CXCL13: a novel biomarker of B-cell return following rituximab treatment and synovitis in patients with rheumatoid arthritis

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Abstract

Objectives. The B-cell chemokine, CXCL13, is a proposed serum biomarker of synovitis in RA. Its behaviour in the context of B-cell depletion therapy and reconstitution was studied during treatment of RA with rituximab.

Methods. Serum samples from 20 RA patients were analysed for CXCL13, RF-IgM and anti-CCP by ELISA before and 2 and 6 months following rituximab treatment. B cells were monitored by flow cytometry. Gene expression in blood and synovial biopsies was determined by qPCR.

Results. Patients with detectable B cells at 6 months had significantly higher levels of CXCL13 and RF-IgM and slightly higher levels of anti-CCP throughout the study, including at baseline, compared with patients with undetectable B cells at 6 months. Conversely, 10 of 12 patients with high baseline CXCL13 had detectable circulating B cells at 6 months, whereas no B cells could be detected at 6 months in patients with low baseline CXCL13. Synovial CXCL13 expression at baseline correlated significantly with serum CXCL13 levels, and the synovium of patients with high serum CXCL13 expressed elevated levels of IL-1β, IL-8, MMP1 and MMP3. In addition, synovial CXCL13 expression correlated significantly with several synovial inflammatory markers.

Conclusions. Serum CXCL13 is predictive of the rate of B-cell repopulation following a course of rituximab in RA. Serum CXCL13 correlates with synovial CXCL13 measured at a single joint, suggesting synovitis as an important source of circulating CXCL13. Within the synovium, CXCL13 expression is highly correlated with markers of synovitis.


Key words: Rheumatoid arthritis, B-lymphocytes, Chemokine CXCL13, Biological markers, Synovitis.

Introduction

RA is characterized by synovial inflammation leading to destruction of cartilage and bone, and by elevated levels of circulating antibodies such as RF and antibodies to citrullinated proteins. [1] In some cases, RA synovia contains lymphocyte aggregates resembling germinal centres [2, 3] that are capable of sustaining immunoglobulin class switching [4] and autoantibody production [5]. Therapeutic options for RA include the anti-CD20 monoclonal antibody, rituximab. Rituximab treatment rapidly depletes circulating B cells, in some cases completely [6, 7], while the extent of synovial B-cell depletion is variable and much less complete [8–10]. Following rituximab treatment, circulating B cells slowly reappear at 6–9 months, although the rate of return varies greatly between patients [11, 12]. B-cell repopulation after rituximab treatment essentially recapitulates normal ontogeny, and echoes the repopulation following bone marrow transplants [13], such that the
earliest B cells to appear have the characteristics of naive, immature cells [11, 12]. The reason for the variable time to repopulation among patients is not understood.

CXCL13 (BCA-1, BLC) is a B cell-specific chemokine thought to play a key role in the organization of germinal centres [14]. Elevated levels of CXCL13 are observed in RA synovia containing lymphoid aggregates compared with the synovia with diffuse infiltration, and CXCL13 independently predicts the presence of such aggregates [15, 16]. Notably, circulating levels of CXCL13 were recently proposed as a biomarker for the severity of RA [17]. However, while CXCL13 is likely to play a central role during repopulation by B cells of inflamed tissues following depletion, there are currently no reports describing the relationship between CXCL13 and B-cell levels during rituximab therapy. We describe here for the first time the effect of rituximab therapy on circulating CXCL13 levels, and show that the rate of return of B cells can be predicted by the level of CXCL13 at baseline. Additionally, correlative studies suggest that the inflamed synovium is at least a partial source of circulating CXCL13 in RA.

Methods

The ARISE trial

The ARISE (Assessment of Rituximab’s Immunomodulatory Synovial Effects) trial was recently described in detail [9]. Briefly, patients received 1 g rituximab i.v. on Day 0, and again on Day 14, in the absence of peri-infusion steroids. Synovial biopsies of the knee or wrist were collected before and 8–9 weeks following treatment. Clinical outcomes were captured as detailed [5]. Blood was collected at the time of the first and second biopsy, and at 6 months following treatment. Clinical outcomes were captured as 28-joint DAS (DAS28). CD19 counts were determined by flow cytometry at baseline and 2-month biopsies, and at 6 months following treatment. Of 24 enrolled patients, 20 patients completed the 6-month protocol and are included in the serum analysis, and for 19 patients synovial biopsies were of sufficient quality for molecular analysis. Local Institutional Review Board (University of California at San Diego Institutional Review Board) approval was obtained, and all patients signed written informed consent before study entry.

