**Universidade de Lisboa**

**Faculdade de Medicina**

# THE ROLE OF B CELLS IN THE EARLY PHASE OF RHEUMATOID ARTHRITIS


**Rita Alexandra Pedra Aguiar de Moura**

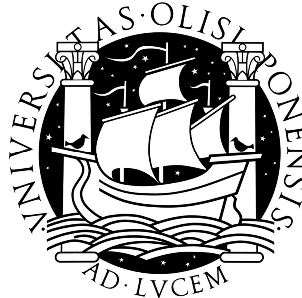

Doutoramento em Ciências Biomédicas

Especialidade em Imunologia

**2011**

**Universidade de Lisboa**

**Faculdade de Medicina**




# THE ROLE OF B CELLS IN THE EARLY PHASE OF RHEUMATOID ARTHRITIS

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**Tese orientada por:**

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**A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina da Universidade de Lisboa em reunião de 21 de Junho de 2011.**

**Dissertação apresentada à Faculdade de Medicina da Universidade de Lisboa, para obtenção do grau de Doutor em Ciências Biomédicas.**

A presente dissertação foi realizada na Unidade de Investigação em Reumatologia do Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal e Centre for Rheumatology, Division of Medicine, University College London, London, United Kingdom.

O trabalho aqui apresentado foi realizado com âmbito no projecto "O papel dos Linfócitos B e dos Neutrófilos na génese e na manutenção da Artrite Reumatóide - implicações no prognóstico e terapêutica", financiado pela Sociedade Portuguesa de Reumatologia e por um *unrestricted research grant* da empresa Roche.

Bolsa de Doutoramento da Fundação para a Ciência e a Tecnologia (referência: SFRH/BD/30247/2006).

*Aos meus pais e irmão*

*To be great, be entire: of what is yours nothing*
*Exaggerate or exclude.*
*Be whole in each thing. Put all that you are*
*Into the least you do.*
*Like that on each place the whole moon*
*Shines, for she lives aloft.*

Translated by Jonathan Griffin in "*Fernando Pessoa - Selected Poems*"

--

*Para ser grande, sê inteiro: nada*
*Teu exagera ou exclui.*
*Sê todo em cada coisa. Põe quanto és*
*No mínimo que fazes.*
*Assim em cada lago a lua toda*
*Brilha, porque alta vive.*

Ricardo Reis,
Fernando Pessoa's heteronym

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# ACKNOWLEDGEMENTS


The experience of going through a PhD is not just one more regular achievement in a student's life. It represents years of work and dedication to a hypothesis that you have decided to prove that it is worth to be studied and eventually make the difference, even if in the end you may come to the conclusion that your hypothesis was wrong. Nevertheless, this is the joy of Science: the uncertainty of what to expect.

I would like to thank all the people that have supported me during this unpredictable and challenging journey to stay in the right direction to try to answer the initial questions I was proposed to solve.

I would like to acknowledge first my supervisor, Professor Doutor João Eurico Fonseca, to have accepted me as a PhD student in his unit and for all the support he gave me, both personal and professionally. Thank you for the guidance in the world of rheumatology and for all the opportunities. Thank you for all the discussions and advices. Thank you for the patience, for listening me whenever I needed, for all my questions, doubts and opinions, for the tolerance every time I was late for a meeting and, above all, thank you for the freedom to organize myself and the work the best way without questioning the method and for believing my word. Thank you for everything.

I would also like to acknowledge Professor Doutor Luís Graça to have accepted to be my supervisor as well and for all the support during the PhD. Thank you for all the scientific advices about immunology, for all the discussions and arguments comparing a T and a B-cell point of view without defining a "winner" (or have we?), but respecting the importance of both participants in immunology. Thank you for all the meetings, for all the suggestions and for all the help to improve the work. Thank you for all the support.

I would like to thank all the members from Rheumatology Department, Hospital de Santa Maria, all the medical doctors and nurses, for the effort to collect the samples I needed to develop my work during the busy hours of a working hospital and for all the support. A very special thanking note to Dr. Ana Maria Rodrigues, Dr. Elsa Sousa, Doutora Helena Canhão, Dr. Joaquim Pereira and Dr. Maria João Saavedra, with whom I have

worked closer, for being present not only professionally, but also during distressful personal moments I had to struggle with during my PhD. There are no words to describe your help, support and friendship. Thank you very much for everything you have done and do. I would like to mention the exact same words of appreciation to Dr. Ana Filipa Mourão, from Rheumatology Research Unit, Instituto de Medicina Molecular, and also affiliated to Rheumatology Department, Hospital Egas Moniz.

I am also very grateful for the opportunity I had to spend a period of time at Centre for Rheumatology, University College London (UCL), in the group of Professor Jonathan Edwards, Doctor Geraldine Cambridge and Doutora Maria José Leandro, where I was able to develop part of my studies during the PhD. I am truly thankful for everything I have learnt, for the life experience and personal growth during my stay in London.

I would like to thank first to Professor Jonathan Edwards for accepting me in the group, for all the scientific discussions during our weekly meetings, for all the lessons on B cells. Thank you for all the support and consideration. I still remember the day you came into the laboratory and asked me how I was going, because we had had a few more patient samples than usual. Thank you for everything.

I would like to specially thank Doctor Geraldine (Jo) Cambridge, for all the support and guidance. Thank you for receiving me in the team and for the warm welcome not only when I arrived, but also throughout all my stay in London for which I am so grateful. Thank you for your enthusiasm for my PhD project and for all the brain-storming discussions we had everyday to achieve the best of data and the results we were having. Thank you so much for your friendship and kindness and those small details so important that made me smile. I cannot forget the gifts I would find on my desk in the morning or after lunch, like the cream custards (our Portuguese “Pastéis de Nata”) to make the distance to my country feel shorter. Thank you for trying to make me feel at home. Thank you for all the teaching, for being there whenever needed, for all the laughter. Thank you for everything.

I also would like to acknowledge Doutora Maria José Leandro, the Portuguese representative of the group, for all the support, for all the discussions and suggestions about the work and for receiving me so well at UCL. Thank you for your critical analysis about

the data, for all the attention during my stay in London, for all the conversations in Portuguese that made me feel closer to Portugal and home.

I would also like to specially thank Doutora Inmaculada de La Torre, my Spanish companion in the lab, who has also become a dear friend. Thank you, Inma, for all the attention since the first minute we have met, for being there always during my stay in London, for all the team work in the lab and for your friendship. Thank you for all the conversations in Spanish, Portuguese and English and for all the giggling, for your great sense of humor, for our private jokes, 9G4. Thank you for all the coffees and meals with a good laugh, after a full day in the lab, thank you for the trust. *Gracias por todo, Inma e un besito muy fuerte de tu amiga portuguesa*.

I would also like to thank all the lab members from Rheumatology Department at UCL who, during my first day at the lab took me out for lunch. Thank you for receiving me as well as your colleague.

A would like to acknowledge Paula Beharry, one of the nicest British persons I have met. Thank you for the jokes and for your joyful spirit, for being surprised for my courage to wear bright colours and for actually enjoying pink!

