EXTENDED REPORT

Clinical efficacy of infliximab plus methotrexate in DMARD naive and DMARD refractory rheumatoid arthritis is associated with decreased synovial expression of TNF α and IL18 but not CXCL12

M van Oosterhout, E W N Levarht, J K Sont, T W J Huizinga, R E M Toes, J M van Laar

Ann Rheum Dis 2005;64:537–543. doi: 10.1136/ard.2004.024927

See end of article for authors' affiliations

Correspondence to:
M van Oosterhout, Leiden University Medical Centre, Department of Rheumatology, C4R, PO Box 9600, 2300 RC Leiden, Netherlands; M.van_Oosterhout@LUMC.nl

Accepted 22 August 2004

Background: Tumour necrosis α (TNF α) blocking agents lead to pronounced clinical effects and reduced synovial infiltrate in rheumatoid arthritis. Laboratory and clinical studies suggest that TNF α independent pathways play a role in the disease.

Objectives: To evaluate the immunopathological effects of combination therapy on rheumatoid synovial tissue in order to identify TNF α independent mechanisms.

Methods: 12 rheumatoid patients, including four DMARD (disease modifying antirheumatic drug) naive patients with early disease, were studied for the effect of combination therapy with infliximab and methotrexate on the synovial infiltrate. Biopsies and clinical assessments (DAS28) were carried out before the first and after the third infusion of infliximab. Synovial inflammation was scored semiquantitatively. Co-expression of CD38⁺ cells was studied by an immunofluorescent double labelling technique.

Results: Marked clinical responses were associated with a global reduction in the synovial infiltrate and expression of cytokines, notably interleukin 18 and TNF α , but low grade disease activity persisted. There was no effect on the expression of CXC chemokine ligand (CXCL12), and germinal centre-like structures were still detectable in synovial tissue in two patients after treatment. CD38⁺ activated T cells were more resistant to treatment than CD38⁺ plasma cells. No differences in clinical response or effects on synovial infiltrate were observed between DMARD refractory and DMARD naive patients.

Conclusions: Persistent expression of CXCL12 and incomplete resolution of lymphocytic infiltrates after infliximab plus methotrexate indicates that TNF α independent mechanisms are operative in rheumatoid arthritis. This may contribute to low grade disease activity, even in DMARD naive patients with early disease.

The introduction of tumour necrosis factor α (TNF α) blocking agents has greatly expanded the therapeutic armamentarium for rheumatoid arthritis. The efficacy and safety of these agents have been established in large clinical trials.^{1–3} They lead to pronounced clinical effects by downregulating local inflammation and delaying joint destruction.⁴ Combination treatment with methotrexate was shown to be more effective than either agent alone,⁵ though 30–40% of patients failed to attain a satisfactory clinical response.

Studies of serial synovial tissue specimens on the effects of TNF α blocking agents on the synovium, the primary site of inflammation in rheumatoid arthritis, have revealed decreased cellularity of the synovial infiltrate,^{6–7} along with effects on chemotaxis of inflammatory cells,^{7–8} on neovascularisation, and on the production of metalloproteinases.⁸ After treatment, a considerable level of synovial infiltration was still demonstrable in these studies despite a good clinical response. These clinical and immunopathological studies raise the question of whether TNF α independent mechanisms play a role in synovitis in rheumatoid arthritis.

In this study we analysed the effect of combination treatment with infliximab and methotrexate on immunohistology of rheumatoid synovial tissue in order to identify TNF α independent mechanisms. We were particularly interested in the expression of the stromal factor CXC chemokine ligand 12 (CXCL12) and its receptor CXCR4, as expression of CXCL12 seemed independent of TNF α in an ex vivo model of rheumatoid arthritis.⁹ We also studied the influence on

cellular composition and changes in production of cytokines, including interleukin (IL) 18, which has proinflammatory capabilities in rheumatoid arthritis.^{10–11}

While synovial tissue studies have so far focused on rheumatoid patients refractory to disease modifying antirheumatic drugs (DMARDs), it is unknown whether similar mechanisms are operative in patients with early rheumatoid arthritis who are DMARD naive. We therefore evaluated the effects of infliximab and methotrexate on the synovial tissue infiltrate not only in patients with longstanding DMARD refractory rheumatoid arthritis but also in newly diagnosed DMARD naive cases. We postulated that synovitis in the latter group would be more amenable to treatment, based on a shorter disease duration and the absence of pretreatment.

