Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization

Diane Sewell\textsuperscript{1}, Zhu Qing\textsuperscript{1}, Emily Reinke\textsuperscript{1}, David Elliot\textsuperscript{2}, Joel Weinstock\textsuperscript{2}, Matyas Sandor\textsuperscript{1} and Zsuzsa Fabry\textsuperscript{1}

\textsuperscript{1}Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53706, USA
\textsuperscript{2}Department of Internal Medicine, University of Iowa, Iowa City, IA 52242, USA

Keywords: experimental autoimmune encephalomyelitis, Schistosoma mansoni, STAT6, T\textsubscript{h}1, T\textsubscript{h}2

Abstract

Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS) characterized by chronic inflammatory demyelination of the central nervous system (CNS). The pathology of EAE involves autoimmune CD4\textsuperscript{+} T\textsubscript{h}1 cells. There is a striking inverse correlation between the occurrence of parasitic and autoimmune diseases. We demonstrate that in mice with \textit{Schistosoma mansoni} ova immunization, the severity of EAE is reduced as measured by decreased clinical scores and CNS cellular infiltrates. Disease suppression is associated with immune deviation in the periphery and the CNS, demonstrated by decreased IFN-\gamma and increased IL-4, transforming growth factor-\beta and IL-10 levels in the periphery, and increased frequency of IL-4 producing neuroantigen-specific T cells in the brain. \textit{S. mansoni} helminth ova treatment influenced the course of EAE in wild-type mice, but not in STAT6-deficient animals. This indicates that STAT6 plays a critical role in regulating the ameliorating effect of \textit{S. mansoni} ova treatment on the autoimmune response, and provides the direct link between helminth treatment, \textit{T}h2 environment and improved EAE. As some intestinal helminthic infections induce minimal pathology, they might offer a safe and inexpensive therapy to prevent and/or ameliorate MS.

Introduction

Autoimmunity and infectious disease do not occur in isolation. The immune repertoire is shaped by environmental exposures, resulting in a pre-existing immune status in every individual (1,2). This pre-existing immune status influences T\textsubscript{h}1- and T\textsubscript{h}2-mediated immune responses (3–5). The lack of serious childhood infections might impair the development of the T\textsubscript{h}1/T\textsubscript{h}2 balance, resulting in susceptibility to chronic immune-mediated diseases (4). This hygiene hypothesis was strongly supported by describing an inverse association between different infectious diseases and atopy (6–10), and has been recently extended to include applicability to autoimmunity (11,12).

Autoimmunity in the central nervous system (CNS) is a complex event, induced and maintained by activated CD4 T\textsubscript{n} cells. Following activation in the periphery, T cells can enter the CNS and persist if they encounter their cognate antigens (13). There are at least two subsets of antigen-experienced T\textsubscript{n} cells. T\textsubscript{n}1 clones secrete primarily IL-2, IFN-\gamma and tumor necrosis factor (TNF)-\beta, and promote inflammation, delayed-type hypersensitivity and cellular immunity. T\textsubscript{n}2 clones produce IL-4, -5, -6, -10 and -13 cytokines, and promote B cell activation and antibody production, and eosinophil and mast cell differentiation. The existence of functionally polarized human T cell responses is also established (14).

CNS-infiltrating T\textsubscript{n} cells in experimental autoimmune encephalomyelitis (EAE) are proinflammatory cytokine-producing T\textsubscript{n}1 cells (15–17). In contrast, oral tolerance to myelin basic protein and natural recovery from EAE is associated with up-regulation of T\textsubscript{n}2 cytokines, IL-4 and TGF-\beta (18–20). Adoptive transfer of neuroantigen-specific T\textsubscript{n}2 cell lines has not resulted in EAE (21). A greatly increased T\textsubscript{n}1 effector cell mass in multiple sclerosis (MS) patients has recently been indicated by the presence of a highly...
IFN-γ-polarized, IL-5+ cytokine profile of proteolipid protein (PLP)-reactive T cells (22).

The contribution of factors inducing Th1 differentiation into the polarized Th1 or Th2 pathway has been controversial. The cytokine profile of innate immunity evoked by different pathogens, the nature of the peptide ligand, the activity of co-stimulatory molecules and the context of varied host genetic backgrounds have been suggested to play a role in this process. Polarized Th1- and Th2-type responses play different roles in protection, with Th1 effective in defense against intracellular pathogens and Th2 against intestinal nematodes. Th1 responses predominate in organ-specific autoimmune disorders, acute allograft rejection and some chronic inflammatory disorders. Th2 responses predominate in transplantation tolerance, chronic graft versus host disease, systemic sclerosis, allergy and atopic disorders. The development of a polarized Th1 or Th2 immune response is also influenced by cross-talk between Th1 and Th2 subsets. Th1 cytokines (IFN-γ and IL-2) inhibit the production of Th2 cytokines (IL-4, IL-5 and IL-10), whereas Th2 cytokines inhibit the production of Th1 cytokines (23). A balanced production of these cytokines is crucial in maintaining a healthy immune system (24-29).