Serum analysis

Serum was stored at –80°C in aliquots and analysed by colorimetric ELISA as follows: RF of the IgM subtype (RF-IgM; Alpco Diagnostics, Windham, NH, USA), anti-CCP IgG (Inova, San Diego, CA, USA) and CXCL13 (BCA-1; R&D Systems, Minneapolis, MN, USA). Standard curves were constructed by regression line fitting on log(absorbance) vs log(concentration). Samples outside standard ranges were re-analysed at more appropriate dilutions. The RF-IgM and anti-CCP quantitation was validated by TheraTest Laboratories (Chicago, IL, USA) with correlation coefficients between the two sites 0.87 and 0.93, respectively (n = 34, both P < 0.0001).

Real-time qPCR analysis

Total blood RNA was isolated from PaxGene tubes (Fisher Scientific, Pittsburg, PA, USA). Aliquots of synovial biopsy tissue were stored at −80°C in RNA-Stat (Tel-Test, Friendswood, TX, USA), and total RNA was isolated using RNeasy Lipid Tissue kits (Qiagen, Valencia, CA, USA). After reverse transcription, the resulting cDNA was subjected to real-time TaqMan qPCR as in Refs [5, 18] using GAPDH as a housekeeper. Briefly, resulting threshold cycle (Ct) data were normalized to standard curves constructed from cDNA from IL-1β-stimulated fibroblast-like synoviocytes [MMP1, MMP3, stromal cell-derived factor-1 (SDF1)], RAMOS (IgM, CD19, CD79A, CD79B), ARH-77 (IgG1), H929 (CD138) or ConA-stimulated human peripheral blood mononuclear cells (PBMCs) (all other genes including GAPDH), yielding cell equivalents. The ratio between the gene product of interest and GAPDH cell equivalents (relative expression units, REUs) is reported.

Statistical analysis

All data are reported as geometric mean (95% CIs). JMP statistical software (SAS Institute, Cary, NC, USA) was employed for all data analysis. Repeated-measures ANOVA followed by contrast testing, paired t-tests where appropriate and parametric correlation analysis were performed on log-transformed data. The Bonferroni P correction was applied for multiple comparisons and correlations. P < 0.05 was considered to be statistically significant.

Results

Effect of rituximab on serum CXCL13

Circulating B cells and other parameters are summarized in Table 1. At 6 months, CD19+ B cells could not be detected in half of the studied patients. There was only a small albeit statistically significant effect on serum anti-CCP IgG at both 2 and 6 months compared with baseline (Table 1). Serum RF-IgM decreased to ~60% of baseline at Month 6 (Table 1). Notably, the selective B cell chemokine CXCL13 was present in serum at high and variable levels at baseline, but was significantly reduced at 2 and 6 months following rituximab treatment (Fig. 1). CXCL13 baseline levels correlated significantly with both anti-CCP IgG (r = 0.66, P = 0.0016) and RF-IgM (r = 0.72, P = 0.0004).

Characteristics of patients with undetectable B cells at 6 months

In order to identify baseline differences between subjects with slower and faster B-cell return following depletion with rituximab, patients were partitioned into groups with...
UNDetectable and detectable B cells at 6 months following rituximab treatment. Anti-CCP IgG, RF-IgM and CXCL13 were all significantly higher at all time-points, including baseline, in patients with detectable circulating CD19⁺ B cells at Month 6 (Fig. 2). In particular, baseline RF-IgM and CXCL13 differences were highly significant (detectable vs undetectable \( P < 0.001 \)). However, the significant decrease in RF-IgM and CXCL13 following rituximab treatment was observed equally in patients with undetectable and detectable B cells at 6 months (time \( P < 0.0001 \) within both populations).

**Baseline serum CXCL13 predicts rate of return of B cells**

The large difference in baseline RF-IgM and CXCL13 between patients with slower and faster returning B cells indicated that either of these parameters might be used to prospectively predict the B-cell rate of return following rituximab depletion. However, examination of the data distribution at baseline revealed that log-transformed serum RF-IgM could be described as one continuous population to which a single normal distribution could easily be fitted (Fig. 3A), thus rendering objective partitioning difficult. In contrast, log-transformed serum CXCL13 data displayed two distinct peaks (Fig. 3B) and could indeed be partitioned into two groups by iterative variance minimization (Fig. 3C) where the geometric mean (95% CIs) of baseline CXCL13 was 330 (259, 421) and 80 (65, 98) pg/ml, \( n = 12 \) and 8, respectively. The cut-off occurred between 112 and 189 pg/ml.

