

# Experimental Granulomatous Colitis in Mice Is Abrogated by Induction of TGF- $\beta$ -mediated Oral Tolerance

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## Summary

In previous studies we showed that a chronic colitis associated with a Th1 T cell response can be induced by the rectal administration of the haptenizing reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS). We report here that oral administration of haptenized colonic proteins (HCP) before rectal administration of TNBS effectively suppresses the ability of the latter to induce colitis. This suppression (oral tolerance) appears to be due to the generation of mucosal T cells producing TGF- $\beta$  and Th2-type cytokines after oral HCP administration. Peyer's patch and lamina propria CD4+ T cells from HCP-fed animals stimulated with anti-CD3/anti-CD28 had a 5–10-fold increase in their production of TGF- $\beta$  and secreted increased amounts of IL-4 and IL-10 but lower levels of IFN- $\gamma$  in comparison to T cells from ovalbumin-fed control animals. In addition, the colons of HCP-fed mice showed strikingly increased TGF- $\beta$  but decreased IL-12 expression by immunohistochemical studies and isolated mononuclear cells from HCP-fed animals secreted less IL-12 heterodimer. Finally, and most importantly, the suppressive effect of orally administered HCP was abrogated by the concomitant systemic administration of anti-TGF- $\beta$  or rIL-12 suggesting a reciprocal relationship between IL-12 and TGF- $\beta$  on tolerance induction in TNBS-induced colitis. In parallel studies we demonstrated that TNBS-induced colitis can be transferred to naive recipient animals with purified CD4+ T cells from the colon of TNBS-treated animals and that such animals develop lethal pancolitis when exposed to very low doses of TNBS. Feeding of HCP suppressed this sensitivity to TNBS, indicating that oral feeding can suppress the response of pre-committed T cells in vivo. These studies suggest for the first time that TGF- $\beta$  production can abrogate experimental granulomatous colitis even after such colitis is established, and thus, that regulation of TGF- $\beta$  levels may have relevance to the treatment of human inflammatory bowel disease.

Oral administration of autoantigens and the consequent induction of oral tolerance have been shown to suppress experimental autoimmune diseases in animal models, such as in experimental autoimmune encephalomyelitis (1, 2), collagen- (3) and adjuvant-induced arthritis (4), diabetes in the non-obese (NOD)<sup>1</sup> mouse (5) and autoimmune uveitis (6, 7). Recent studies have suggested that the dose of fed antigen is an important factor in determining the mechanism of such "oral tolerance". Whereas high doses of antigen have been suggested to favor clonal deletion or clonal anergy of Th1 T cells, low doses of antigen may induce ac-

tive suppression of Th1 T cells resulting in the generation of T cells that secrete TGF- $\beta$ , IL-4, and IL-10 (2, 8, 9). Regardless of the mechanism involved, it is possible that induction of oral tolerance can be used to treat autoimmune disease in humans, and clinical trials exploring this approach for the treatment of multiple sclerosis, rheumatoid arthritis, and uveoretinitis are under way.

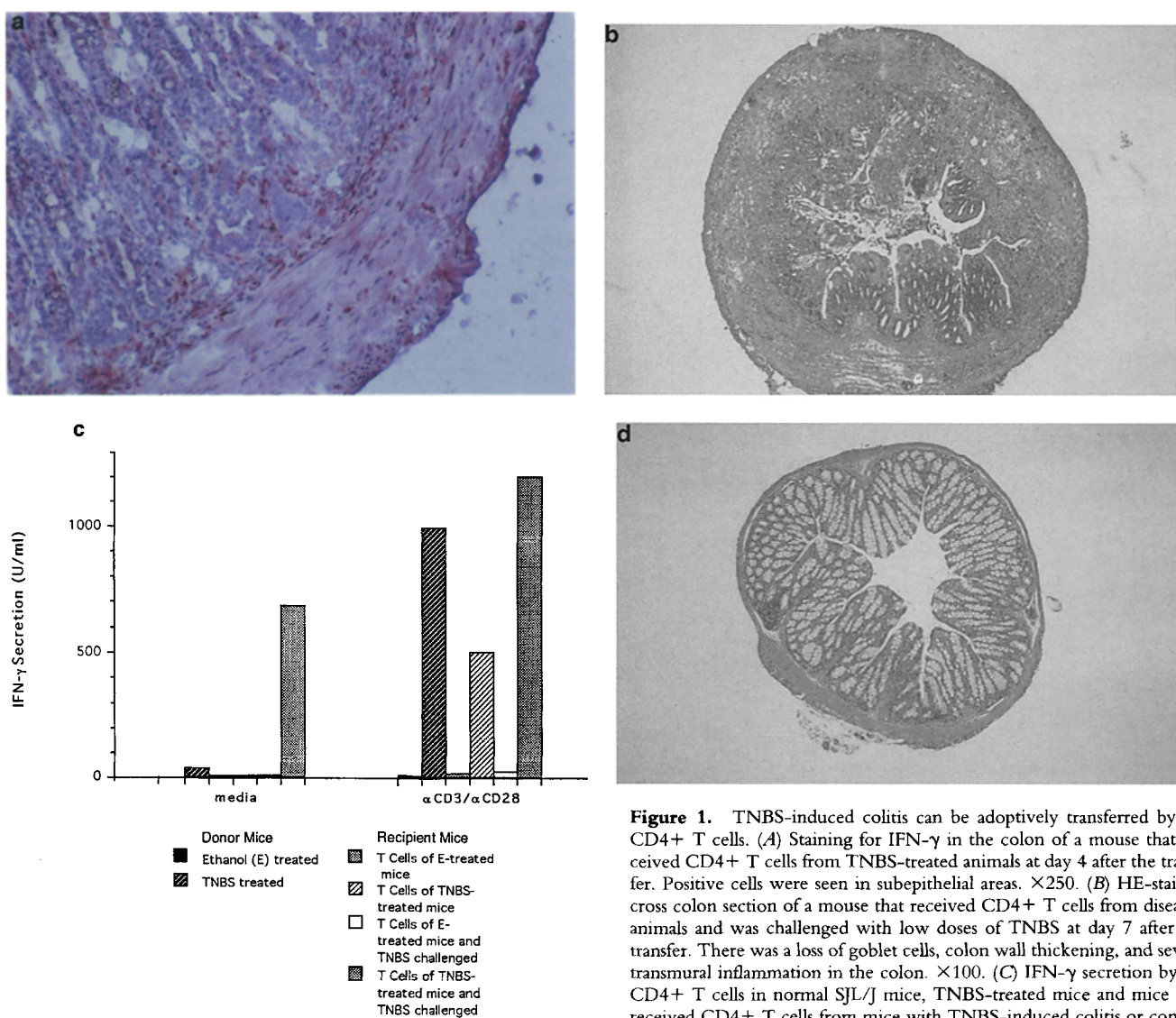
Inflammatory bowel disease (IBD) encompasses Crohn's disease (CD) and ulcerative colitis, the major chronic inflammatory diseases of the gastrointestinal tract in humans (10, 11). Recently, various animal models of chronic intestinal inflammation have been established which will likely provide new insights into the pathogenesis of IBD. These include rats carrying transgenes for HLA-B27 and  $\beta$ 2-microglobulin (12), T cell reconstituted Tg $\epsilon$ 26 mice transgenic for the human CD3 $\epsilon$  gene (13), and mice in which the genes for IL-2 (14), IL-10 (15), G $\alpha$ <sub>12</sub> (16), and the alpha

<sup>1</sup>Abbreviations used in this paper: CD, Crohn's disease; CMFDA, 5-chloromethylfluorescein diacetate; DC, dendritic cells; HPC, haptenized colonic protein; HPF, high power field; IBD, inflammatory bowel disease; LP, Lamina propria; NOD, non-obese; PP, Peyer's patch; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

or beta chain of the T cell receptor (17) have been inactivated by homologous recombination. In addition, a Th1-mediated granulomatous colitis model has been established by the adoptive transfer of normal CD45RB<sup>hi</sup> T cells from BALB/c mice to C.B.-17 scid mice (18). Importantly, the CD45RB<sup>lo</sup> CD4<sup>+</sup> T cell population did not cause disease and, if injected together with the CD45RB<sup>hi</sup> population, prevented disease induction (18); in addition, this prevention could be reversed by adding antibodies to TGF- $\beta$ , suggesting a key negative role for this cytokine in disease induction. TGF- $\beta$  is a pluripotent cytokine that functions as potent inhibitor of T and B cell proliferation and cytokine production by macrophages (19). The importance of this inhibitory function is underscored by the finding that

TGF- $\beta$ <sub>1</sub> null mice show multiorgan inflammation, excessive lymphocytic infiltrations, and early death (20, 21).

