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Pathogenic Nematodes Suppress Humoral Responses to Third-Party Antigens In Vivo by IL-10–Mediated Interference with Th Cell Function

Wiebke Hartmann,* Irma Haben,* Bernhard Fleischer,*† and Minka Breloer*†

One third of the human population is infected with helminth parasites. To promote their longevity and to limit pathology, helminths have developed several strategies to suppress the immune response of their host. As this immune suppression also acts on unrelated third-party Ags, a preexisting helminth infection may interfere with vaccination efficacy. In this study, we show that natural infection with *Litomosoides sigmodontis* suppressed the humoral response to thymus-dependent but not to thymus-independent model Ags in C57BL/6 mice. Thereby, we provide evidence that reduced humoral responses were mediated by interference with Th cell function rather than by direct suppression of B cells in *L. sigmodontis*-infected mice. We directly demonstrate suppression of Ag-specific proliferation in OVA-specific Th cells after adoptive transfer into *L. sigmodontis*-infected mice that led to equally reduced production of OVA-specific IgG. Transferred Th cells displayed increased frequencies of Foxp3+ after in vivo stimulation of Ag-specific proliferation in OVA-specific Th cells after adoptive transfer into *L. sigmodontis*-infected mice. This is potentially detrimental as it might affect the immune response to concurrent infections and to third-party Ags (3). Thus, helminth infections might interfere with vaccine efficacy in the human population (4). Indeed, a reduced humoral and cellular immune response was observed in helminth-infected individuals after cholera (5), bacillus Calmette-Guérin (6, 7), and tetanus toxoid (8–10) vaccination, whereas de-worming increased purified protein derivative-specific immune response (6). Regarding situations of coinfection, helminth-induced immune modulation may interfere with the control of coinfecting parasites but may also help to avoid immune pathology such as development of cerebral malaria (11, 12). Finally, the attenuation of autoimmune diseases has been attributed to preexisting helminth infections (13–15).

To understand the underlying mechanisms, several murine models of helminth infection have been established (16). Infection of mice with *Litomosoides sigmodontis* can be used to model most features of immune response and immune modulation observed in human filarial infections (17, 18). *L. sigmodontis* third-stage larvae (L3) are transmitted to their natural host, the cotton rat (*Sigmodon hispidus*), by the bite of infected mites (*Ornithonyssus bacoti*). Laboratory mice may be infected naturally by the bite of infected mites (19, 20) or artificially by injection of L3 (21) or implantation of different stages such as L3, adults, and first-stage larvae, so-called microfilariae (MF) (22). After infection, L3 migrate during the first 3 d via the lymphatic system to the thoracic cavity (23). They molt to fourth-stage larvae (L4) within 10 d and to young adults within 26–28 d. In the permisive BALB/c strain, *L. sigmodontis* mate and release MF by day 60 postinfection (p.i.) (24). Young adults never reach sexual maturity in the resistant C57BL/6 mice and are removed by granuloma formation by day 60 p.i. (25).

The interference with regulatory T cell function in *L. sigmodontis*-infected BALB/c mice led to improved parasite clearance thus suggesting that indeed *L. sigmodontis* actively suppresses the immune response to itself (26–28). The power of this suppression was demonstrated by the fact that implantation of a single *L. sigmodontis* female adult into resistant DBA/1 mice inhibited the clearance of MF, which under normal circumstances occurs within 3 d in this mouse strain (29). This suppression was not restricted to filarial-specific immune responses but acted on unrelated third-party Ags as well. Surgical implantation of *L. sigmodontis* adults into BALB/c mice reduced induced allergic airway inflammation and hyperreactivity in a model for OVA-induced asthma (30). Also, onset of type 1 diabetes was prevented by injection of L3 and implantation of adults (31), and LPS-induced sepsis was slightly mitigated by adult implantation (22). Finally, it was shown that a preexisting natural *L. sigmodontis* infection prevented the development of cerebral malaria in C57BL/6 mice coinfected with *Plasmodium berghei* ANKA (32).

In this study, we model the helminth-induced interference with vaccination efficiency, using mice that are naturally infected with...
L. sigmodontis via blood-sucking mites. Taking the advantage of defined model Ags, we show that L. sigmodontis L4 drastically suppressed B cell responses specifically to thymus-dependent (TD) Ags and not thymus-independent (TI) Ags. We provide evidence that interference with T cell function contributed to the suppressed TD Ig production within infected mice. In vivo proliferation of OVA-specific Th cell as well as OVA-specific IgG responses were suppressed, and frequency of Foxp3+ in the Th cell population was increased. Although the suppression of transferred OVA-specific Th cells did not depend on host-derived regulatory T cells (Treg), we show that L. sigmodontis-induced IL-10 contributed to the suppression of Th cell proliferation and Foxp3+ induction.

Materials and Methods

Mice, reagents, and Abs

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. C57BL/6 and OT-II mice were obtained from the University Hospital Eppendorf, and cotton rats (Sigmodon hispidus), C57BL6-DEREG, JHT, and IL-10 knockout (ko) mice were bred in the animal facility of the Bernhard Nocht Institute. Animals were kept in individually ventilated cages. Anti-CD4–allophycocyanin or PE (clone RM4-5), anti-CD19–allophycocyanin or PE (clone RM4-5), anti-CD19–allophycocyanin staining sets (EJK-16s) were obtained from eBioscience (San Diego, CA). Mouse IgG2a–PE was from Santa Cruz Biotechnology (Santa Cruz, CA), and CFSE was obtained from Invitrogen (Carlsbad, CA).

