Suppression of chronic streptococcal cell wall-induced arthritis in Lewis rats by liposomal clodronate

P. J. Richards, B. D. Williams and A. S. Williams

Rheumatology Research Laboratory, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK

Abstract

Objectives. To investigate the role of macrophages in the pathogenesis of chronic streptococcal cell wall (SCW)-induced arthritis using liposomal clodronate.

Methods. Female Lewis rats with SCW-induced arthritis received a single intravenous injection of 20 mg of clodronate encapsulated within small unilamellar vesicles (SUVc) 10 days post-arthritis induction.

Results. SUVc significantly suppressed the development of chronic SCW-induced arthritis for up to 26 days after treatment. At this time point, ED1⁺ macrophages were significantly depleted in the liver and ankle joints, although splenic macrophage numbers were not significantly different from control groups. Macrophage elimination induced a significant reduction in local levels of interleukin (IL)-1β, IL-6, tumour necrosis factor-α (TNFα) and matrix metalloproteinase-9 (MMP-9) from ankle joints.

Conclusions. Macrophage elimination by SUVc inhibits local production of IL-1β, IL-6, TNFα and MMP-9, and the pathogenesis of inflammatory arthritis.

KEY WORDS: Streptococcal cell wall-induced arthritis, macrophages, Liposomal clodronate.

In rheumatoid arthritis (RA), continued recruitment and activation of monocytes/macrophages can result in tissue destruction and related pathology [1]. In the inflamed synovium of patients with RA, activated macrophages are found in abundance [2] and are present in strategic sites related to the distribution of destructive pannus [3]. Their secretory products, such as interleukin (IL)-1β, IL-6 and tumour necrosis factor-α (TNFα), dominate the cytokine profile of synovial tissue and fluid [4–9]. Experimental models of arthritis have shown that synovial macrophages are highly activated, they express class II antigens, and secrete tissue-damaging enzymes, TNFα, IL-1β and IL-6, prostaglandins and several reactive oxygen species [10].

The importance of cytokines in the pathogenesis of experimental models of arthritis and RA has been demonstrated using neutralizing monoclonal antibodies. The pre-treatment of murine antigen-induced arthritis with anti-IL-1α/β polyclonal antibodies totally prevented suppression of cartilage proteoglycan synthesis [11]. In both murine type II collagen-induced arthritis and streptococcal cell wall (SCW)-induced arthritis, a single intraperitoneal injection of either anti-TNFα or anti-IL-1α/β suppressed both inflammation and cartilage damage in arthritic joints [12, 13]. The administration of anti-TNFα antibodies to RA patients caused a decrease in markers of disease activity, including swollen joint counts and C-reactive protein levels [14–16], whilst recombinant human IL-1 receptor antagonist demonstrated a beneficial effect on the rate of joint erosion [17].

Previously, we have shown that a single intravenous injection of clodronate encapsulated within small unilamellar vesicles (SUVc) significantly eliminated both systemic and synovial macrophages from rats with established adjuvant arthritis and antigen-induced arthritis [18, 19]. This resulted in a significant reduction in joint swelling and effective amelioration of joint destruction. This phenomenon was attributed to the macrophage-suppressive effects of liposomally encapsulated clodronate, as neither free clodronate nor empty liposomes demonstrated any significant effect upon either macrophage number or disease severity.

The rat SCW-induced arthritis model closely simulates many features of RA [20–24]. A single intraperitoneal injection of group A streptococcal peptidoglycan-polysaccharide (PG-PS) cell wall fragments induces an initial acute exudative inflammatory reaction, followed by a chronic erosive arthritis 2–4 weeks after PG-PS injection [20]. Although the chronic arthritic condition is considered to be a T-cell- and monocyte-mediated immune response [25], studies...
have primarily focused on the role of T cells [26–28]. Although prominent throughout the synovium, lymphocytes are sparse at sites of bone erosion, being heavily out-numbered by fibroblast-like cells and macrophages [29].

In the present study, we investigated the effect of a single intravenous injection of SUVc on the development of chronic SCW-induced arthritis in rats. We examined the influence of SUVc on ED1⁺ cells in ankle joints and also organs of the reticuloendothelial system (RES). Further studies demonstrated the influence of synovial macrophage depletion upon the levels of IL-1β, IL-6, TNFα and matrix metalloproteinase-9 (MMP-9) produced in the joint.

