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Infection with a Helminth Parasite Attenuates Autoimmunity through TGF-β-Mediated Suppression of Th17 and Th1 Responses

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The lower incidence of allergy and autoimmune diseases in developing countries has been associated with a high prevalence of parasitic infections. Here we provide direct experimental evidence that parasites can exert bystander immunosuppression of pathogenic T cells that mediate autoimmune diseases. Infection of mice with Fasciola hepatica resulted in recruitment of dendritic cells, macrophages, eosinophils, neutrophils, and CD4+ T cells into the peritoneal cavity. The dendritic cells and macrophages in infected mice expressed IL-10 and latency-associated peptide, and they had low surface expression of costimulatory molecules and/or MHC class II. Furthermore, most CD4+ T cells in the peritoneal cavity of infected mice secreted IL-10, but not IFN-γ or IL-4. There was a less significant expansion of CD4+Foxp3+ T cells. F. hepatica-specific Tr1-type clones generated from infected mice suppressed proliferation and IFN-γ production by Th1 cells. Infection was associated with suppression of parasite-specific Th1 and Th2 responses, which was reversed in IL-10-defective mice. Infection with F. hepatica also exerted bystander suppression of immune responses to autoantigens and attenuated the clinical signs of experimental autoimmune encephalomyelitis. Protection was associated with suppression of autoantigen-specific IFN-γ and IL-17 production. The suppression of Th1 and Th17 responses and attenuation of experimental autoimmune encephalomyelitis by F. hepatica was maintained in IL-10−/− mice but was reversed by neutralization of TGF-β in vivo. Our study provides evidence that F. hepatica-induced IL-10 subverts parasite-specific Th1 and Th2 responses, but that F. hepatica-induced TGF-β plays a critical role in bystander suppression of autoantigen-specific Th1 and Th17 responses that mediate autoimmune diseases. The Journal of Immunology, 2009, 183: 1577–1586.

Epidemiological and experimental studies suggesting that helminth infections can have a protective effect against the development of both autoimmune and allergic diseases (1) have provoked a major shift in our understanding of T cell regulation. The original hygiene hypothesis suggested that the increasing prevalence of allergies and asthma in industrialized countries may be linked to reduced infections with parasites and bacterial pathogens and was explained on the basis of Th1-Th2 cross-regulation (2, 3). However, this model did not account for the concomitant increase in incidences of many autoimmune diseases (4, 5) and the recently established role of IL-17-producing cells (6, 7), nor the inverse correlation between exposure to Th2-inducing helminths and incidence of allergy (1, 8, 9).

Since the initial description of Th1 and Th2 cells in the mid 1980s and the ensuing evidence of their reciprocal roles in controlling immune responses, a number of additional subtypes of CD4+ T cells have been defined. These include a number of distinct regulatory T (Treg)5 cell subsets, which play a major role in suppressing immune responses to self Ags, thereby maintaining tolerance and preventing autoimmunity (10). Treg cells also function to control anti-pathogen effector T cell responses to limit immunopathology during infection (11). More recently it has been demonstrated that Th17 cells, which develop via cytokine signals distinct from, and antagonized by, products of Th1 and Th2 lineages, are major mediators of inflammation and play a critical pathogenic role in many organ-specific autoimmune diseases (6, 7, 12).

It has already been demonstrated that parasite-induced Treg cells can suppress Th2-mediated allergic responses (13). Furthermore, it has been reported that colonization with Heligmosomoides polygyrus suppresses IL-17 production in the mesenteric lymph node (14). Experimental studies in mouse models have also demonstrated that helminth infections can ameliorate autoimmune diseases (15–17), and although Th2 and anti-inflammatory cytokines have been implicated in protection, the role of Treg cells is still unclear. It has been demonstrated that Treg cells can protect against systemic inflammatory disease in mice (10, 18); however, there is limited evidence that Foxp3+ natural Treg cells can suppress Th17 cells, especially in humans (19). Furthermore, TGF-β has been shown to have both a positive and negative role in driving the development of Th17 cells (20–22). In this study, we provide evidence that a helminth parasite can suppress autoimmune disease through a TGF-β-dependent mechanism.

Abbreviations used in this paper: Treg cell, regulatory T cell; BFA, brefeldin A; DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; ICS, intracellular cytokine staining; LAP, latency-associated peptide; MOG, myelin oligodendrocyte glycoprotein; PEC, peritoneal exudate cell; Tg, transgenic; WT, wild type.

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We previously reported that infection with the liver fluke *Fasciola hepatica* induces polarized Th2 responses in mice, suppressed Th1 responses, and delayed clearance of the bacterial pathogen *Borrelia pertussis* (23, 24). Here we demonstrate that this helminth parasite can modulate dendritic cell (DC) function to induce parasite-specific Treg cells that express IL-10 and TGF-β and inhibit the induction not only of Th1 and Th2 responses to the parasite, but also autoantigen-specific Th1 and Th17 responses that mediate experimental autoimmune encephalomyelitis (EAE).