We have previously described a TH1 model of chronic intestinal inflammation in mice induced by rectal administration of the hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS)(22). In the present study, we demonstrate that TNBS-induced colitis can be adoptively transferred to normal mice by *in vivo* primed lamina propria CD4<sup>+</sup> T cells and that TNBS-induced colitis can be suppressed by TGF- $\beta$ -mediated oral tolerance in mice fed haptenized colonic proteins even after transfer of precommitted T cells. These results suggest that oral tolerance and the regulation of TGF- $\beta$  levels may have relevance to the treatment of human inflammatory bowel disease.



**Figure 1.** TNBS-induced colitis can be adoptively transferred by LP CD4<sup>+</sup> T cells. (A) Staining for IFN- $\gamma$  in the colon of a mouse that received CD4<sup>+</sup> T cells from TNBS-treated animals at day 4 after the transfer. Positive cells were seen in subepithelial areas.  $\times 250$ . (B) HE-stained cross colon section of a mouse that received CD4<sup>+</sup> T cells from diseased animals and was challenged with low doses of TNBS at day 7 after the transfer. There was a loss of goblet cells, colon wall thickening, and severe transmural inflammation in the colon.  $\times 100$ . (C) IFN- $\gamma$  secretion by LP CD4<sup>+</sup> T cells in normal SJL/J mice, TNBS-treated mice and mice that received CD4<sup>+</sup> T cells from mice with TNBS-induced colitis or control ethanol-treated animals 7 d after the cell transfer. Some mice that received

the CD4<sup>+</sup> T cells were challenged 6 d after the transfer with 0.02 mg TNBS and LP CD4<sup>+</sup> cells were isolated the following day. Supernatants of cultured LP CD4<sup>+</sup> T cells were removed after 2 d and analyzed for IFN- $\gamma$  concentration by specific ELISA. A second independent experiment gave similar results. (D) HE-stained cross colon section of a mouse that received CD4<sup>+</sup> T cells from control ethanol-treated animals and was challenged with low doses of TNBS at day 7 after the transfer. There was neither colon wall thickening nor severe transmural inflammation.  $\times 100$ .

## Material and Methods

**Induction of Colitis.** Specific pathogen-free 2–4-mo-old female SJL/J mice were obtained from the National Cancer Institute (NCI, Bethesda, MD) and maintained as described (22). TNBS-colitis was induced as previously described (22).

**Cell Isolation and Purification of Lamina Propria CD4+ T Cells.** Lamina propria (LP) lymphocytes were isolated from freshly obtained colonic specimens using the technique of Van der Heijden and Stok (23) modified as previously described (22). The resultant cells were further enriched by negative selection using mouse CD4+ T cell isolation columns (Isocell; Pierce Co., Rockford, IL) (22). The resultant cells when analyzed by flow cytometry (FACScan<sup>®</sup>; Becton Dickinson, Sunnyvale, CA) contained greater than 85% CD4+ T cells.

**Adoptive Transfer of LP CD4+ T Cells.** LP cells were isolated from ethanol-treated mice and mice with TNBS-induced colitis at day 10 as described above and stained with FITC-labeled rat anti-mouse CD4+ antibodies (PharMingen, San Diego, CA). CD4+ positive cells were then sorted using a FACSTAR Plus<sup>®</sup> (Becton Dickinson) and were >98% pure on reanalysis. CD4+ T cells ( $1.5 \times 10^5$ ) were then injected intravenously into the tail vein of normal SJL/J mice.

**Labeling of CD4+ T Cells.** In some experiments, CD4+ T cells were labeled before transfer by incubating in 15  $\mu$ M 5-chloromethylfluorescein diacetate (CMFDA) (Molecular Probes, Eugene, OR) for 30 min at 37°C and in fresh RPMI for another 30 min, followed by two washes in PBS. Finally, cells were intravenously injected in normal SJL/J mice as described above.

**Immunofluorescence.** To obtain cryosections, tissue samples were taken at indicated time points and put into OCT-compound on dry ice; sections were cut in a dark room according to standard procedures. CMFDA+ cells were visualized on a fluorescence microscope at an excitation wavelength of 490 nm.

**Challenge of Mice That Received CD4+ T Cells with TNBS.** In some experiments, mice that received CD4+ T cells were challenged 6 d after transfer by rectal administration of 0.02 mg TNBS.

**Grading of Histologic Changes.** Tissues were removed at indicated time points and processed as described (22). The degree of inflammation on microscopic cross sections of the colon was graded semiquantitatively from 0 to 5, as previously described (22).

**Generation of Haptenized Colonic Proteins.** The colons of normal SJL/J mice were removed, cut into small strips and mechanically homogenized. After filtration straining through a 40- $\mu$ m nylon cell strainer, remaining cells were spun down and removed. Colonic protein (1 mg) was then incubated in 0.1% TNBS solution for 4 h at room temperature. The resulting solution was dialyzed against 1 $\times$  PBS overnight at 4°C. Haptenization density of proteins was determined by measuring the photospectrometric absorption at 366 nm using the TNBS-molarity index ( $\epsilon_{366} = 1.1 \times 10^4$ ).

**Feeding of Haptenized Colonic Proteins or Control Ovalbumin.** SJL/J mice were fed with 100  $\mu$ g haptenized colonic protein (HCP), TNP-KLH, or control ovalbumin (OVA) in PBS every other day over a period of 10 d using a feeding needle. 0.5 mg TNBS was then administered intrarectally after an additional 7 d.

**Isolation of Peyer's Patch T Cells.** Peyer's patch (PP) cells were dissected free of the adjacent tissue and put on ice in sterile HBSS (Biosource, Rockville, MD) supplemented with 75 mM Hepes, pH 7.2, penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and amphotericin B (0.5  $\mu$ g/ml). PP were rinsed twice in 0.05 mM DTT in Iscove's modified Dulbecco's media (IMDM) and supplemented with 10% heat inactivated FCS, penicillin/streptomycin, 2-ME (50  $\mu$ M; Sigma Chemical), L-glutamine (2 mM), and

amphotericin B, and twice in supplemented IMDM alone. PP were then digested with collagenase and DNase I as described above. Lymphocyte-enriched populations were isolated from the cells at the 40–100% interface. Further enrichment was obtained by negative selection using mouse T cell isolation columns as described above. The resultant cells, when analyzed by flow cytometry (FACScan<sup>®</sup>; Becton Dickinson), contained greater than 85% CD3+ cells.

**Isolation of Spleen Dendritic Cells.** Spleen dendritic cells (DC) were isolated from aseptically removed spleens by digestion with collagenase and DNase I as described for PP T cells above; the resulting suspension was plated on 35-cm plastic tissue culture dishes (Falcon Labware, Oxnard, CA) for 60 min at 37°C. Non-adherent cells were washed free with prewarmed PBS, and adherent cells were cultured for 18 h with supplemented RPMI. DC-enriched cells were then washed free with two rinses of warmed PBS. As assessed by FACS<sup>®</sup>-analysis, the resulting cells were >80% N418 positive. To analyze immune responses to HCP,  $2 \times 10^6$  DC-enriched cells were incubated with HCP (1 mg/ml) overnight at 37°C, after which the DC were washed twice in RPMI and then cultured with  $10^5$  LP CD4+ T cells in 1 ml complete medium. Supernatants were removed at indicated time points and analyzed for cytokine concentrations by specific ELISA.

**Cell Culture of T Cells.** Cell cultures of T cells were performed in complete medium consisting of RPMI-1640 supplemented with 3 mM L-glutamine, 10 mM Hepes buffer, 10  $\mu$ g/ml gentamycin, 100 U/ml each of penicillin and streptomycin, 0.05 mM 2ME, and 10% heat-inactivated FCS. For TGF- $\beta$ , cell cultures of enriched T cells were performed in serum free medium (GIBCO BRL, Gaithersburg, MD) supplemented as above.

