Response to Infliximab Therapy in Ulcerative Colitis is Associated With Decreased Monocyte Activation, Reduced CCL2 Expression and Downregulation of Tenascin C

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Abstract

Background and aims: The cellular mechanisms leading to infliximab therapy response in patients with ulcerative colitis (UC) are incompletely known. We therefore investigated early effects of infliximab therapy on monocytes and associated chemokines linked to clinical therapy response in UC patients.

Methods: Blood and biopsies were obtained from anti-TNF therapy-naïve UC patients (n = 43) before (baseline) and during induction therapy with infliximab. Therapy response was evaluated at Week 14. Expression of monocyte activation markers and levels of chemokines in serum and biopsies were determined. Quantitative proteomic analysis was performed in cultured mucosal biopsies, and obtained data was validated in serum.

Results: In therapy responders, but not in non-responders, infliximab reduced blood monocyte expression of CD14 and CD86, 2 weeks after therapy commenced, relative to baseline. Serum CCL2 levels were decreased only among therapy responders at Week 2 and Week 14, relative to baseline. These data corresponded with lower levels of CD14, CD86 and CCL2 in intestinal tissue in responders as compared with non-responders at Week 14. Proteomic analysis of cultured biopsies showed that infliximab induced a reduction in Tenascin C that predicted downregulation of CCL2. Therapy responders, but not non-responders, had decreased serum Tenascin C levels at Week 2 and Week 14, relative to baseline.

Conclusions: Infliximab therapy response in UC patients is associated with reduced monocyte activation and serum levels of CCL2 2 weeks after therapy commencement. In therapy responders, infliximab influenced Tenascin C, which might be a regulator of CCL2 expression and important for induction of the clinical therapy response.

Keywords: Ulcerative colitis; infliximab; biological therapy; monocyte; CCL2; Tenascin C
Infliximab reduces monocyte activation and recruitment

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) with unknown etiology manifested by an excessive immune response to the normal microbiota. Tumor necrosis factor (TNF) is an important mediator of the pro-inflammatory immune reaction in IBD and is produced by macrophages, monocytes and activated T cells. Anti-TNF antibodies have emerged as powerful therapy options in UC, and among these infliximab has been shown to induce a clinical response in 50–70% of the patients. However, the exact mechanisms behind the clinical response are unknown.

There is evidence that the infliximab therapy response is associated with alterations not only in adaptive immunity but also in cells of the innate immune system. In particular, infliximab induces Fc region–dependent induction of regulatory macrophages that are involved in mucosal healing. Also, a reduction in mucosal CD68+ macrophages as a result of apoptosis has been suggested as a key mechanism for therapeutic success. In relation to this, CD14+ macrophages expressing transmembrane TNF have been shown to indirectly induce apoptosis in T cells as a consequence of infliximab. These data underline the role of mucosal macrophages in the response to infliximab therapy:

Macrophages are derived from circulating monocytes recruited to the mucosa via chemokines. On site, the macrophages themselves express co-stimulatory molecules (such as CD86 and CD80) as well as multiple chemokines in order to attract different cell populations and regulate T-cell differentiation. In inflamed IBD mucosa, gene expression of chemokines has been observed in the form of upregulation of CCL2, CCL3 and CCL4. Anti-TNF therapy has been shown to restore mucosal chemokine expression 4–6 weeks after therapy commencement.

During inflammation, the extracellular matrix (ECM) is an important reservoir for cells and cytokines, but also acts as a transfer of danger signals. The pro-inflammatory ECM glycoprotein Tenascin C (TNC) is absent in most healthy tissues but is expressed during tissue injury, infection and inflammation. TNC has been shown to activate TLR4 signaling, resulting in maintained inflammation in arthritic joint disease. In addition, TNC induces expression of CCL2 and translation of pro-inflammatory cytokines.

Although it is clear that infliximab induces local changes in macrophage functions and regulates chemokine gene signatures, the co-stimulatory molecule CD86 on circulating monocytes and the serum levels of monocyte-related chemokines have not been studied in connection with the infliximab therapy response. Also, mechanistic pathways leading to these changes need to be defined.

Therefore, in this study we aimed to determine the dynamics of circulating monocytes and mucosal macrophage markers as well as to investigate peripheral and local chemokine expression before and during the first 14 weeks of infliximab therapy.