We addressed the possibility of Th1/Th2 cross-talk in a natural immune environment, whether Th2 preconditioning of mice would influence the course of Th1-mediated autoimmunity in EAE. Falcone et al. previously addressed the possibility that a Th2 response against an exogenous, non-self antigen could shift the cytokine profile of encephalitogenic T cells from an inflammatory Th1 to a protective Th2 type by releasing IL-4 in the microenvironment (30).

The cross-regulation of Th1/Th2 balance is documented in several infectious disease contexts. Helminthic parasites, which induce Th2-type inflammation, can modulate Th1 immune responses to unrelated concomitant parasitic, bacterial or viral infections. Patients infected with Schistosoma mansoni mount a Th2-type response to tetanus toxoid instead of the expected Th1 or Th0 response (31,32). Ethiopian immigrants with a high incidence of helminthic infections show a propensity to respond to phytohemagglutinin with Th2, not Th1 cytokines (33). Infection of mice with S. mansoni delays clearance of vaccinia virus and alters response to sperm whale myoglobin (34,35). Mice develop a Th2-type response when infected with Brugia malayi, or when immunized with filarial extract that modulates the Th1 response to non-parasite or microbial antigen. The murine intestinal nematode, Nippostrongylusbrasiliensis, stimulates Th2 activity and delays kidney graft rejection in rats, most likely by cross-regulatory suppression of Th1 activity (36).

These findings have important implications. They suggest that persons harboring helminths are apt to mount a diminished Th1 response when challenged with other antigens. It has been also suggested that helminth infections actively dampen atopy in humans (37-41).

We have developed a model to study the effect of Th2 preconditioning on the course of EAE using S. mansoni ova immunization. Schistosome infections are mild, self-limiting and common in areas of poor sanitation. MS is very rare in these areas, as opposed to the higher incidence of MS and other autoimmune diseases in areas with stringent hygienic standards (42,43). This difference suggests a correlation between an environmental factor (hygienic standards) and Th1-type autoimmune diseases. We demonstrate significant protection from EAE in S. mansoni ova preimmunized animals, indicating that parasitic infections can influence the course of CNS autoimmunity. A direct link between schistosome ova preconditioning, ameliorated EAE and the role of Th2 cytokines in this process is demonstrated by a shift to Th2 cytokines in the periphery and the CNS, and the lack of this effect in STAT6-deficient mice. As some intestinal helminthic infections induce minimal pathology, this approach might offer a new therapeutic option to prevent or ameliorate MS.

Methods

Animals

Female SJL and C57BL6 mice aged 4-6 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). STAT6−/− mice on a C57BL6 background were generated by Dr. Michael Grusby (Harvard University, Boston, MA). All mice were maintained in a VA or UW animal facility. EAE induction was initiated between 6 and 8 weeks of age. All procedures were performed under an approved protocol in compliance with UW Animal Care Guidelines.

Reagents

Murine PLP139-151 (HSLGKWGHPDKF) and murine myelin oligodendrocyte glycoprotein (MOG)35-55 (MEVGWRSPFSRWHLYRNGK) peptides were synthesized by CyberSyn (Lenni, PA). Pertussis toxin was purchased from List Biological (Campbell, CA). Anti-CD3 (145.2C11) was purified as described previously (44). These reagents for ELISA were purchased from PharMingen (San Diego, CA); purified rat anti-mouse IL-2 (clones JES-1A12 and JES-5H4), IL-4 (clones BV4D-24G2), IL-5 (TRFK4 and TRFK5), IFN-γ (clones R4-6A2 and XMG1.2), IL-10 (SXC-1 and JES-16E3) and TGF-β (A75-2.1 and A75-3.1) mAb; recombinant mouse IL-2, IL-4, IL-5, IL-9, IL-10, TGF-β and IFN-γ Streptavidin-conjugated alkaline phosphatase and substrate MUP (4-methylumbellifferyl phosphate) were purchased from Molecular Probes (Eugene, OR).

