

The clinical response to infliximab in rheumatoid arthritis is in part dependent on pre-treatment TNF α expression in the synovium

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Abstract

Objective. To determine whether the heterogeneous clinical response to TNF α blocking therapy in rheumatoid arthritis (RA) can be predicted by TNF α expression in the synovium before initiation of treatment.

Methods. Prior to initiation of infliximab treatment, arthroscopic synovial tissue biopsies were obtained from 143 patients with active RA. At week 16 clinical response was evaluated using the Disease Activity Score (DAS28). Immunohistochemistry was used to analyze the cell infiltrate as well as the expression of various cytokines, adhesion molecules, and growth factors. Stained sections were evaluated by digital image analysis. T-test were used to compare responders (decrease in DAS28 \geq 1.2) with non-responders (decrease in DAS28 $<$ 1.2) and multivariable regression was used to identify the independent predictors of clinical response.

Results. Synovial tissue analysis confirmed our hypothesis that the baseline level of TNF α expression is a significant predictor of response to TNF α blocking therapy. TNF α expression in the intimal lining layer and synovial sublining were significantly higher in responders than in non-responders (P = 0.047 and P = 0.008, respectively). The numbers of macrophages, macrophage subsets, and T cells (all able to produce TNF α) were also significantly higher in responders than in non-responders. The expression of IL-1 β , IL-6, IL-18, IL-10, E-selectin, ICAM-1, VCAM-1, VEGF, and bFGF was not associated with response to anti-TNF α treatment.

Conclusion. The effects of TNF α blockade are in part dependent on synovial TNF α expression and infiltration by TNF α producing inflammatory cells. Clinical response cannot be predicted completely, indicating involvement of other as yet unknown mechanisms.

Tumor Necrosis Factor alpha (TNF α) blocking agents as treatment for rheumatoid arthritis (RA) were developed based on evidence that the pro-inflammatory cytokine TNF α plays an important role in the pathogenesis.[1] Some patients however do not clinically respond to TNF α blockade. At present no factors have been identified that fully explain or predict the differential response.

One explanation for the heterogeneous clinical response may be found in the baseline variability in TNF α expression among individual patients.[2, 3] Genetic studies have suggested that individuals predisposed to high TNF production could show worse responses to anti-TNF α therapy.[4, 5] In contrast, a recent study using an in vitro bioassay suggested that good responsiveness to anti-TNF therapy is associated with significantly higher TNF α bioactivity at baseline compared to non-responding patients.[6] Taken together, it remains to be determined which baseline cytokine profile distinguishes responding from non-responding patients in vivo. Another explanation for the diversity in response may be that inflammatory mediators other than TNF α drive different pathogenetic subsets of RA.

We hypothesized that the pre-treatment TNF α level in the synovium might be related to clinical efficacy, where TNF α blocking therapy could be most effective in patients with high pre-treatment TNF α levels, as previously suggested in a small pilot study.[7] In a prospective study we obtained arthroscopic synovial tissue samples from 143 RA patients prior to initiation of infliximab therapy. We examined the cell infiltrate as well as the expression of cytokines, adhesion molecules, and growth factors to identify predictors of clinical response.

PATIENTS AND METHODS

Patients. Consecutive patients with RA according to the ACR criteria were enrolled in the study. All failed at least two disease modifying anti-rheumatic drugs (DMARDs) including methotrexate (MTX), and had a disease activity score (DAS28) ≥ 3.2 when included in the study. Patients were on stable maximal tolerable MTX treatment (5-30 mg/week). Oral corticosteroids (≤ 10 mg/day) and non-steroidal anti-inflammatory drug (NSAIDs) were allowed if stable for at least one month prior to baseline. Concomitant medication was kept stable throughout the study. Previous use of a TNF blocking agent was an exclusion criterion. The Medical Ethics Committee of the Academic Medical Center, University of Amsterdam approved the protocol. All patients gave written informed consent.

