Synergistic Effect on the Attenuation of Collagen Induced Arthritis in Tumor Necrosis Factor Receptor I (TNFRI) and Interleukin 6 Double Knockout Mice

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ABSTRACT. ***Objective.*** To evaluate any additive effect on attenuation of collagen induced arthritis (CIA) in tumor necrosis factor receptor I (TNFRI) and interleukin 6 (IL-6) double knockout (DKO) mice.

Methods. CIA was induced in wild-type (Wt), TNFRI knockout (TNFRIKO), IL-6 knockout (IL-6KO), and DKO mice. Comparative studies were performed among these different mouse genotypes observing clinical (incidence, arthritis score), histological, radiologic, and immunological aspects.

Results. More than 90% of the Wt, TNFRIKO, and IL-6KO mice developed definite CIA, while only 20% of the DKO mice did so. Severity of arthritis, indicated by the arthritis score, was significantly reduced in both the TNFRIKO and IL-6KO mice compared with the Wt mice. Moreover, the severity of arthritis in the DKO mice was significantly reduced compared with each single KO mouse (by arthritis scores; DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$). In addition, histological and radiologic changes were also significantly reduced in the DKO mice compared with each single KO mouse (by histological and radiologic scores; DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$ and $p < 0.01$ respectively). In immunological studies, serum anti-type II collagen (anti-CII) antibody concentrations were significantly decreased in the DKO mice compared with each single KO mouse (DKO vs TNFRIKO, IL-6KO mice, $p < 0.01$).

Conclusion. Simultaneous blockade of TNFRI and IL-6 showed synergistic rather than additive effects on the attenuation of CIA. Combinations of anti-TNF-α and anti-IL-6 therapy may provide clinical benefits for treatment of rheumatoid arthritis compared with therapy against each single cytokine. (J Rheumatol 2003;30:22–7)

Key Indexing Terms:
TUMOR NECROSIS FACTOR RECEPTOR I
DOUBLE KNOCKOUT MICE
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COLLAGEN INDUCED ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by bone destructive polyarthritis. Although its etiology remains unknown, cumulative evidence suggests that proinflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor (TNF), and IL-6 play a pivotal role in the pathology of RA[1–3]. These proinflammatory cytokines are suggested to be optimal therapeutic targets for RA. Indeed, the outcome of recent clinical trials of anticytokine therapies using neutralizing antibodies, soluble receptors, and other inhibitors encouraged us[4-7]. Among them, anti-TNF therapy using neutralizing antibodies or soluble TNF receptors (TNFR) showed excellent efficacy and is currently accepted as a promising new therapy for RA[5,6]. In addition, a recent study showed that IL-6 blockade by anti-IL-6 receptor (IL-6R) antibody also showed some clinical efficacy for RA, and multicenter clinical trials are proceeding in Japan[7]. However, despite the excellent efficacy, these single cytokine blockade therapies do not always attenuate disease activity sufficiently, and there are still some populations of non-optimal responders for each therapy directed against a single cytokine[8]. In such cases of non-optimal responders for each single cytokine therapy, combination with other types of therapy was suggested to be necessary. Indeed, the combination of anti-TNF-α and anti-T cell (anti-CD4) therapies showed enhanced efficacy in a mouse arthritis model[9].

There may be another potential problem in inhibiting a single cytokine as a therapeutic approach for RA, which relates to the overlapping activities of different cytokines. This problem can possibly be addressed by simultaneous blockade of different cytokines. Partial attenuation of

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collagen induced arthritis (CIA), a mouse model for RA, was reported in TNF-receptor I (TNFRI)[10] and IL-6 deficient mice[11], suggesting that single cytokine blockade may not ameliorate CIA sufficiently.

To investigate whether simultaneous blockade of TNF and IL-6 shows any additive effect on the attenuation of CIA, we carried out a comparative study in wild-type (Wt), TNFRI knockout (TNFRIKO), IL-6 knockout (IL-6KO), and double knockout (DKO) mice, observing clinical, histological, radiologic, and immunological aspects.

