Anti-TNF therapy in rheumatoid arthritis: Searching for mechanisms of effect
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Synovial expression of CD11c and its relationship to response to infliximab in patients with rheumatoid arthritis

Submitted
ABSTRACT

Objective
Expression of CD11c on peripheral blood monocytes of patients with rheumatoid arthritis (RA) has previously been identified as a biomarker predicting clinical response to adalimumab monotherapy, but not in patients using concomitant methotrexate (MTX). CD11c is an adhesion molecule expressed on synovial tissue macrophages and is involved in migration and retention of synovial macrophages. Because the decrease in the number of macrophages in synovium is consistently associated with improvement after effective treatment in RA, we examined the relationship between synovial CD11c expression and the clinical response to anti-TNF antibody therapy.

Methods
In a prospective study, synovial tissue samples were obtained from 22 active biological treatment naïve RA patients on stable MTX before initiation of infliximab treatment. CD11c expression was detected by immunohistochemistry and by double immunofluorescence staining with CD14 and CD68 and related to clinical response.

Results
Overall CD11c expression correlated moderately with TNF expression ($r = 0.48$, $p=0.02$). In the synovial sublining, CD11c expression correlated with CD68+ macrophage numbers ($r=0.47$, $p=0.033$). Higher expression of CD11c was a predictor of clinical response to infliximab treatment and there was a trend for prediction of response for DAS28 at baseline ($p=0.04$ and $p=0.08$, respectively; Nagelkerke $R^2=0.53$).

Conclusion
In line with previous data in peripheral blood, RA patients with a higher expression of synovial CD11c may have a better clinical response to infliximab therapy, also if they use infliximab in combination with MTX.
INTRODUCTION

Disease control has been greatly improved by the use of tumor necrosis factor (TNF) blockade in patients with rheumatoid arthritis (RA). However, 30-40% of the patients do not respond to anti-TNF therapy and side effects may occur. Together with the costs associated with biological treatment there is a need to identify appropriate predictors of response prior to start of TNF inhibition as a means to improve cost-effectiveness.

The CD11c/CD18 complex is part of the β2 integrin family and functions as an adhesion molecule. In humans it is found on monocytes/macrophages, granulocytes and subsets of dendritic cells (DCs) and binds a variety of ligands including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), ICAM-2, fibrinogen and collagen. Transcription profiling using purified peripheral blood monocytes from RA patients identified CD11c as a possible biomarker that was capable of predicting the clinical response to monotherapy with adalimumab. At baseline threshold levels of 40% relative expression of CD11c as compared to all monocytes could distinguish between responders and non-responders with 100% sensitivity and 92% specificity. However, CD11c was shown not to be predictive of the response to adalimumab treatment in patients who used concomitant methotrexate (MTX) therapy, limiting its use as a predictive biomarker to anti-TNF therapy in peripheral blood, as this treatment is mostly used in combination in RA. Previously, CD11c+ cells could not be detected in the synovium of healthy donors, but they were clearly present in the synovium of RA patients. Consecutive tissue sections showed CD11c expression on macrophages in the synovial sublining. These data suggest a role for CD11c in recruitment and/or retention of synovial macrophages. Since inflammatory tissue macrophages are mainly derived from circulating monocytes and a decrease in the number of macrophages in synovium is consistently associated with improvement after effective treatment in RA, CD11c is an interesting candidate to investigate as a synovial biomarker for response. Therefore, we tested the hypothesis that higher expression of synovial CD11c expression predicts a subsequent response to infliximab therapy.

PATIENTS AND METHODS

Patients

Twenty-two RA patients from a previously described cohort were selected for this study based on availability of synovial tissue samples before the start treatment with infliximab (3 mg/kg). Briefly, all patients were diagnosed according to the 1987 ACR classification criteria for RA and had previously failed treatment with at least 2 conventional disease-modifying antirheumatic drugs (DMARDs) and not used anti-TNF treatment previously. All patients provided written informed consent and the study was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam. All patients had active disease, defined as a Disease Activity Score in 28 joints (DAS28) of at least 3.2 before inclusion in the study. Patients were on stable maximally tolerable MTX treatment (7.5-30 mg/week) and the use of oral corticosteroids (≤ 10 mg/ day) and non-steroidal anti-inflammatory drugs (NSAIDs) was allowed if stable for at least one month prior to baseline. Concomitant medication was kept stable throughout the
study. Patients with a DAS28 reduction of ≥1.2 (twice the measurement error of DAS28 over time) were defined as responders, because this constitutes a clinically significant improvement.