EAE Induction

EAE was induced in SJL mice by s.c. flank immunization with 400 µg PLP139-151 peptide emulsified in complete Freund’s adjuvant (CFA) containing 1 mg/ml H37Ra Mycobacterium tuberculosis (Sigma, St Louis, MO). Seven days later, mice received a second immunization with the same protocol. Pertussis toxin, 200 ng (List Biological) was injected i.v. on days 0 and 2. In C57BL6 and STAT6−/− mice, EAE was induced by a single s.c. injection of 100 µg MOG35-55 peptide emulsified in CFA containing 5 mg/ml H37Ra M. tuberculosis; 200 ng pertussis toxin was injected i.p. on days 0 and 2.

Animals were assessed clinically according to standard criteria: 0, normal; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, moribund or death. Mice with a clinical score of 4 for >1 day were euthanized.
**S. mansoni egg isolation and pretreatment**

*S. mansoni* eggs were isolated from infected hamsters as described (45). Two weeks before EAE induction, 10,000 *S. mansoni* eggs were injected i.p. to the experimental groups. A second dose of *S. mansoni* eggs was injected 4 days prior to EAE induction using 5000 eggs i.p. and 5000 s.c. in the flank where PLP peptide was injected. The same volume of PBS was injected in the control groups.

When *S. mansoni* ova treatment was initiated following induction of EAE, 20,000 eggs were injected i.p. to five mice per group, on day 2, 7, 10 or 14 post EAE induction.

**Tissue processing and histology**

For histology and cytokine analysis, mice from experimental and control groups were sacrificed before EAE induction (day 0), at the onset of the disease (day 15) and after recovery (day 45). Brains and spinal cords were used for histological studies. Tissues were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned and stained with H & E.

**Cytokine analysis by ELISA**

Cytokine concentrations were measured in supernatants from anti-CD3-treated (2 μg/ml), 48-h cultures of isolated spleen cells. Costar 3590 96-well plates (Corning, Corning, NY) were coated with primary antibodies, including: anti-IFN-γ (R4-6A2), IL-5 (TRFK5), TGF-β (A75-2.1) and IL-10 (JES5-2A5), all from PharMingen, and IL-4 (11B11) from NIH (Bethesda, MD). All coating antibodies were used at 2 μg/ml concentration except IL-10 and TGF-β, used at 4 μg/ml. After blocking with TBS/1% BSA, serially diluted standards and triplicate samples were added to the plates. Samples were incubated at 4°C overnight. Plates were washed with TBS/Tween and biotinylated secondary antibodies were added. Plates were incubated for 2 h at room temperature. Detection antibodies used were IFN-γ (XMG1.2), IL-4 (BVDF6-2G42), TGF-β (A75-3.1) and IL-10 (SXC-1) (PharMingen), and IL-5 (TRFK-4) prepared in-house from hybridoma. Secondary antibodies were all used at a 1 μg/ml concentration except IL-10, used at 2 μg/ml. Unbound secondary antibody was washed out and a streptavidin–horseradish peroxidase conjugate (1:2000 in TBS/Tween/BSA) was added. Plates were developed using MUP fluorescent substrate and read at 360/465 nm on the HTS 7000 plate reader (Perkin Elmer, Norwalk, CT).

**ELISPOT assays**

ImmunoSpot 96-well plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with 100 μl/well coating antibodies [IFN-γ (R4-6A2) 4 μg/ml or IL-4 (11B11) 2 μg/ml]. Plates were blocked with PBS/1% BSA and washed. Spleen cells (10⁵/well) or brain cells (10³/well) were plated in HL-1 medium supplemented with 1% L-glutamine. Mitomycin C-treated spleen cells from a naive SJL mouse (5 × 10⁵/well) were added to the brain cell wells to act as antigen-presenting cells. PLP₁₃⁹–₁₅₁ peptide antigen (2 μg/ml), concanavalin A (5 μg/ml) or media were added to triplicate wells. Plates were incubated overnight at 37°C. Biotinylated secondary antibodies [IFN-γ (XMG 1.2) 4 μg/ml or IL-4 (BVDF6-2G42) 2 μg/ml; PharMingen] diluted in PBS/Tween/BSA were added to the appropriate plates, at 100 μl/well. Plates were incubated overnight at 4°C. Streptavidin–horse radish peroxidase, 1:2000 in PBS/Tween/BSA, 100 μl/well was added and plates were incubated for 90 min at room temperature. Colored spots were developed by addition of AEC 200 μl/well (Pierce, Rockford, IL). Plates were scanned on an ImmunoSpot analyzer (Cellular Technology, Cleveland, OH) and quantified by Image analysis software.