**Materials and Methods**

**Animals and F. hepatica infection**

Female BALB/c and C57BL/6 mice were purchased from Harlan Olac. IL-10−/− on a C57BL/6 background and DO11.10 OVA TCR transgenic (Tg) mice on a BALB/c background were obtained from The Jackson Laboratory and bred in-house. Mice were housed in individually ventilated cages, and all experiments were performed according to the regulations of the Irish Department of Health, the European Union, and the Ethics Committee of Trinity College Dublin. Mice were infected with *F. hepatica* by oral inoculation with 10 viable metacercariae of *F. hepatica* (Compton Laboratories). This resulted in infection in 100% of animals.

**Induction of EAE and treatment with anti-TGF-β**

C57BL/6 or IL-10−/− mice were each injected s.c. with 150 μg of myelin oligodendrocyte glycoprotein (MOG)35–55 in CFA containing 5 mg/ml Mycobacterium tuberculosis H37Ra (Difco). Mice were injected i.p. with 500 ng of pertussis toxin (Sigma-Aldrich) on days 0 and 2. For treatment with anti-TGF-β, mice were injected i.p. every 2 days from day 0 to day 18 with 100 μg of anti-TGF-β (clone 1D11, reactive to mouse TGF-β1, TGF-β2, and TGF-β3; Bioceros) or with an isotype-matched control Ab (anti-β-galactosidase, GL 113; Bioceros). EAE was scored as follows: 1, limp tail or waddling gait with tail tonicity; 2, waddling gait with limp tail; 3, hind limb weakness; 4, hind limb paralysis. Mice were examined daily in a blinded fashion for signs of EAE. Experiments were terminated when untreated mice with EAE displayed a clinical score of 4.

**Immunofluorescence analysis by FACS**

Peritoneal exudate cells (PEC) were prepared from *F. hepatica* or naive control mice. Cells were recovered and blocked by incubating in Fcγ blocker (BD Pharmingen; 1 mg/ml) for 20 min. Cells were labeled with the following fluorochrome-conjugated anti-mouse Abs: CD11c-PE-Cy7 (P150/90) or CD11c-alkaline phospho- cacin (N418), F4/80-PE-Cy5 (BM8), Gr1-PE-Cy7 (RB6-SC5), CD40-FITC (HM40-3), CCR5-PE (7A4) (eBioscience), Siglec-F-PE (E50-2440), I-A/E-MHC class II)-FITC (2G9), CD80-PE (16-10A1), CD86-FITC (GL1) (BD Biosciences), or with appropriate isotype control Abs. Alternatively, for detection of T cell subsets, PEC were labeled with the following fluorochrome-conjugated Abs: CD4-PE (GK1.5) or CD4-PE-Cy7 (L3T4), CD127-PE-Cy7 (A7R34), CD25-PE-Cy5 (PC61.5), CTLA4-PE (UC 10-4B9) (eBioscience), or with biotinylated T1/ST2 (Millennium Pharmaceuticals) and biotinylated latency-associated peptide (LAP; R&D Systems), followed by streptavidin-allophycocyanin-Cy7 (eBioscience). Intracellular Foxp3 staining was performed according to the manufacturer’s instructions (eBioscience). Briefly, cells were stained for Abs to CD4 and CD25 at 4°C for 30 min. Cells were then fixed, permeabilized, and stained with anti-mouse/rat Foxp3-FITC (FJK-16s) at 4°C for 30 min and washed. Immunofluorescence was analyzed using CellQuest software on a FACSCalibur (BD Biosciences) or using FlowJo software on a CyAn flow cytometer (Dako).

**Intracellular cytokine staining**

To analyze cytokine production in DC, macrophages, and T cells from *F. hepatica*-infected mice, PEC were cultured for 10 h in brefeldin A (BFA; 5 μg/ml), washed, blocked with Fcγ blocker (BD Pharmingen; 1 μg/ml), stained with CD11c, F4/80, or CD4, and then fixed and permeabilized (Fix/Perm cell permeabilization kit; Caltag Laboratories) and stained with Abs specific for and stained with the following fluorochrome-conjugated Abs: IL-10-FITC (JES16E3), IL-4-PE (J5516E3), IL-4-FITC (BV6D24G2), IFN-γ-allophycocyanin (XM11.2) (eBioscience), and TGF-β-PE (TB21) (IQ Products) or with appropriate isotype control Abs. Flow cytometric analysis was performed. Cells were gated on CD11c, F4/80, or CD4.

