Oral Tolerance to Myelin Basic Protein and Natural Recovery from Experimental Autoimmune Encephalomyelitis Are Associated with Downregulation of Inflammatory Cytokines and Differential Upregulation of Transforming Growth Factor β, Interleukin 4, and Prostaglandin E Expression In the Brain

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Summary

Experimental autoimmune encephalomyelitis (EAE) in the Lewis rat is a self-limited inflammatory process localized to the central nervous system that is induced by the injection of myelin basic protein (MBP) in adjuvant. Oral administration of MBP suppresses EAE, and this suppression is mediated by CD8+ T cells that adoptively transfer protection and suppress both in vitro and in vivo by the release of transforming growth factor (TGF) β after antigen-specific triggering. Furthermore, oral tolerance to MBP is enhanced by the concomitant oral administration of lipopolysaccharide (LPS). The present study was undertaken to determine whether the disease course in EAE and its suppression by oral tolerization to MBP is associated with distinct patterns of cytokine expression in the target organ. Detailed immunohistology of the brain was performed at the peak of clinical disease (day 14 after immunization) and after recovery (day 18) in control (ovalbumin [OVA]-fed), MBP-fed, and MBP plus LPS-fed animals. Brains from OVA-fed animals at the peak of disease showed perivascular infiltration with activated mononuclear cells which secreted the inflammatory cytokines interleukins (IL) 1, 2, 6, 8, TNF-α, and interferon γ. The inhibitory cytokines TGF-β and IL-4, and prostaglandin E2 (PGE2) were absent. In MBP orally tolerized animals there was a marked reduction of the perivascular infiltrate and downregulation of all inflammatory cytokines. In addition, there was upregulation of the inhibitory cytokine TGF-β. In MBP plus LPS orally tolerized animals, in addition to upregulation of TGF-β and reduction of inflammatory cytokines, there was enhanced expression of IL-4 and PGE2, presumably secondary to activation of an additional population of immunoregulatory cells. In OVA-fed animals that had recovered (day 18), staining for inflammatory cytokines diminished, and there was the appearance of TGF-β and IL-4. These results suggest that suppression of EAE, either induced by oral tolerization or that which occurs during natural recovery is related to the secretion of inhibitory cytokines or factors that actively suppress the inflammatory process in the target organ.

O ral administration of antigens induces a state of immuno-
logic unresponsiveness, termed oral tolerance (1). We and others have shown that oral administration of autoantigens suppresses experimental autoimmune diseases including

experimental autoimmune encephalomyelitis (EAE)[1] (2–5), uveitis (6), collagen- (7) and adjuvant-induced arthritis (8), and diabetes in the NOD mouse (9). We have also shown

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[1] Abbreviations used in this paper: DTH, delayed type hypersensitivity; EAE,
experimental autoimmune encephalomyelitis; HPF, high power field;
ICAM, intercellular adhesion molecule; MS, multiple sclerosis; PCNA,
proliferating cell nuclear antigen; TF, tissue factor; TM, thrombomodulin.

suppression of allograft rejection by oral administration of splenocytes, and induction of oral tolerance with polymorphic class II MHC peptides in the rat (10).

Oral administration of myelin basic protein (MBP) suppresses EAE and this suppression is mediated by CD8^{+} T cells that adoptively transfer protection and suppress in vitro proliferative responses when stimulated with the tolerizing antigen (2). These MBP-specific CD8^{+} T cells mediate suppression in vitro by the release of TGF-β after triggering by MBP (11). Furthermore, administration of anti-TGF-β in vivo abrogates the protective effects of orally administered MBP (11). Additional studies from our laboratory have shown that administration of LPS orally in conjunction with MBP enhances the protective effect of orally administered MBP on EAE. This enhanced protection was manifested clinically and as measured by suppression of delayed-type hypersensitivity (DTH) responses to MBP. LPS alone or given subcutaneously with oral MBP had no effect (12).

Although we have shown that TGF-β-secreting CD8^{+} T cells are important modulators of EAE after oral tolerization to MBP (11), the effect of oral tolerance on the expression of TGF-β and other cytokines in the brain of animals with EAE is unknown.

Materials and Methods

Induction of Oral Tolerance. Female Lewis rats 6–8-wk-of age were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Preparation of guinea pig MBP, immunization, scoring of clinical disease, and induction of oral tolerance were performed as previously described (12). Rats were fed by gastric intubation using a stainless steel feeding needle (Thomas Scientific, Swedesboro, NJ). The antigens MBP and OVA were dissolved in PBS (Gibco Laboratories, Grand Island, NY) at 1 mg/ml and administered five times before immunization at 2–3-d intervals. LPS (Sigma Chemical Co., St. Louis, MO) from *Escherichia coli* strain 0127:B8 was dissolved in PBS at 1 mg/ml and administered alone as above at a dose of 1 mg per feed, or together with MBP.

