(Anti-)TNF alpha matters in rheumatoid arthritis
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Chapter 3

Responsiveness to anti-TNFα therapy is related to pre-treatment tissue inflammation levels in Rheumatoid Arthritis patients.

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ABSTRACT

Objective.
The response of rheumatoid arthritis (RA) patients to treatment with neutralizing antibodies to TNFα is highly variable. The underlying mechanism for therapy responsiveness is currently unknown. We therefore evaluated the relationship between baseline molecular profiles of synovial tissues from RA patients and the clinical response to treatment with infliximab.

Methods.
Synovial biopsies were obtained by arthroscopy from 18 RA patients with active disease (DAS28 ≥ 3.2) before initiation of treatment with infliximab. All patients were on stable methotrexate treatment. Clinical response at 16 weeks was defined as a reduction in DAS28 of ≥ 1.2, non-response as reduction in DAS28 <1.2. Large-scale gene expression profiling using microarrays was performed on synovial tissue samples. To identify biological processes in synovial biopsies that could discriminate between responders and non-responders, we performed Pathway Analysis on the expression profiles.

Results.
A total of 12 patients responded to therapy, while 6 patients failed to fulfil the response criteria. We identified several biological processes, related to inflammation, which were upregulated in patients who responded to therapy, compared to those who did not show clinical improvement.

Conclusion.
These results indicate that patients with a high level of tissue inflammation are more likely to benefit from anti-TNFα treatment.
Rheumatoid Arthritis (RA) is a chronic inflammatory disease with a striking heterogeneous clinical presentation and disease course, ranging from mild disease to severe pathology with bone and cartilage destruction. We previously described a marked variability in synovial inflammation among RA patients [1,2]. In addition, treatment of RA patients with anti-TNFα antibody therapy results in a variable response [3]. Previous studies have indicated that anti-TNFα treatment reduces tissue cellularity, and serum levels of the proinflammatory molecules S100A12 and IL-7 in responding RA patients [4-7]. However, how the molecular profile in pre-treatment synovial tissue samples relates to the response to anti-TNFα antibody therapy is still unclear.

Here we applied gene expression profiling of synovial biopsies from RA patients before treatment to identify which biological processes associate with the response to therapy.

PATIENTS AND METHODS

Patients.

Synovial tissue was obtained by arthroscopic synovial biopsy from 18 RA patients prior to initiation of anti-TNFα therapy with infliximab (3 mg/kg). 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 active disease defined as a disease activity score (DAS28) ≥ 3.2 when included in the study. Patients were on stable and the study protocol was approved by the Medical Ethics Committees of the Academic Medical Center in Amsterdam. We determined the responder status by the reduction in DAS28 at 16 weeks after start of therapy. RA patients with a reduction of the DAS28 of at least 1.2 were defined as responders, patients not reaching this threshold were defined as non-responders, according to treatment guidelines in the International Consensus Statements for rheumatoid arthritis [8].

Sample preparation, labeling and hybridization.

Total RNA was isolated from synovial tissue using TRIzol reagent (Invitrogen, Breda, The Netherlands) and linearly amplified using the Message Amp. aRNA kit (Ambion, Huntingdon, UK). aRNA was labeled with aminoallyl-dUTP during cDNA synthesis, followed by chemical coupling of the aminoallyl group to Cy3 or Cy5 for the experimental and a common reference sample, respectively. The labeled cDNA transcripts were hybridized together on 43K human cDNA microarrays (Stanford University).

Data filtering and analysis.

Data were stored and pre-analyzed in the Stanford Microarray Database (SMD) [9] at http://genome-www.stanford.edu/microarray. Data are expressed as log2 ratios of fluorescence
intensities of the experimental and the common reference sample. Intensity-dependent normalization using local estimation (“Loess”) was performed separately on each sector of the array. Spots were included in the analysis when in at least 80% of the microarrays a reliable data point was obtained for that element (defined by a regression correlation coefficient $R > 0.6$ for Cy3 and Cy5 pixel intensities, and a signal intensity of 2.5 times the local background for both Cy3 and Cy5). The use of a common reference allows comparison of the expression levels across all samples. Therefore, the expression levels (as log2 ratio’s) were median centered, i.e. each spotted element was expressed relative to the median expression level of that element across all samples. We corrected for array batch differences by applying Singular Value Decomposition in SMD as described before [10]. In short, the eigengene reflecting coating differences was removed. Genes represented more than once on the microarrays were averaged from sequences with the same Unigene identifier. Genes that differed 1.4 fold in expression between the responders and non-responders were analyzed by supervised hierarchical clustering. To visualize the correlation of co-expressed genes we used Treeview (available at http://rana.lbl.gov/EisenSoftware.htm).

Statistical analysis.