I would also like to acknowledge my thesis committee, Professor Doutor Bruno Silva-Santos, Doutora Margarida Carneiro and Doutora Maria Soares, for all the discussions and suggestions during our meetings in order to explore new possibilities about the work to be developed. A special thanking note to Doutora Margarida Carneiro for all her ideas and guidance to better study B cells in rheumatoid arthritis. Thank you for all the meetings we had, for all the discussions and for all the suggestions to innovate the work.

I would also like to thank all my colleagues at Rheumatology Research Unit, from Instituto de Medicina Molecular, the lab people who work with pipettes and are not afraid to use them; the people that pray to have enough cells to be able to work; the people that take the timer with them during lunch, otherwise the experiment will be ruined; the people that check the -80ºC ultrafreezer to be sure it is firmly closed and samples safe; the people who gets frustrated if the cytometer, centrifuge, real-time PCR or any other shared equipment are fully booked in the only available day or time you have to perform your experiments…. Yes, Science and doing a PhD have this kind of consequences to your mental (in)sanity. Nevertheless, as long as there is a question to be answered and curiosity

exists, the scientific spirit will always live. As a matter of fact, this was one of the main reasons for what I have chosen Biology for and to be a scientist.

I would like to specially thank Rita Cascão for all the team work, for the long nights in the lab, for the work discussions, for the companionship, for all the burst of laughs, for our friendship. I believe the two of us together have proven that “insane-in-a-healthy-way” people are able to produce serious work. And because trees die standing up.

I would also like to thank Doctor Saba Abdulghani for all the suggestions and corrections to this thesis, for the careful reading, for all the advices and support. Thank you for the consideration and friendship.

I also would like to acknowledge the colleagues and friends from other departments at Instituto de Medicina Molecular, particularly Letícia Santos, from Molecular Microbiology and Infection Unit, for all the conversations and support in all moments, ups and downs.

I would like to acknowledge Fundação para a Ciência e a Tecnologia (FCT) for the financial support through the PhD fellowship (SFRH/BD/30247/2006).

A very special thank to all my friends, whose support is beyond words. Thank you for being part of my life, for your presence, reassurance and strength. Thank you for the respect and for accepting my choices in life in general and during this journey of PhD, walking side by side with me.

Finally, I would like to deeply thank my family, my parents and my brother, to whom I dedicate this thesis. Thank you for the unconditional love and support, for the endless patience in all occasions, for all the guidance in life. There are no words to describe how much I am grateful for everything you do for me and how much I love you.

*Muito obrigada aos meus pais e irmão, por todo o amor e carinho, pelo apoio incondicional, por tudo o que têm feito por mim ao longo da vida. Obrigada por todo o apoio e pela paciência durante este percurso de doutoramento. Esta tese é dedicada a vocês.*

Thank you all.

# Abbreviations

| | |
|---|---|
| **ACPAs** | Anti-citrullinated protein antibodies |
| **ACR** | American College of Rheumatology |
| **AID** | Activation-induced cytidine deaminase |
| **Anti-CCP** | Anti-cyclic citrullinated peptide |
| **APCs** | Antigen-presenting cells |
| **APRIL** | A proliferation-inducing ligand |
| **BAFF** | B cell activating factor |
| **BAFF-R** | BAFF-receptor |
| **bp** | Base pairs |
| **Bcl-2** | B-cell lymphoma 2 |
| **BCMA** | B cell maturation antigen |
| **BCR** | B cell receptor |
| **Blimp-1** | B lymphocyte-induced maturation protein |
| **BLyS** | B lymphocyte stimulator |
| **BSAP** | B-cell-specific activation protein |
| **β2m** | β2-microglobulin |
| **CD** | Cluster of differentiation |
| **CDRs** | Complementary-Determining Regions |
| **CIA** | Collagen-induced arthritis |
| **C-R** | Concordant relapsing |
| **CRP** | C-reactive protein |
| **CSR** | Class-switch recombination |
| **CXCL13** | Chemokine (C-X-C motif) ligand 13 |
| **CXCR5** | Chemokine (C-X-C motif) receptor 5 |
| **Ct** | Cycle threshold |
| **DAS28** | Disease activity score of 28 joints |
| **DCs** | Dendritic cells |

| | |
|---|---|
| **DMARDs** | Disease modifying anti-rheumatic drugs |
| **DMSO** | Dimethyl-sulfoxide |
| **D-NR** | Discordant non-relapsing |
| **D-R** | Discordant relapsing |
| **EA** | Early arthritis |
| **EBV** | Epstein-Barr virus |
| **ELISA** | Enzyme linked immunosorbent assay |
| **ERA** | Early rheumatoid arthritis |
| **ESR** | Erythrocyte sedimentation rate |
| **EULAR** | European League Against Rheumatism |
| **Fcγ-R** | Fc gamma-receptor |
| **FCS** | Fetal calf serum |
| **FDC** | Follicular dendritic cell |
| **GC** | Germinal center |
| **GPI** | Glucose-6-phosphate isomerase |
| **HAQ** | Health assessment questionnaire |
| **HIV** | Human immunodeficiency virus |
| **HLA** | Human leukocyte antigen |
| **ICOS** | Inducible Costimulator Molecule |
| **IFN-γ** | Interferon-gamma |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **ITP** | Idiopathic thrombocytopenic purpura |
| **LT** | Lymphotoxin |
| **MFI** | Mean fluorescence intensity |
| **MHC** | Major histocompatibility complex |
| **MS** | Multiple sclerosis |
| **MTX** | Methotrexate |
| **NC** | Normal controls |
| **NHL** | Non-Hodgkin's lymphoma |
| **NOD** | Non-obese diabetic |

| | |
|---|---|
| **NSAIDs** | Nonsteroidal anti-inflammatory drugs |
| **OA** | Osteoarthritis |
| **PAD** | Peptidylarginine deiminase |
| **Pax5** | Paired box 5 |
| **PBMC** | Peripheral blood mononuclear cells |
| **PCR** | Polymerase chain reaction |
| **PDN** | Prednisone |
| **RA** | Rheumatoid arthritis |
| **RAG** | Recombination-activating gene |
| **RANKL** | Receptor activator for nuclear factor κB ligand |
| **RF** | Rheumatoid factor |
| **ROS** | Reactive oxygen species |
| **RSS** | Recombination signal sequences |
| **RTX** | Rituximab |
| **SCID** | Severe combined immunodeficiency |
| **SE** | Shared epitope |
| **SF** | Synovial fluid |
| **SHM** | Somatic hypermutation |
| **SLE** | Systemic lupus erythematous |
| **SLZ** | Sulphasalazine |
| **SMZ** | Splenic marginal zone |
| **SS** | Sjögren's syndrome |
| **SSc** | Systemic Sclerosis |
| **TACI** | Transmembrane activator and calcium modulator cyclophilin ligand interactor |
| **TCR** | T-cell receptor |
| **TdT** | Terminal deoxynucleotidyl transferase |
| **TFH** | Follicular helper T cell |
| **Th** | T helper |
| **TLRs** | Toll-like receptors |
| **TNF** | Tumor necrosis factor |

| | |
|---|---|
| **TNFR** | TNF receptor |
| **Tregs** | Regulatory T cells |
| **VAS** | Visual Analogue Scale |
| **VERA** | Very early rheumatoid arthritis |
| **VEA** | Very early arthritis |
| **18S rRNA** | 18S ribosomal RNA |

# SUMÁRIO

A Artrite Reumatóide (AR) é uma doença autoimmune crónica, sistémica e de etiologia desconhecida, que afecta cerca de 1% da população mundial. A AR é caracterizada por uma poliartrite simétrica associada a dor e tumefacção de múltiplas articulações que, se não for tratada, conduz à destruição das articulações, incapacidade funcional, comorbilidades e redução da esperança de vida.

