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Treatment with an Anti-IL-4 Monoclonal Antibody Blocks Suppression of Collagen-Induced Arthritis in Mice by Oral Administration of Type II Collagen¹

Shin Yoshino²

Oral administration of type II collagen (CII) has been shown to suppress collagen-induced arthritis (CIA) in experimental animals. However, the exact mechanism by which CIA is suppressed following administration of CII remains to be investigated, although it was demonstrated that active suppression by regulatory T cells might be involved in the suppression. Therefore, we have examined whether the inhibitory cytokine IL-4 plays a role in the suppression of CIA, by using an anti-IL-4 mAb (11B11 mAb). Mice were fed daily with CII over a period of 10 days before immunization with CII. 11B11 mAb was i.p. injected 30 min before each oral administration of CII. The results showed that treatment with 11B11 mAb markedly blocked suppression of CIA by the oral Ag. The blockade of suppression of CIA by the anti-IL-4 mAb was associated with the blockade of augmentation of IL-4 secretion in CII-fed mice. The treatment with 11B11 mAb also resulted in the prevention of decreases in anti-CII IgG2a Ab production, DTH responses to CII, proliferation of lymphoid cells to CII, and IFN- γ secretion in mice given CII orally. Thus, the neutralization of IL-4 by an anti-IL-4 Ab appears to be effective in blocking suppression of CIA by oral administration of CII, suggesting that IL-4 may be critically involved in its suppression. *The Journal of Immunology*, 1998, 160: 3067–3071.

Collagen-induced arthritis (CIA)³ is an experimental model of autoimmune disease and can be induced in mice (1), rats (2), and primates (3) by immunization with type II native articular cartilage collagen (CII). Many features of CIA resemble those of rheumatoid arthritis in humans (4, 5). It has been shown that both cellular and humoral immune responses to CII are involved in the pathogenesis of CIA. For instance, the disease can be transferred by Abs specific for CII (6, 7). Adoptive transfer of lymphoid cells from animals immunized with CII (8) and of CII-specific T cell lines and clones (9) also transmits the disease.

Oral administration of an Ag induces peripheral immune tolerance to the Ag, termed oral tolerance (10, 11); it is thought to contribute to the prevention of food hypersensitivity (12). Recent studies also indicated that feeding pathogenic Ags was effective in suppressing a variety of autoimmune disorders (13–17). This includes suppression of CIA in mice by feeding them CII (16, 17). Although the exact mechanism of induction of oral tolerance still remains obscure, possibilities include deletion (18) and anergy (19) of Ag-specific lymphocytes, and suppression by inhibitory cytokines including IL-4 secreted from regulatory T cells (20). In the present study, we have investigated a role for IL-4 in suppression of CIA in mice by oral administration of CII, by employing a mAb (11B11 mAb) against IL-4. We show here that treatment with 11B11 mAb was effective in diminishing suppression of CIA by

CII given orally, suggesting a role for IL-4 in down-regulation of the disease.

Materials and Methods

Animals

Female DBA/1J mice, 8 to 9 wk of age, were used in all experiments. The mice were bred in the animal breeding unit of Saga Medical School, Saga, Japan. They were maintained in a temperature- and light-controlled environment with free access to standard rodent chow and water.

Induction of CIA

To induce CIA, 2 mg of CII extracted from native calf articular cartilage (Funakoshi, Co., Tokyo, Japan) was dissolved in 1 ml of 0.1 N acetic acid (AA) and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI). In all, 100 μ l of the emulsion was injected into the base of the tail (day 0). Twenty-one days later, the animals had a booster injection of the same amount of the emulsion at the same site. To evaluate the severity of arthritis, the lesions of the four paws were each graded from 0 to 4 according to the increasing extent of erythema and edema of the periarticular tissue as described by Wood et al. (21). The maximum possible score is 16.

Administration of CII

Mice were fed daily with 1 mg of CII dissolved in 0.5 ml of 0.005 N AA through a syringe fitted with an 18-gauge ballpoint needle from day -10 to day -1 before immunization with CII. A total of 0.5 ml of 0.005 N AA was given as a control.