Specimen Collection. The onset of clinical EAE was day 10–11 after immunization, and the peak of disease was between days 14 and 15. The rats were killed at day 14, their brains were surgically removed, and snap-frozen in liquid nitrogen-chilled isopentane and stored at −80°C.

Antibodies. Murine mAbs were obtained, unless otherwise specified, from Sera-Lab (Accurate Chemicals & Scientific Corp., Westbury, NY). Additional antibodies were produced in our laboratories or obtained from the investigators listed. This panel included mAbs to all rat leucocytes (CD45, OX-1); pan-T marker (CD5, OX-19); TCR-α/β chains (R73; courtesy of Dr. T. Hunig, Martinstried, Germany); T cell subsets (CD4, BWH4; CD8, OX-8); mononuclear phagocytes (ED-1, ED-2); and neutrophils (RP-3; courtesy of Dr. F. Sendo, Yamagata, Japan). Activation of mononuclear or endothelial cells was assessed using antibodies to class II antigens (OX-3); p-55 chain of the IL-2R (CD25, ART-18; courtesy of Dr. T. Diamantstein, Berlin, Germany); intercellular adhesion molecule-1 (ICAM-1, CD54, 1A29; courtesy of Dr. M. Miyasaka, Tokyo, Japan); proliferating cell nuclear antigen (PCNA; Dako Corp., Carpinteria, CA); tissue factor (A1-3) (13) and thrombomodulin (TM-4; courtesy of Dr. H. Salem, Melbourne, Australia) (14); and by labeling for the cytokines IL-1β (C42, Olympus Corp., Lake Success, NY); IL-2 (1D10) (15); IL-4 (Genzyme Corp.,

Boston, MA); IL-6 (R&D, Minneapolis, MN); IL-8 (ICN Biomedicals, Inc., Costa Mesa, CA); IFN-γ (DB-10; courtesy of Dr. P. van der Meide, Rijswijk, Holland); TNF-α (JD10; from Dr. I. McKenzie, Melbourne, Australia); TGF-β (R&D Systems, Inc., Minneapolis, MN); and prostaglandin-E$_2$ (PGE$_2$; Sigma Chemical Co.). Rat Ig-absorbed goat anti–mouse Ig (Sigma Chemical Co.); rabbit anti–goat Ig, and rabbit peroxidase anti-peroxidase (PAP) (Dako Corp.) were obtained commercially. Details of the use of these antibodies, isotype-matched control mAbs and purified rabbit or goat Igs in immunohistologic studies have recently been described (16).

Immunohistology. Brain samples (cerebrum and cerebellum) were harvested from each rat at day 14 or 18 after disease induction (three samples per group per time point). Tissues were frozen in liquid nitrogen and stored at −80°C in preparation for immunohistologic studies, or fixed in neutral-buffered formalin, embedded in paraffin, and sectioned for light microscopy. Cryostat sections were fixed in paraformaldehyde-lysine-periodate for demonstration of leukocytes and activation antigens, or in acetone for the labeling of cytokines, and stained by a three-layer (for polyclonal antibodies) or four-layer (for mAbs) peroxidase-antiperoxidase method as previously described (16).

Data Quantitation and Statistics. Quantitation of discretely-labeled leukocytes in and surrounding cerebral and cerebellar small vessels was undertaken by counting the number of labeled cells (mean ± SD)/100 nucleated cells in each of five consecutive high power fields (HPF, ×400). This allowed correction for variably sized leukocyte collections and vessel diameters in different sections. Three rats per treatment group were studied for each marker. Statistical significance of differences between experimental groups was determined by the Student's t test; differences of $p < 0.05$ were considered significant. Cytokine and endothelial labeling of serial sections from each of these rats was judged semiquantitatively because of the diffusion of reaction products beyond individual cells (cytokines) or because of extensive, often continuous labeling of endothelial or meningeal cell aggregates. Thus, as described previously (16), results of cytokine and endothelial labeling in 20 consecutive fields were judged as (−) absence of labeling; (±) <10 cells per section or trace labeling; (1+) few small foci; (2+) multiple foci; and (3+) multiple large perivascular collections and diffuse submeningeal staining. Evaluation of the slides was performed blindly.

