

**Infection with a helminth parasite attenuates autoimmunity  
through TGF- $\beta$ -mediated suppression of Th17 and Th1 responses<sup>1</sup>**

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## Abstract

The lower incidence of allergy and autoimmune diseases in developing countries has been associated with a high prevalence of parasitic infection. 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 or activation of regulatory dendritic cells and macrophages that expressed IL-10 and TGF- $\beta$ . Furthermore, the majority of T cells in the peritoneal cavity of infected mice secreted IL-10, but not IFN- $\gamma$  or IL-4. *F. hepatica*-specific Treg cell clones generated from infected mice suppressed proliferation and IFN- $\gamma$  production by Th1 cells. Infection was associated with suppression of parasite antigen-specific Th1 and Th2 responses, which was reversed in IL-10-defective mice. Infection of mice with *F. hepatica* also exerted bystander suppression of immune responses to autoantigens and attenuated the clinical signs of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE). Protection was associated with suppression of MOG-specific IFN- $\gamma$  and IL-17 production. The suppression of autoantigen-specific Th1 and Th17 responses and attenuation of EAE by *F. hepatica* was maintained in IL-10<sup>-/-</sup> mice but was reversed by neutralization of TGF- $\beta$  *in vivo*. Furthermore, rTGF- $\beta$  appeared to directly suppress Th1 cells, but indirectly suppress Th17 responses, by inhibiting IL-1 $\beta$  and IL-23 production by DC. Our study provides evidence that *F. hepatica*-induced IL-10 subverts parasite-specific Th1 and Th2 responses, but that *F. hepatica* -induced TGF- $\beta$  plays a critical role in bystander suppression of autoantigen-specific Th1 and Th17 responses that mediated autoimmune diseases.

## Introduction

Epidemiological and experimental studies suggesting helminth infections can have a protective effect against the development of both autoimmune and allergic diseases (1), 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 parasite 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 CD4<sup>+</sup> T cells (Th17 cells) in autoimmune inflammation (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 1980's and the ensuing evidence of their reciprocal roles in controlling immune responses, a number of additional subtypes of CD4<sup>+</sup> T cells have been defined. These include a number of distinct regulatory T (Treg) cell subsets, which play a major role in suppressing immune responses to self antigens, thereby maintaining tolerance and preventing autoimmunity (10). Treg cells also function to control anti-pathogen effector T cell responses in order to limit immunopathology during infection (11). More recently it has been demonstrated that Th17 cells, which develop via cytokine signals distinct from, and antagonised, 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* suppress 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<sup>+</sup> natural Treg cells can suppress Th17 cells, especially in humans (19). Furthermore, TGF- $\beta$  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- $\beta$ -dependent mechanism.

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, *Bordetella 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- $\beta$  and inhibit the induction not only of Th1 and Th2 responses to the parasite. *F. hepatica* infection also suppressed 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 (Bicester, UK). IL-10<sup>-/-</sup> and DO11.10 OVA TCR Tg mice were obtained from Jackson labs, USA and bred in house. Mice were housed in individually ventilated cages and all experiments were performed according to 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 Paddock Laboratories, Berkshire, UK). This resulted in infection in 100% of animals.

### *Induction of EAE and treatment with anti-TGF- $\beta$*

C57BL/6 or IL-10<sup>-/-</sup> mice were each injected s.c. with 150  $\mu$ g of MOG<sub>35-55</sub> in CFA containing 5 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco). Mice were injected i.p. with 500 ng of pertussis toxin (Sigma) on days 0 and +2. For treatment with anti-TGF- $\beta$ , mice were injected i.p. every 2 d from day -1 to day 17 with 100  $\mu$ g anti-TGF- $\beta$  (clone 1D11, reactive to mouse TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3) or with an isotype matched control antibody (anti- -----). EAE was scored as follows: 1, limp tail or waddling gait with tail tonic; 2, waddling gait with limp tail; 3, hind limb weakness; 4, hind limb paralysis. Mice were examined daily in a blind fashion for signs of EAE. Experiments were terminated when un-treated mice with EAE mice displayed clinical score of 4.