MATERIALS AND METHODS

Mice. Animal experiments in this study were approved by the Animal Experimentation Committee of Osaka University and performed in accord with their guidelines. IL-6KO mice were provided by Dr. M. Kopf, Max-Planck-Institut für Immunbiologie, Freiburg, Germany[12]. TNFRIKO mice were provided by Dr. T.W. Mak, Amgen Institute, Ontario Cancer Institute, Toronto, Canada[13]. IL-6KO mice and TNFRIKO mice were backcrossed with DBA/1 mice (Nippon Charles River, Kanagawa, Japan) for 8 generations to introduce CIA susceptibility. Then TNFRI and IL-6 DKO mice were generated by matching these mice. Mice were screened for IL-6 and TNFRI genotype by polymerase chain reaction (PCR) using DNA prepared from tail biopsies as described[12,13]. The Wt, IL-6KO, TNFRIKO, and DKO mice were between 8 and 12 weeks old at the time of first immunization. The ratio between male and female was kept equal in each group. Mice were bred under standard pathogen-free conditions in the Experimental Animal Center of Osaka University Medical School.

Induction of CIA. CIA was induced by established methods[14]. Briefly, mice were immunized by intradermal injection at the base of the tail with 100 µg of bovine type II collagen (CII) (Cosmo Bio, Tokyo, Japan) in 0.1 M acetic acid, emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA). Twenty-one days later, mice were boosted by the same method.

Clinical assessments of arthritis. Every week after the first immunization, during the entire followup period (15 weeks), mice were assessed by 2 independent observers for signs of arthritis. The severity of arthritis was graded on a 0–4 scale as follows: 0 = normal; 1 = swelling and/or redness in only 1 finger; 2 = swelling and/or redness in more than 1 finger; 3 = swelling and/or redness in the entire paw; 4 = deformity and/or ankylosis. Each paw was graded, and the 4 scores were summed, so the maximum possible score per mouse was 16. The group arthritis score was calculated by dividing the total score by the total number of mice that developed definite arthritis. The incidence was expressed as the percentage of the mice that showed visible arthritis.

Histological assessment of arthritis. Histological examinations were carried out as follows. Seven mice were randomly selected from the Wt, TNFRIKO, and IL-6KO groups. In the DKO group, 3 mice that developed definite arthritis were selected. They were killed at the end of the experiment. Both hind paws were removed, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), decalcified in hydrochloric acid and formic acid, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Microscopic evaluation was performed under blinded conditions. The severity of arthritis in each joint was classified as described[15] and graded 0–3 as follows: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.

Radiologic assessment of arthritis. Mice were killed at the end of the experiment. Anteroposterior radiographs of the 4 limbs were obtained using a cabinet soft X-ray apparatus (CMB-2; Softex, Tokyo, Japan). Radiologic changes were evaluated under blinded conditions using a described scoring system[16], with some modification. Each limb was assessed for osteopenia and bone erosion and graded 0–3 as follows: 0 = normal; 1 = mild change; 2 = moderate change; 3 = severe change. The 4 scores were summed, for a maximum possible score per mouse of 12.

Measurements of serum anti-CII antibodies and nonspecific total IgG. Sera from mice of each genotype were obtained at the end of the experiment. Serum anti-CII antibody and nonspecific total IgG concentrations were measured by ELISA as described with some modification[17]. Briefly, 96 well plates were coated with bovine CII antigen solution (2 µg/ml) or anti-mouse IgG (Vector, Burlingame, CA, USA) solution (1 µg/ml) and incubated overnight at 4°C. Nonspecific binding was blocked with PBS containing 1% bovine serum albumin for 1 h at room temperature. Serially diluted serum samples (1:10 to 1:10,000) were added for 2 h at room temperature. Alkaline phosphatase conjugated horse anti-mouse IgG (Vector) or alkaline phosphatase conjugated goat anti-mouse IgG1 or IgG2a (Vector) was also added for 2 h at room temperature. Color development with p-nitrophenylphosphate (Kirkegaard and Perry, Gaithersburg, MD, USA) was monitored at 405 nm with an immunoreader NJ-2300 (Nihon InterMed, Tokyo, Japan). Values from each mouse genotype were shown as relative values compared with the mean value of the Wt group as 100 units.