Treatment and evaluation of clinical response. All patients were treated with infliximab according to the label for RA in a dosage of 3 mg/kg intravenously at baseline, week 2, 6, and subsequently every 8 weeks. The DAS28 was evaluated at baseline, week 4, 8, 12, and 16 by specially trained research nurses.

For the analysis the absolute change in DAS28 (delta DAS28) at week 16 was dichotomized and defined as non-response (delta DAS28 < 1.2) versus response (delta DAS28 ≥ 1.2). The dichotomy of the delta DAS28 (on average comparable with a 20% improvement in DAS28) was chosen because it is applied in daily clinical practice and required for prolongation of reimbursement for TNF α blocking therapy by insurance companies in the Netherlands. Response was evaluated at 16 weeks because a significant improvement is expected to occur within 3 to 4 months, after which alternative treatment should be considered.[8]

Arthroscopy and synovial biopsy. Before the first infliximab infusion patients underwent a mini-arthroscopy under local anesthesia to obtain synovial tissue samples from an actively inflamed knee, ankle, wrist or metacarpophalangeal joint.[9] Biopsies were taken with a 2-mm forceps (Storz, Tuttlingen, Germany) from 6 or more sites within the joint to minimize sampling error. Biopsies were immediately snap frozen en bloc in Tissue Tek OCT (Miles, Elkhart, IN) after collection. Sections of 5 μ m were cut in a cryostat and mounted on Star Frost adhesive glass slides (Knittelgläser, Germany). Slides were stored at -80°C until immunohistochemical staining.

Immunohistochemical analysis. Synovial sections were stained using the following monoclonal antibodies to analyze the infiltrate: anti-CD55 (67:Serotec, Oxford, UK) to detect fibroblast-like synoviocytes (FLS), anti-CD68 (EBM11: DAKO, Denmark) to detect macrophages, anti-CD3 (SK7, Becton Dickinson (BD), CA) for T cells, anti-CD22 (CLB-B-ly/1,6B11, the Netherlands) for B cells and anti-CD38 (HB7, BD) for plasma cells. CD163 (Ber-MAC3; DAKO) was stained to detect resident tissue macrophages; infiltrating macrophages were evaluated by detection of myeloid related proteins MRP8 (8-5c2, BMA biomedical, Augst, SW) and MRP14 (S36.48, BMA biomedical). For the detection of cytokines, adhesion molecules and growth factors we used: anti-human TNF α (52B83; Monosan, Belgium), anti-IL-6 (Nephrology Department LUMC, the Netherlands), anti-IL-10 (23738.111, R&D, UK), anti-IL-18 (2d3b6, MD Biosciences, MN), anti-IL-1 β (2D8, immunoKontakt, UK), anti-ICAM-1 (MEM111, Sanbio, Belgium), anti-VCAM (1G11B1, Sanbio), anti-E-selectin (BBIG-E4, R&D), anti-VEGF (N.010313 C-1, Santa Cruz, CA), and anti-bFGF (F14220, BD). Staining of cellular markers was performed using a three-step immunoperoxidase method.[2] For staining of cytokines, adhesion molecules, and growth factors biotinylated tyramine was used as amplification. For control sections the primary antibody was omitted or irrelevant immunoglobulins were applied.

Digital image analysis. All sections were analyzed at random by trained analysts blinded for clinical outcome. Images were acquired and analyzed by computer-assisted image analysis using a Syndia algorithm on a Qwin-based analysis system (Leica, Cambridge, UK).[10] Per marker 18 high-power fields were analyzed. Cellular markers were expressed as positive cells/mm² (counts/mm²). Staining of cytokines, adhesion molecules, and growth factors was expressed as integrated optical density/mm² (IOD/mm²). CD68+ macrophages and TNF α expression were analyzed separately in the intimal lining layer and the sublining.