Existem diversas doenças autoimunes que são dependentes de células B, principalmente através da produção de autoanticorpos. Estudos anteriores documentaram a importância das células B na patologia da AR através de diversos mecanismos. As células B produzem autoanticorpos, como o factor reumatóide (FR) e anticorpos anti-proteínas citrulinadas (ACPA), que se depositam como complexos imunes nas articulações, causando inflamação. Além disso, as células B podem funcionar como células apresentadoras de antigénio e activar células T, sendo também capazes de produzir citocinas uma vez activadas e participar na organização de estruturas linfóides secundárias.

A descoberta da eficácia da terapêutica de depleção de células B com rituximab (RTX) em doentes com AR veio reforçar a posição chave destas células nesta doença autoimune, tendo colocado as células B no centro da investigação nesta área. Contudo, a maioria dos estudos neste tema tem sido focada em doentes com AR estabelecida, sendo o conhecimento dos mecanismos imunológicos iniciais dependentes de células B muito reduzido.

O principal objectivo deste trabalho foi o estudo do papel das células B na fase muito inicial da AR. Para tal, um grupo de doentes com poliartrite com menos de seis semanas de evolução, não tratada, foi prospectivamente seguido. Foi realizada uma caracterização completa das subpopulações de células B no sangue periférico, analisado o conteúdo de citocinas e quimiocinas no soro e feita uma análise de perfil de expressão génica relacionado com as células B. O acompanhamento clínico destes doentes permitiu a identificação de um subgrupo que evoluiu para AR, que foi classificado como *very early rheumatoid arthritis* (VERA). Os restantes doentes evoluíram para um diagnóstico de

artrite autolimitada, ou para outras doenças crónicas inflamatórias articulares. Os doentes VERA foram avaliados antes do tratamento, após terapêutica de curta duração com dose baixa de corticosteróides e após metotrexato (MTX), de forma a analisar as possíveis influências do tratamento no desenvolvimento dos processos imunológicos mediados pelas células B nas primeiras semanas de evolução da AR.

Nos doentes VERA, foram observadas perturbações nas células B de memória em circulação, nomeadamente uma diminuição da subpopulação de células B de memória *pre-switch* ($IgD^+CD27^+$); um aumento dos níveis séricos de citocinas relacionadas com o recrutamento e a activação de células B, em particular a interleucina (IL)-6, *A proliferation-inducing ligand* (APRIL) e *B cell activating factor* (BAFF); e alterações na expressão de genes relevantes para a homeostasia, desenvolvimento e sobrevivência das células B, nomeadamente BAFF e seus receptores. De salientar que o tratamento com corticosteróides e MTX, embora clinicamente eficaz na redução das manifestações inflamatórias, não pareceu afectar as células B periféricas, ou o padrão de citocinas em circulação.

Adicionalmente, foi analisado um segundo grupo de doentes com AR estabelecida sob terapêutica com RTX. Considerando o importante papel que a citocina BAFF apresenta na maturação e sobrevivência das células B, foi colocada a hipótese de que o balanço entre a expressão do receptor de BAFF (BAFF-R) e o BAFF solúvel poderiam influenciar a relação entre as subpopulações de células B reconstituintes e a recidiva clínica após terapêutica de depleção de células B. Para testar esta hipótese, procedeu-se à análise dos níveis de BAFF no soro, à caracterização fenotípica da reconstituição das subpopulações de células B em circulação e, foi analisada a expressão de superfície do BAFF-R nestas subpopulações, antes e após o tratamento com RTX.

Durante a repopulação das células B, após o tratamento com RTX, a maioria das células B no sangue periférico apresentou um fenótipo *naïve*, verificando-se em simultâneo uma diminuição da frequência das células B de memória em circulação, tal como esperado. Um dos resultados que suscitou interesse foi a observação da redução significativa da expressão do BAFF-R nas células B *naïve* e de memória nos doentes em recidiva, embora não tenham sido detectadas diferenças nos doentes em remissão. Além disso, os níveis de BAFF no soro encontravam-se dentro dos limites normais nos doentes com AR antes do tratamento com RTX e aumentaram significativamente após a terapêutica com RTX. No entanto, não

foi observada qualquer correlação entre a redução da expressão do BAFF-R quer com os números de células B em circulação, quer com os níveis de BAFF no soro. O aumento da frequência das células B de memória *post-switch* ($IgD^{-}CD27^{+}$) no sangue periférico observado nos doentes em recidiva, em comparação com os doentes em remissão, parece sugerir que o restabelecimento da actividade da doença possa estar associado ao processo de recombinação de mudança de classe de imunoglobulina. Deste modo, os resultados observados em doentes com AR após terapêutica de depleção de células B sugerem que factores responsáveis pela redução da expressão do BAFF-R possam estar intimamente ligados a mecanismos associados à geração de células B autoreactivas e ao restabelecimento da actividade da AR.

Em conclusão, os resultados desta tese suportam a existência de alterações nos mecanismos de fisiopatologia associados às células B desde as primeiras semanas de evolução da AR que poderão contribuir para o desenvolvimento da doença. Além disso, os dados obtidos parecem sugerir que uma introdução precoce de terapêuticas direccionadas às células B, depletoras destas células ou dirigidas aos receptores das mesmas ou aos seus ligandos, poderá ser particularmente eficaz na indução da remissão em doentes com AR desde a fase inicial da doença.

**Palavras-chave:** Artrite Reumatóide, Células B, Autoanticorpos, Corticosteróides, Metotrexato, Citocinas, Genes, BAFF-R, Rituximab

# Summary

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease of unknown aetiology that affects around 1% of the world population. RA is characterized by symmetric polyarthritis associated with pain and swelling in multiple joints. If left untreated, RA leads to joint destruction, functional disability, comorbidity and reduced life expectancy.

Many autoimmune diseases are B-cell dependent, mainly through the production of autoantibodies. Previous studies have documented the importance of B cells in RA pathogenesis through diverse mechanisms. B cells produce autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA). These autoantibodies can form immune complexes that deposit in the joints, causing inflammation. Additionally, B cells can function as antigen presenting cells and activate T cells; release cytokines once activated and participate in ectopic lymphoid organogenesis.

The discovery that B cell depletion therapy with rituximab (RTX) was effective in RA patients reinforced B cells key position in this autoimmune disease and placed them in the central stage of research. Nevertheless, the majority of the studies on this field have been focused in established RA patients, while the knowledge on B cell dependent immune mechanisms involved in RA onset is still very scarce.

The main goal of this work was to study the role of B cells in the very early phase of RA. In order to achieve this, a cohort of untreated polyarthritis patients with less than six weeks of disease duration was prospectively followed up. A full characterization of peripheral blood B cell subpopulations, serum cytokine and chemokine environment, and B-cell gene expression profile analysis was performed. The follow up of these patients allowed the identification of a subgroup that evolved into RA. Thus, these patients were classified as very early RA (VERA) patients. The remaining patients either had self-limited forms of arthritis or evolved into other chronic inflammatory joint diseases. VERA patients were evaluated at baseline and after short-term therapy with corticosteroids and methotrexate (MTX) to analyze the possible influence of treatment in the development of B-cell mediated immune processes in the first weeks of RA.