**APC function**

Purified CD4+ T cells from DO11.10 OVA TCR Tg mice were MACS purified. OVA-specific T cells (1 × 10⁶/ml) were cultured with OVA23–39 peptide (New England Peptide; 2 μg/ml) and irradiated PEC or splenic CD11c+ (2 × 10⁶/ml) as APC. After 72 h of incubation, supernatants were removed and IFN-γ, IL-17, IL-4, and IL-10 concentrations determined by ELISA.

**F. hepatica-specific T cell clones**

*F. hepatica*-specific T cell lines were established from PEC of *F. hepatica*-infected mice by co-culture with *F. hepatica* Ag (liver fluke homogenate; 40 μg/ml) for 5 days followed by culture in medium with IL-2 for a further 5–7 days. CD4+ T cell clones were generated by cloning these T cell lines by limiting dilution at 1 cell per well. T cell clones were maintained by 10- to 12-day cycles of culture with *F. hepatica* Ag and APC (irradiated spleen cells, 2 × 10⁶/ml), with IL-2 added after 5 days.

**Ag-specific proliferation and cytokine production**

Spleen cells (2 × 10⁶/ml), lymph node cells (1 × 10⁶/ml), or *F. hepatica*-specific T cell clones (1 × 10⁶/ml) with APC (2 × 10⁶/ml) were cultured with either *F. hepatica* Ag (4–20 μg/ml), MOG peptide (10–100 μg/ml), OVA peptide (20–200 μg/ml), PMA (25 ng/ml; Sigma-Aldrich) and anti-CD3 (0.5 μg/ml; BD Pharmingen) or medium only. After 72 h, supernatants were collected and the concentrations of IL-4, IL-5, IL-10, IL-17, IFN-γ, and latent TGF-β were quantified by ELISA. Latent TGF-β was detected after acid treatment of the samples. Due to the cross-reactivity between murine and bovine TGF-β present in FCS, the amount of total TGF-β present in the culture medium was assayed and subtracted from the total latent TGF-β concentration. Alternatively, cells were cultured in FCS-free X-VIVO 15 medium (Lonza). Proliferation was assessed after 4 days by [3H]thymidine incorporation.

**T cell suppression assays**

Purified OVA-specific CD4+ T cells (1 × 10⁶/ml) from DO11.10 mice were cultured with OVA23–39 peptide (2 μg/ml) and APC (irradiated spleen cells, 2 × 10⁶/ml) either alone or with *F. hepatica*-specific CD4+ T cell clones or CD4+ T cells purified from the peritoneal cavity of *F. hepatica*-infected mice and *F. hepatica* Ag (20 μg/ml) at a 1:1 ratio. The different T cell populations were cultered either in the same well or separated by a semipermeable membrane (transwell). *F. hepatica*-specific CD4+ T cells cultured with *F. hepatica* Ag (20 μg/ml) and APC (irradiated spleen cells) alone acted as negative controls. After 72 h, incubation supernatants were removed and IL-10 and IFN-γ concentrations determined by ELISA. Proliferation was determined by [3H]thymidine incorporation after 96 h of culture.

**Statistical analysis**

Data were compared by an unpaired *t* test or by one- or two-way ANOVA. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups.

