EXTENDED REPORT

TNF receptor gene therapy results in suppression of IgG2a anticollagen antibody in collagen induced arthritis

P Mukherjee, B Wu, L Mayton, S-H Kim, P D Robbins, P H Wooley

Ann Rheum Dis 2003;**62**:707–714

See end of article for authors' affiliations

Correspondence to: Professor P H Wooley, Department of Orthopedic Surgery, Wayne State University, 1 South, Hutzel Hospital, 4707 St Antoine Blvd, Detroit, MI 48201, USA; pwooley@wayne.edu

Accepted 19 December 2002

Background: Therapeutic strategies to block tumour necrosis factor α (TNFα) activity in experimental autoimmune arthritis models and rheumatoid arthritis (RA) have proved highly successful, and provide sustained beneficial effects.
Objective: To examine whether TNFα inhibition has immunological activity beyond the reduction of inflammation in collagen induced arthritis (CIA), an established experimental model of RA.
Methods: Arthritic DBA/1 mice received single periarticular injections of retroviral constructs encoding human TNF receptor (TNF-R) into the affected arthritic paw, at the onset of arthritis. Severity of arthritis, antibodies to collagen type II (CII), and extent of pathological joint damage of arthritic paws were compared between TNF-R and media treated (control) animals 3, 7, 14, 21, and 49 days after disease onset.
Results: Severity of CIA was significantly decreased in TNF-R treated animals compared with controls, 14–34 days after disease onset. Joint destruction was reduced in TNF-R injected joints and in the uninjected contralateral and ipsilateral paws of TNF-R treated animals. Seven days after disease onset, TNF-R treated mice had lower levels of inflammatory Th1 driven IgG2a antibodies to CII ($p<0.05$) than controls. This altered the anticollagen IgG2a:IgG1 ratio towards Th2 driven IgG1.
Conclusions: Local TNF-R gene therapy in CIA appears to have systemic effects on the anti-CII antibodies. The overall influence of TNF-R gene therapy is that it inhibits the progression of CIA mainly by suppressing the inflammatory Th1 response rather than by stimulating a Th2 response. Therefore, periarticular TNF-R gene therapy may have excellent therapeutic potential in RA.

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease manifested by progressive synovial joint inflammation and erosion of the subchondral bone. Interactions between synovial hyperplasia, inflammation, and an altered humoral and cellular immune system characterise the pathology of RA.[1] Collagen induced arthritis (CIA) induced in DBA/1 mice by intradermal injection of collagen type II (CII), is an animal model of RA. Histological appearance of the erosions of cartilage and bone, inflammatory and immune cytokines, genetic and clinical features associated with both RA and CIA are similar.[2] Although the aetiopathogenesis of RA is still unclear, several studies suggest that activated CD4+ T cells have an important role in initiating and perpetuating the chronic inflammation characteristic of this disease.[3–5] It has been suggested that in RA, pathophysiological processes influencing the immune response may be driven by activated Th1 cells with insufficient Th2 cell differentiation to down modulate the ongoing inflammation.[6] Skapenko *et al* showed that CD4+ memory T cells from patients with untreated RA demonstrate an intrinsic abnormality towards differentiating into specific cytokine producing effector cells that might lead to the typical Th1 dominated chronic inflammation in RA.[6] Furthermore, high levels of circulating autoantibodies to CII are always associated with CIA, and seem to be essential for development and propagation of disease. Autoantibodies to CII belonging to the IgG2 subclass have been shown to be most efficient in binding complement, another crucial factor for initiation of joint inflammation in CIA.[7 8] Also, IgG2a production is associated with a Th1 response, whereas IgG1 production is associated with a Th2 response.[9]

Tumour necrosis factor α (TNFα) is a macrophage derived cytokine with multiple inflammatory and immunoregulatory properties. Raised levels of TNFα have been found in the sera and synovial fluid of patients with RA, suggesting it has an important role in the pathogenesis of RA.[2 10] Tissue expression of TNFα and its two receptors (p55 and p75 TNF-R) is seen at several sites within the synovial membrane and the cartilage/pannus junction, indicating that a wide variety of cells may be targets for TNF activity in RA.[11–13] TNFα produced by synovial cells in RA seems to contribute to a cytokine cascade that subsequently leads to increases in interleukin (IL)1β and granulocyte macrophage-colony stimulating factor (GM-CSF) levels.[2 12–14] Studies suggest that neutralisation of TNFα down regulates the production of IL1, IL6, IL8 and GM-CSF, thereby improving the arthritic disease process[11 15–17] Therefore, TNFα represents a suitable target for intervention of the ongoing inflammatory immune process in RA.

Several different treatment immune strategies have been examined for the management of RA. Protein based anti-arthritic treatments have been shown to modulate the pathophysiology of the disease; however, they require frequent parenteral administration and are associated with several adverse side effects.[18 19] Gene therapy, requiring direct intra-articular or periarticular administration of vectors encoding for anti-inflammatory cytokines, avoids the delivery problems

Abbreviations: CIA, collagen induced arthritis; CII, collagen type II; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; PBS, phosphate buffered saline; RA, rheumatoid arthritis; SEM, standard error of mean; sTNF-RI, soluble tumour necrosis factor receptor I; TNFα, tumour necrosis factor α; TNF-R, tumour necrosis factor receptor

associated with protein administration.[19 20] In this study, retroviral vectors encoding for human p55 TNF-R were given periarticularly to paws of CII immunised mice on the day of arthritis onset to elucidate whether anti-inflammatory TNF-R gene therapy could ultimately influence the reactivity of autoimmune lymphocytes in CIA.

MATERIALS AND METHODS

Retroviral vector production

Retroviral construct—namely, MOIN-sTNF-Rc-Ig, encoded a fusion protein consisting of the extracellular domain of human 55 kDa TNFα-R covalently linked to the C_H2 through C_H3 domains of mouse IgG1 heavy chain. The soluble TNF receptor (sTNF-Rc-Ig) was amplified from sTNF-Rc-Ig plasmid using TNFsR5 and Ig3 oligomers.[21 22] The sTNF-Rc-Ig gene was inserted into the *Bam*HI site of the retroviral construct MOIN,[23] resulting in MOIN-sTNF-Rc-Ig. To produce high titre virus, the vector was transfected into Phoenix cells, a 293-based amphotropic packaging cell line, the supernatant harvested 48 hours after transfection, and titres determined by a standard plaque assay.[24]