2. Materials and methods

2.1 Patient population

Patients with UC (n = 43) who were commencing infliximab therapy were consecutively recruited and included in the study at the outpatient clinics at Sahlgrenska University Hospital, Gothenburg, Karlskukhuset, Skövde, and Sodra Alvsborg Hospital, Boras, Sweden. All patients were previously anti-TNF–therapy naive. Clinical and demographic characteristics are presented in Table 1. The disease activity was determined by the Mayo score before therapy commenced (baseline) and at Week 14. All patients had active mucosal inflammation at inclusion with ≥2 in the endoscopic Mayo score, except for one patient who had an endoscopic Mayo score of 1. Infliximab treatment (5 mg/kg) was given at Weeks 0, 2, 6 and 14. Concomitant immunosuppressants were a range of combinations of corticosteroids, thiopurines and 5-ASA, as listed in Table 1.

Therapy response was defined as decrease in Mayo score of ≥3 compared with baseline, with a reduction of at least 1 in endoscopic Mayo score for indication of mucosal healing. Patients not achieving this decrease in Mayo score were considered “non-responders”. The study was performed after receiving written informed consent from all subjects, and the protocol was approved by the Regional Ethical Review Board at the University of Gothenburg.

2.2 Sample specimens

 Serum samples were obtained at baseline, at Week 2 (before treatment dose 2) and at Week 14 (before treatment dose 4) for all included patients. Ethylenediaminetetraacetic acid (EDTA)-treated blood samples for FACS analyses were obtained at baseline and at Week 2. Biopsy specimens (six biopsies per patient) from inflamed sigmoid colon were obtained from 11 of the patients at baseline and from the same area at Week 14 (two biopsies per patient). Two biopsies from each time point were snap-frozen in liquid nitrogen and stored at −80°C. At baseline, the remaining four biopsies were collected and cultured for 24 h in Iscove’s medium supplemented with 5% human AB+ serum, 100 µg/mL gentamicin (Sigma, St Louis, MO) and 3 µg/mL L-glutamine (Sigma). Two biopsies were cultivated with, and two biopsies without, the addition of 1 mg/mL infliximab. At the end of the cell culture, the biopsies and supernatants were collected and duplicate biopsies were pooled and stored at −80°C.

2.3 Isolation of peripheral blood lymphocytes and flow cytometric staining and analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-treated venous blood by density-gradient centrifugation on Ficoll–Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Isolated cells were stained for flow cytometry analysis using combinations of the following antibodies: anti-CD14-fluorescein isothiocyanate (FITC), anti-CD86-PE, anti-TNFRI-PE and 7AAD (all from BD Biosciences, San Jose, CA). Flow cytometry analysis was performed using an LSR II flow cytometer (BD Pharmingen), and at least 10,000 lymphocytes per sample were analyzed, as defined by forward and side scatter. Live monocytes were identified as FSC<SSC<7AAD<CD14+. The data were analyzed using FlowJo software (Treestar Inc, Ashland, OR).

2.4 Protein extraction from biopsies

Mucosal biopsies were transferred to lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 1% Nonidet P40, 0.02% NaN3, 1 mM PMSF and protease inhibitor cocktail, all from Sigma–Aldrich, St Louis, USA) and dispersed using an ULTRA-TURRAX® T25 (IKA® Werke Staufen, Germany) for 20 s. The

Abbreviations:

UC ulcerative colitis
IBD inflammatory bowel disease
TNF tumor necrosis factor
ECM extracellular matrix
TNC Tenasin C
PBMC peripheral blood mononuclear cell
MS mass spectrometry
IPA ingenuity pathway analysis
IPA KB Ingenuity Pathways Analysis Knowledge Base
MAP molecule activity predictor
Table 1. Demographics of anti-TNF therapy-naïve UC patients before infliximab therapy commencement.

<table>
<thead>
<tr>
<th></th>
<th>Responders(^a)</th>
<th>Non-responders(^b)</th>
<th>p-value(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of patients</td>
<td>21</td>
<td>22</td>
<td>&gt;0.99</td>
</tr>
<tr>
<td>Male/female</td>
<td>15/6</td>
<td>16/6</td>
<td>0.13</td>
</tr>
<tr>
<td>Age</td>
<td>29 (19–54)(^d)</td>
<td>38 (18–67)</td>
<td>0.76</td>
</tr>
<tr>
<td>Smoking habit (active/ex-smoker/never)</td>
<td>0/5/16</td>
<td>2/4/16</td>
<td>0.62</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>5 (1–22)</td>
<td>3 (1–24)</td>
<td>0.38</td>
</tr>
<tr>
<td>Mayo score</td>
<td>8 (3–10)</td>
<td>8 (5–11)</td>
<td>0.39</td>
</tr>
<tr>
<td>CRP</td>
<td>3 (1–73)</td>
<td>5 (1–110)</td>
<td>0.14</td>
</tr>
<tr>
<td>Other treatments than anti-TNF:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosteroids, 5-ASA, thiopurines</td>
<td>4</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids, 5-ASA</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Thiopurines, 5-ASA</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids, thiopurines</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5-ASA</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thiopurines</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\)Defined as a decrease in Mayo score ≥3, 12–14 weeks after therapy commencement.