**Intracellular cytokine staining and FACS**

Spleens cells were isolated as previously described (46). Cells were washed and resuspended at 10⁷ cells/ml in RPMI for culture with Golgistop (PharMingen) and lipopolysaccharide (LPS; Sigma, St Louis, MO).

Brains were removed from PBS perfused animals to HBSS and minced using sterile scissors. Samples were transferred to Medicon inserts and processed in a Medimachine for 30 s. The cell suspension was centrifuged at 1000 for 5 min. Cells were re-suspended in 2 ml 50% Percoll (Pharmacia, Piscataway, NY) and overlaid with 30% Percoll. Gradients were centrifuged at 1500 g for 30 min at 4°C. Interfaces were removed, washed and resuspended in RPMI (47).

Spleen or CNS cells were incubated at 1 × 10⁶ (CNS) or 1 × 10⁷ (spleen) cells/ml in RPMI with or without LPS at 5 μg/ml, in the presence of GolgiStop (PharMingen) for 4 h. Cells were surface stained with CD11b antibody (Mac1-Cy5, M1/70) in the presence of anti-CD16 (2.4G2) blocking reagent. Cells were fixed and permeabilized with CytoFix/Cytoperm (PharMingen), and stained with IL-12–phycoerythrin, tumor necrosis factor-α–phycoerythrin or the appropriate isotype control (PharMingen). Samples were analyzed on a FACSCalibur (BD, Mansfield, MA) using CellQuest software as previously described (44,46).

**Statistical analysis**

Statistical analysis was performed using JMP3.2 (Professional Edition) statistical software. Group mean clinical scores were analyzed by paired t-tests. Repeated measures of ANOVA were used for comparison of the mean clinical scores. Mean maximum scores were compared by Student’s t-test and ANOVA. Cytokine concentrations were analyzed by Student’s t-test.

**Results**

*S. mansoni* ova preimmunization improves the clinical course of EAE in SJL mice

To determine whether *S. mansoni* ova preimmunization would modify the course of EAE, 6-week-old female SJL/J mice were injected i.p. with 10,000 ova 14 days, and 5000 ova i.p. and 5000 s.c. 4 days prior to EAE induction. This protocol induces a very strong Th2-type immune response as measured by ELISA for IL-4 cytokines (48–50). EAE was induced by s.c. injection of PLP₁₃⁹–₁₅₁ peptide in CFA. Animals were monitored daily for the development of clinical signs of EAE. Symptoms appeared on day 11 in control EAE groups and peaked at days 15–17 (Fig. 1A). This type of EAE induction results in the development of a relapsing disease, closely approximating the course of MS. We detected a relapse starting at day 30 post-induction, reaching a second
peak at days 32–33 with a mean clinical score of 1.0 ± 0.08 (Fig. 1A). In three independent experiments, mice preimmunized with S. mansoni ova demonstrated a significantly less severe disease course as compared to control EAE mice, indicated by lower daily mean clinical scores (P < 0.0001; Fig. 1B.). The onset of disease occurred on day 13 with a mean maximum clinical score of 1.0 ± 0.06. 

To explore the possibility that schistosome ova treatment can influence the course of EAE when given following initiation of autoimmune response, S. mansoni ova were injected on days 2, 7, 10 and 14 following EAE induction. Figure 2 shows that schistosome ova treatment significantly delayed the development of EAE when ova were applied in the initiation (day 2), but not in the effector (days 7–14), phase of the disease. Together, these studies suggest that schistosome ova preconditioning can modify the outcome of CNS autoimmunity. 

Inflammatory cell recruitment to the CNS is significantly decreased in mice preconditioned with S. mansoni ova and induced for EAE 

Cellular infiltration is a hallmark of EAE. To characterize whether the improved clinical score in EAE is reflected in decreased cellular infiltrates in schistosome ova-treated EAE animals, we induced EAE with or without schistosome ova immunization and examined the animals for histologic evidence of EAE. To study the maximum infiltration of CNS invading cells, tissues from the CNS were taken at day 17 after EAE induction. Figure 3 demonstrates the spinal cord and brain from control or schistosome ova-injected mice with EAE. Figure 3(A and C) demonstrates cellular infiltrates in the brain and spinal cord respectively of animals with EAE. Figure 3(B and D) shows significantly ameliorated cellular infiltration in the brain parenchyma and spinal cord of animals with EAE following S. mansoni ova immunization. Statistically significant differences in both number of infiltrated vessels and the degree of infiltration between control and ova-treated mice is demonstrated in Table 1. Although eosinophils have been indicated in parasitic diseases, there were no eosinophils in the CNS of schistosome ova-treated animals (data not shown). This data indicates that S. mansoni ova preconditioning of experimental animals reduced inflammation in the CNS.