Statistical analysis. Values were expressed as means ± SEM and variance analyses were performed before further statistical analyses. Using a Statview 5.0 statistical package for Macintosh (Abacus, Berkeley, CA, USA), the Mann-Whitney U test was used to assess the significance of the relative arthritis score, incidence, radiologic assessments, and antibody levels. Student t test was used to assess the significance of the histological findings. P values < 0.05 were considered significant.

RESULTS

Incidence and severity of CIA. CIA was induced in each different mouse genotype as described in Materials and Methods. As shown in Figure 1A, mice started to develop arthritis 2 weeks after the first immunization in the Wt, one week later in the TNFRIKO, 3 weeks later in the IL-6KO, and 4 weeks later in the DKO group. At the end of the experiment, all 19 mice had developed arthritis in the Wt, 23 of 24 in the TNFRIKO, 15 of 17 in the IL-6KO group, while CIA was observed in only 4 of 21 DKO mice. Thus, in the DKO mice, the incidence of arthritis was significantly lower compared with the Wt, TNFRIKO, and IL-6KO mice (DKO vs Wt, TNFRI, IL-6KO mice, $p < 0.01$). As shown in Figure 1B, the arthritis score on each single cytokine KO mouse was lower compared with the Wt mice, as previously reported[10,11]. In addition, the arthritis score of the DKO mice that developed arthritis was significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO mice, $p < 0.05$).

Histological changes. Both hind paws were removed at the end of the experiment, and histological changes were evaluated and scored as described in Materials and Methods. As a representative photograph shows (Figure 2), the ankle joint was almost completely destroyed with severe synovitis in the Wt mice (Figure 2, panels A and E). Marked joint destruction and synovitis were detected in the TNFRIKO mice, but were less severe compared with the Wt mice (panels B and F). In the IL-6KO mice, only moderate synovitis was observed (panels C and G), as reported[11]. In contrast, in the DKO mice, the ankle joints were almost completely preserved (panels D and H). The overall histological changes of each mouse genotype are summarized in Table 1. The histological score on each single cytokine KO

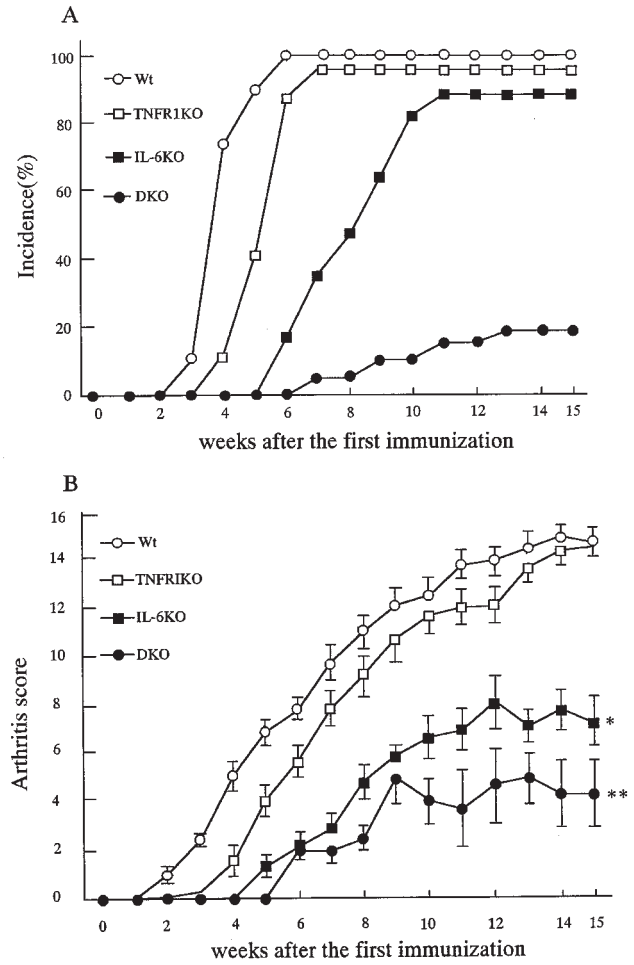

Figure 1. Development of CIA in Wt, IL-6KO, TNFRIKO, and DKO mice. DBA/1 mice of Wt, TNFRIKO, IL-6KO, and DKO mice were immunized with type II collagen in adjuvant, as described in Materials and Methods. Signs of arthritis were recorded every week from the first immunization through the entire followup period (15 weeks). A. Incidence of arthritis. B. Arthritis scores (means ± SEM). *p < 0.05 versus Wt, TNFRIKO mice. **p < 0.05 versus Wt, TNFRIKO, IL-6KO mice at the end of the experiment.

mouse was lower compared with the Wt mice, as reported[10,11]. In addition, the histological score of the DKO mice that developed arthritis was significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO mice, p < 0.05).

