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Effect of a monoclonal antibody against interleukin-4 on collagen-induced arthritis in mice

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1 The present study was undertaken to investigate the effect of a monoclonal antibody (11B11 mAb) against interleukin-4 (IL-4) on collagen-induced arthritis (CIA) in mice.

2 11B11 mAb was daily injected intraperitoneally over a period of 10 days, commencing on the day of immunization with type II collagen (CII).

3 The results showed that the anti-IL-4 mAb markedly augmented both the incidence and the severity of CIA. The augmentation of the disease was associated with a significant increase in anti-CII IgG2a antibody production, proliferative responses of lymph node cells to CII and interferon-γ (IFN-γ) secretion from the lymphoid cells. The production of anti-CII IgG1 antibodies the secretion of IL-4 was markedly reduced in the mAb-treated mice.

4 Thus, the neutralization of IL-4 by 11B11 mAb appears to be effective in augmenting CIA.

Keywords: Collagen-induced arthritis; monoclonal antibody; interleukin-4; interferon-γ

Introduction

Collagen-induced arthritis (CIA) is an experimental model of autoimmune disease and can be induced in mice (Courtenay *et al*., 1980), rats (Trentham *et al*., 1977) and primates (Yoo *et al*., 1988) by immunization with type II native articular cartilage collagen (CII). Many features of CIA resemble those of rheumatoid arthritis in man (Trentham, 1982; Stuart *et al*., 1982b). 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 antibodies specific for CII (Stuart *et al*., 1982a; Wooley *et al*., 1984). Adoptive transfer of lymphoid cells from animals immunized with CII (Trentham *et al*., 1978) and of CII-specific T cell lines and clones (Holmdahl *et al*., 1985) also transmits the disease.

Interleukin-4 (IL-4) was originally identified as a mediator that induces the proliferation of murine B lymphocytes (Howard *et al*., 1982). IL-4 also can differentiate activated B cells into antibody-producing plasma cells (Isakson *et al*., 1982). Cellular analysis revealed that the source of the cytokine was Th2 cells, a subset of $CD4^+$ T cells (Mosmann & Coffman, 1989). More recently, IL-4 has been shown to be crucial in the production of IgG1 and IgE antibodies, while this cytokine inhibits the production of IgG2a antibodies in which Th1 cells play a role (Finkelman *et al*., 1988). These findings suggest that the neutralization of IL-4 by an anti-IL-4 antibody may modulate CIA. However, the previous studies by Joosten *et al*. (1997) failed to demonstrate the modulation of CIA by treatment with an anti-IL-4 monoclonal antibody. Neither IgG1 nor IgG2a antibodies against CII was affected by the IL-4 neutralizing monoclonal antibody. The failure of the modulation of CIA as well as anti-CII antibody production by the anti-IL-4 antibody seen in the studies appeared to be due to the inappropriate timing of the antibody injection. In the studies, the anti-IL-4 monoclonal antibody was injected either on day 29 or day 32 after immunization with CII, when anti-CII antibody production had already been established. Therefore, in the present study, mice were treated with the anti-IL-4 antibody from the time of immunization with CII. The results showed that the treatment with the IL-4 neutralizing antibody was followed by marked augmentation of both the incidence and the severity of CIA. The results also showed that the augmentation of the disease by the anti-IL-4 monoclonal antibody was associated with a decrease in the production of anti-CII IgG1 antibodies, while anti-CII IgG2a antibody production was increased in the animals. The secretion of IL-4 was markedly suppressed in the antibody-treated mice. In contrast, the production of interferon-γ (IFN-γ) was augmented following the administration of the anti-IL-4 antibody.

Methods

Animals

Female DBA/1J mice, 8 to 9 weeks 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 type II collagen extracted from native calf articular cartilage (Funakoshi, Co., Tokyo, Japan) was dissolved in 1 ml of 0.1 N acetic acid and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI); 50 μl of the emulsion containing 50 μg of CII 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 oedema of the periarticular tissue, as described by Wood *et al*. (1969). The maximum possible score is 16.