**Study design and synovial tissue**

Disease characteristics assessed at baseline included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), MTX dose, disease duration, number of prior DMARDs taken, the presence of IgM-Rheumatoid factor (IgM-RF) and anti-citrullinated protein antibodies (ACPAs) as measured by anti-citrullinated peptide 2 enzyme-linked immunosorbent assay (Immunoscan RA, Mark 2 [no. RA-96RT]; Euro-Diagnostica Arnhem, The Netherlands) and the presence of erosions was evaluated by radiographs of hands and feet. All patients were treated with infliximab (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. Before initiation of treatment (i.e. baseline) all patients underwent a mini-arthroscopy under local anesthesia to obtain synovial tissue samples from an actively inflamed knee, ankle or wrist, as previously described.

**Immunohistochemical analysis**

Frozen sections (5 μm) were stained with specific mouse anti-human IgG1 monoclonal antibodies to detect CD11c (mouse IgG1; BD Pharmingen cat: 555391 30481A, San Diego, CA). Bound antibody was detected with a three-step immunoperoxidase method using a biotinylated tyramine amplification method. Stained sections were then randomly analyzed using digital image analysis (DIA), as previously described. Expression levels of CD11c were represented as cell count/mm². CD3+ T cells, CD22+ B cells, CD68+ macrophages, CD163+ resident macrophages, CD38+ plasma cells and CD55+ fibroblast-like synoviocytes and TNF were stained and analyzed as described previously.

Furthermore, double immunofluorescence was performed to analyze co-expression of CD11c with CD68 (for macrophages) and CD14 (for monocyte-derived cells). Sections were incubated with primary monoclonal antibodies against CD11c as described above, and then labelled with goat-anti-mouse Alexa 594 or with goat-anti-mouse Alexa 633 (both from Invitrogen/Molecular Probes, Breda, the Netherlands). Next, sections were blocked with 10% normal mouse serum in PBS/1% BSA. For co-expression of CD68 sections were stained with a specific mouse anti-human IgG2b biotin-conjugated monoclonal antibody to detect CD68 (Biolegend/ITK Diagnostics BV, San Diego, CA) and then incubated with streptavidin Alexa 488 (Invitrogen/Molecular Probes). For co-expression with CD14 sections were stained with a specific mouse anti-human IgG2a monoclonal antibody to detect CD14 (Dakocytomation, Glostrup, Denmark) and then incubated with goat anti mouse IgG2a Alexa 488 (Invitrogen/Molecular Probes). Sections were covered with VectaShield+DAPI mounting medium (H-1200, Vector laboratories, Burlingame, CA) analyzed with a confocal microscope (Leica TCS-SP2). Expression of CD14+ cells and CD68+ macrophages, CD11c+ cells, CD68+CD11c+ double positive cells and CD14+CD11c+ double positive cells was determined by manually counting all single and double positive cells by 2 blinded assessors in 3 randomly chosen high power fields and results were pooled. Percentage of co-expression was determined as the percentage of double
positive cells per total count of CD14+ cells or CD68+ macrophages (i.e. the sum of CD14- or CD68-single and -double positive cells).

**Statistical analysis**
Continuous data were described as mean (SD), if normally distributed, or median (interquartile range [IQR]), if not normally distributed. Categorical data were represented as percentages (%). Independent Student’s t tests or, where appropriate, Mann Whitney U tests were used to compare patient characteristics between responders and non-responders and co-expression of CD68 with CD11c and CD14 with CD11c. The Fisher’s exact test was used to test differences in patient characteristics between responders and non-responders in categorical data and to analyze dichotomized co-expression of CD11c with CD14 or CD68, with a threshold level of 40%. Correlations were assessed with the Pearson product-moment or Spearman rank order correlation coefficients. Logistic regression analysis was used to investigate the predictive value of DAS28 and CD11c levels. A positive cell count of more than 432 positive cells/mm² was considered high expression of CD11c at baseline and a DAS28 more than 5.4 was considered a high DAS28 at baseline. All statistical analyses were performed with SPSS 18.0 software (SPSS, Chicago, IL). A p-value of ≤ 0.05 was considered statistically significant.

**RESULTS**

**Patient characteristics**
Patient characteristics can be found in Table 1. When tested for differences between responders and non-responders, non-responders had longer disease duration at baseline (p= 0.03) and had used more DMARDs previously (p=0.01). Similar to results reported earlier<sup>16</sup>, responders tended to have higher levels of disease activity at baseline (p=0.051).

