The \( \kappa \)-opioid agonist, asimadoline, alters cytokine gene expression in adjuvant arthritis

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Abstract

**Objective.** We have previously found that the \( \kappa \)-opioid agonist, asimadoline, attenuates adjuvant arthritis in a dose-dependent, antagonist-reversible manner. To elucidate possible mechanisms, we investigated the effects of asimadoline (5 mg/kg/day i.p.) or vehicle on *in vivo* cytokine expression and T-cell recruitment in adjuvant arthritis.

**Methods.** Arthritis severity was assessed every 3–4 days for 21 days. Rats were killed on days 0, 13 and 21 post-induction and synovial membrane and inguinal lymph nodes were removed for mRNA extraction. Changes in cytokine mRNA expression were measured using reverse transcription–polymerase chain reaction (RT–PCR) and densitometry. T cells in joints were quantified by immunohistochemistry.

**Results.** Asimadoline significantly decreased arthritis severity at day 13, with a concomitant decrease in synovial membrane expression of cytokines interleukin-17 and transforming growth factor-\( \beta \) (TGF-\( \beta \)) mRNA at day 13, and no change in T cell numbers in the joints of arthritic rats. By contrast, in the inguinal lymph nodes, expression of tumour necrosis factor was increased at day 13 and TGF-\( \beta \) mRNA was increased throughout.

**Conclusion.** An altered balance, therefore, in the pro- and anti-inflammatory effects of TGF-\( \beta \) by asimadoline might explain its striking anti-arthritic actions.

**KEY WORDS:** Cytokines, Opioids, Asimadoline, Adjuvant arthritis, \( \kappa \)-Opioid agonist, Inflammation.

The immune system can be influenced by both endogenous and exogenous opioids [1]. Opioids have been shown to regulate lymphocyte proliferation, antibody production and natural killer cell activity, as well as inhibiting the function of neutrophils, monocytes and macrophages [2]. They exert their effects on the immune system via one of three families of opioid receptors (\( \mu \), \( \kappa \) or \( \delta \)) located on cells of the immune system [1, 3–5]. Pharmacological doses of opioids have been shown to have anti-inflammatory effects. For example, opioids can inhibit carrageenan-induced paw swelling in the rat [6] and can attenuate the development of experimental arthritis [7–10]. Agonists acting at the \( \kappa \)-receptor are more potent immunosuppressive agents than those acting at the \( \mu \)-receptor [11, 12]. In rats, we have shown that opioids acting at the \( \kappa \)-receptor can attenuate adjuvant arthritis, an immunologically mediated inflammatory arthritis [9, 10, 13]. For example, the peripherally selective \( \kappa \)-opioid agonist, asimadoline, attenuated adjuvant arthritis by up to 80% in a dose-dependent, antagonist-reversible manner [13, 14]. However, the mechanism of this effect is not known.

Opioids have been shown to suppress immune cell cytokine production *in vitro*. The prototype \( \kappa \)-opioid agonist, U-50,488H, inhibited gene transcription and production of the cytokines tumour necrosis factor (TNF) and interleukin-1 (IL-1), by macrophages *in vitro* [15]. Our laboratory has shown that asimadoline suppresses TNF production by lipopolysaccharide-stimulated peritoneal macrophages *in vitro* [16]. We have also shown that \( \kappa \)-agonists significantly reduce synovial macrophage and mast cell numbers in adjuvant arthritis [16], as well as inhibit up-regulation of the adhesion molecule, ICAM-1, *in vivo* [17]. The effect of \( \kappa \)-opioid agonists on the expression of TNF and other cytokines in *in vivo* models of inflammation, such as adjuvant arthritis, has not been studied. Furthermore, the effect of \( \kappa \)-agonists on T-cell recruitment has not been examined. Therefore, the aim of this study was to investigate the effects of the \( \kappa \)-opioid agonist, asimadoline, on *in vivo* cytokine expression and T-cell trafficking in adjuvant arthritis.

