A trial of clodronate-liposomes as anti-macrophage treatment in a sheep model of arthritis

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Abstract

Objective

Our previous research has concerned the role of macrophages in joint inflammation in rheumatoid arthritis. We have therefore been interested in liposomes containing clodronate as an anti-macrophage treatment for arthritis. We have used the antigen-induced arthritis model in sheep to evaluate the effect of clodronate liposomes.

Methods

Arthritis was induced in the right hock joint (day 0). We were able to demonstrate uptake of liposomes into macrophages within the inflamed joint lining. On day 7, sheep were given a single intra-articular injection of clodronate liposomes (group 1, n = 10) or saline liposomes (group 2, n = 10). A further 6 sheep (group 3) had no arthritis and no treatment.

Results

No difference in joint diameter was observed between the sheep in group 1 (clodronate) and group 2 (saline treated). Both groups had joint swelling which persisted until the end of the trial (day 20). Histologic scoring was also similar in group 1 and group 2 animals, and both were worse than group 3.

Conclusion

In vitro *studies have shown that interaction of liposomes with neutrophils and monocytes stimulates a respiratory burst. Despite this possible pro-inflammatory effect we did not observe any increase in joint diameter following liposome injection. Thus we were unable to demonstrate a therapeutic effect of a single dose of clodronate liposomes in this large animal model of antigen-induced arthritis.*

Key words

Clodronate-liposomes, treatment of arthritis, sheep model of arthritis.

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Introduction

Rheumatoid arthritis is a granulomatous disease and activated macrophages are a prominent feature of joint lesions and rheumatoid granulomas (nodules) (1). We and others have demonstrated that macrophages infiltrate the joint lining and accumulate at the surface of the synovial lining where they drive inflammatory processes resulting in joint destruction (1, 2). Furthermore, the extent of macrophage infiltration and lining layer thickening correlates with joint damage (3). We have therefore been interested in means of targeting macrophages within the joint lining as a way of treating arthritis. The use of clodronate-containing liposomes appeared to offer a form of anti-macrophage therapy suitable for such a therapeutic trial.

The macrophage suicide technique, using clodronate encapsulated in liposomes (4), was initially described as a way of studying the re-population of the liver and spleen after macrophage depletion, and to demonstrate the importance of liver and spleen macrophages in the immune response to particulate antigens given by the intravenous route (5, 6). These studies have shown that liposomes are selectively taken up by macrophages into phagosomes. Fusion of phagosomes with lysosomes leads to the breakdown of liposomal membranes and to the release of clodronate into the target cell. This results in the death of macrophages. By comparison with other bisphosphonates it was shown that clodronate is most effective for the elimination of macrophages (7).

In order to apply this method in a therapeutic trial, we thought it best to have a model of arthritis in animals big enough to allow easy injection and accurate measurement of joints. We therefore adapted the Dumonde Glynn model of arthritis (8) for use in sheep (9). Using this model, we evaluated the therapeutic potential of liposome-encapsulated clodronate as a drug delivery system designed to eliminate macrophages in the inflamed joint.

Materials and methods

Induction of arthritis

Sheep were immunised 3 times at approximately 2-week intervals with 10 mg ovalbumin in Freund's complete adjuvant and were then skin tested. The skin reaction was scored 24 and 48 hours after the antigen injection to assure their immune status. The protocol was approved by the Animal Ethics Committee.

Trial of treatment with liposomes

Sheep were randomly allocated to three groups. Group 1 (n = 10) and group 2 (n = 10) received an intra-articular injection of 5 mg ovalbumin in 0.5 ml saline in the right hock joint in order to induce arthritis. Group 3 (n = 6) were normal non-arthritic untreated sheep. The left hock joints of all sheep were left untouched and were used as a further negative control. Treatment was given in the chronic phase of arthritis (day 7), in order to avoid the uptake of liposomes into phagocytic cells within the acute joint effusions present at an early stage of the arthritis (10). Group 1 (treatment group, clodronate-liposomes) received an intra-articular injection of 0.5 ml of clodronate-encapsulated liposomes (200 mg/ml), in the right hock joint, while group 2 (control group) received 0.5 ml of saline liposomes. Group 3 (non-arthritic) was left untreated.