Induction and assessment of CIA

Female DBA/1 mice 6–8 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME). Native bovine CII (provided by Dr Marie Griffiths, University of Utah) was solubilised at 2 mg/ml in 0.01 M acetic acid at 4°C overnight and emulsified with an equal volume of CFA (Difco Laboratories, Detroit, MI). Mice were given 100 µl of emulsion containing 50 µg of CII by intradermal injections at the base of their tails. Mice were weighed weekly and overall health status was noted. They were monitored daily for onset and progression of disease. Onset of CIA was determined upon observation of appearance of the first signs of definitive redness, oedema, and erythema in the metatarsal or metacarpal regions of the paws. Mice developing CIA between 20 and 55 days after immunisation were divided on the day of arthritis onset into two groups: (*a*) control (n=24) and (*b*) TNF-R treated (n=22). Arthritic paws of the mice were given periarticular injections of either 100 µl of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, NY) (group 1) or 1.6×10^7 pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of DMEM (group 2) at arthritis onset. Mice were assigned to one of the two groups alternately to normalise the onset date. Periarticular injections were done aseptically by guiding a Hamilton syringe with a 27½ gauge needle at a 45 degree angle into the affected joint. The retroviral vector encoding for TNF-R was injected into the joint once resistance was encountered due to the needle touching the bone in the wrist or ankle joints of the mice. In this manner both the synovial joint and the surrounding muscle cells would be transfected. Arthritic animals were clinically assessed five times a week and paw measurements were recorded three times a week for seven weeks after disease onset and at the start of treatment. An established arthritis scoring system[2] was used to evaluate disease: 0, normal appearance and flexion; 1, erythema and oedema; 2, visible joint distortion; 3, ankylosis detectable on flexion. Each limb was measured with a constant tension caliper (Dyer, Lancaster, PA) and graded, giving a maximum possible score of 12 for each mouse.

Histology

Histological analysis was conducted to determine the extent of joint damage. Front and rear paws were removed post mortem and stored in 10% neutral buffered formalin. Paws were decalcified for 18 days in 10% formic acid, dehydrated, and embedded in paraffin blocks. Sections were cut along a longitudinal axis, mounted, and stained with haematoxylin and eosin. Specimens were cut approximately to the mid-line, and then sagittal central samples mounted for evaluation. This allowed a consistent geographic evaluation. Five to 10 samples were mounted (usually 4–6 samples for each slide), and after staining the slides were permanently bonded with coverslips. A minimum of three separate sections for each specimen was evaluated in a blinded fashion. On the front paws, wrist and metacarpal joints were scored and on the rear paws, ankle and metatarsal joints were scored. Slides were evaluated for the presence of synovitis, pannus formation, marginal erosions, architectural changes (mostly subluxation), and destruction. Synovitis was judged by thickness of the synovial membrane and scored from 0 (<3 cells thick) to 5 (>30 cells thick); pannus was scored as 0 (no pannus formation) to 5 (joint space filled by pannus and extensive pannus proliferation); erosions scored as 0 (no erosions visible) to 5 (loss of visible cartilage and major bone loss due to erosion); architectural changes scored from 0 (normal joint architecture) to 5 (complete fibrosis and collagen bridging). An overall score based on these collective points ranging from 0 (classic normal joint appearance) to 5 (destructive erosive arthritis with major bone remodelling) was then assigned to each section.

Measurement of serum human sTNF-R levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset and administration of treatment. Sera were separated from all samples and stored at −80°C. The levels of human sTNF-R in mouse sera were determined by a quantitative sandwich enzyme immunoassay technique using human sTNF-RI Quantikine ELISA Kit (R&D systems, Minneapolis, MN), according to the manufacturer's protocol. Briefly, 200 µl of sera and serial dilutions of standards were pipetted into wells precoated with murine monoclonal antibody against sTNF-RI and incubated for two hours at room temperature. Plates were washed three times and 200 µl sTNF-RI conjugate was added to each well and incubated for another two hours at room temperature. Unbound antibody-enzyme reagent was removed by washing the plates, and 200 µl of conjugate was added and incubated for 30 minutes. Thereafter, 50 µl of stop solution was dispensed into each well and the plates were read at 450 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA). Negative pre-bleed control sera and human sera with a 1000-fold dilution were titred on each plate to ensure uniformity of the assay.

Measurement of serum anti-type II collagen antibody levels

All mice were pre-bled before CII immunisation and then bled at onset of arthritis, and at 3, 7, 14, 21, and 49 days after disease onset. Sera were separated from all samples and stored at −80°C, and the levels of serum antibovine CII antibodies were determined by enzyme linked immunosorbent assays (ELISAs) as described previously.[25] Briefly, ELISA plates (Nunc-Immuno plates, Denmark) were coated with 100 µl of coating buffer (0.4 M phosphate buffer pH 7.6) containing 3 µg of bovine collagen II, at 4°C overnight. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma, St Louis, MO), and non-specific binding was blocked by PBS containing 5% non-fat milk overnight at 4°C. One hundred microlitres of mouse sera diluted at 1/1000 in 5% milk/PBS was added to each well, except blank wells, and incubated overnight at 4°C. Subsequently, the plates were washed six times in PBS containing 0.05% Tween 20 and incubated with alkaline phosphatase conjugated goat antimouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) at 37°C for one hour. In assays to determine the isotype of the bound antibody, alkaline phosphatase conjugated goat antimouse antibodies specific for IgG, IgG1, or IgG2a were used. Plates were washed six times again and developed for 5–20 minutes in the dark by the addition of

p-nitrophenylphosphate (Sigma, St Louis, MO) as a chromatogen substrate. The resulting optical density was measured at 405 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA). Negative pre-bleed control sera and a standard mouse anti-CII antiserum were titred onto each plate to ensure uniformity of the assay. Antibody binding was expressed as OD_{405} units.

Analysis of local TNFα expression in mice paws

Front and rear paws were removed post mortem at 3, 7, 14, 21, and 49 days after disease onset and treatment. Skin was removed and paws cut into 3–4 pieces and homogenised in lysis buffer using a Polytron tissue homogeniser (Kinematica Inc, Switzerland). Insoluble debris was removed from homogenised tissue by centrifugation at 12 000 *g* at 4°C for 15 minutes. Murine TNFα was measured in tissue lysates by sandwich ELISA. In brief, 96 well ELISA plates (Nunc-Immuno plates, Denmark) were coated overnight with 50 µl of purified rat antimouse TNFα antibody (Pharmingen, San Diego, CA) at 4°C. Plates were washed with PBS/0.05% Tween 20 (Sigma, St Louis, MO), and non-specific protein binding sites were blocked with 10% fetal calf serum in PBS for six hours at room temperature. Samples and standards were added and kept overnight at 4°C. Plates were washed again to remove unbound proteins, and 50 µl biotin conjugated secondary rat antimouse TNFα antibody (Pharmingen, San Diego, CA) was added to each well. After a one hour incubation at 37°C, plates were washed and again incubated at room temperature with an avidin-alkaline phosphatase conjugate (Pharmingen, San Diego, CA). After washing, *p*-nitrophenylphosphate substrate (Sigma, St Louis, MO) was added and plates were developed in the dark for 15–20 minutes. The resulting optical density was measured at 405 nm by an ultraviolet max spectrophotometer (Molecular Devices, CA).

