Concise report

Cytokine pattern in very early rheumatoid arthritis favours B-cell activation and survival

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Abstract

Objectives. B cells play an important role in the perpetuation of RA, particularly as autoantibody-producing cells. The ICs that further develop deposit in the joints and aggravate the inflammatory process. However, B-cell contribution in the very early stage of the disease remains unknown. The main goal of this work was to determine the concentration of cytokines potentially relevant for B-cell activation in serum from very early polyarthritis patients, with <6 weeks of disease duration, who latter on evolved into very early RA (VERA).

Methods. A proliferation-inducing ligand (APRIL), B-cell activating factor (BAFF) and IL-21 levels were measured by ELISA in the serum of VERA, other very early arthritis (VEA), established RA patients and controls. SF samples of established RA were also analysed.

Results. VERA patients have higher levels of APRIL and BAFF as compared with VEA, established RA and controls. Furthermore, APRIL and BAFF levels are also significantly elevated in RA-SF when compared with serum.

Conclusions. The increased levels of APRIL and BAFF in VERA patients suggests that B-cell activation and the development of autoreactive B-cell responses might be crucial in early phases of RA. Therefore, APRIL and BAFF could be promising targets for therapy in the early phase of RA.

Key words: B cells, VERA, Synovial fluid, APRIL, BAFF.

Introduction

B cells play critical roles in RA pathogenesis. They are the source of RFs and anti-CCP autoantibodies, which contribute to IC formation in the joints. These cells are also efficient antigen-presenting cells and contribute to T-cell activation through expression of co-stimulatory molecules. B cells simultaneously respond and produce chemokines and cytokines that promote leucocyte infiltration into the joints, formation of ectopic lymphoid structures, angiogenesis and synovial hyperplasia. Moreover, B-cell depletion therapy with rituximab, as well as the promising results obtained with atacicept in a Phase Ib trial [1], confirmed the importance of these cells in established RA [2, 3]. However, B-cell participation in the early phase of the disease is not yet completely understood. Interestingly, our previous studies showed a significant decrease of pre-switch memory B cells (IgD+CD27+) in peripheral blood of very early RA (VERA) patients, with <6 weeks of disease duration, when compared with controls [4]. In accordance with the results of another group, it is highly likely that this B-cell subset migrates towards the SM, contributing to the onset of the synovitis

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Our hypothesis is that the cytokine environment in early RA favours the recruitment, activation and survival of B cells, and herein we tested this concept in a cohort of very early polyarthritis patients.

Materials and methods

**Patients**

Blood samples were collected from 19 untreated very early polyarthritis patients with <6 weeks of disease duration, who after a minimum follow-up of 3-4 months fulfilled the 1987 ACR criteria for RA [6]. These patients were classified as VERA patients. Further samples were collected 4-6 weeks after starting a low dose of oral CSs (5-10 mg of prednisone) (Time 1) and 4 months after reaching the minimum effective dose of MTX (Time 2) up to a maximum of 20 mg/week required to reduce the 28-joint DAS (DAS-28) to <3.2 [7]. Also, baseline blood samples from VERA patients were compared with 19 other very early arthritis (VEA) patients who, after the same follow-up, did not evolve into RA and with 24 controls. Additionally, 12 blood and 15 SF samples were obtained from MTX-treated established RA patients.

**Cytokine quantification**

A proliferation-inducing ligand (APRIL), B-cell activating factor (BAFF) and IL-21 levels were determined by ELISA (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer’s instructions. Samples were analysed using a plate reader Infinite M200 (Tecan, Männedorf, Switzerland).

**Measurement of autoantibodies**

RF-immunoglobulin M (RF-IgM) was determined in all patients by IMTEC Autoimmune Diagnostics ELISA kit (Human GmbH, Wiesbaden, Germany) according to the manufacturer’s instructions. Serum levels of anti-CCP were measured by the ELIA CCP test system (Phadia GmbH, Freiburg, Germany) and samples were analysed using an ImmunoCAP 100 instrument.

**Statistical analysis**

Statistical differences were determined with non-parametric Kruskal–Wallis and Mann–Whitney tests using GraphPad Prism (GraphPad, San Diego, CA, USA). Correlation analysis was performed using Spearman’s rank correlation coefficient. The Mann–Whitney U test was used for comparisons between two groups.