Materials and methods

Animals
Female inbred Lewis rats were obtained from Charles River, UK. The animals were housed in cages of five, allowed food and water ad libitum, and kept in the Biomedical Services Department for 1 week prior to arthritis induction. The animals were housed in light/ dark cycles of 12 h.

Induction of arthritis
Polyarthritis was induced following a single intraperitoneal injection of group A streptococcal PG-PS cell wall fragments (15 μg rhamnose/g body weight) (Lee Laboratories, GA, USA). The development of arthritis was monitored at regular intervals and expressed as an arthritis index [30] using a scale of 0–4 with a maximum possible score of 16 for individual animals. Inflammation was monitored by measuring ankle diameters (mean of three readings) using a digital micrometer.

Liposome entrapment of clodronate
Small unilamellar vesicles (SUV) encapsulating clodronate (SUVc) were prepared as described previously [18, 31, 32]. SUVc were produced by probe sonication (MSC Soniprep 150, 10 mm probe) of clodronate encapsulated in multilamellar vesicles (MLVs) composed of egg phosphatidylcholine, cholesterol and dipalmityl phosphatidic acid (molar ratio 7:7:1). A mean size of 100 nm was achieved after one 6 m amplitude burst for 10 min. The concentration of encapsulated clodronate within SUV was determined by calculating the amount of tracer ⁹⁹ᵐTc-clodronate remaining using a Wallac 1261 multigamma counter (LKB, UK).

Treatment of SCW-induced arthritis
Ten days after arthritis induction, the animals were divided into four groups (n = 5). At this time (day 10), the animals received a single intravenous injection (2 ml) of either sterile saline (0.9%/v), empty SUV, free clodronate and empty SUV (SUV + c; 20 mg), or SUVc (20 mg).

Histological grading of knee joint sections
At least five rats from each treatment group were killed 26 days after the respective treatments. The ankle joints were removed, trimmed, formalin fixed and decalcified. The joints were embedded in paraffin wax, then sectioned in the sagittal plane at 5 μm and stained with haematoxylin and eosin. All sections were coded prior to assessment to eliminate observer bias and subsequently scored by an independent observer. The sections were graded subjectively using four parameters; degree of cartilage destruction and bone erosions (0–3), severity of synovial infiltration (0–3), inflammatory exudate (0–1), and degree of synovial membrane hyperplasia (0–2).

Safranin O staining
Tissue sections were stained as previously described [33]. Briefly, paraffin wax ankle sections were stained with Harris’s haematoxylin and differentiated in 0.5% acid alcohol. After washing, the sections were stained in 0.02% fast green, rinsed in 1% acetic acid and stained in 0.1% safranin O.

Immunohistochemistry
At least five rats from either the saline or SUVc treatment groups were killed 26 days after the respective treatments. Frozen cryostat sections (10 μm) of spleen and liver were stained as previously described [18]. Briefly, the sections were fixed in acetone for 10 min at 4°C and allowed to air dry. Non-specific staining was blocked by incubation with normal goat serum [NGS; 1:10 in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA)] for 30 min in a humidity chamber at room temperature. The NGS was then removed, and the sections were incubated at room temperature for 1 h with the primary specific antibody, monoclonal mouse anti-rat ED1 (1:200). The control antibody consisted of purified mouse isotype-matched control IgG used at the same concentration as the specific antibody and under identical incubation conditions. After three 1 min washes in PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated rat-absorbed goat anti-mouse IgG (1:50) for 1 h at room temperature. After washing, the peroxidase was developed in 100 ml of PBS containing 50 mg of 3,3′-diaminobenzidine (DAB) and 40 μl of 30% H₂O₂. The control antibody yielded negative results.