**Reagents and Monoclonal Antibodies.** Unconjugated and biotinylated monoclonal rat anti-mouse IL-4 (BVD4-1D11/BVD6-24G2), IL-10 (JES5-2A5/SXC-1), and IFN- $\gamma$  (R4-6A2/XMG1.2)

**Table 1.** Histologic Grading of Colon Sections of HCP- and OVA-fed Mice

	No	Colitis*			
		Mild		Severe	
	0	1	2	3	4
Treatment regimen					
Untreated	10	0	0	0	0
Ethanol	8	2	0	0	0
TNBS	0	0	3	5	2
HCP-fed + TNBS	7	2	1	0	0
OVA-fed + TNBS	0	0	4	5	1
TNP-KLH- fed + TNBS	0	2	5	3	0

Histologic grading of colon sections from mice that were fed control OVA, TNP-KLH or HCP before induction of colitis by TNBS. Colon specimens from 10 mice in each group were taken 7 d after administration of TNBS and histological grading was performed as described (22; see Materials and Methods). Data were pooled from three independent experiments.

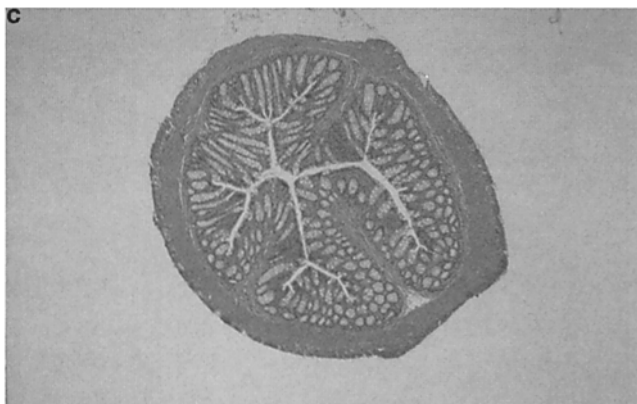
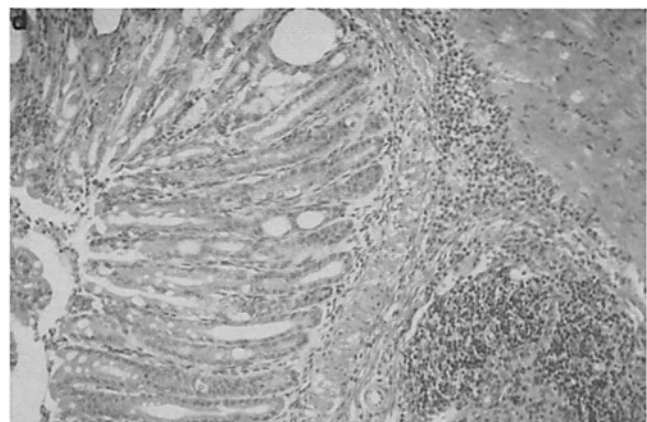
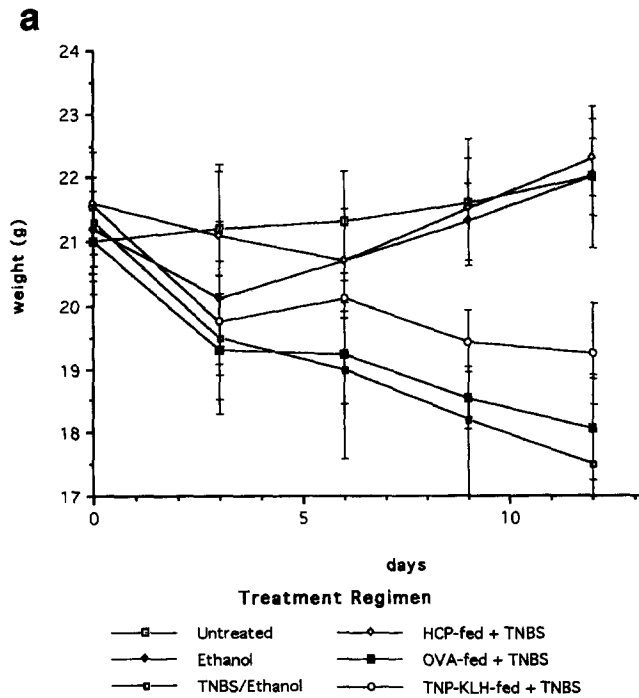
\*See Materials and Methods for grading criteria.

antibodies and recombinant mouse IL-4 ( $1 \times 10^7$  units/mg by CTLL-2.4 assay), IL-10 ( $5 \times 10^5$  units/mg), and IFN- $\gamma$  ( $1 \times 10^7$  units/mg) were purchased from PharMingen and Genzyme Corp. (Cambridge, MA), respectively. For TGF- $\beta$  immunohistologic staining, purified chicken anti-mouse TGF- $\beta$  (R&D Systems, Minneapolis, MN) and biotin-labeled rabbit anti-chicken IgG (Zymed, San Francisco, CA) were used. Purified hamster anti-mouse CD3 $\epsilon$  (clone 145-2C11) and hamster anti-mouse CD28 (clone 37.51) antibodies were obtained from PharMingen. N418 antibodies were kindly provided by Dr. R. Steinman (24).

**Cytokine Assays.** To measure cytokine production, 24-well plates (Costar, Cambridge, MA) were coated with 10  $\mu$ g/ml murine anti-CD3 $\epsilon$  antibody in carbonate buffer (pH 9.6) overnight at 4°C.  $10^5$  LP CD4+ T cells were then cultured in 1 ml of complete medium in precoated or uncoated wells and 1  $\mu$ g/ml soluble anti-CD28 antibody was added to the anti-CD3 $\epsilon$ -coated wells. Cultures were incubated at 37°C in a humidified incubator containing 6% CO $_2$ . After 48 h (after 60 h for TGF- $\beta$ ), culture supernatants were removed and assayed for cytokine concentration (IFN- $\gamma$ , IL-4, and IL-10) by specific ELISA (PharMingen). TGF- $\beta$

concentrations were determined using the Predicta TGF- $\beta$  ELISA Assay (Genzyme Corp.). Optical densities were measured on a ELISA reader (MR 5000; Dynatech Labs., Chantilly, VA) at a wavelength of 490 nm.

**Heterodimer-specific Elispot Assay for Interleukin-12.** Macrophage-enriched cells from the lamina propria were obtained from lamina propria mononuclear cell population using an adherence technique as described for isolation of spleen dendritic cells. In these studies however, macrophage adherent cell population were isolated.  $2 \times 10^5$  macrophage-enriched cells (70% F4/80-positive cells and 25% N418-positive cells) were incubated for 1.5 d in 96-well plates, which had been coated with 5  $\mu$ g/ml rat anti-mouse IL-12 p75 monoclonal antibody (9A5; Hoffmann-LaRoche, Nutley, NJ), in the presence of 30  $\mu$ g/ml Escherichia coli LPS (B4:0111; Sigma Chemical Co., St. Louis, MO). Next, plates were incubated in blocking buffer (3% BSA/1 $\times$  PBS/0.01% Thiomer-sal) overnight at 4°C, washed and 3  $\mu$ g/ml biotinylated rat anti-mouse IL-12 p40 monoclonal antibody (kindly donated by G. Trinchieri) was added and incubation was performed overnight at 4°C. Streptavidine-alkaline phosphatase (AP) (1:1,000 dilution;