We found that VERA patients had disturbances in circulating memory B cells, namely a decrease in the pre-switch memory ($IgD^{+}CD27^{+}$) subpopulation; had increased serum levels of cytokines related with B cell recruitment and activation, in particular interleukin (IL)-6, A proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF); and had alterations in the expression of genes relevant for B cell homeostasis, development and survival, namely BAFF and its receptors. Of note, treatment with corticosteroids and MTX, although clinically effective in reducing inflammatory manifestations, did not seem to affect peripheral B cells or cytokine content in circulation.

Additionally, a second cohort of RA patients with established disease and under B cell depletion therapy with RTX was also analyzed. Considering the important role that BAFF has in B cell maturation and survival, it was hypothesized that the balance between BAFF-receptor (BAFF-R) expression and soluble BAFF could influence the relationship between returning B-cell subpopulations and clinical relapse after B cell depletion therapy. Therefore, serum BAFF levels, a phenotypic characterization of the newly emergent circulating B cell subpopulations during B cell reconstitution and the surface BAFF-R expression were analyzed in circulating B cell subsets before and after RTX treatment.

During B cell repopulation, after B cell depletion therapy, the majority of circulating B cells had a naïve phenotype and simultaneously there was a decrease in the frequency of memory B cells in circulation, as expected. Interestingly, we found that BAFF-R expression was significantly reduced on both naïve and memory B cells in patients at clinical relapse, however, no differences were detected in patients in remission. In addition, BAFF serum levels were within normal range in patients before RTX and significantly increased after RTX treatment. Nevertheless, the reduction in BAFF-R expression was not correlated with B cell numbers or serum BAFF levels. The increased frequency of post-switch memory B cells ($IgD^{-}CD27^{+}$) observed in the blood of relapsing patients in comparison with patients in remission might suggest that the re-establishment of active disease can be associated with an increase in class-switch recombination process. Therefore, the results observed in RA patients after B cell depletion therapy suggest that factors responsible for BAFF-R downregulation might be closely linked with altered thresholds in autoreactive B-cell generation and disease re-establishment.

In conclusion, the results in this thesis support the existence of alterations in the mechanisms associated with B cells' physiopathology since the first weeks of RA onset, which can eventually contribute to RA development. Furthermore, an earlier introduction of B-cell directed therapies, such as B cell depletion, or indirect B-cell targeted therapies affecting B cell receptors or its ligands, might be of beneficial clinical use to induce early remission in RA patients.

**Key-words:** Rheumatoid Arthritis, B cells, Autoantibodies, Corticosteroids, Methotrexate, Cytokines, Genes, BAFF-R, Rituximab

# CHAPTER I

## Introduction

# INTRODUCTION

## 1. Rheumatoid Arthritis

### 1.1. Definition

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease that preferentially affects the joints. RA has a prevalence of 1% worldwide in the adult population [1]. Onset usually occurs between 30 and 50 years of age and it is more frequent in women. RA is characterized by chronic pain and progressive joint damage with high risk of functional disability, which consequently leads to diminished quality of life and premature mortality [2, 3]. If not properly treated RA leads to bone and cartilage destruction with complete loss of joint integrity. This can occur after a period of time that can vary from only a few months to many years, depending on prognostic factors.

Although RA is clinically recognized as an inflammatory process affecting joints, the presence of extra-articular manifestations makes this a systemic disease characterized by different clinical patterns. In general, the disease can affect any joint, however, small joints such as those of hands, wrists and feet are predominantly targeted.

The diagnosis of RA is established according to the 1987 American College of Rheumatology (ACR) criteria [4] and at least four criteria must be present for a classification as RA (Table 1). Common symptoms of RA include symmetrical inflammation of large and small articulations and morning stiffness lasting at least 45 minutes. In most patients, symptoms emerge over a period of weeks to months and are often accompanied by fatigue. Furthermore, the disease progression eventually affects multiple organ systems, such as the heart or lungs, thus contributing to a worse prognosis. In 2010, the ACR and the European League Against Reumatism (EULAR) reviewed and updated the 1987 ACR criteria [5, 6], as summarized in Table 2. However, since this new revisison occurred after the completion of the experimental procedures of this thesis, the diagnosis criteria used for RA classification were those of the 1987 ACR criteria.

**Table 1.** The 1987 American College of Rheumatology criteria for the classification of rheumatoid arthritis.

| Criteria | Definition |
| --- | --- |
| 1. Morning stiffness | Morning stiffness lasting at least one hour, present for at least six weeks. |
| 2. Arthritis of three or more joint areas | At least three joint areas simultaneously with soft-tissue swelling or fluid, for at least six weeks. |
| 3. Arthritis of hand joints | At least one area swollen in a wrist, metacarpophalangeal, or proximal interphalangeal joint, for at least six weeks. |
| 4. Symmetric arthritis | Simultaneous involvement of the same joint areas on both sides of the body, for at least six weeks. |
| 5. Rheumatoid nodules | Subcutaneous nodules observed by a physician. |
| 6. Positive rheumatoid factor | Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects. |
| 7. Radiographic changes | Radiographic changes on hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints. |

A patient is said to have Rheumatoid Arthritis if he/she meets at least four criteria.

Patients with two clinical diagnoses are not excluded.

Designation as classic, definite, or probable Rheumatoid Arthritis is not to be made.

The economic impact of RA can be explained by the high level of functional impairment it causes: 20 to 30% of persons with RA become permanently work-disabled within three years of diagnosis and 50% after ten years [7].

**Table 2.** The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis.

| | **Score** |
|---|---|
| Target population (who should be tested) are patients who: | |
| 1) have at least 1 joint with definite clinical synovitis (swelling) * | |
| 2) with the synovitis not better explained by another disease ** | |
| *Classification criteria for RA* (score-based algorithm). Add score of categories A–D. | |
| A score of ≥ 6/10 is needed for classification of a patient as having definite RA ‡ | |
| **A. Joint involvement** § | |
| 1 large joint # | 0 |
| 2-10 large joints | 1 |
| 1-3 small joints (with or without involvement of large joints) ## | 2 |
| 4-10 small joints (with or without involvement of large joints) | 3 |
| > 10 joints (at least 1 small joint) | 5 |
| **B. Serology** (at least 1 test result is needed for classification) †† | |
| Negative RF *and* negative ACPA | 0 |
| Low-positive RF *or* low-positive ACPA | 2 |
| High-positive RF *or* high-positive ACPA | 3 |
| **C. Acute-phase reactants** (at least 1 test result is needed for classification) ‡‡ | |
| Normal CRP *and* normal ESR | 0 |
| Abnormal CRP *or* abnormal ESR | 1 |
| **D. Duration of symptoms** §§ | |
| < 6 weeks | 0 |
| ≥ 6 weeks | 1 |

* The criteria are aimed at classification of newly presenting patients.

** Differential diagnoses vary among patients with different presentations. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

‡ Although patients with a score of < 6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

§ Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis.

