EXTENDED REPORT

Interleukin 7 stimulates tumour necrosis factor α and Th1 cytokine production in joints of patients with rheumatoid arthritis

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Background: A large number of activated T cells are found in the joints of patients with rheumatoid arthritis (RA). Interleukin 7 (IL7), a T cell growth factor and a regulator of Th1 and Th2 cytokine production, is produced by synovial cells from patients with RA.

Objective: To investigate the effect on proinflammatory cytokine production of synovial fluid mononuclear cells (SFMC) and the mechanism by which IL7 influences CD4+ T cell activity in patients with RA.

Methods: In a cross sectional group of patients with RA, IL7 levels were compared with those of healthy controls and related to disease activity. The effect of IL7 on cytokine production was tested by RA SFMC and on SF CD4+ T cells in the presence of mononuclear cells (MC). Production of tumour necrosis factor α (TNF α), IL1 β , interferon γ (IFN γ), and IL4 was measured by enzyme linked immunosorbent assay (ELISA) and by single cell FACS analysis. Expression of the IL7 receptor α chain on CD4+ T cells (essential for IL7 signalling) was assessed. Direct effects of IL7 on isolated synovial fluid (SF) CD4+ T cells were studied by cytokine analysis. By neutralisation of IL12 in MC cultures, indirect effects of IL7 on T cells through accessory cells were studied.

Results: IL7 serum levels were higher in patients with RA than in healthy controls and correlated positively with C reactive protein levels. IL7 stimulated TNF α production by SFMC and very potently stimulated IFN γ and TNF α production by SF CD4+ T cells. These effects were probably mediated through the IL7 receptor α chain, which was abundantly expressed on SF CD4+ T cells. Besides the direct stimulation of T cell cytokine production by IL7, its action was partly dependent on IL12, indicating that IL7 also stimulates accessory cell function, leading to T cell activation.

Conclusion: IL7 stimulates proinflammatory cytokine production of intra-articular CD4+ T cells and accessory cells from patients with RA. The correlation with measures of disease activity indicates that IL7 might substantially contribute to the perpetuation of Th1 and TNF α mediated proinflammatory responses in patients with RA.

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Activated T cells comprise a large proportion of the inflammatory cells in the joints of patients with rheumatoid arthritis (RA).^{1–3} These cells have been shown to contribute to inflammation by many pathways. Analysis of specific T cell cytokine production has shown a dominance of proinflammatory T helper 1 cell (Th1, defined by interferon γ (IFN γ) and interleukin 2 (IL2) production) over suppressive Th2 cell activity (defined by IL4 production) in the RA joint compared with peripheral blood.^{4–7} Apart from these cytokines, synovial T cells have been shown to produce proinflammatory cytokines such as tumour necrosis factor α (TNF α) and IL17.^{8–9} Furthermore, activated T cells expressing CD25, CD69, and HLA-DR in the RA joint have been shown to express costimulatory molecules such as leucocyte function associated antigen-1, CD40L, CD28, and cell death-inducing molecules such as perforin and granzyme B, molecules which can strongly contribute to the regulation of inflammation and tissue destruction.^{10–12}

Factors which cause differentiation of T cells are also produced in the RA joint. IL12, IL15, and IL18 are, among others, produced by synovial macrophages and induce Th1 cell activity.^{11–14} Furthermore, IL15 has been shown to directly stimulate TNF α production by synovial T cells.¹¹ IL15, in addition stimulates expression of costimulatory molecules on synovial T cells, which in their turn can interact with macrophages and stimulate these cells to secrete TNF α .¹¹ Recently, it has been reported that IL7, in parallel with IL15, is produced by synovial fibroblasts of patients with RA.¹⁵

In humans, IL7, produced by stromal cells at lymphopoietic sites (bone marrow, thymus, spleen, and gut), is a factor known for its role in T and B lymphocyte development.¹⁶ IL7 is a critical growth factor in early human T cell development and has been shown to prime human naive CD4+ T cells, in the absence of accessory cells, for proinflammatory (IFN γ , IL2) and anti-inflammatory cytokines (IL4).^{17–18} IL7 can also stimulate proliferation and, under certain conditions, cytokine secretion of memory T cells.^{19–20} These data suggest that IL7, present in the joints of patients with RA, may be an important immunoregulatory cytokine.