The effect of S. mansoni ova injection on cytokine production—up-regulation of IL-4 and down-regulation of IFN-γ cytokine production from spleen cells in mice following immunization with schistosome ova 

It has been established that infestation with helminthic parasites induces a Th2-type response. Since Th2 cytokines are important in the regulation of Th1 cytokines (23), our next experiments characterized the cytokine profile of schistosome-treated and non-treated control mice, before and 15 days following EAE induction. Spleen cells were cultured with anti-CD3 for 48 h. The supernatants were assayed for IFN-γ, IL-4, IL-5, TGF-β and IL-10 production, (i) to ascertain that the resulting sensitization to the parasite antigens induced Th2 cytokine production and (ii) to analyze the effect of ova treatment on autoimmune disease-induced animals. We analyzed cytokine production before (day 0) and 15 days following EAE induction. Spleen cells from schistosome ova-injected animals produced higher levels of all Th2 cytokines tested, IL-4, TGF-β, IL-10 and IL-5, than unimmunized controls. At the same time, IFN-γ production was significantly inhibited in S. mansoni-treated animals, indicating Th1 down-regulation in these mice (data not shown). The IFN-γ level remained down-regulated and the IL-4 level remained higher in ova-pretreated animals at day 15 post EAE induction (Fig. 4), indicating a continuing Th2 bias in these mice. IL-5 production [previously demonstrated in SJL mice with induced EAE (16)] was not influenced by schistosome ova preconditioning.
These results imply that *S. mansoni* ova pretreatment of mice can shape the profile of autoimmunity-induced cytokine production.

*S. mansoni* ova injection does not affect the course of EAE in STAT6-deficient animals

To further explore the role of T<sub>h</sub>2 cytokines in helminth-induced protection from EAE, we treated STAT6-deficient animals with *S. mansoni* ova and initiated EAE by MOG<sub>35-55</sub> peptide injection in CFA. Because the STAT6 knockout was available on the C57BL6 background, we first needed to demonstrate protection from EAE by schistosome ova was also present in this mouse strain. Then we could ask whether STAT6 gene signaling was involved in schistosome ova protection. MOG peptide-induced EAE in C57BL6 has been described previously in C57BL6 as a chronic, non-remitting form of the disease, in contrast to the relapsing-remitting form in SJL mice (51,52).

It has been demonstrated previously that the STAT6 pathway controls the differentiation of cells into a T<sub>h</sub>2 phenotype and STAT6-deficient mice, with a predominantly T<sub>h</sub>1 phenotype, demonstrate a more severe clinical course of EAE (53). *S. mansoni* ova preconditioning influenced the course of EAE (delayed response and attenuated chronic phase) in control C57BL6 mice, but not in the STAT6-deficient animals (Fig. 5). This data indicates that T<sub>h</sub>2 cytokines, induced via STAT6 signaling, play a critical role in regulating the ameliorating effect of schistosome ova treatment in EAE, and provides the direct link between helminth treatment, T<sub>h</sub>2 environment and improved EAE.
producing IFN-γ.

Cytokine ELISPOT analysis of antigen-specific T cells producing IFN-γ or IL-4 throughout the course of EAE, with or without prior S. mansoni ova immunization

To test whether S. mansoni ova immunization influences the antigen-specific T cell phenotype throughout the course of EAE, we characterized the CD4+ cells that were primed with PLP139-151 peptide with or without prior S. mansoni ova immunization. We evaluated the frequencies of cytokine-producing cells in mice with S. mansoni ova preimmunization; analyzed changes in the frequencies of cytokine-producing cells in mice with concurrent S. mansoni immunization and EAE; and compared the cytokine profile of cells isolated from central (brain parenchyma) and peripheral (spleen) T cell pools throughout the course of EAE with or without S. mansoni ova preimmunization.

