EXTENDED REPORT

T cells, fibroblast-like synoviocytes, and granzyme B+ cytotoxic cells are associated with joint damage in patients with recent onset rheumatoid arthritis

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Objective: To determine immunohistological markers in synovial tissue of patients with early rheumatoid arthritis (RA) which are associated with unfavourable disease outcome.

Methods: Synovial tissue was obtained from 36 patients with RA within 1 year after the initial symptoms and before starting disease modifying antirheumatic drug treatment. Clinical, laboratory, and radiological assessments (Larsen score) were performed at the time of the biopsy and at the end of follow up (mean 58 months, range 38–72). Immunohistological analysis was performed to detect T cells, B cells, plasma cells, fibroblast-like synoviocytes (FLS), macrophages, and granzyme B+ cytotoxic cells. The sections were evaluated by digital image analysis.

Results: Patients were divided into two groups based upon the radiological progression per year of follow up: group I with mild progression (n = 20; Larsen <2 points/year); group II with more severe progression (n = 16; Larsen ≥ 2 points/year). Regression analysis with a univariate model showed that the numbers of granzyme B+ cytotoxic cells (relative risk (RR) = 12, p = 0.003), T cells (RR = 11, p = 0.013), and FLS (RR = 10, p = 0.020) discriminated between groups I and II. A multivariate model demonstrated that the numbers of T cells (RR = 1.2, p = 0.015) and FLS (RR = 1.4, p = 0.013) were independent discriminators between groups I and II.

Conclusion: The numbers of granzyme B+ cytotoxic cells, T cells, and FLS in synovial tissue of patients with RA are related to the severity of joint damage. The data suggest a pathogenetic role for these cells in the process of joint damage.

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by polyarticular and symmetrical arthritis affecting the hands, which often leads to progressive destruction, resulting in functional impairment and disability. At present, RA is thought of as an autoimmune disease, though its exact pathogenesis has not yet been clarified.

There is substantial evidence in favour of an essential role for T cells driving the inflammatory cascade in RA, including the association of HLA-DR4 with disease susceptibility and outcome,[1] the expression of activation markers such as HLA-DR and CD69 by T cells,[2] and the beneficial effect of blockade of costimulatory pathways by CTLA-4Ig treatment.[3] Previous work has shown that T cells are associated with the bone destruction observed in RA through the interaction with osteoclasts via the receptor activator NF-κB ligand/osteoprotegerin (RANKL/OPG) pathway,[4 5] and an increase in RANKL expression is associated with joint erosions.[6]

In addition, macrophages have been identified as key effector cells, based upon their presence in large numbers, their activated phenotype, and the abundant production of cytokines.[7 8] In clinical studies a positive correlation was found between the number of macrophages and the expression of macrophage derived cytokines, on the one hand, and clinical signs of inflammation[8 9] and disease outcome,[10 11] on the other.

Intimal macrophages are found in close association with activated fibroblast-like synoviocytes (FLS), another pivotal player in the pathogenesis of RA.[12] The pattern of activation of FLS is characterised by alterations in the expression of regulatory genes and signalling cascades, as well as by impaired apoptosis. These cells exhibit up regulation of adhesion molecules that mediate attachment to the extracellular matrix, and overexpression of matrix degrading enzymes that mediate the progressive destruction of the joints.[13] FLS and macrophages are the main source of extracellular matrix degrading enzymes.[14 15]

Numerous plasma cells, often surrounding the lymphocyte aggregates, may also be present throughout the synovium, sometimes exceeding the number of infiltrating T cells.[8 9] A considerable number of the plasma cells synthesise and secrete rheumatoid factors and other autoantibodies[16] which may be involved in macrophage activation.[17] Other cells infiltrating the rheumatoid synovium include B cells, mast cells, dendritic cells, natural killer cells, and neutrophils.[8 9]

Recent studies indicate that early intervention may alter disease outcome in RA when instituted before invasive pannus growth and proteinase production have led to loss of bone and cartilage.[18] Therefore, it is important to identify patients at risk for destructive disease as early as possible. Several studies have focused on the factors which correlate with the outcome of RA. Among the predictive factors for a more severe disease course are high disease activity early in the disease,[19] female sex,[20] positive rheumatoid factor (RF),[21] and HLA-DR4.[22 23]

Abbreviations: CRP, C reactive protein; DAS, disease activity score; DMARDs, disease modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; FLS, fibroblast-like synoviocytes; HAQ, Health Assessment Questionnaire; HPF, high powered field; OPG, osteoprotegerin; RA, rheumatoid arthritis; RANKL, receptor activator NF-κB ligand; RF, rheumatoid factor; RR, relative risk

Since genetic background, demographic and clinical determinants, and serological markers can only partially predict disease outcome, this study was undertaken to explore the association between disease severity and cellular markers in synovial tissue of patients with RA of recent onset.