The purpose of this study was to investigate whether IL7 may play a part in proinflammatory or anti-inflammatory intra-articular T (helper) cell regulation. For this reason, IL7 levels in patients with RA were compared with those of healthy controls and related to C reactive protein (CRP) levels, as a marker of disease. Furthermore, synovial fluid mononuclear cell (MC) and T cell activation by IL7 was studied. Because of the linkage of major histocompatibility complex class II molecules (HLA-DR1

Abbreviations: CRP, C reactive protein; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme linked immunosorbent assay; IFN γ , interferon γ ; IL, interleukin; MC, mononuclear cells; RA, rheumatoid arthritis; SF, synovial fluid; SFMC, synovial fluid mononuclear cells; TNF α , tumour necrosis factor α

Figure 1 (A) Serum IL7 levels of patients with RA (n=34) compared with an age and sex matched healthy control group (n=32, HC). Mean (SEM) values are given. Patients with RA showed a significantly higher serum IL7 level than HC ($p < 0.05$). (B) The serum IL7 levels in patients with RA (n=32) correlated with CRP.

and DR4) with RA,²¹ molecules which participate in the stimulation of CD4+ T cells, in particular the role of IL7 in CD4+ T cell activation, were studied.

PATIENTS AND METHODS

Patients

For IL7 assessment, blood samples from 34 consecutive patients with RA visiting our outpatient clinic were taken. Patients fulfilled the 1987 revised American College of Rheumatology criteria²² and ranged in age from 30 to 77 years, with a mean (SD) age of 50 (13) years. Duration of the patients' disease ranged from 1 to 20 years, with a mean disease duration of 5 (4) years. Twenty three patients were rheumatoid factor positive. Eleven patients received methotrexate, seven took sulfasalazine, three were receiving hydroxychloroquine, three were receiving auromyosin, and one was receiving azathioprine. In addition, blood samples were taken from 32 healthy controls ranging in age from 27 to 73 years, with a mean age of 49 (12) years (not statistically different). Serum was taken from the blood samples and frozen at -20°C before analysis. IL7 levels were determined with an IL7 enzyme linked immunosorbent assay (ELISA) kit (Diacor Research, Besancon, France) according to the manufacturer's instructions.

For cell cultures, MC were isolated from the synovial fluid (SF) of 11 randomly selected patients with RA. The patients (nine female, two male) ranged in age from 34 to 66 years, with a mean age of 50 (14) years, and had a disease duration ranging from 2 to 16 years, with a mean disease duration of 8 (5) years. Two patients were receiving methotrexate, three took hydroxychloroquine, three were receiving sulfasalazine, and one was receiving low dose prednisone.

Cell cultures and reagents

SF was diluted 1:1 with Dulbecco's modified Eagle's medium (DMEM, Gibco 074-01600; 24 mM NaHCO_3 , Gibco, NY, USA) containing glutamine (2 mM), penicillin (100 U/ml), and streptomycin sulphate (100 $\mu\text{g}/\text{ml}$; DMEM+), and MC were isolated by density centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden). SFMC were stored in liquid nitrogen for no longer than one year. Viability of the thawed cells, checked by trypan blue exclusion, was always more than 95%. Subsequently, isolated SFMC ($1 \times 10^6/\text{ml}$) were cultured in DMEM + supplemented with 10% pooled human male AB