Statistical analysis

Data were analysed using the SPSS-PC statistical software (SPSS, Chicago, IL). Group comparisons between TNF-R treated and control mice were performed by the two tailed independent *t* test. Comparisons of more than two means were conducted using the one way analysis of variance, and $p<0.05$ was considered to be significant.

RESULTS

Effect of retroviral vector mediated periarticular delivery of TNF-R on collagen arthritis

Onset of arthritic disease was defined as the appearance of definitive signs of oedema and erythema in the paw. Onset of arthritis occurred in a single joint in all animals, and subsequently progressed to other joints. The joint exhibiting the first signs of clinical arthritis received a single periarticular injection of 100 µl of MOIN-sTNF-Rc-Ig containing a viral titre of 1.6×10^7 pfu/ml, at arthritis onset (day 1), while control animals received equal volumes of media into the first affected paw. Progression of arthritis was evaluated until 49 days after onset of arthritis, and the number of paws affected and the mean clinical scores were recorded. MOIN-sTNF-Rc-Ig treatment led to a significant reduction in the number of arthritic limbs affected between 15 and 25 days after disease onset ($p<0.05$) (fig 1A). Mean paw score was significantly reduced ($p<0.05$) in MOIN-sTNF-Rc-Ig treated animals compared with the control animals between 14 and 34 days after disease onset (fig 1B). Overall, these results demonstrate that local delivery of MOIN-sTNF-Rc-Ig significantly ameliorates the arthritic disease process up to 34 days after treatment. These experiments were repeated twice, and consistent results obtained.

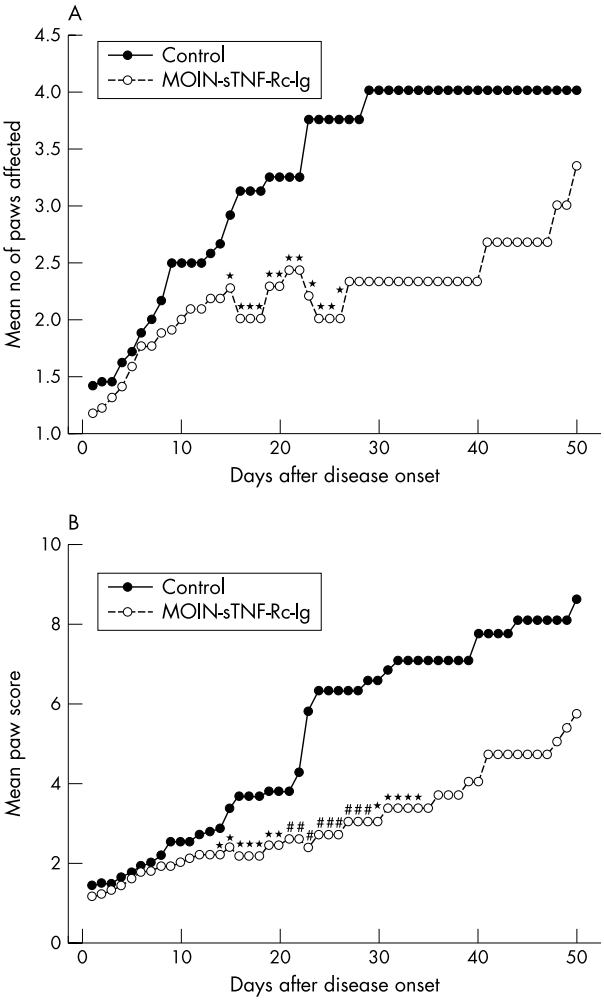

Figure 1 Effect of a single periarticular delivery of retrovirus mediated MOIN-sTNF-Rc-Ig on the clinical severity of CIA. At arthritis onset 100 µl of media alone (n=24) or 1.6×10^7 pfu/ml of MOIN-sTNF-Rc-Ig in 100 µl of media (n=22) was injected periarticularly into the affected arthritic paw. Disease activity represented by (A) the mean number of paws affected and (B) the mean paw score was visually scored up to 49 days after disease onset. Data are expressed as mean for each group. SEM <5% of mean (not shown). Statistically significant differences between the two groups is indicated: *$p<0.05$; #$p<0.02$). The figure shows representative results from two different individual experiments, which gave similar results.

Histological assessment of paws of control and MOIN-sTNF-Rc-Ig treated animals

Examination of the joint pathology demonstrated that the degree of synovitis, erosion, architectural changes of the bone, and overall joint destruction were significantly reduced in the arthritic paws of the MOIN-sTNF-Rc-Ig treated mice compared with the control animals (figs 2 and 3). The MOIN-sTNF-Rc-Ig injected paws showed a considerable reduction ($p<0.05$) in disease pathology compared with media injected control paws. The histological scores also revealed a significant reduction in the degree of joint destruction in the contralateral ($p<0.05$) and ipsilateral ($p<0.01$) paws compared with the control paws. These findings suggest that local periarticular delivery of MOIN-sTNF-Rc-Ig significantly reduces the ongoing disease process in the affected paw, and has marked systemic effects that block the progression of the inflammatory process to the uninjected joints, thereby preventing end stage destructive bone damage in these arthritic joints. This was consistent with levels of IL1β and