PATIENTS AND METHODS

Patients

All patients fulfilled the revised criteria of the American College of Rheumatology for the diagnosis of RA.[24] Only patients with early disease (<1 year disease duration, as measured from the first clinical signs of arthritis) at the time of the baseline biopsy were enrolled in the study. Patients who currently were receiving or had previously received disease modifying antirheumatic drugs (DMARDs) or prednisone were excluded. The study protocol was approved by the ethical committees of the participating centres in the Netherlands and Australia.

Clinical, laboratory, and radiological evaluation

Clinical and laboratory assessment of the patients was performed on the day of the baseline synovial biopsy and after follow up. Clinical data included age, sex, duration of symptoms before the first biopsy procedure, use of DMARDs in the follow up period, tender and swollen joint counts, visual analogue scale for pain and morning stiffness, Health Assessment Questionnaire (HAQ), and disease activity score using 28 joint counts (DAS).[25] Laboratory measurements included the erythrocyte sedimentation rate (ESR), serum levels of C reactive protein (CRP), and RF. At baseline soluble granzyme B levels were determined in paired synovial fluid and blood serum samples, if available.[26 27] Radiographs of hands and feet were obtained at enrolment in the study and after follow up. All *x* ray findings were scored in random order by a ''blinded'' observer using the Larsen method[28] to assess radiological damage. Radiological deterioration was estimated by subtracting the scores at follow up from the entry Larsen scores. To compensate for the variable follow up we calculated the average change in Larsen score per year by dividing the delta change in Larsen score by the number of years of follow up.

Synovial tissue biopsies

Multiple synovial tissue samples (≥6)[29] were obtained by blind needle biopsy (n = 18), or by small needle arthroscopy (n = 18) as described previously.[30] The biopsy specimens were immediately processed en bloc and embedded in Tissue Tek OCT (Miles Diagnostics, Elkhart, IN), snap frozen, and stored in liquid nitrogen. Cryosections 5 mm thick were cut from the frozen specimens and placed on glass slides, air dried overnight, wrapped in laboratory film, and stored at −80°C until stained.

Immunohistological staining

Serial sections were stained using mouse-antihuman antibodies against CD3 (Leu-4, Becton-Dickinson, San Jose, CA) to detect T cells, CD22 (CLB-B-Ly/1, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) to detect B cells, CD38 (Leu-17, Becton-Dickinson) to detect plasma cells, CD55 (Mab67, Serotec, Oxford, UK) to detect FLS, CD68 (EBM11, Dako, Glostrup, Denmark) to detect macrophages, and granzyme B (Gr7, Monosan, The Netherlands) to detect granzyme B+ cytotoxic cells. For all antibodies the staining was conducted, according to a three step immunoperoxidase method, as described previously.[31] Figure 1 provides an example of a sections showing granzyme B+ cells, T cells, and FLS.