**Results**

*F. hepatica* infection modulates DC maturation and function

Following oral infection with *F. hepatica* metacercariae, juvenile parasites migrate from the gut through the peritoneal cavity to the liver. During this migratory period, cells of the peritoneal cavity are exposed to the flukes and their excretory-secretory products. Here we found that infection of mice with *F. hepatica* was associated with infiltration of DC, macrophages, eosi-nophils, neutrophils, and CD4+ T cells into the peritoneal cavity. In comparison with control uninfected mice, there was a substantial increase in the percentage (Fig. 1B) and absolute numbers (Fig. 1A) of CD11c+ cells in infected mice. There was also an increase in the percentage of CD11c+ cells and increases in the absolute numbers of CD11c+ and CD11c+ cells in infected mice. The CD11c+ and CD11c+ cells included two distinct populations based on forward light scatter. CD11c+ is expressed on various DCs, macrophages, eosinophils, and neutrophils. CD11c+ cells are exposed to the flukes and their excretory-secretory products. Here we found that infection of mice with *F. hepatica* was associated with infiltration of DC, macrophages, eosinophils, neutrophils, and CD4+ T cells into the peritoneal cavity. In comparison with control uninfected mice, there was a substantial increase in the percentage (Fig. 1A) and absolute numbers (Fig. 1B) of CD11c+ cells in infected mice. There was also an increase in the percentage of CD11c+ cells and increases in the absolute numbers of CD11c+ and CD11c+ cells in infected mice. The CD11c+ and CD11c+ cells included two distinct populations based on forward light scatter. CD11c+ is expressed on various DCs, macrophages, eosinophils, and neutrophils.
In comparison with control mice, CD11c+ cells from *F. hepatica*-infected animals had significantly lower expression of CD80, CD40, MHC class II (Fig. 2A), and CD86 (not shown) and higher expression of CCR5 (Fig. 2A), a phenotype of immature DC. Furthermore, MHC class II expression on F4/80+ and Siglec-F+ was lower in infected compared with control mice (Fig. 2B). Intracellular cytokine staining (ICS) revealed a very high frequency of IL-10-producing CD11c+ DC in *F. hepatica*-infected mice (Fig. 2C). More than 30% of ex vivo CD11c+ DC from the peritoneal cavity of infected mice were positive for IL-10 when stained with anti-IL-10 without restimulation in vitro. In contrast, IL-10 could not be detected in DC from the peritoneal cavity of naïve control mice (Fig. 2C), and IFN-γ and IL-4 expression by DC was not enhanced by infection (data not shown). Furthermore, DC from *F. hepatica*-infected mice had higher cell surface expression of LAP (Fig. 2C); LAP is bound to TGF-β as part of a latent complex secreted from the cell, and biologically active TGF-β is released by cleavage from LAP, which is retained on the cell surface. A significant proportion of CD11c+ cells also expressed IL-10 and LAP (data not shown). F4/80+ macrophages from the peritoneal cavity of infected mice had high expression of IL-10 and TGF-β detectable by ICS. More than 60% of macrophages from the peritoneal cavity of infected mice expressed TGF-β, compared with 7% from control mice. Macrophages from infected mice also had higher expression of surface LAP than did cells from uninfected control mice (Fig. 2C). Our data demonstrate that infection with *F. hepatica* is associated with local recruitment of DC and macrophages that have low expression of costimulatory and/or MHC class II molecules and express immunosuppressive cytokines. This was a consistent finding in BALB/c and C57BL/6 mice (data not shown).

We next examined the capacity of APC from a local or distant site from *F. hepatica* infection to activate T cells. Total PEC and splenic DC from *F. hepatica*-infected or naïve control mice were isolated and cultured with purified CD4+ T cells from DO11.10 OVA TCR Tg mice and OVA peptide 323–329. PEC from *F. hepatica*-infected mice induced significantly less OVA peptide-induced IL-10, IL-17, and IFN-γ production compared with PEC from naïve control mice (Fig. 3A). Addition of anti-IL-10 enhanced, although not significantly, IL-17 production by OVA-specific T cells from *F. hepatica*-infected mice, but it did not enhance IL-17 or IL-4 production by OVA-specific CD4+ T cells with PEC from *F. hepatica*-infected mice (Fig. 3A). Anti-TGF-β reduced OVA-specific IFN-γ production by CD4+ T cells with PEC from control or infected mice, but it did not affect production of other cytokines. Splenic APC from *F. hepatica*-infected mice promoted significantly greater IL-10 and IL-4 production, but equivalent IFN-γ production, when compared with splenic APC from control animals (Fig. 3B). These data show that APC from the peritoneal cavity of *F. hepatica*-infected mice, but not from a distant site, have a limited capacity to induce effector T cell responses, including IFN-γ production from Th1 cells. The findings also suggest that the defect in APC function of PEC from *F. hepatica*-infected mice is not due to IL-10 or TGF-β production, but may reflect down-regulation of MHC and costimulatory molecules on the APC (Fig. 2).

**Infection with *F. hepatica* induces Treg cells with immunosuppressive function**

Since *F. hepatica* infection induces TGF-β- and IL-10-producing macrophages and IL-10-producing DC and inhibits DC maturation, we examined the possibility that infection with *F. hepatica* was also associated with the induction of Ag-specific

