-specific B cell responses (Fig. 9D, E) were changed upon CTLA-4 blockade. In contrast, S. ratti-specific proliferation of spleen cells and type 2 cytokine production (e.g., IL-3, IL-13, IL-5, and IL-10) were increased upon application of anti–CTLA-4 (Fig. 10A, B). Neutralization of CTLA-4 signaling in naive mice did not induce proliferation or cytokine secretion to S. ratti Ag, thus ruling out nonspecific lymphocyte activation upon application of anti–CTLA-4 (Fig. 10A). Analysis of MLNs revealed similar results (data not shown).

An ongoing S. ratti infection induced Th2-associated cytokines, as well as suppressed the IFN-γ response to CD3 engagement, as we showed previously in C57BL/6 mice (31). Application of mAbs to CTLA-4 induced a further suppression of IFN-γ production by spleen cells, whereas the polyclonal type 2 T cell response to CD3 engagement remained unchanged (IL-3 and IL-10) or increased only moderately (IL-5 and IL-13) (Fig. 10C). These findings strongly suggested that CTLA-4 blockade specifically enhanced parasite-specific type 2 responses during S. ratti infection.
Infection, whereas depletion of Tregs induced a general increase in T cell responses.

Discussion

Initiations of adaptive immune responses usually are accompanied by the activation of a counterregulatory network consisting of regulatory cell types, receptors, and cytokines. Although these regulatory circuits are central for the timely termination of immune responses and the avoidance of immune pathology, they may also counteract the generation of protective immune responses to infectious agents (48, 49).

In this study, we used S. ratti-infected BALB/c-DEREG mice to show that parasitic nematodes induced the expansion of Foxp3+ Tregs that interfere with protective immune responses. We drew this conclusion because of the following evidence: Treg numbers increased rapidly during S. ratti infection, locally and systemically; in vivo depletion of Tregs resulted in accelerated and prolonged mast cell activation that occurred in the context of an increased polyclonal and Ag-specific type 2 cytokine production and Ag-specific proliferation by MLN and spleen cells; and worm burden and larval output during S. ratti infection were drastically reduced in the absence of Tregs.

With regard to the mechanism of protective immunity that was enhanced after Treg depletion in BALB/c-DEREG mice, our results clearly showed that reduced worm burden and larval output upon Treg depletion were due to more efficient expulsion of parasitic adults, because eradication of migrating larvae was unchanged in the absence of Tregs. Our combined findings strongly suggested that this improved expulsion of parasitic adults was mediated by the increased and accelerated mast cell activation observed in the absence of Tregs, because activated mast cells were shown to be the major cell type responsible for parasite expulsion, especially in murine Strongyloides infections (32, 34, 50). This increased mast cell activation again was very likely a direct consequence of the increased Th2 response also observed upon Treg depletion, because the initiation of a potent Th2 response is central for the timely expulsion of Strongyloides adults (36, 51). However, it is also conceivable that Tregs suppressed mast cells directly by cellular interaction via OX40, as was shown recently (52). We are unraveling the exact chain of events leading to improved resistance to S. ratti upon early Treg depletion.

Further evidence for Treg-mediated suppression of the protective immune response to Strongyloides comes from a study in the human system. Montes et al. (53) showed that an increased frequency of CD25+Foxp3+ Tregs in the peripheral blood of human T cell-lymphotropic virus 1/Strongyloides–coinfected individuals resulted in increased worm burden and reciprocally decreased

**FIGURE 8.** CTLA-4 expression during S. ratti infection. Eight- to ten-week-old BALB/c mice were infected s.c. with 1000 S. ratti iL3. Mice were sacrificed at the indicated time points, and MLNs were stained for CD4 (surface), as well as Foxp3 and CTLA-4 (intracellular). A total of 1 × 10^7 lymphocytes was analyzed on a FACSCalibur using CellQuest software. A, Representative dot blots of MLNs derived from S. ratti-infected mice at day 2 p.i., which were stained for CD4 and Foxp3 to distinguish Teffs and Tregs. B, Frequencies of CTLA-4+ cells within the CD4+ Foxp3− Teff and CD4+Foxp3+ Treg populations in naive mice or mice that had been infected for 2, 7, or 14 d. Combined results of two independent experiments (mean ± SEM; n = 9). *p < 0.05, **p < 0.01 compared with naive control group; Student t test.