# "Large joints" refers to shoulders, elbows, hips, knees, and ankles.

## "Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

†† Negative refers to international unit (IU) values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤ 3 times the ULN for the laboratory and assay; high-positive refers to IU values that are > 3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. ACPA = anti-citrullinated protein antibody.

‡‡ Normal/abnormal is determined by local laboratory standards. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

## 1.2. Etiology and pathophysiology

The etiology of RA is not yet fully understood. Different effector pathways and cells are involved in the cascade of events leading to the initiation, progression and persistence of this disease [8, 9] (Figure 1).

A joint consists of two bones covered with cartilage and aligned by a capsule. The inner surface of the capsule corresponds to the synovial membrane. The area between the bones is filled with synovial fluid (SF), which is secreted by fibroblast-like synoviocytes from the synovial membrane. In normal conditions, SF helps to lubricate and support bones during movements. Early changes in rheumatoid synovial tissue are manifested by hyperplasia of synovial cells, edema, vascular proliferation and lymphocyte infiltration, leading to swollen and tender joints. The inflamed synovial membrane begins to grow irregularly, forming invasive pannus tissue that, consequently, invades and destroys cartilage and bone. Importantly, the inflammatory process that occurs is mediated by the activation of intracellular signaling pathways that stimulate the production and release of multiple cytokines, chemokines, growth factors, proteinases and adhesion molecules, which further cause joint destruction and the development of systemic complications.

The inflammatory process that occurs in RA synovium is initiated when an unknown antigenic trigger induces the development of an inflammatory autoreactive response. Antigen-presenting cells (APCs) present antigenic processed peptides to T cells, communicating with them through the major histocompatibility complex (MHC)-T-cell receptor (TCR) interaction and costimulatory signals via CD28-B7 family receptor (CD80/86), thus activating T cells.

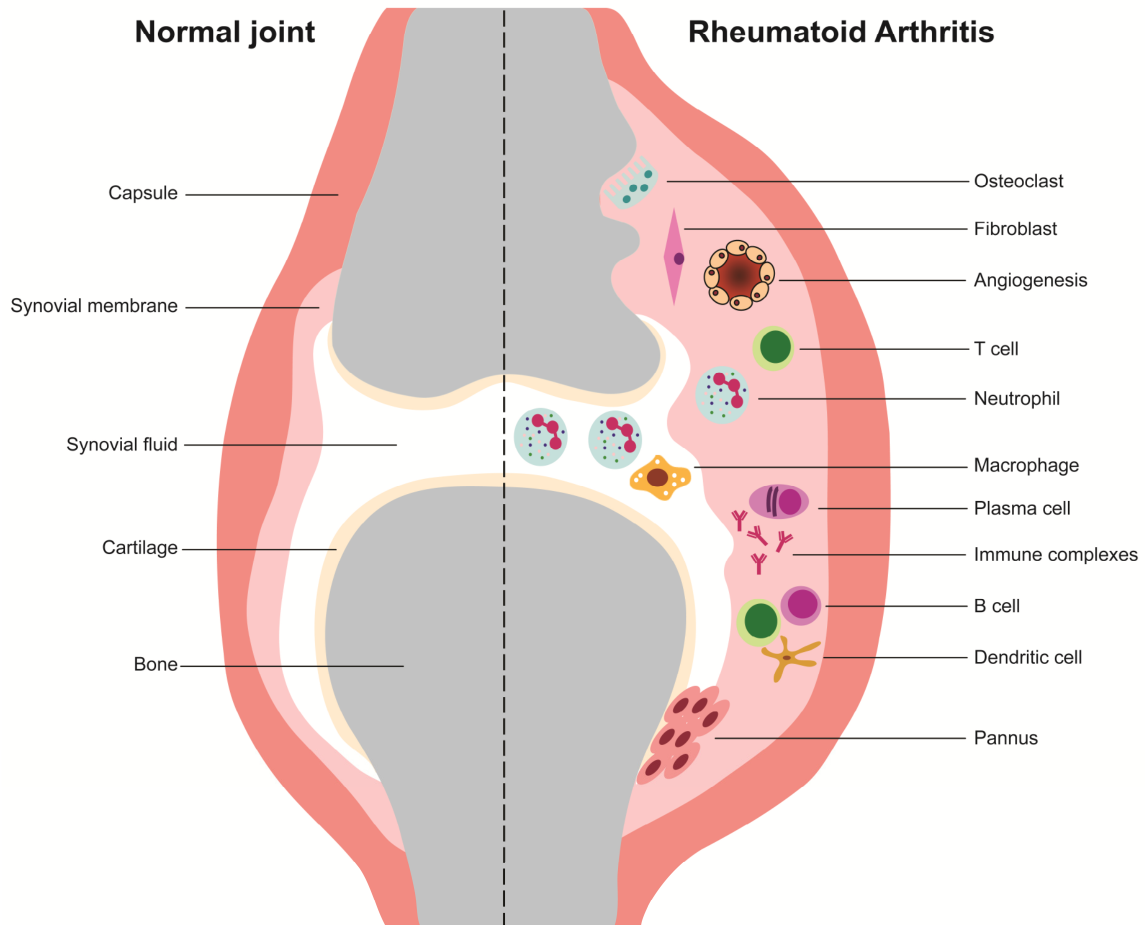


**Figure 1.** *Representative scheme illustrating the differences between healthy and rheumatoid joints.* In RA, the inflammatory process leads to a cellular infiltration not only of the synovial membrane, but also of the synovial fluid, with simultaneous angiogenesis, pannus formation, cartilage and bone erosion, swelling and pain. B cells mainly infiltrate synovial membrane and, once differentiated as plasma cells, are able to produce and release autoantibodies that form immune complexes, further increasing the inflammatory response.

B cells can function both as efficient APCs and antibody producing cells. Autoreactive B cells in RA produce autoantibodies such as rheumatoid factor (RF), that are able to form immune complexes and deposit in joints. These immune complexes activate macrophages through the low-affinity IgG receptor FcγRIIIa [10], leading to the production of several proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β and IL-6. This cytokine environment induces the expression of cell-adhesion molecules, such as VCAM1, by synovial fibroblasts [11, 12], which contributes to an increase of cellular

infiltration towards the joints. Additionally, the local production of metalloproteinases [13], such as MMP3 or MMP9, as well as the production of receptor activator for nuclear factor κB ligand (RANKL) [14] increases cartilage and bone destruction. Thus, a highly regulated immune network seems to contribute to RA development and progression, where autoantibody production and immune complexes deposition seem to be essential for disease initiation.

## 1.3. Environmental risk factors and genetic predisposition

Evidence points to a complex interaction between environmental and genetic risk factors that contribute to RA onset [15]. The female gender, a positive family history and older age are associated with an increased risk for developing RA. Smoking is the best established environmental risk factor for RA [16-18]: in a study that included 13 monozygotic twin pairs discordant for RA and smoking, the smoker was the one who developed disease in 12 out of 13 pairs [19]. Moreover, smoking was shown in several studies to be a risk factor for the presence of autoantibodies: RF or anti-citrullinated protein antibodies (ACPAs) [20]. Interestingly, the observation that RA associated autoantibodies, RF and ACPAs, may be present for more than 10 years before the onset of clinical disease, suggests that risk factors are active several years prior to the development of the disease. Furthermore, potential candidates implicated in an infectious aetiology have also been considered and included Epstein-Barr virus (EBV) [21], *Mycobacterium tuberculosis* [22, 23], *Escherichia coli* [24], *Proteus mirabilis* [25], retroviruses [26] and parvovirus B19 [27]. These associations have been supported by increased antibody titres against the infectious organism present in RA or the possibility of molecular mimicry. However, there has been no consistent evidence that a single infectious agent or other environmental factor explains *per se* the onset of RA.