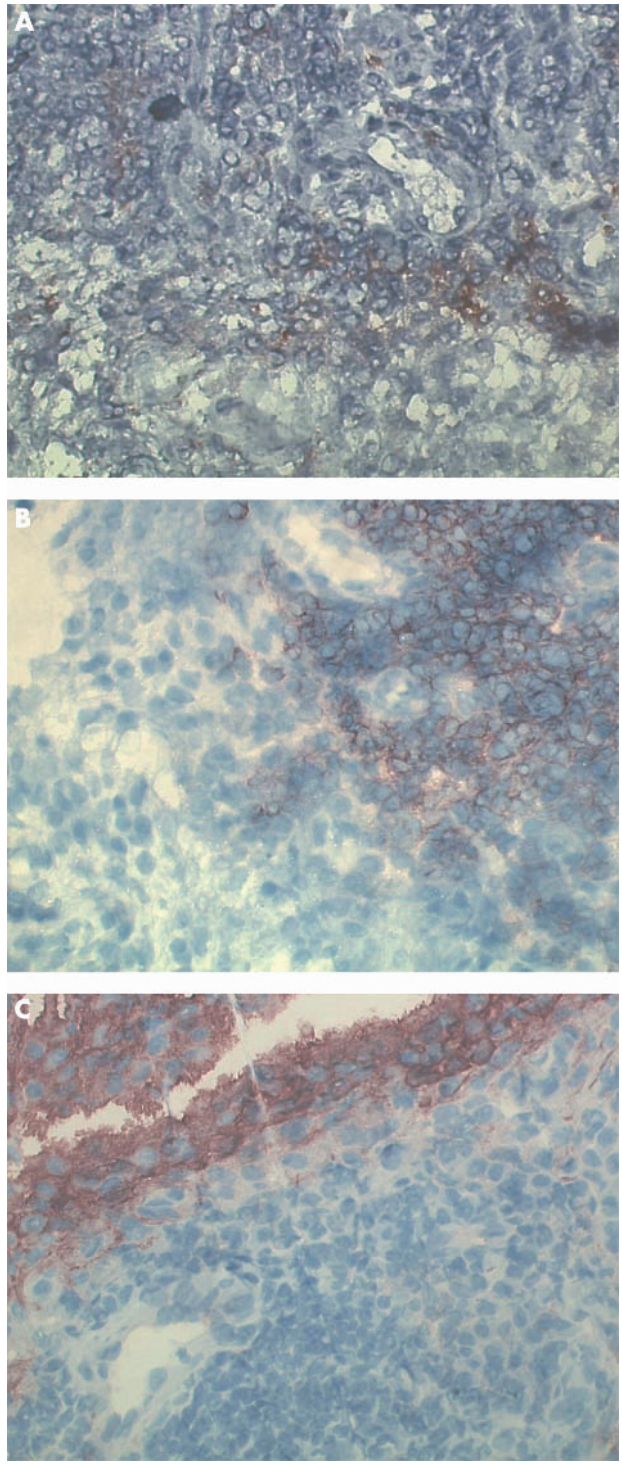

Figure 1 Example of strong positive staining for granzyme B+ cells (A), T cells (B), and fibroblast-like synoviocytes (C) in a patient with unfavourable radiological outcome (×400).

Digital image analysis of synovial tissue

Only synovial tissue samples in which the intimal lining layer was identifiable were included in the study. All sections were coded and analysed in a random order by an independent observer who was unaware of the clinical data (MCK), as described previously.[32] One separate representative region including the intimal lining layer and synovial sublining was chosen for the evaluation of each section. From this region 20 consecutive high power fields (HPFs) were captured and

digitised covering an area of 2.1 mm^2. The HPF images were acquired on a fully automated Leica DMRXA microscope (Leica, Wetzlar, Germany) with a Prior stage table, captured using a three chip CCD (Charged Coupled Device) video-camera (Sony, Tokyo, Japan), and digitised with a Matrox 32 bit colour video digitiser card, using a highly standardised macro program written in the program language QUIPS for the Leica Qwin image analysis software (Leica, Cambridge, UK). The resultant colour images were in a 740×570 pixel RGB format with a 24 bit resolution, enabling the use of 16 581 375 colours. For each acquisition session the microscope, camera, and computer were calibrated according to a standardised procedure, and for each individual marker all images were acquired in one single session. The images obtained were stored using tagged image file compression on a writable CD ROM.

All sections were examined using a computer (Qwin Pro V2.5, Leica, Cambridge, UK) and computer assisted colour video image analysis system operating a specialised algorithm (SYNDIA v1.1) written in the program QUIPS.[32]

Statistical analysis

The distribution of each continuous variable was checked for normality. Non-parametric methods were used for data failing tests for normality. The patients were divided into two groups based on the scores for radiological deterioration for each year of follow up (a cut off value for the change in Larsen's score per year of two points was used). The groups were compared using the Mann-Whitney U test. To assess the baseline characteristics, such as sex, presence of RF, age, and histological markers to predict radiological progression, we used a generalised linear regression model with binary family and log link. Relative risk is for the immunohistological markers given per difference of 100 cells/mm^2. Each variable was included and considered significant when the two tailed p value was <0.05. Variables that were found to be significant were then entered jointly into a generalised linear regression model. Data were analysed using STAT version 7 for Windows.

RESULTS

Clinical features

Thirty six patients (15 (42%) men, 21 (58%) women), participated in the study; mean age at study entry was 64 years (range 28–86). The baseline biopsy was performed at a mean disease duration of 5 months (range 1–12). Follow up examination was performed after a mean of 58 months (range 38–72). Patients were divided into two groups based upon the radiological progression for each year of follow up: group I with minimal progression (n = 20; Larsen <2 points/year); group II with pronounced progression (n = 16; Larsen ≥ 2 points/year). During the follow up period, 7 patients (group I n = 5, group II n = 2) did not receive any DMARDs, 29 patients (group I n = 15, group II n = 14) received at least one DMARD. Of these 29 patients, 16 patients (group I n = 6, group II n = 10) received two or more, and of these 16 patients, 5 patients (group I n = 2, group II n = 3) received three or more DMARDs. None of the patients were treated with biological agents. Table 1 presents the clinical, laboratory, and radiological findings. Twenty one (58%) patients were RF positive and 15 patients (42%) RF negative. In keeping with previous studies,[33] half of the patients had radiological signs of joint damage at study entry (Larsen at study entry 4.4 (0.9) (mean (SEM)); three patients had no signs of damage at the end of follow up. On average, all measures of disease activity had improved at the time of the follow up evaluation. The mean (SEM) DAS score was reduced from 5.8 (0.2) at enrolment to 3.6 (0.3) at follow up ($p<0.0001$). Despite clinical improvement radiological deterioration was seen in almost all patients (Larsen score at follow up 19.2 (3.8); $p<0.0001$).