Results

Cellular Infiltrate and Cytokine Pattern in the Brains of OVA-fed Animals. Lewis rats fed OVA developed clinical EAE at day 10 after immunization. The incidence of the disease was 100% and the average severity at peak of disease (day 14) was 2.8 (range 1–4), consistent with our previously reported observations (12). Oral administration of an irrelevant antigen such as OVA has no effect on EAE and served as a control for animals fed MBP. Immunohistological evaluation of brains harvested from OVA-fed animals at the peak of disease showed dense submeningeal and perivascular infiltrates. As shown in Table 1 and Fig. 1, approximately half of the perivascular mononuclear cells were T cells as demonstrated by positive labeling with antibodies to TCR-α/β (Fig. 1 a) whereas the other half consisted of ED1^{+} macrophages (Fig. 1 c). 20–30% of the infiltrating perivascular mononuclear cells showed evidence of activation with expression of IL-2R (Fig. 1 e), Ia antigens (Fig. 1 g), and PCNA (not shown). Cere-

Table 1. *Perivascular Mononuclear Infiltration and Activation Markers in Brains of EAE vs Orally Tolerized Rats*

	Feeding protocol				
	Naive	OVA	MBP*	MBP + LPS*	LPS
Leukocytes	0.8 ± 0.5	84.8 ± 45.9	8.8 ± 6.3	6.8 ± 4.7	74.5 ± 33.5
TCR-α/β	0.2 ± 0.4	48.2 ± 22.3	6.3 ± 4.1	4.7 ± 2.6	50.6 ± 29.5
CD4$^+$ Cells	0.5 ± 0.4	55.3 ± 28.3	4.6 ± 3.9	3.8 ± 2.9	33.2 ± 18.3
CD8$^+$ Cells	0.1 ± 0.3	15.3 ± 11.6	0.5 ± 0.4	0.6 ± 0.6	12.6 ± 10.1
Macrophages	0.4 ± 0.4	43.3 ± 19.2	4.3 ± 3.9	3.5 ± 2.8	32.8 ± 21.3
IL-2R	0.0	15.3 ± 5.0	0.2 ± 0.2	0.0	12.8 ± 8.4
Ia	0.0	27.8 ± 14.8	2.3 ± 1.5	1.8 ± 1.4	28.5 ± 24.5
ICAM-1	0.0	69.2 ± 38.4	4.8 ± 3.2	5.4 ± 1.9	44.7 ± 23.6

All values represent mean number of labeled cells ± SD/100 nucleated cells in and surrounding cerebral or cerebellar small vessels, 5 HPF/rat brain and three rats per treatment group.
* p <0.001 compared with OVA- or LPS-fed animals.

bral microvessels showed a similar upregulation of Ia expression. Essentially, all mononuclear cells and vascular endothelial cells in OVA-fed animals were ICAM-1$^+$ (Fig. 1 i). Tissue factor procoagulant antigen expression was increased whereas TM expression was essentially absent compared with naive rats (data not shown). In addition, as shown in Table 2 and Fig. 2, staining with antibodies to cytokines revealed strongly positive staining of mononuclear cells for IL-1 (Fig. 2 a), and IL-6 (Table 2). 20–30% of perivascular mononuclear cells were labeled with IL-2 (Fig. 2 e), and a similar proportion was IFN-γ^+ (Fig. 2 g). Approximately half of the perivascular mononuclear cells in OVA-fed animals showed labeling with TNF-α (Fig. 2 c). Most vascular endothelial cells and occasional mononuclear cells were labeled with IL-8 (Fig. 2 i).

There was no detectable staining with anti–TGF-β, IL-4, or PGE (Fig. 3, a, d, and g, respectively). As expected, there was no detectable staining for cytokines or cellular markers in naive animals (negative controls).

Cellular Infiltrate and Cytokine Pattern in MBP-tolerized Animals. As previously reported (12), the clinical expression of EAE was decreased in rats orally tolerized by MBP before immunization, when compared with controls. The incidence of disease was 60% and the average clinical score at the peak of disease was 1.0. Histologically, there was inhibition of inflammatory responses with only small focal leukocytic infiltrates (Fig. 1, b and d). Leukocytes lacked activation markers as evidenced by decreased staining with IL-2R (Fig. 1 f) and PCNA (not shown). The expression of Ia and

Table 2. *Cytokine Expression in the Brain at the Peak of Disease (Day 14)*

	Feeding protocol				
	Naive	OVA	MBP	MBP + LPS	LPS
IL-1	0	2 +	1 +	0	2 +
TNF	0	2 +	0	±	2 +
IL-2	0	2 +	1 +	0	2 +
IFN-γ	0	2 +	0	0	2 +
IL-6	0	2 +	0	0	2 +
IL-8	0	2 +	0	0	2 +
TGF-β	0	0	2 +	2 +	0
IL-4	0	0	±	2 +	0
PGE	0	±	0	2 +	±

