Association Between Parasite Infection and Immune Responses in Multiple Sclerosis

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Objective: To assess whether parasite infection is correlated with a reduced number of exacerbations and altered immune reactivity in multiple sclerosis (MS).

Methods: A prospective, double-cohort study was performed to assess the clinical course and radiological findings in 12 MS patients presenting associated eosinophilia. All patients presented parasitic infections with positive stool specimens. In all parasite-infected MS patients, the eosinophilia was not present during the 2 previous years. Eosinophil counts were monitored at 3- to 6-month intervals. When counts became elevated, patients were enrolled in the study. Interleukin (IL)-4, IL-10, IL-12, transforming growth factor (TGF)-β, and interferon-γ production by myelin basic protein–specific peripheral blood mononuclear cells were studied using enzyme-linked immunospot (ELISPOT). FoxP3 and Smad7 expression were studied by reverse-transcriptase polymerase chain reaction.

Results: During a 4.6-year follow-up period, parasite-infected MS patients showed a significantly lower number of exacerbations, minimal variation in disability scores, as well as fewer magnetic resonance imaging changes when compared with uninfected MS patients. Furthermore, myelin basic protein–specific responses in peripheral blood showed a significant increase in IL-10 and TGF-β and a decrease in IL-12 and interferon-γ–secreting cells in infected MS patients compared with noninfected patients. Myelin basic protein–specific T cells cloned from infected subjects were characterized by the absence of IL-2 and IL-4 production, but high IL-10 and/or TGF-β secretion, showing a cytokine profile similar to the T-cell subsets Tr1 and Th3. Moreover, cloning frequency of $CD4^+CD25^+$ $FoxP3^+$ T cells was substantially increased in infected patients compared with uninfected MS subjects. Finally, Smad7 messenger RNA was not detected in T cells from infected MS patients secreting TGF-β.

Interpretation: Increased production of IL-10 and TGF-β, together with induction of $CD25^+CD4^+$ $FoxP3^+$ T cells, suggests that regulatory T cells induced during parasite infections can alter the course of MS.

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Autoimmune diseases affect 5 to 7% of the adult population in Europe and North America.[1] Included among these diseases is multiple sclerosis (MS), an inflammatory demyelinating condition that affects the central nervous system in which abnormal immune mechanisms induce myelin injury.[2] MS is currently considered a prototypic example of Th1-mediated, organ-specific autoimmunity, with both genetic and environmental factors involved in disease onset.

During recent decades, strong epidemiological evidence has accumulated indicating a steady increase in autoimmune disease incidence in developed countries.[3–5] In addition, differences in autoimmune disease prevalence have also been observed between rural and urban areas within the same country.[6] Because this epidemiological shift occurs in a timeframe too short to be attributable to genetic factors, it is believed that environmental factors could account for changes in the immune repertoire. The decline in infectious disease prevalence has been proposed as the origin for increasing autoimmune disease incidence. A parallel observation in the field of allergy has been called the "hygiene hypothesis."[7]

Parasite infections in humans generate a strongly polarized Th2 reaction,[8] which, in turn, can modulate Th1 immune responses to unrelated antigens, diminishing the strength of the immune response against secondary infections such as malaria and leishmaniasis.[9,10] Likewise, within populations of helminth-endemic areas, an inverse association between parasite infection and allergy has been clearly established.[11] Moreover, in a seminal study, Lynch and colleagues[12] documented allergic symptom recurrence in patients after antiparasite treatment.

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The best evidence in favor of a causal relation between parasite infections and autoimmune diseases derives from animal models. Many examples exist in both spontaneous and induced models of human autoimmune diseases where parasite infection, or products thereof, influence the course of autoimmune pathology. Prior infection with *Schistosoma mansoni* or exposure to nonviable *Schistosoma* ova, for example, reduces both incidence and severity of experimental autoimmune encephalomyelitis (EAE), an animal model for MS.[13,14] Infection with *S. mansoni,* exposure to ova, or exposure to ovum extracts also prevent development of type 1 diabetes in NOD (nonobese diabetic) mice,[15] as well as colitis induced by trinitobenzesulfonic acid, an animal model for Crohn's disease.[16] Collectively, these observations suggest that individuals harboring parasite infections present a diminished Th1 response when challenged with other antigens.

Within this framework, however, the fact that parasite infections might protect from allergy appear paradoxical. Both diseases are typically associated with a Th2 polarized response. Data also show an association between atopic and autoimmune diseases, such as type 1 diabetes and rheumatoid arthritis,[17,18] suggesting common mechanisms underlying parasite infection–mediated protection against both autoimmune and allergic diseases that cannot be accounted merely by Th1 versus Th2 imbalance. Parasite infections may induce regulatory T cells (Treg) secreting high levels of IL-10 or transforming growth factor (TGF)-β (Tr1, Th3),[19] as well as $CD4^+CD25^+$ Treg expressing the FoxP3 transcription factor in the host,[20] and this may represent a potential explanation regarding how exposure to a parasite could alter immune reactivity to stimuli unrelated to its presence.

To better understand the relation between parasite infections and the course of MS, we studied the effect of infections on clinical and radiological disease progression, as well as immune response. This study reports evidence indicating that parasitic infection leads to the development of regulatory cells that produce IL-10 and TGF-β, inhibit T-cell proliferation, and suppress interferon (IFN)-γ production, thus altering the course of MS. To the best of our knowledge, this is the first study to report chronic exposure to parasites as an environmental factor affecting the course of MS in humans.

Subjects and Methods

Patients and Study Design

Twelve patients (8 female and 4 male patients) with a diagnosis of clinically definite relapsing-remitting MS according to Poser's criteria and who presented eosinophilia during the course of disease were assessed in a prospective double-cohort study. Patients were recruited from a larger cohort of 432 regularly followed MS patients and were typical in all respects with the exception of eosinophilia. Any MS patient with a high eosinophil count was included in the study. The mean age was 34 ± 6.8 years, mean extended disability status scale (EDSS) score 2.8 ± 0.6, and mean disease duration 7.3 ± 1.01 years. Intestinal parasites were identified as the cause of eosinophilia. This diagnosis was confirmed in all patients by positive stool examination. Presence of other endemic parasitoses, including trypanosomiasis, leishmaniasis, amebiasis, giardiasis, and toxoplasmosis, was ruled out in the study population using microscopic examination and serological testing. Investigations were always found to be negative, excluding simultaneous presence of these infections.