### *Immunofluorescence analysis by FACS*

PEC were prepared from *F. hepatica* or naïve control mice. Cells were recovered, blocked by incubating in medium with 50% FCS for 30 mins. Cells were labeled with antibodies specific for

mouse CD11c, F4/80, CD80, CD86, CD40, CCR5 or LAP or with appropriate isotype control antibodies. Alternatively for detection of T cell subtypes, PEC were labeled with antibodies specific for CD4, CD25, T1/ST2, IL-10R, CCR5, CD127 or LAP. Intracellular Foxp3 staining was performed to the manufacturer's instructions (eBioscience, San Diego, CA). Briefly cells were stained for antibodies to CD4 and CD25 for at 4°C for 30 min. Cells were then fixed, blocked and permeabilized with anti-mouse/rat Foxp3 (FJK-16s) for 4°C for 30 min and washed. Immunofluorescence was analysed using CELLQuest™ software on a FACScalibur™ (Becton-Dickson, San Jose, CA).

#### *Intracellular cytokine staining*

To analyze cytokine production in DC and T cells from *F. hepatica*-infected mice, PEC were first incubated with 50% FCS in PBS for 20 mins and then stained with anti-CD11c and anti-CD4 respectively, without activation *in vitro*. In some experiments cells were cultured overnight in brefeldin A (BFA; 5µg/ml). Cells were washed, blocked with Fcγ blocker (Pharmingen 1 µg/ml), fixed, permeabilized (Fix and Perm cell permeabilization kit, Caltag) and stained with antibodies specific for IL-10, IL-4, IFN-γ or TGF-β or with an isotype control antibody. Flow cytometric analysis was performed using a FACScalibur™ flow cytometer (Becton Dickinson). Cells were gated on CD11c or CD4 for DC and T-cells respectively.

#### *APC function*

Purified CD4<sup>+</sup> T cells from DO11.10 OVA TCR Tg mice were MACS purified. OVA-specific T-cells (1x10<sup>5</sup>/ml) were cultured with OVA<sub>323-339</sub> peptide (2 µg/ml), and irradiated PEC or splenic CD11C<sup>+</sup> (2x10<sup>6</sup>/ml) as APC. After 72 h incubation, supernatants were removed and IFN-γ, 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 culture with *F. hepatica* antigen (liver fluke homogenate; 10 µg/ml) for 5 days followed by culture in medium with IL-2 for a further 5-7 days. CD4<sup>+</sup> T cell clones were generated by cloning these T cells lines by limiting dilution at 1 cell per well. T cell clones were maintained by 10-12 d cycles of culture with *F. hepatica* antigen and APC (irradiated spleen cells 2 x 10<sup>6</sup> /ml), with IL-2 added after 5 days.

### *Antigen-specific proliferation and cytokine production*

Spleen cells (2 x 10<sup>6</sup>/ml), lymph node cells (1 x 10<sup>6</sup>/ml) or *F. hepatica*-specific T cell clones (1 x 10<sup>5</sup>/ml) with APC (2 x 10<sup>6</sup> /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) and anti-CD3 (0.5 µg/ml; BD Pharmingen, San Diego, Calif.) 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 latent TGF-β present in the culture medium was assayed and subtracted from the total latent TGF-β concentration. Proliferation was assessed after 4 d by <sup>3</sup>H-thymidine incorporation.