L. sigmodontis cycle and experimental infection

The life cycle of L. sigmodontis was maintained in cotton rats (Sigmodon hispidus), the natural reservoir of the nematode. As intermediate host, mites (O. baccoti) were fed on infected cotton rats. Fourteen days after this blood meal, 8- to 12-wk-old C57BL/6, JHT, DEREG, or IL-10 knockout (ko) mice were bred in the animal facility of the Bernhard Nocht Institute. Animals were fed in individually ventilated cages. Anti-CD4–allophycocyanin or PE (clone RM4-5), anti-CD19–allophycocyanin or PE (clone RM4-5), and appropriate isotype controls such as rat IgG2a–allophycocyanin or FITC (clone 1D3), and appropriate isotype controls such as rat IgG2a–allophycocyanin or FITC were purchased from BD Pharmingen (San Diego, CA). Anti-mouse/anti-rat Foxp3–PE and –allophycocyanin staining sets (EJK-16s) were obtained from eBioscience (San Diego, CA). Mouse IgG2a–PE was from Santa Cruz Biotechnology (Santa Cruz, CA), and CFSE was obtained from Invitrogen (Carlsbad, CA).

FIGURE 1. Infection with L. sigmodontis suppresses humoral response to TD Ag. A, A diagram of the experimental setup. Eight- to ten-week-old C57BL/6 mice were either left naive (DNP, closed squares) or naturally infected with L. sigmodontis (DNP + L. s., open squares). Mice were immunized with 200 μg DNP-KLH i.p. An additional control group was infected but not DNP immunized (L. s., open circles). B–E, Serum samples were analyzed for DNP-specific IgG1 (B), IgG2b (C), IgG2c (D), or IgG3 (E) by ELISA at the indicated time points. Results are expressed as mean ± SEM (n = 13) of titer (B) or of arbitrary units (a.u.) (C–E). Combined results from three independent experiments are shown. Asterisks indicate significant difference of the mean of DNP-specific Ig in naive and infected mice after immunization with DNP-KLH: *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test).

Preparation of L. sigmodontis Ag

L. sigmodontis Ag was prepared by homogenization of vital female worms isolated from infected BALB/c mice, followed by centrifugation at 14,000 rpm for 30 min at 4˚C. The supernatant was passed through an 0.22-μm filter and then stored at –80˚C until use.

Model Ag immunization

Naive and infected mice were immunized by i.p. injection of 200 μg alum-precipitated dinitrophenol keyhole limpet hemocyanin (DNP-KLH; Biosearch Technologies, Heidelberg, Germany), 200 μg 4-hydroxy-3-iodo-5-nitrophenylacetate (NIP) conjugated to Ficoll (NIP-Ficoll; Biosearch Technologies), or 500 μg OVA (Sigma, Deisenhofen, Germany). Mice were bled every week by puncture of the tail vein. Blood samples were allowed to coagulate at room temperature for 1 h and centrifuged for 10 min at 15,200 × g. Serum was harvested from the supernatant and stored at –20˚C until further analysis.

ELISA

For the detection of either dinitrophenol (DNP)-, NIP-, OVA-, or L. sigmodontis-specific Ig, ELISA plates were coated overnight with 50 μg/ml DNP-BSA, 1 μg/ml NIP-BSA, 10 μg/ml OVA, or 4 μg/ml L. sigmodontis Ag in carbonate buffer pH 9.6. Plates were washed, blocked by incubation with PBS 1% BSA for 2 h at room temperature, and incubated for 2 h with serum. Plates were washed and incubated for 1 h with HRP-labeled anti-mouse IgM, IgG1, IgG2b (1:1000; Zymed Laboratories), IgG2c, or IgG3 (1:1000; Southern Biotechnology Associates) to detect Ag-specific isotypes in the serum. Plates were washed and developed by incubation with 100 μl tetramethylbenzidine 0.1 mg/ml, 0.003% H2O2 in 100 mM Na2HPO4 pH 5.5 for 2.5 min. Reaction was stopped by addition of 25 μl 2 M H2SO4, and OD450 was measured. Titers were calculated by defining the highest serum dilution in a serial dilution (1:100 to 1:102,400) resulting in an OD450 above the doubled background that was generally below OD450 = 0.15. Arbitrary units were calculated by dividing the OD450 of a fixed
serum concentration (1:50 for IgG2b, 1:100 for IgG3, and 1:400 for IgG2c) to the OD$_{450}$ of the background. Cytokine concentrations in culture supernatants from spleen cells were quantified by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions (Roth, Karlsruhe, Germany).

**Flow cytometry**

The Fc receptors of spleen cells were blocked with mouse serum (5% v/v; Sigma Aldrich) for 10 min on ice. For surface staining, cells were stained with 1:100 dilutions of the indicated mAb for 30 min on ice. Foxp3 expression was determined using PE- or allophycocyanin-anti-mouse Foxp3 Staining Set (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Samples were analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson, Mountain View, CA) using Cell Quest software.