Materials and methods

**Animals**

Male Dark Agouti rats 4–8 weeks old weighing 104–224 g were obtained from the University of Queensland and housed in cages lined with cellulose
bedding and shredded paper in a temperature controlled room (22 ± 1 °C) with a 12 h alternating light and dark cycle. The animals were given food (rat chow, Gordon’s Speciality Stockfeeds, Yanderra, Australia) and water ad libitum for 1–2 weeks prior to, and throughout, the experiments. All experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales, Sydney, Australia.

Induction of adjuvant arthritis
The animals were handled for 1–2 weeks prior to the experiments and every 2–3 days throughout the 21-day study. During this time, food and fluid intake and body weight were monitored. To induce adjuvant arthritis, the rats were anaesthetized with a ketamine (50 mg/kg i.p.; Parnell Laboratories, Alexandria, Australia) and xylazine (5 mg/kg i.p.; Troy Laboratories, Smithfield, Australia) mixture and then injected with Freund’s complete adjuvant (0.1 ml of 10 mg/ml heat killed and dried Mycobacterium butyricum suspension in paraffin oil and mannide monooleate; Difco Laboratories, Detroit, MI, USA) intradermally into the tail base.

Severity of adjuvant arthritis
Disease progress was assessed every 3–4 days post-adjuvant by measuring paw volume by plethysmometry (Ugo Basile, Comerio, Italy), body weight change, and clinical arthritis severity score (as judged by swelling, necrosis and redness) over 21 days. The clinical arthritis severity score utilized a scoring system from 0 to 5; 0 = no arthritis, 1 = mildly swollen, 2 = red and moderately swollen, 3 = swollen with moderate necrosis, 4 = severe necrosis and nodules, 5 = very severe necrosis of entire foot. Gait was evaluated using a scoring system from 0 to 3; 0 = normal, 1 = slight lameness, 2 = lameness with weight bearing on toes only, 3 = non-weight bearing lameness [14, 17]. The same trained observer performed the measurements throughout the study. The rats were euthanased according to institutional guidelines using pentobarbital (60 mg i.p.; Virbac, Sydney, Australia).

Cytokine mRNA expression

Tissue collection. Following death, the inguinal lymph nodes from both sides and synovium from the right paw of each rat were removed, placed in cryotubes and immediately frozen in liquid nitrogen. The samples were stored at −70°C for subsequent RNA extraction.

RNA extraction. Synovial membrane and inguinal lymph node tissues were crushed using a custom-made metal tissue homogenizer and liquid nitrogen on ice. The resulting powder was dissolved in guanidinium isothiocyanate. Total RNA was extracted using the method of Chomczynski and Sacchi [18]. The RNA pellet was dissolved in 25 µl of RNase-free water at 42°C for 15 min. RNA was quantified by diluting 5 µl of RNA in 495 µl of RNase-free water and measuring its absorbance at 260 nm on a QuantaGene spectrophotometer (The Australian Chromatography Company, Australia). Dithiothreitol (DTT, 0.1 m; 0.8 µl; Gibco, Mulgrave, Australia) and 0.1 µl of RNasin (per 20 µl RNA; Promega, Madison, WI, USA) were added to the final RNA preparation and the RNA was stored at −70°C.

cDNA synthesis
To prepare cDNA, the RNA (0.13 µg) was added to 1 µl of 5× first strand buffer (75 mM KCl; 50 mM Tris HCl, pH 8.3; 3 mM MgCl2; Gibco), 2 mM dNTP (10 mM each dATP, dCTP, dTTP and dGTP; Promega), 0.025 µl of 0.0156 U (0.4 µg) random hexamers (Amersham Pharmacia Biotech, Sydney, Australia), 20 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco), 0.001 U RNasin (Promega) and RNase-free water to a final volume of 5 µl per cDNA. The reaction mix was then incubated at 42°C for 60 min to generate cDNA, and the final product was stored at −20°C.