### *T cell suppression assays*

OVA-specific CD4<sup>+</sup> T cells (1 x 10<sup>5</sup>/ml), purified cells from DO11.10 mice were cultured with OVA<sub>323-339</sub> peptide (2 µg/ml) and APC (irradiated spleen cells, 2 x 10<sup>6</sup>/ml) either alone or with *F. hepatica* specific CD4<sup>+</sup> T cells isolated from PEC of mice infected 3 weeks earlier with *F.*

*hepatica*-specific CD4<sup>+</sup> T cell clones and *F. hepatica* Ag (20 µg/ml) at a 1:1 ratio, either in the same well or separated by a semi-permeable membrane (transwell). *F. hepatica*-specific CD4<sup>+</sup> T-cells cultured with *F. hepatica* Ag (20 µg/ml) and APC (irradiated spleen cells) alone acted as further controls. After 72h incubation supernatants were removed and IL-10 and IFN-γ concentrations determined by ELISA. Proliferation was determined by <sup>3</sup>H-thymidine incorporation after 96 h of culture.

#### *Statistical analysis*

Data were compared by 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 significant infiltration of DC and macrophages in the peritoneal cavity (Fig. 1A; Absolute numbers: naive versus infected; DC,  $1.0 \times 10^6$  versus  $24 \times 10^6$ , macrophages  $2.7 \times 10^6$  versus  $8.5 \times 10^6$ ). In comparison with control mice, DC from *F. hepatica*-infected animals had significantly lower expression of the co-stimulatory molecules, CD80, CD86 and CD40, and higher expression of CCR5 (Fig. 1B), a phenotype of immature DC. Intracellular cytokine staining (ICS) revealed a very high frequency of IL-10-producing CD11c<sup>+</sup> DC in *F. hepatica* infected mice (Fig. 1C). More than 30% of *ex vivo* DC from the peritoneal cavity of infected mice were positive for IL-10 when stained with anti-IL-10 without re-stimulation *in vitro*. In contrast, IL-10 could not be detected in DC from the peritoneal cavity of naïve control mice (Fig. 1C) and IFN- $\gamma$  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. 1C); LAP is bound to TGF- $\beta$  as part of a latent complex secreted from the cell, before biologically active TGF- $\beta$  is released by cleavage from LAP, which is retained on the cell surface. F4/80<sup>+</sup> macrophages from the peritoneal cavity of infected mice had high expression of IL-10 and TGF- $\beta$  detectable by ICS. More than 60% of macrophages from the peritoneal cavity of infected mice expressed TGF- $\beta$ , compared with 7% from control mice. Macrophages from infected mice also had higher expression of surface LAP than cells from un-infected control mice (Fig. 1C). These findings suggest that infection with *F.*

*hepatica* is associated with local recruitment of immature DC and macrophages that express immunosuppressive cytokines.

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<sup>+</sup> T cells from DO11.10 OVA TCR transgenic mice and OVA peptide 323-329. PEC from *F. hepatica*-infected mice induced significantly less IL-4, IL-10 and IFN- $\gamma$  production, compared with PEC from naïve control mice (Fig. 1D). Conversely, splenic DC from *F. hepatica*-infected mice promoted significantly greater IL-10 and IL-4 production, but equivalent IFN- $\gamma$  production, when compared with splenic DC from control animals. 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- $\gamma$  production from Th1 cells.

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

Since *F. hepatica* infection induces TGF- $\beta$ - 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 antigen-specific Treg cells. *F. hepatica*-specific CD4<sup>+</sup> 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- $\gamma$  (Fig. 2A). ICS also demonstrated that a very high frequency (more than 70%) of T cells in the peritoneal cavity of *F. hepatica*-infected mice secrete IL-10 in the absence of IL-4 or IFN- $\gamma$  production (Fig. 2B). Phenotypic analysis revealed a high frequency of T cells from the peritoneal cavity of *F. hepatica* infected mice expressed CD25, CTLA-4, T1/ST2, IL-10R and CCR5 (Fig. 2C), markers that are expressed on Treg cells.

However, there was only a modest increase in the frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> in peritoneal cavity (CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> 8.6 ± 0.6 % versus 15.0 ± 1.3 % for control and infected mice respectively). In contrast, the frequency of CD4<sup>+</sup> T cells expressing LAP was significantly enhanced in infected compared with control mice (Fig. 2D). Furthermore, a high percentage of CD4<sup>+</sup> T cells in the peritoneal cavity of control and infected mice expressed intracellular TGF-β. The absolute numbers of TGF-β-expressing CD4<sup>+</sup> T cells were dramatically greater in infected compared with control mice (1.4 x 10<sup>6</sup> versus 0.15 x 10<sup>6</sup> respectively).

