Anti-TNF therapy in rheumatoid arthritis: Searching for mechanisms of effect
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Infliximab treatment depletes dominant T cell clones in synovial tissue and peripheral blood of rheumatoid arthritis patients

In preparation
ABSTRACT

Objectives
T-cells are thought to play a key role in the pathogenesis of rheumatoid arthritis (RA). Anti-TNF therapy has been shown to improve synovial inflammation and progression of joint destruction. The objective of this study was to provide insight into the early effects of infliximab on T-cell clones in synovial tissue and peripheral blood of RA patients.

Methods
Arthroscopic synovial tissue samples (n=7) and paired peripheral blood samples (n=3) were obtained before and 48 hours after infliximab infusion in RA patients. T-cell clones were quantified using next generation sequencing. Clones with a frequency >0.5% of the repertoire were considered dominant.

Results
Within 48 hours the percentage of dominant T-cell clones in synovium decreased from 1.2% (IQR 1.0-4.7%) to 0.45% (IQR 0.2-3.5%), resulting in decreasing the percentage of the T-cell repertoire taken up by dominant clones from 27.1% (IQR 13.3-29.5%) to 4.5% (IQR 3.4-17.2%) (p=0.04). In peripheral blood the percentage dominant clones decreased from 0.15% (range 0.11-0.68%) to 0.05% (range 0-0.06%) The clones were not necessarily identical in synovium compared to peripheral blood: of the clones present in synovium at baseline, the percentage that could be retrieved in peripheral blood at the same time point was 2.1% (0.1-3.9%) (mean,range) and tended to increase after initiation of treatment.

Conclusions
There was a marked and statistically significant reduction of dominant T-cell clones in synovial tissue and peripheral blood, already 48 hours after initiation of infliximab treatment. This profound effect on the T-cell repertoire may add to the disease-modifying properties of anti-TNF antibody treatment.
INTRODUCTION

Since the recognition of synovial tissue as the primary site of inflammation, studying the synovium has provided insight into the pathogenesis of the disease and the mechanism of action of different therapies. Previously, anti-tumor necrosis factor (TNF) therapy has been shown to result in marked reduction of synovial cellularity in both rheumatoid arthritis (RA) and psoriatic arthritis (PsA), as early as 24-48 hours after initiation of treatment. Overall, in these studies the reduction in synovial macrophages and T-cells was the most consistent finding. Interestingly, this reduction in cellularity could not be explained by apoptosis of monocytes/macrophages or lymphocytes in the peripheral blood or synovial tissue, nor could the decrease in synovial macrophages be explained by the decrease of influx of monocytes. Conceivably, decreased expression of adhesion molecules as well as chemokines in the tissue may facilitate cellular egress from the synovium, thereby explaining decreased cellularity in the synovial tissue after therapy. Consistent with this hypothesis, we found that anti-TNF therapy resulted in increased lymphatic vessel formation in the synovium. Furthermore, in peripheral blood CD4+ and CD8+ T-cells or the total lymphocyte count has been shown to increase 24 or 48 hours after infliximab treatment, which might be the result of T-cell egress from the synovium.

T-cells are thought to play a key role in the pathogenesis of rheumatoid arthritis. They are abundantly present in the synovium of RA patients and synovial T-cells express markers of recent activation. Furthermore, modulation of co-stimulation of T-cells with antigen presenting cells by abatacept treatment significantly improves signs and symptoms of RA. The importance of antigen presentation to T-cells is also underlined by the association between RA susceptibility and HLA-DR alleles encoding the shared epitope. We recently developed a new technique using next generation sequencing to provide full-repertoire screening without polymerase chain reaction (PCR)-bias, thus providing an accurate quantitative data on the degree of expansion of individual T-cell clones within the complete T-cell receptor (TCR) repertoire, allowing quantitative comparisons of clonality between different patients and between different compartments of the body.

We used this novel technique to study the early effects of infliximab therapy on the T-cell repertoire in the synovium and peripheral blood.

PATIENTS AND METHODS

Patients

Seven patients from a previously described placebo-controlled, double-blind randomized clinical trial were selected for this study based on availability of synovial tissue samples on baseline and 48 hours after the start of infliximab treatment (3 mg/kg). For three patients paired peripheral blood mononuclear cells (PBMCs) were available. In brief, all patients had active RA, defined as a Disease Activity Score in 28 joints (DAS28) of at least 3.2, despite maximal methotrexate treatment (either 30 mg/week or the maximum tolerable dose). Previously, patients failed treatment with at least one other conventional disease-modifying antirheumatic drug (DMARD). At baseline and after 48 hours all patients underwent mini-arthroscopy under local anesthesia to obtain synovial tissue samples from an actively inflamed knee, as previously
described\(^{20, 21}\). All patients gave written informed consent and the study was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam.