Twelve healthy subjects and 12 uninfected relapsing-remitting MS patients in remission, matched for age, sex, and disease duration, served as control subjects (Table). Healthy individuals were recruited among family members of the infected MS patients. Thorough clinical and neurological examination, as well as standard chemical and hematological laboratory examinations, ruled out the presence of any underlying disorder. Uninfected MS control subjects included in the study presented a clinical disease course similar to that of other MS patients in the clinic. Both eosinophil counts and stool examinations examined in uninfected MS subjects and healthy control subjects were found to be negative at study entry and throughout the entire study. No MS patients were under immunomodulatory or immunosuppressive treatment at any point in the study, and they had not received steroids in the last 3 months before study entry. Severe relapses were treated with intravenous methylprednisolone (1,000mg/day for 3 days), followed by a 2-week tapering course of oral prednisone. Magnetic resonance imaging (MRI) scans and eo-

Table. Clinical Characteristics of Multiple Sclerosis Patients and Control Subjects

Characteristics	Sex Ratio (F/M)	Age (years)	Duration of Disease (years)	EDSS at Entry	Annual Relapse Rate 2 Years before Entry	Duration of Follow-up (years)	Eosinophil Count/mm^3 (%) at Study Entry
Infected MS patients	8:4	34 ± 6.8	7.3 ± 1.01	2.8 ± 0.6	0.76 ± 0.17	4.65 ± 0.85	1358 ± 293(16.9 ± 4.2)
Uninfected MS patients	8:4	34 ± 7.7	6.6 ± 1.06	2.5 ± 0.7	0.90 ± 0.39	4.50 ± 0.69	211 ± 81 (3 ± 1.1)
Healthy control subjects	8:4	34 ± 6.4	—	—	—	—	174 ± 83 (2.5 ± 1)

EDSS = extended disability status scale; MS = multiple sclerosis. Values represent mean ± standard deviation.

sinophil counts were performed more than 30 days after treatment.

Patients were subjected to a comprehensive neurological examination every 3 months, including physical assessment of disease activity and EDSS scoring. If a new relapse occurred, an additional visit was arranged within 72 hours. An exacerbation was defined as the appearance of a new symptom, or worsening of a pre-existing symptom, confirmed on neurological examination and lasting at least 48 hours, in the absence of fever and preceded by stability or improvement for at least 30 days. EDSS values are presented as changes between initial and subsequent examinations.

Fecal samples were analyzed for parasite eggs, species identification, and number of eggs per gram of feces, prepared by formalin-ether sedimentation.

Brain MRIs were performed at 6-month intervals on a 1.5-Tesla Signa Unit (General Electric, Milwaukee, WI). Axial 5mm-thick slices were performed with T2-weighted, proton density, fast spin-echo, fluid attenuation inversion recovery– and T1-weighted sequences, before and after administration of gadolinium (Gd)-diethylenetriamine pentaacetic acid (0.1mmol/kg). Postcontrast images were completed within 15 minutes after Gd injection. The cohort was followed for 55.8 ± 10.3 months.

Immunological evaluations were performed during the last 12 to 18 months of follow-up. At that time, eosinophil count and stool examination were assessed again. Results were similar to those observed at study entry. Assays were always performed on fresh peripheral blood mononuclear cells (PBMCs). All blood draws for evaluation of cytokine secretion by PBMC or Treg isolation were performed at least 90 days after a relapse or a course of steroids.

Antigen Preparation

Because of the large number of PBMCs required for immunological studies in general and for cloning procedures in particular, a single peptide was selected as the main antigen in this study and was used in both groups of MS patients and in control subjects. The peptide selected was $MBP_{83\text{-}102}$ shown in several studies to be an immunodominant epitope of myelin basic protein (MBP). $MBP_{83\text{-}102}$ peptide was synthesized using an automated peptide synthesizer, and purity was assessed using high-pressure liquid chromatography analysis.

Immunoglobulin E Level Assay

Total IgE was calculated using rabbit antihuman IgE antibodies for capture (Dako, Glostrup, Denmark) and biotinylated goat anti–human IgE antibodies for detection (Vector Laboratories, Burlingame, CA). IgE serum concentrations were read from a standard curve constructed using the World Health Organization human serum IgE standard (National Institute for Biological Standards and Control, Hertfordshire, United Kingdom). Assay detection limit was 5IU/ml. Interassay and intraassay variation coefficients were 6.3 and 5.6%, respectively.

Cytokine Production

The number of PBMCs secreting IL-4, IL-10, IL-12, IFN-γ, and TGF-β was measured using single-cell resolution enzyme-linked immunospot (ELISPOT) assays, as described previously.[21] IL-4, IL-10, TGF-β_1, and IFN-γ ELISPOT detection kits were purchased from R&D Systems (Minneapolis, MN), and the IL-12 ELISPOT detection kit from Diaclone (Bensançon, France). The ELISPOT used for IL-12 detection in this study was specific for the p70 heterodimer, which is the bioactive form of IL-12. The number of antigen-specific cytokine-secreting cells was calculated by subtracting the number of spots obtained in zero antigen background control cultures from the number of spots obtained in cultures exposed to stimulating Ag. Data correspond to number of spots per 10^5 mononuclear cells.

MBP peptide-specific T-cell clones (TCCs) were tested for cytokine secretion 7 to 10 days after their last stimulation with feeder cells. Fifty thousand cells per well were stimulated with 1μg/ml of plate-bound anti-CD3 monoclonal antibody (MAb) (OKT3; American Tissue Culture Collection, Rockville, MD). Supernatants were removed at 36 (for IL-4) or 72 hours (for the other cytokines). IL-2, IL-4, IL-5, IL-10, TGF-β, and IFN-γ assays were performed using commercially available enzyme-linked immunosorbent assay kits, purchased from R&D Systems following manufacturer's instructions.

ISOLATION OF $MBP_{83\text{-}102}$-SPECIFIC T-CELL CLONES. MBP peptide-specific TCCs were isolated from peripheral blood as described previously.[22] In brief, 5×10^6 PBMCs were stimulated with 10μg/ml $MBP_{83\text{-}102}$. After 5 to 7 days, cells were recultured in fresh medium containing 50U/ml recombinant human IL-2 (rIL-2; R&D Systems) for an additional week. Restimulation cycles with autologous irradiated PBMCs (3,000 Rads) as antigen-presenting cells (APCs) plus peptide, and expansion with rIL-2 was repeated weekly. After 4 cycles of restimulation and expansion, TCCs were derived by limiting dilutions in 96 round-bottom well microtiter plates (Falcon; Becton-Dickinson Labware, Lincoln Park, NJ) at 1 and 0.3 cells/well, in the presence of MBP peptide and autologous irradiated PBMCs.