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Treatment with 11B11 mAb

The cell line (11B11) for a rat IgG1 mAb against murine IL-4 was kindly provided from the Department of Immunology (Saga Medical School, Saga, Japan). 11B11 mAb was precipitated by ammonium sulphate from ascitic fluid of SCID mice inoculated with the cells and purified with a protein G Sepharose 4FF column (Pharmacia Biotec, Tokyo, Japan). The protein content was quantified by absorbance measurement at 280 nm. The preparation and characterization of 11B11 mAb have been described previously (Ohara & Paul, 1985). Varying doses of 11B11 mAb dissolved in 0.5 ml of PBS were injected once daily i.p. over a period of 10 days, commencing on day 0. As treatment controls, 0.5 ml of PBS only and 0.5 ml of PBS containing normal rat serum IgG, purified as described above, were given to mice.

Measurement of anti-CII antibodies

Blood was collected on day 26 after immunization with CII and sera were heat inactivated at 56°C for 30 min. Anti-CII IgG1 and IgG2a antibodies were measured with an ELISA (Enavall & Perlmann, 1972). In brief, 96-well flat-bottomed microtiter plates were incubated with 100 μl/well of CII (100 μg ml^{-1}) 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 μl of PBS containing 1% ovalbumin (Sigma) at 37°C for 1 h. After washing, the plates were incubated with 100 μl of a 1:600 dilution of each serum sample at 37°C for 30 min. The plates were washed and 100 μl/well of a 1:1,000 dilution of rat anti-mouse IgG1, or IgG2a labelled with alkaline phosphatase (Pharmingen, San Diego, CA), was added and incubated at 37°C for 1 h. After washing, 100 μl 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. The absorbance was then measured at 405 nm in a Titertec Multiscan spectrophotometer (EFLAB, Helsinki, Finland). The results are expressed as absorbance units at OD405 $\pm$ s.e. mean.

Measurement of delayed type hypersensitivity (DTH)

On day 14 after immunization with CII, 10 μg of CII dissolved in 20 μl of 0.005 N acetic acid was injected s.c. into the right footpad. As a vehicle control, 20 μl of 0.005 N acetic acid was injected into the left footpad. The thickness of the right and left footpad were measured with 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 acetic acid 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 μl of RPMI 1640 (Flow Laboratories, Inc., Mclean, VA) containing 1 mM glutamine, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 5×10^{-5} M 2-mercaptoethanol and 1% heat-inactivated autologous mouse serum were added to each microwell followed by the addition of 100 μl of 50 μg ml^{-1} CII. The cells were cultured for 72 h. Each well was pulsed with 0.5 μCi of tritiated thymidine and the cells were cultured for another 16 h. The cultures were harvested onto fibre glass filters with a multiharvester and counted by standard liquid scintillation techniques. Results, expressed in c.p.m., are average of quadruplicate cultures of cells pooled from four mice.

Cytokine measurement

Single cell suspensions from inguinal lymph nodes were resuspended at a final concentration of 5×10^6 cells ml^{-1} and cultured in 1 ml aliquots in 24-well tissue culture plates either in medium alone or with 50 μg ml^{-1} CII. Forty-eight hours later, supernatants were harvested and stored at -70°C until assayed. Cytokine production was quantified by use of sandwich ELISA techniques. Briefly, supernatants were added to 96-well microtitre plates, previously coated overnight at 4°C with 100 μl of anti-IL-4 (4 μg ml^{-1}) and anti-IFN-γ (4 μg ml^{-1}) antibodies (Pharmingen) in 0.1 M $NaHCO_3$ buffer. Plates were then washed twice with PBS containing 0.05% Tween 20, after which non-specific protein-binding sites were blocked by incubation with 100 μl of PBS containing 1% ovalbumin at 37°C for 1 h. After the binding sites had been blocked, 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 μl and incubated at 37°C for 1 h. Plates were washed three times and 100 μl/well biotinylated anti-murine IL-4 (2 μg ml^{-1}) and IFN-α (2 μg ml^{-1}) antibodies (Pharmingen) diluted in PBS/1% ovalbumin were added. After incubation at 37°C for 1 h, the plates were washed three times and 100 μl/well streptavidin-alkaline phosphatase (Pharmingen) were added at 2 μg ml^{-1}. The plates were washed before 100 μl of *p*-nitrophenylphosphate were added to each well. Plates were read at 405 nm with an automatic microplate reader. Cytokine levels were determined with reference to a standard curve constructed with serial dilutions of the standard cytokines and results are expressed in pg ml^{-1}.

