EXTENDED REPORT

Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis

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Background: Previous work identified synovial sublining macrophage numbers as a potential biomarker for clinical efficacy in rheumatoid arthritis.

Objective: To investigate the association between changes in infiltration of synovial macrophages and clinical improvement after antirheumatic treatment.

Methods: 88 patients who participated in various clinical trials were studied. All patients underwent serial arthroscopy before initiation of treatment and after different time intervals. Immunohistochemical and digital image analysis were performed according to standardised procedures to detect changes in CD68+ synovial sublining macrophages in relationship to changes in the 28 joint count Disease Activity Score (DAS28). Statistical analysis was performed using one way analysis of variance, the independent samples *t* test, linear regression, and the standardised response mean (SRM).

Results: For good, moderate, and non-responders, according to the DAS28 response criteria, there was a significant difference in the change in sublining macrophages (mean (SEM) cells/mm^2 −643 (124), −270 (64), and −95 (60), respectively; $p < 0.0003$). There was a significant correlation between the change in the number of macrophages and the change in DAS28 (Pearson correlation 0.874, $p < 0.01$). The change in sublining macrophages explained 76% of the variation in the change in DAS28 ($p < 0.02$). The sensitivity to change of the biomarker was high in patients treated actively (SRM > 0.8), whereas the ability to detect changes in placebo treated patients was weak (SRM < 0.3).

Conclusion: The results suggest that changes in synovial sublining macrophages can be used to predict possible efficacy of antirheumatic treatment.

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The recent increase in the development of a variety of new, targeted treatments clearly raises the need for sensitive biomarkers, which could be used for selection during the development process. The acquisition of optimal tissue samples has been greatly enhanced by technological developments in needle arthroscopy.[1] Reliable microscopic quantification of synovial inflammation has been facilitated by advances in computerised image analysis. By these means, sequential synovial biopsy specimens have recently been analysed in several clinical trials evaluating the effects of disease modifying antirheumatic drugs (DMARDs), biological treatments, and targeted small molecules. These studies suggested consistent associations between the rapidity and magnitude of both the clinical and immunohistological responses. No consensus, however, has previously emerged as to the optimal markers in tissues that are representative of disease activity or sensitive to change.

A prior cross sectional study in 62 patients with rheumatoid arthritis (RA) using stepwise multiple regression analysis showed that scores for local disease activity are particularly associated with the number of macrophages in the synovial sublining as well as the expression of macrophage derived cytokines.[2] Recently, we conducted a randomised trial to try to answer the question of which feature in RA synovial tissue (ST) samples could be used as a biomarker for clinical efficacy in relatively small studies of short duration.[3] Patients received either prednisolone according to the COBRA regimen[4] or placebo for 2 weeks. ST samples were obtained before the start of treatment and at 2 weeks.

Twenty four immunohistological markers were investigated in this study. Each of the end points was statistically analysed using an analysis model of covariance. The model fitted included terms for treatment as a fixed effect and the baseline measurement as a covariate. The aim was to assess the treatment difference. This study identified sublining macrophages as the best biomarker associated with the clinical response to corticosteroids.[3]

The utility of CD68+ macrophages in the sublining layer as a candidate biomarker now requires to be tested across discrete interventions and kinetics. The objective of this study was to investigate the changes in this biomarker after different treatments and after different time intervals in relationship to the clinical response to treatment to validate the analysis of synovial macrophages in clinical studies.

PATIENTS AND METHODS
Patients
ST was obtained by arthroscopy under local anaesthesia at two different times from a clinically active wrist, knee, or ankle joint of each of 88 patients who fulfilled the American College of Rheumatology criteria for RA.[5] Before each arthroscopy the 28 joint count Disease Activity Score

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(DAS28) was calculated.[6] All patients had active disease before initiation of treatment, measured by a tender and swollen joint count of at least six joints in combination with a raised erythrocyte sedimentation rate and/or C reactive protein and/or significant morning stiffness. All patients were included in clinical trials and the complete patient group comprised seven subgroups receiving distinct treatment regimens.

Fifteen patients who were DMARD naïve for at least 28 days started treatment with methotrexate (MTX),[7] at 7.5 mg/week and increased stepwise to 15.0 mg/week in 12 weeks. Synovial biopsy specimens were taken before initiation of treatment and after 112 days of treatment from the same joint.