ISOLATION OF $CD4^+CD25^+$ T-CELL CLONES. $CD4^+CD25^+$ TCCs were obtained as described previously.[23] In brief, $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were single-cell sorted into 96-well plates. The cells were stimulated with 0.05μg/ml phytohemagglutinin (PHA; Calbiochem, San Diego, CA) in the presence of 3×10^4 irradiated autologous PBMCs, 10^4 irradiated JY cells, and 100U/ml rIL-2. Half the medium was replaced on day 7. Cells were split as necessary, and fresh medium containing 100U/ml rIL-2 was added. Cultures were restimulated every 10 to 14 days with 0.05μg/ml PHA, a mixture of 10^4 irradiated autologous and 10^4 allogenic PBMCs, and 100U/ml rIL-2.

Clones growing after 4 to 6 weeks were tested for regulatory activity. Ten days after the last activation, variable numbers of $CD4^+CD25^+$ cells were added to a constant number of $CD4^+CD25^-$ indicator cells (3×10^3 cells/well) to achieve $CD4^+CD25^+$:$CD4^+CD25^-$ ratios of 10:1, 5:1, 2.5:1, and 1:1, respectively. Stimulation was provided by the addition of soluble anti-CD3 and soluble anti-

CD28 (BD Biosciences, San Diego, CA) antibodies, both at a concentration of 5μg/ml, in the presence of 3×10^4/well irradiated PBMCs, depleted of $CD3^+$ T cells as a source of APC. Control wells contained APC, $CD4^+$-$CD25^-$ cells, or $CD4^+CD25^+$ cells exclusively, as well as each of these populations in the absence of anti-CD3/anti-CD28. Additional controls included $CD4^+CD25^+$ cells cultured in the presence and absence of anti-CD3/anti-CD28 stimulation plus recombinant human IL-2 (50U/ml) to confirm anergy. Proliferation was determined at day 6, with [^{3}H] thymidine (ICN Biomedicals, Irvine, CA) added during the last 18 hours of culture. Mean counts per minute ± standard error were calculated for triplicate measurements. To measure IFN-γ production, we removed supernatants from each well before [^{3}H] thymidine addition and analyzed using commercially available enzyme-linked immunosorbent assay kits (R&D systems). For these assays, presort $CD4^+$ T cells were used as additional controls.

Expression of FoxP3 and Smad7

For quantitative assessment of relative messenger RNA (mRNA) levels, total RNA was prepared using TRIzol LS reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions. RNA was reverse transcribed using an M-MLV RT reverse transcription kit with random hexamer primers (Invitrogen). The relative levels of FoxP3 and Smad7 mRNA were determined by real-time polymerase chain reaction on an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). Values obtained were normalized to the amount of glyceraldehyde phosphate dehydrogenase. Primer sequences used were as follows: glyceraldehyde phosphate dehydrogenase: forward 5′-GAAGGTGAAGTCGGAGTC, reverse 5′-GAAGATGGT-GATGGGATTTC; FoxP3: forward 5′-CACCTGGCTGGG-AAAATGG-3′, reverse 5′-GGAGCCCTTGTCGGATGAT-3′; Smad7: forward 5′-CCTTA- GCCGACTCTGCGAACTA-3′, reverse 5′-CCAGATAAT- TCGTTCCCCCTGT.

Statistical Analysis

Differences observed in exacerbation rates, EDSS changes, MRI parameters, cytokine production, and IgE levels between groups were evaluated using the Mann–Whitney–Wilcoxon test. Correlations were determined using Spearman's correlation coefficient. The number of wells producing growth from the cloning of cells derived from infected and uninfected patients, as well as from healthy control subjects, were compared using hierarchical logistic regression analysis. p less than 0.05 were considered statistically significant.

Results

Clinical and Magnetic Resonance Imaging Patient Characteristics

Twelve MS patients with eosinophilia were selected as the study group and were followed for a period of 55.8 ± 10.3 months in a prospective, double-cohort study. All patients presented with high eosinophil counts (see the Table; range, 800–1,800/mm^3), associated with parasitic infection as determined by positive stool examination. Further eosinophil readings were performed at 3- to 6-month intervals without significant changes during follow-up. Three patients were infected with *Hymenolepis nana,* 3 with *Trichuris trichiura,* 3 with *Ascaris lumbricoides,* 2 with *Strongyloides stercolaris,* and 1 with *Enterobius vermicularis.* At the time of eosinophilia detection, egg load values ranged between 1,180 and 9,340 eggs/gm. Eight patients never developed clinical symptoms of parasitic infection, and four patients suffered mild anemia. MS diagnosis always preceded eosinophilia detection by 31.7 ± 5.6 months (range, 24–41 months). At the time of MS diagnosis, all patients presented with normal eosinophil counts; however, stool analysis was not performed. Initial stool examination was performed when eosinophilia was detected. Because none of the patients developed clinical disease as a result of infection, no antihelminthic therapy was indicated. The clinical characteristics of patients and control subjects are summarized in the Table.

Both infected and uninfected MS patients had similar social and economic backgrounds. All lived in low-income neighborhoods with little urban development and high prevalence of helminth infections.

During the 2 years before study enrollment, annual MS relapse rate in parasite-infected patients had been 0.76 ± 0.17/year (median, 0.73), and 0.90 ± 0.36/year (median, 0.90) in uninfected MS patients. No significant differences were observed between groups ($p = 0.256$). Over the 55.8-month study period, 3 clinical relapses were observed in the infected MS group (9 patients remained clinically unchanged), and 56 relapses occurred in the uninfected MS group (Fig 1A). Thus, median annualized relapse rate was 0 in infected MS patients compared with 1.09 in uninfected MS subjects ($p < 0.0001$; see Supplementary Table). Furthermore, only two infected patients showed minimal EDSS changes lasting less than 3 months. EDSS scores did not change in the remaining 10 patients. Conversely, by the end of the follow-up period, 11 of 12 uninfected patients showed an overall increase in baseline EDSS (see Fig 1B).