**Expression of CD11c in the synovium of patients with RA**
Consistent with previous data<sup>11,13</sup>, all patients expressed CD11c in the synovial sublining (Fig. 1A) and a few patients (n=4) also expressed CD11c in the intimal lining layer (Fig. 1B). There were no differences in clinical features between patients with or without expression of CD11c in the intimal lining layer. There was a positive correlation between synovial TNF expression and CD11c expression (r= 0.48, p=0.02) (Fig. 2A) and a positive correlation between CD11c expression in the synovial sublining and the number of CD68+ sublining macrophages (r= 0.47, p=0.033) (Fig. 2B). Baseline expression of CD11c was not related to DAS28, ESR, CRP, MTX dose, disease duration, presence of ACPA, IgM-RF, joint erosions or to the number of CD3+ T cells, CD22+ B cells, CD163+ resident macrophages, CD38+ plasma cells and CD55+ fibroblast-like synoviocytes in synovial tissue at baseline.

All patients showed co-expression of CD11c and CD14+ cells and/or CD68+ macrophages in synovial tissue (Figs. 3A and B). The percentage of CD68+CD11c+ macrophages (i.e. the percentage of CD68 positive macrophages expressing CD11c) was higher than the percentage of CD14+CD11c+ cells (i.e. percentage of CD14 positive cells expressing CD11c) (29.5% [8.9-74.6%])
Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Responders (n=10)</th>
<th>Non-responders (n=12)</th>
<th>p-value</th>
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<tr>
<td>Age (years)</td>
<td>53 (13.5)</td>
<td>61 (11.4)</td>
<td>0.17</td>
</tr>
<tr>
<td>Female (%)</td>
<td>70</td>
<td>58</td>
<td>0.63</td>
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<tr>
<td>DAS28</td>
<td>6.3 (0.95)</td>
<td>5.3 (1.3)</td>
<td>0.051</td>
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<tr>
<td>Erosive disease (%)</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>IgM RF (%)</td>
<td>90</td>
<td>83</td>
<td>1.0</td>
</tr>
<tr>
<td>ACPA (%)</td>
<td>100</td>
<td>83</td>
<td>0.48</td>
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<tr>
<td>ESR (mm/h)</td>
<td>39 (25)</td>
<td>32 (26)</td>
<td>0.54</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>31 (32)</td>
<td>20 (22)</td>
<td>0.37</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>55 [23.5-127]</td>
<td>163 [112-241]</td>
<td>0.03</td>
</tr>
<tr>
<td>Previous DMARDs</td>
<td>1.0 [1.0-2.5]</td>
<td>3.0 [2.0-4.8]</td>
<td>0.01</td>
</tr>
<tr>
<td>Use of prednisone (%)</td>
<td>40</td>
<td>17</td>
<td>0.34</td>
</tr>
<tr>
<td>MTX dose (mg per week)</td>
<td>25 [21-25]</td>
<td>11.3 [7.5-25]</td>
<td>0.11</td>
</tr>
<tr>
<td>CD11c (positive cells/ mm²)</td>
<td>472 [197-642]</td>
<td>309 [102-387]</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Data are represented as mean (SD), 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. Patients were compared, based on clinical response (DAS28 reduction of ≥1.2), with a Fischer’s exact test, unpaired Student t test or Mann–Whitney U test, as appropriate. 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 ≥12.5 IU/ml.

ACPA, anti-citrullinated 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.

**Figure 1.** Representative photographs showing CD11c expression (reddish-brown staining) in RA synovial tissue. (A) CD11c is mainly expressed in the synovial sublining layer (magnification 100 x) (Inlay: Negative isotype control). (B) In a few patients CD11c is expressed in the lining layer (magnification 100 x). (C) Trend toward higher expression of CD11c in responders (n=10) vs. non-responders (n=12) (p=0.056). Median and interquartile range are depicted.
Figure 2. Correlations of (A) CD11c with synovial TNF expression and (B) CD11c in the sublining layer and CD68+ macrophages in the sublining lining layer in 22 RA patients.

Figure 3. Representative photographs showing (A) co-expression of CD11c (Alexa-red) with CD14 (Alexa-green) (Inlay: Negative isotype control) and (B) co-expression of CD11c (Alexa-red) and CD68 (Alexa-green) in the same patient (Inlay: Negative isotype control). DAPI was used to stain the nucleus (blue). (Magnification 100 x). (C) Quantitative analysis of the percentage of co-expression of CD11c with CD14 and CD68 in 22 RA patients. The co-expression of CD11c and CD68 is significantly higher than the co-expression of CD11c and CD14 (p=0.013). Median and interquartile range are depicted. (D) Correlation of the percentage of CD68+CD11c+ cells and DAS28 in 22 RA patients (r=0.52, p=0.017).
vs. 8.4% [3.3-24.6%], (median [IQR]), $p=0.013$) (Fig. 3C). Furthermore, the percentage of CD68+CD11c+ cells correlated with DAS28 at baseline ($r=0.52$, $p=0.017$) (Fig. 3D).