Genetic predisposition is also significant and is a fundamental determinant in RA susceptibility. In fact, findings based on twin studies have estimated the relative contribution of genetic factors to be approximately 50%, leaving the remaining part to environmental and stochastic factors. Genetic research of complex diseases such as RA has had a major contribution from new technologies that allowed for genome-wide association studies of risk alleles [28].

The most important genetic association in RA is with the human leukocyte antigen (HLA)-DR genes, which reside in the MHC and participate in antigen presentation [29-32]. The risk for developing RA is related to the presence of specific alleles of the class II gene HLA-DRB1, that encode a conserved sequence of aminoacids, named the shared epitope (SE), in the third hypervariable region (HVR3) of the class II DRβ1 chain [29]. In fact, this sequence is found in multiple RA-associated DR genes, including DR1, DR4 and DR14 (for example, DRB*0101, DRB*0401, DRB*0404 and DRB*1402, respectively). The location of SE on the MHC molecule suggests that it might have a role in the ability of HLA-DR to bind and present specific arthritogenic peptides, which might cause RA. Indeed, the reported association between HLA-DR alleles with recognition of MHC class II-expressing antigen-presenting cells [33] and T cells [34] in inflamed joints, had led to the idea that MHC class II-dependent T-cell and B-cell activation were major drivers of the disease, thus reinforcing the notion of involvement of adaptive, B-cell and T-cell mediated immunity in RA pathogenesis.

In 2005, PTPN22, a gene that encodes for a tyrosine phosphatase that has a role in T-cell and B-cell signaling, was identified as the second confirmed RA susceptibility gene [35], thus supporting the genetic argument for a T-cell and B-cell contribution to RA [36].

Interestingly, many studies have already documented that the HLA-DRB1 SE and PTPN22 risk alleles are mainly associated with seropositive RA patients for RF, ACPAs or both [31, 32, 37, 38]. Several additional risk alleles for RA have been identified, such as TRAF1-C5 locus [39, 40], STAT4 [41] and OLIG3-AIP3 genes [42].

## 1.4. Prognosis and treatment

Predictors of worse prognosis in early stages of RA include high functional impairment, early involvement of several joints, high erythrocyte sedimentation rate or C-reactive protein level at disease onset, positivity for autoantibodies, namely RF [43] and ACPAs [44], and early radiologic changes [15]. Furthermore, the presence of specific alleles that encode the susceptibility epitope on MHC molecules might also influence the severity of the disease, since the risk of extra-articular and erosive disease is greater if the patients are homozygous [45].

Although RA does not yet have a cure, establishing a diagnosis as early as possible and immediately starting therapy is the basis for a successful management of these patients. The goal of RA treatment should be the remission of symptoms, allowing the preservation of function and quality of life, minimizing pain and inflammation, joint protection and control of systemic manifestations.

Treatment options for RA have been developed to stop or at least attenuate disease progression. Therapies that are used to treat RA are divided into three main classes: nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and disease modifying anti-rheumatic drugs (DMARDs) (both synthetic and biological).

NSAIDs are drugs that provide partial relief of pain and stiffness, but do not alter the disease course [46]. Corticosteroids are potent suppressors of the inflammatory response in RA. Previous studies have confirmed that these drugs are able to decrease the progression of RA and joint damage as detected radiographically [47-49].

The administration of DMARDs to RA patients offers a sustained suppression of inflammation. In fact, early and aggressive treatment with conventional DMARDs, such as methotrexate (MTX), leflunomide, sulfasalazine and hydroxychloroquine can be highly beneficial to avoid inflammation and development of joint erosions. Observational trials have clearly identified MTX as the synthetic DMARD that is most likely to induce a long-term response [50-52]. Nevertheless, combinations of DMARDs have also proven efficacy [53, 54]. When patients do not respond favourably to synthetic DMARDs, treatment with biological DMARDs is initiated. The first line biologics are TNF-inhibitory agents (infliximab, etanercept, adalimumab, certolizumab and golimumab) that act by partly neutralizing circulating and synovial TNF [55]. Other biological agents include abatacept, an anti-CTLA4 immunoglobulin (Ig) inhibitor [56]; tocilizumab, a IL-6 receptor antibody blocker [57]; and anakinra, an IL-1 receptor antagonist that has only a moderate therapeutic effect [58].

Of note, the introduction of rituximab (RTX), a monoclonal antibody that selectively depletes B cells [59, 60], has brought a new interest to the role of B cells in RA pathogenesis. The relevance of B cells in the immune system and their intervention in RA progression is addressed in the following chapters.

# 2. B cells

## 2.1. Origin and development

The human body contains approximately $2\times10^{12}$ lymphocytes, of which 5-15% are B cells. B lymphocytes are small white blood cells (6-10 μm) with a dense nucleus and little cytoplasm that express a membrane-bound Ig and originate and mature in the bone marrow. Discovered in the early 1960's, these cells are important mediators of our immune system, more precisely of the humoral response, through the production and secretion of proteins classified as immunoglobulins or antibodies.

### 2.1.1. Bone marrow

B cells develop in the bone marrow of mammals, but in birds, where they were first studied, these cells develop and mature in a special organ called "*Bursa of Fabricius*" (hence, the designation "B"), which is situated in the cloaca of the animals [61, 62].

In humans, B cells are primarily produced in the fetal liver before birth and in the bone marrow afterwards. B cell development can be detected in human bone marrow from 20 weeks of gestation and continues throughout life [63]. B cells arise from a lymphoid stem cell in the bone marrow and proceed through several maturation stages, during which they express different cell surface markers [64-67]. The earliest distinctive B-lineage cell is the progenitor B cell (pro-B cell) [68-71]. Pro-B cells proliferate in the bone marrow, filling the extravascular spaces between large sinusoids in the shaft of the bone, and further differentiate into precursor B cells (pre-B cells) [72, 73]. Generally, human pre-B cells can be subdivided into large proliferating cells (designated pre-BI) and small postmitotic cells (designated pre-BII) on the basis of cell-cycle analysis [74-76]. The bone marrow phase of B cell development culminates in the production of an immature B cell [77] that is not fully functional.

All B cell differentiation stages in the bone marrow do not require antigen, thus corresponding to the antigen-independent phase of B cell development.

### 2.1.2. Periphery

B cells leave the bone marrow when full maturation is achieved, as signalled by the co-expression of IgM and IgD on the membrane. These naïve B cells, which have never encountered an antigen, circulate in the blood and lymphatic systems and are carried to secondary lymphoid organs (lymph nodes, spleen, Peyer’s Patches), where they reside (Figure 2).