Positive controls: treatment with intra-articular steroid preparations

A further 6 sheep with arthritis were treated with steroid preparations. Steroid group 1 received intra-articular injection of 0.35 ml of Betamethasone (Celestone chronodose, Schering Plough; 5.7 mg/ml) in the right hock joint. Steroid group 2 were treated with 0.5 ml microspheres impregnated with Dexamethasone (gift from Associate Professor Tony Whateley, University of Strathclyde, UK; 4 mg/ml). In both cases the dosage was 2 mg. Treatment was given on day 7.

Joint assessments

The anterior-posterior (AP) diameter of both hock joints was measured in mm with skin calipers using a standardised technique. Triplicate measurements were made and results expressed as the mean of 3 measurements. Measurements were made 2 days apart for 20 days following the induction of arthritis. The results were averaged and expressed as a % dif-

ference [(right AP diameter - left AP diameter)/left AP diameter) x 100] in order to adjust for size variation between individual sheep. Measurements of joint diameter were made by an observer unaware of which sheep were treated.

Histological and pathological analysis
The post mortem examinations and histologic evaluations were carried out blind, i.e. without prior knowledge of which group the animal belonged to. Post mortem synovial joint lining specimens were taken from the right and left hock joints on the last day of the experiment (day 20 post induction of arthritis). Fresh synovial membrane was fixed in 10% buffered formalin and embedded in wax. Sections (4 µm thick) were stained with haematoxylin and eosin.
For each sample, we assigned a total histological score based on semi-quantitative assessments of: (a) lining layer thickness (average of 3 areas, number of cells thick); (b) degree of infiltration with mononuclear cells, where 1 = localised and confined to sublining, 2 = perivascular and sublining, 3 = comprehensive infiltration with inflammatory cells; (c) fibrin deposition, scored 1 = small localised deposits, 2 = large localised deposits, 3 = widespread fibrin deposition; and (d) neutrophils present or not present. Individual scores were added to give a total histological score.
Each specimen was also used for immunohistologic studies. These specimens were embedded in OCT compound (Miles Scientific, Naperville, IL), frozen in isopentane over liquid nitrogen, and stored at -70°C. Frozen sections (8 µm thick) mounted on gelatin-coated slides were fan-dried for 30 minutes and fixed in acetone at 4°C for 7 minutes. The sections were incubated with mouse monoclonal antibody to bovine CD5 (CC17, Serotec, UK) and mouse anti-ovine macrophage CD14 (VPM65, Serotec, UK) antibodies for 30 minutes at room temperature. After washing with PBS, sections were labelled with HRPO conjugated antimouse antibody (DAKO Immunoglobulins, Copenhagen), washed again in PBS and developed with DAB (diaminobenzidine, DAKO). Sections were briefly counterstained with haemotoxylin.

Confocal Microscopy
Sheep were injected in the right hock joint with liposomes constituted with BODIPY-phosphatidyl choline (100 µg, Molecular probe, Eugene, Oregon) seven days after the induction of arthritis so that uptake of liposomes could be traced by immunofluorescence. Sheep were killed at 2, 6, and 24 hours after injection. Sections of synovial membrane (10 µm) were incubated with mouse anti-ovine macrophage CD14 antibodies (Serotec, UK), washed and then incubated with Texas Red goat anti-mouse IgG conjugate (Molecular probe, Eugene, Oregon), washed again and mounted with Prolong mountant (Molecular probe, Eugene, Oregon). These double-labelled sections were then examined by fluorescence microscopy and confocal microscopy to localise liposome uptake, and to see if joint macrophages were effectively targeted by liposomes.

Preparation of liposomes
Clodronate was encapsulated within unilamellar vesicles using the thin film hydration method (11). The lipid components, 40 mg total cholesterol, 80 mg egg phosphatidylcholine (both 99% pure from Sigma, St Louis, USA) and 8 mg phosphatidylserine (Sigma, St Louis, USA) were dissolved in chloroform (10 ml) and dried to a thin film in a 500 ml round bottomed flask. Negatively charged liposomes containing clodronate were formed by the addition of clodronate (200 mg/ml) or normal saline followed by hand shaking of the flask. The preparation was left at room temperature for 2 hours and then probe sonicated for 5 minutes at 4°C. The liposomes were washed 3 times with saline by centrifugation (4°C) and resuspended in a final volume of 2 ml for use. All glassware was sterilised and treated to remove endotoxin and all solutions were filtered.