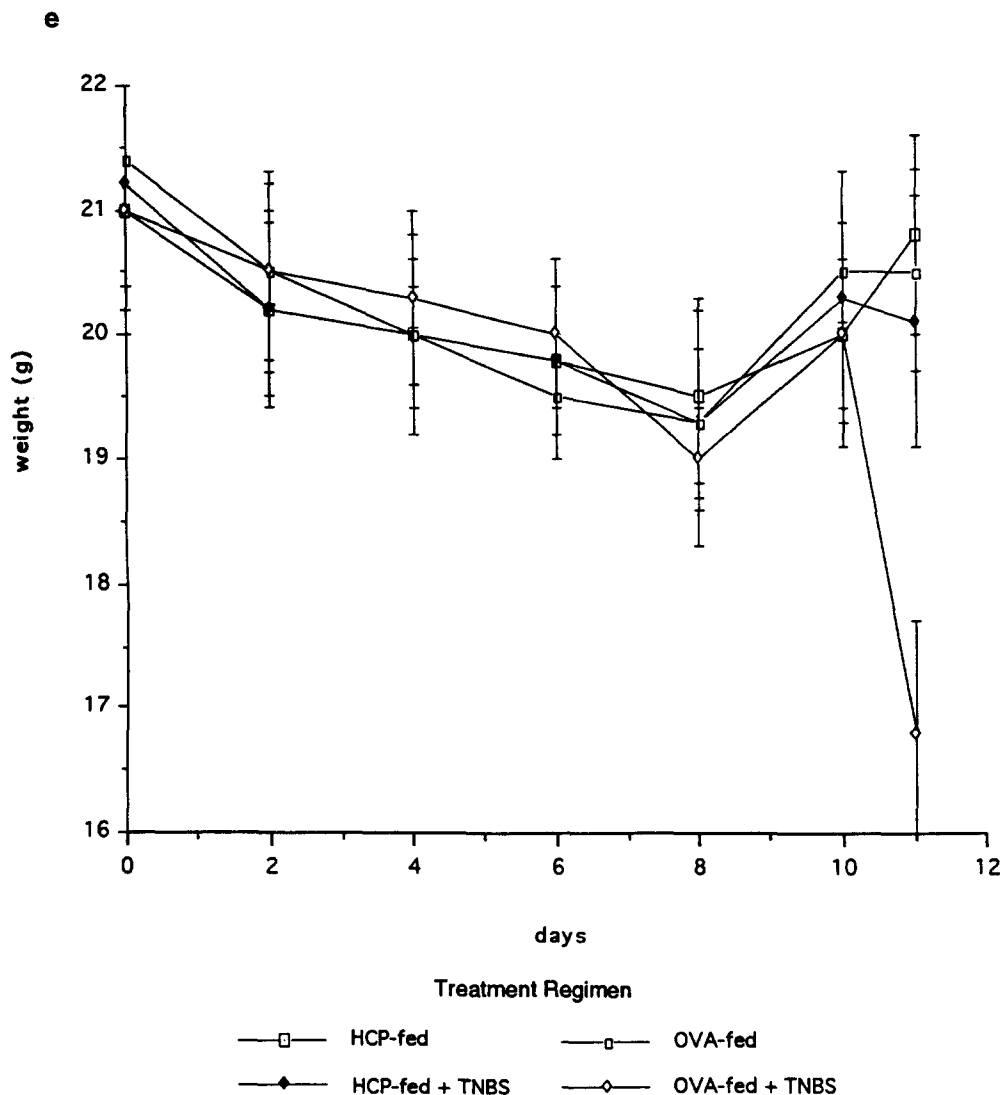
obtained from Zymed) was added for 30 min at 37°C and the AP substrate (Western blue stabilized substrate for AP; Promega, Madison, WI) together with 1% agarose gel was added. Color reaction was allowed to proceed for 36 h. Quantification of spots was performed in 6 mice per group by examining 25 high power fields (HPFs). Under our experimental conditions (magnification of 400), one HPF represented 0.25 mm<sup>2</sup>.

**Proliferation Assays.** Proliferation of cells was assessed by measuring [<sup>3</sup>H]TdR incorporation. In brief, 5 × 10<sup>4</sup> T cells/ml were cultured in flat-bottomed 96-well plates (Costar Corp.) for 72 h; during the last 8 h of culture, 1 μCi of [<sup>3</sup>H]TdR (New England Nuclear, Boston, MA) (specific activity 6.7 Ci/mmol) was added to each well; incorporated [<sup>3</sup>H] radioactivity was measured in a scintillation counter (LS2800; Beckman Instruments, Inc., Fullerton, CA). Each proliferation assay was done in triplicate.

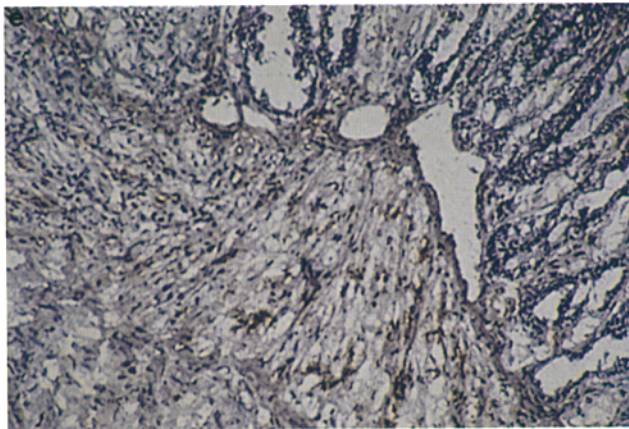
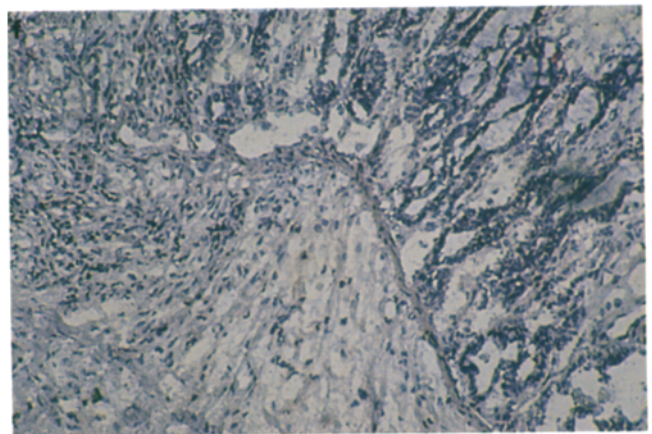
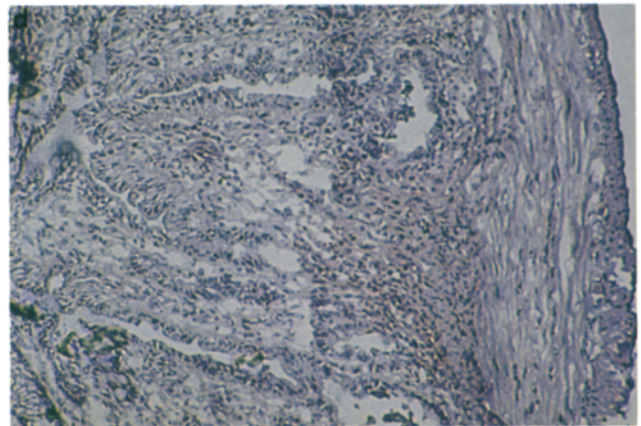
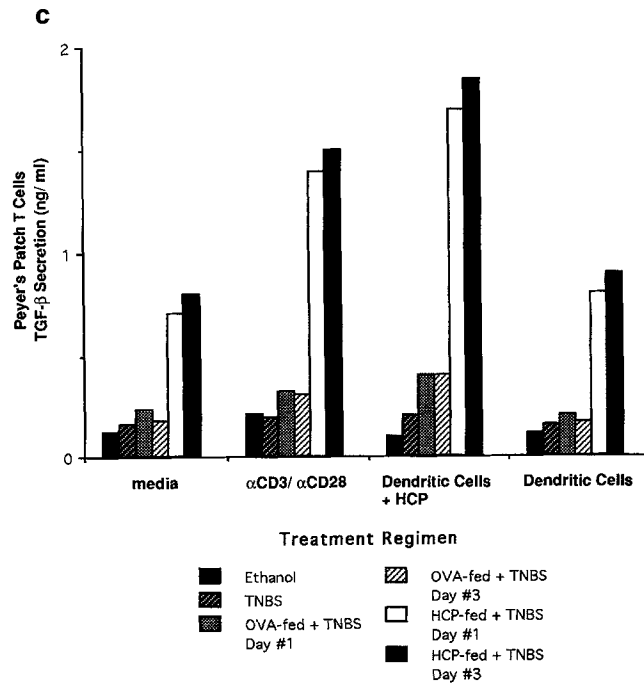
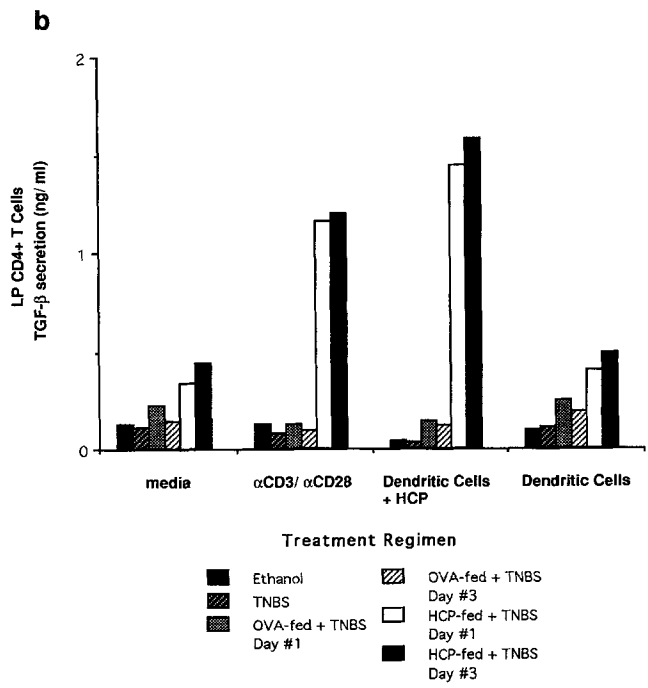
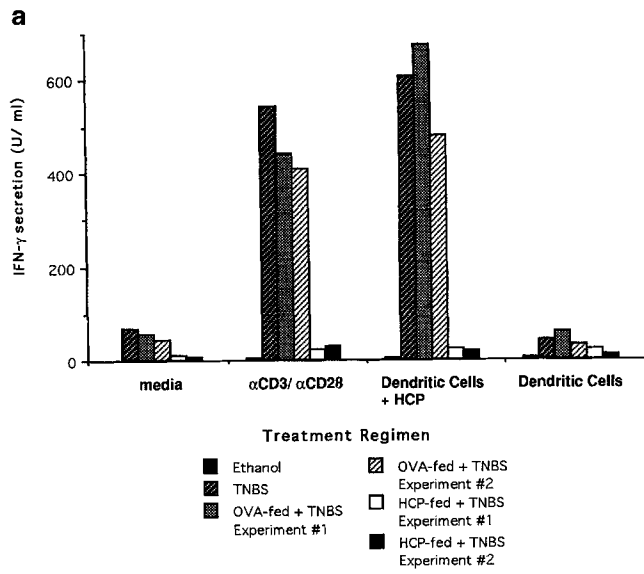
**Immunohistochemistry.** Cryosections (7 μm) were air dried, fixed in cold acetone for 3 min at RT, incubated in 0.6% H<sub>2</sub>O<sub>2</sub>