Primers
Sense and anti-sense oligonucleotide primers used to amplify β-actin, IL-2, interferon-γ (IFN-γ), IL-4, TNF and transforming growth factor-β (TGF-β) have been previously published [19, 20]. IL-17 primers were designed using mRNA sequences from published papers [21] and GenBank. The sequences for the IL-17 primers were as follows: sense = 5′-TGGAC-TCTGAGCCCGATTGA-3′, anti-sense = 5′-GACGGCATGGCCGAATAGA-3′. The primers were checked for specificity using GenBank and the polymerase chain reaction (PCR) product was sequenced to confirm identity. An extensive set of preliminary experiments was undertaken to achieve the optimal conditions for each cytokine (Table 1).

PCR
We used semi-quantitative reverse transcription-PCR (RT-PCR) to measure the expression of relevant cytokines to evaluate the effect of asimadoline treatment. This method was found to show comparable changes in cytokine expression to that using quantitative

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<th>Cytokine expression (% positive control) (mean ± S.E.M.)</th>
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<td>ILN</td>
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<tr>
<td>TNF</td>
<td>34.2 ± 5.3</td>
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<tr>
<td>IFN-γ</td>
<td>43.7 ± 10.2</td>
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<td>IL-1β</td>
<td>9.7 ± 3.5</td>
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<td>IL-2</td>
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<td>IL-4</td>
<td>77.7 ± 8.9</td>
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<td>TGF-β</td>
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PCR [22]. In previous human studies we have found that if the different stages of the RT–PCR process are performed in the same experiment for the samples to be compared, very reliable results are achieved with a variation of 10% [23]. A limited number of samples can be amplified in the same PCR reaction (i.e. 40 samples), thus it is essential to use the same positive control in each reaction so samples with and without treatment can be directly compared. Like immunohistochemistry, RT–PCR is only semi-quantitative, but it has the advantage that it can detect small quantities of cytokines that are expressed weakly, for example in the synovium, if the different stages of the RT–PCR process are performed in the same experiment for the samples to be compared. Very reliable results are achieved with a variation of 10% [23].

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The density of the bands on the negative images of the gels was determined using densitometry (GS-700 densitometer, Biorad Laboratories, CA, USA) and computer analysis (Molecular Analyst Software, Biorad, CA, USA). Background readings of the gels were subtracted from the density of the samples. After correction for background, the density of the samples was expressed as a percentage of the density of positive control samples amplified simultaneously in the PCR reaction with the experimental samples.

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Immunohistochemistry for T cells

For pragmatic reasons, immunohistochemistry was performed on rat ankle sections obtained in a separate experiment. Paraffin sections were cut from pre-fixed ankle blocks at 4 μm on a rotary microtome and set on slides coated with 0.3% gelatin and dried at 56°C. The slides were baked at 80°C for 30 min in an oven prior to dewaxing. The paraffin sections of rat ankle joints were dewaxed in xylene and ethanol and washed with Tris-buffered saline (TBS; Sigma Chemicals).

Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 5 min and the slides were then washed in TBS. The antigen retrieval step consisted of heating the slides in an autoclave at 121°C for 20 min in Tris-EDTA citrate (TEC) retrieval buffer (0.001 M NaEDTA, 0.001 M Tris NaCitrate and 0.002 M Tris base). The slides were rinsed with distilled water and then TBS. The slides were then incubated with skim milk (2% in water) for 15 min, dipped in distilled water and incubated with polyclonal rabbit anti-human CD3 (1:200; Dako,}

Densitometry

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Australia) for 1 h at 37°C. Negative controls were incubated with rabbit serum (Gibco) at the same protein concentration. The slides were washed and then incubated with goat anti-rabbit biotinylated secondary antibody (1:300; Dako, Australia) for 30 min. After further washes with TBS, avidin biotin complex (ABC kit; Vector Laboratories, CA, USA) was applied for 30 min. The slides were again washed and sections developed in 0.01% DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma Chemicals) with 10 µl of hydrogen peroxide (30%) for 5 min and rinsed in TBS. The sections were counterstained in Harris’ haematoxylin for 10 s, washed in water and dipped in Scott’s blue for 5 s. The slides were then washed in water, dehydrated and cleared in alcohol and xylene, respectively, and mounted using DPX (BDH Laboratory Supply, UK).