METHODS

Patients and study design

Patients with rheumatoid arthritis according to the American College of Rheumatology (ACR) criteria¹² and active disease as defined by a 28 joint disease activity score (DAS28) of >4.6 ¹³ were eligible for this study. We recruited eight patients with longstanding rheumatoid arthritis and failed treatment with at least two DMARDs (DMARD refractory) and four

Abbreviations: ACR, American College of Rheumatology; CXCL, CXC chemokine ligand; CXCR, CXC chemokine ligand receptor; DAS28, 28 joint disease activity score; DMARD, disease modifying antirheumatic drug; EULAR, European League Against Rheumatism; IL, interleukin; TNF α , tumour necrosis factor α .

Table 1 Clinical features of patients with DMARD refractory and DMARD naive rheumatoid arthritis

Age (y)	Sex	Erosive disease	RF positive	Disease duration (y)	DMARDs used	EULAR response at second biopsy
<i>DMARD refractory</i>						
56	M	Yes	Yes	11.1	MTX, SASP, lefl, pred, HCQ	Good
45	F	Yes	Yes	10.6	MTX, gold	Moderate
37	F	Yes	Yes	5.2	MTX, SASP, lefl, CsA	Moderate
23	F	Yes	Yes	1.5	MTX, SASP, pred, gold	Moderate
35	F	Yes	No	17.3	MTX, SASP, pred, gold, CsA	No
57	F	Yes	Yes	10.0	MTX, SASP, HCQ, pred, lefl	Moderate
45	F	Yes	Yes	1.6	MTX, SASP, lefl	Good
44	F	Yes	Yes	21.0	MTX, SASP, pred, HCQ	Good
<i>DMARD naive</i>						
35	F	No	Yes	0.2	NA	Good
60	M	Yes	Yes	0.1	NA	Moderate
62	M	Yes	Yes	0.1	NA	Moderate
78	F	Yes	Yes	0.1	NA	Moderate

CsA, cyclosporin; EULAR, European League Against Rheumatism; gold, intramuscular gold salts; HCQ, hydroxychloroquine; lefl, leflunomide; MTX, methotrexate; NA, not applicable; pred, prednisone; SASP, sulfasalazine; y, years.

patients with recent onset rheumatoid arthritis who had not been treated with DMARDs previously (DMARD naive) (table 1). All patients received infliximab (3 mg/kg intravenously) at weeks 0, 2, and 6. DMARD refractory rheumatoid patients were on a stable dose of oral methotrexate (mean 21 mg, range 15 to 25) with a mean duration of methotrexate use at baseline of five months (range 1 to 11). DMARD naive patients were treated with incremental doses of oral methotrexate starting at 7.5 mg at baseline and increasing to 25 mg/week at the time of the third infusion of infliximab. Concomitant treatment with non-steroidal anti-inflammatory drugs (NSAIDs) in a stable dose was permitted. No other DMARDs or intra-articular steroid injections were allowed. Clinical assessments were made by a trained research nurse before the start of treatment and five days (median, range 1 to 10) after the third infusion of infliximab. These assessments included documentation of adverse events, scoring of DAS28, and patient's assessment of general wellbeing and physician's assessment of disease activity using a visual analogue scale (VAS, 0 to 100). Synovial tissue was obtained at arthroscopy on the same day. Blood samples were collected at both visits to determine erythrocyte sedimentation rate (ESR), C reactive protein, and rheumatoid factor. Clinical efficacy was assessed using the EULAR response criteria.¹⁴ The study protocol was approved by the medical ethics committee of the Leiden University Medical Centre and all patients gave their written informed consent.

Arthroscopy

Arthroscopy was done on affected knees in all patients with a small bore 2.7 mm arthroscope (Storz, Tuttlingen, Germany) under local anaesthesia (30 ml of 0.5% or 1% lignocaine (lidocaine) for the suprapatellar or infrapatellar skin portal, respectively). Both baseline and follow up biopsies were taken from the same knee in each patient. The infrapatellar portal was used for introduction of the arthroscope and the suprapatellar portal for the biopsy procedure. At each occasion 20 to 25 pieces of synovial tissue in all were collected from the suprapatellar pouch, the patellar gutters, and the infrapatellar regions using a 2.0 mm grasping forceps (Storz).

