Clinical Immunology (2009) **131**, 98–108

available at www.sciencedirect.com

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Synovial B cells of rheumatoid arthritis express ZAP-70 which increases the survival and correlates with the inflammatory and autoimmune phenotype

Barbara Tolusso [a,1], Maria De Santis [a,1], Silvia Bosello [a], Elisa Gremese [a], Stefania Gobessi [b], Ilaria Cuoghi [a], Michele C. Totaro [a], Giulio Bigotti [c], Carlo Rumi [d], Dimitar G. Efremov [b], Gianfranco Ferraccioli [a,*]

[a] *Division of Rheumatology, School of Medicine, Catholic University of the Sacred Heart, Rome, Italy*
[b] *International Centre for Genetic Engineering and Biotechnology, CNR Campus "Adriano Buzzati-Traverso", Monterotondo Scalo, Rome, Italy*
[c] *Institute of Pathology, Catholic University of the Sacred Heart, Rome, Italy*
[d] *Division of Hematology, Catholic University of the Sacred Heart, Rome, Italy*

Received 6 February 2008; accepted with revision 20 November 2008
Available online 12 January 2009

KEYWORDS
Rheumatoid arthritis;
B cells;
ZAP-70;
Synovial fluid;
BAFF;
IL6

Abstract B cells have acquired an important role in the pathogenesis of rheumatoid arthritis (RA) since B cell depletion allowed to rescue patients poorly responders to TNFα blockers. This study focused on the involvement of ZAP-70 as a bio-marker of B cells immune activation in RA. ZAP-70 expression in synovial fluid (SF) B cells obtained from RA patients was increased compared to SF B cells of osteoarthritis (OA) patients. Moreover we found that ZAP-70 positive/CD38 positive and ZAP-70 positive/CD5 positive B cells were enriched in SF. The analysis of B cell apoptosis *in vitro* showed that the percentage of ZAP-70 negative B cells spontaneously undergoing apoptosis was significantly higher than ZAP-70 positive B cells. The ZAP-70 positive B cell ratio (SF/peripheral blood (PB)) showed a positive correlation with SF autoantibody levels and with local levels of BAFF and IL6. ZAP-70 positive B cells seem to define a subset characterized by increased survival and high relationship with local inflammation and autoimmunity.
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Abbreviations: RA, rheumatoid arthritis; RF, rheumatoid factor; CCP, cyclic-citrullinated peptide; BCR, B cell receptor; CLL, chronic lymphocytic leukaemia; ZAP-70, Zeta-associated protein of 70kDa; SF, synovial fluid; OA, osteoarthritis.

* Corresponding author. Division of Rheumatology, School of Medicine, Catholic University of the Sacred Heart, Rome, Italy. Fax: +39 063503523.
E-mail address: gf.ferraccioli@rm.unicatt.it (G. Ferraccioli).
[1] These authors participated equally to this work.

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doi:10.1016/j.clim.2008.11.011

Introduction

It is increasingly recognized that B cells play a crucial role in rheumatoid arthritis (RA) [1]. This has been derived from the autoantibody signature (rheumatoid factor—RF and anti-cyclic citrullinated peptide—CCP positivity characterizing the most aggressive and progressive form of the disease) as well as by the clinical success obtained in RF positive patients by B cell depletion [2]. The mechanisms underlying the activation and survival pathways of B cells in the early phases of RA are not well known. B cell hyper-reactivity may arise from altered BCR signaling thresholds in response to self-antigens and altered B cell gene expression leading to disregulating B-cell recruitment, differentiation and survival. [3]

Recent studies suggested that B cell receptor (BCR) signal transduction in B cells of hematological malignancies as chronic lymphocytic leukaemia (CLL) may involve ZAP-70 (Zeta-associated protein of 70 kDa) [4–6]. ZAP-70 is constitutively expressed in T and NK cells and it is induced following T cell receptor activation in thymocytes [7]. ZAP-70 has been demonstrated to be also expressed in activated B cells in tonsils [8] and in CLL where it represents a marker of poor prognosis [9–11].The expression of ZAP-70 has been found to enhance BCR signaling and to decrease the rate of the receptor internalization, even if ZAP-70 is expressed at lower levels than Syk in ZAP-70 positive CLL B cells and the phosphorylation of the activating tyrosines in ZAP-70 molecules is inefficient [4]. Moreover ZAP-70 has shown to be involved in survival of CLL B cells. Degradation of ZAP-70 leads to impaired BCR signaling and CLL B cells apoptosis [5].

