Rheumatoid arthritis: predictors of clinical response to TNF blockade
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CHAPTER 3

The Relationship Between Synovial Lymphocyte Aggregates and the Clinical Response to Infliximab in Rheumatoid Arthritis: A Prospective Study

Ruth Klaasen*, Rogier M. Thurlings*, Carla A. Wijbrandts, Arno W. van Kuijk, Dominique Baeten, Daniëlle M. Gerlag, and Paul P. Tak

* Both authors contributed equally to this manuscript

Division of Clinical Immunology and Rheumatology, Academic Medical Center/University of Amsterdam, Amsterdam, the Netherlands

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ABSTRACT

Objective: Some patients with rheumatoid arthritis (RA) exhibit lymphocyte aggregates in the synovium. This study was undertaken to address whether the presence of lymphocyte aggregates before treatment could serve as a biomarker for the clinical response to tumor necrosis factor (TNF) blockade, and to confirm whether the aggregation of synovial lymphocytes is reversible after anti-TNF treatment.

Methods: Synovial tissue biopsy samples were obtained from 97 patients with active RA before the initiation of infliximab treatment. Lymphocyte aggregates in the synovial tissue were counted and also graded for size. Logistic regression analysis was performed to identify whether the presence of lymphocyte aggregates could be a predictor of the clinical response at week 16. Furthermore, the effects of TNF blockade on lymphocyte aggregates were compared between patients with RA and patients with psoriatic arthritis (PsA).

Results: Fifty-seven percent of RA synovial tissue samples contained lymphocyte aggregates, and 32% of the patients had large aggregates. Aggregates were found in 67% of clinical responders compared with 38% of nonresponders. The presence of aggregates at baseline was a highly significant predictor of the clinical response to anti-TNF treatment ($R^2 = 0.10, P = 0.008$). Positivity for lymphocyte aggregates increased the power to predict the clinical response ($R^2 = 0.29$), when analyzed in a prediction model that included baseline disease activity evaluated by the Disease Activity Score in 28 joints, anti–cyclic citrullinated peptide antibody positivity, and synovial TNFα expression. There was a reduction in lymphocyte aggregates after anti-TNF antibody therapy in both RA and PsA.

Conclusion: RA patients with synovial lymphocyte aggregates have, on average, a better response to infliximab treatment than those with only diffuse leukocyte infiltration. Moreover, the aggregation of synovial lymphocytes is reversible after anti-TNF antibody treatment.
Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease of unknown etiology that affects the synovial tissue in multiple joints. Variability in both the cellular features and molecular features of the inflamed synovium, as well as the heterogeneous response to treatment, suggest that RA is a clinical syndrome comprising different pathogenetic subsets. Both the extent and the pattern of synovial lymphocyte infiltration are remarkably variable among different individuals with RA. In some tissues, a diffuse or scarce infiltration of T cells is present, while in others, B and T cells are organized in lymphocyte aggregates that may exhibit germinal center–like features.

It has been proposed that lymphocyte aggregates may be involved in the autoreactive humoral response observed in a subset of RA patients, comprising those who are positive for anti–cyclic citrullinated peptide antibodies (anti-CCP) and/or positive for rheumatoid factor (RF), resulting in amplification and refinement of local autoantibody production. Conversely, other studies have suggested that the presence of lymphocyte aggregates is not directly related to a local germinal center–like humoral response, but rather could be attributed to a phenomenon secondary to the chronic inflammatory processes driving RA. Thus, the role of synovial lymphocyte aggregates in the pathogenesis of RA is still controversial. It is, at present, also unclear whether there is a differential response to treatment between RA patients with synovial lymphocyte aggregates and those without synovial lymphocyte aggregates.

We and other investigators in our group have recently shown that the clinical response to anti–tumor necrosis factor (anti-TNF) therapy in RA is related to the synovial tissue inflammation levels prior to treatment, providing proof of concept that synovial biomarkers may be used to predict the response to treatment.