**Amplification, emulsion PCR, high-throughput sequencing and bioinformatics**

The linear amplification, next-generation sequencing and bioinformatics analyses were described previously\(^{18, 22}\). For all synovial tissue samples and peripheral blood samples >20,000 (bead-bound) TCR\(\beta\) sequences were analyzed. In order to quantitatively compare clonality between different patients and between different compartments of the body TCR frequencies were corrected for the number of reads. In accordance with previous data, clones were considered dominant clones if they consisted of >0.5% of the total repertoire\(^{19}\).

**Statistical analysis**

Data are presented as median and interquartile range (IQR) or range. Changes in number of T-cell clones and contribution of dominant clones to the total repertoire in synovial tissue were analyzed using the Wilcoxon signed ranks test to determine significant changes from baseline. The calculations were performed with IBM SPSS Statistics 19.0 for Windows (SPSS, Chicago, IL)

**RESULTS**

**Clinical characteristics**

Individual baseline patient characteristics are presented in table 1. Sixteen weeks after the initiation of treatment the decrease in DAS28 was 2.5 (IQR, 0.9-3.5) Two patients (29%) had a good response, 4 patients (57%) a moderate response and 1 patient (14%) showed no response according to the European League Against Rheumatism (EULAR) criteria\(^{23}\).

**Changes in dominant T-cell clones in synovial tissue and peripheral blood early after initiation of infliximab treatment**

Of all T-cell clones present in the synovial tissue at baseline, 6.7% (median, IQR 1.9 - 10.7%) could still be detected in the synovium 48 hours after infliximab therapy. At baseline 1.2% (median, IQR 1.0 - 4.7%) of all T-cell clones were dominant clones (Figure 1A). After 48 hours only 0.45% (median, IQR 0.2 - 3.5%) of all synovial clones were dominant clones. Of the clones that were dominant at baseline, only 13.9% (median, IQR 0 - 30.8%) remained dominant after therapy. Overall, the contribution of dominant synovial clones to the total repertoire decreased from 27.1% (median, IQR 13.3 - 29.5%) to 4.5% (3.4 - 17.2%) (\(p = 0.04\)) (Fig. 1B). This space was filled up by smaller clones, resulting in an increased number of T cell clones after infliximab treatment from 1055 (median, IQR 594 - 1736) before to 2145 after treatment (690-2396) (\(p=0.04\)) (Fig 1C).

Infliximab therapy also resulted in depletion of dominant clones in the peripheral blood. As described previously\(^{21}\), the percentage of the T-cell receptor repertoire taken in by dominant clones was lower in peripheral blood than in synovial tissue (0.15%, 0.11% and 0.68% for patient 1, 2 and 3, respectively). Similar to the findings in synovial tissue, there was a decrease in the number of dominant clones (Fig 2A), resulting in an increase in the total number of clones (Fig 2B) after treatment with infliximab.
Figure 1. Changes in T cell clones in synovial tissue after infliximab treatment. (A) Frequency distribution of T-cell clones in synovial tissue. Baseline (white bars) is compared with synovial tissue after 48 hours of infliximab treatment (grey bars). (B) Decrease of the contribution of dominant clones to the total T-cell repertoire after initiation of infliximab treatment in synovial tissue (ST) (p=0.04). Median and interquartile ranges are shown. (C) Increase in the total number of T cell clones 48 hours after initiation of infliximab treatment in synovial tissue (ST) (p=0.04). Median and interquartile ranges are shown.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>Age (years)</td>
<td>58 [44-70]</td>
</tr>
<tr>
<td>Female (%)</td>
<td>100</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>109 [26-143]</td>
</tr>
<tr>
<td>IgM-RF (%)</td>
<td>43</td>
</tr>
<tr>
<td>ACPA (%)</td>
<td>57</td>
</tr>
<tr>
<td>Erosive disease (%)</td>
<td>86</td>
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<tr>
<td>DAS28</td>
<td>6.5 [5.6-7.2]</td>
</tr>
<tr>
<td>ESR (mm/h)</td>
<td>43 [21-82]</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>14.5 [5.3-42.5]</td>
</tr>
<tr>
<td>MTX (mg/week)</td>
<td>16 [9-29]</td>
</tr>
<tr>
<td>Prednisone (%)</td>
<td>71</td>
</tr>
<tr>
<td>NSAIDs (%)</td>
<td>43</td>
</tr>
<tr>
<td>Prior use of DMARDs (n)</td>
<td>2.0 [1.3-2.8]</td>
</tr>
</tbody>
</table>

Data are represented as median [IQR] or n (%), as appropriate. Baseline characteristics of patients with RA treated with infliximab (3 mg/kg) in combination with a stable MTX dose for at least 16 weeks, are described. Presence of erosive joint disease was determined by x-ray. Presence of IgM-RF was defined as serum levels ≥12.5 IU/ml and presence of ACPA was defined as serum levels ≥25 IU/ml. ACPA, anticitrullinated peptide antibody; CRP, C reactive protein; DAS28, disease activity score evaluated in 28 joints; ESR, erythrocyte sedimentation rate; MTX, methotrexate; IgM RF, Immunoglobulin M rheumatoid factor.
There was no overlap of CDR3 regions of the dominant T-cell clones in synovial tissue between patients. In addition, there were great differences in the use of variable (V) and the joining (J) genes in the synovial tissue dominant T-cell clones between patients.