Statistical analysis. Independent Student's t-tests or Mann Whitney U tests were used to detect significant differences in baseline parameters between responders and non-responders. The Chi-square test was employed to compare the percentage erosive, IgM-RF and anti-CCP positive patients in the responder and non-responder groups. Variables with a P value < 0.1 in the univariable analysis were selected as possible predictors in a stepwise backward multivariable logistic regression model. Several exchangeable prediction sets were constructed based on the correlation structure among the potential predictors with strongly correlating predictors in different sets. The model with the highest explained variance according to Nagelkerke is reported. Correlations were assessed with the Pearson product-moment or Spearman rank-order correlation coefficients, whichever was appropriate. SPSS 11.1.4 (Chicago, IL) was used. Because the primary objective of this study was to find a pathogenetic mechanism in synovial tissue relating to clinical response, a per-protocol analysis was performed.

RESULTS

Enrollment, discontinuation and exclusion. One hundred forty-three patients were enrolled. The population was predominantly female (74%), rheumatoid factor positive (71%) with a mean age of 55 ± 13 (\pm standard deviation, SD) years, and a mean disease duration of 125 ± 108 months. Erosions were present in 110 (77%) patients. The mean DAS28 score was 5.9 ± 1.0 at baseline and the mean MTX dose was 18.2 ± 8.5 mg/week. In 3 patients a flare of arthritis at week 6 (n=1) and week 12 (n=2) was the reason for discontinuation. These patients were analyzed as non-responders. Patients (n=18) who violated the protocol or discontinued infliximab therapy (for reasons other than non-response) were excluded.

Synovial tissue exclusion. Following strict quality control, 23 synovial biopsies were excluded by a blinded assessor for absence of an intimal lining layer. After excluding 18 patients from the response analysis and 23 synovial biopsies from the tissue analysis a total of 103 completely evaluable patients remained for the analysis of synovial tissue in combination with clinical response.

Baseline patient characteristics. The remaining group of 103 patients who were analyzed for response in combination with synovial tissue consisted of 71 females and 32 males, of whom 75% were rheumatoid factor positive. The mean age was 55 ± 13 years, and the mean disease duration was 125 ± 110 months. Erosions were present in 79 (77%) patients. The mean DAS28 score was 5.9 ± 1.1 at baseline and the mean MTX dose was 18.8 ± 8.5 mg/week. Corticosteroids were used by 28 (27%) patients with a mean dose of 8.0 ± 2.9 mg/day. On average, patients had failed treatment with 2.2 DMARDs before inclusion in the study. No notable changes occurred in the baseline characteristics of the analyzed 103 patients compared to the baseline characteristics of all 143 enrolled patients (see Table 1).

Table 1. Baseline patient characteristics.

	Analyzed patients (n = 103)	Responders (n = 70)	Non-responders (n = 33)	P-value
Demographics				
Age (years)	55 ± 13	54 ± 13	56 ± 12	0.40
Female (%)	71 (69)	51 (73)	20 (61)	0.21
Disease status				
Disease duration (months)	125 ± 110	123 ± 111	130 ± 110	0.80
Erosive disease (%)	79 (77)	57 (81)	22 (67)	0.10
Rheumatoid factor positive (%)	77 (75)	57 (81)	20 (61)	<i>0.02</i>
Anti-CCP positive (%)	78 (76)	57 (81)	13 (39)	0.08
DAS28	5.9 ± 1.1	6.0 ± 1.0	5.6 ± 1.2	0.07
Patients global score (0-100mm)	60 ± 22	62 ± 21	56 ± 23	0.23
ESR (mm/hr)	34 ± 24	36 ± 23	29 ± 25	0.16
C-reactive protein (mg/dL)	22 ± 28	24 ± 26	19 ± 30	0.36
Drug treatments				
Previous DMARDs	2.2 ± 1.5	2.1 ± 1.4	2.4 ± 1.6	0.42
Methotrexate (mg/wk)	18.8 ± 8.5	19.5 ± 8.2	17.1 ± 8.9	0.18
Receiving corticosteroids (%)	28 (27)	22 (31)	6 (18)	0.22
Receiving NSAIDs (%)	52 (50)	35 (50)	17 (52)	0.89

* Mean values ± standard deviation (SD), median and interquartile range (IQR) or percentages are shown. P values < 0.05 (two-sided) are significant and shown in *Italic*.