**FIGURE 9.** Slightly increased resistance to S. ratti infection upon CTLA-4 blockade. BALB/c mice received 300 μg anti–CTLA-4 or hamster IgG in PBS i.p. Mice were infected with 1000 S. ratti iL3 3 h later. A, Release of S. ratti-derived DNA in the feces over 24 h was quantified at the indicated time points (mean ± SEM, n = 8). B, Mice were sacrificed at days 6, 7, or 8 p.i., and the numbers of adult S. ratti in the small intestine were determined (mean ± SEM, n = 8). Mice were bled at the indicated time points, and MMCP-1 (C), S. ratti-specific IgM (D), and S. ratti-specific IgG1 (E) in the serum were quantified at the indicated time points (mean ± SEM; n = 5). Combined results of two independent experiments. *p < 0.05; Student t test.
Strongyloides Ag-specific IL-5 production compared with patients infected only with Strongyloides.

Although our depletion protocol induced only a transient removal of Tregs throughout the first days of infection, improved protection was observed during wk 3 of primary and even during a second infection 50 d later. Additionally, the increased cytokine production and proliferation response to S. ratti Ag was detected at day 7 p.i., when the Treg compartment started to repopulate. In contrast, using a depletion protocol that led to the absence of Tregs during the peak of infection, we did not observe changes in immune response or host defense. These findings highlighted the central role of Treg-mediated regulation during the priming of an adaptive T cell response, because the transient depletion in the beginning of infection was sufficient to induce increased responses throughout infection and even reinfection, whereas later depletion could not reverse regulatory effects that had been initiated during this first day of infection by the resident Tregs. In line with these findings, Taylor et al. (6) showed that the depletion of Tregs prior to infection with Litomosoides sigmodontis reduced worm burden 60 d later. Depletion of Tregs using anti-CD25 mAb at later time points of infection no longer improved host defense. Only a combination with anti-glucocorticoid–induced TNFR mAb or anti–CTLA-4 mAb treatment resulted in reduced worm burden if Tregs were present during the first 4 wk of infection (16, 54). In line with our results, it was recently shown that Treg depletion in C57BL/6-DEREG mice, specifically during the sensitization phase of experimental allergic airway inflammation, aggravated inflammatory responses (55).

To elucidate the role of CTLA-4 in the regulatory circuits that dampen S. ratti-specific immune responses, we compared the blockade of CTLA-4 to the depletion of all Foxp3+ Tregs within infected mice. Treg depletion led to an increased production of all cytokines analyzed in this study, including IFN-γ that was produced in response to CD3 engagement. In contrast, CTLA-4 blockade specifically increased the ongoing type 2 response and decreased type 1 responses, as reflected by the reduced IFN-γ production in anti–CTLA-4–treated mice. Furthermore, cytokine response to S. ratti Ag and CD3 engagement was increased upon Treg depletion, whereas CTLA-4 blockade predominantly improved the response to S. ratti Ag. This improved S. ratti-specific response resulted in enhanced resistance, as reflected by the reduced intestinal worm burden in anti–CTLA-4–treated mice, although the phenotype was less pronounced and limited to day 7 of S. ratti infection compared with the dramatically improved resistance observed upon complete Treg depletion throughout the course of infection.

The limited effect of CTLA-4 blockade can be explained by the diversity of mechanisms that Tregs use to suppress immune responses, such as the induction of apoptosis by the injection of cAMP, the killing via granzymes, the release of IL-10 and TGF-β, in which CTLA-4–mediated signaling is one out of several (3, 47). Upon neutralization of this single pathway, other means of suppression may remain active, thus resulting in the incomplete effect of anti–CTLA-4 treatment compared with Treg depletion. Furthermore, CTLA-4 was upregulated predominantly on activated Tefs during S. ratti infection, and the experimental setting of anti–CTLA-4 application does not distinguish between blockade of CTLA-4 on Tefs or Tregs. Indeed, Foxp3+ Tefs were shown to represent the majority of CTLA-4+ T cells in L. sigmodontis– (16), Brugia malayi– (8), and S. mansoni (56)-infected mice.
Because the Ag-specific, but not the generalized immune responses, increased upon anti-CTLA-4 treatment in our study, we suggest that this may be due to CTLA-4 expressing nematode-specific Teffs that display increased activation upon application of neutralizing anti-CTLA-4 mAb. In contrast, complete depletion of all Tregs obviously enhances the responses of all possible effector functions, regardless of specificity and differentiation of the T cells.

