Inhibition of neutrophil responses by cyclosporin A. An insight into molecular mechanisms

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Abstract

Objective. Cyclosporin A (CsA) is an effective agent in rheumatoid arthritis (RA), slowing joint damage progression. Its therapeutic effect on T lymphocytes has been studied extensively, but there is little information available about neutrophils, the cells responsible for a substantial proportion of inflammation. A study was performed to investigate the in vitro effects of CsA on neutrophil functions triggered by several agonists and determine whether the drug could counteract the binding of formyl-methionyl-leucyl-phenylalanine (fMLP) to its receptor and/or modulate changes in the intracellular Ca2+ concentration ([Ca2+]i).

Methods. CsA was added to neutrophils 5–50 min before the incubation steps for neutrophil function assays (chemotaxis, superoxide anion production, lysozyme release), calcium measurements and receptor binding experiments.

Results. CsA appeared to be particularly effective in lowering chemotaxis, superoxide anion production and lysozyme release induced by different agonists. However, it did not significantly affect either basal or agonist-stimulated neutrophil [Ca2+]i, and the interaction between fMLP and its receptor.

Conclusions. Because of its in vitro inhibition of neutrophil functions, CsA appears to have considerable potential as an anti-inflammatory drug. Moreover, as it is also a potent immunosuppressive agent, it may reduce the progression of joint damage in RA. More work remains to be done to clarify the molecular mechanism of CsA action on neutrophils.

Key words: CsA, Rheumatoid arthritis, Neutrophil functionality, Cytosolic calcium, Receptor binding assay.

In recent years, several immunosuppressive agents have proved effective in the treatment of rheumatoid arthritis (RA), including azathioprine, methotrexate, cyclophosphamide and cyclosporin A (CsA), a cyclic undecapeptide isolated from the fungi Tolypocladium inflatum and Cylindrocarpon lucidum. Various studies have provided substantial evidence that CsA, at serum concentrations of 100–150 ng/ml, can control the symptoms of active RA, and document the efficacy of the drug in slowing the rate of progression of joint lesions [1–3]. The effects of CsA on T lymphocytes have been studied extensively [4, 5], and evidence has also been reported for its effects on other cell types [6, 7]. In human monocytes and macrophages, CsA induced apoptosis and abolished the inositol 1,4,5-trisphosphate-mediated release of calcium ions from intracellular stores. CsA inhibits nitric oxide synthesis in a fibroblast cell line in vitro [8]. Low-dose CsA inhibited endothelial cell proliferation, chemotaxis and the release of metallopeptinases 2 and 9, both in vitro and in vivo [9]. However, relatively little is known about its effects on neutrophils [10, 11]. In RA, leukocytes accumulate in the synovial fluid and act in synergy with the concomitant release of destructive free oxygen radicals and proteolytic enzymes to destroy connective tissue components, including collagen, elastin, laminin and fibronectin [12]. Neutrophils, in particular, generate arachidonic acid derivatives, including prostaglandins and leukotrienes, which are capable of producing an intense inflammatory response [13]. The present work investigated the in vitro effects of CsA on human neutrophil activation, including several


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antimicrobial functions, namely chemoattractant-induced motility, superoxide anion (O$_2^-$) generation and lysozyme agonist-triggered degranulation. As it has been shown previously that Ca$^{2+}$ is involved in the modulation of neutrophil responses [14], the effect of CsA was also tested on formyl-methionyl-leucyl-phenylalanine (fMLP)-induced changes in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Moreover, the possible interaction of CsA with the chemoattractant receptor was taken into consideration.

Materials and methods

Preparation of human neutrophils

Cells were obtained from the blood of healthy subjects and neutrophils were purified by the standard techniques of dextran (Pharmacia, Uppsala, Sweden) sedimentation, centrifugation on Ficoll–Paque (Pharmacia) and hypotonic lysis of contaminating red cells. The cells were washed twice, resuspended in Krebs–Ringer–phosphate containing 0.1% w/v glucose (KRPG), pH 7.4, at a final concentration of 50 × 10$^6$ cells/ml and kept at room temperature until used. The percentage of neutrophils was >98% pure and >99% viable as determined by the trypan blue exclusion test.