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**Table 1** Clinical information and cytokine levels in healthy controls, VERA, VEA and RA patients

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 24)</th>
<th>VERA (n = 19)</th>
<th>VEA (n = 19)</th>
<th>RA (n = 12)</th>
<th>RA SF (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>40 (13)</td>
<td>50 (17)</td>
<td>40 (13)</td>
<td>63 (10)</td>
<td>57 (10)</td>
</tr>
<tr>
<td><strong>Sex (females/males)</strong></td>
<td>17/7</td>
<td>16/3</td>
<td>15/4</td>
<td>11/1</td>
<td>11/4</td>
</tr>
<tr>
<td><strong>Disease duration, years</strong></td>
<td>NA</td>
<td>&lt;6 weeks</td>
<td>&lt;6 weeks</td>
<td>8 (9)</td>
<td>9 (12)</td>
</tr>
<tr>
<td><strong>DAS-28</strong></td>
<td>NA</td>
<td>6.1 (1.8)</td>
<td>4.1 (1.6)*</td>
<td>4.5 (1.6)*</td>
<td>Baseline: 4.3 (0.8)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Baseline: 4.7 (0.7)*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Last observation: 5.2 (1.0)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.5 (1.0)</td>
</tr>
<tr>
<td><strong>HAQ</strong></td>
<td>NA</td>
<td>1.4 (0.8)</td>
<td>0.8 (0.7)*</td>
<td>0.8 (0.7)*</td>
<td>0 (0.6)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>1.5 (1.0)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.4 (0.8)</td>
</tr>
<tr>
<td><strong>RF positive, %</strong></td>
<td>ND</td>
<td>42</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>67</td>
</tr>
<tr>
<td><strong>Anti-CCP positive, %</strong></td>
<td>ND</td>
<td>32</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45</td>
</tr>
<tr>
<td><strong>IL-21, pg/ml</strong></td>
<td>2612.2 (2726)</td>
<td>11220.3 (3170.0)</td>
<td>16170.0 (3415.0)</td>
<td>12260.0 (1158.0)</td>
<td>464.7 (1178.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20220.0 (4045.0)</td>
<td>20220.0 (3415.0)</td>
<td>20220.0 (1158.0)</td>
<td>464.7 (1178.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>464.7 (1178.0)</td>
<td>246.5 (4787.0)</td>
<td>246.5 (4787.0)</td>
<td>26.2 (25.8)</td>
</tr>
<tr>
<td><strong>APRIL, ng/ml</strong></td>
<td>6.0 (11.3)</td>
<td>18.8 (19.7)</td>
<td>12.5 (8.7)</td>
<td>23.2 (29.9)</td>
<td>5.9 (5.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.9 (5.2)</td>
<td>13.1 (27.6)</td>
<td>13.1 (27.6)</td>
<td>13.1 (27.6)</td>
</tr>
<tr>
<td><strong>BAFF, ng/ml</strong></td>
<td>0.3 (0.6)</td>
<td>0.6 (0.8)</td>
<td>0.5 (0.2)</td>
<td>0.8 (0.3)</td>
<td>0.3 (0.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 (0.4)</td>
<td>0.2 (0.1)</td>
<td>0.2 (0.1)</td>
<td>0.9 (0.6)</td>
</tr>
</tbody>
</table>

All values indicated in table represent mean (s.d.). *The indicated DAS-28 value corresponds to the baseline DAS-28 3V score due to the unavailability of visual analogue scale parameter at baseline in the established RA group of patients. **DAS-28 and HAQ values were compared between VERA and VEA patients with reference to VERA baseline values. Differences were considered to be statistically significant at P < 0.05. NA: not applicable; ND: not determined.
test. Differences were considered to be statistically significant for $P < 0.05$.

**Results**

**Characterization of patients and disease evaluation**

A total of 38 polyarthritis patients with $<6$ weeks of disease duration were consecutively included. VERA patients had a mean (s.d.) age of 59 (17) years; 84.2% were female, 42% were RF positive and 32% anti-CCP positive. The baseline DAS-28 and HAQ were 6.1 (1.8) and 1.4 (0.8), respectively. After treatment with CSs and MTX there was a significant reduction of both DAS-28 and HAQ values (Table 1). VEA patients were classified as having SpA (five cases), SLE (four cases), crystal-induced arthritis (two cases), SS (one case), paraneoplastic polyarthritis related to multiple myeloma (one case) and arthritis associated with HIV infection (one case), and five patients entered spontaneous remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited polyarthritis. These early polyarthritis patients represent a subset of a larger cohort previously described by our group [4]. Furthermore, unpaired blood and SF samples were collected from established RA patients who had similar DAS-28 and HAQ values to those of VERA patients at baseline (Table 1).