Based on examination of 20 HPF/rat and three rats per group and graded semiquantitatively as (0) absence of labeling, (±) trace labeling, (1 +) few small foci, and (2 +) multiple foci.
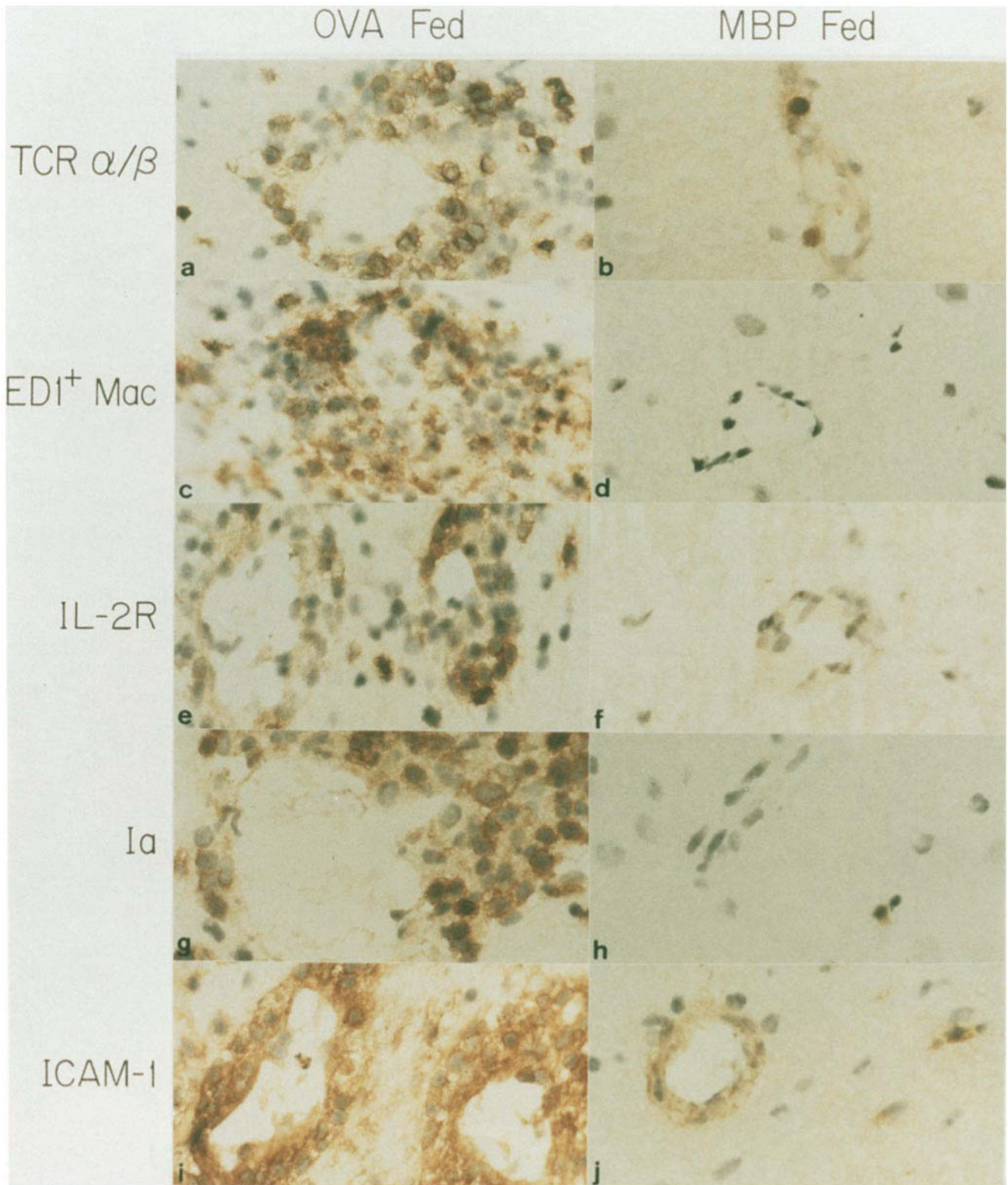
Figure 1. Paired photomicrographs of immunoperoxidase labeling of cryostat sections of cerebri from rats killed on day 14 (peak of disease) after immunization with MBP/CFA. Panels on the left (a, c, e, g, and i) are from control rats fed OVA, and panels on the right (b, d, f, h, and j) are from rats fed MBP (hematoxylin counterstain, ×400): (a and b) TCR-α/β⁺ perivascular mononuclear cells; (c and d) ED1⁺ (macrophages) perivascular mononuclear cells; (e and f) IL-2R⁺ (CD25) perivascular mononuclear cells; (g and h) Ia⁺ mononuclear cells; and (i and j) ICAM-1⁺ (CD54) mononuclear and vascular endothelial cells.
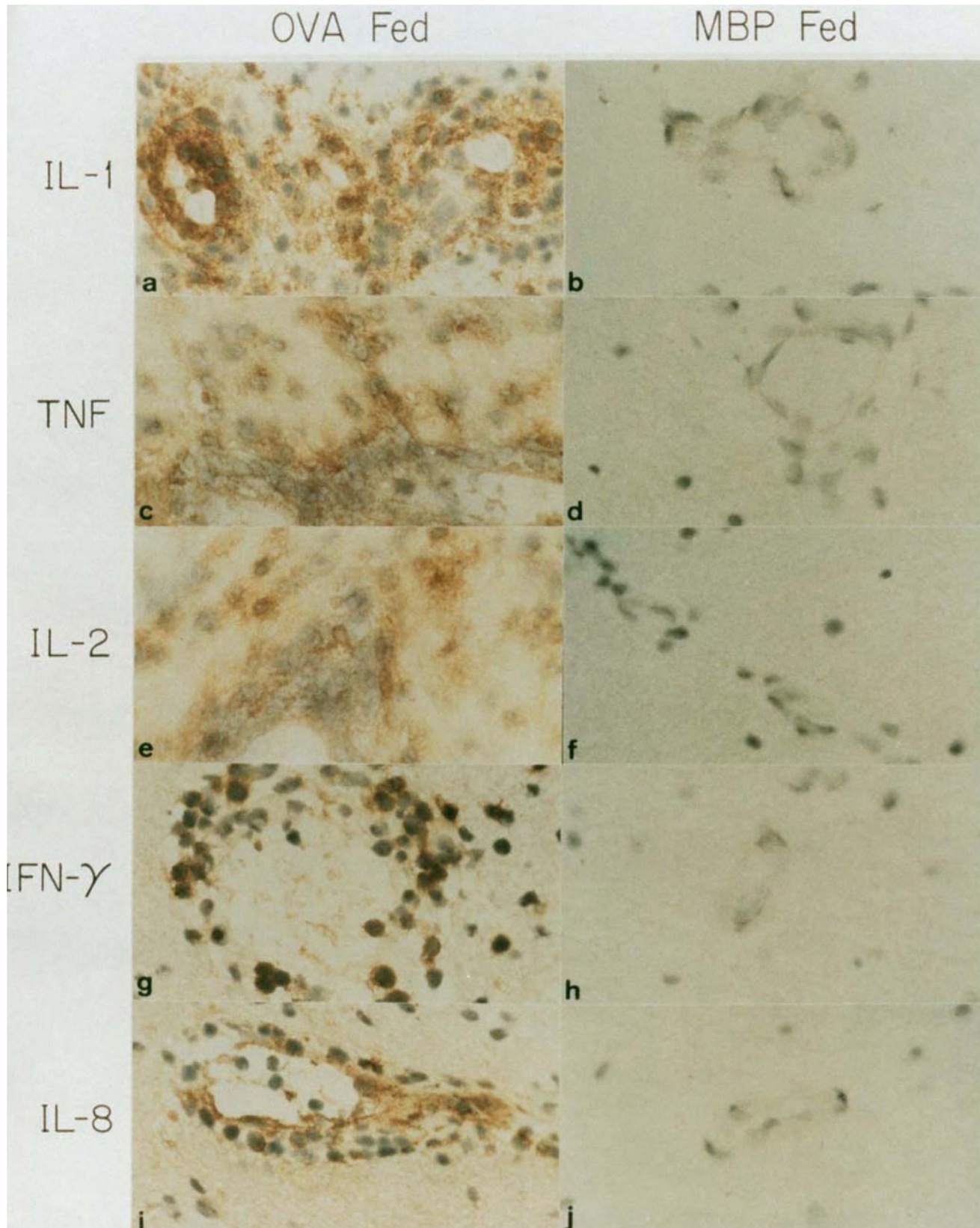
Figure 2. Paired photomicrographs of immunoperoxidase labeling of cryostat sections of cerebri from rats killed on day 14 (peak of disease) after immunization with MBP/CFA. Panels on the left (*a, c, e, g* and *i*) are from