in methanol for 30 min, rehydrated in PBS plus 0.01% Triton-X for 15 min, blocked with 100% normal rabbit serum for 3 h, and then incubated with the primary antibody (rat anti-mouse IFN-γ or chicken anti-mouse TGF-β; 1:100 dilution in 10% normal rabbit serum) overnight at 4°C in a dark humid chamber. Sections were then washed, incubated with the secondary antibody (biotin-labeled rabbit anti-rat IgG or rabbit anti-chicken IgG; 1:100 dilution), washed in PBS, incubated in peroxidase conjugated streptavidine and chromogen (3 amino-9-ethylcarbazide; obtained from Zymed) for 15 min, counterstained with methyl green or hematoxylin, mounted, and analyzed. Samples incubated with isotype-matched control antibodies and without primary antibody served as negative controls.

**In Vivo Administration of Anti-TGF-β Antibodies or rIL-12.** Animals were injected i.p. with 1 mg anti-TGF-β antibodies (murine IgG1; clone 1.D11.16; Celtrix Corp., Santa Clara, CA) or 1 mg control IgG (Jackson ImmunoResearch, West Grove, PA) at in-



**Figure 2.** TNBS-induced colitis can be suppressed by feeding of HCP. (A) Weight changes of untreated SJL/J mice, control ethanol-treated mice, and TNBS-treated mice treated over a period of 12 d. Some of the TNBS-treated mice were pre-fed HCP, TNP-KLH or OVA for 10 d as specified in Materials and Methods; colitis was then induced after an additional 7 d. Weight data from one representative experiment out of three is shown. Each point represents average weight data from four mice. Standard deviations are indicated. (B) Macroscopic appearance of colons of a mouse with TNBS-induced colitis (top row) and mice that were prefed OVA (second row) or HCP (third row) 7 d after administration of TNBS. The bottom row shows a control mouse that was injected with ethanol alone. There was a severe colitis in TNBS-treated mice that were fed OVA but not in those that received HCP. (C) HE-stained colon section of a HCP-fed SJL/J mouse 7 d after administration of TNBS. No severe colitis was observed. However, some colon wall thickening was still present. ×100. (D) HE-stained colon section of a OVA-fed SJL/J mouse 7 d after administration of TNBS. Colitis with loss of goblet cells and lymphocytic infiltrations was present. ×250. (E) Weight changes of SJL/J mice that received 1.5 × 10<sup>5</sup> LP CD4<sup>+</sup> T cells at day 0 and then fed HCP or OVA at days 0, 2, 4, and 6, followed by administration of 0.5 mg TNBS at day 10. Each point represents average weight data from four mice. Standard deviations are indicated.



**Table 2.** Secretion of IL-4 and IL-10 in HCP- and OVA-fed Mice

Treatment regimen	IL-4		IL-10	
	media	$\alpha$ CD3/ $\alpha$ CD28	media	$\alpha$ CD3/ $\alpha$ CD28
	ng/ml		ng/ml	
Untreated	0.5 $\pm$ 0.1	2.5 $\pm$ 0.5	0.4 $\pm$ 0.05	0.8 $\pm$ 0.1
Ethanol	0.4 $\pm$ 0.1	2.3 $\pm$ 0.3	0.3 $\pm$ 0.05	0.7 $\pm$ 0.05
TNBS	0.1 $\pm$ 0.03	0.3 $\pm$ 0.05	0.5 $\pm$ 0.04	0.8 $\pm$ 0.08
HCP-fed + TNBS	0.9 $\pm$ 0.1*	6.9 $\pm$ 0.8*	1.2 $\pm$ 0.1*	4.8 $\pm$ 0.45*
OVA-fed + TNBS	0.2 $\pm$ 0.06	0.4 $\pm$ 0.05	0.3 $\pm$ 0.08	0.6 $\pm$ 0.04

Secretion of IL-4 and IL-10 by LP CD4+ T cells in mice that were fed OVA or HCP before administration of TNBS. LP CD4+ cells were isolated 7 d after administration of TNBS and stimulated with anti-CD3 and anti-CD28. Culture supernatants were removed after 2 d and analyzed for cytokine concentration by specific ELISA.

\*Significantly increased vs. OVA-fed and TNBS control ( $P \leq 0.01$ ).

indicated time points. In some experiments, mice were injected subcutaneously daily with PBS or PBS containing 0.75  $\mu$ g recombinant murine IL-12 ( $2.5 \times 10^8$  Roche units/mg; kindly donated by M. Gately, Hoffmann-LaRoche) at indicated time points.

## Results

**Adoptive Transfer of TNBS-induced Colitis by Lamina Propria CD4+ T Cells to Normal SJL/J Mice.** We have previously described the induction of a chronic granulomatous colitis in normal BALB/c and SJL/J mice by rectal administration of the haptenizing reagent 2,4,6 trinitrobenzene sulfonic acid, a colitis dominated by CD4+ T cells producing Th1-type cytokines (22). To further explore this observation, we initially sought to determine whether the development of TNBS-induced lesions could be adoptively transferred to naive animals with CD4+ T cells. Accordingly, we isolated LP CD4+ T cells from TNBS-treated (diseased) animals or control ethanol-treated animals and injected them into normal SJL/J mice. Whereas recipients of CD4+ T cells from control animals did not develop colitis upon transfer, recipients of CD4+ T cells from diseased animals developed mild colitis. Thus, the colons of the latter animals were found to contain lymphocytic infiltrates, located mainly in the subepithelial areas, that produced IFN- $\gamma$  (Fig. 1 *a*). Moreover, these infiltrates contained injected CD4+ T cells, since CD4+ T cells that were labeled in vitro with

CM-FDA before transfer (see Materials and Methods) were demonstrated by fluorescence microscopy in the colons at the 7-d post-transfer time point (data not shown).