In the follicles of the secondary lymphoid organ, after encountering an antigen, mature B cells transform into large B-blasts and may follow two different pathways. Some proliferate and differentiate into short-living IgM producing plasma cells that are responsible for the early production of antibodies and first-line defence against antigens. A minority of the B-blasts differentiate to form the follicle centre or germinal center (GC) [78, 79]. The non-antigen-triggered naïve B cells form the follicle mantle or mantle zone. The follicle, containing a GC and a mantle zone, is known as a secondary follicle. The inner part of the mantle zone of the B cell follicle is designated the lymphocytic corona and is mainly composed of mature, naïve B cells [80]. The outer part of the mantle zone, called the marginal zone, is populated by different types of cells: macrophages, some T cells, granulocytes, plasma cells and marginal zone B cells [81]. GC are the specialized sites for memory B-cell generation, plasma cells differentiation and where affinity maturation of serum antibodies takes place [82, 83]. GC first appear at day 4-5 post-immunization, achieve their maximum cell number by day 10-11 and decline after three weeks [78].

B cells that form the GC are divided into centroblasts (large noncleaved cells) and centrocytes (small or large cleaved cells). Centroblasts are large, proliferating cells that lack surface immunoglobulin expression and accumulate at one pole of the GC, forming the dark zone. These cells can differentiate themselves into non-proliferating centrocytes that re-express surface immunoglobulin and accumulate at the opposite pole of the GC, known as the light zone [84].

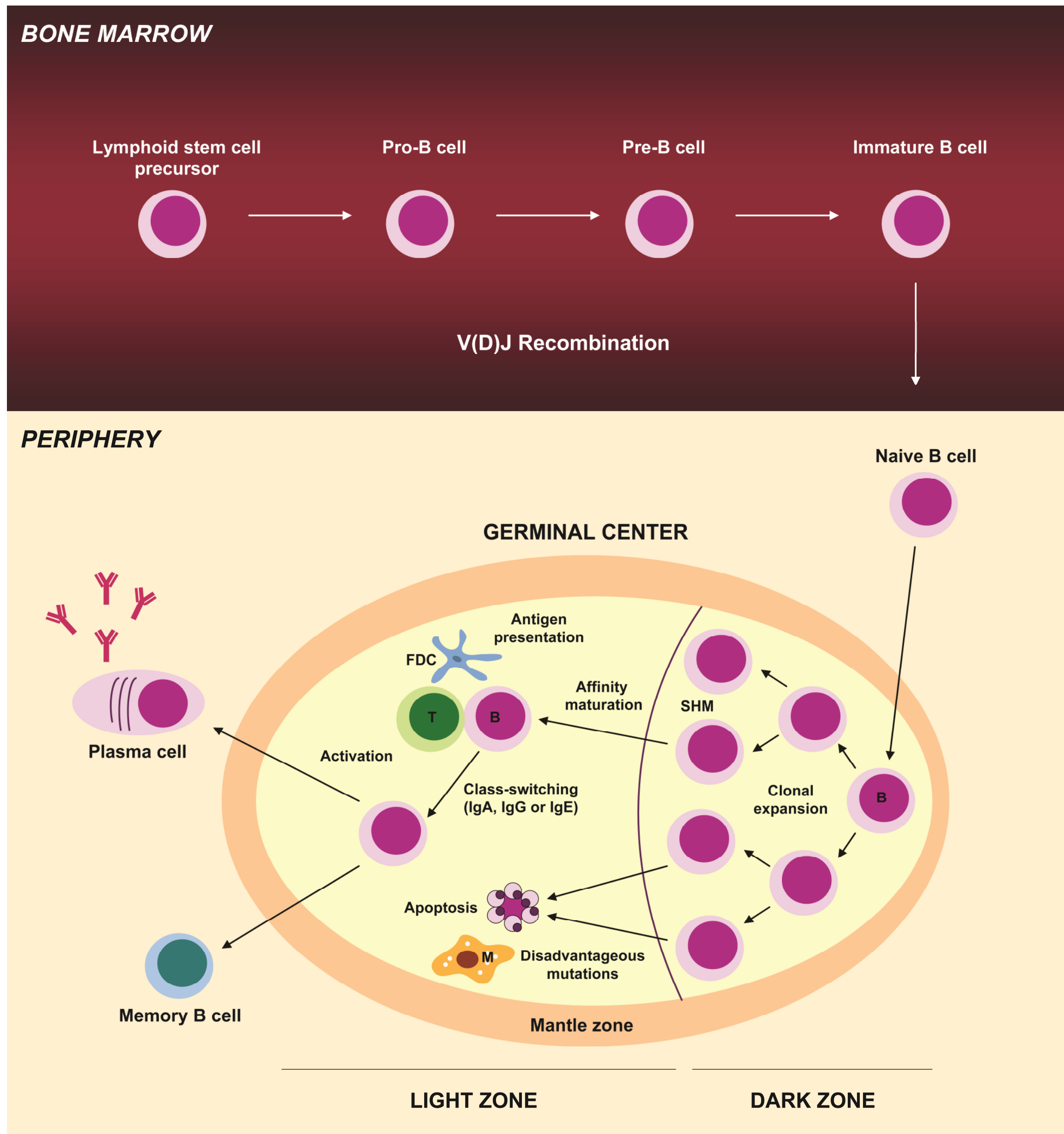


**Figure 2.** *B cell development.*
B cells arise from a lymphoid stem cell in bone marrow and proceed through several maturation stages, during which V(D)J recombination process occurs. When full maturation is achieved, B cells leave bone marrow into circulation and are carried to secondary lymphoid organs (lymph nodes, spleen, Peyer's Patches), where they reside and further differentiate into memory B cells or plasma cells in germinal centers. FDC: follicular dendritic cell, SHM: somatic hypermutation, B: B cell, T: T cell, M: macrophage.

Activation of GC B cells is made by follicular dendritic cells (FDC), which have a pivotal role in promoting B-cell proliferation and differentiation in GC [85, 86]. FDC are

not only very efficient in trapping and retaining antigen-antibody complexes for long periods of time and present them to B cells, but also are involved in B cell recruitment to secondary lymphoid organs with consequent follicle formation and organization through the secretion of the chemokine (C-X-C motif) ligand 13 (CXCL13), a B-lymphocyte chemoattractant [79, 87, 88]. Positive selection of B cells in GC occurs through high-affinity binding to antigens trapped by FDC. These high-affinity B cells receive survival signals from both FDC [86] and T cells [89] located in the light zone, whereas low-affinity B cells non-triggered by antigens die by apoptosis. More than 90% of the GC B cells die as a result of apoptosis [90] and are then phagocytosed and digested by the so-called tingible body macrophages [91-93]. Only GC B cells that are stimulated and able to synthesize high-affinity antibodies survive and may differentiate into long-lived plasma cells or memory B cells.

Long-lived plasma cells are thought to reside mainly in the bone marrow and organs that are directly exposed to foreign antigens (gastrointestinal tract, lungs…) [94], whereas memory cells reside in the follicle mantle or re-circulate freely to survey for secondary antigen exposure. Memory B cells are long-lived and are responsible for the ability of the immune system to learn. During a second encounter with an antigen, memory B cells are able to recognize the antigen to which the organism was previously exposed and induce a more rapid and effective immune response [95].

Since B cell activation and differentiation in the periphery require antigen, this stage comprises the antigen-dependent phase of B cell development. In the absence of antigen-induced activation, naïve B cells in the periphery have a short life span, dying within a few weeks by apoptosis.