control rats fed OVA, and panels on the right (*b, d, f, h,* and *j*) are from rats fed MBP (hematoxylin counterstain, ×400): (*a* and *b*) IL-1β^+ perivascular mononuclear and endothelial cells; (*c* and *d*) TNF-α^+ perivascular mononuclear cells; (*e* and *f*) IL-2$^+$ perivascular mononuclear cells; (*g* and *h*) IFN-γ^+ perivascular mononuclear cells; and (*i* and *j*) IL-8$^+$ submeningeal vascular endothelial and mononuclear cells.
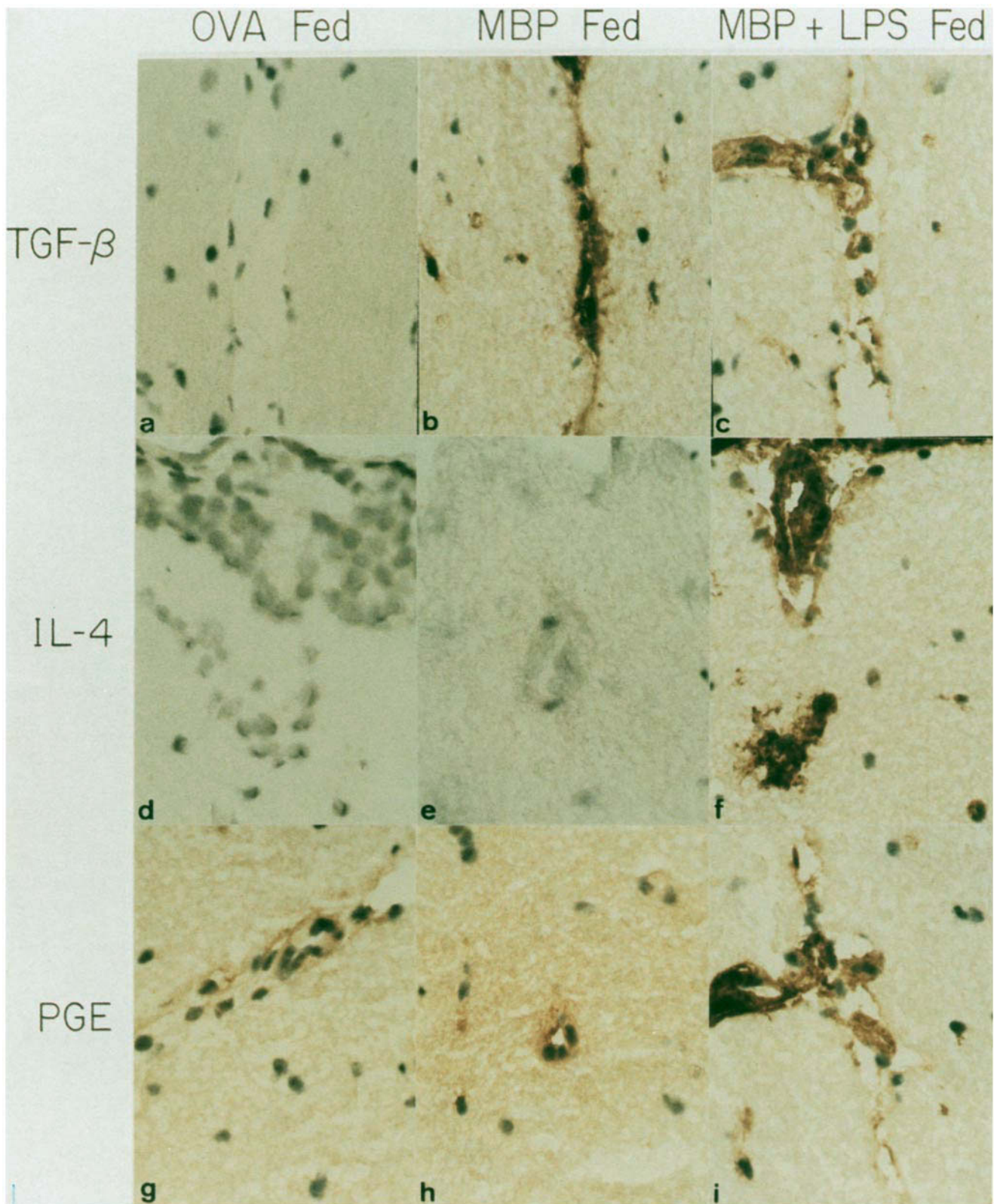
Figure 3. Paired photomicrographs of immunoperoxidase labeling of cryostat sections of cerebri from rats killed on day 14 (peak of disease) after immunization with MBP/CFA. Panels on the left (*a, d,* and *g*) are from control rats fed OVA, middle panels (*b, e,* and *h*) are from rats fed MBP, and panels on the right (*c, f,* and *i*) are from rats fed MBP plus LPS (hematoxylin counterstain, ×400): (*a, b,* and *c*) TGF-β^+ submeningeal vascular endothelial and mononuclear cells; (*d, e,* and *f*) IL-4$^+$ mononuclear and vascular endothelial cells; and (*g, h,* and *i*) PGE$_2^+$ endothelial and mononuclear cells.