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**FIGURE 1.** Macrophages, DC, eosinophils, neutrophils, and CD4+ T cells are recruited to the peritoneal cavity of *F. hepatica*-infected mice. C57BL/6 mice were infected with 10 viable metacercariae of *F. hepatica*. Three weeks after challenge, PEC were isolated from *F. hepatica*-infected and naïve control mice by peritoneal lavage. Cells were stained with Abs against cell surface marker as indicated and cytometric analysis was performed. A. Dot plots show frequency of CD11c, F4/80, GR1, Siglec-F vs forward scatter (FS), and GR1 vs Siglec-F. B. Mean (±SD) absolute numbers of CD11cint, F4/80−, GR1intSiglec-F−, and CD4+ cells in peritoneal cavity of control and *F. hepatica*-infected mice based on total cell counts and percentages for individual control and infected mice (four mice per group). C. Siglec-F vs FS for CD11cint and F4/80− cells. D. F4/80 vs FS gated on GR1intSiglec-F−, GR1−Siglec-F−, GR1− Siglec-F+, and GR1+ Siglec-F+ cells. Results are representative of four mice per group and of two experiments.
Treg cells. F. hepatica-specific CD4+ T cells lines were generated from the peritoneal cavity of infected mice, and these were cloned by limiting dilution. Each of the T cell clones examined secreted IL-10 and low or undetectable concentrations of IL-5 or IFN-γ (Fig. 4A). The T cell lines used to generate the T cell clones expressed CD4 but not Foxp3 (data not shown), suggesting that they are Ag-specific adaptive IL-10-producing Tr1-like Treg cells. ICS also demonstrated that a very high frequency (≥70%) of T cells in the peritoneal cavity of F. hepatica-infected mice secrete IL-10 in the absence of IL-4 or IFN-γ production (Fig. 4B). Phenotypic analysis revealed that a high frequency of T cells from the peritoneal cavity of F. hepatica-infected mice expressed CD25, CTLA-4, T1/2, CCR5 (Fig. 4C), and IL-10R (data not shown), markers that are expressed on T cells (10, 11, 26). The frequency of CD4+ T cells expressing LAP was significantly enhanced in infected compared with control mice (Fig. 4D). Furthermore, a high percentage of CD4+ T cells in the peritoneal cavity of control and infected mice expressed intracellular TGF-β. The absolute numbers of TGF-β-expressing CD4+ T cells were dramatically greater in infected compared with control mice (1.4 × 10^5 vs 0.15 × 10^5, respectively). The frequency of Foxp3+ CD4+ T cells increased from 6% in control mice to ~20% in mice infected with F. hepatica (Fig. 4D). Furthermore, a significant proportion of these cells produced IL-10 and a lower frequency expressed LAP (Fig. 4D).

We next examined T cell responses in the mesenteric lymph node of infected and control mice. Mesenteric lymph node of infected, but not control mice, secreted IL-10 and IL-5, but undetectable IL-4 or IFN-γ following restimulation with F. hepatica Ag ex vivo; the same cells stimulated with PMA and anti-CD3 produced all four cytokines examined (Fig. 5A). However, there was only a modest increase in the percentage of CD4+CD25+ Foxp3+, but not CD4+CD25+Foxp3+, in the mesenteric lymph nodes in infected mice (Fig. 5B). Infection was also associated with a small increase in the percentage of CD4+CD25+CD127−, an alternative phenotype associated with Treg cells (Fig. 5B).

Collectively, these findings suggest that infection with F. hepatica is associated with recruitment or expansion of natural or inducible Foxp3+ Treg cells at the site of infection and to a lesser extent in local lymph nodes, and it is also associated with the induction of Ag-specific adaptive Tr1 cells.

We next examined the ability of T cells from infected mice to suppress Ag-induced proliferation and cytokine production by effect T cells in vitro. CD4+ T cells purified from the peritoneal cavity of F. hepatica-infected mice significantly suppressed proliferation and IFN-γ production by OVA-specific T cells from OVA TCR Tg mice (Fig. 6A). Furthermore, a parasite-specific Treg cell clone generated from a F. hepatica-infected mouse, which secreted IL-10 but not IFN-γ, significantly suppressed IFN-γ production by an OVA-specific Th1 clone (Fig. 6B). Suppression of the Th1 clones was observed when cocultured with the Treg cell clone and across a semipermeable membrane (Fig. 6B). These findings demonstrate that parasite-specific Treg cells from F. hepatica-infected mice exert bystander suppression of Th1 responses to an unrelated Ag via release of soluble factors.

**FIGURE 2.** Regulatory macrophages and DC are recruited to the peritoneal cavity of F. hepatica-infected mice. Mice were infected with 10 viable metacercaire of F. hepatica. Three weeks after challenge, PEC were isolated from F. hepatica-infected and naive control mice by peritoneal lavage. Cells were stained with Abs specific for the indicated cell surface marker and cytofluorometric analysis was performed. A, Expression of CD80, CD40, CCR5, and MHC class II on CD11chigh cells from F. hepatica-infected (solid black line) and control (gray histogram) mice vs isotype-matched control Abs (gray line). B, Expression of MHC class II on F4/80+ and on Siglec-F+ cells from uninfected control (gray histogram) mice vs isotype-matched control Abs (gray histogram) mice vs isotype-matched control Abs (gray line). C, Cells from uninfected control (gray histogram) mice vs isotype-matched control Abs (gray histogram) mice vs isotype-matched control Abs (gray line).
Self Ag-specific Th1 and Th17 cells mediate pathology in a number of autoimmune diseases, including EAE (6, 27–29). Having shown that *F. hepatica* infection induces Treg cells, we examined the influence of *F. hepatica* infection on the development of EAE. Untreated mice developed clinical symptoms of EAE after 12 days and reached clinical scores of between 3 and 4 after 20 days and were sacrificed (Fig. 8A). In contrast, symptoms of EAE did not develop until day 14 in *F. hepatica*-infected mice (Fig. 8A). Furthermore, the severity of disease in these mice was significantly reduced by *F. hepatica* infection.