To verify the presence of hapten-specific CD4+ T cells in the lesions of the recipient mice, we administered to some of the mice low doses of TNBS that were shown in control experiments not to cause inflammation in normal mice. We found that the challenged mice that had received T cells from mice with TNBS-colitis developed a massive pancolitis and died within 36 h (Fig. 1 *b*). Furthermore, analysis of IFN- $\gamma$  secretion by LP CD4+ T lymphocytes from these mice showed very high production of IFN- $\gamma$  (Fig. 1 *c*). In contrast, the colons of challenged mice that had received T cells from ethanol-treated animals appeared histologically normal (Fig. 1 *d*) and LP CD4+ T cells secreted low amounts of IFN- $\gamma$  that were comparable to those produced by CD4+ T cells from normal mice (Fig. 1 *c*). Taken together, these studies provide strong support for the view that TNBS-induced colitis is mediated by IFN- $\gamma$ -producing CD4+ T cells that are present in colonic tissues.

**Active Suppression of TNBS-induced Colitis by Feeding of Haptenized Colonic Proteins.** We next turned our attention to the question of whether TNBS-induced colitis can be suppressed by inducing "oral tolerance" (i.e., unresponsiveness to fed antigen) specific for TNBS-HCP. We generated in vitro HCP using previously described techniques (see Materials and Methods) and obtained colonic proteins with

**Figure 3.** Cytokine production by LP cells in tolerized and nontolerized animals. (A) Secretion of IFN- $\gamma$  by LP CD4+ T cells in mice that were fed control OVA or HCP before administration of TNBS. LP CD4+ T cells were isolated at indicated time points after administration of TNBS and stimulated under various conditions. Supernatants of cultured LP CD4+ cells were removed after 2 d and analyzed for IFN- $\gamma$  concentration by ELISA. (B) Secretion of TGF- $\beta$  by LP CD4+ T cells in ethanol- or TNBS-treated mice at day 3 and in OVA- and HCP-fed animals 1 and 3 d after administration of TNBS. LP CD4+ T cells were stimulated as above, cultured for 2.5 d and culture supernatant was analyzed for TGF- $\beta$  concentration by specific ELISA. (C) Secretion of TGF- $\beta$  by PP T cells in OVA- and HCP-fed animals 1 and 3 d after administration of TNBS. PP T cells were cultured for 2.5 d, and culture supernatant was analyzed for TGF- $\beta$  concentration by specific ELISA. (D) Staining for TGF- $\beta$  in the colon of an OVA-fed mouse 1 d after administration of TNBS. Only weak staining intensity was observed.  $\times 200$ . (E) Staining for TGF- $\beta$  in the colon of a tolerized HCP-fed mouse 1 d after administration of TNBS. There was a high staining intensity in subepithelial areas.  $\times 250$ . (F) Staining analysis in the colon of a tolerized HCP-fed mouse 1 d after administration of TNBS using an IgG control antibody.  $\times 250$ .

**Table 3.** Proliferation Assays of PP T Cells in HCP- and OVA-fed Mice

Treatment regimen	<sup>3</sup> H]Thymidine Incorp.	
	Media	αCD3/αCD28
		<i>cpm</i>
Ethanol	13030 ± 359	89085 ± 876
TNBS	26663 ± 561	154874 ± 12923
OVA-fed + TNBS 1d	21977 ± 765	134021 ± 13211
OVA-fed + TNBS 3d	24121 ± 982	161965 ± 18717
HCP-fed + TNBS 1d	8974 ± 743*	24198 ± 1877*
HCP-fed + TNBS 3d	11865 ± 855*	39478 ± 4876*

Proliferation assays of PP T cells in OVA- or HCP-fed animals were performed 1 and 3 d after administration of TNBS. PP T cells ( $1 \times 10^5$ ) were cultured for 2 d and [<sup>3</sup>H]thymidine was added during the last 8 h of the culture as described in Materials and Methods. Data were pooled from three experiments. Standard deviations are indicated.

\*Significantly reduced compared with corresponding OVA-fed control ( $P \leq 0.01$ ).

very high haptenization densities of 53–74 TNP groups per  $10^5$  Da of protein. Mice were then fed HCP or control protein (ovalbumin [OVA]) for 10 d; then, after an additional 7 d, TNBS was administered per rectum. Whereas sham-fed animals administered TNBS developed severe colitis, diarrhea, and weight loss (Fig. 2 a), mice fed HCP failed to develop diarrhea and weight loss, and their colons appeared macroscopically intact (Fig. 2 b). Furthermore, these mice showed no histologic signs of severe colitis (Fig. 2 c). In contrast, mice fed OVA showed clear macroscopic (Fig. 2 b) and histologic (Fig. 2 d) evidence of colitis that was indistinguishable from that in normal mice given TNBS. Furthermore, TNP-KLH-fed mice showed less weight loss than OVA-fed mice but still exhibited colonic inflammation, as assessed by histologic analysis (Table 1), suggesting that the tolerance observed after feeding of HCP might be at least partially due to the TNP itself.

Since these results showed that TNBS-induced colitis can be prevented by feeding of HCP, we next analyzed whether HCP would also be protective when disease was transferred by LP CD4+ T cells from mice with TNBS-induced colitis. In these experiments, we isolated colonic LP CD4+ T cells from mice with TNBS-induced colitis at day 10, and transferred these cells to normal mice. The recipient mice were fed four times with HCP and then, after an additional 4 d 0.5 mg TNBS was administered per rectum. As shown in Fig. 2 e, oral feeding of HCP suppressed colitis induced by the transfer of in vivo primed CD4+ T cells. These re-

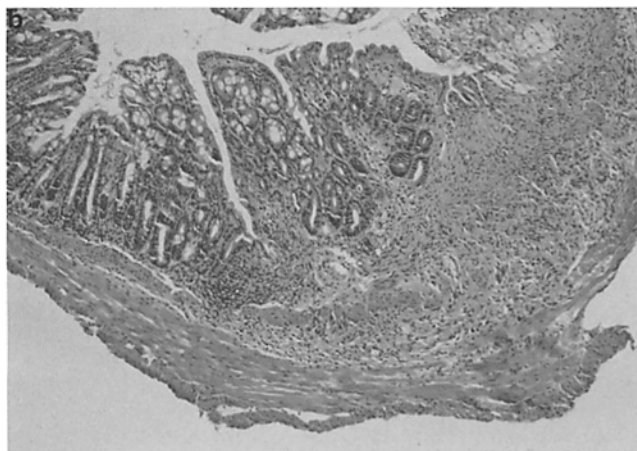
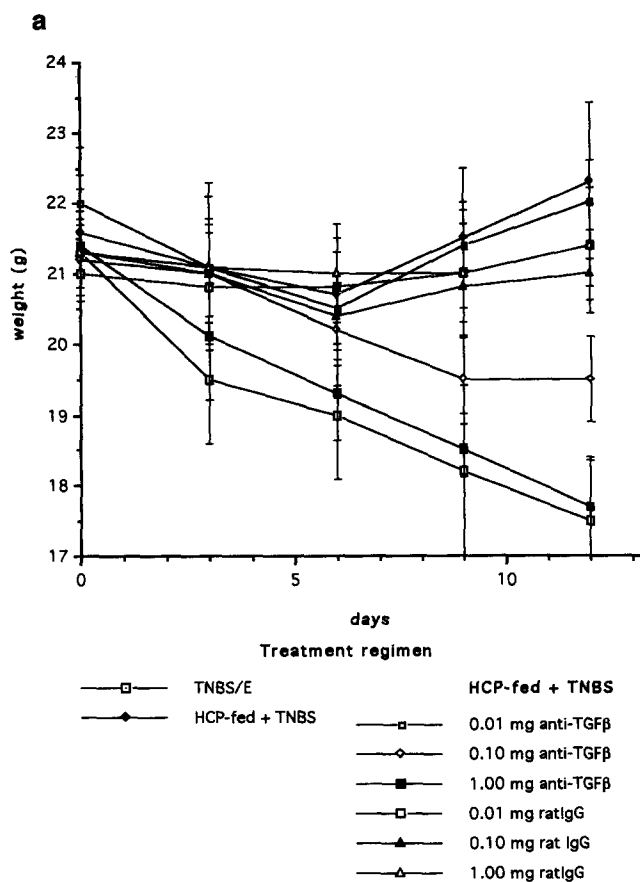
sults suggest that the inflammation secondary to stimulation of preexisting TNBS-specific CD4+ T cells is inhibited by the induction of oral tolerance.

