Proinflammatory cytokine production and cartilage damage due to rheumatoid synovial T helper-1 activation is inhibited by interleukin-4

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Abstract

Objectives—To investigate the role of T helper-1 cell (Th1) activation in the induction of proinflammatory cytokine production and cartilage damage by rheumatoid arthritis (RA) synovial fluid mononuclear cells (SFMNC) and the subsequent possible beneficial role of the T helper-2 cell (Th2) cytokine interleukin-4 (IL-4) in the inhibition of this process.

Methods—SFMNC were stimulated with bacterial antigen (hsp60) to activate Th1 cells. Th1 and Th2 specific cytokine profiles (interferon gamma (IFN γ) and IL-4) and proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor α (TNF α) in the conditioned media were analysed. In addition, the conditioned media were tested for their ability to induce cartilage damage. The same parameters were measured in the presence of IL-4.

Results—Stimulation of SFMNC with bacterial antigen resulted in an increase in IFN γ , IL-1, and TNF α production which was accompanied by the induction of cartilage damage. Th1 activation could be inhibited by IL-4 as shown by a reduction of IFN γ . This was accompanied by a decrease in IL-1 and TNF α production and inhibition of cartilage damage.

Conclusions—Th1 activation is a possible mechanism by which inflammation in RA joints is enhanced. The Th2 cytokine IL-4 inhibits this Th1 activity and may diminish inflammation and induction of cartilage damage in RA joints.

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Rheumatoid arthritis (RA) is characterised by joint destruction as a result of chronic joint inflammation. Many studies indicate that T cells play an important role in the initiation and maintenance of this inflammation.^{1,2} Within the mouse and human T lymphocyte populations, two types of T helper cells have been designated that carry out distinct functions and are characterised by specific cytokine profiles.^{3–5} T helper-1 (Th1) cells participate in delayed type hypersensitivity reactions and cytotoxicity responses and specifically produce interleukin-2 (IL-2) and interferon gamma (IFN γ). T helper-2 (Th2) cells provide more efficient help for B cells to differentiate into Ig

producing cells and specifically produce IL-4. It has been shown that different types of infections are accompanied by either Th2 or Th1 activity.^{6,7} Th1 activity has been demonstrated in joints of patients with bacteria induced arthritis such as Lyme's arthritis and reactive arthritis.^{7–9} Bacterial infections (slow-growing) have been implicated in RA.¹⁰ In RA patients, reactivity of synovial fluid mononuclear cells (MNC) towards bacterial antigens such as the mycobacterial heat shock proteins (hsp) has been demonstrated.^{11–13} Antigens of *Mycobacterium tuberculosis* and *M lepra*, like the mycobacterial hsp60 have been shown to induce Th1 cells selectively.^{14,15} In several studies it has been demonstrated that RA is accompanied by Th1 activity.^{16–19}

In the RA joint, T cells can influence macrophage activity. Stimulated synovial macrophages contribute to inflammatory processes and production of proinflammatory cytokines such as IL-1 and tumour necrosis factor α (TNF α), causing damage to the articular cartilage.^{20–22} In RA peripheral blood monocytes, it has been demonstrated that the Th1 cytokine IFN γ stimulates production of IL-1 and TNF α .²³ Recently, a potential anti-inflammatory capacity of the Th2 cell derived cytokine IL-4 has been described. IL-4 can inhibit the production of IL-1 and TNF α by activated human monocytes and MNC from blood of RA patients and of healthy controls.^{24–26} In addition, IL-4 has been reported to stimulate the production of IL-1 receptor antagonist by monocytes.²⁷ The balance of Th1 and Th2 activity in the RA joint is thus of importance in the regulation of proinflammatory cytokines such as IL-1 and TNF α and in the regulation of cartilage damage. We investigated the contribution of bacterial antigen in the stimulation of RA synovial fluid MNC. Induction of Th1 activity, subsequent production of IL-1 and TNF α , and induction of cartilage damage were studied. In addition, a possible beneficial effect of the Th2 cytokine IL-4 on cartilage damage induced by RA synovial fluid MNC was investigated.