\(^{b}\)Defined as a decrease in Mayo score of ≤2, 12–14 weeks after therapy commencement.

\(^{c}\)Mann–Whitney Test.

\(^{d}\)Data are shown as median (range).

homogenate was incubated for 2 h at 4°C, centrifuged at 10 000 g for 20 min, and the supernatants were analyzed by ELISA for HPRT concentration (Cloud-Clone Corp, USA). HPRT concentrations were used for normalization of chemokines, soluble receptors and TNC.

2.5 Chemokine, soluble receptor and TNC assays
ELISA was used to measure biopsy levels of CD14 and CD86 (Diaclone SAS, Besançon, France). Levels of CCL2, CCL3, CCL4, CXCL1, CXCL10 and TNFRI were measured by cytokine bead array (BD Biosciences). TNC was measured by the Tenascin C Large (Fn III C) ELISA (IBL International GmbH, Hamburg, Germany) and the Human Tenascin C Kit (Meso Scale Discovery, MD®, Rockville, USA). All assays were used according to the manufacturers’ instructions.

2.6 Proteomic analysis
Proteomic analysis using mass spectrometry (MS) was performed at the Proteomics Core Facility at Sahlgrenska Academy, University of Gothenburg, Sweden. To summarize, cultured mucosal biopsies from 3 responders (with and without anti-TNF) and 3 non-responders (with and without anti-TNF) lysis buffer (8 M urea, 4% CHAPS, 0.1% SDS, 10 mM EDTA, 5 mM TCEP) and sonicated in a water bath for 2×20 s with a 20-s rest between bursts. The biopsies were homogenized using a pellet pestle (Sigma–Aldrich Sweden AB, Sweden). The homogenate was incubated for 1 h at room temperature with shaking, followed by centrifugation at 16 000 g for 10 min; an overnight tryptic digestion was carried out at 37°C. The protein concentration was determined using the Pierce BCA Protein Assay (Thermo Scientific Inc., IL, USA). Digested peptides were labelled with TMT (tandem mass tag) following the manufacturer’s instructions (Thermo Scientific Inc.) for a sixplex Isobaric Mass Tagging Kit. Two groups of six labelled samples were produced (one from cultured biopsy samples with and without anti-TNF from three responders and the other one from three non-responders) and mixed to form sixplex sets. The complexity of each sixplex set was reduced by fractionation using strong cation exchange chromatography. Each fraction was then desalted and subjected to nano-liquid chromatography-MS/MS using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, Inc., Waltham, MA, USA) mass spectrometer interfaced with an Easy-nLC autosampler (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a nano-LC column, 200×0.075 mm, packed in-house with 3-μm Reprosil-Pur C18-AQ particles (Dr. Maisch, Ammerbuch, Germany). The separation used a gradient of up to 80% of acetonitrile in 0.2% formic acid, starting with 5:95 acetonitrile to 0.2% formic acid. Ions were injected into the LTQ-Orbitrap Velos mass spectrometer (under a spray voltage of 1.6 kV in positive ion mode, with MS scans at 30 000 resolution (at m/z 400), mass range of m/z 400–1 800, and MS/MS scans at 7 500 resolution (at m/z 400) with a mass range of m/z 120–2 000 and stepped high energy collision dissociation (HCD) energies of 25%, 35% and 45%) for the top 10 most abundant doubly or multiply charged precursor ions in each MS scan. The fractionated sample was analyzed twice, excluding identified peptides from the first run in the second analysis.

All resulting spectra per TMT set were merged for relative quantification and protein identification using Proteome Discoverer version 1.3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with the Mascot search engine (Matrix Science Ltd, London, UK) using the human Swissprot Database version 2.3 from October 2010. The following settings were selected: MS peptide tolerance of 10 ppm, MS/MS tolerance of 100 molecular mass units, tryptic peptides accepted with one missed cleavage, variable modifications of methionine oxidation, cysteine methylthiolation, and fixed modifications of methionine oxidation, cysteine methylthiolation, and fixed modifications of N-terminal TMT6plex and lysine TMT6plex. The peptide threshold was set to 1% False Discovery Rate by searching against a reversed database, and identified proteins were grouped by sharing the same sequences to minimize redundancy. For TMT quantification, the ratios of the TMT reporter ion intensities in MS/MS spectra were derived with tolerance as 80 ppm for the most confident centroid peak; only peptides unique for a given protein were considered, excluding those common to other isoforms or proteins of the same family. The quantification was normalized using the protein median.