Immunohistochemistry

Immediately after harvesting, synovial tissue was collected en bloc in a mould, embedded in Tissue-Tek OCT (Miles, Elkhart, Indiana, USA), and stored in liquid nitrogen (−180°C) until sectioning. Sections (5 µm) were cut on a

cryostat (Leica, Rijswijk, Netherlands), mounted on glass slides (Star Frost, Knittelgläser, Braunschweig, Germany), and stored at −70°C until immunohistochemistry was done in a single session. Serial sections were stained with the following monoclonal antibodies: anti-CD3 (M0740, Dako, Denmark), anti-CD4 (F0818, Dako), anti-CD8 (M0707, Dako), anti-CD19 (M0740, Dako), anti-CD68 (M0718, Dako), anti-CD38 (34768, Becton-Dickinson, San Jose, California, USA), anti-CD55 (M2156, CLB, Netherlands), anti-CXCL12 (MAB350, R&D Systems, UK), anti-CXCR4 (MAB170, R&D Systems), anti-TNFα (MAB610, R&D Systems), rabbit anti-human IL1 (LP712, Genzyme, Cambridge, Massachusetts, USA), and anti-IL18 (D043-3, MBL, Nagoya, Japan). All antibodies were mouse anti-human unless indicated otherwise.

The immunohistochemical staining procedure was carried out as follows. Slides were warmed to room temperature, fixed in acetone (99.5%; Merck, Darmstadt, Germany) for 10 minutes, and then air dried for 20 minutes. After each step the samples were washed with phosphate buffered saline (PBS; apotheek LUMC, Leiden, Netherlands) and all incubations were done at room temperature. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide (Merck) in PBS containing 0.1% sodium azide (Merck) for 20 minutes. The monoclonal antibodies were diluted in PBS with 1% bovine serum albumin (BSA; ICN Biomedicals Inc, Aurora, Colorado, USA) and incubated for 60 minutes. For control sections, PBS, matching isotype, and conjugate controls were applied. The detection of the monoclonal antibodies involved the use of affinity purified and horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Dako), goat anti-mouse IgG2a (Dako), goat anti-rabbit-HRP (Biosource, Camarillo, California, USA), and swine anti-goat-HRP (Biosource). The biotinyl tyramide/streptavidin-HRP amplification system (NEN Life Science Products Inc, Boston, Massachusetts, USA) was used to enhance the HRP staining. The HRP conjugated antibodies were diluted in PBS/BSA (1%) with 10% normal human serum (NHS, Bloedbank LUMC, Leiden, Netherlands) as blocking serum, and incubated for 30 minutes. The biotinyl tyramide was then diluted in dilution buffer (NEN Life Science Products) and incubated for 30 minutes. HRP activity was detected using hydrogen peroxide as a substrate and aminoethylcarbazole (AEC, Sigma, St Louis, Missouri, USA) as a dye. After washing with distilled water, the sections were counterstained with Mayer's Hämalaunlösung (Merck), and mounted with Kaiser's glycerol gelatine (Merck).

Table 2 Clinical response to combination treatment with infliximab and methotrexate in all patients, DMARD refractory patients, and DMARD naive patients before and after three infusions of infliximab

	All patients (n = 12)			DMARD refractory RA (n = 8)			DMARD naive RA (n = 4)			
	Before	After	p Value*	Before	After	p Value*	Before	After	p Value*	p Value†
DAS28	5.86	3.90	<0.01	5.61	3.90	<0.01	6.36	3.90	<0.01	0.17
SJC	8.7	3.7	<0.01	7.13	4.50	0.11	11.8	2.00	0.06	0.04
TJC	11.3	4.7	<0.01	9.25	5.00	0.05	15.5	4.00	0.02	0.04
ESR	37.8	19.7	<0.01	28.5	16.3	<0.01	56.3	26.5	0.28	0.28
CRP	38.6	5.5	<0.01	15.5	4.8	0.01	84.8	7.0	0.18	0.76
VAS pat	62.8	26.5	<0.01	67.0	28.9	<0.01	54.5	21.8	0.04	0.68
VAS phys	53.3	22.8	<0.01	54.9	27.8	<0.01	50.3	12.8	0.05	0.44
MS (min)	70.0	17.9	0.05	65.6	13.8	<0.01	78.8	26.3	0.35	0.99
IgM RF	51.4	36.1	0.03	54.0	33.4	0.07	47.0	40.7	0.18	0.54

*Wilcoxon's signed rank test between first and second biopsy within groups.