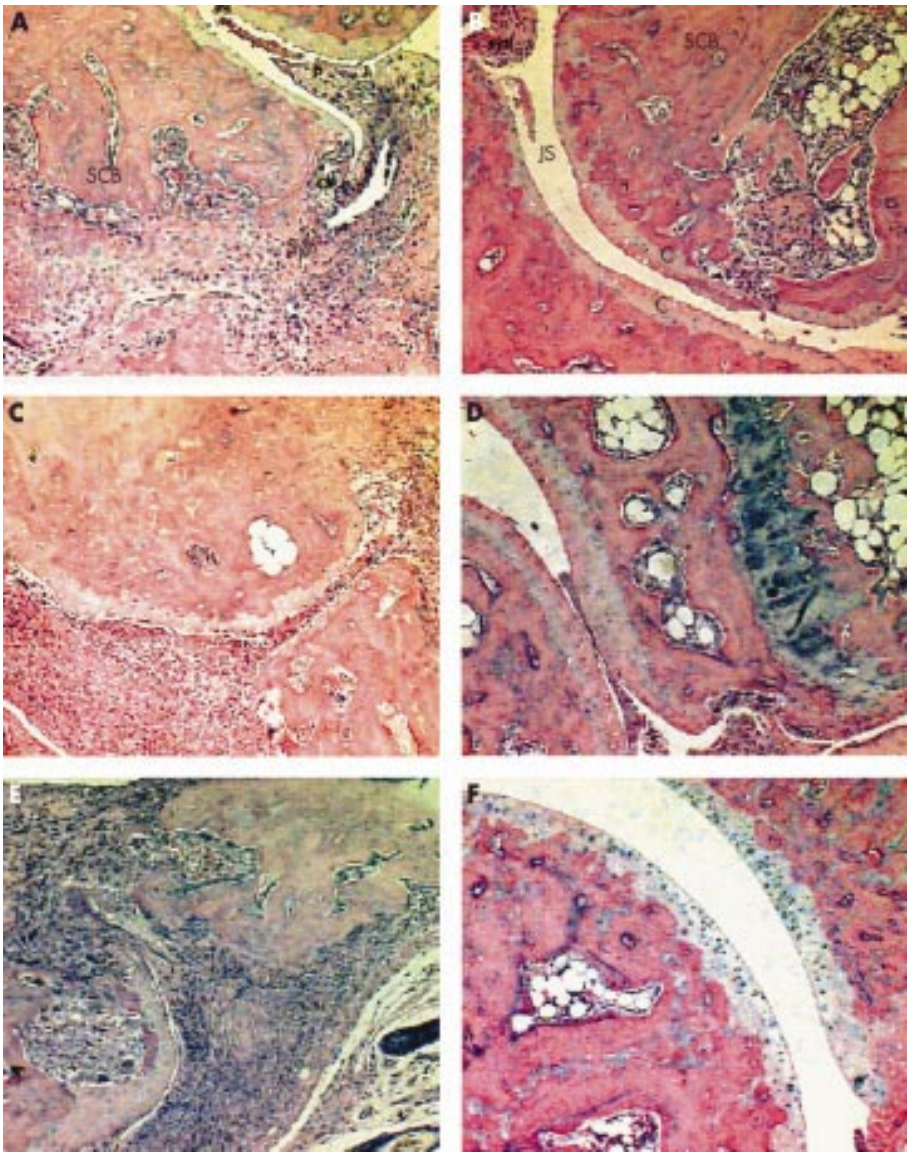

Figure 2 Histopathology of injected, contralateral, and ipsilateral joints. Histological evaluation of control injected (A), contralateral (C), and ipsilateral (E) and MOIN-sTNF-Rc-Ig injected (B), contralateral (D), and ipsilateral (F) arthritic joints were conducted at 49 days after disease onset and at the start of treatment. Media injected control joints show severe synovial inflammation with clear pannus attachment and erosions extending into the subchondral bone, indicating destructive erosive disease. Similar pathological changes are seen in the control arthritic contralateral and ipsilateral joints. Injected, contralateral, and ipsilateral arthritic joints of MOIN-sTNF-Rc-Ig treated mice show a lesser degree of both synovial inflammation and destructive disease, indicating a relatively normal joint architecture. Haematoxylin and eosin staining (magnification ×100) used for all sections. JS, joint space; SCB, subchondral bone; CPJ, cartilage/pannus junction; Syn, synovium; P, pannus.

TNFα mRNA measured in the paws. The contralateral and ipsilateral paws of the MOIN-sTNF-Rc-Ig treated animals had lower levels of both these cytokines than the injected and the similar paws of the control animals (data not shown).

Effect of retroviral gene therapy delivering TNF-R on anti-CII antibody levels

Serum samples were obtained from both control and treated animals at 3, 7, 14, 21, and 49 days after disease onset. The effect of MOIN-sTNF-Rc-Ig treatment on the humoral response to CII at these different times was determined by assaying the sera for antibodies to bovine CII (fig 4). Total immunoglobulin levels were lower in MOIN-sTNF-Rc-Ig treated animals than in controls ($p<0.05$) three weeks after onset of arthritis and administration of treatment (fig 4A). Subsequently, the isotype of the anti-CII antibody response was assessed at different times. Lower anti-CII IgG levels were seen in MOIN-sTNF-Rc-Ig treated animals than in controls ($p<0.05$) seven days after treatment (fig 4B). No significant differences were seen in the anti-CII IgG1 levels between the two groups (fig 4C); however, the anti-CII IgG2a titres were significantly lower ($p<0.05$) in sera from MOIN-sTNF-Rc-Ig treated mice than in controls seven days after arthritis onset (fig 4D). At that time, because anti-CII IgG1 levels were similar in the two groups but anti-CII IgG2a titres were markedly decreased in the TNF-R treated mice, a significant shift ($p<0.005$) in the anti-CII IgG2a:IgG1 ratio towards IgG1 was seen (fig 5). These observations suggested that although periarticular anti-inflammatory TNF-R gene therapy does not cause an isotype switch of the anti-CII autoantibody, it leads to an alteration in the anti-CII IgG2a:IgG1 ratio towards IgG1 by predominantly down regulating the Th1 mediated IgG2a response rather than up regulating the Th2 driven IgG1 response. The suppressive effect on IgG2a antibodies is consistent with an influence on the Th1 cells rather than Th2 cells. We have demonstrated that periarticular administration

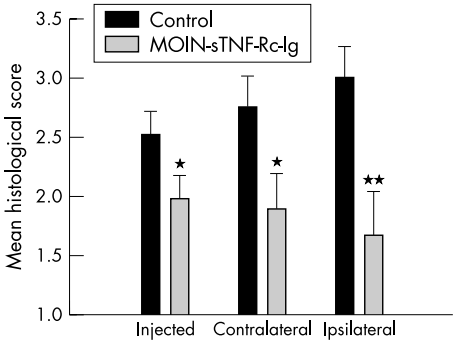

Figure 3 Histological scoring of arthritic joints. Arthritic joints were analysed by histology using haematoxylin and eosin staining for extent of joint damage and assigned a histological score (as described in "Materials and methods"). Each column represents the mean scores (SEM) for the total number of mice in each group. *p < 0.05; **p<0.01.