Fifteen patients who were DMARD naïve for at least 28 days started receiving leflunomide (LEF) after the first arthroscopy,[7] at a loading dose of 100 mg/day during the first 3 days and then 20 mg/day thereafter. Synovial biopsy specimens were obtained before initiation of treatment and after 112 days of treatment from the same joint.

Ten patients who were receiving stable DMARD treatment (MTX, sulfasalazine, hydroxychloroquine, LEF, or a combination of these drugs) for at least 28 days were given 2 weeks' oral treatment of prednisolone at 60 mg/day for 7 days followed by 40 mg/day for another 7 days.[3] Before treatment and at 2 weeks synovial biopsy specimens were taken from the same joint.

Infliximab treatment was started in 20 patients. These patients were receiving stable MTX for at least 28 days before infliximab treatment and stable prednisone was allowed at ≤10 mg/day. Patients were dosed with 3 mg/kg infliximab on day 1 (directly after the first arthroscopy) and on day 15.[8] Arthroscopies were performed before initiation of treatment and, on average, after 28 days.

Ten patients with active RA who were receiving stable DMARD treatment for at least 28 days were treated with a specific CCR1 antagonist.[9] Patients were allowed to use stable low dose prednisone ≤10 mg/day. They underwent arthroscopy directly before and after 14 days of treatment.

Some of the above mentioned clinical trials were placebo controlled studies.[3 8 9] Therefore this allowed us to include a control group of 18 patients who underwent serial synovial biopsies at different intervals while they were receiving stable DMARD treatment (mostly MTX). These patients received a placebo treatment as detailed in the referred studies.

Arthroscopy

Synovial tissue was obtained by arthroscopy under local anaesthesia in all patients at baseline (before treatment) and directly after completion of each treatment strategy. Arthroscopies, tissue sampling, and storage were performed according to standardised procedures, as described previously in detail.[7]

Immunohistochemistry

Synovial tissue sections were stained with a monoclonal mouse-antihuman anti-CD68 antibody (EBM11; Dako, Glostrup, Denmark). Sections with non-assessable tissue, defined by the absence of an intimal lining layer, were omitted before analysis. For control sections, the primary antibodies were omitted or irrelevant isotype matched mouse antibodies were applied. Staining was performed according to a three step immunoperoxidase method as previously described in detail.[10]

Digital image analysis

Stained slides were evaluated by computer assisted image analysis. All sections were coded and analysed in a random order by an independent observer who was unaware of the clinical data, as described previously.[11] For each patient the change in the number of total positive cells per square millimetre ST was calculated for sublining macrophages, and intimal macrophages were also evaluated.

Statistical analysis

Mean differences between independent groups were determined by one way analysis of variance and the independent samples t test; significant differences were confirmed by non-parametric tests. The association between clinical measurements and the immunohistochemical results was quantified using Pearson's correlation coefficient. The immunohistochemical factors with significant correlation coefficients ($p<0.05$) were entered into a linear regression analysis, testing for variables capable of explaining clinical response. The immunohistochemical markers were assigned as the independent variable. The probability of a significant association between the independent and dependent variables was defined as $p<0.05$.

Standardised response means (SRMs) of the clinical scores and immunohistochemical scores were calculated to evaluate the ability of these measurements to detect changes over time in the different study groups. The SRM is the mean change in a score in a defined period of time divided by the standard deviation of the change in that score. An SRM of >0.8 is considered high, representing a change of at least four fifths of a standard deviation of the change in the score. An SRM of 0.5 is defined as having a moderate potential to detect changes, and an SRM of 0.2 as having low potential.[12]

RESULTS
Clinical features

Eighty eight patients were included and analysed in this study. Table 1 shows their baseline disease activity and treatment characteristics. All patients had active disease at baseline as measured by the DAS28 (mean (SEM) 6.02 (0.11)). On average, there were no differences in age, sex, and disease duration between the different subgroups; for further demographic and clinical characteristics we refer to

Table 1 Treatment characteristics and baseline disease activity

Treatment	Active/control group	Number of patients	Interval (days)	DAS28 baseline Mean (range)
Methotrexate	Active	15	112	6.15 (4.50–7.83)
Leflunomide	Active	15	112	6.05 (4.38–7.31)
Prednisone	Active	10	14	6.26 (4.58–7.98)
Infliximab	Active	20	27	6.07 (4.14–8.21)
CCR1 antagonist	Active	10	14	5.84 (4.31–6.95)
Stable MTX	Control	6	2	5.97 (4.22–8.21)
Stable DMARD	Control	12	14	5.69 (3.43–7.59)
Overall		88	53 (5)*	6.02 (0.11)*

*Mean (SEM).