Our first goal was to evaluate the appearance of PLP139-151+ specific T cells in the spleen and brain parenchyma in animals with ongoing EAE. On day 17 following EAE induction an increased frequency of IFN-γ-producing cells is present in both spleen and brain (Fig. 6). This profile illustrates a highly polarized (IFN-γ^highIL-4^low) neuroantigen-specific T_h1 response in these experimental animals. When mice were preimmunized with S. mansoni ova, an increased frequency of cells carrying a more T_h2-like phenotype (PLP specific, IFN-γ^lowIL-4^high) was detected in the CNS. At the same time, an increased frequency of IL-4-producing cells with specificity for the PLP antigen could also be detected in the spleen; however, the frequency of neuroantigen-specific, IFN-γ^high^ cells did not decrease significantly. This emphasizes the shift to a more T_h2-polarized immune response following S. mansoni ova immunization. The fact that the frequency of IFN-γ^producing cells was significantly decreased in the brain parenchyma, but not in the spleen, suggests local brain tissue regulation of cytokines during EAE.

These data illustrate the modifying effect of helminth ova immunization on the generation of autoimmune T cells.

Reduced IL-12, but unchanged TNF-α, expression by CD11b+ macrophages in the CNS tissue from mice with concomitant S. mansoni ova conditioning and EAE

Antigen-specific T cells constitute a very small portion of infiltrating leukocytes in EAE or MS lesions (47,54). Recruited inflammatory cells account for the majority of infiltrating cells and play a crucial role in CNS damage (55). Macrophage recruitment to the inflamed CNS is required for primed antigen-specific T cells to execute a T_h1 effector program in EAE (56).

Results described above suggested that S. mansoni-treated mice were deficient in recruiting and/or activating macrophages in the CNS during EAE due to decreased IFN-γ production in the brain tissue. To address this issue, mononuclear cells were isolated from CNS tissue of EAE and schistosome ova-pretreated EAE mice, and CD11b+ cells were analyzed with intracellular flow cytometry detecting IL-12 and TNF-α cytokines. Figure 7 illustrates that when compared with EAE controls, S. mansoni ova-pretreated mice showed a sharply reduced percentage of IL-12-producing, CD11b+ cells in the CNS during EAE attacks (29.4 ± 4.5 versus 15.3 ± 3.1%, mean ± SD, n = 3). In contrast, percentages of CD11b+ TNF-α+ cells were relatively stable between the two groups of mice (41.3 ± 5.5 versus 40.4 ± 2.5%, mean ± SD, n = 3). These data suggest that IL-12 produced by CD11b+ macrophages is important in maintaining CNS autoimmune functions in EAE.

Discussion

The data described above demonstrate that pretreatment of mice with S. mansoni ova can attenuate the clinical course of actively induced EAE. Furthermore, schistosome ova treatment after induction of EAE significantly delayed the development clinical symptoms when ova were applied in the initiation, but not in the effector, phase of the disease. The recruitment of inflammatory cells in EAE lesions is also less severe in S. mansoni ova-treated mice than in untreated, EAE-induced groups. The amelioration of EAE is partly due to the effect of S. mansoni eggs on the T_h1/T_h2 balance in the hosts because splenocytes from schistosome egg-treated mice...
Fig. 5. Schistosome ova pretreatment improves the clinical course of EAE in wild-type, C57BL/6 mice but not in STAT6-/- mice. Groups of C57BL/6 mice (A) and STAT6-/- mice (B) were pretreated 14 and 4 days prior to induction of EAE (open circles) or not pretreated (filled diamonds). Animals were scored daily beginning at 6 days following disease induction. S. mansoni ova pretreatment improved the clinical course in C57BL/6 mice (A). In the STAT6-/- mice, schistosome ova showed no effect on the clinical course of EAE (B). This experiment suggests that induction of Th2 cytokines through the STAT6 pathway contributes to the protection mediated by schistosome ova. Data represents one experiment of three (three to four mice per group) with similar results. There was no significant difference between schistosome ova-pretreated and untreated STAT6-/- mice on any day in any of the three experiments. In the C57BL6 mice, schistosome ova pretreatment provided statistically significant protection on days 11–15 and 23–28, p < 0.05.

Fig. 6. ELISPOT analysis of IFN-γ- and IL-4-producing cells isolated from spleen and brain of SJL mice in response to ex vivo stimulation with PLP. EAE was induced in both naive and S. mansoni ova-pretreated groups, and cytokine-producing cells were quantified at the peak of disease, day 17. S. mansoni ova induced an increase in both IFN-γ- and IL-4-producing PLP antigen-specific spleen cells. In the brain, IL-4-producing cells were increased with a concomitant decrease in the IFN-γ cell frequency. (A) Representative wells demonstrating the pattern of increased Th2 cytokine-producing cells in both brain and spleen as a result of ova pretreatment. (B) Mean counts ± SE of six wells representing two animals at each condition. Media control values are presented to show specificity of the assay.
secretes ~2.5 times more IL-4 cytokine than the control group before the induction of EAE. Local PLP antigen-specific CD4+ T cells from the CNS parenchyma exhibited a more significant modulation in their cytokine production than cells from the spleen. T cell responses against foreign antigen (S. mansoni ova immunization) and autoantigen (neural antigen: PLP) might be different. T cell responses to autoantigens might consist of low-affinity clones that remain following deletion of high-affinity auto-reactive T cells from the repertoire (57–60). T cell affinity has been implicated as an important factor in differentiation of Th1/Th2 cells (58,61). The possibility that IL-4 plays an important role in down-regulation of neural antigen-specific autoreactive cells in local tissues remains to be determined.