Dihydrorhodamine oxidative burst assay
Cell suspensions (500 µl monocytes) were incubated at 37°C for the required time with 50 µl Dihydrorhodamine (1 mM), plus 150 µl negatively charged liposomes; or 50 µl FMLP (formyl methionyl-leucyl-phenylalanine) diluted with 100 µl saline; or 150 µl saline. Mean fluorescence was measured by FACScan™ analysis (12).

Statistical analysis
Anova (Statview, single factor) were performed to assess differences between the AP diameter, and Student's t-test for the histological scores of the different treatment groups and in the coagulation test. We considered values of less than 0.05 to be statistically significant.

Results
Preliminary studies
Incubation of liposomes containing fluorescein acetate with whole heparinised blood followed by FACScan analysis confirmed the uptake of liposomes into monocytes. This was maximal at 4 hours. Incubation of clodronate liposomes with cultured macrophages resulted in the depletion of cell numbers in comparison with saline liposomes, thus confirming the ability of clodronate liposomes to kill macrophages as has been described (13) (data not shown). Cells loaded with the dye dihydrorhodamine 123 (12) showed increased fluorescence upon interaction with liposomes, indicating the presence of a respiratory burst (Fig. 1). This suggested that liposomes could potentially have a pro-inflammatory effect.
Injection of Bodipy-labelled liposomes into inflamed sheep hock joints and fluorescence microscopy carried out on synovial samples removed at 2, 6 and 24 hours showed maximum uptake in the 6 hour sample. Liposomes had dispersed by 24 hours. Figure 2 shows confocal microscopic images from inflamed synovium from a sheep hock joint injected with Bodipy-liposomes 6 hours previously. These experiments indicated that injected liposomes do gain access to synovial macrophages.

Treatment of arthritis with clodronate liposomes
Injection of 5 mg ovalbumin into the right hock joint of ovalbumin-sensitised sheep resulted in acute swelling of the joint as shown by a 20 - 30% increase in joint diameter (Table I). The most acute swelling abated after 24 - 48 hours and the arthritis entered a more chronic phase. Sheep in group 1 were treated

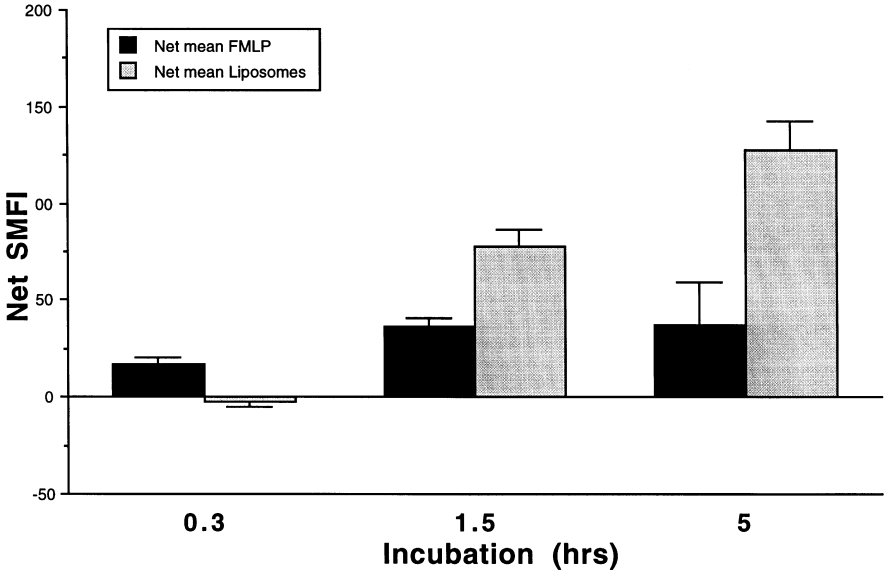

Fig. 1. Figures represent net mean fluorescence ± s.d. of monocytes loaded with dihydrorhodamine and exposed to liposomes (measured by Facscan™ analysis). Net figures for fluorescence intensity were calculated as the test mean minus the mean without stimulation. The bacterial peptide FMLP (formyl methionyl-leucyl-phenylalanine) was used as a positive control. Results shown are the mean of 3 experiments ± s.d. SMFI = specific mean fluorescence intensity. Monocyte FMLP and liposomes results were not significantly different (ANOVA, $p > 0.05$). The neutrophil FMLP and liposomes results (not shown) were similar and showed a significant difference only at 0.3 hours (ANOVA, $p = 0.002$).