**Analysis of OT-II cell proliferation in vivo**

Single-cell suspensions were prepared from pooled spleens from OT-II mice. For in vivo proliferation, OT-II cells were suspended in 10 ml sterile PBS (5 × 10^5 cells). After addition of 200 μl 0.5 mM CFSE, cells were incubated for 10 min at 37°C. Labeling reaction was stopped by addition of 40 ml 3% FCS in PBS, and cells were washed three times. CFSE-labeled transgenic OT-II spleen cells (2 × 10^6) were injected intra- peritoneally in naive or L. sigmodontis-infected (day 14 p.i.) mice (C57BL/6, DEREG, or IL-10 ko) as recipients. For Treg depletion, naive and infected DEREG mice received 1 μg human diphtheria toxin (DT; Merck, Darmstadt, Germany) 1 d before and at the day of adoptive transfer. For TGF-β neutralization, naive and infected mice received 100 μg anti-TGF-β (clone 1D11; R&D Systems, Wiesbaden, Germany) or isotype control i.p. at the day of adoptive transfer and 1 d after stimulation of the cells with OVA peptide. The day after transfer, mice received 50 μg OVA323–339 peptide or 500 μg OVA protein i.p. Mice were sacrificed 48 h later and spleen cells isolated and stained with anti-CD4 Ab. The number of proliferation cycles was calculated by CFSE dilution.

**Adoptive transfer of B cells**

Spleens were prepared from naive or L. sigmodontis-infected (day 14 p.i.) C57BL/6 mice, and B cells were isolated by negative selection using a B cell separation kit according to the manufacturer’s recommendation (Miltenyi Biotech, Bergisch Gladbach, Germany). Purity was verified by subsequent staining with allophycocyanin- or FITC-labeled anti-CD19 and was found to be >95%. Freshly isolated B cells (1 × 10^5) were injected into the tail vein of B cell-deficient JHT mice.

**In vitro stimulation of lymphocytes**

Spleen cells were prepared from naive and infected mice 72 h after adoptive transfer of OT-II T cells. Spleen cells (5 × 10^6) were cultivated in 96-well round-bottom plates for 72 h at 37°C and 5% CO₂ in RPMI 1640 medium supplemented with 10% FCS, l-glutamine (2 mg/ml), and gentamicin (50 μg/ml). For stimulation, cells were either incubated with medium alone or with 1 μg/ml anti-CD3 (145-2C11), 0.1 to 10 μg/ml OVA323–339 peptide (JPT Peptide Technologies, Berlin, Germany), or 12.5 μg/ml L. sigmodontis Ag in quadruplicate. Supernatants were collected and stored at −20°C until analysis.

**Statistical analysis**

All statistical tests were performed using Prism software (GraphPad Software, San Diego, CA), and p values <0.05 (Student t test) were considered statistically significant.

**Results**

L. sigmodontis infection suppresses the humoral response to third-party Ags

Several lines of evidence suggest that an ongoing nematode infection interferes with humoral response to third-party Ags (3). To analyze this phenomenon in detail, we compared the humoral response to the model Ag DNP-KLH in naive and L. sigmodontis-infected mice (Fig. 1A). DNP-KLH immunization of noninfected mice led to the production of DNP-specific IgG, predominantly IgG1 but also IgG2b, IgG2c, and IgG3 in the serum, as expected (Fig. 1B–E, closed squares). An ongoing L. sigmodontis infection caused a clear reduction in the humoral response to DNP-KLH regarding all IgG isotypes (Fig. 1B–E, open squares). Quantification of DNP-specific IgM was not possible because of a strong cross-reaction of L. sigmodontis-specific IgM with DNP (data not shown).

To differentiate whether the impaired humoral response to the TD Ag in L. sigmodontis-infected mice was due to impaired B cell or impaired Th cell function, we repeated the experiment using the TI B cell Ag NIP-Ficoll (Fig. 2A). Although the exact mechanism of TI immunization is still under investigation, it is agreed that the highly polyvalent epitope induces a potent cross-linking of the BCR that allows a partial B cell activation and also class switch in the absence of Th cell-mediated costimulation (34). NIP-Ficoll immunization in the absence of any adjuvant induced an NIP-specific IgG response consisting again predominantly of IgG1 but also IgG3 and IgG2c (Fig. 2B–D, closed squares). This response, however, was not suppressed but even slightly increased in L. sigmodontis-infected mice (Fig. 2B–D, open squares). Because these results strongly suggest that nematode infection did not interfere with B cell function directly, we performed adoptive transfer experiments to verify this notion.

**FIGURE 2.** Unchanged humoral response to TI Ag in L. sigmodontis-infected mice. A, A diagram of the experimental setup. Eight- to ten-week-old C57BL/6 mice were either left naive (NIP, closed squares) or naturally infected with L. sigmodontis (NIP + L. s., open squares). Mice were immunized with 200 μg NIP-Ficoll i.p. An additional control group was infected but not NIP immunized (L. s., open circles). B–D, Serum samples were analyzed for NIP-specific Ig by ELISA at the indicated time points. NIP-specific serum IgG1 (B), IgG2c (C), or IgG3 (D) was measured by ELISA. Results are expressed as mean ± SEM (n = 10) of titer (B) or of arbitrary units (a.u.) (C, D). Combined results from two independent experiments are shown.
Th cells and not B cells are the target of L. sigmodontis-mediated suppression of humoral responses

We transferred B cells that were derived from either naive or L. sigmodontis-infected mice into naive but B cell-deficient JHT mice, thus creating mice that contained naive T cells and either naive B cells or nematode primed B cells (Fig. 3A). These recipient mice were immunized subsequently with DNP-KLH, and DNP-specific IgG1 in the serum was quantified. B cells derived from naive and L. sigmodontis-infected mice mounted similar responses to the TD Ag DNP-KLH (Fig. 3B). This result shows that exposure to nematode infection before vaccination did not suppress B cell function in the presence of a Th cell population that had never been in contact with L. sigmodontis.