Treatment with 11B11 mAb

The cell line (11B11) for a rat IgG1 mAb against murine IL-4 was kindly provided by the Department of Immunology, Saga Medical School. 11B11 mAb was precipitated by ammonium sulfate from ascitic fluid of SCID mice inoculated with the cells and purified using a protein G-Sepharose 4FF column (Pharmacia Biotech, Tokyo, Japan). The preparation and characterization of 11B11 mAb have been described previously (22). Two milligrams of 11B11 mAb dissolved in 0.5 ml of PBS was injected daily i.p. 30 min before each administration of CII. As treatment controls, 0.5 ml of PBS only and 0.5 ml of PBS containing 2 mg of normal rat serum, IgG purified as described above, were given to mice.

Measurement of anti-CII Abs

Blood was collected on day 20 after immunization with CII and sera were heat inactivated at 56°C for 30 min. Anti-CII IgG1 and IgG2a Abs were

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³ Abbreviations used in this paper: CIA, collagen-induced arthritis; 11B11 mAb, anti-IL-4 mAb; CII, type II collagen; DTH, delayed-type hypersensitivity; AA, acetic acid.

measured using an ELISA (23). In brief, 96-well flat-bottom microtiter plates were incubated with 100 μ l/well of CII (100 μ g/ml) at 37°C for 1 h and washed three times with PBS containing 0.05% Tween-20. The wells were then blocked by incubation with 100 μ l of PBS containing 1% OVA (Sigma, St. Louis, MO) at 37°C for 1 h. After washing, plates were incubated with 100 μ l of a 1:600 dilution of each serum sample at 37°C for 30 min. The plates were washed, and 100 μ l/well of a 1:1000 dilution of rat anti-mouse IgG1 or IgG2a labeled with alkaline phosphatase (PharMingen, San Diego, CA) was added and incubated at 37°C for 1 h. After washing, 100 μ l of 3 mM of *p*-nitrophenylphosphate (Bio-Rad Laboratories, Hercules, CA) was added per well and the plates were incubated in the dark at room temperature for 15 min. Absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results were expressed as absorbance units at OD₄₀₅ \pm SEM.

Measurement of DTH

On day 12 after immunization with CII, 10 μ g of CII dissolved in 20 μ l of 0.005 N AA was injected s.c. into the right footpad. As a vehicle control, 20 μ l of 0.005 N AA was injected into the left footpad. The thickness of the right and left footpad were measured using dial gauge calipers calibrated with 0.01-mm graduations (Ozaki MFG, Tokyo, Japan) immediately before and 24 h after the challenge injection. The increase in left footpad thickness was subtracted from the increase in right footpad thickness to give the value due to the specific response to CII. There was minimal footpad swelling at 24 h in response to CII and 0.005 N AA in nonimmunized mice.

Proliferation assay

Mice were killed 14 days after immunization with CII and single cell suspensions were prepared from their inguinal lymph nodes. A total of 5×10^5 cells, in 100 μ l of RPMI 1640 (Flow Laboratories, Inc., McLean, VA) containing 1 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 5×10^{-5} M 2-ME, and 1% heat-inactivated autologous mouse serum were added to each microwell followed by the addition of 100 μ l of 12.5, 25, and 50 μ g/ml CII. The cells were cultured for 72 h. Each well was pulsed with 0.5 μ Ci of tritiated thymidine, and the cells were cultured for another 16 h. The cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques. Results, expressed in cpm, are average of quadruplicate cultures of cells pooled from four mice.

Cytokine measurement

Single cell suspensions from inguinal lymph nodes removed on days 7, 14, and 21 after immunization with CII were resuspended at a final concentration of 5×10^6 cells/ml and cultured in 1-ml aliquots in 24-well tissue culture plates either in medium alone or with 50 μ g/ml of CII. Forty-eight hours later, supernatants were harvested and stored at -70°C until assayed. Cytokine production was quantified using sandwich ELISA techniques. Briefly, supernatants were added to 96-well microtiter plates, previously coated overnight at 4°C with 100 μ l of anti-IL-4 (4 μ g/ml) and anti-IFN- γ (4 μ g/ml) Abs (PharMingen) in 0.1 M NaHCO₃ buffer. Plates were then washed twice with PBS containing 0.05% Tween-20, after which nonspecific protein-binding sites were blocked by incubation with 100 μ l of PBS containing 1% OVA at 37°C for 1 h. After blocking, the plates were washed three times and samples and standards (recombinant murine IL-4 and IFN- γ) (PharMingen) were added to each well in a volume of 100 μ l and incubated at 37°C for 1 h. Plates were washed three times, and 100 μ l/well biotinylated anti-murine IL-4 (2 μ g/ml) and IFN- γ (2 μ g/ml) Abs (PharMingen) diluted in PBS/1% OVA was added. After incubation at 37°C for 1 h, the plates were washed three times and 100 μ l/well of streptavidin-alkaline phosphatase (PharMingen) was added at 2 μ g/ml. The plates were washed before 100 μ l of *p*-nitrophenylphosphate was added to each well. Plates were read at 405 nm using an automatic microplate reader. Cytokine levels were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines, and results are expressed in pg/ml.