The direct association between Th2 cytokines and helminth ova-induced protection from EAE was further suggested by the absence of any S. mansoni ova-induced protection from EAE in STAT6-deficient animals (Fig. 5). STAT6-deficient mice experience a more severe clinical course of EAE (53). S. mansoni ova preconditioning influenced the course of EAE in C57BL6 mice, but not in STAT6-deficient animals, indicating that the STAT6 signaling plays a critical role in the ameliorating effect of schistosome ova treatment in EAE, and provides a direct link between helminth ova treatment, the Th1,2 environment and improved EAE. Microbial compounds from S. mansoni have been shown to induce the development of DC2 and to promote development of Th1,2 cells (62). The role of DC2 in helminth-induced protection from EAE remains to be explored.

Our data further emphasizes the role of IL-4 in the outcome and severity of EAE. IL-4 had been previously correlated with a polarized Th2 immune response, crucial in the differentiation of Th1,0 cells toward the Th1,2 pathway (63–66). IL-4 knockout mice are defective in development of Th1,2 cells (67). The disruption of the IL-4 gene in EAE-resistant stains such as BALB/c results in susceptibility to the induction of EAE (68). IL-4-deficient C57BL6 mice, and to a lesser extent IL-4-deficient BALB/c mice, developed a more severe form of EAE, with more extensive pathologic involvement of the spinal cord, in parallel with an increased expression of pro-inflammatory cytokines in the CNS. EAE could also be inhibited by local delivery of IL-4 using retrovirus-transfected T cells (69). Systemic administration of IL-4 ameliorated the clinical course of EAE, and inhibited the production of IL-2 and TNF-α (Th1) in SJL mice (70). These studies and ours suggest that a predominantly Th2 cytokine microenvironment protects from development of EAE. Genetic predisposition toward Th2 may also protect against the induction of EAE/MS (71).

The administration of helminth ova prior to EAE induction can lead to a significant degree of protection from disease, associated with a decreased number of IFN-γ-producing cells in the CNS and a greatly reduced level of leukocyte infiltration into the CNS. A strong Th1,2 down-regulation in ova-preimmunized animals was detected by decreased IFN-γ production. The schistosome ova-induced Th1,2 bias was maintained during EAE as indicated by the fact that IFN-γ remained down-regulated and IL-4 remained higher in ova-pretreated animals at day 15 of EAE.

Fig. 7. Decreased IL-12 but unchanged TNF-α expression by CD11b+ cells in the CNS tissue from mice with S. mansoni ova pretreatment and EAE. SJL mice were pretreated with S. mansoni ova 14 and 4 days prior to EAE induction. EAE was induced by s.c. immunization with PLP_{135-151} peptide emulsified in CFA. Pertussis toxin was administered at day 0 and 2. Animals were analyzed at the peak of disease (day 17, control mean score 2.5 and S. mansoni ova pretreated mean score 1.0). Mononuclear cells were isolated from CNS and spleen tissues, and CD11b+ cells were analyzed with intracellular staining and flow cytometry, detecting IL-12 and TNF-α cytokines. Compared with EAE controls, S. mansoni ova-treated mice showed a sharply reduced percentage of CD11b+cells producing IL-12 in the CNS during EAE attacks (29.4 ± 4.5 versus 15.3 ± 3.1%, mean ± SD, n = 3). In contrast, the percentage of CD11b+TNF-α+ cells was similar between the two groups (41.3 ± 5.5 versus 40.4 ± 2.5%, mean ± SD, n = 3). Numbers are representative quadrant percentages.
IFN-γ has been shown to exacerbate MS (72–74). Antibodies to IFN-γ-inducing factor inhibited the production of IFN-γ and suppressed clinical EAE (75). However, IFN-γ treatments have also been shown to be protective against EAE in SJL mice (76) and IFN-γ knockout mice are still susceptible to EAE (77,78). Interestingly, IFN-γ has been shown to shape the immune invasion profile of leukocytes in the CNS by regulating chemokines (79). The possibility that the chemokine profile is changing in brain tissue due to schistosome ova treatment is currently under investigation in our laboratory.