The objective of this study was to investigate the relationship between synovial lymphocyte aggregates and the response to anti-TNF therapy. Therefore, in a prospective study, we evaluated 97 patients with active RA starting infliximab treatment. Arthroscopic synovial biopsy samples were obtained before treatment, and the presence of synovial lymphocyte aggregates at baseline was assessed for any association with the clinical response at week 16. In addition, we obtained serial synovial biopsy samples before and after treatment in a subset of the RA patients, to confirm the previously reported effects of TNF blockade on synovial lymphocyte aggregates. To assess whether changes in synovial lymphocyte aggregates after TNF-blocking therapy are specific to rheumatoid synovial tissue, we also analyzed such changes after adalimumab treatment in a small cohort of patients with psoriatic arthritis (PsA).

PATIENTS AND METHODS

Patients
To examine the relationship between the presence of synovial lymphocyte aggregates and the response to anti-TNF treatment, we obtained synovial tissue samples from 97 patients with RA before initiation of infliximab treatment. The baseline features of the larger cohort, including the presence of lymphocyte aggregates, have been described previously. Patients were selected for the present analysis based on the availability of evaluable synovial tissue at baseline, combined with standardized follow up data on the response to infliximab treatment. The relationship between baseline synovial TNFα expression and clinical response in these patients has been reported previously.
All patients were being treated with stable dosages of methotrexate (5–30 mg/week) and had never taken biologic agents. In addition, all patients had active disease, defined by a Disease Activity Score in 28 joints (DAS28) of ≥ 3.2. Use of oral corticosteroids (≤ 10 mg/day) and non-steroidal anti-inflammatory drugs was allowed if the dosage had not been changed within 1 month prior to baseline. Intra-articular steroid injections within the month prior to baseline were not allowed.

Disease characteristics and the presence of IgM-RF and anti-CCP (as measured by the second-generation anti-CCP enzyme-linked immunosorbent assay; Immunoscan RA [Mark 2], NO.RA-96RT from Eurodiagnostica, Arnhem, The Netherlands) were assessed at baseline. All patients were administered intravenous infusions of infliximab in a dose of 3 mg/kg at baseline and at weeks 2 and 6, and subsequently one every 8 weeks. We determined the responder status by evaluating the reduction in the DAS28 after 16 weeks of therapy. RA patients with a reduction in the DAS28 of at least 1.2 (twice the measurement error of the DAS28 over time) were defined as responders, representing a clinically significant improvement. The clinical response was also determined according to the European League Against Rheumatism (EULAR) response criteria.

To investigate the effect of anti-TNF therapy on synovial lymphocyte aggregates, we analyzed serial synovial tissue samples from 15 patients who underwent arthroscopy in the same joint before treatment and 28 days after treatment; in 10 patients, we also performed arthroscopy 48 hours after the first infliximab treatment. To determine whether the synovial tissue response to anti-TNF antibody therapy is specific to RA or whether it is a more general phenomenon, we analyzed serial synovial biopsy samples from a second cohort, comprising 9 patients with active PsA who underwent arthroscopy and had never taken biologic agents. These patients were evaluated at baseline and at 28 days after the start of treatment with adalimumab (40 mg every other week). The clinical response in patients with PsA was defined by a decrease in the DAS28 score of at least 1.2 at 12 weeks.

All patients gave their written informed consent to participate. The study was approved by the Medical Ethics Committee of the Academic Medical Center at the University of Amsterdam.

Synovial biopsy and assessment of lymphocyte aggregates
All patients, under local anesthesia, underwent a mini arthroscopy of an actively inflamed knee, wrist, or ankle, as described previously in detail, and samples of the synovial tissue were studied. The following monoclonal antibodies were used to analyze the lymphocytic cell infiltrate: anti-CD3 (SK7; Becton Dickinson, San Jose, CA) to detect T cells, and anti-CD22 (CLB-B-ly/1, 6B11; Sanquin Research, Amsterdam, 3218, The Netherlands) to detect B cells. Anti-CD21–long isoform (anti-CD21L; a kind gift from Dr. Y. J. Liu, M. D. Anderson Cancer Center, Houston, TX) was used for detection of follicular dendritic cells (FDCs). Staining of cellular and cytokine markers was performed as described previously. The presence of lymphocyte aggregates was assessed on anti-CD3–stained sections. The presence of FDCs in lymphocyte aggregates was assessed at 3 different levels of the tissue. The size and number of lymphocyte aggregates and the presence of T or B cell aggregation were assessed at 2 different levels of the tissue, at least 50 µm apart, on sequential sections stained with CD3 and CD22. Thus, multiple sections representing different levels of a tissue block, consisting of at least 6 biopsy specimens, were examined to further minimize sampling error.
Aggregates were counted and graded on a 4-point scale (range 0–3) according to the number of cells in their diameter, as described previously\(^\text{19}\). We calculated the total number of aggregates per section and the mean aggregate diameter per section. Grade 2 and grade 3 aggregates were termed large lymphocyte aggregates, while grade 1 aggregates were termed small lymphocyte aggregates. Germinal center–like structures were defined as lymphocyte aggregates containing FDCs.