To perform the reciprocal experiment, we transferred naive B cells into B cell-deficient mice that were either naive or L. sigmodontis infected and immunized these mice with DNP-KLH (Fig. 3C). Thus, we created recipient mice that contained either naive or nematode primed T cells but naive B cells at the moment of immunization with DNP-KLH. In this experiment, the response to DNP-KLH was again suppressed in the presence of nematode primed T cells although all mice contained naive B cells (Fig. 3D). This result suggests that ongoing L. sigmodontis infection suppressed Ig production of B cells by interference with Th cell-mediated costimulation.

It should be noted that we analyzed mice that were L. sigmodontis infected and received naive B cells in the second setting (Fig. 3C). Thus, the transferred naive B cells were not only exposed to Th cells that had been primed by L. sigmodontis for 14 d but also exposed directly to L. sigmodontis at the moment of immunization (Fig. 3C). Although the priming of B cell responses is completed within the first days of immunization, we cannot formally rule out that exposure to L. sigmodontis infection during this short time contributed to the impaired humoral response. Therefore, we intended to demonstrate the impaired Th cell function in nematode infection directly.

L. sigmodontis infection suppresses proliferation of OVA-specific Th cells and subsequent production of OVA-specific IgG in vivo

As a source for model Ag-specific Th cells, we used the OT-II mouse strain. OT-II T cells are transgenic for an MHC class II-restricted TCR that is specific for the chicken OVA323–339 peptide in association with H-2Kd (35). We transferred CFSE-labeled OT-II cells into naive or L. sigmodontis-infected mice. OT-II T cells were stimulated after 1 d of circulation within the recipient mice by injection of their cognate Ag (Fig. 4A). Activation of OT-II T cells was quantified after an additional 2 d of in vivo stimulation by analysis of proliferation (i.e., CFSE dilution of labeled CD4+ cells in the spleen) (Fig. 4B, 4C). OT-II T cells that had been stimulated within naive mice displayed significantly more division cycles compared with OT-II T cells that had been stimulated within L. sigmodontis-infected mice (Fig. 4D). Analysis of other lymphoid organs such as mesenteric and inguinal lymph nodes revealed similar results (data not shown). Consequently, the frequency (Fig. 4E) and the absolute number of OT-II T cells (Fig. 4F) within the spleen were reduced in L. sigmodontis-infected mice compared with naive mice.

**FIGURE 3.** Suppressed humoral TD response of naive B cells upon adoptive transfer into L. sigmodontis-infected mice. A, Untouched B cells were purified from the spleens of naive mice or mice that had been infected with L. sigmodontis for 14 d and transferred i.v. (1 × 107) into naive B cell-deficient JHT mice. JHT mice were immunized with 200 μg DNP-KLH i.p., and DNP-specific Ig was measured in the serum at time points indicated in the diagram. B, Shown is the titer of DNP-specific IgG1 in JHT mice reconstituted with B cells derived from naive (B cells naive, closed squares) or infected (B cells L. s., open squares) mice. C, B cells isolated from the spleens of naive C57BL/6 mice were transferred into B cell-deficient JHT mice that were either naive or had been infected with L. sigmodontis for 14 d. Subsequently, JHT mice were immunized with 200 μg DNP-KLH, and DNP-specific IgG1 was measured in the serum. D, Shown is the titer of DNP-specific IgG1 in B cell-reconstituted naive (JHT naive, black squares) or infected (JHT L. s., open squares) JHT mice. All results are expressed as mean titer ± SEM (n = 8 for all groups) and represent the combined data of two independent experiments. Asterisks indicate significant differences of the mean: *p < 0.05 (Student t test).
FIGURE 4. Suppressed proliferation of OVA-specific OT-II T cells in L. sigmodontis-infected mice. A, A diagram of the experimental setup. C57BL/6 mice were either left naive or naturally infected with L. sigmodontis. CFSE-labeled OT-II cells were transferred i.v. 14 d later and activated by i.p. application of 50 μg OVA peptide 1 d later. Mice were sacrificed 2 d after in vivo stimulation. Splenocytes were stained for CD4, and proliferation was quantified by dilution of CFSE in CD4+ T cells. B and C, Shown are representative dot plots of spleen cells used for the identification and gating of CFSE+CD4+ OT-II T cells and the respective histograms used for analysis of OT-II T cell division cycles. Markers M1–M4 indicate 0–3 division cycles. D, Shown is the frequency of OT-II T cells that did not divide or divided once, twice, or three times and more after stimulation in either naive (black bars) or infected (open bars) C57BL/6 mice. E and F, Total frequency (E) and number (F) of OT-II T cells recovered from the spleens of naive (black bars) or L. sigmodontis-infected (open bars) mice are shown. Results are combined from five independent experiments and expressed as mean ± SEM (n = 18). Asterisks indicate significant differences of the mean: *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test). G, The frequency of OT-II T cells displaying three and more division cycles is shown in correlation to the worm burden of the mouse they had been stimulated in. Each symbol represents a single mouse. Please note that naive mice are inserted in the graph with a worm burden of zero.

As we performed natural, vector-mediated infections, we cannot control the number of infective larvae transferred and encountered interexperimental differences in worm burden. The correlation of dividing OT-II T cells to worm burden in the individual mice of all experiments performed clearly shows that proliferation was suppressed in all L. sigmodontis-infected mice compared with naive mice. The magnitude of suppression did not increase with increasing worm burden but remained constant within the SD of our assay (Fig. 4G). Taken together, these results show that L. sigmodontis infection, once successfully established, directly impairs Ag-specific proliferation of Th cells in vivo, independent of the individual worm burden.