Quantification of T cells

Stained cells were counted in high-power fields (×400) by means of a 0.02 mm² graticule fitted in the eyepiece of the microscope. Up to 10 fields were counted for all samples (coefficient of variation = 15%) by two independent observers blind to the treatments (sections from some non-arthritic rats did not have enough tissue to count 10 fields). Negative controls for each individual ankle joint were also included. The number of cells was expressed per mm² of joint tissue.

Statistical analysis

Inflammation. Previous studies in our laboratory have shown that asimadoline significantly reduces measures of arthritis severity, assessed by oedema, radiology and histology, to a similar extent [13]. For pragmatic reasons, radiology and histology could not be performed in the present study. Thus, the three indices of arthritic damage used were oedema, clinical arthritis severity and gait.

Successive paw volumes (oedema) were averaged from bilateral measures. All measurements were made over the entire 21-day observation of the time course of the adjuvant arthritis and every measurement was normalized relative to the measurement of day 0. Maximum disease severity was at day 21. Thus, the averaged paw volumes, clinical arthritis severity and gait for each rat on days 13 and 21 were then adjusted to control (vehicle-treated animals) values on day 21 (100%).

All graphical plots show mean values with standard error of the mean (S.E.M.). Multiple comparisons for each variable were made by performing a repeated measures ANOVA [the factors [groups and days] being considered fixed and where the F-ratios indicated significant heterogeneity (P < 0.05)].

Cytokines. For each sample on a gel, densities were corrected for background. The density of the samples was then expressed as a percentage of the density of positive control samples amplified simultaneously in the PCR reaction with the experimental samples. To express the data relative to non-arthritic control values, the individual values on days 13 and 21 were normalized relative to the averaged day 0 measurements (essentially non-arthritic rats). Differences between means were calculated using a one-way ANOVA.

If a significant difference was found (P < 0.05) after ANOVA, post-hoc t-tests utilizing Fisher’s multiple comparison test were performed. All statistical computations were performed using the Number Crunching Statistical System (NCSS, UT, USA).

Results

Time course of adjuvant arthritis

The animals were judged to be arthritic if their paw volume increased more than two standard deviations from the mean of the non-arthritic control group at day 21. Using this criterion, all but two rats treated with Freund’s complete adjuvant developed arthritis. The arthritic animals groomed themselves well and maintained their original body weight throughout the progress of the disease (day 0 vs day 21: 203 ± 4 vs 209 ± 10 g). Paw volume, arthritis severity and gait scores were significantly increased in the untreated rats at days 13 and 21 compared with day 0, P < 0.05 (Fig. 1).

Effect of asimadoline on adjuvant arthritis

Compared with untreated rats, asimadoline significantly reduced paw volume at day 13 (P < 0.05), but not at day 21. Arthritis severity scores and gait followed a similar pattern to paw volume (Fig. 1).

Effect of asimadoline on cytokine expression

The expression of cytokine mRNA at day 0 is summarized in Table 1. The cycle numbers for the PCR experiments for each tissue are also shown. In general,
cycle numbers to generate PCR product at the exponential stage of product accumulation were higher in the synovium than inguinal lymph nodes, indicating lower levels of cytokine mRNA in the synovial tissue. The patterns of cytokine mRNA expression in both the synovium and inguinal lymph nodes are presented in Figs 2 and 3, respectively.

In the synovium, asimadoline significantly reduced mRNA expression of IL-17 \( (P < 0.05) \) and TGF-\( \beta \) \( (P < 0.05) \) at day 13 compared with untreated rats (Fig. 2C, E). Asimadoline also significantly reduced mRNA expression of TGF-\( \beta \) \( (P < 0.05) \) in the synovium at day 21 compared with untreated rats (Fig. 2E). Although asimadoline reduced mRNA expression of TNF \( (P = 0.056; \text{Fig. } 2A) \) and IL-4 \( (P = 0.069; \text{Fig. } 2F) \), it did not quite reach the 5% level of significance. Similarly, there was a trend for a reduction in IFN-\( \gamma \) expression \( (P = 0.13) \) by asimadoline (Fig. 2B).