serum (Red Cross Blood Transfusion Centre, Utrecht, The Netherlands). SFMC were cultured for five days in the absence or presence of different concentrations of IL7 (Genzyme, Cambridge, USA, specific activity 1×10^7 U/mg), antihuman IL12 (p40/p70) neutralising monoclonal antibody (clone C8.6, IgG1, BD/Pharmingen, San Diego, CA), or IgG1 isotype control (BD/Pharmingen, San Diego, CA), followed by specific T cell stimulation with CD3/CD28 for 24 hours (1 $\mu\text{g}/\text{ml}$; CLB-T3/4.E and CLB-CD28, respectively, CLB Reagentia, Amsterdam, The Netherlands).²³ Subsequently, cytokine production during this 24 hour period was determined by ELISA and by FACS analysis (see below).

To analyse IL7 α receptor (CD127) expression on CD4+ T cells from the synovial fluid, SFMC were triple stained with CD3-PerCP (BD/Pharmingen, San Diego, CA, USA), CD4-FITC (BD/Pharmingen, San Diego, CA, USA), and CD127-PE (Immunotech, Marseille, France).

To test the direct effects of IL7 on CD4+ T cells, these cells were isolated from the SFMC by means of microbead activated cell sorting according to the manufacturer's instructions (Mylteni Biotec, Bergisch Gladbach, Germany). Briefly, SFMC were incubated with a cocktail of monoclonal antibodies directed against non-T cells and against CD4- T cells, followed by binding of a secondary microbead coupled antibody to the primary antibodies. Microbead labelled cells were then removed by binding to a magnet. The purity of the CD4+ T cell population was checked by FACS analysis and was more than 95%. The CD4+ T cells obtained were almost all CD45RO+ (memory) T cells (FACS analysis, >90%; data not shown). CD4+ T cells (5×10^5 cells/ml) were costimulated (to mimic costimulation by MC) with soluble CD3/CD28 monoclonal antibodies (1 $\mu\text{g}/\text{ml}$, see above) for a total of 10 days (refreshed with medium at day 5), with or without IL7 (10 ng/ml). Subsequently, cells were washed three times at day 10 and seeded at a concentration of 1×10^6 cells/ml for a short (24 hours) restimulation with CD3/CD28. Cytokine production during these 24 hours was measured by ELISA (see below).

Cytokine analysis

For TNF α , IL1 β , IFN γ , and IL4 analysis, media were harvested and freed of cellular material by centrifugation (five minutes, 900 g), frozen in liquid nitrogen, and stored at -20°C . TNF α , IL1 β , IFN γ , and IL4 protein in the culture supernatant were

Figure 2 IL7 stimulates TNF α production by RA SFMC. SFMC (1×10^6 /ml) were incubated without or with IL7 (10 ng/ml) for five days. Mean (SEM) values are given (n=4). Asterisks indicate significant difference compared with control culture (*p<0.05).

determined by ELISA according to the manufacturer's instructions (Biosource Europa, Nivelles, Belgium).

For TNF α , IFN γ , and IL4 analysis at the single cell level, FACS analysis was performed. Cells were cultured as described for cytokine secretion. To improve intracellular detection of cytokines, protein secretion was inhibited by the addition of 10 μ g/ml Brefeldin A (ICN Pharmaceuticals, Costa Mesa, CA, USA) during the last two hours of culture. Subsequently, cells were collected and T cells stained with CD3-PerCP and

CD4-FITC (CLB Reagentia, Amsterdam, The Netherlands). The cells were then fixed and permeabilised with a fixation and permeabilisation kit, according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA, USA). Fluorochrome labelled anti-TNF α , anti-IFN γ , anti-IL4, or isotype control antibodies (BD/Pharmingen, San Diego, CA, USA) were added during the permeabilisation step (1 μ g/ml) to stain intracellular cytokines. Fluorescence was analysed with a FACScan (Becton Dickinson). The quadrant markers for the bivariate dot plots were set based on the autofluorescence control, and verified with isotype control antibodies.