2.7 Bioinformatic analysis of protein data
Data were analyzed using IPA (Ingenuity pathway analysis) (Ingenuity® Systems, http://www.ingenuity.com). Prior to the analysis, up- or downregulation of proteins as a result of anti-TNF were
calculated for responders and non-responders. Only proteins significantly up- or downregulated (p-values < 0.05) were uploaded and analyzed. IPA uses the Ingenuity Pathways Analysis Knowledge Base (IPA KB), a manually curated database of protein interactions from the literature. These proteins and their association with the IPA KB are used to generate networks and perform functional pathway analyses. In addition, the molecule activity predictor (MAP) was used to anticipate probable events that may happen downstream of the changes in the pathway.

2.8 Statistical analysis

The Wilcoxon signed rank test was used to evaluate differences between two sets of paired samples, and the Mann–Whitney U test was performed to evaluate differences between two groups, using GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA). For the proteomic analysis, differences between groups were calculated with Welch’s t-test. Those p-values < 0.05 were considered statistically significant.

3. Results

3.1 Early changes in circulating monocyte marker expression was linked to clinical therapy response

To monitor early changes in monocyte activation, cell surface expression of CD14, CD86 and TNFRI on circulating monocytes 2 weeks after infliximab treatment commenced with baseline. Monocytes were identified as FSC<8SSC<7AADCD14< cells, and the median fluorescence intensity (MFI) of CD14, CD86 and TNFRI was determined (Figure 1A). There was a decreased expression level of CD14 on monocytes among responders 2 weeks after therapy commenced, while the expression of CD14 tended to increase in non-responders (Figure 1B). Similarly, the expression of CD86 on monocytes was reduced in responders but increased in non-responders 2 weeks after therapy commenced, as compared with baseline (Figure 1C). No changes in the expression profile of TNFRI were detected after 2 weeks of therapy in either responders or non-responders (Figure 1D). Also, no differences in frequencies of monocytes among total PBMCs could be detected between baseline and Week 2 for responders (12.2% (7.7–18.7) vs. 11.9% (7.8–16.9), p = 0.63) or non-responders (13.4% (8.3–21.4) vs. 12.1% (9.8–13.9), p = 0.28).

When studying the MFI of CD14, CD86 and TNFRI expression on circulating monocytes after 14 weeks of therapy (compared with baseline), neither therapy responders nor non-responders demonstrated reduced cell surface markers expression. In fact, a slight increase in TNFRI expression was detected among therapy non-responders (Table 2).

In order to investigate whether the altered activation profile of peripheral monocytes reflected events in the intestinal tissue, the same receptors were analyzed in the colonic mucosa at baseline and 14 weeks after therapy commenced for 11 of the patients. At baseline there were no differences in protein levels of CD14, CD86 and TNFRI between therapy responders (n = 7) and non-responders (n = 4) (Table 3). However, at Week 14, responders had lower protein levels of CD14 and CD86 in biopsies than non-responders, indicating more activated tissue-resident macrophages in therapy non-responders (Figure 2).

3.2 Infliximab treatment induced short- and long-term changes in serum levels of monocyte-related chemokines

Chemokines recruiting monocytes or being produced by macrophages were analyzed in serum in order to assess the effects of infliximab on cell recruitment. Serum levels of CCL2, CCL3, CCL4, CX3CL1 and CXCL10 were determined at baseline, Week 2 and Week 14. At baseline, there were no differences in serum levels of

Figure 1. Surface receptor expression on circulating monocytes in UC patients undergoing infliximab therapy. The surface phenotype of freshly isolated circulating monocytes was examined using flow cytometry. PBMCs were isolated from UC patients before the first anti-TNF treatment (baseline) and at Week 2. (A) Monocytes were identified as FSC<8SSC<7AADCD14< cells, and the median fluorescence intensity (MFI) was determined for CD14, CD86 and TNFRI. Histograms show antibody staining (dark gray) and fluorescence minus one control (light gray). MFI for CD14 (B), CD86 (C) and TNFRI (D) was determined for therapy responders and non-responders. Each symbol represents the MFI of the examined cell populations of one individual. Connecting lines show values from samples taken from the same individual.
the chemokines between responders and non-responders (Figure 3). CCL2 was found to be reduced at Week 2 and Week 14, respectively, as compared with baseline in therapy responders but not in non-responders (Table 4). Likewise, CXCL10 was reduced in therapy responders at Week 14 as compared with baseline. However, CXCL10 was reduced at Week 2 as compared with baseline in both therapy responders and non-responders (Table 4). No changes were identified at any time point for CCL3 and CX3CL1, while CCL4 was significantly reduced in both patients groups and stayed low over time, regardless of therapy response (Table 4).