during this phase at day 7 by injection of 0.5 ml clodronate liposomes into arthritic joints. Comparison with group 2 controls injected with saline liposomes showed no significant difference between the two groups ($p = 0.9$, ANOVA) (Table I). Joints in both groups were still swollen in comparison to non-arthritic sheep when the experiment was terminated at day 20: group 1, 16.3 ± 0.1, group 2, 9.9 ± 0.1, group 3, -0.7 ± 0.6 ($p < 0.01$, t-test, for both group 1 and group 2 vs group 3). The results for sheep in group 1 treated with clodronate liposomes could also be compared with positive controls treated with steroid preparations which were more effective ($p < 0.01$, ANOVA, Table I).

Post mortem synovial specimens were assessed for thickening of the synovial lining, the intensity of the inflammatory cellular infiltrate and the extent of fibrin deposition (Fig. 3). There was no significant difference in these individual parameters or in the overall total histologic score (mean ± SEM, clodronate-liposomes 9.8 ± 0.5, control saline liposomes 6.7 ± 0.2, $p = 0.6$, t-test). Results for both types of liposomes were also significantly worse than for the non-arthritic group, (total score 1.8 ± 0.1, t-test $p < 0.001$ for both group 1 and 2). Histological scores for steroid-treated joints were not statistically superior to the clodronate-liposome-treated joints (betamethasone ($n = 3$) total score 6.3 ± 1.1, t-test, $p = 0.08$; dexamethasone ($n = 3$), total score 5.0 ± 0.6, t-test, $p = 0.16$).

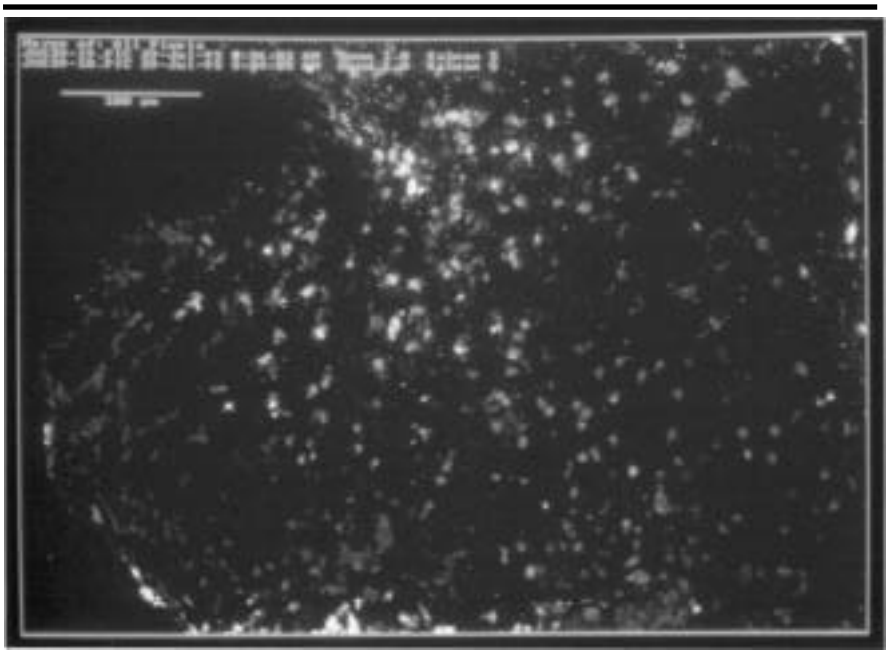

Fig. 2. Confocal microscopic images of inflamed synovial lining from one sheep hock joint injected with Bodipy-liposomes 6 hours previously. Red fluorescence shows macrophages labelled with antibody to sheep CD14 and texas-red; green fluorescence identifies cells that have taken up Bodipy-liposomes; yellow (green plus red) cells are macrophages that have taken up Bodipy-liposomes.