Patients and methods

PATIENTS

This study was carried out with synovial fluid (SF) MNC from the inflamed knees of 15 successive patients (eight women, seven men) with RA as defined by the 1987 revised ARA

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criteria.²⁸ Patients were aged 40–83 years (mean 58 (SD 13) years). Mean disease duration was 11 (8) years (range 1–24). Eleven patients were rheumatoid factor positive; four were negative. Eleven patients were being treated with non-steroidal anti-inflammatory drugs, seven were receiving slow acting drugs and three were taking prednisone.

MONONUCLEAR CELL ISOLATION

SF was diluted 1:1 with Dulbecco's Modified Eagle's medium (D-MEM, Gibco 074-01600; 0.81 mmol/l SO_4^{2-} ; 24 mmol/l NaHCO_3) containing glutamine 2 mmol/l, penicillin 100 U/ml, and streptomycin sulphate 100 $\mu\text{g/ml}$. SFMNC were subsequently isolated by density centrifugation using Ficoll-Paque (Pharmacia). Viability of the cells was checked by trypan blue exclusion and was always more than 95%.

PRODUCTION OF CONDITIONED MEDIA

For production of conditioned media, SFMNC were cultured in a concentration of $5 \cdot 10^5$ cells/ml in a final volume of 1 ml in 24 well plates in culture medium (see above). Cells were cultured alone, with hsp60²⁹ 10 $\mu\text{g/ml}$ as a potential Th1 stimulus (National Institute for Public Health and Environmental Protection, Bilthoven, The Netherlands), with IL-4 2 and 20 U/ml as a Th2 cell specific cytokine (Genzyme) or the combination of this bacterial antigen and IL-4. After four days, conditioned media were harvested and made cell free by centrifugation, frozen in liquid nitrogen and stored at -80°C .

MEASUREMENT OF CYTOKINES IN CONDITIONED MEDIA

$\text{IFN}\gamma$, IL-4, $\text{TNF}\alpha$, and IL-1 were determined by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Medgenix, Flerus, Belgium for $\text{IFN}\gamma$, $\text{TNF}\alpha$, IL-1 β ; CLB, Amsterdam, The Netherlands for IL-4). The limits of detection of the assay were 1 IU/ml for $\text{IFN}\gamma$, 1 pg/ml for IL-4, 10 pg/ml for $\text{TNF}\alpha$, and 10 pg/ml for IL-1.

CARTILAGE TISSUE CULTURE

Macroscopically normal postmortem cartilage was obtained from the central part of human femoral knee condyles within 24 hours after death of the donor. Donors were aged 47–85 years (mean 65 years) ($n = 8$). As far as could be determined from the patients' medical records, these donors had not been treated for joint disorders. Pieces of cartilage, as thick as possible, were cut from condyles of both knees, excluding underlying bone. The slices obtained were kept in phosphate buffered saline (PBS, pH 7.4). Within one hour after dissection they were cut into squares, weighed aseptically (5–15 mg, accuracy 0.1 mg) and transferred to the culture medium (see above). Individual explants were cultured randomly, eightfold, in

96 well microtitre plates (200 $\mu\text{l/well}$). They were precultured for 24 hours, the media were refreshed, then during a culture period of four days the effects of the addition of IL-4, conditioned media of SFMNC, or SFMNC (coculture) on cartilage proteoglycan (PG) turnover were tested by determination of the rate of synthesis and release of PG. IL-4 was added in concentrations of 2 and 20 U/ml. Conditioned media of SFMNC were tested in concentrations of 5% (v/v). Isolated RA SFMNC were cocultured with cartilage in a concentration of $2 \cdot 10^3$ cells/ml.