Statistical analysis

Paired sample *t* tests and Mann-Whitney U tests were used when appropriate. A Spearman test was used for correlation analysis. Data were judged to be significant at p<0.05.

RESULTS

Serum levels of IL7 are increased in patients with RA compared with healthy controls

Serum levels of IL7 in patients with RA (n=34) were on average almost fivefold higher than in an age and sex matched healthy control group (n=32, p<0.05, fig 1A). IL7 serum levels did not differ between rheumatoid factor positive or negative patients. CRP levels, available in 23 patients with RA, correlated positively with serum IL7 levels ($r_s=0.406$, p<0.05, fig 1B).

IL7 stimulates TNF α production by SFMC and TNF α and IFN γ production by SF T cells

Because IL7 is produced by fibroblasts from RA joints,¹⁵ the effect of IL7 on synovial fluid MC and T cell activation was investigated. IL7, at concentrations of 10 ng/ml, significantly stimulated TNF α production by SFMC (fig 2, on average 157%). IL1 β production was undetectable and was not stimulated by IL7. IFN γ production by SFMC from only one donor was stimulated by IL7 (from <10 to 138 pg/ml), whereas IL4 in each case remained undetectable. To analyse if T cells were modulated in their responsiveness, culture of SFMC for five days with IL7 was followed by T cell stimulation for 24 hours. Upon this T cell stimulation, increased TNF α and IFN γ

Figure 3 Effect of IL7 on cytokine production by RA synovial T cells. (A) SFMC (1×10^6 /ml) were incubated with or without IL7 (10 ng/ml) for five days, followed by T cell-specific stimulation with CD3/CD28 for 24 hours. Secretion of TNF α , IFN γ , and IL4 during this 24 hour period was assessed by ELISA. Mean (SEM) values are given (n=8). Asterisks indicate significant difference compared with control culture (*p<0.05, **p<0.01). (B) A dose range of IL7 is shown. Mean (SD) values of two separate experiments are given. Note the difference in scale of the y axis for IFN γ and TNF α compared with IL4.

Figure 4 Effect of IL7 on cytokine production by synovial T cells. SFMC (1×10^6 /ml) from patients with RA were incubated with or without IL7 (10 ng/ml) for five days, followed by specific T cell stimulation with CD3/CD28 for 24 hours. Cytokine production during this 24 hour period was assessed intracellularly by FACS analysis. CD3⁺ T cells were gated and cytokine expression of CD4⁺ (CD4-FITC, horizontal axis) and CD4⁻ T cells was analysed by phycoerythrin labelled antibodies. A representative patient (A) and the average of three patients (B) is shown. Asterisks indicate significant difference compared with control culture ($p < 0.05$).

production by IL7 were seen (fig 3A). IL4 production was not significantly influenced by IL7 pretreatment. Similar effects were seen with peripheral blood mononuclear cells from patients with RA (data not shown). The effect on TNFα and IFNγ production was already evident at very low levels of IL7 and was dose dependent (fig 3B).

Previously we have shown that cytokine secretion induced through CD3/CD28 stimulation of MC is more than 90% T cell-specific.²⁴ Furthermore, analysis of individual T cells showed that the increase in TNFα and IFNγ production was associated with an increase in the number of TNFα and IFNγ expressing CD4⁺ T cells (fig 4A, representative donor; fig 4B, means). Less than 5% of CD3⁻ cells produced IFNγ (on average 4.9%) or TNFα (on average 2.4%). No significant change in the

IL4-producing CD4⁺ T cells was found. To a smaller extent the increase in TNFα, IFNγ, and IL4 production was due to IL7 stimulated cytokine secretion by CD3⁺CD4⁻ T cells (on average from 3.9 to 10.6%, 4.5 to 10.3%, and 0.43 to 0.93% of total T cells, respectively).