*LP CD4+ T Cells and PP T Cells in HCP-fed Tolerized Mice Display Reduced Proliferative Capacity and Secrete High Amounts of TGF-β, IL-4, and IL-10.* To determine the mechanisms underlying the tolerance induced by feeding of HCP, we next focused on the capacity of intestinal LP cells in HCP- and OVA-fed mice to produce various cytokines. Since previous studies had shown that TNBS-induced colitis is critically dependent on IL-12 levels (22), we first analyzed the ability of macrophage-enriched LP cells from HCP-fed tolerized mice to secrete functionally active IL-12 heterodimer. We found that cells from the LP of HCP-fed mice secreted strikingly less IL-12 heterodimer than those from the lamina propria of OVA-fed mice ( $4.2 \pm 0.8$  vs.  $69 \pm 12$  spot forming cells per HPF). Furthermore, as assessed by immunohistochemistry, there was a marked reduction in IL-12 heterodimer expression by macrophages in the colon of HCP-fed mice compared with those from OVA-fed mice (data not shown) suggesting that IL-12-dependent Th1 cell development was suppressed in HCP-fed mice.

This hypothesis was supported by the analysis of IFN-γ production in tolerized mice 7 d after TNBS administration. In these studies, we stimulated LP CD4+ T cells with anti-CD3/anti-CD28 and measured the concentration of IFN-γ in culture supernatants. As shown in Fig. 3 a, LP CD4+ T cells from TNBS-treated mice that were fed OVA produced large amounts of IFN-γ at day 7 (compared with mice not treated with TNBS), while T cells from mice fed HCP produced strikingly lower amounts of IFN-γ. In contrast, as shown in Table 2, secretion of IL-4 and IL-10 was significantly higher in HCP-fed mice than in OVA-fed mice ( $P \leq 0.01$ ).

In additional studies, we measured TGF-β production by LP CD4+ and PP T cells in OVA- and HCP-fed mice. In this case, we stimulated both LP CD4+ T cells and PP T cells either with anti-CD3/anti-CD28 or with isolated dendritic cells loaded with HCP. As shown in Fig. 3, b and c, TGF-β production by both cell populations and both methods of stimulation was strongly increased in cells from mice fed HCP compared with cells from mice fed OVA. Additional studies showed that FACS®-purified, anti-CD3/anti-CD28-stimulated CD4+ T cells from the lamina propria of HCP-fed mice produced higher levels of TGF-β than those from OVA-fed mice (1.2 ng/ml vs. 0.1 ng/ml). To obtain direct evidence of TGF-β production in vivo, we performed immunohistochemical staining for this cytokine. As shown in Fig. 3, d–f, there was an increase in staining intensity for TGF-β by LP cells in HCP-fed animals compared with OVA-fed control animals after TNBS challenge. Finally, we measured the proliferation of PP T cells from HCP and OVA-fed mice. We found that PP T cells from mice fed HCP when stimulated with anti-CD3 and anti-CD28, proliferated significantly less than cells from mice fed OVA ( $P \leq 0.01$ ) suggesting that the proliferation of these cells was actively suppressed by increased TGF-β secretion (Table 3).





**Figure 4.** In vivo administration of antibodies to TGF- $\beta$  abrogates the suppressive effects of feeding HCP. (A) Weight changes of SJL/J mice that received 0.01–1 mg of anti-TGF- $\beta$  antibodies at days 0, 4, 7, and 11 after oral feeding with HCP. Mice receiving rat IgG served as control. Weight data from one representative experiment out of two is shown. Each point represents average weight data from five mice. Standard deviations are indicated. (B) HE-stained colon section of a HCP-fed SJL/J mouse that was treated with 1 mg TGF- $\beta$ -antibodies as described above 9 d after administration of TNBS. Severe colitis was present.  $\times 150$ .

*Administration of Anti-TGF- $\beta$  In Vivo Abrogates the Protective Effects of Orally Administered HCP.* If, indeed, increased TGF- $\beta$  production was responsible for the oral tolerance to HCP, pretreatment of mice with anti-TGF- $\beta$  should abro-

gate tolerance and allow development of TNBS-induced lesions. To explore this possibility, we treated mice in vivo with anti-TGF- $\beta$  or control IgG on days 0, 4, 7, and 11 after feeding HCP. As shown in Fig. 4 a, anti-TGF- $\beta$  treatment abrogated the protective effects of feeding of HCP on TNBS-induced colitis in a dose-dependent manner. The anti-TGF- $\beta$ -treated mice displayed clinical and histologic signs of colitis (Fig. 4 b) similar to those observed in TNBS-treated mice. In addition, cytokine profiles of LP CD4<sup>+</sup> T cells in HCP-fed, anti-TGF- $\beta$ -treated mice were similar to those of mice given TNBS intrarectally in the absence of feeding HCP (Table 4). These studies, together with the TGF- $\beta$  production studies described above, strongly suggest that TGF- $\beta$  mediates the protective effect of feeding of HCP in TNBS-induced colitis.

*Suppression of TNBS-induced Colitis by Feeding of Haptenized Colonic Proteins Can Be Abrogated by High Doses of rIL-12.* In further studies, we tested whether increased levels of IL-12 could counteract the suppressive effect of oral antigen administration. To test this hypothesis, we systemically administered IL-12 to mice that were fed HCP and then examined the effects of such treatment on the clinical course of the TNBS-induced disease. As depicted in Fig. 5, when high doses of IL-12 were administered, a striking reduction of the suppression of TNBS-induced colitis by feeding of HCP was seen. In contrast, systemic administration of antibodies to IL-12 augmented the suppressive effects seen after feeding of HCP in TNBS-induced colitis (data not shown). These results establish the reciprocal functional relationship between IL-12 and TGF- $\beta$  on tolerance induction in TNBS-induced colitis.

*Systemic Administration of TGF- $\beta$  Prevents TNBS-induced Colitis.* Since the above data implied the importance of TGF- $\beta$  levels on the clinical course of TNBS-induced colitis, we wanted to determine in a final series of studies if systemic administration of TGF- $\beta$  would be sufficient to prevent TNBS-induced colitis. Accordingly, we treated mice every other day with recombinant TGF- $\beta$ , starting one day before administration of TNBS over a period of 11 d. As shown in Fig. 6, high doses of TGF- $\beta$  abrogated diarrhea and weight loss in TNBS-treated animals, suggesting that high levels of TGF- $\beta$  are sufficient to prevent TNBS-induced colitis.

## Discussion

In the present study we explore the cellular mechanisms that regulate experimental TNBS-induced colitis. First, we show in adoptive transfer studies that TNBS-induced colitis can be mediated by CD4<sup>+</sup> T cells that home to colonic tissue. Second, we demonstrate that TNBS-induced colitis can be actively suppressed via oral tolerance by feeding of HCP and that such tolerance is critically dependent on TGF- $\beta$  production by intestinal cells. Third and finally, we show that colitis due to transferred CD4<sup>+</sup> T cells can be suppressed by feeding of HCP, indicating that the oral tolerance mechanism affects precommitted T cells and thus