Next we asked if the suppressed proliferation of OVA-specific Th cells within L. sigmodontis-infected mice would result in equally reduced OVA-specific B cell responses. To this end, naive or L. sigmodontis-infected mice were supplemented with OVA-specific Th cells by adoptive transfer of OT-II cells and vaccinated with complete OVA protein in PBS 1 d later (Fig. 5A). As observed upon stimulation with the OVA323-339 peptide, analysis of OT-II T cell division 2 d later revealed suppressed proliferation of OVA-specific Th cells within L. sigmodontis-infected mice (Fig. 5B). Analysis of the humoral response showed that the production of OVA-specific IgG by host-derived B cells was also significantly suppressed in L. sigmodontis-infected mice compared with naive mice (Fig. 5C). As we injected OVA without any adjuvant such as alum, the only Th cells available for provision of B cell help in significant frequencies are the transferred OT-II T cells. These results suggest that the suppressed OVA-specific IgG response that we observed within L. sigmodontis-infected mice was partly due to the equally suppressed proliferation and thus reduced number of OVA-specific Th cells available although we cannot exclude that direct suppression of B cells by the ongoing L. sigmodontis infection also contributed to the reduced OVA-specific IgG response.

L. sigmodontis infection induces Th2 cytokine production and Foxp3 expression in OVA-specific Th cells in vivo

Nematode-induced suppression of Th cell proliferation in vivo was established after 3 d of OT-II T cell circulation within infected mice. To understand the mechanism of this rapid suppression, we analyzed the phenotype of resident and transferred T cells. We did not observe a decrease in the frequency of follicular Th cells (36) within either resident T cells or OT-II T cells in L. sigmodontis-infected mice as indicated by unchanged expression of CXCR5, CD40-L, ICOS, or PD-1 (data not shown). To analyze cytokine production by resident and transferred Th cells, we activated OT-II T cells within naive and L. sigmodontis-infected mice by application of OVA323-339 as described earlier (Fig. 6A). Spleen cells were harvested 2 d later and restimulated in vitro, using either anti-CD3 to activate all splenic T cells, L. sigmodontis Ag to activate resident nematode-specific T cells, and OVA323-339 for the selective activation of transferred OT-II T cells. The Th2-associated cytokines IL-5 and IL-3 (37) were increased upon nematode infection in both L. sigmodontis-specific, resident T cells and OVA-specific, transferred OT-II T cells after 3 d of circulation within the L. sigmodontis-infected mice (Fig. 6B, 6C). Infection did not affect IFN-γ production in resident or transferred T cells (Fig. 6E) but induced a striking increase in IL-13 and IL-10 production by L. sigmodontis-specific, host-derived T cells (Fig. 6D, 6F).

Next we analyzed the frequency of Treg in resident or transferred T cells as indicated by expression of the transcription factor Foxp3 in unlabeled or CFSE-labeled CD4+ T cells, respectively (Fig. 7A). L. sigmodontis infection induced no significant increase in the ratio of Treg within the resident CD4+ T cell population at day 17.
mediated induction of both Foxp3+ Treg and IL-10 has been shown. As helminth-specific stimulation within naive wt and IL-10–deficient (IL-10 ko) mice (Fig. 9). Foxp3+ OT-II T cells did not divide at all or underwent a single division cycle in both naive and infected mice, whereas we observed up to five division cycles in Foxp3+ OT-II T cells (data not shown).

Taken together, these results show that L. sigmodontis infection induced no increase in the frequency of resident Treg but a significant production of IL-13, IL-5, IL-3, and IL-10 by L. sigmodontis-specific, resident T cells. The phenotype of OT-II T cells was changed upon circulation within L. sigmodontis-infected mice: transferred OT-II T cells showed OV A-specific production of IL-5 and IL-3 and increased frequency of Foxp3. As helminth-mediated induction of both Foxp3+ Treg and IL-10 has been shown to modulate immune responses in other systems (2), we asked if they would contribute to the observed suppression of OT-II T cell proliferation.

**Suppression of Th cell proliferation in L. sigmodontis-infected mice is not mediated by resident Treg**

To analyze whether resident Treg mediated the observed suppression of OT-II T cell proliferation in L. sigmodontis-infected mice, we used the DEREG (depletion of Treg) mouse strain. DEREG mice are transgenic for a bacterial artificial chromosome expressing a DT receptor–enhanced GFP (eGFP) fusion protein under the control of the foxp3 gene locus (38). Thus, they allow the detection of Foxp3+ Treg due to eGFP expression and their depletion by application of DT.

DEREG mice were either left naive or infected with L. sigmodontis. To compare the impact of nematode infection on Th cell activation in the presence or absence of Treg, resident Treg were depleted by application of DT starting 1 d before adoptive transfer of CFSE-labeled OT-II T cells (Fig. 8A). Depletion was controlled by quantification of Foxp3 expression in CD4+ T cells at the time point of in vivo stimulation (Fig. 8B) and at the time point of analysis (Fig. 8C). For analysis, spleens were harvested 2 d after the application of OVA peptide, and proliferation of OT-II T cells was measured by dilution of CFSE. Please note that CFSE-labeled dividing OT-II T cells and eGFP+ Treg could be distinguished within the FL-1 channel as the fluorescence intensity of the eGFP was 100-fold lower than the fluorescence intensity of the CFSE used for OT-II T cell labeling (Fig. 8D, 8F). DT-induced depletion of Foxp3+ Treg at the moment of stimulation was comparable and almost complete in naive and L. sigmodontis-infected mice (Fig. 8B). The Treg compartment started to repopulate within 2 d, whereby Foxp3+ expression preceded GFP expression (Fig. 8C, 8F) as described previously (39).

