Alterations on peripheral blood B-cell subpopulations in very early arthritis patients

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Abstract

Objective. To characterize circulating B-cell subpopulations of arthritis patients with <6 weeks of disease duration.

Methods. Peripheral blood samples were collected from very early untreated polyarthritis patients, with <6 weeks of disease duration, for flow cytometric evaluation of B-cell subpopulations. Samples from patients who were later diagnosed as RA [very early RA (VERA)] were also collected 4–6 weeks after starting a low dose of prednisone (5–10 mg) and 4 months after reaching the minimum effective dose of MTX. A matched healthy group was used as a control.

Results. VERA patients have a lower percentage of total peripheral blood memory B cells (CD19+CD27+) and a significant decrease in the frequency of circulating pre-switch memory B cells (CD19+IgD+CD27+) as compared with controls. Therapy with corticosteroids or MTX was unable to restore the normal frequencies of these B-cell subpopulations. A significant decrease in peripheral pre-switch memory B cells is equally observed in other early arthritis patients. Furthermore, no significant differences are found in the frequencies of CD4+ and CD8+ T cells in all patient groups.

Conclusions. In very early polyarthritis patients, there is a reduction in circulating pre-switch memory B cells. The reasons that may account for this effect are still unknown. Short-term corticosteroids and MTX do not seem to have a direct effect on circulating B-cell subpopulations in VERA patients.

Key words: Rheumatoid arthritis, B cells, Corticosteroids, Methotrexate, Autoimmunity.

Introduction

RA is a chronic, systemic autoimmune disease of unknown aetiology affecting ~1% of the world population. RA is characterized by symmetric polyarthritis associated with pain and swelling in multiple joints that, if left untreated, ultimately leads to joint destruction [1].

The very early inflammatory reaction that occurs in the rheumatoid synovium is mainly constituted by neutrophils [2, 3]. However, this first inflammatory infiltrate quickly leads to increased expression of inflammatory cytokines, chemokines and adhesion molecules, inducing the recruitment of B cells, T cells and macrophages [4–6]. In fact, it is this secondary cell infiltrate that supports the persistence of the inflammatory response and mediates cartilage and bone destruction. Although RA has long been considered as a T-cell-centred disorder, recent evidence suggests that B cells do play an important role in the onset and perpetuation of this disease [7]. B cells function both as IL-producing cells and antigen presenting cells that activate T cells [8, 9] and are also responsible for the
production of autoantibodies [10–12], such as RF. RF can interact with FcγRIIa (CD16) receptors on monocytes and macrophages inducing the production of TNF [13]. Moreover, B-cell depletion therapy with rituximab, a mAb directed to CD20, have confirmed the importance of these cells in established RA [14]. Several studies have shown that following B-cell depletion in patients with RA, there is clinical and serological improvement that parallels with a decrease in RF levels [15]. Despite the evidence for a critical role of B cells in established RA, the knowledge on the participation of these cells in the early phase of the disease is still scarce. In addition, the effect of commonly used DMARDs, such as MTX, on B cells is also largely unknown.

The major goal of this study is to characterize circulating B-cell subpopulations in very early arthritis patients (VERA) and in other very early arthritis (VEA) patients when compared with healthy donors, and also to evaluate whether corticosteroids and MTX therapies have an impact on the frequencies of these cell subsets.