The presence of new enlarging T2 MRI lesions, with or without contrast enhancement, was evaluated over time. In infected MS patients, 14 new or enlarging T2 MRI lesions were detected, 4 of which enhanced after Gd injection. In 6 patients, scans remained unchanged throughout the study period. In contrast, new or enlarging T2 MRI lesions occurred in all uninfected MS patients during the course of the study, with 164 new or enlarging T2 MRI lesions registered, 78 of which enhanced after Gd injection (see Figs 1C, D).

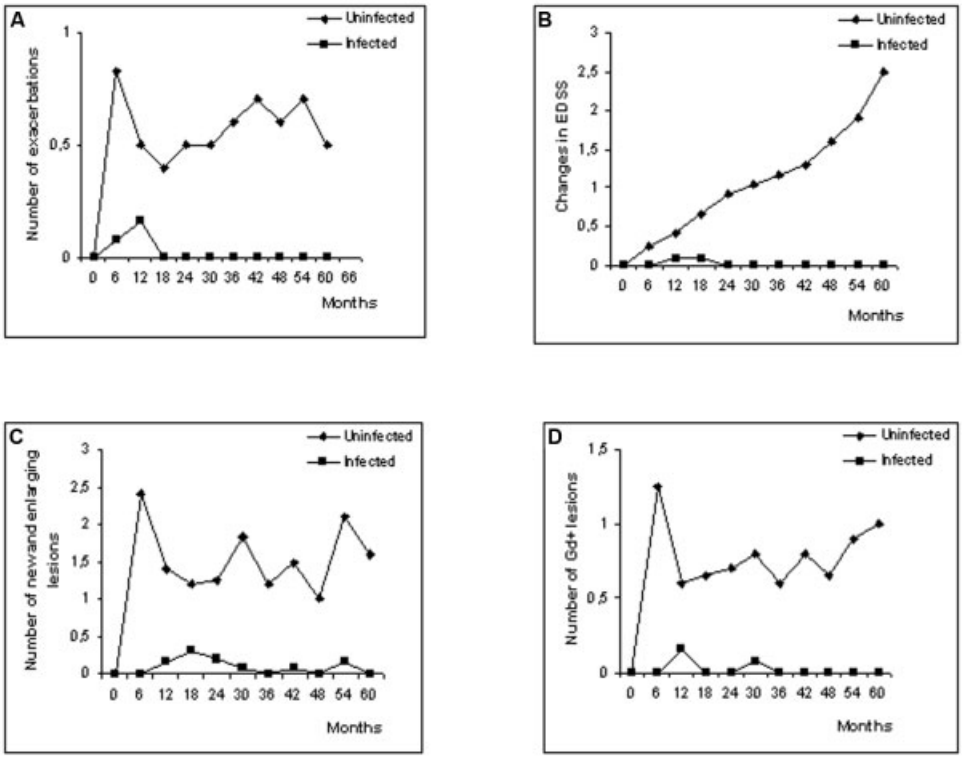

Fig 1. Number of exacerbations (A) and changes in extended disability status scale (EDSS; B) and magnetic resonance imaging (C and D) parameters observed over time in parasite infected (squares) *and uninfected* (diamonds) *multiple sclerosis (MS) patients. Gd = gadolinium.*

All relapses observed in infected MS patients and 48 exacerbations (86%) observed in uninfected MS subjects were treated with intravenous methylprednisolone followed by a short tapering course of oral prednisone. The scant number of exacerbations observed in the infected MS group (n = 3) during this study precludes statistical analysis of treatment impact on the results of this investigation.

In infected MS individuals, the median total IgE level (1,800IU/ml; range, 525–3,300) was significantly greater ($p = 0.001$) than in healthy subjects (285IU/ml; range, 170–1,508) or in uninfected MS patients (380IU/ml; range, 213–2,100), remaining constant throughout follow-up. Furthermore, total IgE concentrations showed positive correlation with the total number of parasite eggs per gram in feces ($r = 0.48$; $p = 0.01$). Concentrations of parasite-specific IgE were not investigated. The limited number of patients infected with each different type of parasite precludes appropriate statistical analysis regarding impact of specific helminth species of immunological response. Parasite infections are associated with increased production of TGF-β and IL-10 and reduced production of IFN-γ and IL-12.

Helminth infections have been shown to promote cytokine production associated with a Th2-type response.[8] Conversely, MS is characterized by an inflammatory response associated with the production of Th1-type cytokines, such as IFN-γ.[2] To test whether parasite infection influences antigen-specific T-cell phenotype during the course of MS, we characterized cytokine production in PBMC using ELISPOT assays. As shown in Figures 2A and 2B, the number of $MBP_{83\text{-}102}$ peptide–specific IL-10 and TGF-β secreting cells was significantly greater in samples collected from parasite-infected MS patients, compared with those obtained from uninfected MS patients or control subjects ($p < 0.0001$). At the same time, as illustrated in Figures 2C and 2D, parasite-infected MS patients showed significantly reduced numbers of IL-12– and IFN-γ–secreting cells compared with uninfected MS patients or control subjects ($p = 0.0001$). No significant difference in the number of IL-4–secreting cells was observed between patient groups.

Regulatory T-Cell Isolation and Characterization

To further examine potential mechanisms explaining immune response changes in parasite-infected MS patients, we obtained MBP peptide–specific TCCs with