We next examined T cell responses in the mesenteric lymph node of infected and control mice. CD4<sup>+</sup> T cells from the mesenteric lymph nodes of infected, but not control mice, secreted IL-10 and IL-5, but undetectable IL-4 or IFN-γ following re-stimulation with *F. hepatica* antigen *ex vivo*; the same cells stimulated with PMA and anti-CD3 produced all four cytokines examined (Fig. 3A). However, there was only a modest increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>, but not CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>, in the mesenteric lymph nodes in infected mice (Fig. 3B). Infection was also associated with a small increase in the percentage of CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>, an alternative phenotype associated with natural Treg cells (Fig. 3B). Collectively these findings suggest that infection with *F. hepatica* promotes the induction of antigen-specific adaptive Treg cells that express immunosuppressive cytokines.

We next examined the ability of T cells from infected mice to suppress antigen-induced proliferation and cytokine production by effector T cells *in vitro*. CD4<sup>+</sup> 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. 4A). Furthermore, a parasite-specific Treg 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 4B). Suppression of the Th1 clones was observed when co-cultured with the Treg clone and across a

semi-permeable membrane (Fig. 4B). These findings demonstrate that parasite-specific Treg cells from *F. hepatica*-infected mice exert bystander suppression of Th1 responses to an unrelated antigen via release of soluble factors.

*Suppression of parasite-specific Th1 and Th2 responses during F. hepatica infection is mediated by IL-10*

In order to examine the role of IL-10 in parasite-induced suppression of immune responses *in vivo*, C57BL/6 and IL-10<sup>-/-</sup> mice were infected with *F. hepatica* and antigen-specific cytokine production was examined. Mesenteric lymph node cells from WT mice infected with *F. hepatica* produced IL-10 and IL-5, but not IFN- $\gamma$  when stimulated *in vitro* with *F. hepatica* antigen (Fig. 5A). In contrast, significant concentrations of IFN- $\gamma$  and enhanced IL-5 were detected in antigen-stimulated lymph node cells from IL-10<sup>-/-</sup> 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 (Fig. 5B) and IFN- $\gamma$  (not shown) was 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<sup>-/-</sup> when compared with WT mice (Fig. 5B). These findings demonstrate that suppression of parasite-specific Th1 and Th2 responses in *F. hepatica*-infected mice is mediated by IL-10.

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

Self-antigen-specific Th1 and Th17 cells mediate pathology in a number of autoimmune diseases, including EAE (6, 25-27). 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. 6A). In contrast, symptoms of EAE did not develop until day 14 in *F. hepatica* infected mice (Fig. 6). 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. 6B). In untreated mice, significant concentrations of IL-17 and IFN- $\gamma$ , but no 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- $\gamma$  and significantly higher concentrations of MOG-specific IL-10 (Fig. 6B).

*F. hepatica* mediated suppression of autoantigen-specific Th1 and Th17 responses and the attenuation of EAE is mediated by TGF- $\beta$ , but not by IL-10

In order 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<sup>-/-</sup> mice with and without infection with *F. hepatica*. Consistent with the immunosuppressive properties of IL-10, EAE was exacerbated in IL-10<sup>-/-</sup> compared with WT mice (Fig. 7A) and this was associated with elevated MOG-specific IL-17 production (Fig. 7B). However, infection with *F. hepatica* significantly attenuated the clinical signs of EAE in IL-10<sup>-/-</sup> as well as WT mice (Fig. 7A). Furthermore, infection with *F. hepatica* significantly suppressed MOG-specific IL-17 and IFN- $\gamma$  in IL-10<sup>-/-</sup>, as well as in WT mice, with a coincident elevation in MOG-specific IL-10 production in WT mice (Fig. 7B). These data indicate that endogenous IL-10 does play a protective role in preventing the development of EAE, but demonstrate that that suppression of pathogenic

autoantigen-specific T cells and the clinical signs of EAE by *F. hepatica* is not mediated by IL-10.