Materials and methods

Patients

Blood samples were obtained from 46 untreated polyarthritis patients (Rheumatology Department, Hospital de Santa Maria, Lisbon) with <6 weeks of disease duration. Twenty-two of these patients later on fulfilled the ACR criteria for RA [16]. These patients were classified as VERA patients and further samples were collected at 4–6 weeks after starting a low dose of oral prednisone (5–10 mg) (Time 1) and 4 months after reaching the minimum effective dose of MTX, up to a maximum of 20 mg/week, which was needed to reduce the 28-joint disease activity score (DAS28) to <3.2 (Time 2) [17]. The baseline blood samples from VERA patients were compared with 24 VEA patients and 29 healthy donors who were used as controls. The HAQ [18] was applied to all patients and the DAS28 was calculated in all patients who fulfilled the ACR criteria for RA. The local ethics committee (Comissão de Ética do Hospital de Santa Maria) approved the study and all patients signed an informed consent. Patient’s management was done in accordance with the standard practice and the study was conducted in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

Antibodies

Immunophenotyping of B and T cells in peripheral blood and peripheral blood mononuclear cells (PBMC) samples was performed using matched combinations of anti-human murine mAbs conjugated to FITC, phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). Isotype control antibodies were used for each fluorophore. For B-cell analysis, combinations of anti-CD19 conjugated to PerCP (clone 4G7, BD Biosciences, San Jose, CA, USA) or APC (HIB19, eBioscience, San Jose, CA, USA), anti-IgD conjugated to FITC or PE (IA6-2, BD Biosciences) and anti-CD27 conjugated to PE or APC (O323, eBioscience) were used. T cells were identified with anti-CD3 PerCP (SK7, BD Biosciences), anti-CD4 FITC (MEM-241, Immunotools, Friesoythe, Germany) and anti-CD8 APC (MEM-31, Immunotools).

Whole-blood staining

Approximately 5 ml of whole blood was collected by venipuncture into tubes containing ethylenediamine tetra-acetic acid. Erythrocytes were lysed with FACS Lysing Solution (BD Biosciences) and cells were stained, incubated for 20 min at 4°C, washed and stored in the dark at 4°C until analysed by flow cytometry. Frozen PBMC samples of patients were also used for staining protocol in order to establish the reproducibility of flow cytometry data from fresh and frozen samples. A total of 200 000 cells/sample were acquired with a FACSCalibur (BD Biosciences). Data were analysed with FlowScalibur (TreeStar, Stanford University, CA, USA). Absolute cell counts were calculated from differential leucocyte count determined at each time point for all patients.

PBMC isolation

PBMCs were isolated from 20 ml of heparinized whole blood following density gradient centrifugation with Percol (Amersham, Stockholm, Sweden). Cell viability was estimated with Trypan Blue (Sigma, St. Louis, USA). Cells were frozen in 1 ml/10⁷ cells RPMI-1640 (Invitrogen), 40% fetal calf serum (Invitrogen), Paisley, UK), 40% fetal calf serum (Invitrogen), 10% dimethyl-sulphoxide (Sigma) and stored at −80°C until further use.

Measurement of autoantibodies

RFs (IgM, IgG and IgA) and anti-cyclic citrullinated peptide (anti-CCP) were determined at baseline in VERA patients and also at Times 1 and 2 for VERA patients. IgM-RF, IgG-RF and IgA-RF were measured in the serum by IMTEC Autoimmune Diagnostics ELISA test system kits (Human GmbH, Wiesbaden, Germany) according to the manufacturer’s instructions and samples were processed using a ChemWell 2910 automated analyser. Serum levels of anti-CCP were measured by ELISA CCP test system (Phadia GmbH, Freiburg, Germany) and samples were analysed using an ImmunoCAP 100 instrument.

Statistical analysis

Statistical differences were determined using one-way analysis of variance and Bonferroni’s multiple comparison tests using GraphPad Prism (GraphPad, San Diego, CA, USA). For populations that did not follow Gaussian distribution, the Kruskal–Wallis non-parametric test was used. Differences were considered statistically significant for P<0.05.