**APRIL and BAFF levels are increased in VERA patients at baseline**

At baseline, APRIL and BAFF levels were significantly higher in VERA patients as compared with both VEA and controls (Fig. 1A). No differences were observed between treated or untreated VERA patients, or between VEA and controls (Table 1). Moreover, no significant differences in IL-21 levels could be observed in VERA when compared with either VEA patients and controls, or after therapy with CSs and MTX (data not shown). Furthermore, there was no correlation between DAS-28, anti-CCP or RF autoantibodies and APRIL and BAFF serum concentrations (data not shown).

**VERA patients have higher APRIL and BAFF levels in comparison with established RA patients**

In order to compare early RA with the chronic phase, we also analysed established RA serum samples. Importantly, VERA patients, in baseline and even after MTX treatment, had higher circulating levels of both APRIL ($P < 0.05$) and BAFF ($P < 0.001$) in comparison with established RA. However, regarding IL-21, no differences could be found in VERA when compared with established RA (data not shown). Furthermore, no significant differences could be observed in established RA in comparison with controls (data not shown).

**Established RA-SF has increased levels of APRIL, BAFF and IL-21**

To verify whether in established RA patients a B-cell activation environment could be present in the joint fluid despite lower APRIL and BAFF serum levels, we tested the same cytokine panel in established RA-SF. APRIL, BAFF and IL-21 levels were in fact increased locally in the joints of established RA patients in comparison with RA serum (Fig. 1B).

**Discussion**

In the present work, a cytokine pattern favouring B-cell activation is observed in RA patients with $<6$ weeks of disease duration, when compared with other causes of VEA and established RA. Our previous results demonstrated a significant decrease of pre-switch memory B cells (IgD$^+$CD27$^+$) in peripheral blood of VERA patients [4]. This is in agreement with the report from another group referring to a migration of this B-cell subset towards the SM [5]. In fact, in established RA, ectopic germinal centre-like structures develop in the inflamed synovial tissue that support survival of B cells and autoantibody production [9]. Therefore, we also analysed established RA-SF samples and depicted the presence of a cytokine-based B-cell survival environment that could explain the maintenance of potentially autoreactive B cells in the synovium. Our results demonstrated that VERA patients have increased APRIL and BAFF levels when compared with VEA and controls. Interestingly, APRIL was also increased in VERA patients’ serum as compared with established RA and its levels were even higher in RA-SF suggesting a local up-regulation in the synovium. APRIL affects not only the class-switch recombination process [10–12], but also plasma cell differentiation and survival [13, 14], which could thus explain the maintenance of autoreactive B cells in the joints [15]. Actually, a highly positive association between the infiltration of plasma cells and SF levels of APRIL has been demonstrated in RA patients [15, 16]. BAFF, similar to APRIL, is a fundamental B-cell survival factor and we have also detected increased serum levels in VERA patients when compared with established RA. Moreover, BAFF was also significantly elevated in RA-SF in comparison with RA serum. Previous studies have demonstrated that BAFF increases the chemokine (C-X-C motif) ligand 13 (CXCL13)-dependent chemotaxis of memory B cells through BAFF receptor (BAFF-R) triggering [17]. Therefore, increased BAFF levels in RA could support the migration of pre-switch memory B cells towards RA synovium, thus justifying the decrease of this B-cell subpopulation in circulation. Furthermore, increased IL-21 levels in RA-SF support local plasma cell differentiation and autoantibody production [18]. Additionally, therapy with neither CSs nor MTX affected cytokine production in VERA patients. However, the patients with established RA that we have studied were on chronic treatment with MTX and low-dose CSs and this might have influenced the serum levels of APRIL and BAFF. The effect of low-dose CSs and MTX on cytokine production in RA patients is still controversial [19, 20]. So, an absence of effect of short-term therapy with CSs and MTX on the cytokines analysed was not entirely unexpected in VERA, but an effect on chronic-treated patients might in fact occur.
Conclusions

In conclusion, we have shown increased APRIL and BAFF levels in VERA and in RA-SF, which could hypothetically support the maintenance, expansion, activation and survival of autoreactive B cells from the first weeks of disease onset. Therefore, we suggest that APRIL and BAFF may be potential promising treatment targets in the very early phase of RA.

**Rheumatology key messages**

- APRIL and BAFF levels are increased from the first weeks of RA onset.
- APRIL and BAFF may be potential promising treatment targets in VERA.
Acknowledgements

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References