DETERMINATION OF RATE OF PROTEOGLYCAN SYNTHESIS

As a measure of PG synthetic activity of the chondrocytes, incorporation of sulphate was determined during a four hour period before termination of the experiment. Procedures were followed as described previously using $^{35}\text{SO}_4^{2-}$ as a tracer and cetylpyridinium chloride to precipitate glycosaminoglycan (GAG) in a papain digest of cartilage explants.³⁰ The total sulphate incorporation rate was calculated from the $^{35}\text{SO}_4^{2-}$ incorporation rate and the specific activity of the medium, and was normalised to the wet weight of the sample. The rate of incorporation of sulphate is expressed as nmoles of sulphate incorporated per hour per gram wet weight of tissue (nmol/h·g).

DETERMINATION OF PROTEOGLYCAN RELEASE

To determine the release of PG, medium from the four day culture period (that is, after the one day of preincubation) was analysed for GAG content. GAG was stained and precipitated with alcian blue dye solution; chondroitin sulphate (Sigma, C4384) was used as a reference. Procedures were as described previously.³¹ The GAG content was normalised to the wet weight of the samples, and expressed as milligrams released over four days (day 2 till 5), per gram wet weight of tissue (mg/g).

CALCULATIONS AND STATISTICAL ANALYSIS

Because of focal differences in composition and bioactivity of the cartilage, the average of eight samples from one donor, taken at random and handled individually, was taken as representative of that donor. Mean values obtained from several donors, with the SEM, are given. Statistical evaluation was performed by the use of Wilcoxon paired sample test. Significance was accepted at a value of $p < 0.05$.

Results

BACTERIAL ANTIGEN STIMULATION OF SFMNC

Addition of conditioned media from bacterial antigen stimulated SFMNC resulted in an inhibition of proteoglycan (PG) synthesis in seven of 10 patients. Figure 1 shows a mean inhibition of 42% for these seven patients. Conditioned media from MNC of three

Figure 1 Effects of hsp60 stimulation on synovial fluid mononuclear cells. Left axis: effect of conditioned media from these cells on inhibition of proteoglycan (PG) synthesis, expressed as percentage of untreated cartilage. ● = Patients that respond to hsp60 stimulation by inhibiting PG synthesis ($n = 7$). ○ = Non-responding patients with respect to PG synthesis ($n = 3$). + = mean value of group. Right axis: production of interferon gamma ($\text{IFN}\gamma$) (U/ml), interleukin-4 (IL-4), IL-1, and tumour necrosis factor α ($\text{TNF}\alpha$). Horizontal bars represent indicated detection limits; symbols below these do not represent actual values.

Figure 2 Effects of conditioned media from synovial fluid mononuclear cells (MNC) of responding patients ($n = 7$) on PG synthesis (measured as sulphate incorporation) (left axis) and corresponding interferon gamma ($\text{IFN}\gamma$) concentrations (right axis). Untreated cartilage (C), and cartilage cultured with conditioned media of MNC, unstimulated and stimulated with bacterial antigen (hsp) treated with different concentrations of IL-4 (0, 2 and 20 U/ml). Discontinuous values for individual patients are connected in order to display changes per patient.

patients did not significantly influence PG synthesis, and addition of conditioned media from untreated SFMNC did not influence PG synthesis significantly (fig 2).

SFMNC of patients inhibiting PG synthesis in response to bacterial antigen had increased levels of $\text{IFN}\gamma$, IL-1, and $\text{TNF}\alpha$ (20 U/ml, 71 pg/ml, and 751 pg/ml on average, respectively). IL-4 levels remained below the limit of

detection (fig 1). In the conditioned media both of SFMNC not influencing PG synthesis in response to bacterial antigen, and of untreated SFMNC, levels of $\text{IFN}\gamma$, IL-4, IL-1, and $\text{TNF}\alpha$ remained below the limit of detection (fig 2, and data not shown).

EFFECTS OF IL-4 ON SFMNC INDUCED CARTILAGE DAMAGE AND CYTOKINE PRODUCTION

The addition of IL-4 to SFMNC reduced the inhibition of PG synthesis induced by conditioned media from these SFMNC stimulated with bacterial antigen, both significantly and dose dependently, by up to 82% ($n = 7$) (fig 2). The enhanced $\text{IFN}\gamma$ levels of these SFMNC were also inhibited by IL-4 significantly and dose dependently, by up to 53% (fig 2).