Results

Disease assessment and autoantibody production

A total of 46 polyarthritis patients with <6 weeks of disease duration were evaluated. Twenty-two patients,
18 females and 4 males, with a mean age of 46.9 (16.3) years (range 23–77 years) fulfilled the ACR criteria for RA later on and were classified as VERA patients. At baseline, 10 of the VERA patients were RF positive, 6 of whom had anti-CCP antibodies (analysis performed up to 6 weeks after onset). All the RF-negative patients simultaneously lacked anti-CCP antibodies. A quantitative analysis of the production of RF and anti-CCP was also performed (Table 1). Patients with detectable levels for RF were positive for both IgM- and IgG-RF. Only two of the patients were positive for IgA-RF (data not shown). Interestingly, although not statistically significant, the mean levels of IgG-RF and anti-CCP decreased with therapy (Table 1). After therapy with corticosteroids and MTX, a clinical response associated with the decrease in DAS28 score ($P = 0.0019$ and $P = 0.0068$, respectively) could be observed. In the remaining group of 24 other VEA subjects, 16 females and 8 males, with a mean age of 44.8 (18.5) years (range 19–87 years), patients were later classified as having SLE (4), crystal-induced arthritis (3), PsA (2), colon adenocarcinoma (1), multiple myeloma (1), PMR (1), arthritis associated with HIV infection (1), arthritis associated with Crohn’s disease (1), unremitting undifferentiated arthritis (2), unremitting ReA (2) and 6 patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as a self-limited form of arthritis. For this study, 29 healthy controls, 16 females and 8 males, with a mean age of 39.8 (13.6) years (range 22–63 years) were also analysed.

**VERA patients have a reduced memory B-cell subpopulation irrespective of therapy**

The main B-cell memory subsets were analysed, depending on their IgD and CD27 expression, being classified as pre-switch memory B cells (IgD$^{−}$/CD27$^{−}$) and post-switch memory B cells (IgD$^{−}$/CD27$^{+}$). The frequencies of total peripheral blood B cells (CD19$^{+}$), naïve B cells (CD19$^{+}$/CD27$^{−}$), pre-switch memory B cells (CD19$^{+}$/CD27$^{−}$), post-switch memory B cells (CD19$^{+}$/CD27$^{+}$), total memory B cells (CD19$^{+}$/CD27$^{+}$) and plasma cells (CD19$^{+}$/CD27$^{hi}$) from VERA patients were compared with the same populations of healthy donors (Fig. 1). The frequency of total B cells at baseline was similar between VERA patients and healthy controls, being the average of circulating B cells gated in total lymphocytes of 11.69% (6.85) and 11.37% (5.69), respectively (Fig. 2A). Furthermore, corticosteroids and MTX did not affect the frequency of total B cells. Also, the analysis of absolute cell numbers of total B cells confirmed this result (Fig. 2A). Naïve B cells were significantly higher in VERA patients without treatment when compared with controls (Fig. 2B) and their percentages tended to return to normal values after corticosteroid and MTX treatment, although this effect was not statistically significant. In addition, the analysis of absolute cell counts of this B-cell subpopulation did not show any statistically significant difference between groups (Fig. 2B). Both controls and VERA patients had comparably very low levels (<3%) of circulating plasma cells (Fig. 2C). VERA patients had significantly lower frequencies of pre-switch memory B cells when compared with controls (Fig. 3A), irrespective of therapy. Importantly, this observation was also confirmed by a decrease in the absolute numbers of this B-cell subpopulation (Fig. 3B). In contrast, no statistically significant differences were observed in post-switch memory B cells between VERA patients and controls (Fig. 3C). A lower percentage of total memory B cells was observed in untreated VERA patients as compared with controls (Fig. 3D) and no effect after MTX treatment was observed. Furthermore, no correlation was found between the age of the patients and the percentages or absolute cell numbers of pre-switch and total memory B cells (data not shown). No correlation was found between DAS28 and the percentages or absolute cell numbers of pre-switch and total memory B cells at all time points (data not shown). Moreover, in order to verify whether an association existed between peripheral B-cell abnormalities, particularly in the memory B-cell pool, with the presence or absence of autoantibodies in the serum, circulating B-cell subsets were analysed at baseline comparing seronegative and seropositive VERA patients both for RF (IgM and IgG) and anti-CCP, but no statistically significant differences were observed (data not shown).