We hypothesized that in autoimmune diseases, such as RA, ZAP-70 could be involved in modulating B cell activation, assessed as activation markers and decreased apoptosis. We report here that ZAP-70 is expressed in B cells obtained from the SF and synovial tissue of RA patients, but not in B cell rich areas of osteoarthritis (OA) tissues and that CD38, CD5 and CD23 positive B cells of RA patients are enriched in ZAP-70 protein. Moreover, we show that B cells carrying ZAP-70 present an increased survival *in vitro* compared to ZAP-70 negative B cells.

Patients and methods

Patients and samples collection

After obtaining the informed consent, peripheral blood (PB) samples from 29 RA and 5 OA patients were analyzed. SF and synovial tissues were collected through joint aspiration and synovial tissue biopsy ultrasound-guided in the same patients.

All RA patients satisfied the American College of Rheumatology classification criteria for RA [12] and had a mean disease duration of 6.0 ± 6.8 years (11 patients had a disease duration <1 year, early-RA). Patients with long standing RA were taking non-steroidal anti-inflammatory drugs (NSAIDs) and methotrexate (15–20 mg/weekly) but not steroids or Tumor Necrosis Factor (TNF)-α blockers. Patients with early RA were taking NSAIDs. Subjects with knee OA, free of therapy, and healthy controls ($n=13$) were considered the control group.

Laboratory parameters recorded for RA patients were SF and PB (peripheral blood) anti-CCP autoantibodies (ELISA method, Axis-shield Diagnostics, Dundee, UK), SF and PB IgM and IgA RF autoantibodies (ELISA method, Orgentec Diagnostika, Mainz, Germany), erythrocyte sedimentation rate (ESR), C reactive protein (CRP), white blood cell (WBC) count and white synovial fluid cell (WSFC) count (using an emocytometer). Disease activity (measured by disease activity score-DAS44) was assessed at the time of samples collection. RA patients with DAS44 $>$ 2.4 having active disease [13].

Tonsil B cells were obtained from a tonsil of a subject undergoing tonsillectomy for chronic tonsillitis and CLL B cells were obtained from a ZAP-70 negative CLL patient (5.6% of ZAP-70 positive B cells, measured by flow-cytometry technique).

B cell isolation

Mononuclear cells from RA and OA PB and SF samples were isolated on a Ficoll gradient centrifugation (Cederlane, Ontario, Canada). B cells were isolated by positive selection from mononuclear cells using anti-CD19 Microbeads (Miltenyi Biotec, Auburn CA, USA). The purity of the selected B cells was evaluated by staining with anti-CD19-ECD conjugated antibody (Beckman Coulter, Marseille, France), followed by flow cytometry analysis on a EPICS® XL Cytometer (Beckman Coulter, Marseille, France). The preparations so obtained are referred to as highly purified B cells (>95%).

Mononuclear cells from tonsil and PB of the CLL patient were isolated by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) and the B cells were purified by negative selection using anti-CD3, anti-CD14 and anti-CD16 mouse monoclonal antibodies (kindly provided by Prof. F. Malavasi) and Dynabeads coated with pan anti-mouse antibody (Dynal Biotech, Oslo-Norway). The purity of the selected B cell populations was evaluated by staining with anti-CD5 R-phycoerythrin (R-PE)-conjugated and anti-CD19-fluoresceine isothiocyanate (FITC)-conjugated antibodies (Becton Dickinson (BD) Biosciences, Franklin Lakes-NJ), followed by flow-cytometry analysis on a FACSCalibur flow cytometer (BD). The purity of tonsil and CLL B cells was 89% and $\geq$98%, respectively.

Flow cytometry (FC) analysis

Fresh PB or SF samples were processed within few hours after sample collection. 100 microliters of PB containing approximately 5×10^5 WBC or 5×10^5 mononuclear cells derived from SF in 100 μL of phosphate-buffered saline (PBS) was first incubated in the dark at room temperature for 20 min with anti-CD3-phycoerythrin-cyanine (PC) 5, anti-CD56-PC5, anti-CD19-ECD and with anti-CD38-FITC or anti-CD5-FITC or anti-CD23-FITC to label cell surface markers (Beckman Coulter, Marseille France). The cells were fixed and permeabilized using the IntraPrep kit (Beckman Coulter, Marseille France) according to the manufacturer's instructions and incubated with anti-ZAP-70-PE monoclonal antibody for 30 min (clone SBZAP, Beckman Coulter, Fullerton CA). All cell samples were analyzed on properly compensated EPICS® XL Cytometer, using the EXPO32 software (Beckman Coulter, Marseille, France). The expression of ZAP-70 in CD19 positive cells (B cells) was measured according to the gating strategy published by Crespo et al. [14]. The first gate was set on

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