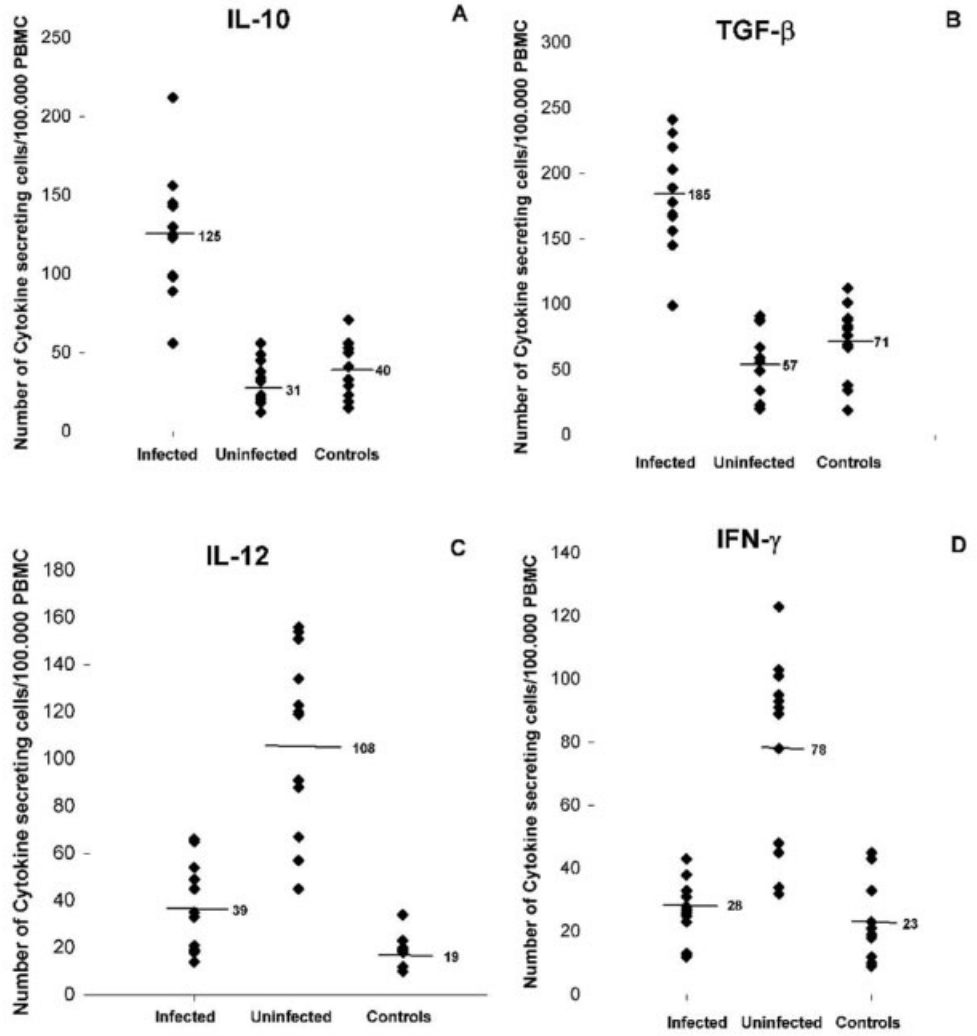

Fig 2. Number of cytokine-secreting cells per 10^5 peripheral blood mononuclear cells (PBMCs) in parasite-infected multiple sclerosis (MS) patients, uninfected MS patients, and healthy control subjects. Horizontal lines *indicate mean group values. IFN = interferon; IL = interleukin; TGF = transforming growth factor.*

Th3/Tr1 regulatory cell phenotype from both infected and uninfected MS patients; their frequency and cytokine production were analyzed. Th3/Tr1-like TCC cloning frequency was significantly greater in parasite-infected MS patients compared with those not infected (Fig 3A). A total of 48 TCCs from infected MS patients were extensively analyzed for cytokine secretion. The first group of 36 TCCs showed high IL-10 (10 TCCs, 480–1,300pg/ml) production, high TGF-β (14 TCCs, 550–1,750pg/ml) production, or both (12 TCCs), and lack of IL-2 (see Fig 3B). To rule out any possibility that the lack of IL-2 secretion may have been due to IL-2 consumption, we harvested culture supernatants from 12 Th3/Tr1-like TCCs at earlier time points, that is, 1, 2, 4, 8, 12, and 24 hours after stimulation. IL-2 was not detected at any of these time points, indicating that IL-2 absence was not due to consumption during culture. A second group of 12 TCCs showed strong IL-2 production (500–2,800pg/ml), but lacked IL-10 and TGF-β (see Fig 3C). Both types of TCCs produced IL-5 (220–460pg/ml), but little IFN-γ (0–100pg/ml) or IL-4 (0–80pg/ml).

Forty-two TCCs were isolated from uninfected MS patients. Of these, only 5 were characterized by strong production of IL-10 (2 TCCs, 550–1,200pg/ml), TGF-β (2 TCCs, 600–1,150pg/ml), or both (1 TCC). No IL-2 and IFN-γ production was observed. In contrast, 37 TCCs were characterized by high IL-2 (480–3,200pg/ml) and IFN-γ (350–3,300pg/ml) production and absence of IL-10 and TGF-β. Thirty of 42 TCCs isolated produced IL-5 (200–480pg/ml),

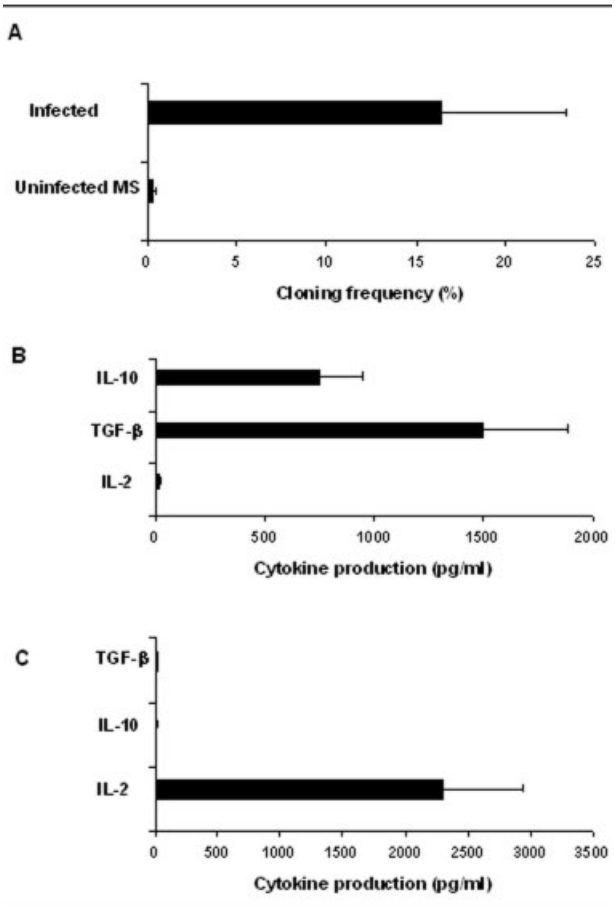

Fig 3. (A) T-cell clones (TCCs) with Th3/Tr1-like phenotype cloned as described in Subjects and Methods. Differences in cloning frequency between infected and uninfected multiple sclerosis (MS) patients were highly significant ($p < 0.0001$). *(B) Production of interleukin (IL)-2, IL-10, and transforming growth factor (TGF)-β by* $MBP_{83\text{-}102}$ *peptide–specific TCCs. A representative TCC producing high amounts of IL-10 and TGF-β, but no IL-2. Each* bar *represents average cytokine secreted from three different wells. (C) A representative TCC predominantly producing IL-2, but not producing IL-10 or TGF-β. Each* bar *represents average cytokine secreted from three different wells. MBP = myelin basic protein.*

but little IL-4 (60–80pg/ml). Collectively, these findings suggest that during parasite infections, cytokines secreted by MBP-specific TCCs play an important role in the clinical events observed during the course of MS.