To determine which biological processes were represented by the difference in expression levels (fold change) of all genes in responders compared to non-responders, we applied Gene Ontology analysis in the PANTHER database at http://PANTHER.appliedbiosystem.com [11]. The statistical tool utilizes the fold change of all genes to define the probability that the functional category distribution (biological processes) was drawn randomly, estimated by the Mann–Whitney Rank-Sum Test (U-test). A Bonferroni correction was applied to adjust for multiple comparisons. Bonferroni-corrected P values $< 0.05$ were considered significant.

RESULTS

Transcriptome analysis of synovial biopsies prior to anti-TNFα therapy.

We analyzed the expression profiles of synovial tissues from 18 patients with RA prior to treatment with infliximab. The patient characteristics are summarized in Table 1. None of the clinical parameters before treatment differed significantly between responders and non-responders.

To identify biological processes that are associated with response to therapy, we calculated the fold difference in transcript levels for all genes between responders and non-responders. Subsequently, we performed a Gene Ontology analysis in the Panther database based on the expression of all genes (see methods).
This analysis resulted in the identification of nine biological processes that were more actively expressed in the responding patients: Immunity and defense, T-cell mediated immunity, Cell surface receptor mediated signal transduction, MHCII-mediated immunity, Cell adhesion, Cytokine and chemokine mediated signaling pathway, Cell adhesion-mediated signaling, Signal transduction, and Macrophage-mediated immunity (Table 2). These processes all indicate a higher level of cellularity and inflammatory activity in responding patients, which was confirmed by an increased expression of CD3 and CD163 in the synovial tissues detected by immunohistochemistry (p<0.05, data not shown).

To visualize the transcript expression patterns of synovial tissues, we performed a one-way hierarchical clustering. By separating responders and non-responders we could identify genes associated with responsiveness to treatment with anti-TNFα. 

Table 1. Baseline patient characteristics.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Responders (n=12)</th>
<th>Non-Responders (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (range)</td>
<td>Mean 50 (30-71)</td>
<td>59 (37-85)</td>
</tr>
<tr>
<td>Sex: Female, Male</td>
<td>9 F, 3 M</td>
<td>3 F, 3 M</td>
</tr>
<tr>
<td>Disease characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP (mg/dL) (range)</td>
<td>mean 28 (3-110)</td>
<td>13 (5-49)</td>
</tr>
<tr>
<td>ESR (mean, range)</td>
<td>mean 35 (2-95)</td>
<td>38 (8-82)</td>
</tr>
<tr>
<td>Rheumatoid factor titer (kAU/L) (range)</td>
<td>mean 201 (1-516)</td>
<td>74 (1-213)</td>
</tr>
<tr>
<td>ACPA titer (kAU/L)</td>
<td>mean 1093 (1-4181)</td>
<td>1384 (1-6264)</td>
</tr>
<tr>
<td>Disease duration in months</td>
<td>69 (7-209)</td>
<td>119 (29-314)</td>
</tr>
<tr>
<td>Erosions</td>
<td>N=11</td>
<td>N=5</td>
</tr>
<tr>
<td>DAS28</td>
<td>6 (4.7-7.7)</td>
<td>5.3 (3.7-7.2)</td>
</tr>
<tr>
<td>Methotrexate dose (mg/week) (range)</td>
<td>Mean 22 (12.5-30)</td>
<td>19 (7.5-30)</td>
</tr>
<tr>
<td>Prednisone (≤10 mg/day)</td>
<td>N=5</td>
<td>N=0</td>
</tr>
</tbody>
</table>

Mean values and range are shown. CRP = C-reactive protein, ESR = Erythrocyte sedimentation rate, ACPA = anti-citrullinated peptide antibodies.

Table 2. Biological processes associated with responsiveness to treatment with anti-TNFα.

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Number of Genes</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated in responders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunity and defense</td>
<td>718</td>
<td>9.30E-10</td>
</tr>
<tr>
<td>T-cell mediated immunity</td>
<td>111</td>
<td>1.75E-07</td>
</tr>
<tr>
<td>Cell surface receptor mediated signal transduction</td>
<td>700</td>
<td>2.27E-04</td>
</tr>
<tr>
<td>MHCII-mediated immunity</td>
<td>15</td>
<td>7.83E-04</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>340</td>
<td>1.56E-03</td>
</tr>
<tr>
<td>Cytokine and chemokine mediated signaling pathway</td>
<td>126</td>
<td>2.95E-03</td>
</tr>
<tr>
<td>Cell adhesion-mediated signaling</td>
<td>196</td>
<td>5.58E-03</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>1662</td>
<td>1.62E-02</td>
</tr>
<tr>
<td>Macrophage-mediated immunity</td>
<td>65</td>
<td>2.56E-02</td>
</tr>
<tr>
<td>Protein biosynthesis</td>
<td>208</td>
<td>1.08E-02</td>
</tr>
<tr>
<td>Upregulated in non-responders</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This analysis resulted in the identification of nine biological processes that were more actively expressed in the responding patients: Immunity and defense, T-cell mediated immunity, Cell surface receptor mediated signal transduction, MHCII-mediated immunity, Cell adhesion, Cytokine and chemokine mediated signaling pathway, Cell adhesion-mediated signaling, Signal transduction, and Macrophage-mediated immunity (Table 2). These processes all indicate a higher level of cellularity and inflammatory activity in responding patients, which was confirmed by an increased expression of CD3 and CD163 in the synovial tissues detected by immunohistochemistry (p<0.05, data not shown).
with a correlated expression pattern in these groups. From the expression of genes shown in Figure 1a and the enlarged cluster of inflammation-related genes (Figure 1b) it is clear that almost all patients with a high transcript level of inflammation-related genes responded to infliximab. These transcripts included those encoding CD163, S100A8, several HLA class II and immunoglobulin genes, integrins and chemokines, which we previously associated with high inflammatory RA tissues [2].