We also investigated differences between VERA patients and controls in circulating T cells, namely total

**Table 1** Characteristics of VERA patients and other VEA patients

| Clinical parameter | VERA (n = 22) | | | | |
|--------------------|--------------|--------------|--------------|--------------|
|                    | Baseline     | Visit 1      | Visit 2      | VEA (n = 24) |
| DAS28              | 6.083 (1.629)| 4.268 (1.568)*| 3.079 (1.659)*| NA           |
| HAQ                | 1.335 (0.730)| 0.900 (0.687)| 0.808 (0.746)| 0.908 (0.651)|
| IgM-RF, U/ml       | 26.8 (9.2)   | 28.3 (13.8)  | 19.6 (4.5)   | 0            |
| IgG-RF, U/ml       | 356.3 (375.9)| 333.1 (240.9)| 175.7 (207.9)| 0            |
| Anti-CCP, U/ml     | 111.9 (115.6)| 101.1 (60.7) | 51.4 (20.6)  | 0            |
| Leucocyte counts ($\times 10^9$/l) | 7.452 (2.432) | 7.727 (2.531) | 6.789 (2.226) | 6.955 (2.155) |

Baseline: before any treatment; Visit 1: after 4–6 weeks with 5–10 mg prednisone; Visit 2: 4 months after reaching the minimum effective dose of MTX. $^*$Differences are considered statistically significant for $P < 0.05$. All values indicated represent the mean (s.o.). NA: not applicable.
(CD3+), CD4+ and CD8+ T cells, and whether corticosteroids and MTX could have some effect on these populations. In VERA patients, no significant differences were found in the frequencies of CD4+ and CD8+ T cells as compared with controls, or after treatment (data not shown).

Although 22 VERA patients were selected for this study, 5 patients were lost to follow-up, technical problems occurred with the processing of the samples in 4 patients and 3 patients missed one of the appointments. Thus, the number of patients considered at each time point (n) is indicated together with the appropriate data in all the figures.

**VEA patients have a diminished pre-switch memory B-cell subset at baseline**

Other very early polyarthritis patients evaluated with <6 weeks of disease duration, who later were diagnosed as having types of arthritis other than RA, were followed in this study for comparison with VERA patients. VEA patients did not show any statistically significant difference in both frequencies and absolute numbers of total B cells (Fig. 4A), naïve B cells (Fig. 4B) or plasma cells (Fig. 4C) when compared with controls. However, similar to what was observed in VERA patients, VEA patients had a significantly (P < 0.05) lower frequency of pre-switch and total memory B cells as compared with controls (Fig. 5). Interestingly, the frequency of pre-switch memory B cells was similar in both VEA and VERA patients without treatment, being the average of 6.94% (4.64) and 5.33% (3.82), respectively (Fig. 5A). The analysis of absolute numbers of pre-switch memory B cells in the peripheral blood of VEA patients confirmed a statistically significant reduction in this B-cell subpopulation (Fig. 5B). No other statistically significant results were obtained with post-switch memory B cells (Fig. 5C), or with CD4+ and CD8+ T cells (data not shown). The subanalysis of the six patients who entered spontaneously into remission depicted the same pattern. As noted for VERA patients, no correlation was found between the age of VEA patients and the percentages or absolute cell numbers of pre-switch and total memory B cells (data not shown).

**Discussion**

Several studies have documented the presence of B cells in the rheumatoid synovium [5, 19–23] and reinforced the importance of these cells in RA progression. However, little is known about peripheral blood B-cell subpopulations and their functions in the very early phase of the disease.