Previous investigations in rodents have demonstrated that helminth infection leads to the development of $CD4^+CD25^+$ T cells expressing transcription factor forkhead box P3 (FoxP3).[24] Moreover, recent studies have shown that the cloning frequency and effector functions of $CD4^+CD25^+$ T cells are significantly reduced in MS patients compared with healthy control subjects.[23] For this reason, cloning frequency of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells was also analyzed in infected and uninfected MS patients, as well as in healthy control subjects. As shown in Figure 4A, cloning frequency of $CD4^+CD25^-$ cells was similar in uninfected MS patients, parasite-infected MS patients, and healthy control subjects ($p = 0.68$ to 0.15), whereas cloning frequency of $CD4^+CD25^+$ cells in parasite-infected patients was similar to that of healthy control subjects ($p = 0.81$), but significantly greater than in uninfected MS patients ($p < 0.0001$). In addition to being a marker for Treg cells, expression of CD25 (the α chain of the IL-2 receptor) is also an indicator of cell activation. Therefore, it is possible that some isolated $CD4^+$-$CD25^+$ TCCs represent effector cells rather than Treg cells. Expression of FoxP3 in $CD4^+CD25^+$ T cells correlates with their ability to function as Treg cells. Its expression was ascertained using quantitative polymerase chain reaction. As illustrated in Figure 4B, $CD4^+CD25^+$ isolated TCCs expressed significantly greater levels of FoxP3 mRNA compared with $CD4^+CD25^-$ cells.

Regulatory properties of $CD4^+CD25^+$ Treg TCCs isolated from parasite-infected MS patients were further investigated by testing their ability to suppress proliferative responses and IFN-γ secretion by $CD4^+CD25^-$ cells. To do this, we stimulated $CD4^+CD25^-$ T cells with anti-CD3 and anti-CD28 MAbs and added increasing numbers of autologous $CD4^+CD25^+$ cells. As illustrated in Figure 4C, $CD4^+CD25^+$ Treg cells from infected MS subjects were able to suppress the proliferation of indicator $CD4^+CD25^-$ T cells, titrating from high to low ratios of $CD4^+CD25^+$:$CD4^+CD25^-$. The magnitude of this suppressive effect was significantly greater for $CD4^+CD25^+$ Treg cells derived from infected MS patients than those obtained from uninfected MS subjects ($p < 0.0001$–0.003). As reported previously, $CD4^+CD25^+$ T cells failed to proliferate in response to anti-CD3/anti-CD28 stimulation.[25] The addition of IL-2 on day 0 of culture breaks the lack of response of the $CD4^+CD25^+$ population. Furthermore, $CD4^+CD25^+$ Treg cells suppressed the proliferation of $CD4^+CD25^-$ T cells in response to PHA (75 ± 15%, at ratio 10:1) and to plate-bound anti-CD3 stimulation (82 ± 12%, at ratio 10:1), suggesting that the suppressive effect of $CD4^+CD25^+$ Treg cells is independent of APCs. In addition, $CD4^+CD25^+$ Treg cells from infected MS patients were also capable of suppressing the production of IFN-γ by $CD4^+CD25^-$ T cells activated by anti-CD3/anti-CD28 MAbs (see Fig 4D). Again, the inhibitory effect was significantly greater in $CD4^+CD25^+$ T cells derived from infected MS subjects than in those obtained from uninfected MS patients ($p < 0.0001$–0.001).