Random locomotion

Random locomotion was evaluated with a 48-well microchemotaxis chamber (BioProbe, Milan, Italy), using the method of Zigmond and Hirsch [15], by estimating the distance (in micrometres) which the leading front of the cell migrated after 90 min of incubation at 37°C. A 3 µm pore size filter (Millipore, Rome, Italy) was used to separate the upper and lower compartments. The distance migrated was 37 ± 4 µm (s.e.) in 15 separate experiments. Assays were carried out in duplicate under each experimental condition.

Chemotaxis

Chemotaxis was studied by adding the chemoattractant to the lower compartment. The chemotactic factors used were: fMLP (Sigma, St Louis, MO, USA) 10$^{-8}$ M in KRPG containing 1 mg/ml of bovine serum albumin (KRPG-A, Sigma) or casein (Hammarsten; Merck, Darmstadt, Germany) 2 mg/ml in KRPG-A. The fMLP stock solution [10$^{-2}$ M in dimethylsulphoxide (DMSO, Sigma)] was diluted before use in KRPG-A. The casein stock solution (10 mg/ml in KRPG) was diluted before use in KRPG-A. Assays were performed in duplicate under each experimental condition. The DMSO did not interfere with any of the biological assays performed.

Superoxide anion production

Production of O$_2^-$ was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome c modified for microplate-based assays [16]. The tests were carried out in a final volume of 200 µl containing 4 × 10$^5$ neutrophils, 100 nmol cytochrome c (Sigma) and KRPG. At zero time the stimulant was added and the plates were incubated in a microplate reader (Ceres Bio-TeK Instruments, Winooski, VT, USA) with compartment T set at 37°C. Absorbance was recorded at wavelengths of 550 and 468 nm. The difference in absorbance at the two wavelengths was used to calculate the number of nanomoles of O$_2^-$ produced, using a molar extinction coefficient for cytochrome c of 15.5 mm$^{-1}$ cm$^{-1}$. The stimulants employed were phorbol 12-myristate 13-acetate (PMA, Sigma) 100 or 10 ng/ml, fMLP 10$^{-6}$ or 2.5 × 10$^{-8}$ M or zymosan (Sigma) opsonized with a pool of fresh human sera (OZ) 2 or 1 mg/ml. When needed, neutrophils were pre-incubated with cytochalasin B (Sigma) 5 µg/ml for 5 min prior to activation by fMLP or OZ. Assays were performed in triplicate under each experimental condition.

Enzyme assay

Release of neutrophil granule enzymes was evaluated by determining lysozyme activity modified for microplate-based assays [16]. Cells (3 × 10$^8$) were incubated in microplate wells in the presence of the stimulus for 15 min at 37°C. The plates were then centrifuged for 5 min at 400 g and the lysozyme was quantified nephelometrically by the lysis rate of Micrococcus lysodeikticus (Sigma) cell wall suspension. Enzyme release was expressed as the net percentage of total enzyme content released by 0.1% Triton X-100. Total enzyme activity was 85 ± 1 µg/1 × 10$^5$ cells/min. The reaction rate was measured with a microplate reader at 465 nm. Spontaneous release was less than 10%. The degranulating agents used were PMA (100 or 10 ng/ml) or fMLP (10$^{-6}$ or 2.5 × 10$^{-8}$ M) in KRPG. When required, cells were preincubated with 5 µg/ml cytochalasin B for 10 min prior to activation by fMLP. Assays were performed in duplicate under each experimental condition.

Changes in [Ca$^{2+}$$]_i$

Neutrophils were loaded with 2 µM Fura 2-AM (Sigma) for 30 min in the dark at 37°C, then washed and resuspended in KRPG, and tested for fluorescence with a Perkin-Elmer spectrofluorometer equipped with 2 ml cuvettes, stirring apparatus and a thermostatic unit. The evaluation of basal or stimulated levels of Ca$^{2+}$ was carried out as described by Grynkiewicz et al. [17] using the equation: [Ca$^{2+}$]$_i$ = K$_D$(37°C) × (F – F$_{min}$/F$_{max}$ – F) where F$_{min}$ and F$_{max}$ were determined as previously described [14]. Briefly, for each sample fluorescence calibration was carried out in the presence of 2.5 mm EGTA and 0.05% Triton X-100 to obtain the minimum fluorescence (F$_{min}$) and of 5.0 mm CaCl$_2$ to obtain the maximum fluorescence (F$_{max}$). F corresponded alternatively to the basal fluorescence of loaded cells or to the peak after stimulation. K$_D$ at 37°C was 224 nm.