Table 1 Clinical data on the study patients with rheumatoid arthritis

	Baseline	Follow up
Group I (n = 20)		
Tender joint count	11 (1–26)	2 (0–18)
Swollen joint count	8 (1–24)	2 (0–13)
HAQ	2.0 (0–3.0)	0.3 (0–2.3)
DAS	6.0 (3.2–7.8)	2.7 (1.3–6.7)
CRP (mg/l)	58 (8–307)	5 (1–96)
ESR (mm/1st h)	69 (17–109)	12 (2–66)
Larsen score	2.0 (0–18)	7.0 (0–45)
Group II (n = 16)		
Tender joint count	11 (3–26)	6 (0–26)
Swollen joint count	8 (2–27)	4 (0–28)
HAQ	1.4 (0.5–2.8)	0.9 (0–2.6)
DAS	5.5 (4.6–7.4)	4.0 (1.7–7.4)
CRP (mg/l)	41 (3–80)	7 (1–34)
ESR (mm/1st h)	54 (14–120)	20 (8–60)
Larsen score	1.0 (0–17)	22 (12–99)

The data represent median (range). The mean follow up was 58 months (range 38–72).

Immunohistological findings in relation to progression of joint damage

Table 2 summarises the demographic, clinical, immunohistological, and radiological data. Generally, there was increased cellularity in the synovial tissue of patients with more severe progression of joint destruction. There were significantly more FLS in the synovium of patients with more destructive disease than in patients with less progression of radiological signs of joint damage (change in Larsen score <2 points/year: 158 (2–1473) FLS/mm^2 (median (range)); change in Larsen score ≥ 2 points/year: 441 (35–2405) FLS/mm^2; $p = 0.03$). Although the numbers of B cells, plasma cells, and granzyme B+ cells tended to be increased in group II, comparison by Mann-Whitney U test did not show statistical significance. Of interest, there was no clear cut difference in macrophage infiltration between the two patient groups.

Regression analysis was used to identify prognostic markers of joint damage, as described previously.[21 34] Regression analysis using a univariate model identified female sex (relative risk (RR) = 10.7; $p = 0.015$), FLS

Table 2 Demographic data, rheumatoid factor, Larsen score, and immunohistochemical analysis (cells/mm^2) at baseline. Data are divided according to radiological progression into two groups: group I (Larsen score <2 points per year of follow up) and group II (Larsen score ≥ 2 points/year of follow up)

	Group I (n = 20)	Group II (n = 16)
Sex (M/F)	14/6	1/15
Age, mean (SD)	66 (13)	62 (13)
Follow up (months)	47 (22–69)	66 (34–72)
Rheumatoid factor (+/−)	9/11	12/4
Larsen score	2.0 (0–18)	1.0 (0–17)
Macrophages		
Synovial sublining	2155 (103–5735)	2190 (192–6765)
Intimal lining layer	249 (71–671)	307 (8–1381)
Fibroblast-like synoviocytes	158 (2–1473)	441 (35–2405)
B cells	53 (0–2854)	182 (0–3456)
Plasma cells	187 (8–3013)	410 (1–1212)
T cells	469 (0–1733)	480 (137–2013)
Granzyme B+ cytotoxic cells	12 (0–63)	25 (0–346)

Data represent median (range) unless stated otherwise.

(RR = 1.7; p = 0.02), T cells (RR = 2.7; p = 0.013), and granzyme B+ cells (RR = 7.2; p = 0.003), as discriminators for unfavourable radiological outcome (group II, Larsen score ≥2 points per year).

In addition, a multivariate model identified female sex (RR = 78.2; p = 0.017), FLS (RR = 1.5; p = 0.013), and T cells (RR = 1.2; p = 0.015) as independent predictors of unfavourable radiological outcome (table 3).