**Table 4.** Production of Cytokines in Anti-TGF- $\beta$ -treated SJL/J Mice

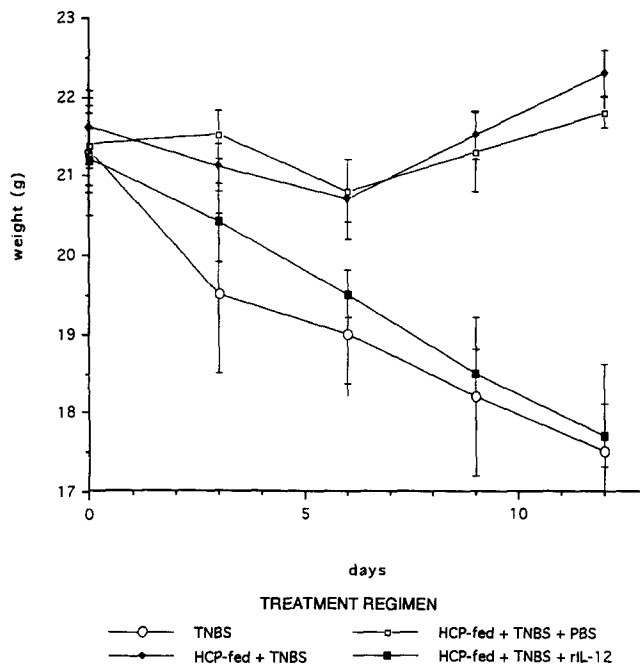
Group	IFN- $\gamma$		IL-4		IL-10	
	Media	CD3/28	Media	CD3/28	Media	CD3/28
Ethanol (E)	4	12	0.4	2.0	0.4	0.8
E + 1 mg $\alpha$ TGF- $\beta$	10	25	0.2	0.8	0.2	0.5
TNBS	55	534	0.1	0.3	0.4	0.7
HCP-fed + TNBS						
No $\alpha$ TGF- $\beta$	3	27	1.0	4.9	1.5	5.1
0.1 mg $\alpha$ TGF- $\beta$	14	76	0.8	2.0	1.2	3.1
1.0 mg $\alpha$ TGF- $\beta$	63	421	0.2	0.5	0.3	0.6
0.1 mg rat IgG	4	20	0.8	4.6	1.2	4.4
1.0 mg rat IgG	3	27	1.0	4.9	1.6	4.9

HCP-fed or unfed mice were treated with ethanol or TNBS and some mice received injections with antibodies to TGF- $\beta$  at days 0, 4, 7, and 11. LP CD4+ cells were isolated 9 d after administration of TNBS or ethanol. Culture supernatants were removed after 2 d and analyzed for cytokine concentration by specific ELISA. A slight increase in IFN- $\gamma$  production was found in ethanol-treated mice that were given anti-TGF- $\beta$  compared with ethanol-treated mice. A second independent experiment gave similar results.

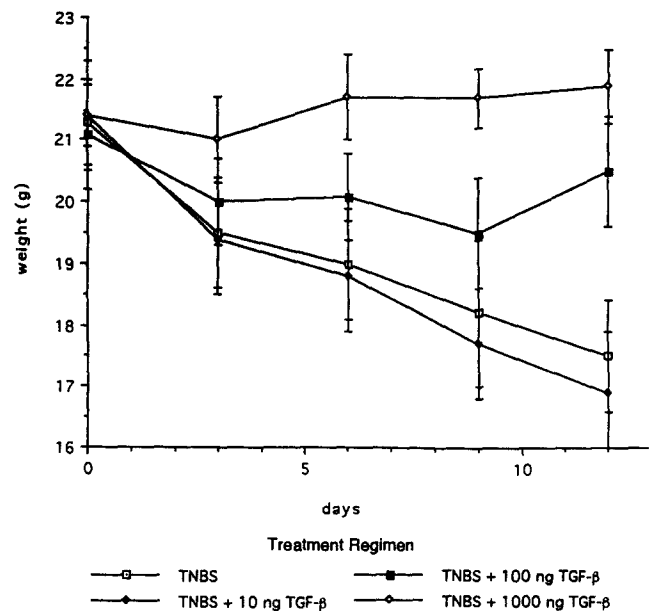
may be relevant to the treatment of inflammatory bowel disease in humans.

Inflammatory bowel diseases comprising Crohn's disease (CD) and ulcerative colitis are thought to be immunologically mediated diseases of the gastrointestinal tract (11). Re-

cently, several murine models of chronic intestinal inflammation have been described that mimic some characteristics of CD in humans including transfer of CD45RB<sup>hi</sup> T cells from Balb/c mice into scid mice (18) and hapten reagent-induced colitis by TNBS (22). Both models show some histologic similarity (transmural inflammation, granuloma formation, infiltration of CD4+ T cells, and macrophages) to



**Figure 5.** Administration of rIL-12 abrogates the suppressive effects of feeding HCP. Weight changes of HCP-fed SJL/J mice that were systemically treated with rIL12 (days -1, 1, 3, 5, 7, and 9) after administration of TNBS. Weight data from one experiment out of two is shown. Each point represents average weight data from four mice. Standard deviations are indicated.



**Figure 6.** Systemic administration of rTGF- $\beta$  prevents TNBS-induced colitis. Weight changes of SJL/J mice that were treated with rTGF- $\beta$  at days -1, 1, 3, 5, 7, 9, and 11 after administration of TNBS. Weight data from one representative experiment out of two is shown. Each point represents average weight data from five mice. Standard deviations are indicated.

CD in humans and CD4+ T cells in both diseases reveal a Th1 cytokine pattern that is consistent with the cytokine pattern found in CD (Fuss, I., M. Neurath, M. Boirivant, J. Klein, C. de la Motta, S. Strong, C. Fiocchi, and W. Strober, manuscript in preparation). In the CD45RB<sup>hi</sup> transfer model, it was shown that treatment of mice with antibodies to IFN- $\gamma$  ameliorates disease, whereas in the TNBS model, it was shown that anti-IL-12 (which downregulates IFN- $\gamma$  production) abrogates disease even after the disease is well established.

Further insight into the regulation of experimental colitis was gained in the present studies of TNBS-induced colitis in which the effect of induction of oral tolerance to TNBS-haptenated colonic proteins on TNBS-induced colitis was determined. In particular, we showed that prefeeding of HCP to mice not only prevented TNBS-induced colitis at the clinical and histologic level but also prevented the Th1-type T cell response that accompanies and presumably mediates this colitis. In addition, regarding the mechanism of the oral tolerance, we showed that tolerization to HCP was accompanied by elevated production of Th2-type cytokines and strikingly increased TGF- $\beta$  secretion in mucosal tissues (in PP and LP) *in vivo*. Finally, we found that oral tolerance was abrogated in mice administered anti-TGF- $\beta$  and that systemic administration of TGF- $\beta$  prevented TNBS-induced disease. These results indicate clearly that TGF- $\beta$  is a necessary condition for the establishment of oral tolerance to TNBS-haptenated colonic proteins.

In further studies, we showed that T cell-enriched LP and PP cells from tolerized animals produce high amounts of TGF- $\beta$  upon *in vitro* stimulation and displayed reduced proliferative capacities. The observation that fed HCP prevented TNBS-induced colitis via induction of cells producing TGF- $\beta$  supports and extends the work of Miller et al. (25) who demonstrated the presence of bystander suppression mediated by TGF- $\beta$  after feeding of antigen. Weiner (9, 25–27) has recently proposed that mucosally derived TGF- $\beta$ -producing CD4+ T cells are a special T

helper subset that is induced upon low-dose oral antigen administration, provides mucosal T helper function and downregulates Th1 T cell function. After their induction, such cells may migrate to other tissues including the lamina propria, where they exert TGF- $\beta$ -mediated suppressive effects on CD4+ T cells and macrophages that might otherwise cause inflammation. This suppressor mechanism could explain the protective effect of feeding of TNBS-haptenated proteins on TNBS-induced colitis. In particular, since TGF- $\beta$  has been shown to be a potent inhibitor of IL-12-induced Th1 development (28), TGF- $\beta$ -producing cells generated upon feeding may prevent IL-12-dependent Th1 cell development upon which TNBS-induced colitis is critically dependent (22). Furthermore, oral tolerance induction led to a striking reduction of IL-12 heterodimer secretion by LP cells that might further account for the suppression of Th1 cytokine production. Finally, taken together with the fact that administration of exogenous IL-12 abrogates tolerance induction, the results suggest a reciprocal functional relationship between IL-12 and TGF- $\beta$  on tolerance induction in TNBS-induced colitis.

The concept of induction of oral tolerance mediated by TGF- $\beta$  has important implications to the treatment of autoimmune diseases generally: for instance, it has been shown that one can suppress PLP peptide-induced experimental autoimmune encephalitis by feeding MBP or antigen-induced arthritis by feeding collagen type II. These findings have led to the design of a rational immunotherapy for patients with rheumatoid arthritis (29) and multiple sclerosis (30) and promising clinical trials are currently in progress. The present data, in showing that TNBS-induced colitis can be actively suppressed by feeding HCP via induction of TGF- $\beta$  secretion in the mucosal tissues and that oral tolerance can also abrogate experimental colitis when disease was transferred by LP CD4+ T cells, suggest that induction of oral tolerance has therapeutic relevance to immunologic diseases of the gastrointestinal tract, such as Crohn's disease.

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