To investigate if serum levels of chemokines reflected the intestinal expression, the same chemokines were analyzed in the colonic mucosa at baseline and 14 weeks after therapy commenced. At baseline there were no differences in any chemokine between therapy responders and non-responders (Table 3). At Week 14, responders had lower protein levels of CCL2 and tended to have lower levels of CCL3, CCL4 and CX3CL1 in biopsies, as compared with non-responders (Figure 4).

3.3 Proteomic analysis revealed Tenascin C as an important modulator of CCL2 expression
To further investigate the mechanistic pathway leading to the reduced chemokine expression associated with infliximab therapy response, a proteomic analysis of biopsies obtained from six patients (three responders and three non-responders) was performed. Biopsies were cultured with and without infliximab in vitro in order to identify

Table 2. Surface receptor expression on circulating monocytes at baseline and Week 14 for patients undergoing infliximab therapy.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Baseline</th>
<th>Week 14</th>
<th>p-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 (MFI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>5575 (2927–14136)(^b)</td>
<td>5572 (2442–11292)</td>
<td>0.38</td>
</tr>
<tr>
<td>Non-responder</td>
<td>3359 (2467–6771)</td>
<td>5754 (3068–8437)</td>
<td>0.15</td>
</tr>
<tr>
<td>CD86 (MFI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>2862 (2062–3996)</td>
<td>4074 (2496–5600)</td>
<td>0.32</td>
</tr>
<tr>
<td>Non-responder</td>
<td>2536 (1947–2823)</td>
<td>2693 (2367–3664)</td>
<td>0.20</td>
</tr>
<tr>
<td>TNFRI (MFI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>897 (656–1176)</td>
<td>559 (414–1545)</td>
<td>0.76</td>
</tr>
<tr>
<td>Non-responder</td>
<td>668 (617–1276)</td>
<td>958 (547–2114)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

\(^a\)Wilcoxon signed rank test.
\(^b\)Data are shown as median (25–75 percentile).

Table 3. Mucosal levels of surface receptors, chemokines and TNC at baseline for patients undergoing infliximab therapy.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Sample Type</th>
<th>Responder</th>
<th>Non-responder</th>
<th>p-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14 (ng/mL)</td>
<td></td>
<td>0.4 (0.3–0.9)</td>
<td>0.8 (0.4–1.4)</td>
<td>0.44</td>
</tr>
<tr>
<td>CD86 (ng/mL)</td>
<td></td>
<td>0.2 (0.1–0.3)</td>
<td>0.3 (0.1–0.4)</td>
<td>0.15</td>
</tr>
<tr>
<td>TNFRI (ng/mL)</td>
<td></td>
<td>10.2 (3.1–18.3)</td>
<td>12.4 (2.1–24.3)</td>
<td>0.79</td>
</tr>
<tr>
<td>CCL2 (pg/mL)</td>
<td></td>
<td>5.1 (2–17)</td>
<td>3.7 (2.7–14.2)</td>
<td>0.74</td>
</tr>
<tr>
<td>CCL3 (pg/mL)</td>
<td></td>
<td>3.7 (1.7–6.9)</td>
<td>2.5 (0.9–7.4)</td>
<td>0.84</td>
</tr>
<tr>
<td>CCL4 (pg/mL)</td>
<td></td>
<td>6.6 (2.3–38)</td>
<td>2.8 (2.3–22.4)</td>
<td>0.57</td>
</tr>
<tr>
<td>CX3CL1 (pg/mL)</td>
<td></td>
<td>2.6 (1.4–7.7)</td>
<td>0.9 (0.8–4.4)</td>
<td>0.48</td>
</tr>
<tr>
<td>CXCL10 (pg/mL)</td>
<td></td>
<td>13.1 (4.4–37.5)</td>
<td>12.3 (5.1–16.4)</td>
<td>0.65</td>
</tr>
<tr>
<td>TNC (pg/mL)</td>
<td></td>
<td>2416 (719–6718)</td>
<td>1463 (1050–4064)</td>
<td>0.71</td>
</tr>
</tbody>
</table>

\(^a\)Wilcoxon signed rank test.
\(^b\)Data are shown as median (25–75 percentile).
regulation of mucosal protein expression as a consequence of the addition of anti-TNF antibodies. Interestingly, significantly up- or downregulated proteins differed between therapy responders (number of proteins = 43, Table S1) and non-responders (number of proteins = 37, Table S2), with no proteins in common for both groups.