In the presence of natural Treg frequencies, the transferred OT-II T cells displayed the same suppressed proliferation in L. sigmodontis-infected DEREG mice compared with naive mice (Fig. 8E) that we observed in C57BL/6 mice (Fig. 4D). This was characterized by an increased ratio of OT-II T cells that did not divide or just underwent a single division cycle and a reciprocally decreased ratio of OT-II T cells dividing three or four times in L. sigmodontis-infected mice. Transient depletion of resident Treg at the moment of in vivo T cell stimulation induced an increased proliferation of transferred OT-II T cells: 20–30% OT-II T cells went through four and more division cycles within naive and L. sigmodontis-infected mice. This alternation of resident Treg obviously accelerated OT-II T cell division by one round in both naive and infected mice alike. Therefore, we interpreted the increased frequency of OT-II T cells that divided two times in infected Treg-depleted mice not as restoration of suppressed proliferation but as a consequence of this accelerated proliferation in the absence of Treg. Also, transient depletion of resident natural Treg during the first days of L. sigmodontis infection that we achieved by DT injection 1 d before infection did not restore the OT-II T cell proliferation in these mice at day 17 p.i. (Supplemental Fig. 1).

Taken together, these results show that resident Treg suppressed the proliferation of adoptively transferred OT-II cells in general. Resident Treg did not contribute to the nematode-induced suppression of Th cell proliferation in L. sigmodontis-infected mice.

**Nematode-induced IL-10 mediates Foxp3 induction and contributes to suppression of OT-II T cell proliferation**

To analyze the impact of nematode-induced IL-10 (Fig. 6F) on the suppression of Th cell proliferation, we repeated the in vivo stimulation of OT-II T cells in naive and L. sigmodontis-infected wt and IL-10–deficient (IL-10 ko) mice (Fig. 9A). IL-10 deficiency in recipient mice did not induce a generalized increase of proliferation, as no significant difference between OT-II T cells that underwent three and more division cycles in naive wt and IL-
10 ko mice was observed (Fig. 9B, 9C). Whereas the suppression of OT-II T cell proliferation was clearly visible in naive wt mice compared with \textit{L. sigmodontis}-infected wt mice (Fig. 9B), proliferation was partially restored in \textit{L. sigmodontis}-infected IL-10 ko mice (Fig. 9C). Although still significantly less OT-II T cells divided more than three times within infected IL-10 ko mice in comparison with naive IL-10 ko mice, the magnitude of this difference was lower compared with infected and naive wt mice. Consequently, the total frequencies of OT-II T cells recovered from the spleens of infected and naive IL-10 ko mice were not different, whereas infected wt mice again displayed the significant reduction in OT-II frequency in comparison with noninfected wt mice (Fig. 9D) that we described earlier (Fig. 4E).

Suppression of proliferation was only partly restored in IL-10-deficient \textit{L. sigmodontis}-infected mice, but the expansion of Foxp3$^+$ T cells within the transferred OT-II T cell population was completely abolished in the absence of host-derived IL-10 (Fig. 9E). The partially restored OT-II T cell proliferation and abolished Foxp3 induction in the IL-10 ko mice were not due to different worm burden, as the numbers of L4 in the thoracic cavity were comparable at the day of analysis (Fig. 9F). Moreover, worm burden did not affect the magnitude of suppression in general, as we have shown earlier (Fig. 4G).

The incomplete restoration of Th cell proliferation within nematode-infected mice in the absence of endogenous IL-10 strongly suggests that other nematode-induced regulatory effectors contributed to this suppression. Neutralization of TGF-\(\beta\) by application of a neutralizing anti–TGF-\(\beta\) mAb during in vivo stimulation of OT-II T cells, however, did not restore the suppressed proliferation but induced a general increase in OT-II T cell proliferation in both naive and infected mice (Fig. 9G). In conclusion, these results show that nematode-induced IL-10 but not host-derived Treg or TGF-\(\beta\) contributed the observed suppression of OT-II T cell proliferation cells within \textit{L. sigmodontis}-infected C57BL/6 mice.

**Discussion**

Several lines of evidence suggest that preexisting helminth infections interfere with the immune response to third-party Ags and thus with vaccination efficiency (1–3). In the current study, we use mice that were naturally infected with the pathogenic nematode \textit{L. sigmodontis} to dissect the chain of events leading to this helminth-induced suppression.

First, we demonstrate that model Ag immunizations of \textit{L. sigmodontis}-infected mice resulted in drastically reduced titers of Ag-specific Ig. Thereby, all isotypes (i.e., the Th1-associated isotype...
IgG2c as well as the Th2-associated isotype IgG1) were equally suppressed. These findings show that *L. sigmodontis* infection induced a generalized suppression of B cell function and not a polarization toward a Th2 response. Such polarization to a Th2-dominated humoral response was reported for preexisting infections (40), or naturally infected with *L. sigmodontis* by exposure to infected mites (open squares). CFSE-labeled OT-II cells were transferred and activated as described in Fig. 4. Mice were sacrificed and spleen cells stained for CD4 and Foxp3. B and D, Shown are representative dot plots for CFSE and CD4 used to differentiate between resident CFSE T cells (B) and transferred CFSE T cells (D) and representative histograms showing Foxp3 expression in these T cell populations. C and E. The frequency of Foxp3 in either resident CD4 CFSE spleen cells (C) or in the transferred CD4 CFSE OT-II T cells (E) is shown. Each symbol represents one mouse, results were combined from four experiments, the bar indicating the mean (n = 13 to 15). Asterisks indicate significant difference of the mean: **p < 0.01 (Student t test).