Cross-regulation of T<sub>1</sub>/T<sub>2</sub> cytokines is a paradigm that is currently under considerable scrutiny. There is ample evidence that this cross-regulation can exist in an infectious environment. Recently it has been shown that concurrent enteric helminth infection modulates inflammation and reduces helicobacter-induced gastric atrophy in mice (80). The same group demonstrated that the decrease of helicobacter-induced atrophy correlated with a reduction in mRNA for cytokines and chemokines associated with a T<sub>1</sub>-type inflammatory response. Furthermore, ongoing helminth (S. mansoni)-induced T<sub>2</sub> response was shown to influence the outcome of a T<sub>1</sub>-inducing protozoan parasite Toxoplasma gondii infection by regulating the IFN-γ, TNF-α and NO response (81).

Because cytokines are often multifunctional and redundant, the results from cytokine knockout studies may be difficult to interpret, especially in a complicated disease like EAE (82). There are potential problems created by systemic administration of cytokines or antibodies to cytokines, including short half-life, systemic toxicity and access to target organs, especially the CNS. It is still unclear when, where and which cytokines or combinations of cytokines are needed due to the complicated function and cross-regulation of cytokines. In this study, S. mansoni eggs appear to induce a more physiological T<sub>1</sub>/T<sub>2</sub> microenvironment, which is inhibitory to the encephalitogenic T<sub>1</sub> cell-mediated immune cascade. Changes in IL-12-producing cell phenotypes were also detected in treated animals (Fig. 7). S. mansoni ova-treated mice showed a sharply reduced percentage of CD11b<sup>+</sup>IL-12<sup>+</sup> cells in the CNS during EAE. In contrast, percentages of CD11b<sup>+</sup>TNF-α<sup>+</sup> cells were relatively stable between the two groups. These data suggest that IL-12 produced by CD11b<sup>+</sup> macrophages is important in maintaining CNS autoimmune functions in EAE. S. mansoni ova might also regulate CD11b<sup>+</sup> IL-12-producing cells. The identity of these cells is not known at this time; they certainly must be further characterized.

Individuals with predominant T<sub>2</sub> responses against egg antigens have less severe egg-associated morbidity than those with predominantly T<sub>1</sub> responses, thus a T<sub>2</sub> predisposition is advantageous to the human host. We detected granulomas in the peritoneal cavity and the liver of ova-treated animals surrounded by healthy tissue, indicating minimal pathology induced by our treatment (data not shown). From an evolutionary perspective, people living in areas with a high prevalence of helminth infections might be positively selected because of their adaptively advantageous T<sub>2</sub> responses. More importantly, many helminth parasites can survive in the host for many years (83). Long-term exposure to helminth antigens, especially during childhood, may have a deep impact on maturation of the host immune system.

**Immunomodulation of EAE by helminth ova immunization**

MS patients have significantly fewer T<sub>1</sub>2-mediated allergic diseases than would be expected (84). The reduced prevalence of allergic diseases in MS patients appears to be related to increased IL-12 production (85). Interestingly, helminth infections have also been suggested to protect people from allergic diseases. Some argue that under the pressure of helminth infections, allergens become minor stimuli to T<sub>1</sub>2 effector cells such as mast cells (3).

In summary, the results of this study provide evidence that T<sub>1</sub>2 diseases can modulate the development of T<sub>1</sub>1 diseases by influencing the cytokine environment of immune competent cells. This study suggests that long-term infections with helminths in childhood might deeply impact the maturation of T<sub>1</sub>1 and T<sub>1</sub>2 cells. Exposure to helminths could be an important factor in suppressing the development of T<sub>1</sub>1 cell-mediated autoimmune diseases in adulthood.

**Acknowledgements**

This work was supported by the National Multiple Sclerosis Society (grant RG3113A1/1 to Z. F.) and the National Institutes of Health (grant RO1 NS 37570-01A2 to Z. F.).

**Abbreviations**

CNS = central nervous system
CFA = complete Freund's adjuvant
EAE = experimental autoimmune encephalomyelitis
LPS = lipopolysaccharide
MOG = myelin oligodendrocyte glycoprotein
MUP = 4-methylumbelliferonyl phosphate
MS = multiple sclerosis
PLP = proteolipid protein
TGF = transforming growth factor

**References**

Immunomodulation of EAE by helminth ova immunization