Table 2 Mean (SEM) change in DAS28, mean (SEM) change in the number of intimal lining CD68+ macrophages, mean (SEM) change in the number of sublining CD68+ macrophages, and the mean percentage change in sublining CD68+ macrophages compared with baseline for each substudy

Treatment (No; interval)	Change in the number of intimal lining CD68+ macrophages Mean (SEM)	Change in the number of sublining CD68+ macrophages Mean (SEM)	Change for sublining CD68+ macrophages Mean %	Change in DAS28 Mean (SEM)
Prednisolone start (10; 14)	−35 (46)	−492 (89)	−51	−2.15 (0.40)
Infliximab (Remicade) (20; 28)	−100 (30)	−275 (84)	−47	−1.39 (0.28)
Leflunomide start (15; 112)	−28 (17)	−286 (112)	−38	−1.29 (0.31)
Methotrexate start (15; 112)	−44 (17)	−292 (106)	−41	−1.36 (0.25)
CCR1 antagonist (10; 14)	−192 (81)	−338 (131)	−44	−0.72 (0.27)
Stable DMARD (12; 14)	+40 (38)	+152 (107)	+ 13	−0.23 (0.10)
Stable MTX (6; 2)	−71 (29)	+26 (158)	+ 22	+0.07 (0.28)
Active treatment grouped (70; 53)	−76 (17)	−321 (46)	−44	−1.37 (0.14)
Controls grouped (18; 10)	+3 (29)	+110 (87)	+ 17	−0.13 (0.12)

the published data of all subgroups.[3] [7–9] The 70 patients who started active treatment showed, on average, a significant change in the DAS28 of −1.37 (0.14) (mean (SEM)) after treatment (p<0.001), whereas the DAS28 remained the same in the 18 control patients (change in DAS28 −0.13 (0.12); table 2).

The number of good, moderate, and non-responders was calculated for all patients according to the DAS28 response criteria.[13] In total, 11 patients fulfilled the criteria for good responders (that is, improvement >1.2 and DAS28 at end point ⩽3.2), 35 patients were moderate responders (that is, improvement >0.6 and ⩽1.2 and DAS28 at end point ⩽5.1, or improvement >1.2 and DAS28 at end point >3.2), and 42 patients were considered non-responders.

Changes in sublining macrophages are associated with clinical improvement

Table 2 shows the changes in macrophage numbers in the intimal lining layer and sublining as well as the percentage changes in sublining macrophages compared with baseline, in relationship to changes in the DAS28 for the various trials. There was a significant difference in the mean (SEM) change in the number of sublining macrophages between good responders (−643 (124)), moderate responders (−270 (64)), and non-responders (−95 (60)) (one way analysis of

variance, p<0.0003; between group differences were determined by t tests and confirmed by non-parametric tests). The intimal macrophages tended to be decreased in all groups. There were no significant differences in the mean change from baseline between the three response groups for the number of intimal macrophages (fig 1).

Of interest, the mean change in the number of sublining macrophages for each substudy was highly correlated with the mean change in DAS28, according to the two tailed Pearson correlation coefficient (Pearson correlation 0.874, p<0.01) (fig 2). There were no correlations between the change in intimal macrophages and the change in DAS28 (data not shown). Linear regression analysis, weighted for the number of patients in each substudy, showed that the mean change in sublining macrophages could significantly explain 76% of the variance in the mean change in DAS28 grouped for each substudy (p<0.02).

Changes in sublining macrophages may predict active treatment

To determine the sensitivity to change, SRMs of the changes after treatment were calculated. Figure 3 shows that the SRMs in individual substudies were high for both sublining macrophages and DAS28 after active treatment. When patients from all active treatment substudies were grouped
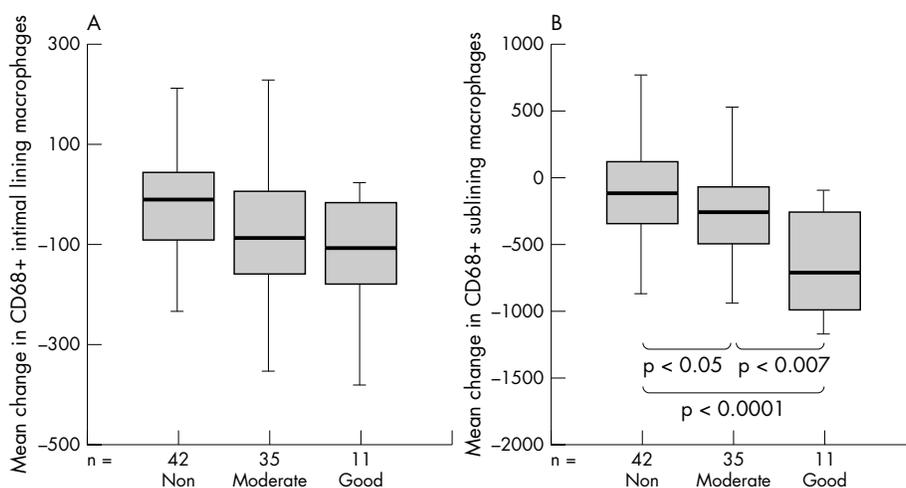