We next examined the possible role of TGF- $\beta$  in parasite-mediated suppression. Administration of a neutralizing anti-TGF- $\beta$  antibody *in vivo* to un-infected mice with EAE had little effect on the course of disease (Fig. 8A). Infection with *F. hepatica* significantly attenuated the clinical symptoms of EAE in mice treated with a control antibody. The protective effect of the infection on development of EAE was reversed by the administration of anti-TGF- $\beta$  *in vivo*; *F. hepatica* infected mice treated with anti-TGF- $\beta$  had more severe symptoms than non-infected mice or infected mice treated with a control antibody (Fig. 8A).

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- $\beta$  production (Fig. 8B). Furthermore, surface expression of LAP, the amino-terminal domain of the TGF- $\beta$  precursor peptide, was enhanced on CD4<sup>+</sup> T cells from spleens of *F. hepatica*-infected with EAE, when compared with cells from non-infected mice with EAE (Fig. 8C). The reversal by anti-TGF- $\beta$  of the protective effect of *F. hepatica* and the increase severity of EAE correlated with enhancement of MOG-specific IL-17 production (Fig. 8B). These findings suggest that in the absence of endogenous and parasite-induced TGF- $\beta$ , *F. hepatica* infection may exacerbate EAE by enhancing the induction of pathogenic T cells, but that in the presence of a functional TGF- $\beta$  response, *F. hepatica* suppresses EAE by inhibiting pathogenic T cells.

#### *TGF- $\beta$ suppresses IFN- $\gamma$ production by T cells and IL-12, IL-23 and IL-1 $\beta$ production by DC*

In order to investigate the possible mechanisms of TGF- $\beta$  mediated suppression, we examined the influence of rTGF- $\beta$  on Th1 and Th17 responses *in vitro*. We found that low concentrations

of rTGF- $\beta$  significantly suppressed *in vitro* production of IFN- $\gamma$  by MOG-specific T cells from mice with EAE (Fig. 9A). In contrast, these physiological concentrations of rTGF- $\beta$  had no direct effect on MOG-specific IL-17 production.

Suppression of T cell responses can also be induced at the level of the APC, especially through the innate cytokines that promote the development and expansion of effector T cells. Although IL-6 and TGF- $\beta$  are considered to be the key cytokines that induce differentiation of Th17 cells from naïve T cells (20, 28), we and others have shown that IL-23 and IL-1 play a critical role in IL-17 production, especially from memory T cells (29, 30). Furthermore, IL-1RI<sup>-/-</sup> and IL-23<sup>-/-</sup> mice are resistant to the induction of EAE (30, 31). Conversely, IL-12 has an established role in promoting Th1 responses. Here, we found that rTGF- $\beta$  induced significant dose dependent inhibition of LPS-induced IL-12p40, IL-23 and IL-1 $\beta$  by DC (Fig. 9B). Our findings suggest that *F. hepatica* attenuates EAE by inducing TGF- $\beta$  production, which inhibits the production of innate cytokines that promote the induction and expansion of Th17 and Th1 cells and also has a direct inhibitory effect on IFN- $\gamma$  production by Th1 cells.

## 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 a less clear cut but re-emerging evidence of a role for Th1 cells (25-27). 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 antigen-specific adaptive Treg cells, suppresses the generation of autoantigen-specific Th1 and Th17 cells and attenuates the induction of EAE through a TGF- $\beta$ -dependent mechanism.