Results

Treatment with 11B11 mAb blocks suppression of CIA by oral administration of CII

The incidences of CIA in mice injected i.p. with PBS and subsequently orally given AA were 0, 27, 73, and 93% on days 20, 23, 27, and 30 after immunization with CII, respectively (Table I). Oral administration of CII following i.p. injection of PBS decreased incidence of the disease. The highest incidence of arthritis

Table I. Incidence of CIA in mice^a

Days After Immunization	Treatment			
	PBS/AA ^b	PBS/CII	11B11/CII	Rat IgG/CII
20	0/15 (0) ^c	0/15 (0)	0/15 (0)	0/15 (0)
23	4/15 (27)	0/15 (0)*	3/15 (20)	0/15 (0)
27	11/15 (73)	2/15 (13)*	8/15 (53)**	1/15 (7)
30	14/15 (93)	6/15 (40)*	12/15 (80)**	3/15 (20)
35	14/15 (93)	7/15 (47)*	12/15 (80)**	6/15 (40)
40	14/15 (93)	7/15 (47)*	12/15 (80)**	6/15 (40)

^a Mice were s.c. immunized with CII at the base of the tail on day 0 and boosted on day 21. Either AA or CII was orally administered daily from day -10 to day -1. 11B11 mAb was i.p. injected 30 min before each oral administration of CII. PBS and rat IgG were injected as controls. The incidence of arthritis was examined on the indicated days. Data are representative of three experiments.

^b i.p. injected/fed.

^c Number of mice with arthritis/total number of mice used (% of incidence).

p* < 0.05 v. PBS/AA; *p* < 0.05 v. PBS/CII, χ^2 analysis.

in the CII-fed animals was 47% on day 35. When mice were treated with 11B11 mAb before feeding CII, the incidence of joint inflammation increased up to 80% on day 30. Treatment with control rat IgG failed to affect its incidence.

Oral administration of CII also markedly suppressed the severity of CIA (Fig. 1). However, suppression of the joint inflammation was significantly diminished by treatment with the neutralizing mAb against IL-4. There was no effect of normal rat IgG on the down-regulation of arthritis by the oral Ag.

Treatment with 11B11 mAb blocks suppression of anti-CII IgG2a Ab production by oral administration of CII

Feeding CII was followed by marked suppression of anti-CII IgG2a Ab production (Fig. 2). The levels of anti-CII IgG1 Abs were not affected by oral Ag. Treatment with 11B11 mAb markedly reduced the CII-specific IgG1 Ab levels. On the other hand, the reduction of anti-CII IgG2a Ab production in CII-fed mice was significantly diminished by mAb treatment. Normal rat IgG failed to modulate levels of both of the isotypes of anti-CII Abs in animals fed CII.

Treatment with 11B11 mAb blocks suppression of footpad DTH response to CII by oral administration of CII

Footpad DTH response to CII was markedly reduced by oral administration of CII (Fig. 3). The reduction of DTH response was

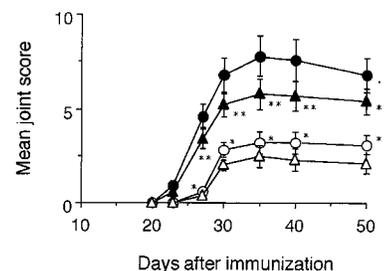


FIGURE 1. Treatment with 11B11 mAb diminishes suppression of the severity of CIA in mice by oral administration of CII. Mice were s.c. immunized with CII at the base of the tail on day 0 and boosted on day 21. Either AA or CII was orally administered daily from day -10 to day -1. 11B11 mAb was i.p. injected 30 min before each oral administration of CII. PBS and rat IgG were injected as controls. The severity of arthritis was examined on days 20, 23, 27, 30, 35, 40, and 50. ●, PBS injected and AA fed (PBS/AA); ○, PBS injected and CII fed (PBS/CII); ▲, 11B11 mAb injected and CII fed (11B11/CII); △, rat IgG injected and CII fed (rat IgG/CII). Bars show the mean \pm SEM of 4 to 14 arthritic mice. Data are representative of three experiments. *, Indicates *p* < 0.05 vs PBS/AA; **, *p* < 0.05 vs PBS/CII, Student's *t* test.