If bacterial antigen did not stimulate SFMNC to inhibit PG synthesis ($n = 3$) (fig 1), no changes were observed on addition of IL-4 (data not shown). In particular, addition of IL-4 to these non-responding SFMNC did not produce a change in $\text{IFN}\gamma$ levels, which remained below the limit of detection.

Figure 3 shows the effects of IL-4 on IL-1 and $\text{TNF}\alpha$ levels in conditioned media of SFMNC that responded to bacterial antigen (inhibited PG synthesis). A dose dependent statistically significant reduction in IL-1 and $\text{TNF}\alpha$ by up to 68% and 30%, respectively, was seen upon addition of IL-4. IL-1 and $\text{TNF}\alpha$ levels from the conditioned media of non-responding SFMNC, not inhibiting PG synthesis, remained below the limit of detection (data not shown).

EFFECTS OF IL-4 ON SFMNC INDUCED CARTILAGE DAMAGE IN COCULTURE

No direct effect of IL-4 on human articular cartilage PG synthesis or release was observed (data not shown). Figure 4 shows the effects of SFMNC in coculture with cartilage on PG turnover. On addition of unstimulated SFMNC to cartilage explants, no change in PG synthesis was observed. Addition of IL-4 to these cocultures of untreated cells and cartilage explants did not alter PG synthesis. Stimulation of the cocultures with bacterial antigen resulted in a statistically significant inhibition of PG synthesis (mean 45%). This inhibition during coculture was restored to a statistically significant extent upon addition of IL-4. PG release from the cartilage did not change as a result of coculturing with SFMNC (fig 4), and was not influenced by bacterial antigen stimulation or IL-4.

Discussion

Many bacterial antigens have been reported to induce Th1 cell activation. In patients with *Chlamydia* and *Yersinia* induced reactive arthritis, T cell clones from the synovial fluid that were reactive with *Chlamydia* and *Yersinia* antigens displayed a Th1 subtype.^{5,6} Similar results were obtained with *Borrelia burgdorferi* antigens and synovial cells of patients with

Figure 3 Percentage inhibition (▨) by interleukin-4 (IL-4) of production of IL-1 (left) and tumour necrosis factor α (TNFα) (right) by hsp60 activated synovial fluid mononuclear cells (mean, SEM (n = 7)). □ = Production of IL-1 and TNFα after hsp60 stimulation (100%). Statistically significant inhibition: *p < 0.05.

Lyme's arthritis.⁷ These data suggest that the inflammatory process during reactive and septic arthritis is accompanied by Th1 activation. Similarly, RA is suggested to be associated with Th1 activity.¹⁷⁻¹⁹ In RA patients, reactivity towards preparations of *Mycobacterium tuberculosis* and *Escherichia coli* has been demonstrated.^{11-13, 32} Reactivity to the mycobacterial hsp60, which we used in the present study, has also been shown in those studies. Several studies have demonstrated that isolated T cell clones specific for this mycobacterial antigen belong functionally to the Th1 subtype.^{7, 14, 15} We attempted to activate Th1 cells with mycobacterial hsp60 as a potential Th1 stimulus to investigate the role of Th1 activation in the induction of pro-inflammatory cytokine production (IL-1 and TNFα) and cartilage damage. Elucidation of the mechanism of this Th1 induced immune response will contribute to a better understanding of the pathogenesis of RA and other arthritides.

We have demonstrated that activation of Th1 cells by bacterial antigen within a population of RA SFMNC was accompanied specifically by the production of IFNγ (not of IL-4), and was associated with an increase in the production of IL-1 and TNFα. As a result of this activation, cartilage damage was induced. This occurred in coculture, and indirectly when the effects of conditioned media of stimulated SFMNC were tested on cartilage turnover. In the present study (data not shown) and earlier studies using neutralising antibodies, we have shown that both IL-1 and TNFα activity produced during this stimulation are, indeed, predominant inducers of cartilage damage.³³ It appeared that not all patients responded to the bacterial antigen, regardless of disease parameters or medication as had been reported previously.³³