†Mann-Whitney test for change in clinical indices between DMARD naive and DMARD refractory patients.

CRP, C reactive protein; DAS28, 28 joint disease activity score; DMARD, disease modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; IgM RF, rheumatoid factor in arbitrary units (normal range 0–5); MS, morning stiffness (minutes); RA, rheumatoid arthritis; SJC, swollen joint count; TJC, tender joint count; VAS pat, visual analogue scale for general wellbeing (0–100); VAS phys, visual analogue scale for disease activity assessed by physician (0–100).

Double labelling procedure

For further phenotypic characterisation of CD38 cells, double labelling studies were carried out to investigate co-expression of CD38 plus CD3 and CD38 plus CD138 (syndecan, a marker of plasma cells), respectively, employing immunofluorescent techniques. We used unconjugated mouse anti-human CD3 and CD138 (M7228, Dako) and biotinylated mouse anti-human CD38 (187–030, Ancell, Bayport, Minnesota, USA) as primary antibodies. Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies were used where appropriate. Cross reaction of the goat anti-mouse secondary antibody was prevented by carrying out the TRITC and FITC procedures consecutively. Appropriate blocking of free epitopes was achieved by 10% normal mouse or human serum in PBS. The weak FITC signal of CD3 and CD138 was enhanced using a third (FITC labelled) antibody directed against the goat (FITC labelled) immunoglobulins. Finally, tissue sections were mounted in vectastain (Vector Laboratories, Burlington, California, USA) and photographed on the same day to avoid fading of the signal.

Semiquantitative scoring of inflammation

Stained sections were coded and randomly analysed. All areas of each biopsy section were scored blindly by two independent observers (MO and NL). At least two samples per patient per time point were scored semiquantitatively for all markers, from 1 to 4 (1 = lowest, 4 = highest level of expression). The scoring system was calibrated for each marker separately because some markers are more abundantly expressed than others. All differences between the observers were resolved by mutual agreement. Interobserver agreement reached 90% and differed by one point at the most. Double staining of the synovial tissue cells was scored independently by two blinded observers and given as a percentage by counting at least 100 cells, either single positive for the two markers or double positive.

Statistical analysis

Wilcoxon's signed rank test was used to determine the statistical significance of the differences between the first and the second biopsy (SPSS for Windows, version 10.0.7, SPSS Inc, Chicago, Illinois, USA). Differences between groups were tested using a Mann-Whitney test. Correlations were expressed using Spearman's rank correlation coefficient. Probability (p) values of <0.05 were considered significant.

RESULTS

Clinical efficacy

As shown in table 1, four patients had a good response, seven had a moderate response, and one had no response according to the EULAR response criteria.¹⁴ When taken as a group, all clinical indices improved significantly (table 2). There was no difference in clinical response between DMARD refractory and DMARD naive patients except for a more pronounced decrease in swollen and tender joint counts in the patients with early rheumatoid arthritis (p = 0.04 for both variables). Arthroscopic procedures were well tolerated and no side effects were observed.

Immunohistochemistry

The two groups did not differ in expression of cellular markers and cytokines at baseline. When taken as a group, expression of most markers decreased significantly with the exception of CD55⁺ cells (lining layer) and CXCL12, which remained unaltered overall (table 3). Notably, reduced expression of IL18 and TNF α was observed in both groups (p<0.01), as was expression of most cell markers including CD38⁺, commonly used as a plasma cell marker. The changes were not significantly different between the two groups. Figure 1 shows the synovial infiltration before and after three infusions of infliximab, as shown by expression of CD3, CD38, IL18, and CXCL12.

Double labelling

In standard monochrome immunohistochemical analysis of the tissue sections we observed a decrease in both CD38 and CD3 staining in DMARD refractory and DMARD naive patients during treatment. To further characterise the CD38⁺ cells we carried out an immunofluorescent staining procedure for CD38/CD3 and CD38/CD138 double positive cells (activated T cells and plasma cells, respectively). In the whole group, 50.3% of CD38 positive cells co-expressed CD138 at baseline, compared with 51.7% after treatment (p = 0.23), while 36.8% of CD38 cells co-expressed CD3 at baseline and 49.9% after treatment (p<0.01). There was no difference between DMARD refractory and DMARD naive patients related to the changes of CD38/CD138 (p = 0.81) or CD38/CD3 (p = 0.21). Figure 2 shows immunofluorescent double staining after treatment in one patient, with different staining patterns of CD3 and CD138 single positive and double positive populations.