Much of the focus to date on Treg cells induced by parasites has been on natural Treg cells. CD4<sup>+</sup>CD25<sup>+</sup> Treg cells have been shown to contribute to immune suppression during malaria infection, allowing the parasite to escape from host protective immune responses (32). It has also been demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> Treg cells maintain *Leishmania major* persistence after resolution of dermal lesions in resistant mice (33). Studies in humans have shown that individuals with chronic parasitic infections develop prominent anti-inflammatory networks, leading to an attenuation of antigen-specific immune responses to both the parasite and unrelated pathogens (1), suggesting that the parasites can not only suppress host immune responses against itself, but can exert bystander suppression against third party antigens. Further experimental evidence for this was provided by the demonstration that CD4<sup>+</sup>CD25<sup>+</sup> T cells induced by the gastrointestinal



nematode *Heligmosomoides polygyrus* suppressed allergic responses to OVA or DerP1 in sensitized animal, a process in which Foxp3 expression and TGF- $\beta$  production has been implicated, but which was IL-10 independent (13). Our study revealed that infection with *F. hepatica* is associated with induction of adaptive antigen-specific Treg cells, which suppress host immune response to the parasite and to unrelated antigens.

It has previously been reported that infection with *F. hepatica* induces immunosuppression *in vivo* and that this can compromise 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 (34). 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 Treg cells. Indeed we found that infection of mice with *F. hepatica* induced a very high frequency of IL-10-secreting CD4<sup>+</sup> Treg cells. Furthermore, *F. hepatica*-specific IL-4, IL-5 and IFN- $\gamma$  production was enhanced in IL-10<sup>-/-</sup> 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 antigen, 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* by was mediated in part by IL-4 (35). In the present the suppression of pathogenic T cells that promote autoimmunity was not mediated by IL-10, but was reversed by neutralization of TGF- $\beta$  *in vivo*. A high proportion of macrophages and CD4<sup>+</sup> Treg cells in the *F. hepatica* infected mice expressed TGF- $\beta$ . A

protective role for TGF- $\beta$  is consistent with the demonstration that expression of TGF- $\beta$  is associated with recovery of mice from clinical disease in the relapsing remitting EAE model (36). However, TGF- $\beta$  has been shown to be involved in both the development and regulation of Th17 responses. It has been demonstrated that TGF- $\beta$ , together with IL-6 can promote the differentiation of murine Th17 cells from naive CD4<sup>+</sup> T cells (20, 28). Furthermore, recent reports have shown that in association with IL-21 or IL-1 and IL-23, low concentrations of TGF- $\beta$  promote human Th17 cell differentiation (37-39). However, it has also been reported that while TGF- $\beta$  can synergize with IL-6 and IL-21 to enhance Th17 differentiation, high concentrations of TGF- $\beta$  suppress Th17 differentiation and enhance Foxp3 expression (21). In addition, we have recently demonstrated that virus-induced TGF- $\beta$  can suppress antigen-specific Th1 and Th17 cells in patients chronically infected with hepatitis C virus (22). In the present study, we found that rTGF- $\beta$  suppressed cytokine production MOG-specific Th1 cells, but not Th17 cells, *in vitro*. However, consistent with our findings with human monocytes (22), rTGF- $\beta$  significantly inhibited TLR agonist-induced IL-12p40, IL-23 and IL-1 $\beta$  production by murine DC. Since IL-23 and IL-1 are crucial for the differentiation and proliferation of Th17 cells (6, 30), this suggests that parasite-induced TGF- $\beta$  may exert suppression of autoantigen-specific Th17 cells by inhibiting the innate cytokine 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- $\beta$  may exert regulatory control on the induction and expansion of Th1 and Th17 cells and the development of EAE. Thus, our study provide direct experimental evidence that parasite-induced regulatory cells and their immunosuppressive cytokines can suppress pathogenic T cells that mediate autoimmune diseases.

## Footnotes

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<sup>5</sup>Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; Th17 cell, IL-17-producing CD4<sup>+</sup> T cell; Treg cell, regulatory T cell; ICS, Intracellular cytokine staining.