Paraffin wax ankle sections were prepared and stained using a modification of the avidin-biotin system previously described [34]. Briefly, endogenous peroxidase was quenched with 1% H₂O₂ followed by a 15 min incubation with 10% normal serum. Suppression of endogenous avidin binding activity was performed by successive 15 min incubations with avidin and biotin blocking reagents. The sections were incubated at room temperature for 1 h with primary specific antibodies (Table 1). The control antibodies consisted of purified mouse and goat isotype-matched control IgG used at
the same concentration as the specific antibodies and under identical incubation conditions. The sections were then incubated with the appropriate biotinylated secondary antibody for 1 h, followed by high-sensitivity streptavidin–HRP conjugate, and developed using the HRP–DAB cell and tissue staining kit (R&D Systems, UK). Both frozen and paraffin wax sections were counterstained with Harris’s haematoxylin and analysed by light microscopy. The control antibodies yielded negative results.

**Image analysis**

Immunoperoxidase staining of spleen, liver and ankle joint sections was analysed quantitatively using the method previously described (18, 35). Briefly, the slides were imaged using a Leica DMLB light microscope (Milton Keynes, UK) and analysed with the computer-based image analysis system Improvision Density Slicing (OpenLab, Coventry, UK). The illumination voltage, camera set-up and calibration parameters were kept constant throughout all measurements. Minimum object boundaries were defined with 35 pixels and manual correction of selected fields was performed to achieve a complete match between the visual screen mask and the original microscope fields. In each slide, at least three consecutive representative fields were evaluated, and the mean area of brown immunoperoxidase staining determined. A total of three rats per treatment group was analysed.

**Statistical analysis**

The two-tailed Student’s t-test was used to determine whether ankle swelling was significantly different between treatment groups and also to determine whether the area of positive staining in immunohistochemically stained tissue sections was significantly different between saline- and SUVc-treated rats. The Mann–Whitney U-test was used to determine whether the arthritic index and histological grading were significantly different between treatment groups. A P value of < 0.05 was considered statistically significant. Values are expressed as the mean ± standard error of the mean (S.E.M.).

**Results**

**Effect of SUVc on ankle swelling and arthritis**

On day 10 (day of treatment), there was no significant difference in ankle swelling (6.5 ± 0.1 nm) (Fig. 1A) or arthritis index (2.9 ± 1) (Fig. 1B) between the treatment groups. When the rats treated with either saline (control) or empty liposomes were compared, there was no significant difference in ankle swelling or arthritis index at any time point.
When the rats treated with SUV + c or saline were compared, ankle swelling was significantly lower from day 26 (7.2 ± 0.1 vs 8.3 ± 0.3; P < 0.03) to day 32 (7.7 ± 0.1 vs 8.5 ± 0.2; P < 0.008) and the arthritis index was significantly lower at day 28 (4.2 ± 0.4 vs 7.4 ± 1.2; P < 0.04) only. When the rats treated with SUVc or saline were compared, ankle swelling was significantly lower at all time points from day 18 (6.2 ± 0.1 vs 7.5 ± 0.3; P < 0.0008) to day 36 (6.4 ± 0.1 vs 8.5 ± 0.3; P < 0.00002) and the arthritis index was significantly lower at all time points from day 16 (2.2 ± 1.6 vs 5.4 ± 0.8; P < 0.03) to day 36 (4 ± 1.2 vs 8 ± 0.9; P < 0.008).

**Histopathological effects**

The experiments were terminated on day 36. At this time point, the control ankles exhibited lesions characteristic

![Image](image1.png)

**Fig. 2.** Safranin O and fast green staining of paraffin wax sections of ankle joints isolated from rats at day 36 following treatment with either saline (control) or SUVc on day 10. (A) The saline-treated rat ankle joint shows development of the erosive pannus (p) which is associated with the destruction of articular cartilage (c) and subchondral bone (b) in both the tibia (T) and talus (t). Extensive inflammatory infiltrate is evident within the bone and pannus (arrow heads) having destroyed the majority of subchondral bone and articular cartilage. Cellular infiltrate and fibrinous matter are seen within the joint space (js). (B) The SUVc-treated rat ankle joint shows almost total absence of any inflammatory infiltrate within the subchondral bone (b) or synovium (s). The pannus (p) is greatly diminished and the articular cartilage (c) surface has remained intact in both the talus (t) and tibia (T). Although still present, the amount of cellular infiltrate within the joint space had decreased (js) (original magnification ×40).