Although CTLA-4 expression on Tregs was not modulated during S. ratti infection, we observed a rapid increase in Treg numbers locally in the draining LN and systemically in the spleen. Due to the rapidly expanding Teff population, no robust increase in Treg ratio within the CD4+ T cell population was achieved. In line with our findings, other gut-dwelling nematodes (7, 9), tissue-dwelling filariae (6, 8), and trematode infections (11) predominantly induced the expansion of Treg numbers.

There is accumulating evidence that these Tregs exert dual functions during infection. On the one hand, they actively suppress potentially harmful immune responses and avoid immune pathology. On the other hand, their suppressive activity interferes with host defense. The central role of Tregs in the control of immune pathology in murine helminth infections is supported by several studies: S. mansoni-infected mice displayed increased liver pathology if Tregs were depleted or Treg expansion was restricted (11, 20). Also, the development of severe hypersplenomegaly syndrome in chronic S. mansoni infection correlated to the ratio of Tregs/activated T cells (57). Trichuris muris isolates that induced a more pronounced Treg expansion upon infection displayed prolonged survival within their murine hosts. However, the depletion of CD25+ Tregs by anti-CD25 mAb led to increased pathology without reducing the worm burden (7). Furthermore, one study using C57BL/6-DEREG mice to specifically deplete Foxp3+ Tregs without affecting CD25+ Teffs during an intestinal nematode infection, reported increased type 2 responses upon Treg depletion. Again, this improved immune response did not translate into improved host defense but instead increased the inflammatory pathology of the intestine (19).

In sharp contrast, our result using BALB/c-DEREG mice strongly suggested that Tregs specifically interfered with the protective immunity to S. ratti infection, because their depletion increased resistance without any evidence for immune pathology. Improved resistance to nematode infection in the absence of Tregs has only been demonstrated for L. sigmodontis-infected BALB/c mice (6). Interestingly, the suppression of immune responses to unrelated Ags that is often observed upon helminth infection was also shown to be associated with Tregs in some studies. Using models of adoptive transfer or depletion by anti-CD25 mAb, Tregs have been identified as one of the helminth-induced cell populations responsible for silencing the pathology in OVA-induced asthma (58–61) or counteracting the protective immune response to Plasmodium yoelii in a coinfection model (62).

It would be tempting to attribute a predominantly beneficial role of Treg function to certain features, such as chronic infection or tissue-migrating parasites, but the data accumulated so far do not reveal such patterns. Obviously, Tregs silence helminth-specific immune response, because their depletion or functional inactivation was shown to increase adaptive-immune responses in the majority of murine helminth infection models. However, beneficial and deleterious functions of this Treg-mediated immune suppressed seem to occur simultaneously. The final outcome depends on parameters that remain to be elucidated. One factor may be the presence of Treg-independent counterregulatory pathways, such as regulatory cytokines produced by Teffs. In this context, the increased IL-10 production that we observed in Treg-depleted mice upon S. ratti infection may contribute to the control of immune pathology in the context of an improved type 2 immune response as was shown for Trichinella spiralis–infected mice (10).

In conclusion, to our knowledge ours is the first study to show that transient, but specific, depletion of Tregs during the first days of S. ratti infection clearly increased the resistance to infection and did not interfere with the generation of a protective memory response. Thus, despite the dual role of Tregs in infections, within the experimental system of murine S. ratti infection, we provide strong evidence that Treg expansion contributes to immune evasion of this pathogenic nematode.

Disclosures
The authors have no financial conflicts of interest.

References