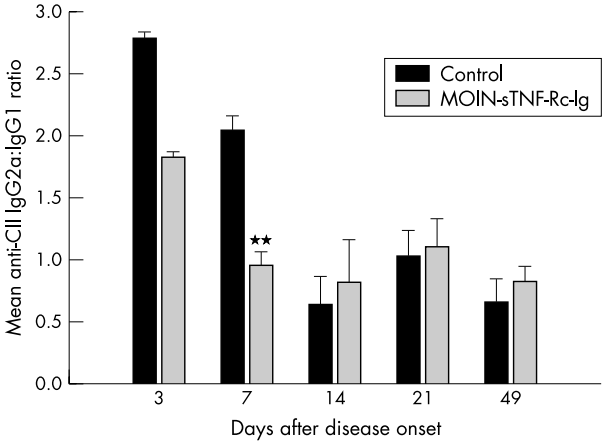

Figure 5 Effect of a single periarticular delivery of MOIN-sTNF-Rc-Ig on the antibovine CII IgG2a:IgG1 ratio. Titres of anti-bovine CII IgG1 and IgG2a were measured by ELISA. The anti-CII IgG2a:IgG1 ratio 3, 7, 14, 21, and 49 days after disease onset is represented. Each column represents the mean ratios (SEM) for the total number of mice in each group for each time. n = 5 for both the media and MOIN-sTNF-Rc-Ig treated mice. **p<0.005.

of retroviral construct MOIN encoding for marker gene *Lac*Z into the arthritic paw had no effect on the clinical disease in CIA or the anti-CII antibody profile, suggesting that the above observations were a result of TNF-R and not an effect of the retroviral vectors (data not shown)

Measurement of local TNFα expression in control and MOIN-sTNF-Rc-Ig treated animals

Local expression of TNFα in the joints of MOIN-sTNF-Rc-Ig treated and control animals was measured by ELISA at 3, 7, 14, 21, and 49 days after treatment and disease onset. It was noted that the TNFα levels in the MOIN-sTNF-Rc-Ig injected paws were slightly higher than in media injected control joints, although these differences were not statistically significant (fig 6A). Paradoxically, it was observed that local TNFα levels in the uninjected arthritic ipsilateral joints of the MOIN-sTNF-Rc-Ig were lower than the ipsilateral joints of the control animals at all times, achieving statistically significant values 14 days after disease onset (fig 6B). Similar results were also seen in the arthritic contralateral joints (data not shown). Consequently, when the local TNFα expression in all the

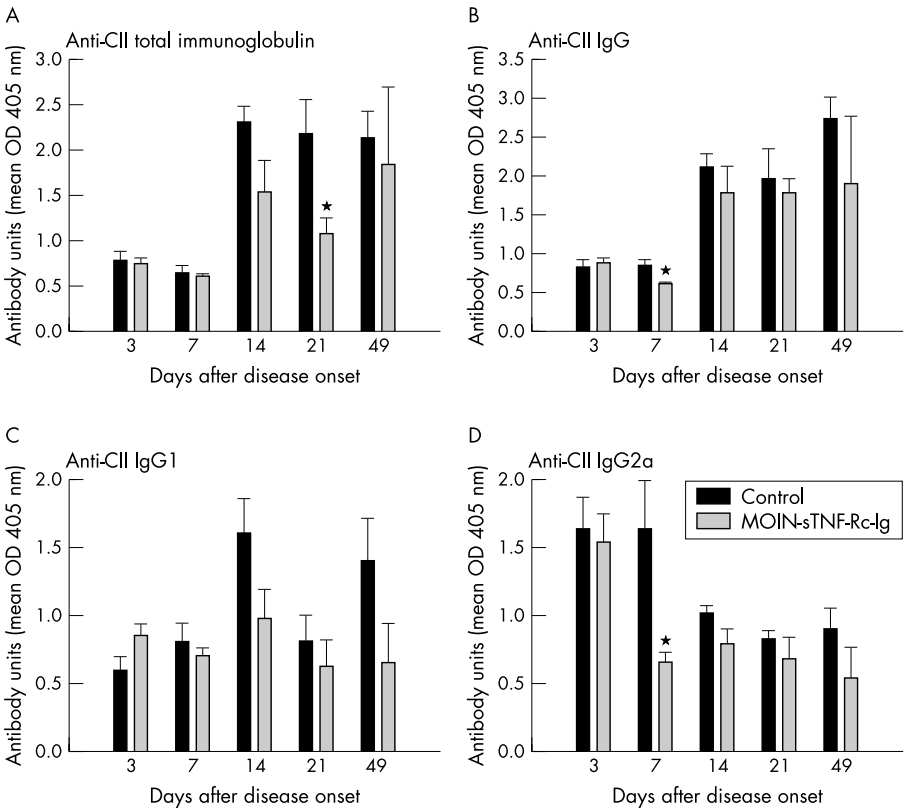

Figure 4 Effect of periarticular delivery of MOIN-sTNF-Rc-Ig on the antibody profile to bovine CII. At arthritis onset mice received single periarticular injections of MOIN-sTNF-Rc-Ig or media into the affected arthritic paw. Serum samples were collected 3, 7, 14, 21, and 49 days after arthritis onset. Titres of antibodies to bovine CII were measured by ELISA. Each column represents the anti-bovine CII (A) immunoglobulin; (B) IgG; (C) IgG1; and (D) IgG2a levels in the treated or control sera at these times. Values are means (SEM) for the total number of mice in each group for each time. n=5 for both the media and MOIN-sTNF-Rc-Ig treated mice. *p<0.05.