**Fig. 2.** mRNA expression (as a percentage of day 0 values) for (A) TNF, (B) IFN-\( \gamma \), (C) IL-17, (D) IL-2, (E) TGF-\( \beta \) and (F) IL-4 in the synovium of rats with adjuvant arthritis treated with either 5 mg/kg/day asimadoline or vehicle i.p. Filled panels represent vehicle-treated rats, open panels represent asimadoline-treated rats. *Significant difference from vehicle-treated rats \( (P < 0.05; n = 6–8) \).
By contrast, IL-2 expression remained unchanged with asimadoline treatment (Fig. 2D).

In the inguinal lymph nodes, an extra-articular site thought to reflect the early stages of the disease, asimadoline increased mRNA expression of TNF ($P < 0.05$) at day 13 and TGF-β ($P < 0.05$) at days 13 and 21 (Fig. 3A, E). Asimadoline decreased mRNA expression of IL-17 at day 21 compared with untreated rats ($P < 0.05$) (Fig. 3C). Expression of IFN-γ, IL-2 and IL-4 remained unchanged with asimadoline treatment on both days 13 and 21 (Fig. 3B, D, F).

**Effect of asimadoline on T cell numbers**

In a separate group of rats, asimadoline treatment did not change the number of T cells at either day 13 or day 21 in the synovium of rats with adjuvant arthritis.
Asimadoline treatment did not prevent the increase in synovial T cell numbers observed in adjuvant arthritis. Thus, the significantly reduced mRNA expression of IL-17 in the synovium at day 13, together with the trend for reduced synovial IFN-γ transcripción, in the asimadoline-treated rats probably reflects suppression of synovial T-cell activation by opioids. Our results support literature findings that opioids affect T-cell function (see [1] for a review) and that β-endorphin suppresses lymphocyte chemotactic factor release by peripheral blood mononuclear cells [28]. Alternatively, our results may also reflect a decreased number of monocytes and less co-stimulation, with no direct effect on T cells. The observed reduction in cytokine expression with no change in T cell numbers is consistent with studies in rheumatoid arthritis patients in which treatments such as leflunomide and methotrexate showed a reduction in cytokine expression with no change in T cell numbers [29].

The inguinal lymph node is an important site of immune activity in adjuvant arthritis, particularly in the first few days [30–33]. The overall lack of changes in cytokine expression at day 13 compared with day 0 in the inguinal lymph node suggests that changes may have occurred before day 13 of adjuvant arthritis. The increase in TNF mRNA expression in the inguinal lymph nodes at day 13 with asimadoline treatment compared with vehicle-treated controls may reflect a delay in disease development, compatible with a reduction in clinical arthritis score at day 13, but not at day 21. Alternatively, TNF may be promoting an extra-articular pro-inflammatory effect.

TGF-β is present in the synovium of rheumatoid arthritis patients and animal models of inflammatory arthritis. Its role, however, remains elusive. TGF-β is a predominantly macrophage-derived cytokine, which has been shown to have both pro- and anti-inflammatory effects, depending on the site of action [34]. For example, TGF-β has been shown to have both pro- and anti-inflammatory effects in collagen-induced arthritis and streptococcal cell wall arthritis in mice. In one study, extra-articular injection of TGF-β protected against collagen-induced arthritis and anti-TGF-β treatment exacerbated the disease [35, 36]. In contrast, intra-articular injection of TGF-β has been shown to augment collagen-induced arthritis [37] or induce synovitis in normal rats [38]. These data suggest that intra-articular TGF-β may be pro-inflammatory, while extra-articular TGF-β may be anti-inflammatory [39]. Our results show that asimadoline treatment changed TGF-β expression in a way that would maximize the anti-inflammatory effects of these opposing TGF-β functions. Asimadoline decreased synovial (intra-articular) TGF-β expression and increased inguinal lymph node (extra-articular) TGF-β expression. An altered balance in the pro- and anti-inflammatory effects of TGF-β by asimadoline might explain its striking anti-arthritic actions. This hypothesis requires further testing using TGF-β blocking strategies and in vitro experimentation.