**Statistical analysis**

The primary analysis was focused on the comparison of the clinical response between patients with either small or large aggregates and patients without aggregates. Furthermore, we analyzed separately whether the presence of germinal center–like structures is related to the clinical response to anti-TNF antibody therapy. The chi-square test was used to compare patient characteristics between those patients with diffuse synovitis and those patients with lymphocyte aggregates. For comparison of continuous variables, we used the t-test or, if the data were skewed, the Mann-Whitney U test. To examine the relationship between clinical features and synovial parameters and the clinical response to anti-TNF treatment, we performed univariate Cox logistic regression, the Kruskal-Wallis test with the post hoc Games-Howell test, and linear regression analysis, as appropriate.

To assess whether the presence of lymphocyte aggregates increased the predictive power to determine an association with clinical response to infliximab in a combined prediction model, we analyzed a multivariable prediction model that consisted of TNF\(\alpha\) expression in the synovial lining at baseline, positivity for anti-CCP, and the DAS28 at baseline. We performed stepwise forward and backward multivariable logistic regression analyses to obtain estimates of the odds ratios, with outcome measures expressed as the natural log of the regression coefficient (eB). Collinearity diagnostics were performed to analyze the presence of multicollinearity. Hosmer and Lemeshow tests were performed to assess the goodness of-fit. Wilcoxon’s signed rank test was used for analysis of the lymphocyte aggregates in paired biopsy specimens. SPSS version 16.0 for Windows (SPSS, Chicago, IL) was used for all statistical analyses.

**RESULTS**

**Characteristics of the RA patients at baseline**

Ninety-seven RA patients were analyzed. Demographic and clinical features are shown in Table 1. All patients used methotrexate. Oral low-dose corticosteroids were used by 27% of the patients. On average, patients failed treatment with 2.1 disease-modifying anti-rheumatic drugs (DMARDs) prior to inclusion in the study.

**Clinical improvement after infliximab treatment**

Sixteen weeks after initiation of treatment with infliximab, the mean ± SD DAS28 decreased from 5.9 ± 1.1 to 4.2 ± 1.3 (P < 0.0001); the mean ± SD change in the DAS28 was 1.7 ± 1.3. Sixty-three of the 97 RA patients (65%) experienced a decrease in the DAS28 of ±1.2. Twenty-two patients (23%) had a good response to infliximab treatment according to the EULAR response criteria, 51 patients (53%) had a moderate response according to the EULAR response criteria, and 24 patients (25%) did not fulfill the EULAR response criteria.
Association of the presence of synovial lymphocyte aggregates with the acute-phase response

Of the 97 patients with RA, 42 (43%) had diffuse synovial inflammation, 24 (25%) had small lymphocyte aggregates, and 31 (32%) had large lymphocyte aggregates. The synovial tissue of patients with large aggregates contained significantly more small aggregates compared with those with only small aggregates or those with only diffuse synovitis (P = 0.019). Disease duration and use of corticosteroids were not related to the presence of synovial lymphocyte aggregates.

Of the 97 synovial biopsy samples, 89 could be evaluated by staining for the presence of FDCs. Seven samples (8%) showed CD21L-positive staining, which was observed within large aggregates in all 7 samples. Separate clusters of T cells and B cells were found in 7 of the 31 patients with large aggregates, but separate clusters were not detected in small aggregates. Patients with aggregates had a higher erythrocyte sedimentation rate and higher C-reactive protein level compared with those without aggregates (P = 0.005 and P < 0.05, respectively). As reported previously, the presence of circulating auto-antibodies was not related to the presence of synovial lymphocyte aggregates.