Discussion

The prevailing view of rheumatoid arthritis is that it is a T-lymphocyte driven disease. Despite this the evidence for the involvement of T-lymphocytes is mainly indirect, and therapies directed against T lymphocytes have met with limited success (14). The principal alternative view is that rheumatoid arthritis is mainly a disease of disordered macrophage function (15). In both models, the activated synovial macrophage is the cell which orchestrates those inflammatory events which culminate in joint destruction (1, 16). Macrophage production of pro-inflammatory cytokines such as IL-1 and TNF are critical events, and inhibition of such macrophage-produced cytokines is therapeutic (17).

The use of antibodies to TNF has been shown to be effective in a double-blind clinical trial (18). This suggests that a therapeutic attack targeted directly at macrophages should be an effective treatment for inflammatory joint diseases such as rheumatoid arthritis. Consequently we undertook a trial of clodronate liposomes as anti-macrophage treatment in an animal model of arthritis. The sheep was chosen as it provided a model with joints of sufficient size to be readily injected and measured.

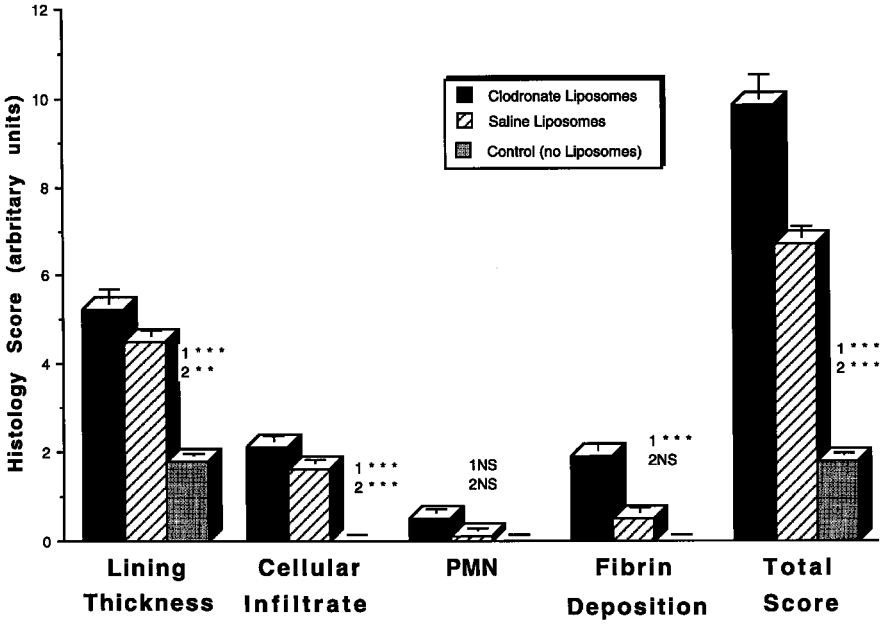

Fig. 3. Histological scores. Individual measures and total scores for sheep injected with clodronate-liposomes (group 1, treatment n = 10), saline-liposomes (group 2, control n = 10) or untreated (no liposomes, group 3, n = 6), where *** is $p < 0.001$ and **$p < 0.01$, and 1 indicates group 1 vs group 3, and 2 indicates group 2 vs group 3.

Table I. Mean difference in anterior-posterior (AP) hock joint diameter (%) in sheep with antigen-induced arthritis (day 0) who on day 7 were given either a single intra-articular injection of clodronate liposomes (group 1), saline liposomes (group 2) or no treatment.

			Positive controls		
Days	Clodronate-lip. Group 1	Control Group 2	Betamethasone Steroid 1	Dexamethasone Steroid 2	Control Group 3
-2	-0.1 ± 0.1	0.6 ± 0.1	-1.7 ± 0.5	-1.3 ± 0.5	2.0 ± 0.3
-1	0.7 ± 0.2	0.1 ± 0.1	-0.67 ± 0.7	-1.3 ± 0.5	-1.2 ± 0.3
1	19.3 ± 0.6	25.1 ± 0.6	25.7 ± 1.0	31.7 ± 2.5	0.7 ± 0.3
3	20.1 ± 0.1	22.5 ± 0.5	20.7 ± 1.8	19.0 ± 3.3	0.5 ± 0.2
5	18.4 ± 0.2	21.9 ± 0.9	21.0 ± 8.9	18.7 ± 3.0	0.0 ± 0.2
8	22.4 ± 0.3	19.4 ± 0.7	13.7 ± 6.7	11.3 ± 2.5	0.0 ± 0.3
10	18.5 ± 0.1	17.5 ± 0.9	8.0 ± 7.2	9.7 ± 2.4	0.7 ± 0.3
12	17.6 ± 0.1	16.1 ± 0.7	8.7 ± 3.9	9.3 ± 1.9	-0.7 ± 0.4
14	16.0 ± 0.3	14.6 ± 0.8	9.3 ± 2.3	6.7 ± 0.7	-0.5 ± 0.3
16	12.8 ± 0.5	13.4 ± 0.9	8.0 ± 1.5	5.0 ± 0.3	-0.8 ± 0.2
18	14.6 ± 0.3	10.3 ± 0.6	7.3 ± 1.7	5.0 ± 0.6	0.0 ± 0.4
20	16.3 ± 01	9.9 ± 0.7	7.0 ± 2.0	6.3 ± 0.5	-0.7 ± 0.6