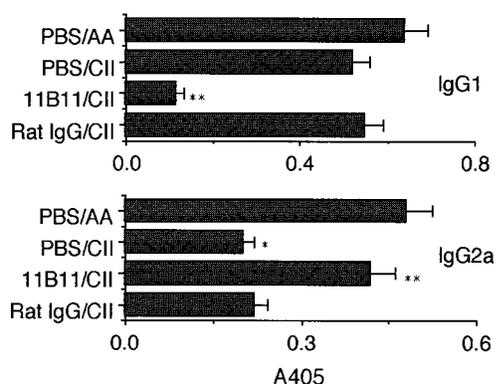


FIGURE 2. Treatment with 11B11 mAb diminishes suppression of anti-CII IgG2a Ab production in mice by oral administration of CII. Mice were s.c. immunized with CII at the base of the tail on day 0. Either AA or CII was orally administered daily from day -10 to day -1. 11B11 mAb was i.p. injected 30 min before each oral administration of CII. PBS and rat IgG were injected as controls. Sera were measured on day 20 for the measurement of anti-CII IgG1 and IgG2a Abs as described in *Materials and Methods*. PBS/AA, PBS injected and AA fed; PBS/CII, PBS injected and CII fed; 11B11/CII, 11B11 mAb injected and CII fed; Rat IgG/CII, rat IgG injected and CII fed. Bars show the mean \pm SEM of seven mice. Data are representative of three experiments. *, Indicates $p < 0.05$ vs PBS/AA; **, $p < 0.05$ vs PBS/CII, Student's *t* test.

significantly reversed by treatment with the anti-IL-4 mAb, while mice given control IgG showed no effect on the cellular immune response to CII.

Treatment with 11B11 mAb blocks suppression of the proliferative response to CII by oral administration of CII

Reduced proliferative responses of lymphoid cells to CII were observed in mice fed CII (Table II). When the animals were treated with 11B11 mAb, reduced cell proliferation was markedly diminished. Control rat IgG failed to affect the proliferative responses.

Treatment with 11B11 mAb blocks augmentation of IL-4 secretion and suppression of IFN- γ production by oral administration of CII

Table III shows the effect of 11B11 mAb on the secretion of IL-4 and IFN- γ from lymphoid cells in mice fed CII. The level of IL-4

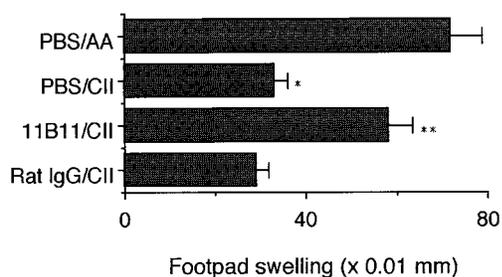


FIGURE 3. Treatment with 11B11 mAb diminishes suppression of the footpad DTH response to CII in mice by oral administration of CII. Mice were s.c. immunized with CII at the base of the tail on day 0. Either AA or CII was orally administered daily from day -10 to day -1. 11B11 mAb was i.p. injected 30 min before each oral administration of CII. PBS and rat IgG were injected as controls. The DTH response to CII was examined on day 12 as described in *Materials and Methods*. PBS/AA, PBS injected and AA fed; PBS/CII, PBS injected and CII fed; 11B11/CII, 11B11 mAb injected and CII fed; Rat IgG/CII, rat IgG injected and CII fed. Bars show the mean \pm SEM of seven mice. Data are representative of three experiments. *, Indicates $p < 0.05$ vs PBS/AA; **, $p < 0.05$ vs PBS/CII, Student's *t* test.