Interestingly, when comparing the high and low baseline CXCL13 groups, there was no difference in circulating B cells at baseline or at 2 months following rituximab treatment (Fig. 3D). However, at 6 months, all the patients in the low baseline CXCL13 group had undetectable B cells, whereas 10 of 12 patients in the high baseline CXCL13 group had circulating B cells that could easily be detected \( (P < 0.0001) \). Expression of the B-cell-associated markers CD79A (Fig. 3E) and IgM heavy constant chain (Fig. 3F) was also significantly different in total blood cDNA, as was expression of CD19 and IgG1 heavy constant chain (data not shown). On the other hand, expression of CD79B, a shared marker for B cells and plasma cells [19], was not different between the two groups at any time point, nor was IgA1 heavy constant chain (data not shown). As expected from the earlier described correlations with circulating CXCL13, anti-CCP IgG and RF-IgM serum levels were significantly higher at baseline in the high baseline CXCL13 group than in the low CXCL13 group, and remained so at 2 and 6 months following rituximab treatment, while RF-IgM was significantly \( (P < 0.0001) \) lowered to the same degree within both groups (data not shown). There was no difference in baseline DAS-28 between the low and high baseline CXCL13 groups [geometric mean (95% CI) 6.3 (5.5, 7.1) and 6.3 (5.8, 6.9), respectively], and rituximab lowered DAS-28 in both groups to a similar

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**Table 1** Overall effect of rituximab in the ARISE trial

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 months</th>
<th>6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating CD19⁺ cells, mean (95% CI), n/μl</td>
<td>113⁺ (78, 165)</td>
<td>1 (0.8, 1.4)*</td>
<td>2.9 (1.2, 6.9)*</td>
</tr>
<tr>
<td>No. of patients with undetectable CD19⁺ cells</td>
<td>0/20</td>
<td>7/20</td>
<td>10/20</td>
</tr>
<tr>
<td>RF-IgM, mean (95% CI), U/ml</td>
<td>19 176 (8553, 42 990)</td>
<td>13 375 (6286, 28 457)*</td>
<td>11 190 (4872, 25 703)*</td>
</tr>
<tr>
<td>RF-IgM (% of baseline)</td>
<td>100</td>
<td>68.8 (61.6, 79.0)</td>
<td>58.4 (45.1, 75.5)</td>
</tr>
<tr>
<td>Anti-CCP IgG, mean (95% CI), U/ml</td>
<td>8402 (2756, 25 618)</td>
<td>7816 (2623, 23 288)*</td>
<td>7337 (2424, 22 208)*</td>
</tr>
<tr>
<td>Anti-CCP IgG (% of baseline)</td>
<td>100</td>
<td>93.0 (87.4, 99.0)</td>
<td>87.3 (77.4, 98.5)</td>
</tr>
<tr>
<td>DAS-28, mean (95% CI)</td>
<td>6.3 (5.8, 6.8)</td>
<td>(4 months) 4.6 (4.0, 5.4)*</td>
<td>4.6 (4.1, 5.2)*</td>
</tr>
</tbody>
</table>

⁺n = 20. *P < 0.025 to baseline by paired t-test on log-transformed data.
The ability of baseline CXCL13 levels to predict return of detectable circulating CD19+ B cells at 6 months was then examined. Using the cut-off described above, a patient with high CXCL13 levels at baseline could be predicted to have detectable B cells at 6 months with a sensitivity of 80% and a specificity of 100%.

Since baseline circulating CXCL13 correlated with synovial CXCL13 and appeared to co-vary with synovial inflammation, the association between synovial CXCL13 and synovial inflammatory biomarker expression was next examined. A total of 18 synovial biomarkers were tested for their association with CXCL13, yielding a Bonferroni-adjusted critical $P$-value of 0.0027. Significant correlations were observed with IL-1β, IL-6, IL-8, MMP1, MMP3, IgM heavy and Ig-κ light constant chain, BAFF, CD19, CD79A, CD138 and CD3E (Table 2). On the other hand, not all inflammatory biomarkers correlated with CXCL13. The synovial expression of TNF-α, CCL21, IgG1 and IgA1 heavy constant chains, SDF1 or APRIL were not significantly associated with synovial CXCL13 expression (Table 2). Taken together, these findings are consistent with the notion that synovial CXCL13 production reflects the degree of synovial inflammation, and in turn constitutes a significant source of circulating CXCL13 protein.