IPA analysis of infliximab-regulated proteins identified a network in which TNF influenced TNC, which in turn regulated CCL2 and CXCL10 (Figure 5). A reduced TNC expression was detected (protein expression with/without infliximab; median fold change 0.89, p < 0.05) as a consequence of infliximab, which predicted decreased

Table 4. Serum levels of chemokines at baseline, Week 2 and Week 14 for patients undergoing infliximab therapy.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Baseline</th>
<th>Week 2</th>
<th>p-value</th>
<th>Week 14</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>169 (103–228)</td>
<td>132 (83–172)</td>
<td>0.03</td>
<td>126 (74–188)</td>
<td>0.009</td>
</tr>
<tr>
<td>Non-responder</td>
<td>118 (87–177)</td>
<td>133 (80–219)</td>
<td>0.22</td>
<td>128 (73–147)</td>
<td>0.16</td>
</tr>
<tr>
<td>CCL3 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>11.4 (7.8–15.7)</td>
<td>8.8 (5.5–16.8)</td>
<td>0.92</td>
<td>11.6 (4.8–16.7)</td>
<td>0.70</td>
</tr>
<tr>
<td>Non-responder</td>
<td>12.9 (9.1–25.2)</td>
<td>13.9 (6.4–18.3)</td>
<td>0.62</td>
<td>7.9 (3.9–15.3)</td>
<td>0.25</td>
</tr>
<tr>
<td>CCL4 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>84 (42–116)</td>
<td>49 (23–64)</td>
<td>0.001</td>
<td>43 (19–60)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Non-responder</td>
<td>80 (53–177)</td>
<td>66 (29–105)</td>
<td>0.02</td>
<td>44 (24–66)</td>
<td>0.001</td>
</tr>
<tr>
<td>CX3CL1 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Responder</td>
<td>12.5 (5.7–25.9)</td>
<td>21.3 (7.1–27.5)</td>
<td>0.59</td>
<td>17.2 (0–34.9)</td>
<td>0.67</td>
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<tr>
<td>Non-responder</td>
<td>25.8 (0.3–43.6)</td>
<td>15.2 (0.4–11.7)</td>
<td>0.37</td>
<td>11.6 (1–24.3)</td>
<td>0.24</td>
</tr>
<tr>
<td>CXCL10 (pg/mL)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Responder</td>
<td>213 (148–288)</td>
<td>105 (87–137)</td>
<td>&gt;0.0001</td>
<td>158 (111–202)</td>
<td>0.02</td>
</tr>
<tr>
<td>Non-responder</td>
<td>224 (164–383)</td>
<td>137 (90–222)</td>
<td>0.01</td>
<td>157 (107–190)</td>
<td>0.63</td>
</tr>
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*p-value (Wilcoxon signed rank test) for baseline vs. Week 2.
*p-value (Wilcoxon signed rank test) for baseline vs. Week 14.
Data are shown as median (25–75 percentile).

IPA analysis of infliximab-regulated proteins identified a network in which TNF influenced TNC, which in turn regulated CCL2 and CXCL10 (Figure 5). A reduced TNC expression was detected (protein expression with/without infliximab; median fold change 0.89, p < 0.05) as a consequence of infliximab, which predicted decreased

Figure 3. Baseline levels of serum chemokines for infliximab therapy responders and non-responders. Serum was obtained before therapy commenced, and the levels of CCL2, CCL3, CCL4, CX3CL1 and CXCL10 were analyzed by cytokine bead array. Each symbol represents one individual.

Figure 4. Chemokine expression in intestinal biopsies 14 weeks after infliximab therapy initiation. Biopsies were obtained from the sigmoid colon at Week 14. The biopsies were dispersed, and protein levels of CCL2, CCL3, CCL4, CX3CL1 and CXCL10 were analyzed by cytokine bead array. Protein concentrations were normalized to HPRT. Each symbol represents one individual.
CCL2, CXCL10 and IL-8 expression in therapy responders (Figure 5A). TNC was also detected in biopsies from non-responders, but was not regulated in the presence of infliximab (median fold change 1.32, \( p = 0.85 \)). Since no changes were detected, no predictions of outcome as a consequence of infliximab could be made via IPA for non-responders (Figure 5B). Levels of TNC in uncultivated biopsies were similar for responders and non-responders (Table 3).