Clinical response. Seventy of 103 patients (68%) were DAS28 responder and 33 (32%) non-responder at week 16. The clinical efficacy matched the expected 60-70% responders observed in previous randomized controlled trials with infliximab in RA patients. The mean change in DAS28 was 1.84 ± 1.26 at week 16. For non-responders the average delta DAS28 was 0.45 ± 0.58 compared to 2.52 ± 0.89 for responders. This difference was highly significant ($P < 0.001$).

All baseline patient characteristics were also tested for differences between responders and non-responders. The DAS28 at baseline was nearly significant and was included in the prediction model ($P = 0.07$). The same holds for anti-CCP positivity ($P = 0.08$). Finally, there were significantly more rheumatoid factor positive patients in the responder compared to the non-responder group ($P = 0.020$), (see Table 1).

Baseline synovial TNF α expression and the number of major TNF α producing cells are associated with clinical response (Δ DAS28 \geq 1.2) after 16 weeks of TNF α blocking therapy. Scatter diagrams of the synovial predictors in individual patients in both response groups are presented in Figure 1. TNF α expression levels were higher in the synovial sublining of responders compared to non-responders ($P = 0.008$) (Figure 1 A). Similarly, TNF α expression in the intimal lining layer was higher in responders compared to non-responders ($P = 0.047$). Table 2 shows the associations of synovial markers and response.

Because macrophages are known to be the main TNF α producing cells in the synovium, the number of macrophages was studied. The mean number of CD68+ sublining macrophages was significantly higher in responders than in non-responders ($P = 0.029$) (Figure 1 C). Similarly, the number of CD163+ resident tissue macrophages as well as the number of infiltrating MRP8+ and MRP14+ macrophages was higher in responders ($P = 0.017$, $P = 0.018$ and $P = 0.024$, respectively) (Figure 1 D). Finally, the median number of CD3+ T cells was higher in responders ($P = 0.001$) (Figure 1 B).

Table 2. Associations of studied synovial predictors with clinical response.

	Responders	Non-responders	P value
	N= 70	N= 33	
	Delta DAS28 score		
	≥ 1.2	<	
Cytokines (IOD/mm²)			
TNF α lining	48214 (29116-88971)	36376 (23394-54271)	<i>0.047</i>
TNF α sublining	77947 (38710-123535)	46033 (19890-78129)	<i>0.008</i>
IL-1 β	56278 (31063-81655)	54484 (32098-129041)	0.814
IL-6	50537 \pm 41939	38364 \pm 28527	0.135
IL-10	131106 \pm 87359	115964 \pm 83953	0.415
IL-18	9530 \pm 12086	7009 \pm 7470	0.289
Cellular markers (counts/mm²)			
CD55	652 \pm 418	694 \pm 389	0.895
CD3	149 (66-385)	47 (22-163)	<i>0.001</i>
CD68 lining	374 \pm 253	295 \pm 184	0.130
CD68 sublining	576 \pm 428	387 \pm 338	<i>0.029</i>
CD163	1100 \pm 432	878 \pm 433	<i>0.017</i>
CD22	54 \pm 99	47 \pm 111	0.756
CD38	284 \pm 384	325 \pm 556	0.661
MRP8	139 (35-294)	53 (19-135)	<i>0.018</i>
MRP14	159 (34-526)	51 (18-166)	<i>0.024</i>
Adhesion molecules (IOD/mm²)			
ICAM	30790 \pm 33016	22321 \pm 21706	0.195
VCAM	80065 \pm 52630	70701 \pm 44574	0.387
E-Selectin	37651 \pm 40134	30488 \pm 29972	0.373
Growth factors (IOD/mm²)			
VEGF	18252 \pm 78689	5776 \pm 5372	0.374
BFGF	209 \pm 736	64 \pm 163	0.276

* Data are presented as mean \pm SD or median (interquartile range) whichever appropriate.

P values < 0.05 (two-sided) are significant and shown in *Italic*.