Figure 1 Mean values of the change compared with baseline in (A) the number of CD68+ macrophages in the intimal lining layer and (B) the synovial sublining for, respectively, non-responders, moderate responders, and good responders according to the DAS28 response criteria in the total study group.
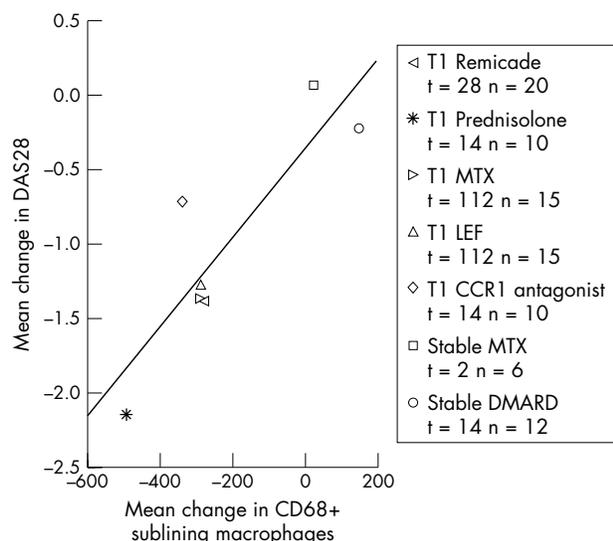
Figure 2 Correlation between the mean change for each substudy in the number of CD68+ sublining macrophages and the mean change in DAS28 (p<0.01, Pearson correlation 0.874, weighted linear regression p<0.02, R^2 =0.755, 95% confidence interval (95% CI) 0.001 to 0.005). t, interval between the first and second measurement, n, number of patients; TI, treatment initiation.

Figure 3 Standardised response mean (SRM) of the change in DAS28 (SRM DAS28) and the number of CD68+ macrophages in the sublining (SRM CD68sl) for the individual studies (t, interval between the first and second measurement; n, number of patients, TI, treatment initiation) and grouped for patients who received initiation of an active compound (n = 70) and placebo (n = 18). The solid line indicates the 0.5 SRM cut off point (moderate), the dotted line indicates the 0.8 SRM cut off point (high).

(n = 70), the SRMs of the change in both the DAS28 and sublining macrophages were high (1.16 and 0.83, respectively). In the control group where the second biopsy was performed after 2 days (n = 6) the SRM for both DAS28 and sublining macrophages was close to zero (0.10 and 0.07, respectively). In the control group where the second biopsy was performed after 2 weeks (n = 12), the SRM for the DAS28 was −0.64 and the SRM for sublining macrophages 0.40. When both control groups were grouped the SRM for the change in DAS28 was −0.23 and the SRM for the change in sublining macrophages was 0.30, consistent with the notion that the biological marker is less susceptible to placebo effects or expectation bias than clinical evaluation.

DISCUSSION

The results of this study show that changes in numbers of synovial sublining macrophages correlate with clinical improvement independently of the therapeutic strategy. Furthermore, this study demonstrates that the change in the numbers of sublining macrophages may be used to explain clinical outcome. Of importance, the data indicate that the change in the number of sublining macrophages could be used as a sensitive biomarker to predict possible efficacy of a new antirheumatic treatment.