**Relationship between CD11c and clinical response to infliximab in RA**

There was a clear trend towards higher expression of synovial CD11c at baseline in responders compared to non-responders (472 [197-642] counts/mm$^2$; median [IQR] vs. 309 [102-387], respectively, $p=0.056$) (Fig. 1C). Logistic regression analysis revealed that CD11c was significantly correlated with clinical response ($\Delta$DAS$\geq$ 1.2) to infliximab treatment and there was a trend for such a correlation with DAS28 at baseline ($p=0.04$ and $p=0.08$, respectively; Nagelkerke $R^2=0.53$). Sex, age, disease duration at baseline and ACPA status did not contribute to explaining the response to infliximab therapy in this study. Consistent with these results, dividing patients into 4 groups (1. patients with low CD11c and low DAS28 at baseline, 2. patients with low CD11c and high DAS28 at baseline, 3. patients with high CD11c and low DAS28 at baseline and 4. patients with high CD11c and high DAS28 at baseline) showed the highest percentage of responders in group 4 (100%) and the lowest percentage of responders in group 1 (12.5%). The percentage of responders in group 2 and 3 was of intermediate magnitude (Fig. 4).

No difference could be found in the number of CD14+CD11c+ cells or CD68+CD11c+ macrophages between responders and non-responders ($p=0.79$ and $p=0.67$, respectively).

Dichotomized data on co-expression, using a threshold level of 40% as described previously $^{11}$, did not reveal a statistically significant difference between responders and non-responders either.

**DISCUSSION**

In line with previous data based on peripheral blood analysis$^{10}$, the results of this study suggest that patients with higher synovial CD11c expression and higher DAS28 levels at baseline respond better to infliximab therapy than patients with lower synovial CD11c expression and lower DAS28 levels at baseline; of note, the patients used concomitant MTX. Although anti-TNF therapy has proven to be effective in many RA patients, there is still a need for validated biomarkers to predict the clinical response$^{23}$. Recently, we have developed a model that can

![Figure 4](image-url) **Figure 4.** Patients with high CD11c expression at baseline and high DAS28 at baseline had a higher percentage of response than patients with low CD11c and low DAS28 at baseline.
partly predict response to TNF blockade in RA patients. The addition of other variables to this model may increase the prediction of response. Although our results await confirmation in a larger cohort, this study clearly suggests that CD11c is an interesting candidate to further refine this prediction model in the context of stratified medicine.

Furthermore, this study confirms previous work showing expression of CD11c in the synovium and its relationship to the number of CD68+ macrophages. As expected there was also a correlation with TNF expression, as CD68+ macrophages are a major source of TNF in the synovium. In addition, the percentage of CD68+CD11c+ macrophages was higher than the percentage of CD14+CD11c+ cells in synovial tissue, suggesting that the same stimuli that drive the differentiation of monocytes into CD68+ macrophages also drive CD11c expression in RA synovial tissue. This might be the consequence of more systemic inflammation since a higher percentage of CD68+CD11c+ macrophages in the synovial tissue was associated with a higher DAS28.

Previous work in peripheral monocytes of RA patients already has shown that higher expression of CD11c is associated with higher expression levels of inflammatory markers in monocytes; the increment in CD11c expression in the dermis significantly correlated with psoriatic lesion progression. Furthermore, in mice CD11c expression increased on spleen monocytes after a sterile systemic inflammatory stimulus. Taken together, these data suggest that CD11c expression is higher under inflammatory conditions and as such may contribute to migration and/or retention of inflammatory cells in the target tissue. In agreement with this, we have previously reported that responsiveness to infliximab treatment is related to pre-treatment tissue inflammation.

In conclusion, our findings reveal a significant relationship between higher expression of synovial CD11c at baseline and the subsequent clinical response to infliximab therapy, making CD11c an interesting candidate which could help to further refine our current prediction model. The work presented here provides the rationale to test this in the future in independent cohorts.

**REFERENCE LIST**


7. Ingalls RR, Golenbock DT. CD11c/CD18, a transmembrane signaling receptor for