Soluble granzyme B levels in relation to progression of joint damage

To provide more insight into the possible role of granzyme B+ cytotoxic cells, we measured soluble granzyme B (produced by these cells) in body fluids. Paired synovial fluid and serum samples were available from seven patients participating in the biopsy study. In three patients with minimal progression (group I) the median serum level was 362 µg/ml (range 160–357) and the median granzyme B level in synovial fluid was 253 µg/ml (30–308) (fig 2). In these patients granzyme B+ cytotoxic cells were absent in synovial tissue (median 0 cells/mm^2, range 0–1). In four patients with more severe radiological progression (group II), granzyme B serum levels were similar to those in group I (median 362 µg/ml, range 121–939), but granzyme B levels in synovial fluid were markedly increased (median 489 µg/ml, range 55–1337). Moreover, these patients had increased numbers of granzyme B+ cytotoxic cells in the synovium (19 cells/mm^2 (1–47)). Thus, although statistical analysis could not be performed owing to the small sample size, these data are consistent with the results from the larger biopsy study and support the notion that granzyme B+ cytotoxic cells are associated with progression of joint destruction.

DISCUSSION

We confirmed in 36 patients with recent onset RA (symptoms <1 year) that female sex is predictive for an unfavourable disease outcome after a mean follow up of 54 months. In the synovium retrieved in the first year of the disease we found that the presence of granzyme B+ cells, T cells, and FLS at baseline is associated with the severity of radiological deterioration over the follow up period.

Table 3 Regression analysis of the immunohistochemical analysis (cells/mm^2) at baseline. Patients were divided into two groups: group I (Larsen score <2 point per year of follow up) and group II (Larsen score ≥2 points per year of follow up)

Characteristics	RR	95% CI	p Value
Sex			
Male	1		
Female	10.7	1.6 to 72.6	0.015
Rheumatoid factor			
Negative	1		
Positive	2.1	0.9 to 5.5	0.103
Age	0.9	0.9 to 1.0	0.400
Macrophages			
Synovial sublining	1.0	1 to 1	0.997
Intimal lining layer	1.0	0.9 to1.3	0.282
B cells	1.0	1 to 1.1	0.283
Plasma cells	1.0	0.9 to 1.1	0.986
Fibroblast-like synoviocytes	1.7	1 to 2.7	0.020
T cells	2.7	1 to 4.4	0.013
Granzyme B+ cytotoxic cells	7.2	1.8 to 15.8	0.003
Final model			
Sex	78.2	2.2 to 2797.7	0.017
Fibroblast-like synoviocytes	1.5	1.1 to 2.0	0.013
T cells	1.2	1.0 to 1.5	0.015

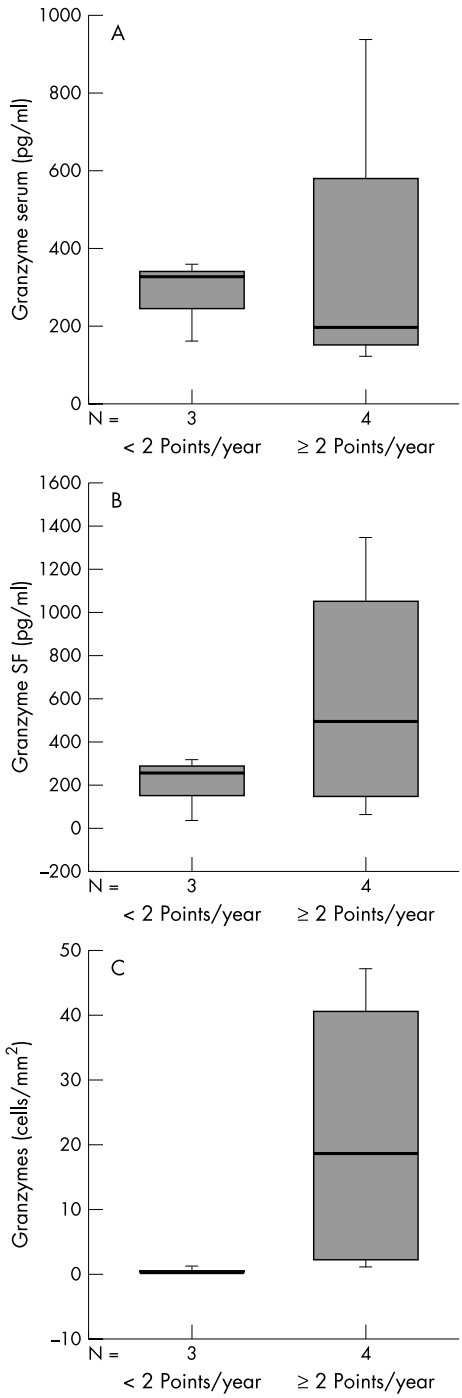

Figure 2 Box plots of the measurements in paired serum, synovial fluid, and synovial tissue of patients with early RA with more favourable (n = 3) and unfavourable (n = 4) radiological outcome. Depicted are (A) granzyme B in serum samples, (B) granzyme B in synovial fluid, and (C) granzyme B+ cells in synovial tissue.