Table II. Proliferative responses of lymphoid cells to CII^a

Treatment	Proliferation (cpm)		
	CII (μ g/ml)		
	12.5	25	50
PBS/AA ^b	38,420 \pm 2,644	55,921 \pm 4,315	58,193 \pm 5,386
PBS/CII	11,692 \pm 8,452	16,193 \pm 1,369	18,317 \pm 2,016
11B11/CII	28,479 \pm 1,633	33,680 \pm 2,522	38,572 \pm 3,482
Rat IgG/CII	13,032 \pm 1,528	14,945 \pm 1,686	16,033 \pm 1,475

^a Mice were s.c. immunized with CII at the base of the tail on day 0. Either AA or CII was orally administered daily from day -10 to day -1. 11B11 mAb was i.p. injected 30 min before each administration of CII. PBS and rat IgG were injected as controls. Proliferative responses of lymphoid cells to the indicated doses of CII were measured on day 20. Background counts of CII-immunized lymphoid cells without CII added were between 1500 and 2500 cpm. Values are shown as mean \pm SEM of quadruplicate samples from culture supernatants of cells pooled from four mice.

^b i.p. injected/fed.

in CII-fed mice was 2.29 times higher than that in AA-fed mice when the cytokine was measured on day 7. On day 14, cytokine production in mice fed CII was 1.42 times greater than that in those fed AA. In contrast, there was no significant difference in IL-4 production between CII- and AA-fed mice on day 21. When the animals fed CII were treated with 11B11 mAb, the levels of IL-4 markedly dropped. In contrast, oral administration of CII resulted in suppression of the production of IFN- γ on days 7, 14, and 21. Suppression of IFN- γ production was significantly diminished by treatment with anti-IL-4 mAb.

Discussion

The present study demonstrates that treatment with the anti-IL-4 mAb 11B11 mAb (22) prevented the suppression of CIA in mice by the oral administration of CII, implying that IL-4 may play a role in its suppression. It was previously shown that feeding CII induced tolerance, resulting in the reduction of the incidence as well as the severity of CIA in mice (16, 17) and rats (24). However, a role for IL-4 in oral tolerance has not been shown previously, although active suppression by regulatory cells appeared to be involved in the down-regulation of CIA following oral administration of CII (16, 17). Other animal models of autoimmune diseases, including experimental autoimmune encephalomyelitis (13), experimental autoimmune uveoretinitis (14), and experimental autoimmune myasthenia gravis (15) have also been shown to be down-regulated by feeding a respective pathogenic autoantigen. Some of the studies demonstrated that inhibitory cytokines, including IL-4 and TGF- β , may play a role in suppression of the diseases by oral Ags (25, 26), based on the observation that the production of these cytokines is up-regulated both in vivo and in vitro following oral administration. More recently, Miller et al. demonstrated that anti-TGF- β sera abrogated protection of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein, suggesting a role for TGF- β in its suppression (27). However, no studies have directly shown in vivo the role of IL-4 in the modulation of autoimmune diseases by oral pathogenic Ags. To our knowledge, this is the first report of the effect of an anti-IL-4 Ab on the suppression of an autoimmune disease by an oral pathogenic Ag.

Oral administration of CII significantly suppressed production of the isotype of IgG2a, but not IgG1 Abs to CII. Suppression of anti-CII IgG2a Ab production by the oral Ag appears to be supported by the finding by Khare et al. that feeding an immunodominant peptide of CII suppresses the production of the isotype IgG2a of CII-specific Abs, although they also showed that anti-CII IgG1

Table III. The secretion of IL-4 and IFN- γ from lymphoid cells^a

Treatment	Cytokine Level (pg/ml)					
	Day 7		Day 14		Day 21	
	IL-4	IFN- γ	IL-4	IFN- γ	IL-4	IFN- γ
PBS/AA ^b	125 \pm 27	1668 \pm 184	246 \pm 24	2833 \pm 279	224 \pm 19	2492 \pm 264
PBS/CII	286 \pm 30*	322 \pm 34*	350 \pm 28*	415 \pm 45*	240 \pm 26	394 \pm 42*
11B11/CII	16 \pm 2**	1183 \pm 121**	24 \pm 3**	2033 \pm 197**	58 \pm 5**	1835 \pm 137**
Rat IgG/CII	260 \pm 28	304 \pm 24	378 \pm 32	435 \pm 37	210 \pm 24	362 \pm 28
11B11/AA	104 \pm 12	1892 \pm 243	220 \pm 16	2442 \pm 360	236 \pm 23	2238 \pm 268

^a Mice were s.c. immunized with CII at the base of the tail on day 0. Either AA or CII was orally administered daily from -10 to day -1. 11B11 mAb was i.p. injected 30 min before each administration of CII. PBS and rat IgG were injected as controls. IL-4 and IFN- γ secreted from lymphoid cells were measured on days 7, 14, and 21. Values are shown as mean \pm SEM of quadruplicate samples from culture supernatants of cells pooled from four mice.