**Discussion**

A role for B cells in the pathobiology of at least a subset of RA patients has been established by the clinical efficacy of rituximab. However, the exact mechanism by which B-cell depletion diminishes disease activity remains elusive. Downstream B-cell-related products such as circulating autoantibody levels are only partially albeit significantly lowered [20], a finding that was repeated in the current study. In addition, the extent of B-cell depletion in the synovium is quite variable compared with the near complete and consistent depletion of circulating B cells [8–10]. A similar variability is observed in bone marrow B-cell populations [8, 21], supporting the hypothesis that tissue-residing B cells might be partially protected from the effects of rituximab. Thus, trafficking and repopulation patterns of B cells following rituximab
are relevant to the mechanism of action of rituximab and potentially the pathogenesis of RA. Following treatment, the time to B-cell return varies greatly from patient to patient, and in a minority of cases can be delayed past 104 weeks [22]. We have identified the chemokine, CXCL13, as a novel serum predictor of the rate of return of B cells following rituximab treatment.

CXCL13 is a B-cell attractant and is crucial for germinal centre formation. Follicular Th cells also exhibit chemotactic responses towards CXCL13 [23]. Elevated levels of synovial CXCL13 in RA are associated with the presence of synovial lymphoid aggregates that resemble germinal centres [15, 16], although CXCL13 production is also observed in some synovial tissues with diffuse lymphoid infiltration [24]. Serum CXCL13 was earlier proposed as a disease activity biomarker in RA and could distinguish between active and quiescent disease [17], which agrees well with our finding that patients with elevated circulating CXCL13 levels have significantly higher levels of synovial inflammatory biomarkers. While the source of circulating CXCL13 is uncertain, we show here for the first time that serum CXCL13 protein levels and synovial CXCL13 mRNA expression co-vary to a highly significant degree, suggesting that the inflamed synovium constitutes an

**Fig. 3** Partitioning of baseline serum CXCL13 into two distinct populations and their properties. A single distribution can be fitted to RF-IgM data (A), but not to CXCL13 data (B) which represents two distinct populations. Digits above bars in (A) and (B) represent numbers in group. (C) The resulting partition for CXCL13 (n = 12 and 8 for high and low CXCL13, respectively). (D–F) Patients with high baseline circulating CXCL13 regenerate B cells more quickly following rituximab treatment. Groups partitioned as in (C) were examined for (D) numbers of circulating B cells, (E) blood CD79A mRNA and (F) IgM heavy constant region mRNA before and after rituximab treatment. Geometric mean (95% CIs), n = 12 (high) and 8 (low). (C–F) Note logarithmic scales. *P < 0.05 between patient groups by repeated-measures ANOVA and contrast testing on log-transformed data.
important source of the circulating protein. It should be noted that the synovial CXCL13 measurement is from a single joint with active disease. There are likely a number of cell types that contribute to CXCL13 synthesis in the RA synovium, including not only follicular dendritic cells [15, 16] and lymphoid aggregate-associated T cells [25], but also fibroblast-like synoviocytes (FLSs) and endothelial cells [16] as well as macrophage-like cells [26].

The large number of synovial inflammatory markers that correlate with synovial CXCL13 synthesis implies that CXCL13 might be used as a surrogate marker for synovitis. Interestingly, expression of CCL21, another chemokine frequently associated with lymphoid aggregates and germinal centres [14], did not significantly correlate with CXCL13. On the other hand, the two highest correlations of CXCL13 synovial expression were with expression of the FLS secreted proteases, MMP1 and MMP3. This could be an indication of significant interplay between FLS and other cells secreting CXCL13, or CXCL13 itself, although such a relationship remains speculative. The absence of an association of CXCL13 with CCL21 and the expression of CXCL13 in synovia lacking germinal centre-like aggregates in this RF-positive cohort is consistent with these chemokines being necessary but not sufficient for aggregate formation. The strong correlation of CXCL13 expression with MMP expression suggests a model wherein CXCL13 expression is associated with and perhaps induced by synovial inflammation, yet its effect on synovial aggregate formation or maintenance is dependent on additional factors. Thus we propose a CXCL13 high synovial phenotype of uncertain aetiology, the utility of which is the ability to detect an active synovitis phenotype by measurement of a circulating factor, CXCL13, rather than sampling synovia.