Receptor binding experiments

Neutrophils (2 × 10$^6$/sample) were incubated for 15 min at 37°C in the presence of 6 nM [3H]fMLP (specific
activity 71 Ci/mmol; NEN, Milan, Italy). Unlabelled fMLP (10 μM) was used to determine non-specific binding. At the end of incubation, the samples were rapidly washed with ice-cold buffer and filtered through Whatman GF/B filters with a cell harvester (Brandel; Gaithersburg, MD, USA). Radioactivity bound to the filters was measured by liquid scintillation spectrometry (LS 6500; Beckman, Galway, Irish Republic).

**Measurement of cell viability**

To assess possible cytotoxic effects of the tested drug, the amount of cytoplasmic lactate dehydrogenase (LDH) was determined by measuring the rate of oxidation of NADH (Fluka, Milan, Italy). The change in absorbance was followed at 340 nm [18].

**Treatment with CsA**

When required, 10^{-12} to 10^{-5} M CsA (kindly provided by Novartis Farma, Origgio, Italy) was added to the cells alone, simultaneously with the triggers or 5–50 min before the incubation steps for neutrophil functional assays, calcium measurements and receptor binding experiments. CsA stock solutions (10^{-3} M in DMSO) were diluted in KRPG before use.

**Statistical analysis**

One-way analysis of variance and Bonferroni multiple comparison, using the Sigma Stat (Jandel Scientific, Erkrath, Germany) computer program, were used for statistical analysis of the data (level of significance, P < 0.05).

**Results**

Release of the cytoplasmic enzyme LDH was used as an indicator of cell viability. In none of the experiments described below was the percentage of total LDH release more than 3%. Preliminary experiments were carried out to establish the dose–response effects of fMLP, PMA, casein and OZ on neutrophil activation. The maximal active concentrations were 10^{-6} M fMLP, 100 ng/ml PMA or 2 mg/ml OZ, while 2 × 10^{-8} M fMLP, 10 ng/ml PMA and 1 mg/ml OZ were the doses at which half the maximal effect was reached. Treatment with CsA alone (time range 0–50 min depending on the analysis) did not have significant effects on the cells (not shown). Similarly, when CsA was added simultaneously with the triggers, it did not induce significant changes in cell function, at least with respect to the variations studied (not shown). In the light of the above observations, tests were carried out routinely, after a period of CsA preincubation.

**Effect of CsA on neutrophil motility**

When CsA was tested for its ability to influence random locomotion, the compound showed slight but not statistically significant (P > 0.05) effects at all of the concentrations tested (10^{-12} to 10^{-6} M) (data not shown). Conversely, when concentrations of CsA ranging from 10^{-12} to 10^{-6} M were tested for their ability to influence directed migration, dose-dependent inhibition of fMLP- and casein-induced chemotaxis (Fig. 1A and 1B respectively) were observed. The inhibition became statistically significant (P < 0.05) at a drug concentration of 10^{-11} M, reaching about 80% at the highest concentration (P < 0.01) for both the agonists. As the reduction was almost always independent of the duration of preincubation, only the 20-min results are reported.

CsA was not per se a chemotaxin for neutrophils.

**Effect of CsA on neutrophil respiratory burst**

We studied the effect of CsA on O_2^- production triggered by maximally and submaximally effective agonist concentrations. Increasing doses of CsA (10^{-9} to 10^{-6} M) did not inhibit the respiratory burst activated by either 100 ng/ml PMA or 2 mg/ml OZ (data not shown), while the 10^{-6} M fMLP-activated response was inhibited in a dose-dependent manner (Fig. 2). This effect was more evident without cytochalasin B treatment (P < 0.05 beginning at 10^{-9} M CsA) (Fig. 2A) than with it (P < 0.05 beginning from 10^{-7} M CsA) (Fig. 2B). As the reduction was almost always independent of the duration of preincubation, only the 20-min results are reported.