Table 1. Histological scores in each mouse genotype. Values are expressed as means ± SEM.

Genotype (No. of Mice Studied)	Histological Score
Wt (7)	2.63 ± 0.28
TNFRIKO (7)	2.00 ± 0.20*
IL-6K0 (7)	1.43 ± 0.37**
DKO (3)	0.67 ± 0.33***

* p < 0.05 versus Wt mice. ** p < 0.05 versus Wt, TNFRIKO mice. *** p < 0.05 versus Wt, TNFRIKO, and IL-6KO mice.

Radiologic changes. The summary of radiologic changes (osteopenia and bone erosion) of each mouse genotype is shown in Table 2. Each single cytokine KO mouse showed significant reductions in the radiologic scores compared with the Wt mice. The DKO mice showed significantly lower radiologic scores than those of each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO, p < 0.01), concordant with their histological changes.

Serum anti-CII antibody and nonspecific total IgG levels. Serum anti-CII antibody and nonspecific total IgG levels were measured by ELISA. As shown in Table 3, each single cytokine KO mouse showed significantly lower anti-CII antibody levels in total IgG, IgG1, and IgG2a than those of the Wt mice (p < 0.01). Serum anti-CII antibody levels of the DKO mice in total IgG, IgG1, and IgG2a were significantly lower compared with each single cytokine KO mouse (DKO vs TNFRIKO, IL-6KO, p < 0.01). However, nonspecific total IgG levels were not significantly decreased in each single cytokine KO and DKO mouse.

DISCUSSION

We performed a comparative study in Wt, TNFRIKO, IL-6KO, and DKO mice induced with CIA, observing clinical, histological, radiologic, and immunological findings. Partial attenuation of CIA was observed in the TNFRIKO mice as described[10]. In addition, more obvious partial attenuation of CIA was also observed in the IL-6 KO mice, as we reported previously[11]. However, we could not observe complete protection from CIA in the IL-6KO mice as Alonzi, *et al* reported[18]. Although we cannot provide a definite reason for the difference between their results and ours, 2 points are worth consideration. First, the immunization protocols were different. We used complete Freund's adjuvant (CFA) for both immunizations, while they used CFA for the first immunization but incomplete Freund's adjuvant for the second. Second, the genetic background of the IL-6KO mice might be different. The mice used by Alonzi, *et al* were backcrossed with DBA/1J for 5 generations, while our mice were backcrossed for 8 generations. Therefore, the induction of CIA (or immunization) in our experimental system might be stronger than in theirs, and our mice might have been more susceptible to CIA. Indeed, almost all the littermate Wt mice used in the present study developed CIA as severe as DBA/1J mice, whereas a lower incidence and milder severity of CIA were reported by Alonzo, *et al*.

We also observed enhanced attenuation of CIA in the DKO mice compared with each single cytokine KO mouse. Interestingly, almost all the mice of each single cytokine KO group developed definite CIA, as reported[10,11], while only 20% of the mice in the DKO group developed CIA. Moreover, only marginal changes were observed in the histologic and radiologic examinations of the DKO mice. Thus, the effect on the attenuation of CIA in the DKO mice appears to be synergistic rather than additive. Although it is