Synovial fluid CD4⁺ T cells express the IL7α receptor and respond directly to IL7

Down regulation of the IL7α receptor (CD127) has been shown upon maturation of naive neonatal CD4⁺ T cells to cytokine secreting memory cells.¹⁸ Because most SF T cells express the memory phenotype (CD45RO⁺) we analysed CD127 expression levels on these cells. CD127 was abundantly expressed on synovial CD4⁺ T cells from patients with RA (fig 5A). The

Figure 5 (A) Expression of the IL7 receptor α chain (CD127) on isolated synovial CD4+ T cells (black histogram) compared with control staining (open histogram). (B) Isolated CD4+ T cells (5×10^5 /ml) were stimulated for 24 hours with CD3/CD28 alone, and cytokine secretion was assessed. Mean (SEM) values of three patients are given. Asterisks indicate significant difference compared with control culture ($p < 0.05$).

expression was comparable with peripheral blood memory CD4+ T cells and higher than that of naive peripheral blood T cells (data not shown). The functionality of the IL7 α receptor was shown by incubating isolated CD4+ T cells with IL7. IL7 not only significantly increased TNF α and IFN γ but also IL4 production, although IL4 levels were much lower (fig 5B).

IL7 also stimulates T cell responses through accessory cells

In addition to the direct effect of IL7 on T cells, other pathways of proinflammatory cytokine induction might exist as well.

IL7 has been shown to activate cytokine secretion by monocytes which express CD127²⁵ (this study, data not shown). To analyse the involvement of IL12, which is mainly produced by synovial macrophages and a potent stimulator of Th1 cell activity, anti-IL12 neutralising antibodies (5 μ g/ml) were added to SFMC during stimulation with IL7. This resulted in an inhibition of IL7 stimulated IFN γ production by more than 50% ($p < 0.01$), in unchanged TNF α production, and in enhanced IL4 production (97%, $p < 0.05$, fig 6). In an identical control experiment (RA SFMC, $n = 3$) it was shown that these effects were specific, because IL7-induced IFN γ

Figure 6 Effect of IL12 neutralising antibodies (aIL12) on IL7-stimulated cytokine production by RA synovial T cells. SFMC (1×10^6 /ml) were incubated without (open bars) or with IL7 (10 ng/ml, black bars) and IL12 neutralising antibodies (5 μ g/ml, grey bars) for five days, followed by a T cell-specific stimulation with CD3/CD28 for 24 hours. Secretion of TNF α , IFN γ , and IL4 was assessed by ELISA. Mean (SEM) values of eight patients are given. Asterisks indicate significant differences compared with control cultures ($p < 0.05$). † Indicates significant differences between IL7 and IL7/anti-IL12 cultures ($p < 0.01$).

production (from 8.7 (4.4) to 19.0 (2.5) ng/ml) was inhibited by anti-IL12 (to 12.0 (4.7) ng/ml), but not by irrelevant isotype control antibodies (5 µg/ml, to 22.7 (0.6) ng/ml). The isotype control antibodies also did not change IL7-induced TNFα production.

DISCUSSION

In humans, IL7 has been recognised as a factor regulating B and T cell development, myelopoiesis, and activating monocytes/macrophages, natural killer cells, and (cytotoxic) T cells.¹⁶ Little is known about the role of IL7 in patients with RA. Nurse-like cell clones from the bone marrow of patients with RA have been shown to secrete IL7 and to promote survival and enhance the function of B cells.²⁶ Our study shows that IL7 may have a proinflammatory role in patients with RA. IL7 was increased in serum from patients with RA compared with healthy controls and correlated with a marker of inflammation (CRP). The source of IL7 in RA serum is not known, but may include stromal cells in lymphopoietic tissues and cells at the inflammatory sites.¹⁶ For the latter it has been shown that synovocytes of patients with RA can produce IL7.^{15, 16} The relation between IL7 and markers of inflammation has also been found in patients with juvenile RA, in whom levels of IL7 in plasma were significantly higher than in healthy children and correlated with markers of inflammation (plasma IL6 levels, CRP) and disease (systemic features).²⁷ In support of the role of IL7 in increasing the acute phase responses is the stimulatory effect of IL7 on TNFα production by RA SFMC (this study) and the previously described effects on IL6 and IL1β production by monocytes/macrophages. TNFα, IL6, and IL1 are all cytokines which can increase acute phase responses.²⁵