In order to verify the proteomic data, TNC levels were measured in serum at baseline, Week 2 and Week 14. For therapy responders, there was a marked decrease in levels of TNC both at Week 2 and Week 14, as compared with baseline (Figure 6A and B). In contrast, no changes in serum levels of TNC were detected for non-responders (Figure 6A and B). At Week 14, no differences in TNC levels in the colonic mucosa were detected between responders and non-responders (Figure 6C). No correlation between changes in serum levels of CCL2 and CXCL10 vs. TNC from baseline to Week 2 or 14 could be detected (data not shown).

4. Discussion

In this study we demonstrate that an early decrease in monocyte activation and reduction in serum levels of CCL2 are associated with infliximab therapy response. In conjunction with this, we report decreased levels of circulating TNC in therapy responders, indicating reduced pro-inflammatory effects imposed by the danger signal TNC.

CD14+ macrophages, producers of pro-inflammatory cytokines and chemokines, are recruited from the circulation and accumulate in the intestinal mucosa during inflammation.22 Already in the circulation, blood monocytes express the activation molecule CD86 and the LPS co-receptor CD14; however, no CD80 expression can be detected until the cells reach the periphery.11 We found that responders to infliximab therapy have reduced levels of CD86 and CD14 on monocytes 2 weeks after therapy initiation, suggesting that the monocytes of these patients are less responsive to inflammatory stimuli when reaching the intestinal mucosa. This could be of importance for dampening the inflammatory reaction, because local immune modulatory mediators generating tolerance are absent at inflammation.22 We also showed that levels of CD86 and CD14 on monocytes returned to baseline at Week 14, irrespective of response to the therapy, demonstrating that the altered activation profile of circulating monocytes was transient and reverted over time. In contrast, the expression of CD86 and CD14 in the mucosa was lower among responders than non-responders at Week 14, indicating that the effects of infliximab therapy persisted over time in the intestinal milieu of therapy responders.

Because chemokines are important both for recruitment and activation of leukocytes,23 we studied five chemokines related to macrophages. CX3CL1 (fractalkine) is produced by epithelial cells, recruits monocytes and T cells, and also functions as an adhesion marker.24,25 No changes in serum levels were detected for this chemokine, indicating that infliximab did not have any effects on its expression or that the effects were only local. CCL3 (mip-1\( \alpha \)) and CCL4 (mip-1\( \beta \)) are produced by macrophages, and the production is induced by TNF. Both chemokines are important for the recruitment of T cells, monocytes, neutrophils and NK cells, and mucosal levels are increased in UC patients with active disease.26 Interestingly, a striking downregulation of CCL4 was detected at Week 2 and Week 14 compared with baseline, irrespective of therapy response. Thus, anti-TNF therapy exerted effects not only in therapy responders but also in non-responders, reducing the expression of CCL4, although this was not linked to therapy response. Correspondingly, it has been shown that patients with Crohn’s disease who are not responding to anti-TNF therapy downregulate mucosal gene expression of several genes such as IL-6 and CD69.27 Accordingly, far from all anti-TNF–induced alterations of immune factors can be linked to therapy response, and it is important to identify the true response parameters.

The two chemokines that could be linked to therapy response were CCL2 (mcp-1) and CXCL10 (IP-10). These are produced by inflammatory cells and stromal cells, recruit leukocytes, and are induced in inflamed UC mucosa.26,28 Also, CCL2-deficient mice show reduced severity of 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis compared with wild type mice.29 A randomized phase II clinical study of CXCL10-blocking antibody (BMS-966557) for treatment of moderately to severely active UC was potentially effective.30 Together these data support the importance of CCL2 and CXCL10 in the control of intestinal inflammation. However, it is unclear why some patients downregulate these chemokines (responders) while others do not (non-responders).