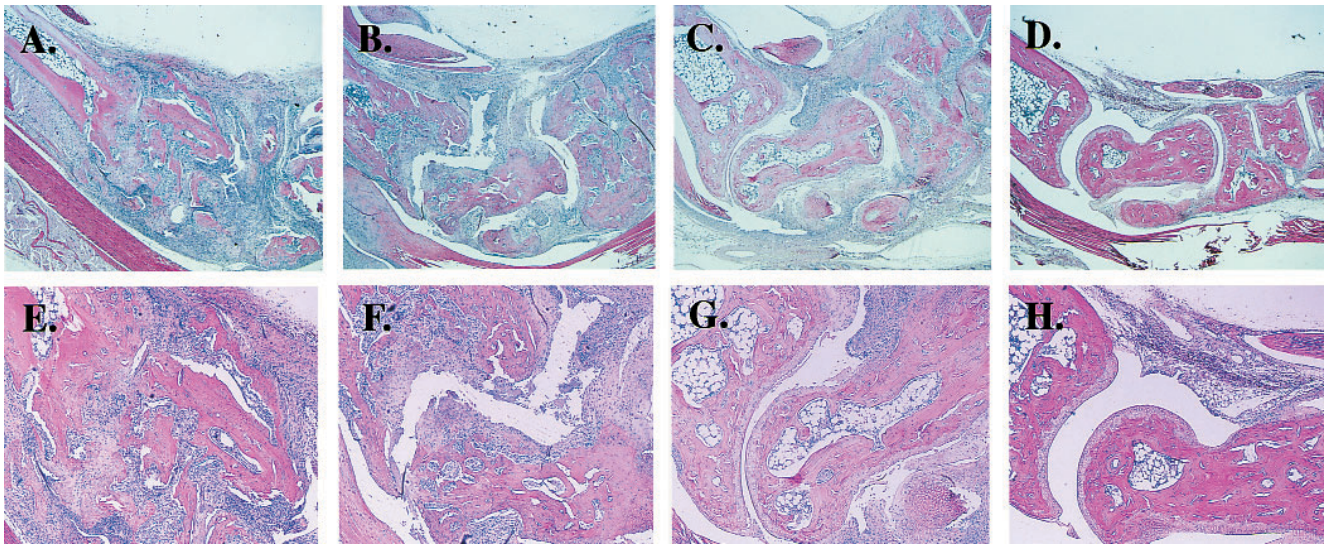

Figure 2. Histopathological evaluations of CIA in Wt, IL-6KO, TNFRIKO, and DKO mice were performed at the end of the experiment. Each photograph shows an ankle joint of a hind limb stained with hematoxylin-eosin. A and E show paws of Wt mice with CIA (A, original magnification ×20; E, original magnification ×40). In A and E, ankle joints are almost completely destroyed by severe synovitis in Wt mice. B and F show paws of TNFRIKO mice with CIA (B, original magnification ×20; F, original magnification ×40). In B and F, marked joint destruction and synovitis are shown, but are less severe compared with Wt mice. C and G show paws of IL-6KO mice with CIA (C, original magnification ×20; G, original magnification ×40). In C and G, only moderate synovitis is shown. D and H show paws of DKO mice with CIA (D, original magnification ×20; H, original magnification ×40). In G and H, almost normal joint organization is preserved.

Table 2. Radiologic changes in each mouse genotype. Values are expressed as means ± SEM.

Genotype (No. of Mice Studied)	Osteopenia	Bone Erosion
Wt (19)	9.21 ± 0.56	10.07 ± 0.38
TNFRIKO (24)	7.04 ± 0.66*	8.83 ± 0.62*
IL-6KO (17)	3.82 ± 0.78**	4.88 ± 1.04**
DKO (21)	0.68 ± 0.25***	0.95 ± 0.49***

* $p < 0.05$ versus Wt mice. **$p < 0.05$ versus Wt, TNFRIKO mice.
*** $p < 0.01$ versus Wt, TNFRIKO, and IL-6KO mice.

not clear, there is a possible explanation for such synergistic effects on the attenuation of CIA in the DKO mice. Proinflammatory cytokines such as TNF, IL-1, and IL-6 are well known to show redundancy in several biologic activities. In addition, since the individual cytokines do not act independently, but interact with each other, synergistic effects are often observed in *in vitro* studies. For example, the synergistic effect on acute phase protein production in combinations of IL-6 and TNF-α has been reported. TNF-α alone does not induce serum amyloid A (SAA), but in combination with IL-6 shows enhanced production of SAA in human hepatoma cell lines[19]. In contrast, IL-6 alone does not induce plasminogen activator inhibitor type 1 in Hep G2 cells, but markedly enhances its induction in combination with TNF-α[20]. Thus, simultaneous blockade of TNF and IL-6 may turn off such synergistic biologic activities, which relate to the pathogenesis of CIA, resulting in some synergistic effect on the attenuation of CIA.