![Image](image2.png)

**Fig. 3.** Immunohistological analysis in paraffin wax sections of ankle joints isolated from rats at day 36 following treatment with either saline (control) or SUVc on day 10. Resident macrophages and osteoclasts present within the ankle joints of rats treated with saline (A) or SUVc (B) were detected using the marker ED1. A large, significant reduction in all ED1+ cells was observed in SUVc-treated rat ankle joints. ED1+ cells are identified by brown HRP staining (original magnification ×40).
of chronic erosive arthritis (Fig. 2A). Synovial thickening and pannus formation over the surface of the articular cartilage, extensive inflammatory cell infiltrate and oedema were observed in ankle sections from control animals. This was accompanied by cartilage destruction and subchondral bone erosion which was most apparent within the epiphyseal and metaphyseal regions of the distal tibia and calcaneus and also the talus. Present throughout the synovium and at sites of bone erosion were large numbers of mononuclear cells, spindle-shaped fibroblast-like cells and neutrophils. Numerous giant multinucleated osteoclasts and cuboidal osteoblasts were found attached directly to the surface of subchondral bone. Large amounts of fibrous tissue were also observed within the joint spaces.

Synovial macrophage depletion achieved by a single dose of SUVc resulted in the preservation of joint integrity (Fig. 2B). The extensive subchondral bone erosion and resorptive cell populations observed in control animals was greatly diminished as was the destruction of the articular cartilage. Pannus formation was absent from the surface of articular cartilage. Histological grading demonstrated a significant reduction in cartilage destruction and bone erosion \( (P < 0.001) \) and in inflammatory cell infiltrate \( (P < 0.001) \) when compared with saline-treated rats (Table 2).

**Identification of \( ED1^+ \) cells**

On day 36, extensive \( ED1^+ \) staining of both macrophages and osteoclasts was seen throughout the synovial tissue and bone of control ankle joints (Fig. 3A). Macrophages were seen in large numbers within the inflamed synovium and at sites of bone erosion. Osteoclasts were specifically located at the surface of mineralized bone. Both cell types were seen in close association with neutrophils and fibroblast-like cells. Synovial macrophage depletion resulted in a significant reduction in \( ED1^+ \) staining (98%, \( P < 0.01 \)) in both synovial tissue and bone compared with control animals (Figs 3B, 4). Also on day 36, extensive \( ED1^+ \) staining of macrophages was observed throughout the liver and spleen of both control and SUVc-treated animals. A significant reduction in \( ED1^+ \) staining (46%, \( P < 0.01 \)) was observed in livers from SUVc-treated animals (Fig. 4).

**Identification of cytokines**

In control ankle joints, IL-1\( \beta \) was identified as the most abundant cytokine (Fig. 5A). It was observed throughout the synovial tissue and bone and was often present in the cytoplasm of chondrocytes in the articular cartilage. IL-1\( \beta \) was found predominantly at bone/pannus junctions in association with macrophages, osteoclasts and neutrophils. IL-6 staining was associated with osteoblasts present within and on the surface of eroded bone in the calcaneus (Fig 5B). Cytoplasmic staining for IL-6 was identified within neutrophils and macrophages. Less TNF\( \alpha \) was found, concentrated mainly at cartilage and bone/pannus junctions (Fig. 5C), and in the synovial tissue.

A significant reduction in IL-1\( \beta \) \( (P < 0.01) \), IL-6 \( (P < 0.05) \) and TNF\( \alpha \) \( (P < 0.05) \) was observed in ankles of SCW rats previously depleted of synovial macrophages (Fig. 5D–F, Table 3). All cytokines were totally

**Table 2.** Histological grading of the ankle joints isolated from rats 26 days after treatment with either saline, SUV + c or SUVc

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline (n = 5)</th>
<th>SUV + c (n = 5)</th>
<th>SUVc (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage destruction/bone erosion (0–3)</td>
<td>3</td>
<td>2.6 ± 0.3</td>
<td>1.2 ± 0.4*</td>
</tr>
<tr>
<td>Inflammatory cell infiltrate (0–3)</td>
<td>3</td>
<td>1.6 ± 0.3*</td>
<td>0.8 ± 0.2*</td>
</tr>
<tr>
<td>Synovial membrane hyperplasia (0–2)</td>
<td>2</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Exudate (0–1)</td>
<td>1</td>
<td>1</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