Our results demonstrate, for the first time, that VERA patients have a lower pre-switch memory (CD19+IgD+CD27+) B-cell subset as compared with controls and that treatment with corticosteroids and MTX does not affect this B-cell subpopulation. However, this difference does not appear to be specific of VERA patients, since other early arthritis patients with the same disease duration show a similar pattern. As a consequence, the reduction of pre-switch memory B cells seems to be an early manifestation of polyarthritis.

It has been demonstrated that adult circulating B cells can be separated into three subpopulations on the basis of CD27 and IgD expression: IgD+CD27− naïve B cells, IgD−CD27− and IgD−CD27− memory B cells [24–26]. Klein et al. [27] have also described that IgM+IgD+CD27+ B cells carried somatically hypermutated antibodies, which indicates that this population is in fact a memory B-cell subset. However, the functional differences and characteristics between the two memory B-cell subpopulations remain to be clearly elucidated. In a study performed by Shi et al. [28], data were obtained that helped to clarify the differences between memory B-cell subsets.
Therapy with corticosteroids and MTX does not affect circulating total B cells, naïve B cells or plasma cells of VERA patients. Data from flow cytometry analysis of peripheral blood B cells from VERA patients without treatment and after therapy with corticosteroids and MTX. Total B cells (CD19⁺) (A) were gated on total lymphocytes and represented are the frequencies and absolute cell numbers. Naïve B cells (CD19⁺IgD⁺CD27⁻) (B) and plasma cells (CD19⁺CD27high) (C) were gated in CD19⁺ B cells. Differences are considered statistically significant for $P < 0.05$. 

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**A**

![Graph](image1.png)

**B**

![Graph](image2.png)

**C**

![Graph](image3.png)
In fact, it was demonstrated that IgD⁺CD27⁺ are unclass-switched memory B cells that play a crucial role in secondary immune response by producing high-affinity IgM in the early phase of infections and IgD⁺CD27⁺ are class-switched memory B cells that mainly express surface IgG and IgA isotypes. These findings were reinforced by the discovery that activation-induced cytidine deaminase, which is essential for class-switch recombination process [29, 30], was spontaneously expressed in IgD⁺CD27⁺ B cells, but was not found in IgD⁺CD27⁺ memory B cells [28].

CD27 is now an important marker for analysis of B-cell differentiation in diseases characterized by disturbances in B-cell development. In fact, distinct types of abnormal
B-cell homeostasis have been documented in some autoimmune diseases and other immunodeficiency disorders. Of interest, previous reports had already mentioned a decrease in IgD⁺CD27⁺ memory B-cell subset in patients with SLE [31], primary SS [32–34] or SSc [35]. Also, a reduction in circulating memory B cells was demonstrated in patients with X-linked Hyper-IgM syndrome [36], chronic granulomatous disease [37] and HIV infection [38]. Hence, lower levels of circulating memory B cells seem to be hallmark linked with chronic inflammation rather than an exclusive feature of autoimmune conditions. Furthermore, in a study performed by Hansen et al. [39], it was shown that the generation of the peripheral B-cell memory subset in SS patients seems to be particularly affected by abnormalities in post-recombination events. Our observations suggest that changes in the B-cell memory subset also occur in very early stages of RA and other polyarthritis. In VERA patients, despite the
clinical response induced by corticosteroids and MTX, no effect of these treatments was reflected in changes of IgD+CD27+ memory B-cell levels.