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## FIGURE LEGENDS

**Figure 1.** Regulatory DC and macrophages are recruited to the peritoneal cavity of *F. hepatica* infected mice. BALB/c mice were infected with 10 viable metacercariae of *F. hepatica*. (A-C) Three weeks after challenge, PEC were isolated from *F. hepatica*-infected and naïve control mice by peritoneal lavage. Cells were stained with antibodies specific for CD11c, F4/80 and co-stimulatory molecules and cytofluorometric analysis performed. (A) Frequency of CD11c<sup>+</sup> DC and F4/80 macrophages populations in PEC from control and *F. hepatica* infected mice. (B) Expression of CD80, CD86, CD40 and CCR5 on CD11c<sup>+</sup> cells from *F. hepatica* infected (solid black line) and control (grey histogram) mice versus isotype-matched control antibodies (grey line) (C) PEC from un-infected control (grey histogram) and *F. hepatica* infected (black line) mice were surface labelled with anti-CD11c, F4/80 or LAP, before fixing and permeabilising for ICS with anti-IL-10 and anti-TFG- $\beta$ . (D). PEC and CD11c<sup>+</sup> cells spleen from *F. hepatica* infected or naïve control (Cont.) mice were co-cultured with purified CD4<sup>+</sup> T-cells from DO11.10 mice and OVA (2  $\mu$ g/ml). Supernatants were removed after 72 h and the concentrations of IFN- $\gamma$ , IL-4 and IL-10 quantified by ELISA \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus control by unpaired *t*-test

**Figure 2** Infection with *F. hepatica* induces antigen-specific Treg cells. (A) *F. hepatica*-specific CD4<sup>+</sup> T cell clones were generated from PEC of *F. hepatica* infected mice. T cell clones were stimulated with APC and antigen and after 3 d supernatants were removed and the concentrations of IFN- $\gamma$ , IL-5 and IL-10 quantified by ELISA. (B) PEC from naïve and *F. hepatica*-infected mice were stained with anti-CD4, before fixing and permeabilising and staining with anti-IL-10, anti-IL-4, anti-IFN- $\gamma$  or isotype control antibodies and FACS performed. (C) PEC from *F.*

*hepatica* infected (grey line) and naïve control (black histogram) mice were incubated with anti-CD4, anti-CD25, anti-CTLA-4, anti-T1/ST2, and IL-10R and anti-CCR5. Cells were gated on CD4. (D) PEC from *F. hepatica* infected and naïve control mice were overnight with BFA, before staining for surface CD4, CD25 and LAP and intracellular TGF- $\beta$ . Numbers represent % of CD4<sup>+</sup> cells.

**Figure 3. Infection with *Fasciola hepatic* induces antigen-specific IL-10 producing T cells in the mesenteric lymph node cells.** (A) Mesenteric lymph node cells were recovered 3 weeks post *F. hepatica* infection or from control naïve mice. Cells were stimulated *ex vivo* with *F. hepatica* antigen, medium or PMA and anti-CD3, as positive control. Supernatants were removed after 72 h and the concentrations of IFN- $\gamma$ , IL-4, IL-5 and IL-10 quantified by ELISA (B) Mesenteric lymph node cells from naïve control and *F. hepatica*-infected mice were stained with anti-CD4, anti-CD25 and anti-CD127 and intracellularly with anti-Foxp3 and FACS analysis performed. Cells were gated on CD4. Numbers are % of CD4<sup>+</sup> T cells.

**Figure 4. *F. hepatica*-induced Treg cells suppress IFN- $\gamma$  production by Th1 cells.** (A) CD4<sup>+</sup> T cells purified from DO11.10 Tg mice (Teff) were cultured with OVA peptide (2  $\mu$ g/ml) and APC alone or in the presence of CD4<sup>+</sup> T cells purified from the peritoneal cavity of mice *F. hepatica*-infected mice (Treg cells). Supernatants were removed after 72 h and the concentrations of IFN- $\gamma$  determined by ELISA; proliferation was determined after 4 d. \*\*\* $P < 0.001$  versus Teff alone (B) An OVA-specific Th1 clone was cultured with OVA peptide (2  $\mu$ g/ml) and APC alone or the presence of the Tr1 clone FhH5 (generated from *F. hepatica* infected mice) either in a co-culture (Cocult.) or in a transwell (Trans.). Supernatants were removed after 72 h and the concentrations of IL-10 and IFN- $\gamma$  determined by ELISA. \*\*\* $P < 0.001$  versus Th1 alone by ANOVA.