The most challenging observation in this study is perhaps the relationship between the initial number of T cells and unfavourable disease outcome in RA. Until recently, the possible role of T cells in the pathogenesis of joint destruction has been controversial.[2] The recently proposed interaction between RANKL+ T cells and RANK+ osteoclasts[4 35 36] might provide the mechanism by which T cells promote erosive

disease. Consistent with this view, RANKL expression was seen predominantly in synovial tissue from patients with active RA, particularly within areas of lymphocyte infiltration.[6 37] In addition, the expression of OPG, which inhibits osteoclastogenesis by acting as a soluble decoy receptor for RANKL, is very low in the synovium of patients with RA.[37]

We also observed a relationship between the number of FLS in the initial synovial biopsy samples and joint destruction. Of interest, FLS also express RANKL and might thus activate osteoclasts by the same mechanism as that described above.[36] Previous work has shown that cultured rheumatoid FLS may efficiently induce osteoclastogenesis in association with up regulated expression of RANKL and decreased production of OPG.[38] In addition to stimulating osteoclasts, FLS might also directly promote joint degradation.[12 15] They serve as source of factors that mediate joint destruction: cytokines, metalloproteinases, serine proteinases, and cathepsins, together with various small molecules that increase vascular permeability and enhance the inflammatory response. It has been suggested that the interaction between FLS and macrophages through ligand pairs like CD97/CD55[39] and the expression of adhesion molecules such as VCAM-1 facilitating attachment to extracellular matrix[40] might be involved in the destructive process.

Granzyme B+ cytotoxic cells were the third cell type found to be associated with the development of destructive disease. Granzyme B is a serine protease that is stored in the granules of effector memory T cells, such as activated cytotoxic T cells and natural killer cells. The results from this study were supported by the association between increased expression of granzyme B+ cells (in the group with more destructive disease) and raised synovial fluid levels of extracellular granzyme B. Previously, we have shown that soluble granzyme levels are specifically increased in the serum and synovial fluid of patients with established RA compared with disease controls.[27] In addition, the number of granzyme B+ cells is specifically increased in rheumatoid synovial tissue.[31] The relationship between granzyme B and destruction might be explained by the extracellular activity of the enzyme when released from cytotoxic cells. It has been shown previously that granzyme B can degrade extracellular matrix.[41 42] Of interest, granzyme B+ cells were also shown to be present at the invasive front, the pannus-cartilage junction.[42] Further support for the role of granzyme B producing cells in the destructive process comes from the recent observation that granzyme B levels are highest in patients with early RF positive RA when compared with other arthritides, and this is independently predictive of the development of erosions.[43]

Previous work suggested a relationship between synovial tissue macrophages and an unfavourable course of RA.[10 44 45] We could not confirm these results. The present study is larger than previous studies and we selected patients with earlier disease at baseline. In addition, immunohistological markers for FLS and cytotoxic cells were included that were not previously studied, and investigated in a prospective study design. Moreover, we investigated the simultaneous associations of immunohistological variables with a specific outcome variable instead of analysing each variable separately, because these variable are highly correlated with each other. Therefore, we believe that the results shown here are valid. Of interest, the same approach did disclose a relationship between synovial macrophage infiltration and scores for local disease activity,[9] supporting the view that the pathogenesis of synovial inflammation and joint destruction might be partly different.[10]

Taken together, this prospective study shows a positive relationship between cell infiltration by T cells and FLS, on the one hand, and joint destruction, on the other, which might be explained in part by stimulation of osteoclasts. In addition, we found increased granzyme B+ cells in patients with more destructive disease; this might be explained by the role of granzyme B in degradation of extracellular matrix. Interference with these pathways may help to protect the joints against destruction.