* \( P < 0.001 \) vs saline controls (Mann–Whitney U-test).
FIG. 5. Immunohistological analysis in paraffin wax sections of ankle joints isolated from rats at day 36 following treatment with either saline (control) or SUVc on day 10. Localized production of cytokines within the ankle joints of rats treated with saline (A, B, C) or SUVc (D, E, F) was detected using antibodies to IL-1β (A and D), IL-6 (B and E) and TNFα (C and F). A significant reduction in all cytokines tested was observed in SUVc-treated rat ankle joints. Cytokines and proteinase are identified by brown HRP staining (original magnification ×80).

FIG. 6. Immunohistological analysis in paraffin wax sections of ankle joints isolated from rats at day 36 following treatment with either saline (control) or SUVc on day 10. Localized production of MMP-9 was identified within the ankle joints of rats treated with saline (A and B) or SUVc (C and D) using a polyclonal antibody. In saline-treated rats, MMP-9 was seen in the cytoplasm of osteoblasts on the surface of osteoid tissue (A) and in fibroblast-like cells in the synovium (B). The cytoplasmic staining of MMP-9 was significantly reduced throughout the ankles of SUVc-treated rats (original magnification ×80).
absent from the bone, but staining for IL-1 and TNFz was sparse within the synovium.

**Identification of MMP-9**

In control ankle joints, positive staining for MMP-9 was identified within the cytoplasm of fibroblast-like cells found throughout the synovium (Fig. 6A) and in osteocytes, osteoblasts present within and on the surface of eroded bone mainly in the calcaneus (Fig. 6B).

MMP-9 was significantly reduced (P < 0.05) in both bone (Fig. 6C) and synovium (Fig. 6D, Table 3) of ankles of SCW rats previously depleted of synovial macrophages.

**Discussion**

Our study clearly demonstrates that a single intravenous injection of SUVc (20 mg) is effective in suppressing the onset of chronic SCW-induced arthritis in female Lewis rats. This is associated with the elimination of both hepatic and synovial macrophages, as well as a reduction in IL-1β, IL-6, TNFz and MMP-9 produced in the joint.

Activated macrophages at sites of inflammation, specifically in the synovial tissue, are highly phagocytic [10] and are therefore capable of ingesting liposomes. Previous studies using experimental models of arthritis have demonstrated selective depletion of synovial macrophages following intra-articular injection of MLVc [36–42]. This resulted in the prevention and amelioration of chronic arthritis, thus implicating a central role for local macrophage populations in the pathogenesis of this disease. Systemic administration of MLVc was also effective in reducing synovitis and inflammation in chronic rat adjuvant arthritis [43, 44]. However, in these studies the reduction in clinical and histological signs of arthritis was attributed to the elimination of macrophages from organs of the RES (mainly the liver, spleen and lymph nodes), and not the inflamed synovium. A possible explanation for this is that MLVs, because of their size, fail to accumulate in inflamed joints after systemic administration [45].

In addition to MLVc, SUVc are also effective in eliminating macrophages from organs of the RES after intravenous injection [18, 31]. As with MLVc, SUVc significantly reduce inflammation in rat antigen-induced arthritis [18] and adjuvant arthritis [19]. A single intravenous injection of SUVc (20 mg) was more effective in increasing the rate of resolution of experimental arthritis than an equivalent dose of MLVc. This increase in efficacy was due to a significant depletion of resident macrophage populations present within the inflamed synovial tissue, a probable result of greater accumulation of the smaller vesicles in the inflamed joint [45]. In the present study, we further utilized this property of SUVc in order to investigate the role of macrophages in the onset of chronic SCW-induced arthritis. We observed that on day 36, ED1+ macrophages were significantly depleted in livers (46%) and ankle joints (98%) of SUVc-treated rats, but not spleens. Previously, we have demonstrated that 3 days after a single intravenous administration of SUVc, macrophages from the spleen, liver and joint of rats with antigen-induced arthritis were significantly depleted by 71, 93 and 92%, respectively [18]. Studies have shown that the elimination of both systemic and synovial macrophages after liposomal clodronate administration is reversible, and complete repopulation takes 2–4 weeks [31, 40, 41]. Therefore, it is likely that the increase in splenic and hepatic ED1+ staining observed in the present study was due to macrophage repopulation. The sustained reduction in ED1+ synovial macrophages (98%) indicates repopulation had not occurred and we therefore speculate that ED1+ synovial macrophages are integral to the pathogenesis of SCW-induced arthritis.