Analysis of transcription factor binding sites.
To gain more insight into the differential activation of gene expression in tissues from responders versus non-responders, we performed a search for transcription factor binding sites that are enriched in the regulatory regions of the genes expressed at different levels in responders and non-responders. We investigated whether the genes with at least a 1.4 fold increase in expression (86 genes) in the responders overrepresented specific transcription factor binding sites.

![Figure 1. A) Visualization of expression of 189 genes with at least a 1.4 fold difference in expression between responders and non-responders (indicated at the top). Red indicates a high level of expression, green a low expression level, black indicates intermediate expression and grey indicates missing data points. The red bar at the right indicates a cluster of correlated genes (R=0.79) with a higher expression in responding patients' samples. B) Enlarged view of genes with a high correlation across the patient samples (R=0.79), indicating increased expression of inflammation-related genes in the responder group. See color figures page 215.](image-url)
TNF response and inflammation

2. binding sites in the 300 base pair upstream region of these genes resulted in the identification
3. of two members of the Ets family of transcription factors, Elf-1 (p=10^{-4.9}), and Ets2 (p=10^{-3.2}).
4.
5. Using the same criteria, genes with a 1.4 fold lower expression in responders (103 genes) over-
6. represented transcription factor binding sites for factors that are not related to inflammation.
7. The most significant factor was *Myc-associated zinc finger–related factor* (MAZR, p=10^{-6.4})
8.
9. **DISCUSSION**
10.
11. In this report we showed that patients who subsequently responded to anti-TNFα therapy
12. showed an increased baseline transcription of genes involved in inflammatory processes,
13. compared to patients not fulfilling the response criteria. This difference between responders
14. and non-responders corresponds with the heterogeneity in expression profiles of RA synovial
15. tissues, which reflects a difference in inflammation-related genes, confirming our previous find-
16. ings on synovial tissues from patients undergoing surgical joint replacement [2].
17.
18. Recently, the effect of infliximab treatment was analyzed by gene expression profiling of
19. synovial tissues from 10 patients. Within a subgroup of 4 patients that showed pre-treatment
20. positivity of TNFα by immunohistochemistry, treatment with infliximab resulted in a reduction
21. of a number of biological processes, i.e. immune response, cell communication, signal trans-
22. duction and chemotaxis [13]. In our analysis, we determined that these (and other) processes
23. could be used to distinguish between responders and non-responders already at baseline. The
24. clinical characteristics show a trend towards higher levels of inflammation markers (CRP, RF,
25. DAS) and more patients on steroids in the responder group, but this does not reach statistical
26. significance. Here we show that a pathway analysis based on the expression of thousands of
27. genes has more power to detect significant differences in inflammatory markers than a small
28. number of clinical characteristics.
29.
30. One patient did not respond to TNFα blockade, despite a high level of inflammation. It may be
31. anticipated that such patients either require a higher dose of infliximab or that the disease is
32. driven by other pro-inflammatory processes, in addition to, or independent of TNFα.
33.
34. The identification of the Ets family of transcription factors, overrepresented by genes with a
35. higher expression in responders, is in line with the finding that a high level of inflammation
36. is associated with responsiveness, since Ets2 activation is involved in macrophage survival
37. and pro-inflammatory cytokine production [14]. In contrast, genes with a lower expression in
38. responders did not reveal an important contribution of transcription factors associated with
inflammation. Instead, we identified enriched binding sites for the factor MAZR, also known as PATZ. The activity of this transcription factor is associated with transcriptional repression, and may therefore be responsible for the low expression of genes harboring a MAZR-binding site in

In conclusion, this study indicates that different processes are at play in responding versus non-responding patients, suggesting that the pathology may be driven by distinct mechanisms.

Acknowledgements

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REFERENCES