Table 1. Except where indicated otherwise, values are the mean ± SD. Anti-CCP = anti–cyclic citrullinated peptide antibody; DAS28 = Disease Activity Score in 28 joints; ESR = erythrocyte sedimentation rate; DMARDs = disease-modifying antirheumatic drugs; NSAIDs = nonsteroidal antiinflammatory drugs.

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<th>Characteristics of patients with rheumatoid arthritis (n=97)</th>
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Predictive power of the presence of synovial lymphocyte aggregates at baseline in relation to the clinical response to infliximab treatment in RA

Aggregates were present in 42 (67%) of 63 clinical responders (defined as a decrease in the DAS28 of ≥1.2) and in 13 (38%) of 34 non-responders (P = 0.007 between groups). Univariate Cox logistic regression analysis confirmed that the presence of lymphocyte aggregates at baseline was related to the clinical response (R² = 0.10, P = 0.008). The positive predictive value was 76% and the negative predictive value was 50%. Subsequently, we analyzed the patients for clinical response to treatment according to the EULAR response criteria. Aggregates were present in 16 (73%) of the 22 EULAR good responders, in 30 (59%) of the 51 EULAR moderate responders, and in 9 (38%) of the 24 patients who did not respond to infliximab treatment according to the EULAR response criteria (Figure 1A). Kruskal-Wallis analysis and a post hoc test (Games-Howell test) showed that lymphocyte aggregates were significantly more often present in good responders as compared with non-responders (95% confidence interval [95% CI] -0.69, -0.01; P = 0.041) (Figure 1A). Furthermore, patients were analyzed for treatment response according to the absolute decrease in the DAS28. Univariate linear regression analysis showed that the presence of lymphocyte aggregates was predictive of the absolute decrease in the DAS28 (R² = 0.041, P = 0.026) (Figure 1B). Separate analyses of only those synovial tissue samples with large aggregates suggested a relationship with clinical response, but the relationship with aggregate size did not reach statistical significance when the clinical response was analyzed as categories of improvement according to the EULAR criteria (P = 0.52) (Figure 1C) or dichotomously as a
decrease in the DAS28 of ≥1.2 ($R^2 = 0.025, P = 0.19$). However, the presence of large lymphocyte aggregates was a significant predictor of the clinical response when the response was analyzed as the absolute decrease in the DAS28 ($R^2 = 0.033, P = 0.041$) (Figure 1D). The presence of FDCs in large aggregates, as determined by the expression of CD21L, was not predictive of the clinical response; these were present in only 7 of 97 biopsy samples. Taken together, our findings indicate that the presence of lymphocyte aggregates (defined as one group with either small or large aggregates) is a significant predictor of the response to infliximab treatment in RA.

**Contribution of lymphocyte aggregates to a combined model for the prediction of clinical response to infliximab**

In a recent study in which the patients from the current study were included, we identified synovial TNFα expression, the baseline DAS28, and the presence of anti-CCP antibodies as predictors of the clinical response to infliximab 12. The dichotomy of the DAS28, when applied as a measure of clinical response using a decrease of ≥1.2 to define improvement, was chosen because it is used in daily clinical practice and is required for prolongation of reimbursement for TNF blocking therapy by insurance companies in The Netherlands. Analysis of this combined prediction model in our previous study showed that the model was modestly predictive of the clinical response to infliximab at week 16 ($R^2 = 0.19$) (with inclusion of baseline TNFα expression, $P = 0.009$, eB 1.1, 95% CI 1.1, 1.5; with inclusion of anti-CCP positivity, $P = 0.044$, eB 2.8, 95% CI 1.0, 9.2; and with inclusion of baseline DAS28, $P = 0.040$, eB 1.6, 95% CI 1.0, 2.6). The results from our current study showed that the addition of lymphocyte aggregates as a variable into this model improved the prediction of the clinical response to infliximab ($R^2 = 0.29$, by forward stepwise method). Collinearity diagnostics showed that all variables had a tolerance of > 0.95 and a variance inflation factor close to 1, indicating that no significant multi collinearity had occurred. The variables were included in the following order in the prediction model: inclusion of baseline TNFα expression ($P = 0.016$, eB 1.2, 95% CI 1.0, 1.4), lymphocyte aggregates ($P = 0.023$, eB 3.3, 95% CI 1.2, 9.4), anti-CCP positivity ($P = 0.019$, eB 4.0, 95% CI 1.36, 12.8), and, finally, baseline DAS28 ($P = 0.048$, eB 1.6, 95% CI 1.0, 2.7). The positive predictive value of the model was 85% and the negative predictive value was 53%. A backward stepwise method yielded the same results.