Results

Effect of 11B11 mAb on CIA

When mice were treated with PBS, one of 12 mice (8%) showed signs of arthritis on day 26 and the incidence of arthritis increased up to 58% by day 35 (Table 1). On the other hand, 4 of 12 mice (33%) treated with 1 mg of 11B11 mAb began to develop arthritis on day 18 and all mice had joint

Table 1 Treatment with 11B11 mAb augmented the incidence of collagen-induced arthritis

Days after immunization	*Treatment*		
	PBS	*11B11*	*Rat IgG*
14	0/12 (0)[#]	0/12 (0)	0/12 (0)
18	0/12 (0)	4/12 (33)	0/12 (0)
22	0/12 (0)	9/12 (75)	0/12 (0)
26	1/12 (8)	12/12 (100)	0/12 (0)
30	3/12 (25)	12/12 (100)	3/12 (25)
35	7/12 (58)	12/12 (100)	6/12 (50)
40	7/12 (58)	12/12 (100)	7/12 (58)

Mice were s.c. immunized with CII at the tail base on day 0 and boosted on day 21 as described in Methods. 11B11 mAb (1 mg) was injected daily i.p., from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. The incidence of arthritis was examined on the days indicated. Data are representative of three experiments. [#]% incidence.

inflammation on day 26. The incidence of arthritis in mice treated with control rat IgG was similar to that in animals treated with PBS. The severity of the disease was also greater in 11B11 mAb-treated mice than in either PBS- or rat IgG-treated animals at all times examined (Figure 1). When mice were treated with 0.04, 0.2 and 1 mg of 11B11 mAb, the incidence of arthritis, as well as the severity of the disease, were augmented in a dose-related fashion (Table 2).

Effect of 11B11 mAb on anti-CII antibody production

To determine whether the augmentation of CIA by 11B11 mAb was associated with the production of anti-CII antibodies in the serum, the two isotypes IgG1 and IgG2a of CII-specific antibodies were measured by ELISA. As shown in Figure 2, treatment with 11B11 mAb markedly suppressed the production of anti-CII IgG1 antibodies. In contrast, anti-CII IgG2a antibody production was significantly enhanced by the anti-IL-4 antibody. No effect of rat IgG on the formation of either isotype of anti-CII antibody was observed.

Effect of 11B11 mAb on the footpad DTH response to CII

The effect of the neutralization of IL-4 by 11B11 mAb on the footpad DTH response to CII was examined. The results are shown in Figure 3. Treatment with the anti-IL-4 mAb led to marked augmentation of the DTH response to the antigen. Treatment with rat IgG failed to affect the footpad DTH response to CII.

Effect of 11B11 mAb on the proliferative response to CII

The effect of 11B11 mAb on the proliferative response to CII of lymph node cells was also investigated. As shown in Figure 4, the proliferation to CII of lymph node cells in mice treated with the anti-IL-4 mAb was greater than that in those treated with either PBS or rat IgG.