Overlap of T cell clones between synovial tissue and peripheral blood before and after infliximab therapy

At baseline, comparison of TCR sequences showed that only a small percentage of the synovial tissue clones were also present in the analysis of peripheral blood samples. This overlap was 2.1%, 3.9% and 0.1% for patient 1, 2 and 3, respectively. To test whether synovial clones migrated to the peripheral blood we analyzed overlap of the synovial tissue at baseline and peripheral blood after infliximab treatment. Compared to the baseline peripheral blood sample, the analysis in peripheral blood at 48 hours showed only a slightly higher number of overlapping clones (3.8%, 4.7% and 3.3% for patient 1, 2 and 3, respectively) (Fig 2C). Of note, only a small percentage of the T cell clones that decreased in the synovial tissue could be detected in the peripheral blood after 48 hours.

DISCUSSION

The results of this mechanistic study suggest that infliximab therapy leads to depletion of dominant clones present in the synovial tissue and peripheral blood of RA patients very early.
after initiation of treatment. In synovial tissue, this resulted in a statistically significant decrease in the contribution of dominant clones to the total T-cell repertoire. Whether the depletion of dominant T-cell clones after treatment with infliximab is treatment-specific or the result of a decrease in disease activity remains to be elucidated. Previously, we did not see a decrease in the degree of expansion of T-cell clones in the synovium after treatment with methotrexate for 6 months, but it should be noted that treatment with methotrexate was ineffective in these patients. Furthermore, from the current study we cannot exclude the possibility that the depletion of dominant clones is a transient effect. Consistent with the notion that the inflamed synovium can provide a specific niche for the retention and/or proliferation of certain T-cell clones, new clones might form in the synovium at a later time points. Of note, a previous study using spectratyping did report the reduction of BV6-containing expansions in peripheral blood after infliximab treatment after 4 and 12 months of therapy.

In accordance with our previous data, the synovium contained many oligoclonal expansions of synovium-specific T-cells (i.e. TCR sequences could not be detected in peripheral blood at baseline). Interestingly, some TCR sequences, that were specific for the synovium at baseline, could be detected in the peripheral blood 48 hours after treatment with infliximab.

Previous studies in sheep showed that T-cells can leave the site of inflammation via the afferent lymph and after a period of residency in lymph nodes, enter efferent lymph sinuses, which drain into efferent lymph vessels, and via the thoracic duct, back into the peripheral blood. Importantly, many of these T-cells are capable of secreting inflammatory cytokines like IFN-γ and/or IL-17, proving that polarized T-cell subsets not only traffic from blood into inflamed sites, but also leave the inflammatory site via the afferent lymph. In addition, cytokine activated T cells (Tck cells), a model for RA synovial T-cell function, showed upregulation of VLA-4 and increased migratory responsiveness to its principal ligand VCAM-1. VCAM-1 is highly expressed on synovial fibroblasts and VCAM-1 expression in the synovial tissue is reduced after anti-TNF treatment. This suggests that anti-TNF treatment may diminish cellularity in the synovium by decreasing T-cell retention in the synovial tissue e.g., by reducing integrins expressed by fibroblast-like synoviocytes. In this study the percentage of synovium-specific T-cell clones (i.e. not detectable in peripheral blood at baseline) that could be detected in the peripheral blood 48 hours after infliximab therapy was small. This suggests that if egress from the synovium occurs after infliximab infusion, there is retention and/or apoptosis of T-cells in the lymphatic system after egress from the synovium and few T-cells recirculate into the peripheral blood. On the other hand, we cannot exclude the possibility that the effects of infliximab therapy differ between T-cells and monocytes/macrophages, and that anti-TNF antibody therapy directly interferes with T-cell migration towards the synovial compartment.

Obviously, this study has its limitations. We could only study a limited number of patients, as the clinical trial design was quite challenging with serial arthroscopy within 48 hours. In addition, we cannot exclude the possibility that the depletion of dominant clones was the result of regression to the mean. However, in our previous study we found similar numbers of dominant clones and a similar impact of these dominant clones on the repertoire, suggesting that the decrease is indeed anti-TNF mediated. Moreover, we previously reported a larger
percentage of overlap between synovial tissue biopsies obtained from both inflamed knees within a 10-day interval from one patient than in this study\(^9\), suggesting that the reduction in degree of expansion was the result of TNF blockade.

In conclusion, this study shows that treatment with infliximab results in rapid depletion of dominant T-cell clones in both synovial tissue and peripheral blood, which might help explain the disease-modifying properties of this treatment.

REFERENCE LIST


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