To establish whether the recovery in function of

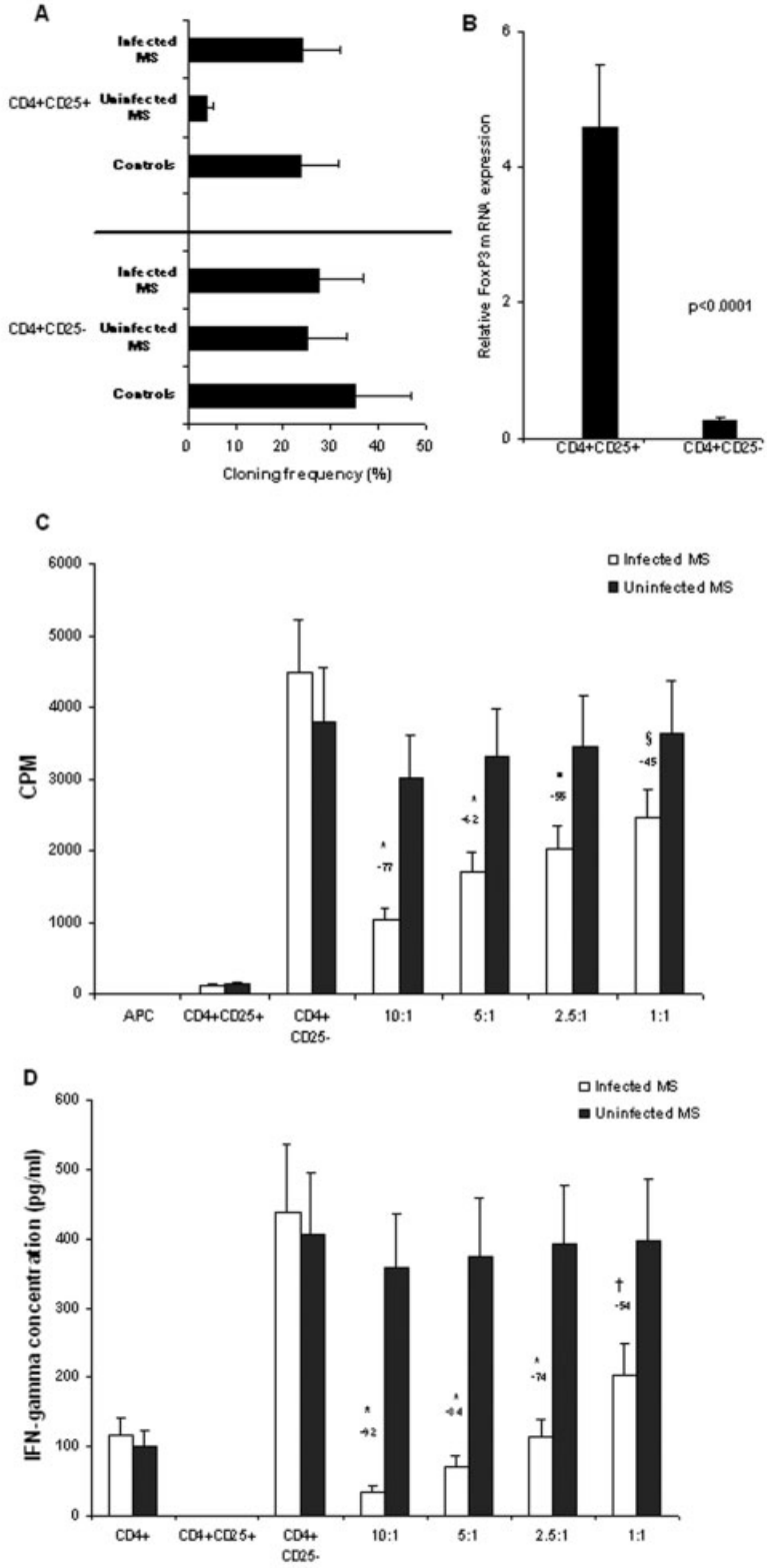

Figure 4.

$CD4^+CD25^+$ T cells induced by parasite infections was cell contact dependent or cytokine mediated, we stimulated $CD4^+CD25^+$ Treg cells from healthy control subjects, uninfected MS patients, and infected MS patients with plate-bound anti-CD3 MAb and anti-CD28 MAb in the presence of exogenous IL-2.

Under these stimulation conditions, no detectable levels of IL-10 or TGF-β could be measured, although both cytokines were produced in all cultures of $CD4^+CD25^-$ cells alone and cocultures stimulated under similar conditions. Furthermore, addition of anti–IL-10 and anti–TGF-β MAbs to cultures did not result in loss of suppressor function by $CD4^+CD25^+$ Treg cells. These findings were observed in the $CD4^+CD25^+$ T cells isolated from all three groups of individuals under study, and they collectively indicate that regulation by $CD4^+CD25^+$ Treg cells is not mediated by these cytokines. Supporting these observations, and as described previously,[26] transwell experiments demonstrated that prevention of cell contact abolished the regulatory function that $CD4^+CD25^+$ Treg cells exert on $CD4^+CD25^-$ T cells.

Differential expression of Smad7 may prove to be crucial for identification of cells with regulatory functions. Favoring this hypothesis, blockade of TGF-β–mediated signaling induced by overexpression of Smad7 in T cells has been shown to enhance antigen-induced inflammation.[27] Interestingly, gut parasite infections have been associated with the secretion of TGF-β, correlating with downregulation of Smad7.[28] Both Smad6 and Smad7 are Smad family members expressing inhibitory capacity, and different studies suggest that T cells that do not express Smad7 may be able to function as regulatory T cells. For this reason, in this initial study, differential Smad7 expression was analyzed in TCCs secreting TGF-β versus TCCs not secreting TGF-β derived from parasite-infected MS patients. Figure 5 shows that whereas Smad7 was highly expressed in cells lacking TGF-β production, Smad7 mRNA was scarcely detected in TGF-β–producing cells.

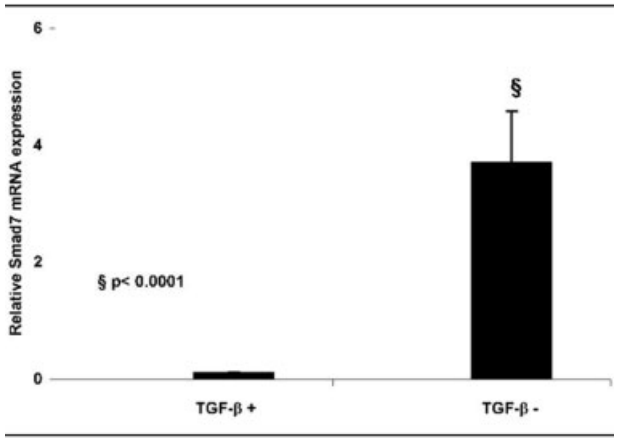

Fig 5. Smad7 expression in transforming growth factor (TGF)-β–producing cells and in cells lacking TGF-β production. Data expressed as Smad7 messenger RNA (mRNA) relative to glyceraldehyde phosphate dehydrogenase (GAPDH), presented as mean ± standard error of mRNA expression in 12 T-cell clones (TCCs) producing TGF-β and in 10 TCCs not producing TGF-β. §p = 0.0001.

Discussion

These results demonstrate that during a follow-up period of 4.6 years, parasite-infected MS patients showed a significantly lower number of relapses, minimal changes in disability scores, and significantly lower MRI activity compared with uninfected MS individuals. Parasite-driven protection was associated with induction of Treg cells secreting the suppressive cytokines IL-10 and TGF-β, as well as $CD4^+CD25^+$ $FoxP3^+$ T cells displaying significantly increased suppressive function. These findings provide evidence to support autoimmune downregulation secondary to parasite infections in MS patients through the action of regulatory cells, whose effects extend beyond the response to the invading agent.

We are aware of certain limitations in the design of this prospective observational study: It is unblinded for judgment of the occurrence of relapses, as well as radiological and immunological assessments, and the number of patients studied is limited.