ICAM-1 by microvessels was limited (Fig. 1, *h* and *j*). In addition, staining with antibodies to cytokines revealed absent to weak staining with IL-1 (Fig. 2 *b*), IL-2 (Fig. 2 *f*), IL-6, IL-8 (Fig. 2*j*), IFN-γ (Fig. 2 *h*), and TNF-α (Fig. 2 *d*). However, in contrast to OVA-fed animals, there was positive staining with anti-TGF-β in MBP tolerized animals (Fig. 3 *b*).

Cellular Infiltrate and Cytokine Pattern in MBP Plus LPS Tolerized Animals. Feeding LPS alone did not affect EAE either clinically or immunohistologically (Table 1). The cytokine pattern of staining for IL-1, -2, -6, and -8, TNF-α, and IFN-γ was identical in LPS-fed animals to that seen in OVA-fed animals. However, in animals fed LPS orally in conjunction with MBP, there was added protection clinically (incidence 20%, average score at the peak of disease 0.2), which was consistent with our previously reported observations (12). Immunohistologic examination of brain sections at day 14 showed that the decrease in inflammatory response was identical to that seen in MBP-fed rats (Table 1). The cytokine staining pattern showed decreased labeling for IL-1, -2, -6, and -8, TNF-α, and IFN-γ, but increased staining for TGF-β, IL-4, and PGE$_2$ (Fig. 3, *c*, *f*, and *i*, respectively). Thus, in comparison with animals fed MBP alone, animals fed with MBP plus LPS showed an increased expression of IL-4 and PGE$_2$ in the brain in addition to the increase in TGF-β seen with MBP feeding. There was normal TM expression and no upregulation of tissue factor expression in these rats.