Analyzing the mechanisms underlying the generalized suppression of Ig response in *L. sigmodontis*-infected mice, we provide the following evidence that defective T cell help rather than direct interference with B cell function mediated defensive B cell responses in our system: 1) The responses to TI Ags that do not require T cell help were alike in naive and *L. sigmodontis*-infected mice. Although this does not exclude that B cells were suppressed directly in TD vaccination, it clearly shows that B cell function was not suppressed in this T cell-independent system. 2) Naive B cells failed to respond to TD Ags upon transfer into *L. sigmodontis*-infected mice. 3) Nematode primed B cells derived from *L. sigmodontis*-infected mice responded as efficiently as naive B cells to TD Ag immunization upon transfer into naive hosts. 4) TCRtg OT-II T cells that we used as model Th cells displayed significantly reduced Ag-specific proliferation, altered cytokine production, and increased Foxp3 expression upon stimulation in *L. sigmodontis*-infected mice. 5) The reduced expansion of OVA-specific Th cells within *L. sigmodontis*-infected mice was correlated to reduced production of OVA-specific IgG. Although we cannot exclude that direct suppression of B cells by the ongoing *L. sigmodontis* infection also contributed to the reduced OVA-specific IgG response, this result suggests that the reduced number of OVA-specific Th cells available at least contributed to defective OVA-specific IgG response.

To understand how the established *L. sigmodontis* infection mediated suppression of Th cells, we focused on Treg and IL-10 because there is accumulating evidence that these are central mediators of *L. sigmodontis*-induced suppression of the immune response toward itself (26–29, 43) and to third-party Ags (30–32, 44, 45) in different systems. Also, human studies strongly support the notion that IL-10 is a key mediator of nematode-induced immune suppression (46–49).

We rule out a central role for resident Treg in the infected host because their specific depletion at day 13 p.i. did not restore proliferation of transferred OT-II T cells in *L. sigmodontis*-infected mice. Although transient Treg depletion during the in vivo stimulation of transferred OT-II cells resulted in a generalized increase in Ag-specific proliferation of OT-II T cells in both naive and infected mice, the suppressed proliferation of OT-II T cells within infected mice in direct comparison with naive mice was still present albeit at a higher level. It is, however, conceivable that resident Treg had initiated suppressive circuits in the very beginning of the infection. Taylor et al. (28) showed that depletion of Treg before infection with *L. sigmodontis* reduced worm burden 60 d later, whereas Treg depletion by application of anti–CD25 mAb alone at later time points of infection did not improve resistance (26, 27). In this setting, it was necessary to additionally interfere with other regulatory pathways by application of anti–CTLA-4 (27) or anti–GITR mAb (26) to improve resistance. Consenting with this study, we have shown that the depletion of Treg in *Strongyloides ratti*-infected BALB/c DEREG mice increased resistance to infection only if performed during the first days of infection, whereas depletion at later time points had no beneficial impact (50). Addressing this possibility, we also rule out a contribution of such secondary regulatory mechanisms induced by the presence of natural Treg during the first days of infection, as transient depletion of Treg during the first days of infection did not restore the *L. sigmodontis*-induced suppression of OT-II T cell proliferation at day 17 p.i. in the current study (Supplemental Fig. 1).

We observed a dramatic increase in *L. sigmodontis* Ag-specific IL-10 production in infected mice, and we provide evidence for a contribution of this IL-10 to the *L. sigmodontis*-induced suppression of OT-II T cell proliferation. Although proliferation of OT-II T cells was not completely restored in *L. sigmodontis*-infected IL-10-deficient mice, suppression was less pronounced. The end-point analysis of OT-II T cell frequency in the host spleen...
clearly revealed that reduction in infected mice compared with naive mice was significant in wt mice and no longer significant in IL-10–deficient mice. As we did not observe complete restoration of Th cell function within *L. sigmodontis*-infected mice in the absence of IL-10, clearly other factors play a role. While contribution of nematode-induced TGF-β together with IL-10 has been shown to suppress inflammatory responses in mice infected with the gut-dwelling nematode *Heligmosomoides polygyrus* (51–53), in vivo neutralization of TGF-β did not restore OT-II T cell proliferation in our system.

Regarding other mechanism of suppression, Treg and CD19+ B cells have been shown to mediate suppression of OVA-induced asthma in *H. polygyrus*-infected mice independently of IL-10 (54, 55). Treg were involved in *H. polygyrus*-mediated interference with the protective immune response to *Plasmodium yoelii* infection (56), whereas protection of NOD mice from the onset of diabetes was independent of both Treg and IL-10 (57). The role of TGF-β, however, was not analyzed in these studies (54–57). A population of inducible Foxp3+ Treg (iTR35) that mediate suppression via IL-35 and independent of IL-10 has been described recently (58). As iTgR35 were also induced at the site of a nematode infection (i.e., present in T cells derived from the large intestine of *Trichuris muris*-infected mice), it would be interesting to analyze their contribution in *L. sigmodontis*-mediated suppression.