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REFERENCES

1 **Van Zeben D**, Hazes JM, Zwinderman AH, Cats A, Schreuder GM, D'Amaro J, *et al.* Association of HLA-DR4 with a more progressive disease course in patients with rheumatoid arthritis. Results of a followup study. *Arthritis Rheum* 1991;**34**:822–30.
2 **Choy EH**, Panayi GS. Cytokine pathways and joint inflammation in rheumatoid arthritis. *N Engl J Med* 2001;**344**:907–16.
3 **Moreland LW**, Alten R, Van Den BF, Appelboom T, Leon M, Emery P, *et al.* Costimulatory blockade in patients with rheumatoid arthritis: a pilot, dose-finding, double-blind, placebo-controlled clinical trial evaluating CTLA-4Ig and LEA29Y eighty-five days after the first infusion. *Arthritis Rheum* 2002;**46**:1470–9.
4 **Bolon B**, Shalhoub V, Kostenuik PJ, Campagnuolo G, Morony S, Boyle WJ, *et al.* Osteoprotegerin, an endogenous antiosteoclast factor for protecting bone in rheumatoid arthritis. *Arthritis Rheum* 2002;**46**:3121–35.
5 **Goldring SR**, Gravallese EM. Pathogenesis of bone lesions in rheumatoid arthritis. *Curr Rheumatol Rep* 2002;**4**:226–31.
6 **Crotti TN**, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M, *et al.* Receptor activator NF-κB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. *Ann Rheum Dis* 2002;**61**:1047–54.
7 **Burmester GR**, Stuhlmuller B, Keyszer G, Kinne G. Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum* 1997;**40**:5–18.
8 **Kraan MC**, Versendaal H, Jonker M, Bresnihan B, Post W, 't Hart BA, *et al.* Asymptomatic synovitis precedes clinical manifest arthritis. *Arthritis Rheum* 1998;**41**:1481–8.
9 **Tak PP**, Smeets TJM, Daha MR, Kluin PM, Meijers KA, Brand R, *et al.* Analysis of the synovial cellular infiltrate in early rheumatoid synovial tissue in relation to disease activity. *Arthritis Rheum* 1997;**40**:217–25.
10 **Mulherin D**, FitzGerald O, Bresnihan B. Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. *Arthritis Rheum* 1996;**39**:115–24.
11 **Soden M**, Rooney M, Whelan A, Feighery C, Bresnihan B. Immunohistological analysis of the synovial membrane: search for predictors of the clinical course in rheumatoid arthritis. *Ann Rheum Dis* 1991;**50**:673–6.
12 **Firestein GS**. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? *Arthritis Rheum* 1996;**39**:1781–90.
13 **Pap T**, Muller-Ladner U, Gay RE, Gay S. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res* 2000;**2**:361–7.
14 **McCachren SS**. Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. *Arthritis Rheum* 1991;**34**:1085–93.
15 **Tak PP**, Bresnihan B. The pathogenesis and prevention of joint damage in rheumatoid arthritis. Advances from synovial biopsy and tissue analysis. *Arthritis Rheum* 2000;**43**:2619–33.
16 **Mellbye OJ**, Vartdal F, Pahle J, Mollnes TE. IgG and IgA subclass distribution of total immunoglobulin and rheumatoid factors in rheumatoid tissue plasma cells. *Scand J Rheumatol* 1990;**19**:333–40.
17 **Abrahams VM**, Cambridge G, Lydyard PM, Edwards JC. Induction of tumor necrosis factor alpha production by adhered human monocytes: a key role for Fcgamma receptor type IIIa in rheumatoid arthritis. *Arthritis Rheum* 2000;**43**:608–16.
18 **Boers M**, Verhoeven AC, Markusse HM, van de Laar MAFJ, Westhovens R, van Denderen C, *et al.* Randomised comparison of combined step-down prednisolone, methotrexate and sulphasalzine with sulphasalazine alone in early rheumatoid arthritis. *Lancet* 1997;**350**:309–18.