Figure 3 illustrates CsA inhibition upon triggering of neutrophils with submaximal doses of the agonists. In particular, when cells were triggered with 2.5 × 10^{-8} M fMLP (Fig. 3A), time- and dose-dependent inhibition was observed. Five minutes of preincubation was statistically different (P < 0.05) from 20 min at all the concentrations tested. The concentration effect became statistically significant (P < 0.05) after 10 and 20 min of preincubation with 10^{-9} M CsA or after 5 min of preincubation with 10^{-8} M CsA. The highest inhibition (about 60%, P < 0.001) was seen at 10^{-7} M CsA and with 20 min of preincubation. When cells were activated with 10 ng/ml PMA (Fig. 3B) or 1 mg/ml OZ (Fig. 3C), only time-dependent inhibition was seen. Five minutes of preincubation was statistically different (P < 0.05) from 20 min at all the concentrations tested. A concentration of 10^{-9} M was needed to significantly (P < 0.05) reduce O_2^- production, which remained unchanged up to 10^{-6} M Data which were not statistically significant are omitted.

CsA was not per se able to induce superoxide anion production by neutrophils.

**Effect of CsA on neutrophil granule secretion**

With regard to granule enzyme release (Fig. 4), CsA elicited marked concentration- and time-dependent inhibition with maximally effective agonist concentrations of 10^{-6} M fMLP (Fig. 4A) and 100 μg/ml PMA (Fig. 4B). Five minutes of preincubation was statistically different (P < 0.05) from 20 min at all the concentrations tested. The highest inhibition (about 50%, P < 0.001) was seen at 10^{-9} M CsA and with 20 min of preincubation. Time- and concentration-independent inhibition was seen when cells were activated with

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**References**

Fig. 1. Effect of CsA concentration on neutrophil chemotaxis induced by 10^{-8} M fMLP (A) or 2 mg/ml casein (B). Cells were incubated with different concentrations (10^{-12} to 10^{-5} M) of CsA for 5, 10 or 20 min before the chemotactic assay. The distance of chemotaxis was 83 ± 2 and 95 ± 4 μm, induced by fMLP and casein respectively in 15 separate experiments. Standard errors are within 10% of the mean values. Statistical significance is reported in the text. *Concentration at which the effect of CsA became statistically significant (P < 0.05).

Fig. 2. Effect of CsA concentration on O_2^− generation triggered by maximal fMLP concentration in neutrophils not treated with cytochalasin B (A) and treated with cytochalasin B (B). Cells were incubated with different concentrations (10^{-3} to 10^{-6} M) of CsA for 5, 10 or 20 min before the stimulus was applied. The rate of O_2^− generation was 30 ± 2 nmol/2 × 10^6 cells/5 min and 35 ± 2 nmol/2 × 10^6 cells/10 min for fMLP or PMA respectively in 15 separate experiments. Standard errors are within 10% of the mean values. Statistical significance is reported in the text. *Concentration at which the effect of CsA became statistically significant (P < 0.05); **P < 0.001.

2 mg/ml OZ (Fig. 4C). The concentration effect became statistically significant (P < 0.05) after 20, 10 and 20 min of preincubation with 10^{-9}, 10^{-8} and 10^{-7} M CsA respectively. Data which were not statistically significant are omitted.

CsA was not per se a secretagogue for neutrophils.

Effect of CsA on intracellular Ca^{2+} levels

The effects of CsA on basal and agonist-induced Ca^{2+} intracellular changes were tested at concentrations of 1 μM and 25 nM fMLP. Preincubation of cells with CsA (time range 10–30 min) did not significantly (P > 0.05) affect either basal or agonist-stimulated neutrophil [Ca^{2+}],. Control and 20-min preincubation effects are reported in Table 1.