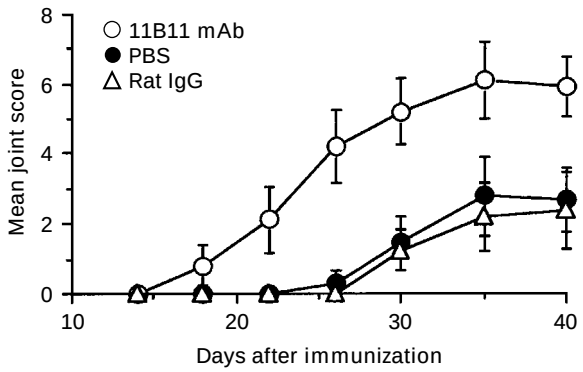

Figure 1 Treatment with 11B11 mAb augmented the severity of collagen-induced arthritis. Mice were immunized (s.c.) with CII at the tail base on day 0 and boosted on day 21 as described in Methods. 11B11 mAb 1 mg was injected daily i.p. from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. The severity of arthritis was evaluated on days 14, 18, 22, 26, 30, 35, and 40. The mean and s.e.mean (vertical lines) of 12 mice are shown. Data are representative of three experiments.

Table 2 Effects of varying doses of 11B11 mAb on collagen-induced arthritis

11B11 (mg)	*Incidence of arthritis*	*Mean joint score*
0 (PBS)	1/10 (10)[#]	0.3±0.30
0.04	3/10 (30)	1.2±0.54
0.2	7/10 (70)	2.4±0.80
1.0	10/10 (100)	5.2±0.63

Mice were immunized s.c. with CII at the tail base on day 0 and boosted on day 21 as described in Methods. The indicated doses of 11B11 mAb were injected daily, i.p., from day 0 to day 10. PBS was injected as a control. The incidence and the severity of arthritis were examined on day 26. Data are representative of three experiments. [#]% incidence.

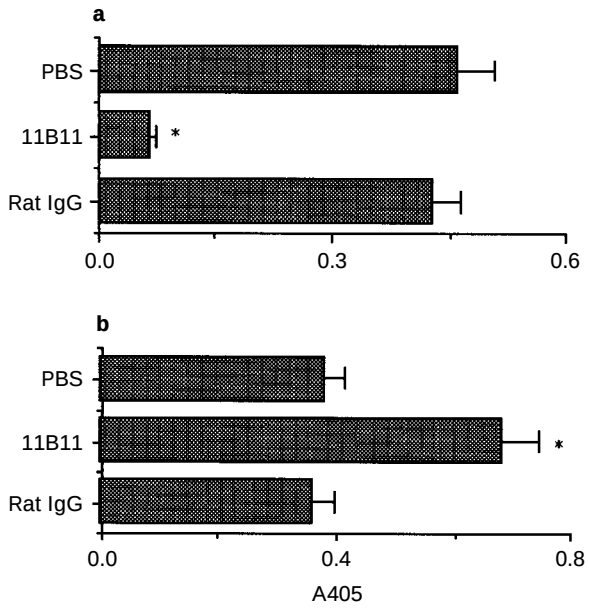

Figure 2 Treatment with 11B11 mAb (a) suppressed the production of anti-CII IgG1 antibodies but (b) augmented the production of anti-CII IgG2a antibodies. Mice were immunized s.c. with CII at the tail base on day 0 and boosted on day 21 as described in Methods. 11B11 mAb 1 mg was injected daily i.p. from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. Serum samples were collected on day 26 and individually assayed for anti-CII IgG1 and IgG2a antibodies by ELISA. Columns show the mean±s.e. mean of 5 mice. Data are representative of three experiments. $^{*}P<0.05$ versus PBS-treated mice, Student's *t* test.

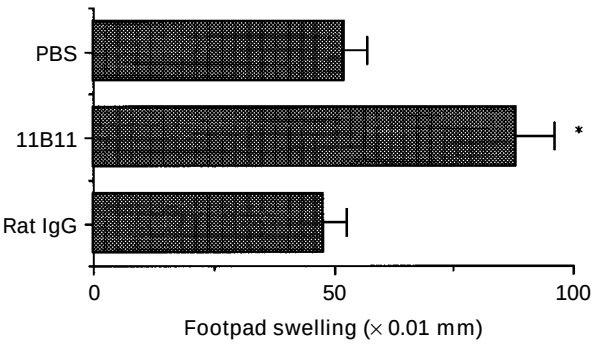

Figure 3 Treatment with 11B11 mAb augmented footpad DTH responses to CII. Mice were immunized s.c. with CII at the tail base on day 0. 11B11 mAb 1 mg was injected daily, i.p., from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. On day 14 after immunization, CII was injected s.c. into the footpad and 24 h later the footpad thickness was determined as described in Methods. Columns show the mean±s.e.mean of 7 mice. Data are representative of three experiments. $^{*}P<0.05$ versus PBS-treated mice, Student's *t* test.