19 **van Leeuwen MA**, van Rijswijk MH, van der Heijde DM, Te Meerman GJ, van Riel PL, Houtman PM, *et al*. The acute-phase response in relation to radiographic progression in early rheumatoid arthritis: a prospective study during the first three years of the disease. *Br J Rheumatol* 1993;**32**(suppl 3):9–13.
20 **Van Zeben D**, Breedveld FC. Prognostic factors in rheumatoid arthritis. *J Rheumatol Suppl* 1996;**44**:31–3.
21 **Van Zeben D**, Hazes JM, Zwinderman AH, Cats A, van der Voort EA, Breedveld FC. Clinical significance of rheumatoid factors in early rheumatoid arthritis: results of a follow up study. *Ann Rheum Dis* 1992;**51**:1029–35.
22 **Van Zeben D**, Hazes JM, Zwinderman AH, Cats A, Schreuder GM, D'Amaro J, *et al*. Association of HLA-DR4 with a more progressive disease course in patients with rheumatoid arthritis. Results of a followup study. *Arthritis Rheum* 1991;**34**:822–30.
23 **Visser H**, le Cessie S, Vos K, Breedveld FC, Hazes JM. How to diagnose rheumatoid arthritis early: a prediction model for persistent (erosive) arthritis. *Arthritis Rheum* 2002;**46**:357–65.
24 **Arnett FC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1987;**31**:315–24.
25 **van der Heijde DM**, van't Hof MA, van Riel PL, van de Putte L. Disease activity score. *Ann Rheum Dis* 1992;**51**:140.
26 **Spaeny-Dekking EH**, Hanna WL, Wolbink AM, Wever PC, Kummer AJ, Swaak AJ, *et al*. Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol* 1998;**160**:3610–16.
27 **Tak PP**, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE. The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). *Clinical Exp Immunol* 1999;**116**:366–70.
28 **Pincus T**, Larsen A, Brooks RH, Kaye J, Nance EP, Callahan LF. Comparison of 3 quantitative measures of hand radiographs in patients with rheumatoid arthritis: Steinbrocker stage, Kaye modified Sharp score, and Larsen score. *J Rheumatol* 1997;**24**:2106–12.
29 **Dolhain RJEM**, ter Haar NT, de Kuiper R, Nieuwenhuis IG, Zwinderman AH, Breedveld FC, *et al*. Distribution of T cells and signs of T cell activation in the rheumatoid joint: implications for semiquantitative comparative histology. *Br J Rheumatol* 1998;**37**:324–30.
30 **Youssef PP**, Kraan MC, Breedveld FC, Bresnihan B, Cassidy N, Cunnane G, *et al*. Quantitative microscopic analysis of inflammation in rheumatoid arthritis synovial membrane samples selected at arthroscopy compared with samples obtained blindly by needle biopsy. *Arthritis Rheum* 1998;**41**:663–9.
31 **Tak PP**, Kummer JA, Hack CE, Daha MR, Smeets TJM, Erkelens GW, *et al*. Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue. *Arthritis Rheum* 1994;**37**:1735–43.
32 **Kraan MC**, Haringman JJ, Ahern MJ, Breedveld FC, Smith MD, Tak PP. Quantification of the cell infiltrate in synovial tissue by digital image analysis. *Rheumatology (Oxford)* 2000;**39**:43–9.
33 **van der Heijde DM**. Joint erosions and patients with early rheumatoid arthritis. *Br J Rheumatol* 1995;**34**:74–8.
34 **van Zeben DJ**, Hazes JM, Zwinderman AH, Vandenbroucke JP, Breedveld FC. Factors predicting outcome of rheumatoid arthritis: results of a followup study. *J Rheumatol* 1993;**20**:1288–96.
35 **Gravallese EM**, Manning C, Tsay A, Naito A, Pan C, Amento E, *et al*. Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum* 2000;**43**:250–8.
36 **Kotake S**, Udagawa N, Hakoda M, Mogi M, Yano K, Tsuda E, *et al*. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 2001;**44**:1003–12.
37 **Haynes DR**, Barg E, Crotti TN, Holding C, Weedon H, Atkins GJ, *et al*. Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls. *Rheumatology (Oxford)* 2003;**42**:123–34.
38 **Takayanagi H**, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, *et al*. Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum* 2000;**43**:259–69.
39 **Hamann J**, Wishaupt JO, Van Lier RAW, Smeets TJM, Breedveld FC, Tak PP. Expression of the activation antigen CD97 and its ligand CD55 in rheumatoid synovial tissue. *Arthritis Rheum* 1997;**42**:650–8.
40 **Muller-Ladner U**, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, *et al*. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. *Am J Pathol* 1996;**149**:1607–15.
41 **Froelich CJ**, Zhang X, Turbov J, Hudig D, Winkler U, Hanna WL. Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. *J Immunol* 1993;**151**:7161–71.
42 **Ronday HK**, van der Laan WH, Tak PP, De Roos JA, Bank RA, TeKoppele JM, *et al*. Human granzyme B mediates cartilage proteoglycan degradation and is expressed at the invasive front of the synovium in rheumatoid arthritis. *Rheumatology (Oxford)* 2001;**40**:55–61.
43 **Goldbach-Mansky R**, Suson S, Hoxworth J, Hack CE, Peters K, El-Gabalawi HS, *et al*. Elevated serum granzyme B levels are associated with erosions in patients with early rheumatoid arthritis. *Arthritis Rheum* 2000;**43**(suppl):S155.
44 **Mulherin D**, FitzGerald O, Bresnihan B. Clinical improvement and radiological deterioration in rheumatoid arthritis: evidence that the pathogenesis of synovial inflammation and articular erosion may differ. *Br J Rheumatol* 1996;**35**:1263–8.
45 **Yanni G**, Whelan A, Feighery C, Bresnihan B. Synovial tissue macrophages and joint erosion in rheumatoid arthritis. *Ann Rheum Dis* 1994;**53**:39–44.