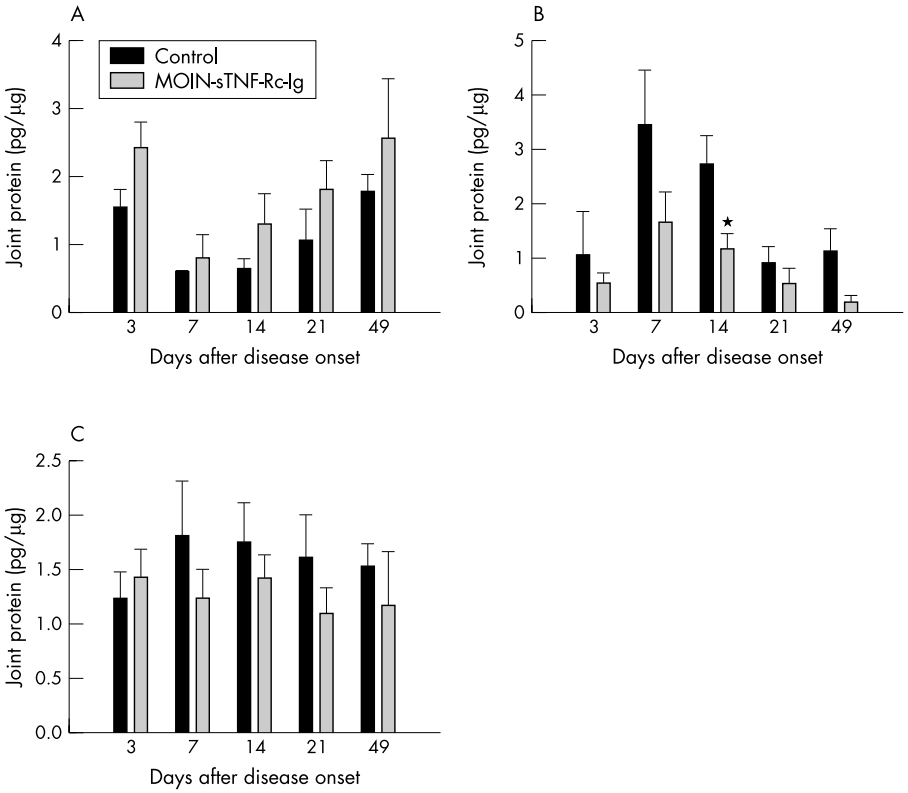

Figure 6 Effect of a single periarticular delivery of MOIN-sTNF-Rc-Ig on the local TNFα levels. Local TNFα levels present in tissue lysates obtained from (A) injected (B) ipsilateral paws of control and MOIN-sTNF-Rc-Ig treated mice, harvested at 3, 7, 14, 21, and 49 days after disease onset were measured by ELISA. Contralateral joints showed results very similar to the ipsilateral joints. The cumulative levels of local TNFα expression in the injected, ipsilateral, and contralateral joints of the treated and control animals are represented in (C). Each column represents the mean (SEM) TNFα levels for the total number of mice in each group for each time. n = 5 for both the media and MOIN-sTNF-Rc-Ig treated mice. *p<0.05.

injected, contralateral, and ipsilateral joints was cumulatively analysed, an overall reduction in the TNFα was observed in MOIN-sTNF-Rc-Ig treated animals compared with controls from 7 to 49 days after disease onset (fig 6C). These results showed that MOIN-sTNF-Rc-Ig was not completely successful in lowering the TNFα levels in the first joints to be affected. This can be explained by the markedly raised levels of proinflammatory cytokines[26 27] associated with the onset of clinical disease and inflammation in CIA. It appears that our therapeutic treatment given at the onset of arthritis cannot effectively lower the already raised levels of TNFα present in the joint at disease onset but can significantly reduce TNFα levels in uninjected joints as they become affected, leading to overall disease amelioration.

Because local MOIN-sTNF-Rc-Ig treatment had beneficial systemic effects we assayed the expression of human TNF-R levels in sera over the time course of the experiment. Serum human sTNF-R levels from both control and MOIN-sTNF-Rc-Ig treated mice obtained at different times were measured by ELISA. Three days after disease onset and MOIN-sTNF-Rc-Ig administration, the human sTNF-R level in the circulation was 16.7 pg/ml. This value fell to 12.7 pg/ml seven days after disease onset and treatment and thereafter was undetectable at 14, 21, and 49 days after onset of disease. Therefore, the beneficial systemic effects of periarticular TNF-R gene therapy on the anti-CII antibodies and local TNFα expression in uninjected paws correlated well with the ability to measure circulating levels of human TNF-R. However, local expression of human TNF-R in the joints of the MOIN-sTNF-Rc-Ig could not be detected, possibly owing to either the rapid diffusion of the protein or the dilution effects during paw protein extraction. Therefore, although local expression of human TNF-R was below the level of detection in our assay system, periarticular TNF-R therapy resulted in a generalised reduction in the local TNFα levels in the treated animals.

DISCUSSION

CIA and RA have several common immunological and pathological features,[28] including the involvement of inflammatory cytokines in the arthritic aetiology. IL1 levels are increased in mice with CIA. Whereas the administration of recombinant IL1 to mice with CIA aggravates the disease process,[2 29] treatment with IL1 receptor antagonist protein inhibits both RA and CIA,[30–32] thus implicating IL1 as one of the mediators of the inflammatory process in this disease.[33] IL2 and IL6 are also associated with the immunopathogenesis of CIA.[34 35] These results provide compelling evidence that many cytokines participate in an immune network regulating the pathogenesis of RA. The onset of clinical symptoms and inflammation in CIA is highly dependent upon a predominantly Th1 response, characterised by the presence of anticollagen IgG2a antibodies and the proinflammatory cytokines TNFα and interferon γ.[26 27] TNFα participates in the cytokine cascade by up regulating IL1β and GM-CSF levels, leading to synovial inflammation and joint erosion.[2 15 17 20] Anti-TNFα monoclonal antibody therapy has been shown to ameliorate disease significantly both in clinical trials with patients having longstanding RA and in experimental arthritis.[36] Our results support previous findings[2 37–39] and indicated that TNF-R delivered locally at the arthritic paw using a retroviral vector inhibited the disease by reducing the number of paws affected and the severity of arthritis. Histology revealed that while TNF-R injected paws showed significant local effects, including reduced bone damage and a marked improvement in disease, more pronounced protective effects occurred in the uninjected paws. This suggests that the therapeutic treatment given at the onset of

disease may not completely down regulate the raised TNFα levels and inflammatory process already initiated in the affected joint, but may exert a systemic effect that reduces TNFα secretion and the development of the inflammatory process in other joints, as they become affected. This hypothesis is supported by the lower levels of local TNFα expression found in uninjected arthritic paws compared with the injected arthritic paws.

The systemic effects of the local retrovirus mediated TNF-R gene therapy correlate well with the circulating serum human TNF-R levels in treated mice. The observation of reduced joint erosions and bone destruction in treated animals is new, because Joosten *et al* reported that TNFα blockade by intraperitoneal injections of TNF binding protein only improved joint inflammation and had little effect on cartilage and bone destruction,[40] whereas IL1 inhibition had a greater effect on reducing bone destruction than TNFα blockade. Possibly, the periarticular route of delivery of the retroviral vectors in our study was effective in concentrating TNF-R within the joint and thereby reducing bone destruction. Because TNFα blockade leads to down regulation of IL1,[15–17] which in turn may inhibit cartilage erosion and bone resorption, the successful outcome of TNF-R gene therapy seen in our study might well be expected.