As in Lyme's and reactive arthritis, Th1 inflammatory responses to many pathogenic

micro-organisms are beneficial to patients suffering from these infections: Th1 activation supports cell mediated cytotoxicity that is necessary to combat these micro-organisms. In contrast, in diseases in which autoreactive Th1 responses to self antigens predominate, this immune response is harmful to the patient. In several animal models for autoimmune diseases, such as multiple sclerosis and insulin dependent diabetes, it has been demonstrated that autoimmune responses are Th1 mediated.³⁴ Downregulation of Th1 activity and upregulation of Th2 activity by administration of neutralising IFNγ antibodies or IL-4 has been shown to prevent the induction and cause remission of inflammatory responses in these experimental autoimmune diseases.³⁴ Similarly, it has been shown that remission of collagen induced arthritis can be stimulated by addition of IL-4, whereas spontaneous remission is accompanied by a Th2 induced phenomenon (IgE production³⁵).

In RA synovitis, Th2 cells represent a minority of synovial T helper cells¹⁶⁻¹⁹ and IL-4 is absent.³⁶ We investigated the effects of IL-4 on Th1 activation of RA SFMNC in our (human) culture system, and demonstrated that Th1 induced cartilage damage, as measured by proteoglycan synthesis, was inhibited by the addition of IL-4. This inhibition was accompanied by downregulation of IFNγ production and was associated with a reduction in IL-1 and TNFα. Proliferation resulting from the bacterial antigen stimulation (stimulation index on average 7.2, data not shown) was also inhibited dose dependently and statistically significantly up to 44% by IL-4. Upon bacterial antigen stimulation, no changes in proteoglycan release was observed. This last observation may reflect the fact that release and synthesis of proteoglycans are differentially regulated, as has been shown for IL-1 effects on cartilage proteoglycan turnover.³⁷ Separate experiments using conditioned media with higher levels of IL-1 and TNFα during similar culture periods have been shown to induce GAG release (data not shown). In the present study, levels of IL-1 and TNFα may have been too low to induce GAG release.

Whether Th1 autoreactivity is a major cause of joint damage in RA has not been shown. Nevertheless, the predominance of Th1 cells in RA joints¹⁶⁻¹⁹ has been demonstrated. The present study indicated that Th1 activation in vitro induces proinflammatory cytokine production and cartilage damage. In addition, our findings suggest a possible beneficial role of IL-4 in RA patients. This idea is in agreement with the findings of recent studies demonstrating the potential of IL-4 to inhibit synovial cell proliferation³⁸ and to prevent joint destruction, including articular cartilage degradation, by downregulation of metalloproteinase production of monocytes-macrophages and chondrocytes.³⁹⁻⁴¹ Our results substantiate and extend the work of others showing that proinflammatory cytokine production by RA peripheral blood MNC, monocytes, and synovial tissue explants can be inhibited by IL-4.^{25, 26, 42} However, IL-4 has also

Figure 4 Effect of IL-4 0, 2 or 20 U/ml on cartilage proteoglycan synthesis (measured as sulphate incorporation) (left) and release (measured as release of glycosaminoglycan (GAG)) (right) from cartilage cocultured with synovial fluid mononuclear cells unstimulated or stimulated with hsp60 (▨) (means, SEM (n = 6)). □ = Control synthesis and release. Statistically significant inhibition. *p < 0.05.

been reported to be unable to suppress IL-1 and TNF α production.²⁵⁻⁴³ This discrepancy may result from differences in the states of activation or differentiation of the target cell used. The present study has shown that IL-4 was able to inhibit production of these cytokines by SFMNC upon antigen induced Th1 activation—a relevant observation, as Th1 cells, in addition to IL-1 and TNF α , predominate in RA joints.^{16-19, 22} On the basis of our observation of IL-4 induced inhibition of Th1 activity, proinflammatory cytokine production, and cartilage damage, we conclude that administration of IL-4, and maybe more effectively the upregulation of Th2 cell activity, may be considered for treatment of RA.

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