Correlations

Comparison of changes in clinical indices with the decrease in expression of synovial markers yielded a correlation

Figure 1 Immunohistochemical analysis of the synovial infiltrate before (panels A, C, E, G) and after (panels B, D, F, H) three infusions of infliximab. Expression of CD38 (panels A and B), CD3 (panels C and D), IL18 (panels E and F), and CXCL12 (panels G and H). A–D, G, and H original magnification $\times 100$; E and F original magnification $\times 250$.

between CD3 and CRP ($p = 0.62$, $p = 0.04$) while TNF α showed a trend towards correlation with DAS28 ($p = 0.59$, $p = 0.07$). Correlations were also found between CXCR4 and CD68 ($p = 0.69$, $p = 0.03$), CXCR4 and TNF α ($p = 0.76$, $p = 0.01$), and CD3 and CD4 v CD8 ($p = 0.66$ and 0.61 ; $p = 0.04$ and 0.05 , respectively).

DISCUSSION

This study was conducted to evaluate the immunopathological effects of combination therapy with infliximab and methotrexate on rheumatoid synovitis, in order to identify TNF α independent mechanisms. Our results show that marked clinical responses were associated with a global reduction in the synovial infiltrate and expression of

cytokines but that low grade disease activity persisted, clinically as well as at the tissue level. More specifically, though we observed a reduction in the synovial infiltrate with macrophages (CD68 $^{+}$), T and B cells (CD3 $^{+}$ and CD19 $^{+}$, respectively), and plasma cells (CD38 $^{+}$), the resolution was incomplete. Also no differences in clinical and synovial responses could be found between DMARD refractory and DMARD naive patients. As large clinical trials have shown only a small additional improvement after seven weeks of infliximab treatment we do not expect further changes in the synovial inflammation with continued treatment.

The incomplete resolution of synovitis in treated patients, together with the data from ex vivo and in vitro studies suggesting TNF α independency of CXCL12,^{9, 15} prompted us to

Table 3 Semiquantitative score of synovial tissue before and after three infusions of infliximab in all patients, DMARD refractory patients, and DMARD naive patients: scores for cellular markers, cytokines, and chemokine CXCL12 and its receptor CXCR4

	All patients (n=12)			DMARD refractory RA (n=8)			DMARD naive RA (n=4)			p Value†
	Before	After	p Value*	Before	After	p Value*	Before	After	p Value*	
CD3	2.48	1.99	<0.01	2.36	2.00	0.06	2.72	1.98	<0.01	0.11
CD4	2.89	2.58	0.08	3.00	2.56	0.03	2.60	2.60	1.00	0.44
CD8	2.45	1.80	<0.01	2.42	1.79	<0.01	2.50	1.83	0.24	0.97
CD19	2.06	1.44	0.07	2.08	1.39	0.09	2.00	1.60	0.71	0.63
CD38	2.37	1.90	<0.01	2.39	1.92	0.03	2.33	1.86	0.01	0.70
CD55	2.68	2.55	0.59	2.87	2.60	0.46	2.28	2.43	0.66	0.37
CD68	2.63	2.04	<0.01	2.79	2.07	0.02	2.33	1.98	0.24	0.69
IL1	2.96	2.22	<0.01	3.00	2.20	<0.01	2.88	2.25	0.10	0.78
IL18	2.27	1.23	<0.01	2.43	1.36	<0.01	2.00	1.00	0.04	0.87
CXCL12	2.46	2.54	0.72	2.46	2.55	0.72	2.33	2.50	0.70	0.97
CXCR4	2.90	2.45	0.04	3.00	2.62	0.10	2.72	2.14	0.19	0.82
TNF α	2.30	1.20	<0.01	2.23	1.23	0.01	2.43	1.14	0.02	0.64

*Wilcoxon's signed rank test between first and second biopsy within groups.
†Mann-Whitney test for change in synovial markers between DMARD naive and DMARD refractory patients.
CXCL, CXC chemokine ligand; CXCR, CXC chemokine ligand receptor; DMARD, disease modifying antirheumatic drug; IL, interleukin; RA, rheumatoid arthritis; TNF α , tumour necrosis factor α .