Baseline TNF α is the only significant synovial predictor of response. The relationship between TNF α expression and clinical response was confirmed by stepwise backward multivariable logistic regression analysis. Due to multicollinearity among predictors ten exchangeable bivariate prediction sets were constructed consisting of 1) TNF α expression in either the intimal lining layer or synovial sublining with 2) one out of five cellular markers. This revealed that TNF α expression in the synovial sublining was the only independent, early determinant of therapy response ($P = 0.011$), explaining just about 10% of the variance in response to therapy ($R^2 = 0.099$, according to Nagelkerke, odds ratio (OR) = 1.013). Likewise, TNF α expression in the intimal lining layer rather than synovial sublining explained 9% of the variance in response to therapy ($P = 0.020$), ($R^2 = 0.089$, OR = 1.017). Thus, after adjustment for TNF α in a bivariate logistic regression model including either the numbers of CD68+ sublining macrophages, CD163+, MPR8+, MRP14+ macrophages, or CD3+ T cells, these cellular markers were no longer significantly associated with response. The removal of these cellular markers from the model is consistent with the notion that these cells are the main producers of TNF α .

Contribution of disease activity at baseline to the prediction of response. To assess the value of a combined prediction model consisting of both clinical and synovial data we added the relevant clinical variables (DAS28 score, the presence of IgM-RF or anti-CCP antibodies) to the 10 bivariate synovial models. With stepwise backward multivariate logistic regression this resulted in a significant prediction model with TNF α expression in the synovial sublining ($P = 0.008$, OR = 1.014) and the DAS28 ($P = 0.031$, OR = 1.611) with an increased explained variance of 17% ($R^2 = 0.172$). A similar analysis except with TNF α expression in the intimal lining layer ($P = 0.023$, OR = 1.017) resulted in a significant model including the DAS28 ($P = 0.044$, OR = 1.588) and the presence of anti-CCP antibodies ($P = 0.046$, OR = 3.038) ($R^2 = 0.182$).

Baseline IL-1 β , IL-6, IL-18, and IL-10 expression is not associated with response to TNF α blocking therapy. In addition to TNF α , we studied the expression of other cytokines such as IL-18 which is believed to operate mainly upstream of TNF α , while IL-1 β and IL-6 on the other hand appear to act mainly downstream of TNF α . Despite the known interplay of these cytokines with TNF α we found no relationship between synovial expression levels of either of these cytokines and clinical response. We did find, however, a significant correlation between the level of IL-1 β expression and TNF α expression ($r = 0.306$, $P = 0.014$). The expression level of IL-10 was not different between response groups (see Table 2).

Baseline expression of adhesion molecules and angiogenic factors is not associated with response to TNF α blocking therapy. In accordance with their function in leukocyte recruitment a significant correlation was found between the number of sublining macrophages on the one hand and the expression of the vascular adhesion molecules E-selectin (Spearman's rho correlation coefficient 0.275-0.536, $P < 0.01$) on the other. However, the level at which the adhesion molecules were expressed in the synovium at baseline did not differ between responders and non-responders. Furthermore, VEGF is known to mediate angiogenesis and is regulated by pro-inflammatory cytokines like TNF α . However, no association was found between VEGF expression and response (Table 2). Similarly, no difference was found in the expression of bFGF.

DISCUSSION

The primary objective of this study was to investigate whether immunohistologic assessment of the cell infiltrate and cytokine expression in the synovium prior to initiation of TNF α blocking therapy could predict clinical response in patients with RA. We could confirm our hypothesis that the level of synovial TNF α expression is a significant early predictor of the response. This was shown by increased TNF α expression levels in both the intimal lining layer and synovial sublining of responding compared to non-responding patients. In line with these findings, there was increased infiltration by macrophages, including both CD163+ resident tissue macrophages and MRP8+ and MRP14+ infiltrating macrophages, as well as T cells in responders versus non-responders. It is important to note that these cells are the main source of TNF α in the synovium of RA patients.[11]