^b i.p. injected/fed.

* p < 0.05 vs PBS/AA; ** p < 0.05 v. PBS/CII, Student's t test.

Ab production was reduced (28). Reduced anti-CII IgG2a Ab production in CII-fed mice was significantly reversed by treatment with 11B11 mAb. This finding may at least in part explain the mechanism of diminished suppression of CIA in CII-fed mice by 11B11 mAb treatment since anti-CII IgG2a Abs have been shown to mediate CIA. For instance, injection of purified anti-CII IgG2a Abs can induce arthritis in mice (29). IgG2a but not IgG1 Abs are known to fix complement, and complement fixation is required for the induction of CIA (30).

The correlation between diminished suppression of CIA by 11B11 mAb and diminished reduction of anti-CII IgG2a Ab production by the mAb suggests that CIA may be mediated by Th1 cells, a subset of CD4⁺ T cells, since IgG2a Ab production is Th1 cell dependent (31). Furthermore, this appears to be supported by the result that the effect of 11B11 mAb on CIA in CII-fed mice was associated with blockade of down-regulation of the footpad DTH response to CII that was mediated by Th1 cells (32). Not only anti-CII IgG2a Abs but also CII-specific lymphoid cells, cell lines, and clones have been shown to transmit CIA (8, 9).

Oral administration of CII resulted in augmentation of secretion of the Th1 cytokine IL-4 when the cytokine was measured on days 7 and 14, although the degree of the augmentation of the cytokine secretion appeared to be greater on day 7 than on day 14. There was no significant effect of the oral Ag on IL-4 secretion on day 21. In contrast, production of the Th1 cytokine IFN- γ was markedly suppressed by the oral Ag on days 7, 14, and 21. These results suggest that feeding CII modulates IL-4 cytokine secretion temporally, while oral Ag affects IFN- γ production persistently. Augmented IL-4 secretion in CII-fed mice appears to have contributed to the suppression of CIA, the DTH response to CII, anti-CII IgG2a Ab production, and IFN- γ secretion, since IL-4 has been shown to suppress these Th1 responses (33). Treatment with 11B11 mAb markedly reduced secretion of IL-4, while suppression of IFN- γ production was significantly diminished by the anti-IL-4 Ab. Therefore, the increased IFN- γ production as well as the decreased IL-4 production following mAb treatment might have resulted in blocking suppression of CIA, anti-CII IgG2a Ab production, and the DTH response to CII, since IFN- γ up-regulates Th1 responses (34). Our results are supported by the previous finding that anti-IL-4 Abs increase DTH responses as well as IFN- γ secretion, but decrease IL-4 secretion (35), although the effect of the Abs on secretion of both of the cytokines in orally tolerized animals has not been examined previously.

Friedman and Weiner demonstrate that low doses (<1 mg) of oral Ags favor cytokine-mediated active suppression and high doses (>5 mg) favor anergy (36). Our results suggesting a role of IL-4 in the induction of tolerance to CII following oral adminis-

tration of the 1-mg dose of CII appear to support their findings. Furthermore, as shown in the present study, the data that the suppression of the proliferative response to CII in CII-fed mice was diminished following the 11B11 mAb treatment also suggest that active suppression may be at least in part involved in the suppression.

In summary, treatment with an anti-IL-4 mAb appears to be effective in blocking suppression of CIA by oral administration of CII. Blockade of the suppression of CIA by the mAb was associated with a decrease in augmented IL-4 secretion in CII-fed mice, suggesting that the cytokine may play a role in its suppression. Treatment with 11B11 mAb was also associated with abrogation of the suppression of Th1 responses, including anti-CII IgG2a Ab production, DTH responses to CII, and IFN- γ secretion.

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