The central finding of the current report is that serum levels of CXCL13 at baseline can distinguish two groups of patients that responded very differently to rituximab in terms of replenishing their B cells. To our knowledge, this is the first report to link circulating CXCL3 levels with B-cell regeneration following rituximab treatment in any disease. The utility of CXCL13 as a predictor of the rate of B-cell return, as well as a biomarker of inflammatory burden, may well be of use in other pathological conditions and should be investigated. Importantly, however, circulating CXCL13 is not proposed to be a specific marker for RA, since elevated serum levels have been described in patients with autoimmune diseases such as lupus [27, 28], multiple sclerosis [29] and myasthenia gravis [30]; in certain malignancies [31–33]; and in some infectious conditions [34, 35]. Nevertheless, CXCL13 may be a useful serum biomarker of synovitis in the context of RA and other inflammatory arthritides. Patients with a significant inflammatory burden, and with elevated serum levels of CXCL13 and perhaps other B-cell survival factors, may promote early B-cell regeneration more easily. Although chemokine antagonists have not had great success in RA clinical trials, the close relationship of CXCL13 and other inflammatory markers begs the question of the usefulness of CXCL13 itself as a therapeutic target. Interestingly, in mouse models of arthritis, CXCL13 is up-regulated in inflamed joints [36], and the course of disease is significantly limited in animals treated with anti-CXCL13 antibodies [37] or lacking CXCL13 receptors [38].

The ARISE study was principally designed to evaluate the synovial effects of rituximab.
In conclusion, RA patients with more rapidly returning B cells following rituximab depletion have elevated baseline circulating levels of CXCL13 that could be objectively partitioned into two groups that had significantly different numbers of circulating B cells at 6 months after treatment. Serum levels of CXCL13 at baseline correlated significantly with synovial CXCL13 mRNA, suggesting that the inflamed synovium contributes to circulating protein. Furthermore, both serum and synovial CXCL13 levels co-varied with expression of other biomarkers of synovitis. Hence, CXCL13 constitutes a novel RA biomarker, both as an indicator of baseline synovitis and for predicting the rate of return of B cells after depletion with rituximab.

### Table 2: Correlations among synovial expression of inflammatory markers and CXCL13

<table>
<thead>
<tr>
<th>Synovial gene expressed</th>
<th>Correlation with synovial CXCL13</th>
<th>Correlation P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP1</td>
<td>0.890*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>MMP3</td>
<td>0.870</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>BAFF</td>
<td>0.794</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>CD19</td>
<td>0.721</td>
<td>0.0005*</td>
</tr>
<tr>
<td>CD138</td>
<td>0.725</td>
<td>0.0005*</td>
</tr>
<tr>
<td>Ig-xLC</td>
<td>0.723</td>
<td>0.0005*</td>
</tr>
<tr>
<td>CD3E</td>
<td>0.703</td>
<td>0.0008*</td>
</tr>
<tr>
<td>Ig-MHC</td>
<td>0.697</td>
<td>0.0009*</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.686</td>
<td>0.0012*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.682</td>
<td>0.0013*</td>
</tr>
<tr>
<td>CD79A</td>
<td>0.682</td>
<td>0.0013*</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.676</td>
<td>0.0015*</td>
</tr>
<tr>
<td>IgA1HC</td>
<td>0.623</td>
<td>0.0044</td>
</tr>
<tr>
<td>IgG1HC</td>
<td>0.515</td>
<td>0.0239</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.485</td>
<td>0.0353</td>
</tr>
<tr>
<td>APRIL</td>
<td>0.363</td>
<td>0.1266</td>
</tr>
<tr>
<td>CCL21</td>
<td>0.226</td>
<td>0.3516</td>
</tr>
<tr>
<td>SDF1</td>
<td>0.028</td>
<td>0.9109</td>
</tr>
</tbody>
</table>

*Coefficient of parametric correlation of log-transformed synovial gene expression at baseline, determined by qPCR; n = 19. *P < 0.0027, the Bonferroni-adjusted critical P of 0.05 for 18 comparisons.

### Rheumatology Key Messages
- In RA patients, high serum CXCL13 predicted faster return of B cells after rituximab.
- Serum CXCL13 protein was highly correlated with synovial CXCL13 expression.
- Serum and synovial CXCL13 correlated with synovial biomarkers suggesting CXCL13 as a biomarker of synovitis.

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