Figure 5. Mechanistic network analysis of biopsies cultured in vitro in the presence of anti-TNF. Biopsies from inflamed intestinal mucosa were obtained from three responders and three non-responders at baseline, cultured for 24h (with or without 1\( \mu \)g/ml infliximab) and analyzed by MS quantitative proteomics. Significantly up- or downregulated proteins were studied by IPA. Data show networks for infliximab therapy responders (A) and non-responders (B). The molecule activity predictor (MAP) was used to identify upregulated proteins (red nodes), downregulated proteins (green nodes) or detected but not regulated proteins (gray nodes). Blue colours show anticipated inhibition generated by MAP. Red lines indicate anticipated induction. Stronger colours indicate higher regulation of the complex. Infliximab, acting on TNF, is indicated in the network.
In order to explore this, we performed proteomic analysis of biopsies cultured with or without infliximab. Proteomic analyses enable discovery of previously unknown proteins that can be linked to a defined process. It is, however, necessary to verify the data after detection because the proteomic tool may only be considered an indicator, not a validator. Other protein analyses, such as western blotting or ELISA, only measure predefined proteins. Here, we wanted to perform an unbiased assay to explore the impact of infliximab at the initial stage; thus, we cultivated biopsies with and without a low dose of infliximab (1 µg/ml) for 24 h and found a downregulation of TNC in therapy responders. By using the molecule activity predictor in IPA, it was predicted that a reduction in TNC downregulates CCL2, CXCL10 and TLR4; thus, TNC is closely linked to inflammation. Previous work has indeed shown that TNF enhances TNC expression, leading to higher CCL2 expression, both in IBD and rheumatoid arthritis.\(^\text{30,31}\) Also, it has been shown that TNF enhances TNC expression, leading to higher CCL2 expression.\(^\text{32}\) In addition, patients with Crohn’s disease responding to infliximab therapy have been shown to express reduced levels of TNC in the subepithelial mucosal layer at Week 10 and Week 52 post therapy commencement.\(^\text{33}\) However, TNF has not been studied among patients with UC undergoing anti-TNF therapy, and TNC levels in serum samples have not been determined. Moreover, there are no previous studies of TNC levels after a short duration of anti-TNF therapy, or comparing TNC levels between therapy responders and non-responders. In order to confirm the proteomic data, we studied TNC levels in serum from patients taken at 2 weeks and 14 weeks post therapy commencement (compared with baseline). A reduction in TNC levels was evident for therapy responders, but not for non-responders, even by Week 2. Consequently, downregulation of the pro-inflammatory molecule TNC is important for induction of infliximab therapy response. TNC is expressed by a large variety of cells, including fibroblasts, glial cells, endothelial cells, epithelial cells and macrophages,\(^\text{34–37}\) and has been found to localize both to the lamina propria and the subepithelium.\(^\text{38}\) The expression itself is induced through a range of signaling pathways, including ROS/Nrf2, ERK1/2, β-catenin/Wnt and Ras/MAPK,\(^\text{39–41}\) and the pathway/cell type important for anti-TNF therapy response warrants further investigation. However, we could not establish a correlation between the reduction in levels of TNC, CCL2 and CXCL10—which may be due to the low number of study subjects, or it might be that Week 2 and 14 are suboptimal sampling time points.

What are the clinical implications for these results? First, we have identified several parameters (such as CD86 and CD14 expression on circulating monocytes, together with serum levels of CCL2 and TNC) to be indicators of early response to infliximab therapy. Second, we have recognized parameters that are being affected by the therapy, but are not linked to the therapy response. It is crucial to identify markers associated with a response before or during the early treatment phase. Thus, this study might be of value for the development of clinical biomarkers to identify a therapy response in patients undergoing infliximab therapy. Additionally, this study brings valuable insight into the mechanisms of the infliximab therapy response, which in the future might aid in appropriately identifying patients who are good candidates for successful therapy. However, there are limitations to this study. In particular the small sample size of biopsy specimens and biopsy experiments made it impossible to perform correlation studies in biopsy specimens and between biopsy and serum, even though samples originated from the same patients. For the proteomic study, the dose of infliximab and the duration of biopsy culture were chosen empirically, and these should be evaluated in more detail and compared with the in vivo situation. Also, concomitant therapies may confound data, and a more stringent study without parallel therapies would be desirable. However, concomitant therapies did not seem to have an impact on the anti-TNF therapy outcome in our study cohort.

In conclusion, we have demonstrated that the infliximab therapy response is associated with a reduced expression of activation molecules on circulating monocytes, along with lower levels of CCL2 and TNC in the serum 2 weeks after therapy commencement. The presence of infliximab downregulated TNC \textit{in vitro}, which predicted decreased CCL2 in therapy responders but not in non-responders. Thus, infliximab induced downregulation of TNC, which may be associated with, or act in parallel with, reduced monocyte activation and less chemokine secretion in therapy responders. These cellular differences may partially explain the different clinical outcomes of infliximab therapy in therapy responders and non-responders.

**Supplementary material**

Supplementary data to this article can be found online at: [http://www.ecco-jcc.oxfordjournals.org/](http://www.ecco-jcc.oxfordjournals.org/)

**Conflict of interest statement**

None declared.
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Statement of authorship: LÖ, MKM and HS contributed to the conception and design of the study. MKM and SI performed the experiments. HS, AB, AL and KAU recruited and enrolled patients in the study. All the authors contributed to the analysis and interpretation of data. LÖ and MKM wrote the manuscript, and HS, SI, AB, AL and KAU critically reviewed it and approved the final draft.

Competing interests: The authors have no competing interests.

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