The attenuation of clinical signs of disease by *F. hepatica* infection was associated with suppression of MOG-specific Th1 and Th17 cells (Fig. 8B). In uninfected mice, significant concentrations of IL-17 and IFN-γ, but not IL-10, were detected in supernatants of spleen cells stimulated ex vivo with MOG peptide. In contrast, T cells from *F. hepatica* infected mice secreted significantly less MOG-specific IL-17 and IFN-γ and significantly higher concentrations of MOG-specific IL-10 (Fig. 8B). Furthermore, TGF-β production by medium or MOG-stimulated spleen cells was significantly enhanced in mice infected with *F. hepatica* (Fig. 8C).

Infection with *F. hepatica* suppresses MOG-induced Th1 and Th17 cells and attenuates EAE through the induction of Treg cells

To examine the mechanism of suppression of autoantigen-specific T cell responses, we first examined the role of IL-10. EAE was induced in C57BL/6 WT and IL-10−/− mice with and without infection with *F. hepatica*. Consistent with the immunosuppressive properties of IL-10, EAE was exacerbated in noninfected IL-10−/− compared with WT mice (Fig. 9A), and this was associated with elevated MOG-specific IL-17 production (Fig. 9B). However, infection with *F. hepatica* significantly attenuated the clinical signs of EAE in IL-10−/− as well as WT mice (Fig. 9A), suggesting that the protective influence of *F. hepatica* on the clinical course of EAE is retained in the absence of IL-10. Furthermore, infection with *F. hepatica* significantly suppressed MOG-specific IL-17 and IFN-γ in IL-10−/−, as well as in WT mice, with a coincident elevation in MOG-specific IL-10 production in WT mice (Fig. 9B). These data indicate that endogenous IL-10 does play a protective role in preventing the development of EAE, but they demonstrate that suppression of pathogenic autoantigen-specific T cells and the clinical signs of EAE by *F. hepatica* are not mediated by IL-10.

We next examined the possible role of TGF-β in parasite-mediated suppression. Administration of a neutralizing anti-TGF-β Ab in vivo to noninfected mice with EAE had little effect on the course of disease (Fig. 10A). Infection with *F. hepatica* significantly attenuated the clinical symptoms of EAE in mice treated with a control Ab. Administration of the TGF-β Ab completely reversed the protective effect of the infection on the development of EAE. The *F. hepatica*-infected mice treated with anti-TGF-β had more severe EAE than did infected mice treated with a control Ab or noninfected mice treated with anti-TGF-β (Fig. 10A). The protective effect of *F. hepatica* infection was associated with a reduction in MOG-specific IL-17 and enhancement of endogenous and MOG-specific TGF-β production (Fig. 10B). The data from the TGF-β ELISA need to be interpreted with caution, as unlike the data in Fig. 6, culture medium with FCS was used for this experiment, with the background values subtracted. However, surface expression of LAP, the amino-terminal domain of the TGF-β precursor peptide, was also enhanced on CD4+ T cells from spleens of *F. hepatica*-infected EAE, when compared with infected with *F. hepatica*, and Ag-specific cytokine production was examined. Mesenteric lymph node cells from wild-type (WT) mice infected with *F. hepatica* produced IL-10 and IL-5, but not IFN-γ or IL-17, when stimulated in vitro with *F. hepatica* Ag (Fig. 7A). In contrast, significant concentrations of IFN-γ and enhanced IL-5 were detected in Ag-stimulated lymph node cells from IL-10−/− mice. Furthermore, when compared with control mice, IL-10 and IL-5 concentrations were elevated in the peritoneal fluid of *F. hepatica*-infected WT mice, but IL-4, IL-17 (Fig. 7B), and IFN-γ (not shown) were undetectable. In contrast, significant concentrations of IL-4 and higher concentrations of IL-5 were detected in the peritoneal fluid of *F. hepatica*-infected IL-10−/− mice when compared with WT mice (Fig. 7B). These findings demonstrate that suppression of parasite-specific Th1 and Th2 responses in *F. hepatica*-infected mice is mediated by IL-10.
cells from noninfected mice with EAE (Fig. 10C), thus confirming enhanced TGF-β in *F. hepatica*-infected mice. The reversal by anti-TGF-β of the protective effect of *F. hepatica* and the increased severity of EAE correlated with enhancement of MOG-specific IL-17 production (Fig. 10B). Although we do not have a definitive explanation for the latter findings, it is possible that in the absence of the antiinflammatory effect of TGF-β, the net effect of the helminth infection may be proinflammatory, thereby promoting pathogenic T cells and EAE. Alternatively, the Ab treatment may not have completely neutralized the helminth-induced TGF-β, and any residual TGF-β may have enhanced Th17 responses and EAE. This is consistent with the suggestion that low-dose TGF-β promotes Th17 differentiation, whereas high-dose TGF-β inhibits Th17 responses (12).