Cytokine Pattern in Brains of OVA, MBP, and MBP plus LPS-fed Animals after Recovery (Day 18). In OVA-fed animals, examination of brain sections taken on day 18 after immunization (corresponding with clinical recovery), showed persistent submeningeal and perivascular infiltrates. Staining for IL-2 and IFN-γ was markedly diminished, but staining for TNF-α was still present. However, in contrast to day 14, the leukocytic infiltrates and associated vessels were now heavily stained for TGF-β and IL-4. In MBP-fed animals on day 18, inflammatory cells around small vessels were now stained for IL-4 in addition to the TGF-β staining noted on day 14. In MBP plus LPS-fed animals, there was persistent staining for IL-4, TGF-β, and PGE. These results are summarized in Table 3.

Discussion

Perivascular infiltration of mononuclear cells into the central nervous system is a pathologic hallmark for EAE and multiple sclerosis (17). Consistent with previous reports (18), our data show perivascular infiltrates consisting primarily of CD4$^+$ T cells and macrophages and increased Ia expression on cerebral endothelial cells in the brains of animals with EAE. It is unclear whether increased Ia expression on vascular endothelial cells plays a role in the disease pathogenesis, or whether it is a byproduct of exposure to activated T cells.

We also found evidence of activation in the perivascular infiltrates as seen by increased expression of IL-2R and PCNA. Furthermore, the infiltrates stained positively for IL-1 and TNF-α, which are produced by activated macrophages and synergize with IFN-γ in upregulation of class I, class II, and ICAM-1 molecules and have potent activating effects on endothelial cells (19). The increased expression of IL-1, -2, TNF, and IFN-γ at the height of clinical disease is compatible with findings in murine chronic relapsing EAE (CREAE) (20) and in multiple sclerosis (MS) brains (21–23). IL-1 and TNF downregulate TM expression on endothelial cells and stimulate endothelial cells in vitro to secrete tissue factor (TF), causing a conversion of the endothelial cell surface from an anticoagulant to a procoagulant state leading to local fibrin deposition (24, 25). Our findings of decreased TM and increased TF expression on the surface of endothelial cells in the brains of OVA-fed animals at the height of disease, provide a demonstration of such cytokine interactions in vivo and support the findings previously described in vitro.

Although various cells in the central nervous system are capable of cytokine production (26), we found the expression of these cytokines to be localized around the inflammatory infiltrates. This anatomic location of cytokine expression correlates with their function in cell recruitment,

Table 3. *Cytokine Expression in the Brain after Recovery (Day 18)*

	Feeding protocol			
	Naive	OVA	MBP	MBP + LPS
IL-2	0	±	0	0
TNF-α	0	1+	0	0
IFN-γ	0	±	0	0
TGF-β	0	2+	2+	2+
IL-4	0	2+	2+	2+
PGE$_2$	0	±	±	2+

Based on examination of 20 HPF/rat and three rats per group and graded semiquantitatively as (0) absence of labeling, (±) trace labeling, (1+) few small foci, and (2+) multiple foci.

activation, and differentiation, and supports their role in the pathogenesis of the disease. Recent reports on the importance of adhesion molecules on endothelial cells for lymphocyte migration and homing (27, 28) suggest that vascular endothelial cells play an active role in disease pathogenesis, which is consistent with our finding of increased ICAM expression in the OVA-fed group and decreased expression in the MBP-fed and MBP plus LPS fed groups.

We also found increased expression of IL-6 and IL-8 in OVA-fed animals with EAE. IL-6 is produced by a variety of cells and is a potent inducer of B cell differentiation (29). Increased production of IL-6 has been reported in several autoimmune diseases (30, 31) and has been described in EAE (32). The increased production of IL-6 in EAE could explain the finding of raised Ig levels in the CSF of EAE animals and by analogy in MS patients. The present report is the first demonstration of increased IL-8 expression in animals with EAE. IL-8 stimulates neutrophil chemotaxis (33) and can be secreted by activated T cells, monocytes (34), and vascular endothelial cells (35). The expression of IL-8 correlates well with the presence of neutrophils in inflammatory infiltrates.