We also demonstrated that periarticular delivery of TNF-R had a marked effect on the humoral response to bovine CII, with a reduction of anti-CII total IgG levels, and moreover a decrease in the anticollagen IgG2a antibody. Anti-CII IgG2a plays a crucial part in the immunopathogenesis of CIA.[7 8] Further, deposition of IgG antibodies and complement fixation occurs immediately before the development of overt arthritis.[41] In our study, reduction in levels of anti-CII IgG2a led to an alteration in the ratio of IgG2a and IgG1 in favour of Th2 driven anti-CII IgG1 at seven days after disease onset. Various studies have reported an activated Th1 response in the absence of an adequate counterbalancing Th2 response before the clinical onset of CIA[42 43] and that a switch from a Th1 to Th2 profile leads to a clinical improvement in both CIA and RA.[33 37 44] We show that local TNF-R gene therapy significantly lowered the Th1 mediated IgG2a antibody response early in the disease, but did not influence the Th2 driven IgG1 antibody levels. Because the anti-CII IgG1 levels were not affected and only anti-CII IgG2a titres were reduced, isotype switching of the autoantibody was not apparent. Instead, a shift towards IgG1 rather than a switch in the anti-CII IgG2a:IgG1 ratio was observed. The alleviation of the clinical signs caused by TNF-R therapy up to 34 days after disease onset can be attributed mainly to the down regulation of the Th1 mediated inflammatory response rather than to the up regulation of a Th2 driven IgG1 response, in agreement with the finding of Horsfall *et al* that continuous administration of IL4 improved CIA by suppressing the Th1 response without any effect on Th2 responses.[27]

In this study the reduction in anti-CII IgG2a levels was not sustained beyond seven days and, also, clinical improvement started diminishing 34 days after treatment. This is consistent with our finding that the human TNF-R levels were undetectable in the circulation eight days after disease onset and MOIN-sTNF-Rc-Ig treatment. This might be an effect of the single administration of MOIN-sTNF-Rc-Ig given at disease onset in our study. In unpublished studies we have shown that retroviral vectors encoding a marker *Lac*Z gene, injected periarticularly into the affected joints at arthritis onset showed good *Lac*Z transduction into the injected joint, but had no effect on the clinical disease or on the anti-CII antibody profile in CIA. Thus, these findings indicate that the outcome of MOIN-sTNF-Rc-Ig treatment on CIA is an effect of the anti-inflammatory TNF-R rather than the retroviral vector. A previous study showed paradoxical effects after adenovirus mediated delivery of TNF-R, with amelioration of arthritis 10 days after treatment followed by a rebound in disease despite the presence of bioactive TNF-R fusion protein.[36] This discrepancy with our findings may be due to different vectors and routes of delivery of the anti-inflammatory cytokines. The retroviral vectors in our study were given periarticularly, whereas the previous study used an intravenous route. Additionally, the delayed rebound inflammation observed in the latter study might possibly be attributed to the immunogenic effects associated with the use of adenoviruses.[45 46] A previous report indicated that periarticular delivery of viral IL10 by adenoviral vectors inhibited CIA development in both the injected and uninjected paws, and significantly reduced the antibodies to autologous CII while having no effect on the circulating antibodies to heterologous CII.[20] These differences suggest that cytokines other than TNF are critical to the development of autoantibodies in this model.

In conclusion, these findings suggest that local TNF-R treatment has systemic effects that inhibit the production of the proinflammatory cytokine TNFα in the uninjected arthritic joints and suppress Th1 cells without up regulating the Th2 cells, eventually leading to an overall improvement in CIA. Future studies will be done to evaluate whether repeated periarticular administrations of retrovirus mediated TNF-R might have a more sustained effect on the humoral response and clinical progression of disease in CIA. Thus, retroviral vector mediated periarticular delivery of the anti-inflammatory cytokine TNF-R may be a suitable strategy for alleviating disease in patients with RA.

Authors' affiliations
P Mukherjee, P H Wooley, Department of Immunology and Microbiology, Wayne State University School of Medicine Detroit, MI 48201, USA
B Wu, L Mayton, P H Wooley, Department of Orthopaedic Surgery, Wayne State University School of Medicine Detroit, MI 48201, USA
S-H Kim, P D Robbins, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA

REFERENCES

1 **Gay S**, Gay RE, Koopman WJ. Molecular and cellular mechanisms of joint destruction in rheumatoid-arthritis - 2 cellular mechanisms explain joint destruction. Ann Rheum Dis 1993;52(suppl):S39–47.
2 **Wooley PH**, Dutcher J, Widmer MB, Gillis S. Influence of a recombinant human soluble tumor-necrosis-factor receptor Fc fusion protein on type-II collagen-induced arthritis in mice. J Immunol 1993;151:6602–7.
3 **Sewell KL**, Trentham DE. Pathogenesis of rheumatoid arthritis. Lancet 1993;341:283–6.
4 **Schulze-Koops H**, Davis LS, Haverty TP, Wacholtz MC, Lipsky PE. Reduction of Th1 cell activity in the peripheral circulation of patients with rheumatoid arthritis after treatment with a non-depleting humanized monoclonal antibody to CD4. J Rheumatol 1998;25:2065–76.
5 **Janossy G**, Duke O, Poulter LW, Panayi G, Bofill M, Goldstein G. Rheumatoid arthritis - a disease of lymphocyte-T-macrophage immunoregulation. Lancet 1981;ii:839–42.
6 **Skapenko A**, Wendler J, Lipsky PE, Kalden JR, Schulze-Koops H. Altered memory T cell differentiation in patients with early rheumatoid arthritis. J Immunol 1999;163:491–9.
7 **Myers LK**, Rosloniec EF, Cremer MA, Kang AH. Collagen-induced arthritis, an animal model of autoimmunity. Life Sci 1997;61:1861–78.
8 **Brand DD**, Marion TN, Myers LK, Rosloniec EF, Watson WC, Stuart JM, *et al*. Autoantibodies to murine type II collagen in collagen-induced arthritis - a comparison of susceptible and nonsusceptible strains. J Immunol 1996;157:5178–84.
9 **Apparailly F**, Verwaerde C, Jacquet C, Auriault C, Sany J, Jorgensen C. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. J Immunol 1998;160:5213–20.
10 **Hopkins SJ**, Meager A. Cytokines in synovial-fluid. 2. The presence of tumor necrosis factor and interferon. Clin Exp Immunol 1988;73:88–92.
11 **Charles P**, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, *et al*. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. J Immunol 1999;163:1521–8.
12 **Husby G**, Williams RC. Synovial localization of tumor necrosis factor (TNF-gamma) in patients with rheumatoid-arthritis [abstract]. Clin Res 1988;36:A879.
13 **Chu CQ**, Field M, Feldmann M, Maini RN. Localization of tumor necrosis factor-alpha in synovial tissues and at the cartilage pannus junction in patients with rheumatoid arthritis. Arthritis Rheum 1991;34:1125–32.