Figures represent the mean difference in AP diameter between left (normal) and right (arthritic) hock joints, expressed as a % ± SEM. Lines at day 0 and day 7 indicate the induction of arthritis and the therapeutic joint injection respectively. There was no significant difference between group 1 (clodronate treated arthritic sheep, n = 10) and group 2 (saline treated arthritic sheep, n = 10), p = 0.9, ANOVA. At the end of the experiment (day 20), both groups were still worse than the non-arthritic controls (n = 6) in group 3 ($p < 0.01$ respectively, Student's t-test).

Both of the steroid-treated groups were significantly better than group 1 treated with clodronate liposomes ($p < 0.01$, ANOVA). Steroid group 1 sheep (n = 3) were treated with a standard intra-articular steroid preparation, celestone chronodose (betamethasone, Schering Plough). Steroid group 2 sheep (n = 3) were treated with microspheres impregnated with dexamethasone. In both cases the dosage was 2 mg. Dexamethasone microspheres were a gift from Associate Professor Tony Whateley, University of Strathclyde, U.K.

Our data from this trial has demonstrated that clodronate-liposomes, as an anti-macrophage treatment, did not show a significant therapeutic effect in antigen-induced arthritis in sheep when injected intra-articularly on a single occasion. Van Lent *et al.* (10) reported that a single dose of clodronate-liposomes given by intra-articular injection was effective in mice with antigen-induced arthritis. Efficacy for the depletion of synovial macrophages and therapy was dependent upon injecting clodronate liposomes in the chronic phase of arthritis.

Clodronate liposomes were given to sheep in this study after the initial acute phase of inflammation and consumption of liposomes by the acute inflammatory infiltrate of neutrophils is therefore an unlikely explanation for the lack of therapeutic effect in this study. Other authors have demonstrated efficacy when clodronate liposomes were given intravenously to rats with adjuvant arthritis (19), but not when clodronate liposomes were administered intra-articularly to rats with antigen-induced arthritis, similar to our results. These results suggest that successful treatment with clodronate liposomes might be influenced by the animal species and type of arthritis induced.

We confirmed that the clodronate liposomes we manufactured were capable of eliminating macrophages *in vitro*. We were also able to demonstrate that such liposomes rendered fluorescent with BODIPY were readily taken up by macrophages throughout the inflamed synovial lining of sheep with antigen-induced arthritis. Other authors have demonstrated the ability of such liposomes to deplete synovial macrophages (21, 22). Thus, failure to access synovial macrophages is also an unlikely explanation of the lack of therapeutic effect demonstrated in this trial.

Trials conducted to date in animals with antigen-induced arthritis have showed efficacy in mice (10), but not in rats (19) or sheep with the same type of arthritis. Therefore, there could be species-specific effects as well as effects from the model of arthritis chosen, e.g. adjuvant arthritis versus antigen-induced arthritis. Our data is derived from relatively few animals. However, they are large animals

whose joints are easily observed and measured and the complete lack of any apparent therapeutic effect suggests that larger numbers of animals would not have resulted in a statistically significant outcome. It is also possible that a single dose of clodronate liposomes was insufficient, and that multiple doses would be effective, for example, against the rapid recruitment of fresh macrophages into the joint lesion. In summary, although this trial did not show a therapeutic effect, the concept of anti-macrophage therapy in arthritis remains attractive, and efforts to refine methods for the therapeutic targeting of macrophages for the treatment of arthritis should continue.

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