Effect of CsA on fMLP binding

The effect of different concentrations of CsA (range 10^{-8} to 10^{-6} M) and various drug exposure times (range 10–30 min) on fMLP binding to neutrophils was evaluated. Our experimental conditions did not affect the interaction between fMLP and its receptors. Control and 20-min preincubation effects are reported in Table 2.
Fig. 3. Effect of CsA concentration on $O_2^-$ generation triggered by submaximal agonist concentrations: $2.5 \times 10^{-8}$ M fMLP (A), 10 ng/ml PMA (B) and 1 mg/ml OZ (C). Cells were incubated with different concentrations ($10^{-10}$ to $10^{-6}$ M) of CsA for 5, 10 or 20 min before the stimulus was applied. The rate of $O_2^-$ generation was $15 \pm 2, 17 \pm 2$ and $13 \pm 1$ nmol/2 x 10^6 cells for fMLP (5 min), PMA (10 min) and OZ (20 min) respectively in 15 separate experiments. Standard errors are within 10% of the mean values. Statistical significance is reported in the text. *Concentration at which the effect of CsA became statistically significant ($P < 0.05$); **$P < 0.001$.

Discussion

Despite the large number of studies using CsA in RA and the immunological effects of CsA, no studies report the effect that this drug has on neutrophils, which appear to play a substantial role in joint destruction as they invade the synovium in RA and release proteolytic enzymes.

The synovial pannus, the first compartment, consists of hypertrophic synovium infiltrated predominantly with mononuclear leukocytes. The pannus may contribute to the propagation of inflammation in several ways. First, plasma cells in the pannus are a source of rheumatoid factor as well as anti-collagen antibodies in RA. The immune complexes these antibodies form in the joint may activate complement, leading to generation of C5a and chemotraction of neutrophils. Monocytes and synoviocytes in the pannus then release proinflammatory cytokines, some of which may recruit and activate neutrophils [19]. The rheumatoid pannus is located at the margins of the joint cartilage—the site of characteristic periarticular erosion—and appears to be active in joint destruction. The pannus contains relatively few neutrophils, but these cells have been identified at the pannus–cartilage border, suggesting a role for neutrophils in the pannus-mediated component of cartilage destruction [20].

In contrast to the synovium, in RA the joint space is filled almost exclusively with neutrophils. Indeed, the destructive capacity of neutrophils has long been known, and the presence of considerable numbers of neutrophils in the synovial fluid of patients with RA supports a role for these cells in the pathogenesis of joint destruction. There are many lines of evidence that neutrophils contribute to the pathogenesis of RA [21, 22]. Numerous studies have addressed the question whether the neutrophils involved in RA are fundamentally different from non-rheumatoid neutrophils in terms of either function or molecular expression. In general, peripheral blood neutrophils from RA patients exhibit evidence of prior activation compared with neutrophils from control subjects and synovial fluid neutrophils from rheumatoid patients demonstrate further evidence of prior activation. Felzmann et al. [23] studied a panel of function-associated receptor molecules and demonstrated that a number of these, including FcγR1 and the complement receptors CR1, CR3 and CR4, were up-regulated in rheumatoid patients; moreover, Spisani et al. [24, 25] showed defective neutrophil membrane protein expression in these patients, indicating that neutrophil phagocytosis—which is strongly activated by the persistence of high levels of immune complexes in circulation—probably causes an imbalance in the expression of different surface receptor proteins. Goulding and Guyre [26] studied FcR signalling in rheumatoid vs normal peripheral neutrophils; they showed that, whereas the overall measure of neutrophil activation (i.e. basal $[Ca^{2+}]_i$) was increased in RA compared with control neutrophils, FcR signalling was increased in neutrophils from patients with quiescent RA but decreased in patients with active disease. According to Jones et al. [27] leukocyte migration into rheumatoid joints is increased, although several studies have shown that peripheral blood rheumatoid neutrophils leave chemotaxis unaffected or decrease it. Other studies support increased activation of neutrophils in RA, including enhanced peripheral degranulation [28] and superoxide anion generation [29].

The main goal of the present research was to determine whether CsA affects some important neutrophil functions—possibly the molecular mechanisms involved—and, if so, how. Because there is little information about the effects of CsA on the biochemical machinery of neutrophils, we carried out a series of experiments on cells from healthy subjects. We analysed the in vitro effect of CsA on a wide range of neutrophil functions triggered by different stimuli acting either through specific cell surface receptors (e.g. fMLP and zymosan) or by postreceptor signalling (e.g. casein and PMA). It is generally accepted that receptor and non-receptor agonists use different transduction mechanisms to activate chemotaxis and free radical production [14, 30]. According to our results, CsA seems to be particularly effective in lowering chemotaxis when neutrophils are activated by the optimal doses of two agonists, fMLP and casein. Superoxide anion production, in contrast, seems more affected by CsA when submaximal doses of agonists are used, and inhibition appears independent of the stimulus used. CsA, moreover, inhibits lysosomal release in a concentration- and time-dependent manner.