Our findings also suggest that the combination of anti-TNF and anti-IL-6 therapy may have some clinical benefits for the treatment of RA in some cases. The outcome of recent clinical trials of therapies directed against single cytokines, including anti-TNF and anti-IL-6 therapies, appears to be excellent, and some are currently accepted as

Table 3. Serum anti-type II collagen (anti-CII) antibody titers and nonspecific total IgG levels in each mouse genotype. Values from each mouse genotype are shown as relative values compared with the mean value of the Wt group as 100 units.

Genotype (No. of Mice Studied)	Total IgG	IgG1	IgG2a	Nonspecific IgG
Wt (16)	100 ± 11.80	100 ± 20.56	100 ± 14.19	100 ± 17.45
TNFRIKO (17)	42.71 ± 10.26†	48.01 ± 13.28†	43.89 ± 10.11†	111.36 ± 23.07
IL-6KO (14)	65.35 ± 11.84†	52.68 ± 15.08†	76.57 ± 14.33†	88.41 ± 23.16
DKO (16)	13.29 ± 4.22††	9.58 ± 2.21††	13.58 ± 4.45††	95.56 ± 9.95

† $p < 0.01$ versus Wt mice. †† $p < 0.01$ versus Wt, TNFRIKO, and IL-6KO mice.

promising new therapy for RA[5-7]. However, despite the excellent efficacy, these single cytokine blockade therapies do not always sufficiently attenuate disease activity, and there are still some populations of non-optimal responders for each single cytokine therapy[8]. In such cases of non-optimal response for single cytokine therapy, combination with other types of therapy is suggested to be necessary. Indeed, the combination of anti-TNF-α and anti-T cell (anti-CD4) therapies showed enhanced efficacy in a mouse arthritis model[9].

There may be another potential problem in inhibiting a single cytokine as a therapeutic approach for RA, which relates to the overlapping activities of different cytokines. For example, we described a patient with severe RA who did not respond optimally to anti-TNF-α antibody therapy[21]. The patient showed lymphadenopathy and underwent a biopsy of the swollen lymph nodes to eliminate the possibility of malignancy. When we assessed TNF-α and IL-6 mRNA expression in the lymph nodes by reverse transcription PCR, only enhanced IL-6 but not TNF-α mRNA expression was observed. In addition, anti-TNF-α antibody could not inhibit spontaneous *in vitro* IL-6 production of the lymph node block culture, indicating IL-6 production in the lymph node might be independent of TNF-α. In such a case of a non-optimal response, it would be necessary to block not only TNF-α but also IL-6 simultaneously.

As for the immunological aspects, serum anti-CII antibody concentrations in the DKO mice were significantly decreased compared with each single cytokine KO mouse and were less than 10% of the Wt mice. This suggests that the DKO mice might be in an immunocompromised state. However, nonspecific total IgG concentrations in serum were not significantly decreased in the DKO mice compared with each single KO mouse. As well, we observed no infections in DKO mice or single KO mice during the whole experiment period. Thus the risk of infection in the DKO mice may not be higher than in each single KO mouse, although they were bred under standard pathogen-free conditions. However, since recent reports note that infections, particularly opportunistic infections including tuberculosis, are the most serious adverse effects of anti-TNF-α therapy[22,23], we should observe great caution in the use of anticytokine combination therapy.

We observed synergistic effects on the attenuation of CIA in TNFRI and IL-6KO mice, and CIA was almost completely prevented in the DKO mice. Recently, Bendele, *et al* reported synergistic effects of IL-1 receptor antagonist and PEG soluble TNFRI therapy on the amelioration of CIA[24]. Thus our findings suggest a clinical benefit of the combination of anti-TNF-α and anti-IL-6 therapies compared with therapy against each single cytokine in some cases of non-optimal responses to single cytokine therapy, although treatment experiments using combination of neutralizing antibodies to TNF-α and IL-6 must still be carried out in mice with established CIA.

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