**Discussion**

There is increasing awareness of important interactions between endogenous and exogenous opioids with the immune system [1]. Few studies have demonstrated that opioids have important effects on immune function in vivo. The present study confirms our previous findings that the peripherally selective κ-agonist, asimadoline, attenuates adjuvant arthritis. In this study we showed that asimadoline may exert its anti-inflammatory actions via altered mRNA expression of pro- and anti-inflammatory cytokines in the synovium and inguinal lymph nodes of rats with adjuvant arthritis.

The synovium is the site where pannus formation and joint destruction are observed in both adjuvant arthritis and rheumatoid arthritis. The reduced expression of IL-17, a pro-inflammatory cytokine, at day 13, but not day 21, in the synovial membrane is consistent with the clinical signs of adjuvant arthritis (e.g. oedema) which begins to resolve after 21 days.

TNF is a pro-inflammatory cytokine thought to play a pivotal role in rheumatoid arthritis [25]. Our in vivo findings showing a trend for a reduction in synovial TNF mRNA expression with asimadoline treatment support our previous studies in vitro showing reduced TNF production by rat peritoneal macrophages [16], and those of others demonstrating reduced TNF production and transcription by a macrophage cell line by the selective κ-opioid agonist U50,488H [15]. The present study is the first to show such effects in vivo and warrants further study.

The changes in synovial TNF and TGF-β expression, both predominantly macrophage-derived cytokines, may be due to suppression of macrophage activation or to reduced numbers of synovial macrophages. We have previously shown that asimadoline reduces macrophage trafficking to the joints of rats with adjuvant arthritis [16]. In addition, the up-regulation of ICAM-1, which is thought to be stimulated by pro-inflammatory cytokines such as TNF and IFN-γ, has been shown to be reduced by the κ-opioid agonist PD117302 [17]. The reduction in TNF expression in the synovium observed in this study may explain the asimadoline-related suppression of ICAM-1 up-regulation (a TNF-dependent function) and macrophage trafficking in the synovium.

IL-17 is a newly described, pro-inflammatory, T cell cytokine with the potential to play an important role in inflammatory arthritis. We have previously shown that IL-17 mRNA is expressed over the time course of adjuvant arthritis [26]. We show here, for the first time, that asimadoline reduces the mRNA expression of this cytokine in the synovium at day 13 by more than 50%, concordant with a decrease in paw swelling. Such effects may contribute to the unique anti-inflammatory properties of κ-opioids [27].

(vehicle vs asimadoline: day 13: 565 ± 259 vs 610 ± 169 cells/mm²; day 21: 1012 ± 214 vs 896 ± 248 cells/mm²).
One limitation of the present study is that changes in mRNA expression may not reflect a proportional change in protein in the synovium. In a preliminary study, we found that there is secreted TNF protein in synovial cultures on day 17 post-adjuvant (K. A. Bush, unpublished observations). Once rat antibodies to all the cytokines of interest are available, in particular IL-17 and TGF-β, studies are warranted to confirm that similar changes in cytokine proteins do occur.

In summary, we have found that the peripherally selective κ-opioid agonist, asimadoline, reduces synovial membrane cytokine production in rats with adjuvant arthritis, with no effect on T-cell recruitment to the joint. These results from an in vivo model, support our previous work and that of others which demonstrate κ-opioid suppression of inflammatory cell immune function. To date, κ-opioids have only been used clinically as analgesics and their anti-inflammatory and anti-arthritic actions have largely gone unrecognized. The majority of specific centrally acting κ-agonists have shown little analgesic efficacy and the prevalence of central side-effects has ruled out many of these compounds [40]. Asimadoline is a peripherally selective κ-opioid agonist, which lacks these side-effects. Its analgesic actions have only been assessed in post-operative pain in patients undergoing knee surgery [41]. Our studies are the first to evaluate the anti-arthritic actions of asimadoline [13]. κ-opioid suppression of inflammatory immune cell function may therefore be clinically advantageous in immune diseases such as inflammatory arthritis.

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