**Decrease in synovial lymphocyte aggregates after TNF blockade in both RA and PsA**

Serial synovial tissue samples were obtained from 15 RA patients before the initiation of infliximab treatment and on day 28 after treatment. In 9 of the 15 patients, lymphocyte aggregates were present at baseline. After 28 days, the number of aggregates decreased in 6 of these 9 aggregate-positive patients (Figure 2A) and the size of the aggregates decreased in 7 patients, whereas we observed an increase in the number of aggregates in 3 patients (Figure 2A) and an increase in the size of the aggregates in 2 patients. These reductions in the number and size of the aggregates from baseline to day 28 did not reach statistical significance, possibly due to the relatively small number of patients as well as the variability in response. We also obtained synovial biopsy tissue from 9 of these patients at 48 hours after the first administration of infliximab, 6 of whom had lymphocyte aggregates at baseline. In 5 of the 6 aggregate-positive patients, there was a trend toward a decrease in the size and number of lymphocyte aggregates within 48 hours after the first infusion with infliximab (Figure 2B).
To determine whether the decrease in the number and size of the lymphocyte aggregates after treatment was specific to RA or was, perhaps, a more general phenomenon, we studied the synovial tissue response to adalimumab treatment in 9 patients with PsA. This patient cohort was compared with patients with PsA (n = 9) who received placebo. In 7 of the 9 patients treated with adalimumab, lymphocyte aggregates were present at the time of the baseline biopsy. In 6 of 7 patients, there was a decrease in the size and number of lymphocyte aggregates 28 days after the initiation of adalimumab treatment (P = 0.028 and P = 0.043, respectively, versus baseline) (Figure 2C). Because lymphocyte aggregates are found in both RA and PsA, and because they appear to decrease after anti-TNF therapy in both inflammatory forms of arthritis in a similar way, we subsequently pooled the data from all patients with inflammatory arthritis whose synovial tissue was positive for lymphocyte aggregates and compared the values before and after anti-TNF treatment, using the same time points, to get more statistical power. In these 16 patients, there was a significant decrease in the size and number of synovial lymphocyte aggregates 28 days after the start of anti-TNF therapy (P = 0.028 and P = 0.044, respectively, versus baseline) (results not shown and Figure 2D), consistent with the observations described in previous reports.

**DISCUSSION**

Since the clinical response to anti-TNF therapy is heterogeneous, there is a clear need for biomarkers that can identify different pathogenic subsets that are associated with the response.
to or lack of response to TNF-antagonist therapy. We have recently provided proof of concept to confirm that synovial biomarkers predictive of the response to anti-TNF therapy might be identified. In the present study, we investigated the relationship between the pretreatment presence of synovial lymphocyte aggregates and the primary clinical response to infliximab treatment, in a prospective study of a large cohort of well-characterized patients with RA. The results revealed a highly significant relationship between the presence of synovial lymphocyte aggregates at baseline and the primary clinical response defined at 16 weeks. When the presence of synovial lymphocyte aggregates was added into a combined prediction model with synovial TNFα expression, the DAS28 at baseline, and the presence of anti-CCP antibodies, the presence of lymphocyte aggregates increased the prediction of response from 19% to 29%.

Of interest, the aggregation of synovial lymphocytes was also shown to be reversible after anti-TNF antibody treatment, both in patients with RA and in patients with PsA.