Although absence of IL-10 alone only partly restored proliferation of OT-II T cells, the observed increase in the frequency of Foxp3+ T cells within the transferred OT-II T cells was completely abrogated. The nematode-induced induction of Foxp3 in TCRtg DO11.10 T cells was shown previously upon implantation of *Brugia malayi* L3 and adults into the peritoneum of BALB/c mice (59) and also upon treatment with *H. polygyrus* excretory-secretory proteins (60). Whereas the latter study clearly showed that Foxp3 was induced de novo by transfer of sorted strictly Foxp3+ DO11.10 reporter mice (60), we transferred TCRtg T cells containing resident Foxp3+ T cells (3.91 ± 0.14% of CD4+). Therefore, we cannot distinguish between expansion of resident Treg and de novo Foxp3 induction. We provide evidence that increased frequency of Foxp3+ OT-II was not a consequence of reduced proliferation of Foxp3+ OT-II in *L. sigmodontis*-infected mice, as IL-10 deficiency completely abrogated Foxp3 induction whereas proliferation was only partly restored. If the frequency of Foxp3+

**FIGURE 8.** No restoration of OT-II T cell proliferation upon Treg depletion in *L. sigmodontis*-infected mice. A, A diagram of the experimental setup. C57BL/6-DEREG mice were either left naive or naturally infected with *L. sigmodontis*. Resident Treg were depleted by application of DT (1 µg i.p.) at days 13 and 14 p.i. CFSE-labeled OT-II cells were transferred at day 14 p.i. and activated as described in Fig. 4. B and C, Frequency of splenic Foxp3+ in CD4+ T cells at days 15 and 17 p.i. after depletion of Treg (+DT) in comparison with untreated DEREG mice (−DT) are shown. D and F, Representative dot blots showing eGFP/CFSE expression of the CD4+ population in untreated (−DT) and DT-treated (+DT) recipient mice are shown. Please note that discrimination of CFSE+ CD4+ OT-II T cells and eGFP+ Treg is possible because of different intensities of CFSE and eGFP in FL1. E and G, The frequency of OT-II T cells that did not divide or divided one to three or four and more times after stimulation in either naive (black bars) or infected (open bars) C57BL/6-DEREG mice without DT treatment (E) or after DT treatment (G) is shown. Results are combined from three independent experiments and expressed as mean (±SEM, n = 6). Asterisks indicate significant differences of the mean: *p < 0.05, **p < 0.01 (Student t test).
OT-II T cells had reflected the decreased proliferation of Foxp3^− OT-II T cells and thus reduced dilution of Foxp3^+ OT-II T cells, a partial restoration of proliferation would have induced partial and not complete restoration of the original Foxp3^+ frequency.

Taken together, our results suggest the following chain of events: Acute *L. sigmodontis* infection induced IL-10 production by host-derived leukocytes in C57BL/6 mice. This IL-10–induced increase of Foxp3 in Th cells, together with other mediators that remain to be elucidated, interfered with proliferation of Th cells. As OVA–specific Th cells displayed no modulation in receptors significant for Th cell function such as CXCR5 or CD40L, it is most likely that the reduced number of Th cells available resulted in inefficient help for B cells responding to TD Ags. This inefficient T cell help contributed to the reduced TD humoral response, whereas B cells responding to TI Ags remained unchanged in function.

Our study focused on the impact of acute *L. sigmodontis* infection on the humoral response using the semipermissive C57BL/6 mouse strain (61). Preliminary experiments show that *L. sigmodontis* infection interfered with Th cell proliferation and humoral response to TD but not TI model Ags also in the fully permissive BALB/c mouse strain, which allows the analysis of effects induced by chronic *L. sigmodontis* infection (W. Hartmann and I. Haben, unpublished observations).

Finally, we would like to emphasize our observation that successful acute infection of C57BL/6 mice at day 14 induced suppression of Th cell proliferation independent of the actual worm burden. This finding strongly suggests that responsible *Litomosoides*-derived factors would either act in a dose-independent manner or are abundantly available. We are currently analyzing the suppression conferred by different *L. sigmodontis* larval stages in both acute and chronic infection as well as the duration of suppression after treatment or resolution of infection. These studies will help to design more efficient vaccination schemes for the human population in areas of endemic helminth infections.

**FIGURE 9.** Partial restoration of OT-II T cell proliferation in IL-10–deficient *L. sigmodontis*-infected mice. A, A diagram of the experimental setup. C57BL/6 wt or IL-10 ko mice were either left naive or naturally infected with *L. sigmodontis*. CFSE-labeled OT-II cells were transferred and activated as described for Fig. 4. B and C, The frequency of OT-II T cells that underwent the indicated numbers of cell divisions after stimulation in either naive (black bars) or infected (open bars) wt mice (B) or IL-10 ko mice (C) is shown. Results are expressed as mean ± SEM (*n* = 13). D, Total frequency of OT-II T cells recovered from spleens of infected (open squares) and naive (closed triangles) wt and IL-10 ko mice is shown. E, Frequency of Foxp3^+ T cells within the transferred OT-II T cell population recovered from infected (open squares) and naive (closed triangles) wt and IL-10 ko mice is shown. F, Number of worms in the pleural cavity in wt and IL-10 ko mice at day 17 p.i. is shown. E and F, Each symbol represents a single mouse, the bar indicating the mean (*n* = 12 to 13). Results are combined data from four experiments. G, CFSE-labeled OT-II cells were transferred in either infected (open squares) or naive (closed triangles) mice that had not (control) or had been treated with 100 μg anti–TGF-β mAb (clone 1D11) i.p. at day 14 and day 16 p.i. Shown is the frequency of OT-II T cells in the spleen. Data are combined results from three independent experiments; each symbol represents one mouse (*n* = 8 to 10). Asterisks indicate significant differences of the mean: *p* < 0.05, **p** < 0.01, ***p*** < 0.001 (Student *t* test).
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Disclosures

The authors have no financial conflicts of interest.

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