Effect of 11B11 mAb on the secretion of IL-4 and IFN-γ from lymph node cells

Table 3 shows the effect of 11B11 mAb on the secretion by lymph node cells of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. Treatment of mice with the mAb markedly suppressed IL-4 secretion, while the secretion of IFN-γ was significantly enhanced by the anti-IL-4 antibody.

Discussion

The present study demonstrates that treatment with the monoclonal antibody 11B11 mAb against IL-4 (Ohara & Paul, 1985), that was commenced on the day of immunization with CII, was followed by marked augmentation of both the incidence and the severity of CIA in mice (Courtenay *et al*., 1980). Importantly, it is of note that 33% of 11B11 mAb-treated mice showed signs of joint inflammation before the second immunization with CII. In general, a booster injection of CII is essential for the induction of autoimmune arthritis (Courtenay *et al*., 1980). Indeed, in our experiments none of the mice treated with either PBS or control rat IgG developed arthritis before day 21 when the animals had the second injection of the cartilage component antigen.

The production of anti-CII IgG1 antibodies that needs the help of Th2 cells (Boom *et al*., 1988) was markedly reduced by treatment with 11B11 mAb. Conversely, the mAb treatment was effective in enhancing anti-CII IgG2a production which is dependent on Th1 cells (Jain *et al*., 1996). These results appear to be supported by previous findings, that IL-4 augments Th2 cell-dependent antibody production, but suppresses Th1 cell-dependent antibody production (Finkelman *et al*., 1988). Therefore, the augmentation of CIA by the neutralizing mAb to IL-4 seems to be due to an increase in the production of anti-CII IgG2a antibodies. The important role of anti-CII IgG2a antibodies in CIA has been shown previously. For instance, injection of purified anti-CII IgG2a antibodies can induce arthritis in mice (Kerwar *et al*., 1983; Hirofuji *et al*., 1985). Oral administration of type II collagen suppresses CIA in mice and in these animals the production of the IgG2a isotype of CII-specific antibodies was downregulated (Thompson *et al*., 1993). IgG2a but not IgG1 antibodies are known to fix complement and complement fixation is required for the induction of CIA (Morgan *et al*., 1981). We have more recently found that antibody-mediated footpad swelling in mice in which the Arthus reaction plays a role is augmented by 11B11 mAb (unpublished observations).

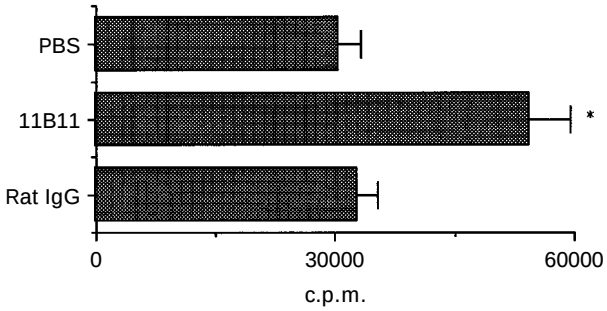

Figure 4 Treatment with 11B11 mAb augmented the proliferative responses to CII. Mice were immunized s.c. with CII at the tail base on day 0. 11B11 mAb (1 mg) was injected daily, i.p., from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. On day 14 after immunization, proliferative responses of lymph node cells to CII were determined as described in Methods. Columns show the mean ± s.e.mean of quadruplicate cultures of cells pooled from 4 mice. Data are representative of three experiments. $^{*}P<0.05$ versus PBS-treated mice, Student's *t* test.