Our findings appear, at first sight, to be in clear contrast with those from a previous study in which synovial lymphocyte aggregates were not identified as a predictor of the response to anti-TNF therapy. There are several differences between the 2 studies that may help to resolve the apparent discrepancy. First, in the other study, lymphocyte aggregate–positive patients were defined by the presence of large aggregates rather than the presence of either small and/or large aggregates. In our study, we did not find a statistically significant relationship between the presence of aggregates and the response to infliximab therapy (defined according to the EULAR criteria or defined dichotomously as a decrease in the DAS28 of ≥1.2) when only large aggregates were taken into account, although there was a trend toward significance. Second, in contrast to our study, the patient cohort in the other study was more heterogeneous, in that patients with early, untreated RA, along with DMARD inadequate responders and anti-TNF inadequate responders, were included. Those patients were also treated with a different DMARD background medication. During follow up, patients were sequentially treated with gold salts, methotrexate, leflunomide, or different TNF blockers depending on the DAS28. It is likely that the markedly large number of variables in that study may have made it difficult to detect the relationship between the presence of synovial lymphocyte aggregates and the response to anti-TNF therapy. Third, in the other study, the patients with synovial lymphocyte aggregates had a significantly longer disease duration compared with those without lymphocyte aggregates. Of importance, a high proportion of them had failed previous treatment with other TNF antagonists, and thus represented a therapy-resistant subgroup whose data were followed up in a subsequent study. Therefore, enrichment of the cohort of patients with synovial lymphocyte aggregates with the subset of patients who had previously failed TNF blockade may have been an important confounding factor. In our study, we only included patients who had failed treatment with at least 2 conventional DMARDs, including methotrexate; previous use of TNF antagonists was an exclusion criterion. Fourth, duration of followup after the initiation of anti-TNF therapy was variable in the other study, with a mean followup of 43 months. In contrast, we chose to select a fixed end point at 16 weeks to ascertain the primary response to infliximab treatment, since the secondary response defined at later time points could have been influenced by totally unrelated mechanisms, including the development of human anti-chimeric antibodies against infliximab or human anti-human antibodies against adalimumab.

The use of a very stringent study design allowed us to identify synovial lymphocyte aggregates as a highly significant predictor of the response to infliximab therapy. The
relationship observed between synovial lymphocyte aggregates and the response to anti-TNF therapy is consistent with previous circumstantial evidence that indicated 1) a correlation between synovial lymphocyte aggregates and synovial inflammation\(^9\),\(^10\), and 2) a relationship between synovial inflammation and the response to anti-TNF therapy\(^11\),\(^12\). When the presence of lymphocyte aggregates was added into a combined prediction model for the prediction of the clinical response to infliximab, it increased the explained variance of response to 29%.

Consistent with the clinical experience, in which it has been observed that the response to TNF blockade is not a dichotomous phenomenon\(^26\), there was no distinct threshold value for scores for lymphocyte aggregates in the synovium of patients with RA. Therefore, the predictive value of the presence of synovial lymphocyte aggregates alone or in combination with other tested predictors of response is statistically significant and of great scientific interest, but cannot be translated into a predictive test in individual patients.

The results presented herein also confirm that aggregation of synovial lymphocytes represents a reversible phenomenon after resolution of inflammation, which is consistent with previous observations in patients with RA\(^21\) and patients with PsA\(^9\),\(^20\), and supports the notion that lymphocyte aggregates are formed secondary to the inflammatory process. Previous studies have shown that TNF blockade results in decreased expression of cytokines\(^27\), chemokines\(^28\), and adhesion molecules\(^29\), which are all required for secondary lymphoid organ formation. The decrease in lymphocyte aggregates after anti-TNF treatment is consistent with the protective effect of TNF blockade on joint destruction, since previous work has shown that the presence of small lymphocyte aggregates is related to the development of bone erosions\(^30\),\(^31\).

Taken together, our findings reveal a highly significant relationship between the presence of synovial lymphocyte aggregates at baseline and the primary clinical response to anti-TNF antibody treatment, but the clinical response cannot be predicted completely, thus indicating the involvement of other, as yet unknown mechanisms. Future work should expand the search for other biomarkers and molecular networks (for instance, with the use of microarray analysis) as well as combinations of clinical variables, to achieve an effective approach that would increase the percentage of patients exhibiting a robust response to TNF blockade.

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