Previous work suggested an association between the number of synovial macrophages and joint destruction in RA.[14] Moreover, analysis of the synovial cell infiltrate demonstrated a positive correlation between scores for local disease activity and the number of macrophages as well as expression of macrophage derived cytokines (tumour necrosis factor α (TNFα) and interleukin (IL) 6) in rheumatoid ST, suggesting that macrophage numbers are associated with clinical signs of inflammation.[2] In keeping with this concept, macrophage numbers are increased in clinically affected joints compared with clinically non-affected joints of patients with RA.[15] It appears likely that various pathogenetic mechanisms might lead to a final common pathway resulting in synovial macrophage activation. This may cause increased production of a variety of proinflammatory cytokines and chemokines, which play a part in the development of
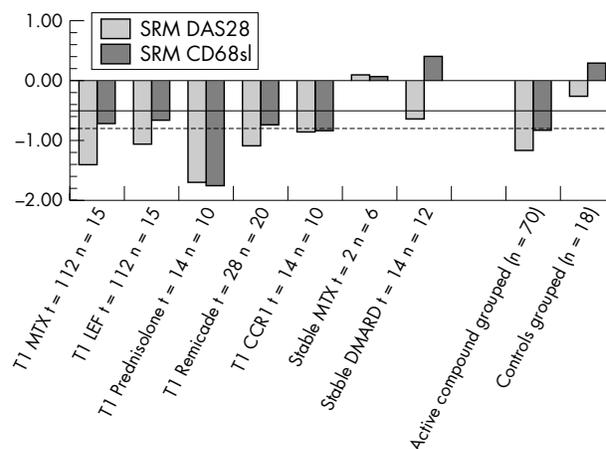
symptoms like pain and swelling.[16–18] The importance of macrophage derived cytokines for the clinical expression of the disease is underlined by the beneficial effect of strategies aimed at targeting TNFα, IL1, and IL6.[19–21] Of note, most of the macrophages in actively inflamed joints are localised by far in the synovial sublining rather than in the intimal lining layer.[22]

The results presented here are consistent with previous studies examining the effects of treatment with DMARDs and biological agents at the site of inflammation. It has been shown that various effective agents like gold, sulfasalazine, MTX, and LEF can reduce macrophage infiltration in RA ST.[7 23–25] Moreover, a predominant reduction in macrophage numbers of the synovial membrane has been reported in patients who achieved clinical remission as defined by the American College of Rheumatology criteria, induced by the use of various DMARDs.[26] Similarly, treatment with anti-TNFα antibodies and the IL1 receptor antagonist reduced macrophage numbers.[8 27 28] The same results were obtained using a specific CCR1 antagonist.[9]

It should be noted that inflammatory cells other than macrophages may also be reduced after antirheumatic treatment, depending on the specific mechanism of action and the duration of treatment. Obviously, these specific cell types and their mediators of inflammation also need to be evaluated in studies focusing on the mechanism of action of targeted treatments. The immunohistological variables may correlate with each other to a certain extent. Recently, we conducted a study to provide a greater understanding of the changes in the ST alongside clinical response by using a known clinically effective treatment, prednisolone.[3] The analysis model of covariance showed that clinically effective prednisolone treatment was particularly associated with a marked reduction in macrophage infiltration in the RA ST after 2 weeks of treatment. Comparable results were obtained after infliximab treatment.[8]

As a result of these observations, we investigated whether this biomarker might exhibit similar changes after different therapeutic regimens and after varying lengths of treatment. The results of this study show that this is the case. There is a highly significant correlation between changes in sublining macrophages and clinical improvement, independent of the specific treatments studied. The changes may be observed as

early as 14 days after initiation of effective treatment, but treatment for more prolonged periods results in a more pronounced decrease in macrophage infiltration. Patients who are good, moderate, or non-responders according to the DAS28 response criteria differ significantly in the changes in the number of sublining macrophages. Additionally, it is possible to explain the change in DAS28 based upon the change in sublining macrophages, which implies a direct relationship between macrophages and clinical measures of disease activity.

Moreover, we investigated the sensitivity to change of this biomarker after active treatment or placebo. According to the SRM, the sensitivity to change after active treatment is good for both the DAS28 and sublining macrophages. The SRMs calculated for changes in DAS28 and sublining macrophages after placebo treatment suggest that the biological marker may be less susceptible to placebo effects or expectation bias than clinical evaluation. This might be explained by the subjective components included in clinical measures of disease activity.[29] This notion is supported by a previous study in an independent patient cohort using semiquantitative analysis, showing unaltered immunohistological scores in serial synovial biopsy specimens obtained after placebo treatment.[30]

In conclusion, the results of this study indicate that synovial sublining macrophages might be used as a biomarker for the evaluation of new antirheumatic treatments. In addition to providing insight into the mechanism of action of treatment, this approach may help to screen for possible efficacy.

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