**Figure 5. Suppression of parasite-specific Th1 and Th2 responses by *F. hepatica* is mediated by IL-10.** C57BL/6 and IL-10<sup>-/-</sup> mice were infected with *F. hepatica* and were sacrificed 3 weeks later. Un-infected control (Cont) C57BL/6 and IL-10<sup>-/-</sup> mice were sacrificed at the same time. (A) Mesenteric lymph node cells (1x10<sup>6</sup>/ml) were stimulated with *F. hepatica* antigen (4 and 20 µg/ml), or with medium only. Supernatants were removed after 72 h and concentrations of IFN-γ, IL-10 and IL-5 determined by ELISA. Data represents the mean (± SE) cytokine concentrations from 4 individual mice. (B) Peritoneal fluid was centrifugation and the concentrations of IL-10, IL-5 and IL-4 in the supernatants determined by ELISA. (IFN-γ was undetectable in peritoneal fluid). \**P* < 0.01, \*\* *P* < 0.05, \*\*\**P* < 0.001 versus Control by ANOVA

**Figure 6. Infection with *F. hepatica* suppresses Th1 and Th17 responses and attenuates the clinical symptoms of EAE.** EAE was induced in C57BL/6 mice by immunization with MOG<sub>35-55</sub> in CFA on day 0, with PT injected i.p. on days 0 and 2. C57BL/6 mice were infected with 10 metacercariae of *F. hepatica* the day before induction of EAE (A). Clinical scores were assessed daily and mice sacrificed at 20 d for detection of T cell responses. \* *P* < 0.05, \*\**P* < 0.01 infected versus untreated by ANOVA. (B) Spleen cells were stimulated with MOG peptide (10 or 100 µg/ml) or medium only 72 h supernatants were removed and analyzed for IL-17, IFN-γ and IL-10 by ELISA. \* *P* < 0.05, \*\**P* < 0.01 versus untreated by ANOVA.

**Figure 7. 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<sup>-/-</sup> mice, one group of which were left un-treated and another infected with *F. hepatica* one 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- $\gamma$ , IL-17 and IL-10 by ELISA. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  infected versus untreated by ANOVA

**Figure 8. Attenuation of EAE by *F. hepatica* is mediated by TGF- $\beta$ .** EAE was induced in C57BL/6 mice which were treated with anti-TGF- $\beta$  or a control antibody, a proportion of which were infected with *F. hepatica*. (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- $\beta$  by ELISA. (C) Expression of LAP on CD4<sup>+</sup> T cells from spleens of untreated mice with EAE (grey histogram) and from mice infected with *F. hepatica* before induction of EAE (block line).

**Figure 9. rTGF- $\beta$  suppresses IL-12p40, IL-23 and IL-1 $\beta$  production by DC and IFN- $\gamma$  production by T cells.** (A) Spleen cells from mice with EAE were stimulated in vitro with MOG (50 µg/ml) in the presence of increasing concentrations for TGF- $\beta$ . Supernatants were removed after 3 d and IFN- $\gamma$  and IL-17 concentrations quantified by ELISA. \*  $P < 0.05$ , \*\*\*  $P < 0.001$  versus MOG-stimulated cells without TGF- $\beta$  by ANOVA. (B) BMDC were stimulated with LPS (100 ng/ml) in the presence of medium only or increasing concentrations of rTGF- $\beta$ . After 24 h supernatants were removed and IL-12p40, IL-23 and IL-1 $\beta$  concentrations quantified by ELISA. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  versus LPS without TGF- $\beta$  by ANOVA.