**Discussion**

There is now convincing evidence that exposure to certain infectious agents or their products may reduce the symptoms of allergy and asthma in humans (1, 8, 9). This was initially explained on the basis of suppression of Th2-mediated allergic reactions by pathogen-induced Th1 responses. There are also reports from studies with mouse models that infection with helminth parasites can suppress not only Th2-mediated allergic disorders, but also autoimmune diseases (15–17). This is more difficult to explain on the
basis of Th1-Th2 cross-regulation, since parasites have traditionally been associated with Th2 responses, whereas autoimmune diseases are thought to be mediated by Th17 cells (6, 7, 12), with less clear but reemerging evidence of a role for Th1 cells (27–29).

However, it has recently been reported that immunosuppression by a parasite infection is associated with the induction of natural Treg cells (13). Here, we demonstrate that a helminth infection induces Ag-specific adaptive Treg cells, suppresses the generation of autoantigen-specific Th1 and Th17 cells, and attenuates the induction of EAE through a TGF-β-dependent mechanism.

Much of the focus to date on Treg cells induced by parasites has been on natural Treg cells. CD4⁺CD25⁺ Treg cells have been shown to contribute to immune suppression during malaria infection, allowing the parasite to escape from host protective immune responses (30). It has also been demonstrated that CD4⁺CD25⁺ Treg cells maintain Leishmania major persistence after resolution of dermal lesions in resistant mice (31). Studies in humans have shown that individuals with chronic parasitic infections develop prominent antiinflammatory networks, leading to an attenuation of Ag-specific immune responses to both the parasite and unrelated pathogens (1), suggesting that the parasite cannot only suppress host immune responses against itself, but can exert bystander suppression against third-party Ags. Further experimental evidence for this was provided by the demonstration that CD4⁺CD25⁺ Treg cells induced by the gastrointestinal nematode H. polygyrus suppressed allergic responses to OVA or DerP1 in sensitized animals, a process in which Foxp3 expression and TGF-β production have been implicated, but which was IL-10 independent (13). Our study revealed that infection with *F. hepatica* is associated with expansion or recruitment of Foxp3⁺ Treg cells and induction of adaptive Ag-specific Treg cells, which suppress host immune response to the parasite and to unrelated Ags.

**FIGURE 5.** Infection with *F. hepatica* induces Ag-specific IL-10-producing T cells in the mesenteric lymph node cells. A, Mesenteric lymph node cells were recovered 3 wk after *F. hepatica* infection or from control naive mice. Cells were stimulated ex vivo with *F. hepatica* Ag, medium, or PMA and anti-CD3, as positive control. Supernatants were removed after 72 h, and the concentrations of IFN-γ, IL-4, IL-5, and IL-10 were quantified by ELISA. Results are mean values for triplicate cultures for four mice per group and are representative of two experiments. B, Mesenteric lymph node cells from naive control and *F. hepatica*-infected mice were stained with anti-CD4, anti-CD25, and anti-CD127 and intracellularly with anti-Foxp3 and FACS analysis was performed. Cells were gated on CD4. Numbers are percentage of CD4⁺ T cells.

**FIGURE 6.** *F. hepatica*-induced Treg cells suppress IFN-γ production by Th1 cells. A, CD4⁺ T cells purified from DO11.10 Tg mice (Teff) were cultured with OVA peptide (2 μg/ml) and APC alone or in the presence of CD4⁺ T cells purified from the peritoneal cavity of *F. hepatica*-infected mice (Treg cells). Supernatants were removed after 72 h, and the concentrations of IFN-γ were determined by ELISA; proliferation was determined by BrdU; proliferation was determined after 4 days. ***p < 0.001 vs Teff alone. B, An OVA-specific Th1 clone was cultured with OVA peptide (2 μg/ml) and APC alone or the presence of the Th1 clone FhH5 (generated from *F. hepatica*-infected mice) either in a coculture (Cocult.) or in a transwell (Trans.). Supernatants were removed after 72 h, and the concentrations of IL-10 and IFN-γ were determined by ELISA. Results are mean values for triplicate cultures and are representative of three experiments. ***p < 0.001 vs Th1 alone by ANOVA.
It has previously been reported that infection with *F. hepatica* induces immunosuppression in vivo and that this can compromise not only host effector immune responses to the parasite, but also to other pathogens (23, 24). Furthermore, it has been demonstrated that *F. hepatica* products stimulate IL-10 production by macrophages (32). The findings of the present study demonstrate that infection with *F. hepatica* induced IL-10 production by DC and inhibited their maturation, a phenotype associated with the induction of Tr1 cells. Indeed, we found that infection of mice with *F. hepatica* induced a very high frequency of IL-10-secreting CD4+ Treg cells. Furthermore, *F. hepatica*-specific IL-4, IL-5, and IFN-γ production was enhanced in IL-10−/− mice, suggesting that Th1 and Th2 responses during the helminth infection were constrained by parasite-induced IL-10.
Our findings also demonstrated that regulatory cells induced by *F. hepatica* could exert bystander suppression of immune responses against unrelated Ag, including Th1 and Th17 responses against an autoantigen. It has recently been reported that suppression of IL-17 production in mesenteric lymph nodes following colonization with *H. polygyrus* was reversed by blocking IL-4 and IL-10 (14). This is consistent with our previous report that *F. hepatica* induced suppression of Th1 responses to the bacterial pathogen *B. pertussis* was mediated in part by IL-4 (23). In the present study, the suppression of pathogenic T cells that promote autoimmunity was not mediated by IL-10, but was reversed by neutralization of TGF-β in vivo. A high proportion of macrophages and CD4+ Treg cells in the *F. hepatica*-infected mice expressed TGF-β. A protective role for TGF-β is consistent with the demonstration that expression of TGF-β is associated with recovery of mice from clinical disease in the relapsing-remitting EAE model.