At the time of clinical recovery, OVA-fed animals showed expression of TGF-β and IL-4 around the blood vessels and mononuclear infiltrates. Biologically active TGF-β is secreted by antigen-activated T cells and by mononuclear phagocytes, and is generally a negative regulator of immune responses (36). We have previously found that the injection of anti–TGF-β serum into Lewis rats immunized with MBP/CFA resulted in an increased severity and duration of disease, which suggests that TGF-β plays a role in natural recovery from EAE (11). Furthermore, Karpus and Swanborg (37) have isolated CD4$^+$ postrecovery cells from Lewis rats with EAE that suppress EAE via adoptive transfer and that suppress in vitro via the secretion of TGF-β. The present results are the first demonstration that TGF-β is expressed de novo in the brains of recovering animals and suggest that these CD4$^+$ postrecovery cells may be acting at the target organ. The relationship of these TGF-β secreting CD4$^+$ postrecovery cells to TGF-β secreting CD8$^+$ T cells generated after oral tolerance remains unknown at this time. Of note is that the administration of exogenous TGF-β suppresses EAE and other experimental autoimmune diseases (38–40).

In addition to TGF-β, IL-4 also appeared in the inflammatory infiltrates of recovering OVA-fed EAE animals. The intracerebral cytokine staining patterns observed in this study are consistent with the reported immunoregulatory effects of IL-4 in vitro (41). The expression of IL-4 may indicate the presence of IL-10–producing cells. We were unable to stain for IL-10 in the present study because of the current lack of antibodies to rat IL-10. Of note, is that both IL-4 and IL-10 are known to downregulate Th1 functions in mice (42, 43), and, based upon recent reports, it is likely that Th1 and Th2 type cells exist in the rat (44, 45). Thus, recovery from EAE may be associated both with the activation of TGF-β–secreting cells and of IL-4–secreting Th2 type cells that regulate Th-1 type cells and inhibit their secretion of proinflammatory cytokines. Whether IL-4 alone mediates the immunosuppressive effect, or whether it is a marker for Th2 cells that act through the elaboration of other immunosuppressive cytokines such as IL-10 (42, 43), is unknown.

In MBP orally tolerized animals, we found marked inhibition of the inflammatory infiltrate, downregulation of cytokines associated with immune activation, and upregulation of TGF-β. Thus, IL-1, -2, -6, and -8, TNF-α, and IFN-γ expression was diminished, whereas there was prominent staining for TGF-β. The presence of TGF-β in the brains of MBP-fed but not OVA-fed animals at the height of disease corroborates our previous findings that oral tolerance to MBP in the Lewis rat is actively mediated (2). Of particular note is that IL-4 was not detected in MBP orally tolerized animals on day 14, which suggested that suppression of EAE induced by MBP feeding was not merely due to the earlier activation of natural recovery mechanisms.

In MBP plus LPS orally tolerized animals, there was an increased expression of IL-4 and PGE$_2$ in the brain on day 14, in addition to the increase in TGF-β expression seen in MBP-fed animals. During the recovery phase, MBP plus LPS–fed animals continued to show expression of PGE$_2$. A regulatory role for PGs in EAE has been postulated, since animals treated with indomethacin, an inhibitor of PG synthesis, developed a more severe form of EAE (46) and PGE$_2$ inhibits MBP-stimulated proliferation of MBP-sensitized lymphocytes in vitro (47). In the present study, we found PGE$_2$ to be present in the infiltrates of MBP plus LPS–fed animals but not in the other groups, even during recovery, which suggests that it does not play a role in natural recovery from EAE. Local adjuvant effects of LPS in gut-associated lymphoid tissue may result in the activation of PGE$_2$ producing regulatory cells since PGE$_2$ was found in the brains of MBP plus LPS fed but not in MBP-fed animals, and since the synergistic effect of LPS on oral tolerance is seen with oral but not with subcutaneous administration of LPS (12).

The results we have obtained further implicate a role for actively mediated suppression as opposed to clonal anergy (48) after oral tolerance to MBP in the Lewis rat EAE model and identify locally secreted TGF-β as a mediator of our previously described bystander suppression associated with oral tolerance (49). The differential expression of TGF-β, IL-4, and PGE$_2$ appear to represent different classes of immunoregulatory cells that participate in suppression. This is true both for natural recovery from EAE and for orally induced tolerance. The finding of IL-4 in the target organ during natural recovery and in MBP plus LPS orally tolerized animals implicates a Th-2 type response as participating in suppressing inflammation. Characterization of the cells that secrete TGF-β, IL-4, and PGE$_2$ and the mechanisms by which they are triggered is now required. Our results suggest that active suppression by inhibitory cytokines may represent an important mechanism of tolerance maintenance in the host and have implications for the treatment of autoimmune diseases in humans by oral tolerance to autoantigens (50).

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