The continued presence of PG-PS in chronically inflamed tissue is important for the maintenance of SCW-induced arthritis, possibly through activation of macrophages [20, 46–49]. PG-PS has been identified in phagocytes in joint tissue for up to 90 days after the initial intraperitoneal injection [46]. Similarly, PG-PS persists for at least 180 days in livers and spleens where it is believed to be slowly degraded and redistributed to articular sites for periodic reactivation of arthritis [49–52]. Macrophage depletion following SUVc treatment may have therefore resulted in reduced distribution of degraded PG-PS and removal of the main effector cells in inflamed joints to which the redistributed PG-PS is targeted.

In an attempt to characterize further the role of macrophages in local inflammation and joint destruction in SCW-induced arthritis, we investigated the effects of macrophage depletion on the level of locally produced cytokines. Following macrophage depletion, IL-1β, IL-6 and TNFz were almost totally eliminated from bone and synovium in ankle joints. The elimination of these cytokines correlated well with the observed reduction in inflammatory parameters. Resident ED1+ macrophages are therefore an essential requirement for the local expression of cytokines involved in both inflammation and joint destruction.

In addition to cytokines, we also demonstrated intense staining for the gelatinase, MMP-9 (gelatinase B) in fibroblast-like cells and osteoblasts in inflamed control joints. Following synovial macrophage depletion, positive staining for MMP-9 was greatly diminished.

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**Table 3. Image analysis of peroxidase-stained ankle joints isolated from rats 26 days after treatment with either saline or SUVc**

<table>
<thead>
<tr>
<th>Target</th>
<th>Staining index (Saline n = 3)</th>
<th>Staining index (SUVc n = 3)</th>
</tr>
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<tbody>
<tr>
<td>IL-1</td>
<td>18.71 ± 5000</td>
<td>696 ± 340**</td>
</tr>
<tr>
<td>IL-6</td>
<td>10.821 ± 4905</td>
<td>50*</td>
</tr>
<tr>
<td>TNF</td>
<td>1759 ± 407</td>
<td>664 ± 393*</td>
</tr>
<tr>
<td>MMP-9</td>
<td>12.612 ± 4960</td>
<td>861 ± 658*</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

*P < 0.05, ** P < 0.01 vs saline controls (Student’s t-test).
throughout the joint, suggesting that local MMP-9 expression may be mediated by macrophages. The activity and expression of MMP-9 is both induced and increased in osteoblast-like cells [53] and rheumatoid synovial fibroblasts [54] following stimulation with IL-1 and TNFα. Thus, the observed reduction in MMP-9 production may be the consequence of almost complete inhibition of IL-1β and TNFα in the joint.

Although our studies cannot differentiate between the relative importance of macrophage elimination in the RES and in the joint, it is clear that macrophage elimination by a single dose of intravenous SUCVc inhibits the production of mediators within the joint which are implicated in cartilage and bone erosion. In a recent clinical trial, Barrera et al. [55] reported depletion of CD68⁺ cells in the synovial lining of patients with RA. This was accomplished by intra-articular administration of liposomal clodronate and was accompanied by a significant decrease in the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. This study highlights the importance of local macrophage depletion within the joint to attain therapeutic benefit.

In conclusion, we have established that both local and systemic ED1⁺ macrophages are important cell populations to the progression of chronic SCW-induced arthritis. They are pivotal to the localized production of both cytokines and proteolytic enzymes involved in inflammatory arthritis. Following intravenous administration, SUCVc effectively eliminates macrophages from the RES and the synovial joint. This has now been demonstrated in several experimental models of arthritis. The potential of SUCVc-based therapies for the treatment of human inflammatory disorders such as RA has been highlighted in this study.

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