Consistent with the clinical experience that the response to TNF α blockade is not a dichotomous phenomenon, [12] there was no distinct threshold value in TNF α expression in the synovium of RA patients. Multivariate logistic regression analysis of synovial markers showed that TNF α expression in the sublining could explain about 10% of the variance in response to therapy. After adjusting for disease activity at baseline this further increased to 17%. Hence, the predictive value of synovial TNF expression is statistically significant, but overall limited. This clearly indicates that variables other than synovial TNF α expression are involved as well. The results presented here show for the first time that the expression of IL-1 β , IL-6, IL-18, IL-10, E-selectin, ICAM-1, VCAM-1, VEGF, and bFGF is not associated with clinical response to anti-TNF α treatment. Thus, the features of synovial inflammation at baseline in responders to anti-TNF therapy compared to non-responders do not merely represent a greater amount of inflammation, but the results presented here underscore the importance of specific inflammatory pathways. Future work should expand the search for other biomarkers and molecular networks to better understand the variable response to anti-TNF α therapy.

The search for predictors of response is important in the context of personalized medicine, which may be an effective approach to increase the percentage of patients exhibiting a robust response to a given treatment. Previous work has clearly suggested that RA consists of different pathogenetic subsets, leading to common signs and symptoms associated with what we at present define clinically as RA.[2, 3, 13] Thus, it is conceivable that for instance TNF α expression is more important in some patients than in others, and that TNF blockade could be more effective in the former. Although we are as yet not able to predict the response sufficiently to select patients who are likely to have a beneficial response to a specific treatment before initiation of therapy and to guide treatment decisions, the results of the present study do provide proof of principle that this might be achieved by further optimization of the biomarkers or perhaps combinations with other clinical and biological variables that need to be identified. The relevance of this approach is underscored by the expanding array of biological therapies and their costs. [14]

There is no evidence that simple measurement of plasma TNF α levels can be used to predict clinical response to TNF α blockade. As it appears logical that patients producing high levels of TNF α at the site of inflammation are more likely to benefit from TNF α blockade than those with lower TNF α levels, we focused on the synovium as the primary target of RA. The association between pre-treatment TNF α expression in the synovium and clinical response to 3 mg/kg of infliximab described here in 103 RA patients confirms and extends a trend observed in a pilot study in RA patients where 4 out of 8 patients who met the ACR50 response criteria at 2 weeks after initiation of infliximab (10 mg/kg) therapy were those with the highest levels of TNF α expression in the synovium, as shown by immunohistochemistry.[7] Of interest, the findings are also in accordance with a recent study suggesting that clinical response at 52 weeks is associated with significantly higher circulating TNF α bioactivity (measured by an in vitro bioassay) at baseline in responding compared to non-responding patients.[6] Together, these studies support the notion that the initial clinical response to TNF α blockade is related to pre-

treatment levels of TNF α production. In patients who initially respond, but loose response over time, other mechanisms like formation of antibodies against the drug may be operative.[15]

In conclusion, the results presented here show proof of principle that the heterogeneous response to TNF α blockade is associated with TNF α expression in the inflamed synovium. Future work should expand the search for other biomarkers and molecular networks as well as combinations with clinical variables. Thus, the prediction of how a patient will respond might come in reach of the treating physician.

Legend to Figure 1.

Figure 1. **A)** The median synovial sublining TNF α expression was higher in responders compared to non-responders ($P = 0.008$). **B)** The median number of CD3+ T cells in responders versus non-responders ($P = 0.001$). **C)** The mean number of CD68+ sublining macrophages was also higher in responders 576 ± 428 , than in non-responders 387 ± 338 ($P = 0.029$). **D)** CD163+ macrophages in responders versus non-responders ($P = 0.017$).

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Conflict of interest statement

C. A. Wijbrandts, M.G.W. Dijkgraaf, Maarten C. Kraan, Marjolein Vinkenoog, Tom. J. Smeets, Huib Dinant, Koen Vos, Willem F. Lems, Gerrit Jan Wolbink, Dorine Sijpkens, and Ben A. C. Dijkmans have no conflicts of interest. Prof. P. P. Tak has served as a consultant for Abbott, Amgen, Centocor, and Wyeth.

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