14 **Alvarogracia JM**, Zvaifler NJ, Brown CB, Kaushansky K, Firestein GS. Cytokines in chronic inflammatory arthritis. 6. Analysis of the synovial-cells involved in granulocyte-macrophage colony-stimulating factor production and gene-expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-alpha. J Immunol 1991;146:3365–71.
15 **Brennan FM**, Jackson A, Chantry D, Maini R, Feldmann M. Inhibitory effect of TNF-alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. Lancet 1989;ii:244–7.
16 **Haworth C**, Brennan FM, Chantry D, Turner M, Maini RN, Feldmann M. Expression of granulocyte-macrophage colony-stimulating factor in rheumatoidarthritis – regulation by tumor necrosis factor-alpha. Eur J Immunol 1991;21:2575–9.
17 **Butler DM**, Maini RN, Feldmann M, Brennan FM. Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. Eur Cytokine Netw 1995;6:225–30.
18 **Moreland LW**, Pratt PW, Bucy RP, Jackson BS, Feldman JW, Koopman WJ. Treatment of refractory rheumatoid-arthritis with a chimeric anti-Cd4 monoclonal-antibody - long-term follow-up of Cd4+ T cell counts. Arthritis Rheum 1994;37:834–8.
19 **Evans C**, Robbins PD. Prospects for treating arthritis by gene-therapy. J Rheumatol 1994;21:779–82.
20 **Whalen JD**, Lechman EL, Carlos CA, Weiss K, Kovesdi I, Glorioso JC, *et al*. Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws. J Immunol 1999;162:3625–32.
21 **Ghivizzani SC**, Lechman ER, Kang R, Tio C, Kolls J, Evans CH, *et al*. Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. Proc Natl Acad Sci USA 1998;95:4613–18.
22 **Peppel K**, Crawford D, Beutler B. A tumor-necrosis-factor (TNF) receptor-IgG heavy-chain chimeric protein as a bivalent antagonist of TNF activity. J Exp Med 1991;174:1483–9.
23 **Kim SH**, Yu SS, Park JS, Robbins PD, An CS, Kim S. Construction of retroviral vectors with improved safety, gene expression, and versatility. J Virol 1998;72:994–1004.
24 **Mittereder N**, March KL, Trapnell BC. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 1996;70:7498–509.
25 **Wooley PH**, Whalen JD. The influence of superoxide scavenging compound Ctc-23 on type-II collagen-induced arthritis in mice. Agents Actions 1992;35:273–9.
26 **Arend WP**, .Dayer JM. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor alpha in rheumatoid arthritis. Arthritis Rheum. 1995;38:151–60.
27 **Horsfall AC**, Butler DM, Marinova L, Warden PJ, Williams RO, Maini RN, *et al*. Suppression of collagen-induced arthritis by continuous administration of IL-4. J Immunol 1997;159:5687–96.
28 **Wooley PH**, Chapedelaine JM. Immunogenetics of collagen-induced arthritis. Crit Rev Immunol 1987;8:1–22.
29 **Phadke K**, Carlson DG, Gitter BD, Butler LD. Role of interleukin 1 and interleukin 2 in rat and mouse arthritis models. J Immunol 1986;136:4085–91.
30 **Gabay C**, Marinova-Mutafchieva L, Williams RO, Gigley JP, Butler DM, Feldmann M, *et al*. Increased production of intracellular interleukin-1 receptor antagonist type I in the synovium of mice with collagen-induced arthritis: a possible role in the resolution of arthritis. Arthritis Rheum 2001;44:451–62.
31 **Muller-Ladner U**, Roberts CR, Franklin BN, Gay RE, Robbins PD, Evans CH, *et al*. Human IL-1Ra gene transfer into human synovial fibroblasts is chondroprotective. J Immunol 1997;158:3492–8.
32 **Bendele AM**, Chlipala ES, Scherrer J, Frazier J, Sennello G, Rich WJ, *et al*. Combination benefit of treatment with the cytokine inhibitors interleukin-1 receptor antagonist and PEGylated soluble tumor necrosis factor receptor type I in animal models of rheumatoid arthritis. Arthritis Rheum 2000; 43:2648–59.
33 **Chabaud M**, Page G, Miossec P. Enhancing effect of IL-1, IL-17, and TNF-alpha on macrophage inflammatory protein-3alpha production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. J Immunol 2001;167:6015–20.
34 **Banerjee S**, Wei BY, Hillman K, Luthra HS, David CS. Immunosuppression of collagen-induced arthritis in mice with an anti-IL-2 receptor antibody. J Immunol 1988;141:1150–4.
35 **Takagi N**, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, *et al*. Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. Arthritis Rheum 1998;41:2117–21.
36 **Quattrocchi E**, Walmsley M, Browne K, Williams RO, Marinova-Mutafchieva L, Buurman W, *et al*. Paradoxical effects of adenovirus-mediated blockade of TNF activity in murine collagen-induced arthritis. J Immunol 1999;163:1000–9.
37 **Mageed RA**, Adams G, Woodrow D, Podhajcer OL, Chernajovsky Y. Prevention of collagen-induced arthritis by gene delivery of soluble p75 tumour necrosis factor receptor. Gene Ther 1998;5:1584–92.
38 **Bendele AM**, McComb J, Gould T, Frazier J, Chlipala E, Seely J, *et al*. Effects of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) alone and in combination with methotrexate in adjuvant arthritic rats. Clin Exp Rheumatol 1999;17:553–60.
39 **Bendele AM**, McComb J, Gould T, Frazier J, Chlipala ES, Seely J, *et al*. Combination benefit of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) and dexamethasone or indomethacin in adjuvant arthritic rats. Inflamm Res 1999;48:453–60.
40 **Joosten LA**, Helsen MM, Saxne T, van de Loo FA, Heinegard D, van den Berg WB. IL-1 alpha beta blockade prevents cartilage and bone destruction in murine type II collagen-induced arthritis, whereas TNF-alpha blockade only ameliorates joint inflammation. J Immunol 1999;163:5049–55.
41 **Stuart JM**, Tomoda K, Yoo TJ, Townes AS, Kang AH. Serum transfer of collagen-induced arthritis. II. Identification and localization of autoantibody to type II collagen in donor and recipient rats. Arthritis Rheum 1983;26:1237–44.
42 **Boissier MC**, Chiocchia G, Bessis N, Hajnal J, Garotta G, Nicoletti F, *et al*. Biphasic effect of interferon-gamma in murine collagen-induced arthritis. Eur J Immunol 1995;25:1184–90.
43 **Mauri C**, Williams RO, Walmsley M, Feldmann M. Relationship between Th1/Th2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. Eur J Immunol 1996;26:1511–18.
44 **Chu CQ**, Londei M. Induction of Th2 cytokines and control of collagen-induced arthritis by nondepleting anti-CD4 Abs. J Immunol 1996;157:2685–9.
45 **Smith TA**, Mehaffey MG, Kayda DB, Saunders JM, Yei S, Trapnell BC, *et al*. Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. Nat Genet 1993;5:397–402.
46 **Yang Y**, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA 1994;91:4407–11.