Since B cells differentiate into memory or plasma cells [26], the reduced frequency of circulating memory B cells in both VERA patients and other early arthritis patients could be explained by a skewing towards plasma cell differentiation, or by an increase in naïve B-cell population, thus resulting in less memory B cells. However, in our study, we did not find any statistically significant difference in the frequencies of circulating plasma cells when comparing both VERA and VEA patients with controls, although we might not exclude the possibility of this B-cell subpopulation being increased in the bone marrow, where it mainly resides [40], or in the rheumatoid synovium. Disturbances in the naïve B-cell subpopulation have been observed in other autoimmune conditions associated simultaneously with a decrease in circulating memory B cells. In fact, SS [34, 41] and SSc [35] patients have a predominance of CD27− naïve B cells and a reduced frequency of CD27+ memory B cells in circulation. In our study, VERA patients had an increased naïve B-cell subpopulation observed at baseline as compared with controls, returning this B-cell subset to normal values upon corticosteroids and MTX therapy. Nevertheless, this effect was not statistically significant. Moreover, there was not a statistically significant difference between absolute cell counts of this B-cell subpopulation in VERA patients as compared with controls. Also, similar results were observed in VEA patients when analysing naïve B cells. Considering our results, we hypothesize that during the initial phase of arthritis, circulating pre-switch memory B cells are recruited to the synovial membrane, where the production of high-affinity IgM is induced, which can react with antigens (self and non-self, depending if it is an autoimmune condition or not) and lead to inflammation. Importantly, it has been demonstrated that in established RA patients there is an accumulation of both pre-switch IgD+CD27+ and post-switch IgD−CD27+ memory B cells in the synovial membrane, which supports our hypothesis [42]. Furthermore, there is also the possibility that in the initial
phase of arthritis, pre-switch memory B cells are recruited towards secondary lymphoid organs, where they consequently become activated, thus leading to a decrease in the circulating pool.

In addition, corticosteroids and MTX did not affect the levels of the other B-cell subpopulations in circulation, or CD4+ or CD8+ T-cell frequencies. Corticosteroids are frequently administered to RA patients and their use can cause redistribution of lymphocyte populations [43], since long-term, low-dose corticosteroid therapy induces a decrease in B-cell counts [44]. However, our results indicate that short-term low doses of corticosteroids do not appear to affect B-cell counts. On the other hand, the effect of MTX on circulating blood cells in autoimmune conditions is controversial [45–47]. In a study performed by Bohm [48], it was observed that SLE patients treated with short-term MTX had slightly increased levels of total CD3+, CD4+ and CD8+ T cells, whereas monocytes and B cells remained stable. However, long-term MTX treatment decreased absolute numbers of both B and T cells. Moreover, a decrease in autoantibody levels accompanied the B-cell response to long-term MTX. Nevertheless, several studies by Lacki and co-workers [49–52] state that there are no significant differences in the percentage of CD3+, CD4+ and CD8+ T cells in RA patients treated with long-term MTX, although a decrease in B-cell levels is observed. Our results seem to indicate that T- and B-cell subsets are not affected by short-term treatment with MTX in the VERA patients.

Conclusions

In summary, in the first few weeks of arthritis onset there seems to be an alteration in the frequency of circulating memory B cells, particularly pre-switch memory B cells, as compared with controls. In addition, the short-term use of corticosteroids and MTX does not seem to affect circulating B-cell subpopulations in the VERA patients. However, since other early arthritis patients, who did not fulfill the ACR criteria for RA, also had a decrease in pre-switch memory B cells before any treatment was started, it seems that this effect is not unique and specific to the initial phase of RA. Further studies are required for a better understanding of the biological meaning of the reduction in the memory B-cell pool in an early arthritis condition.

Rheumatology key messages

- In very early polyarthritis patients, there is a reduction in pre-switch memory B cells.
- Short-term therapy with corticosteroids and MTX does not affect circulating B-cell subpopulations.

Acknowledgements

The authors would like to acknowledge João Cavaleiro and Ana Margarida Nery for technical assistance and Peter Lipsky for helpful discussions.

Funding: This work was supported by a grant from Sociedade Portuguesa de Reumatologia/Schering-Plough 2005. R.A.M. was funded by Fundação para a Ciência e Tecnologia (FCT) SFRH/BD/30247/2006, and M.M.S.-C. by Marie Curie Intra-European Fellowship LIF-025885 and a EULAR Young Investigator Award.

Disclosure statement: The authors have declared no conflicts of interest.

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B-cell subpopulations in very early arthritis patients