Table 3 Effect of 11B11 mAb on the secretion of IL-4 and IFN-γ from lymph node cells

Group	*IL-4* (pg ml^{-1})	*IFN-γ* (pg ml^{-1})
PBS	286 ± 22	3563 ± 285
11B11	42 ± 7*	6279 ± 488*
Rat IgG	260 ± 18	3712 ± 358

Mice were immunized s.c. with CII at the tail base on day 0. 11B11 mAb 1 mg was injected daily, i.p., from day 0 to day 10. PBS and 1 mg of rat IgG were injected as controls. On day 14 after immunization, lymph nodes were removed and the secretion of IL-4 and IFN-γ from the lymphoid cells was examined by sandwich ELISA as described in Methods. Values are the mean ± s.e.mean of quadriplicate samples from culture supernatants of cells pooled from 4 mice. Data are representative of three experiments. $^{*}P<0.05$ versus PBS-treated mice, by Student's *t* test.

Recently, Joosten *et al*. (1997) showed that when the treatment with 11B11 mAb commenced on day 29 or on day 32 after immunization with CII it failed to affect CIA as well as the production of anti-CII IgG1 and IgG2a antibodies, although anti-IL-10 antibodies or a combination of anti-IL-4 and anti-IL-10 antibodies were effective in enhancing CIA. These results suggest that anti-IL-4 antibodies themselves lack the ability to modify ongoing immune responses to CII. Unfortunately, in this study the effect of 11B11 mAb on CIA, by treating mice with the mAb before immune responses to CII were established, was not investigated.

As shown in our experiments, the footpad DTH response to CII was enhanced by the treatment with 11B11 mAb. Because DTH responses are known to be mediated by Th1 cells (Fong & Mosmann, 1989), the enhancement of the DTH response to CII by the anti-IL-4 mAb also appears to support the finding that Th1 cell-mediated responses, such as the production of CII-specific IgG2a antibodies, are involved in CIA.

The secretion of IL-4 was markedly decreased in 11B11 mAb-treated mice, while the antibody treatment increased IFN-γ secretion. These results are consistent with the finding that anti-IL-4 antibodies suppress IL-4 secretion but enhance IFN-γ production in a murine model of parasite infection (Cheever *et al*., 1995). The results also seem to support the hypothesis that IL-4 suppresses IFN-γ secretion but augments IL-4 production (Finkelman *et al*., 1988). Since IFN-γ plays a crucial role in Th1 cell-mediated responses (Cher & Mosmann, 1987), the enhancement of anti-CII IgG2a antibody production is probably due to the increase in IFN-γ secretion. In contrast, the decrease in IL-4 secretion in 11B11 mAb-treated mice seems to lead to a suppression of anti-CII IgG1 antibody production, in which the cytokine plays a role.

Alternatively, the augmentation of CIA by 11B11 mAb may also be due to the blockade of the inhibition of the production of proinflammtory cytokines by IL-4. For instance, in experimental animals, this cytokine has been shown to inhibit the production of IL-1 (Essner *et al*., 1989; Vannier *et al*. 1992), IL-6 (Yanagawa *et al*., 1991; Szabo *et al*., 1991), IL-8 (Standiford *et al*., 1990) and tumour necrosis factor-α (Hart *et al*., 1989; Te Velde *et al*., 1990) that all play a role in inflammation. IL-4 is also known to prevent the production of superoxide as well as reactive nitrogen intermediates (NO) (Bogdan *et al*., 1991). Furthermore, in the patients with rheumatoid arthritis, IL-4 suppresses the production of the

above cytokines and reduces bone resorption (Miossec *et al*., 1994; Sugiyama *et al*., 1995).

In summary, treatment with an anti-IL-4 mAb was effective in augmenting CIA. The augmentation of the joint inflammation by the mAb was associated with increases in Th1 responses including IFN-γ secretion and anti-CII IgG2a antibody prodution that appeared to be critically involved in the disease. It is also possible that the augmented CIA in mice treated with the IL-4-neutralizing mAb may be due to the abrogation, by the mAb, of the inhibition of the production of proinflammatory cytokines by IL-4.

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