Fig 4. $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from parasite-infected multiple sclerosis (MS) patients, as well as from uninfected MS patients and healthy control subjects, were cloned as described in Subjects and Methods. (A) Differences in cloning frequency of regulatory $CD4^+CD25^+$ cells between infected and uninfected MS patients were highly significant ($p < 0.0001$), whereas no differences were observed in the ability to generate clones from $CD4^+CD25^-$ cells ($p = 0.68$). (B) $CD4^+CD25^+$ cell expressed greater levels of FoxP3 messenger RNA (mRNA) compared with $CD4^+CD25^-$ cells. Results are expressed as levels of FoxP3 mRNA relative to glyceraldehyde phosphate dehydrogenase (GAPDH), and represent mean values ± standard error of mRNA expression in 26 $CD4^+CD25^+$ and 19 $CD4^+CD25^-$ T-cell clones (TCCs) isolated from parasite-infected MS patients. (C) $CD4^+CD25^+$ TCCs isolated from parasite-infected (white bars) *MS patients mediate suppression of proliferation induced by anti-CD3/anti CD-28 in $CD4^+CD25^-$ target cells. By contrast, $CD4^+CD25^+$ T cells from uninfected* (black bars) *MS patients exhibit significantly less suppressor activity. Percentage inhibition is indicated above each bar. Significant difference was observed between infected and uninfected MS patients. *$p < 0.0001$; ■$p = 0.0008$; §$p = 0.003$. (D) $CD4^+CD25^+$ cells isolated from parasite-infected MS patients are potent suppressors of interferon (IFN)-γ secretion induced by anti-CD3/anti-CD28 in $CD4^+CD25^-$ target cells. As previously described for proliferation assays, $CD4^+CD25^+$ TCCs isolated from uninfected MS patients showed significantly less suppressor activity. Percentage inhibition is indicated above each bar. Significant difference was observed between infected and uninfected MS patients *$p < 0.0001$; †$p = 0.001$.*

Several studies in human and animal models have shown the ability of helminths to alter immune responses. Extracellular parasites similar to those considered in this investigation characteristically induce predominantly Th2 responses, together with downregulation of proinflammatory cytokines, whereas intracellular parasites generally elicit a Th1 response profile.[29] Likewise, eosinophils and IgE products generated as a consequence of helminth infection are also characteristic and contribute to its control. Because of easier control of infection duration and parasite load, results from animal models are more clear than those observed in human studies. Recently, La Flamme and colleagues[14] demonstrated that a preestablished infection with *S. mansoni* significantly reduced incidence and delayed onset of EAE. This altered disease progression was associated with significant induction of IFN-γ and IL-12 responses. However, in contrast with our findings, helminth infection did not alter myelin oligodendrocyte glycoprotein MOG-specific IL-10 levels produced by splenocytes. Moreover, in parasite-infected animals, infiltrating macrophages were absent from inflammatory lesions, suggesting that schistosomiasis may inhibit EAE by altering macrophage activation or effector functions. Interestingly, the authors hypothesized that the maintenance of IL-10, but not of IFN-γ, could indicate a development or expansion of Th3 or Treg, a possibility also suggested by this study's results.

There is clear evidence from animal models and human studies that naturally occurring Tr1 cells inhibit different autoimmune diseases in vivo, probably through their ability to control T-cell proliferation and influence on the cytokine milieu.[30] Thus, in EAE, Tr1 cells are induced in vivo by the administration of soluble protein antigens, which are known to generate T-cell tolerance, reversing ongoing disease.[31] Likewise, adoptively transferred T cells genetically modified to produce high levels of IL-10 or Tr1 cells generated in vitro result in marked and prolonged inhibition of EAE progression and demyelination.[32,33] Interestingly, transferred Tr1 cells are able to induce a host-derived immune Tr1 response, capable of inhibiting autoreactive T-cell IFN-γ production, providing ultimate long-term inhibition of the disease.[32]

There is also good evidence that regulatory Th3 cells can be induced after oral exposure to antigens, and that their function is TGF-β dependent.[34,35] Thus, T cells cloned from the mesenteric lymph nodes of mice tolerized with MBP, secreting IL-4, IL-10, and TGF-β were capable of suppressing ongoing EAE, induced either by MBP or PLP. These regulatory properties were abrogated when mice received anti–TGF-β antibodies, suggesting a critical role for TGF-β in immune response suppression.[35] Moreover, transfer of T cells transfected with TGF-β significantly delayed and ameliorated EAE development,[36] and $CD4^+CD25^+$ Treg cells have been described in human and mice.[26,37,38] Our results, as reported previously, demonstrate that cloning frequency of $CD4^+CD25^+$ T cells was significantly reduced in uninfected MS patients compared with healthy control subjects. Furthermore, Treg cells show altered effector function as demonstrated by their inability to regulate IFN-γ secretion and T-cell proliferation.[23] These changes are not unique to MS. Indeed, $CD4^+CD25^+$ T cells isolated from peripheral blood of rheumatoid arthritis patients are also functionally defective in their ability to suppress proinflammatory cytokine secretion.[39] Interestingly, treatment of these patients with anti–tumor necrosis factor-α restored regulatory T-cell capacity to inhibit cytokine production. In parasite-infected MS patients, regulatory T-cell–mediated suppression and cloning frequency reach levels similar to those observed in healthy subjects.

Evidence for Treg generation during parasite infection is now emerging, offering an explanation for the mechanism by which infected hosts exhibit altered immune responsiveness to bystander antigens.[40] Thus, parasites may lead to increased Treg numbers or activity, either by generating new cells or by activating/expanding existing cells.[19,24,41]

On recognition of microorganisms by innate immune cells such as macrophages and dendritic cells, diverse signaling pathways are activated. One of these recognition mechanisms is mediated by Toll-like receptors, which have recently emerged as key molecules responsible for recognizing specific conserved components of different infectious organisms. For example, recent experiments have shown that lysophosphatidylserine from parasite eggs activated Toll-like receptor 2 at the dendritic cell surface, promoting Tr1 cell development.[42]

Smad7 is a key intracellular antagonist of TGF-β–mediated signaling, preferentially expressed in Treg cells.[43] Indeed, intestinal T cells mediating inflammatory bowel disease strongly express Smad7, and blocking its activity in these cells results in a T-cell population with regulatory function.[27] Coinciding with these observations, our results show that Smad7 expression was not detected in TGF-β–producing cells induced during parasite infections. Likewise, recent studies have demonstrated that in *Toxoplasma gondii* orally infected mice, intraepithelial lymphocytes secrete increased amounts of TGF-β, modulating inflammatory disease activity, an effect linked to Smad7 downregulation.[28] Collectively, these observations provide a basis for further investigations on Smad/TGF-β signaling mechanisms during parasite infections.

Parasites inhabit immune-competent hosts for long periods and can therefore develop modulatory molecules generating strong antiinflammatory responses destined to enhance their survival. Further investigation is warranted to identify which molecules cause immunomodulatory effects that dampen inflammatory reactions normally occurring in autoimmune diseases. Also, induction of a regulatory antiinflammatory network generated by persistent parasite infections may offer a potential explanation for environment-related suppression of MS development in areas with low disease prevalence.

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