Figure 2 Immunofluorescent double staining in a DMARD refractory patient after treatment. FITC staining for CD3 (panels A and C) and CD138 (panels D and F). TRITC staining for CD38 (panels B and E) and double staining for CD38/CD3 (C) and CD38/CD138 (F). Original magnification $\times 80$. DMARD, disease modifying antirheumatic drug; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

investigate the synovial expression of CXCL12, a stromal derived factor with distinct pro-inflammatory capacities.^{16–19} We found that it was not affected by treatment. This confirms and extends previous studies suggesting the presence of TNF α independent pathways in synovial inflammation.²⁰ Two observations in our study add to the latter hypothesis. The first was that a significant proportion of CD38 positive cells were shown to be (presumably activated) T cells^{21–22} that were relatively more resistant to treatment. The persistence of CXCL12, which is involved in chemoattraction and homing of T cells in synovial tissue,¹⁸ may play a role in this phenomenon, as chemoattraction through TNF α is markedly reduced by infliximab.⁸ Second, in the standard immunohistochemical and the immunofluorescent double staining analysis it was noticeable that germinal centre-like structures in the synovial tissue of two DMARD refractory patients persisted (see fig 2) despite infliximab and methotrexate, possibly contributing to the chronic disease course.²³ The same TNF α independent mechanisms which contribute to the formation and persistence of germinal centre-like structures in the synovial tissue in our study may be responsible for the recently observed non-responsiveness to infliximab treatment of rheumatoid nodules.²⁴

Treatment with infliximab and methotrexate almost abrogated the expression of TNF α and IL18, which indicates that TNF α was effectively downregulated at the synovial level. This is the first evidence of therapeutic downregulation in synovial tissue of IL18, an IL1 related cytokine produced by macrophages and synoviocytes with chemotactic and angiogenic properties.^{10–11–25} As a result, TNF α blockade also interrupts the synergistic effects between TNF α and IL18.^{26–27}

Our results are in keeping with previous studies showing that synovitis in early disease is generally indistinguishable from late stage disease.²⁸ There was no difference in histological response between DMARD refractory and DMARD naive patients. Fewer markers reached significance in patients with early rheumatoid arthritis, probably because of the small sample size. Treatment responses were thus similar at the level of synovial tissue, at least for the markers tested, and did not confirm our hypothesis of superior treatment responsiveness in DMARD naive rheumatoid arthritis. This is more striking considering the fact that naive patients had also just started on methotrexate, while refractory patients were already on a stable dose at baseline. Methotrexate inhibits synovial infiltration²⁹ and concomitant treatment with methotrexate may limit the reduction in cellularity caused by infliximab in DMARD refractory patients. On the other hand all four patients with early rheumatoid arthritis in our study were rheumatoid factor positive, and three already had erosive disease at the time of diagnosis—both unfavourable prognostic factors. It is conceivable that the results of synovial infiltrate analysis after infliximab and methotrexate would have been different in patients with non-erosive, rheumatoid factor negative, DMARD naive early disease. The semiquantitative scoring method, especially with a limited number of observations, is relatively insensitive to small changes, and caution is needed in the interpretation of the results. Nevertheless, we feel that the consistency and magnitude of the immunohistological changes (comparable to similar studies in the past) make our results valid and identify CXCL12 as a TNF α independent factor.

Conclusions

Our study shows marked effects of infliximab plus methotrexate on synovial pathology, notably IL18 and TNF α , but not on the expression of CXCL12. Together with the limited reduction of activated T cells and the persistence of germinal centre-like structures after treatment, our data indicate that

TNF α independent mechanisms are operative in rheumatoid arthritis, both in early untreated disease and in late stage disease. In CXCL12 we have identified for the first time a proinflammatory cytokine in rheumatoid arthritis that persists in spite of a marked clinical response to anti-TNF α treatment.

Authors' affiliations

M van Oosterhout, E W N Levarht, T W J Huizinga, R E M Toes, J M van Laar, Department of Rheumatology, Leiden University Medical Centre, Leiden, Netherlands

J K Sont, Department of Medical Decision Making, Leiden University Medical Centre

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