Because of the rather low production of TNFα, we could not identify the source of TNFα production by single cell analysis. This TNFα production may result from direct stimulation of T cells (this study) or direct stimulation of macrophages.²⁴ Furthermore, because IL7 can enhance expression levels of costimulatory molecules (for example, CD69), it might mediate T cell dependent regulation of TNFα by macrophages, similar to IL15.¹¹ Hence, IL7 might contribute to increased T cell and macrophage TNFα production in patients with RA, and promote joint inflammation and destruction.^{8, 28, 29}

Although IL7 has been shown to have a role in the IL4 production of naive CD4+ T cells,¹⁸ the present data indicate that IL7 has a minor effect on IL4 production of RA SF memory CD4+ T cells and merely stimulates proinflammatory T cell activity. In the presence of accessory cells IL7 stimulates Th1 cytokine secretion (IFNγ) without having a significant influence on Th2 cell (IL4) activity, which is in agreement with T cell cytokine profiles found in the RA joints.^{4-6, 28} These data for RA SF are also in line with the effects of IL7 on IFNγ and IL4 production of isolated peripheral blood CD4+ T cells from healthy subjects, demonstrating primarily Th1 cell activation by IL7.¹⁹

The effect of IL7 on RA CD4+ SF T cells present in SFMC was, at least partly, directly on the T cells. SF CD4+ T cells expressed the IL7 receptor α chain and were, when isolated, strongly stimulated in their TNFα and IFNγ production by IL7. In addition to the direct effect of IL7 on CD4+ T cells, part of the effect of IL7 on Th1/Th2 cytokine production was mediated by the production of IL12 because blockade of IL12 resulted in a significant decrease in Th1 cell activity (IFNγ production) and increase in Th2 activity (IL4 production). This IL12 is probably produced by antigen presenting cells, in particular monocytes/macrophages which are major producers.¹⁴ The present data also suggest that in patients with RA, IL7 may give rise to IL4 production, in the absence of stimulatory accessory cells, or when the activity of synovial accessory cells is modulated, for example by IL12 blockade.

T cell TNFα production (after pretreatment with IL7 and T cell stimulation) seems to be regulated differently from IFNγ production because, in contrast with IFNγ production, this TNFα is not blocked by anti-IL12. In agreement with this we found that IL10 similarly blocked IL7-induced IFNγ production, but not TNFα production (data not shown). Because IL10 inhibits many cytokines produced by macrophages, including IL12, this indicates that the dichotomy between TNFα and IFNγ may not be dependent on factors produced by accessory cells. T cell TNFα production may be more dependent on direct T cell stimulation by IL7, or IL7-induced T cell-accessory cell membrane interactions.

In addition to the effect of IL7 on CD4+ T cells, cytokine production by RA CD4- T cells was stimulated by IL7 as well. This is in agreement with the stimulation of cytotoxic activity of CD8+ T cell clones by IL7 which has been shown previously,³⁰ and it indicates that IL7 might stimulate a generalised arthritogenic T cells response in RA.

As far as we know, this study is the first to show an association between IL7 and a marker of RA disease activity and demonstrates that IL7 stimulates SFMC and synovial (CD4+) T cells, producing proinflammatory responses associated with secretion of TNFα and IFNγ. This effect is achieved by direct binding to the IL7α receptor on T cells and indirectly through accessory synovial mononuclear cells. The immunostimulatory ability of IL7 and the presence of IL7 in RA joints indicate that IL7 can substantially contribute to the continuing inflammatory response in RA and that patients with RA might benefit from treatment strategies in which inhibition of IL7 is considered.

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