We have shown previously that fMLP increases neutrophil $[Ca^{2+}]_i$ by interacting with a specific membrane receptor [14], and that this effect is an essential trigger for fMLP-induced degranulation and...
FIG. 4. Effect of CsA concentration on granule secretion triggered by 10⁻⁶ M fMLP (A), 100 ng/ml PMA (B) and 2 mg/ml OZ (C). Cells were incubated with different concentrations (10⁻¹⁰ to 10⁻⁶ M) of CsA for 5, 10 or 20 min before stimulation. The rate of lysozyme release was 45 ± 4, 40 ± 3 and 38 ± 3% of 10⁷ cells for fMLP (15 min), PMA (15 min) and OZ (20 min) respectively in 15 separate experiments. Standard errors are within 10% of the mean values. Statistical significance is reported in the text. *Concentration at which CsA effect becomes statistically significant (P<0.05); **P<0.001.

| Table 1. Effect of CsA on basal and fMLP-induced changes in [Ca²⁺], in neutrophils |
|---------------------------------|---|---|---|
| Time of preincubation          | [CsA] | [fMLP] | [Ca²⁺] (% of basal) |
|                                |      |      |                      |
| −                               | −    | 1 µM | 526 ± 38 (n=8)       |
| −                               | −    | 25 nM| 233 ± 20 (n=6)       |
| 20 min                          | 1 µM | 1 µM | 532 ± 29 (n=4)       |
| 20 min                          | 1 µM | 25 nM| 232 ± 34 (n=6)       |

Basal [Ca²⁺], in neutrophils was 91 ± 8 nm.

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<th>Table 2. Effect of CsA on fMLP binding to human neutrophil receptors</th>
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Data are mean ± SEM of four separate determinations. fMLP binding under control conditions: 10.5 fmol/1 x 10⁶ cells.

superoxide production, whereas it is not required for chemotaxis. Because the molecular mechanism whereby CsA influences the neutrophil response is still unknown, we verified whether the drug could counteract the binding of fMLP to its receptor and/or modulate [Ca²⁺] changes. Times of preincubation of cells with CsA were chosen on the basis of parallel experiments revealing the inhibitory effect of the drug on chemotaxis, degranulation and superoxide production. Within an interval of 0–30 min, CsA did not significantly impair specific fMLP binding. Therefore only CsH—and not CsB, C, D, E [31] or A (present work)—appears to act as a competitive formyl peptide receptor antagonist. Previous data [32] suggested a long-term effect (20 h) of CsA on receptor desensitization in vascular smooth cells, though no direct evidence has been reported. It has been demonstrated that CsA may interfere with neutrophil degranulation induced by Ca²⁺ ionophores such as A23187 and ionomycin [33]. The mechanism of action, however, is unclear, as the effect of CsA on ionophore-induced Ca²⁺ fluxes towards the cytosolic compartment was not measured. Because the fMLP interaction with the specific receptor is known to potently increase neutrophil [Ca²⁺], mainly by mobilizing intracellular stores [14], we studied the interference of CsA in this receptor/agonist-modulated effect. In our experimental model, fMLP-induced elevation of [Ca²⁺], was not counteracted by CsA at any concentration, no matter how long the cells were exposed to the compound. Similarly, CsA did not show any significant effect on [Ca²⁺], in unstimulated neutrophils.

Taken together, these observations lead us to conclude that the inhibition of neutrophil activity observed in patients during CsA therapy can be ascribed to the drug’s direct action on the neutrophils. The molecular mechanisms by which CsA exerts its inhibitory role on cell chemotaxis, degranulation and superoxide generation have not yet been elucidated, and other transduction mechanisms remain to be studied as potential targets of the drug.

We conclude that, because of its inhibitory effects on neutrophil activity in vitro, CsA could be used as an anti-inflammatory drug in the control of joint damage in RA. Further studies are needed to show whether RA neutrophils function differently from healthy cells and to what extent CsA affects their biochemical machinery and function.
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