**FIGURE 9.** Suppression of Th1 and Th17 responses and the protective effect of *F. hepatica* infection in EAE are independent of IL-10. EAE was induced in C57BL/6 and IL-10−/− mice, one group of which was left untreated and another infected with *F. hepatica* 1 day before induction of EAE. A, Clinical scores were recorded and mice were sacrificed on day 20 for assessment of T cell responses. B, Spleen cells were stimulated with MOG peptide (10 or 100 µg/ml) or medium only. After 72 h, supernatants were removed and analyzed for IFN-γ, IL-17, and IL-10 by ELISA. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 infected vs untreated (by ANOVA). Results are mean values for triplicate cultures for four mice per group (cytokines) or five mice per group (EAE) and are representative of three experiments.

**FIGURE 10.** Attenuation of EAE by *F. hepatica* is mediated by TGF-β. EAE was induced in 24 C57BL/6 mice, 12 of which were treated with anti-TGF-β and 12 with a control Ab. Six mice in each group were infected with *F. hepatica*, and two groups of six were not infected. A, Clinical scores were recorded for 21 days. B, Spleen cells were stimulated with MOG peptide (10 or 100 µg/ml) or medium only. After 72 h, supernatants were removed and analyzed for IL-17 and TGF-β by ELISA. TGF-β concentrations represent values after subtraction of the background levels in the culture medium (1200 pg/ml). C, Expression of LAP on CD4+ T cells from spleens of untreated mice with EAE (gray histogram) and from mice infected with *F. hepatica* before induction of EAE (black line). Results are mean values for triplicate cultures for four mice per group (cytokines) or five mice per group (EAE) and are representative of two experiments.
TGF-β has been shown to be involved in both the development and regulation of Th17 responses. It has been demonstrated that TGF-β, together with IL-6, can promote the differentiation of murine Th17 cells from naive CD4+ T cells (20, 34). Furthermore, recent reports have shown that in association with IL-21 or IL-1 and IL-23, low concentrations of TGF-β promote human Th17 cell differentiation (35–37). However, it has also been reported that while TGF-β can synergize with IL-6 and IL-21 to enhance Th17 differentiation, high concentrations of TGF-β suppress Th17 differentiation and enhance Foxp3 expression (21). Additionally, we have recently demonstrated that virus-induced TGF-β can suppress Ag-specific Th1 and Th17 cells in patients chronically infected with hepatitis C virus (22). We have also found that TGF-β can suppress TLR agonist-induced IL-12p40, IL-23, and IL-1β production by human monocytes (22), as well as murine DC (K. P. Walsh and K. H. G. Mills, unpublished observations). Since IL-23 and IL-1 are crucial for the differentiation and proliferation of Th17 cells (6, 38), this suggests that parasite-induced TGF-β may exert suppression of autotnigen-specific Th17 cells by inhibiting the innate cytokines that promote their development or expansion.

Our findings demonstrate that infection with *F. hepatica* suppresses effector Th1 and Th2 responses against the parasite through regulatory mechanisms involving IL-10, but that high concentrations of parasite-induced TGF-β may exert regulatory control on the induction and expansion of Th1 and Th17 cells and the development of EAE. Thus, our study provides direct experimental evidence that parasite-induced regulatory cells and their immunosuppressive cytokines can suppress pathogenic T cells that mediate autoimmune diseases.

**Disclosures**

Kingston Mills is a co-founder and shareholder in Opsona Therapeutics Ltd, a start-up company involved in the development of anti-inflammatory drugs. Louis Boon is a founder and shareholder in Bioceros BV.

**References**