## 2.2. Antibody production

Antibodies, also called immunoglobulins, are the antigen-binding proteins present on the B cell membrane and secreted by plasma cells. The main function of an antibody is to recognize a foreign antigen exhibited by an invading pathogen or present at the surface of an altered cell, like a tumour cell, and facilitate the clearance and elimination of that antigen. Antibodies can eliminate pathogens by several mechanisms including

opsonization, complement activation and direct lysis of bacteria and neutralization of viruses and toxins to prevent their entry into host cells.

An antibody molecule has a common structure of four peptide chains that consist of two identical light (L) chains and two identical heavy (H) chains, which are linked by disulfide bonds. The first 100-110 amino acids of the amino-terminal region of a light or heavy chain vary greatly among antibodies. These segments of highly variable sequence are called *variable* (V) regions: $V_L$ in light chains and $V_H$ in heavy chains. The carboxyl-terminal region of both light and heavy chains consists of relatively constant sequences, designated as *constant* (C) regions: $C_L$ on the light chain and $C_H$ on the heavy chain. Most of the differences among antibodies fall within areas of the V regions called Complementary-Determining Regions (CDRs) and these CDRs, on both light and heavy chains, constitute the antigen-binding site of the antibody molecule.

There are two light chain types, kappa (k) and lambda (λ). In humans, 60% of the light chains are kappa and 40% are lambda [96]. A single antibody molecule contains only one light chain type, either k or λ, but never both. The heavy chain of a given antibody molecule determines the class of that antibody or isotype. There are five different heavy-chain constant region sequences: μ, δ, γ, ε and α; hence in humans, the five major classes of antibodies are: IgM (μ), IgG (γ), IgA (α), IgD (δ) and IgE (ε), each with different structural and functional properties.

### 2.2.1. Mechanisms of antibody diversity

One of the most remarkable features of our immune system is its ability to respond to an apparently limitless array of foreign antigens. The major mechanisms that are responsible for the enormous diversity of antibodies ($>10^{10}$ possibilities) produced by our immune system are random rearrangements of the genes that codify the variable region (antigen binding domain) of immunoglobulins.

Antibody diversity is achieved by V(D)J recombination, somatic hypermutation (SHM) and class switching. Three separate multigene families, situated on different chromosomes, encode immunoglobulin light and heavy chains. In germ-line DNA, each of these multigene families contains several coding sequences, called gene segments that are separated by noncoding regions. During B cell maturation, these gene segments are rearranged and

brought together to form functional immunoglobulin genes [97, 98]. The k and λ light-chain families contain V (for *variable*), J (for *joining*) and C (for *constant*) gene segments and the rearranged VJ segments encode the variable region of the light chains. The heavy-chain family contains V, D (for *diversity*), J and C gene segments and the rearranged VDJ gene segments encode the variable region of the heavy chain. In each gene family, C gene segments encode the constant region. The V(D)J recombination is an ordered site-specific DNA rearrangement process [99] that occurs in developing lymphocytes in the bone marrow and is initiated by two lymphocyte-restricted specific proteins recombination-activating gene (RAG)-1 and RAG-2 [100-103], which together form an endonuclease responsible for DNA double-stranded breaks at recombination signal sequences (RSS) [104, 105] (Figure 3). RAG proteins are expressed at high levels during ontogeny, but their expression diminishes in immature B cells and is usually absent in recirculating mature naïve B cells [106, 107]. In certain pathologies, however, like in autoimmune diseases, RAG expression may be upregulated [108, 109]. The importance of RAG for V(D)J recombination is supported by the fact that mutations in RAG genes abolish recombination activity and block differentiation of both B and T cells, leading to different forms of immune deficiency [110-112]. RSS, which are recognized by RAG in the initial event of the recombination process, are comprised of a highly conserved heptamer and nonamer sequences, separated by a relatively nonconserved spacer region of either 12 or 23 base pairs (bp) [105, 113]. A gene segment with 12-bp spacer can only join with a segment flanked by 23-bp spacer (12-23 bp rule) [104, 114].

During variable-region gene rearrangements the heavy-chain genes rearrange first, followed by light-chain rearrangements. The generation of a functional immunoglobulin heavy-chain gene requires two separate rearrangement events within the variable region. The first step of recombination is a D to J association. The resulting D-J segment then moves next to and joins a V segment to generate a VDJ unit that encodes the entire variable region. Light-chain DNA rearrangements occur by the joining of a functional V segment to a functional J-C segment. The combinatorial diversity generated by V(D)J recombination is further augmented by another enzyme, terminal deoxynucleotidyl transferase (TdT) [115-117], that randomly adds up to 20 nucleotides, called N additions, to D-J and V-DJ junctions.

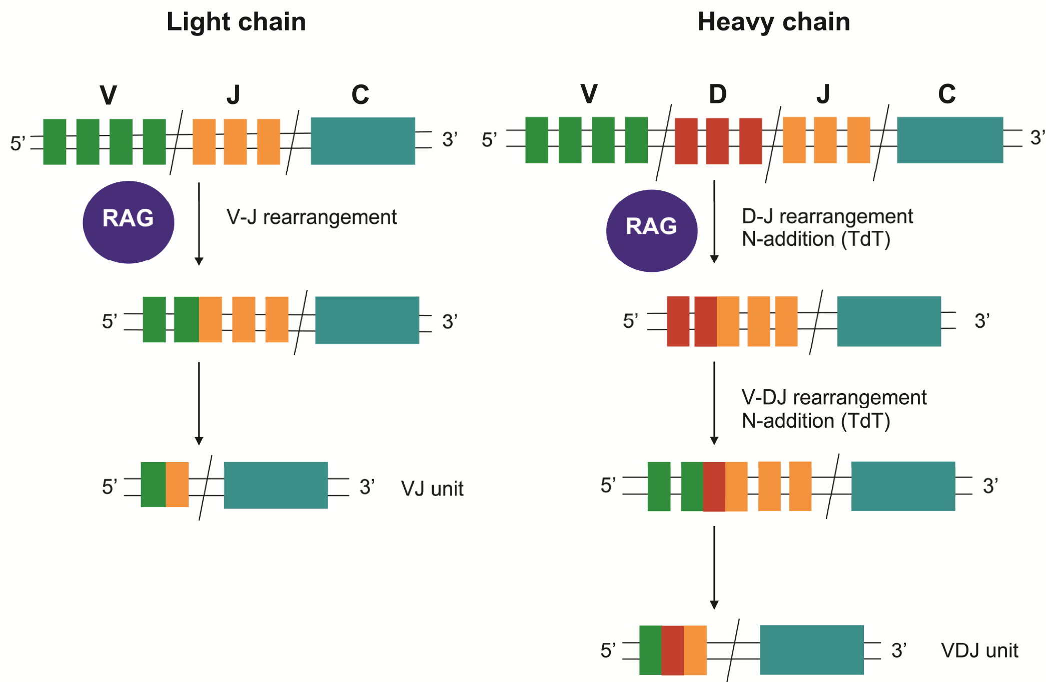


**Figure 3.** *V(D)J recombination process in light and heavy chains.*
During VDJ recombination, the gene segment rearrangements that occur in both light and heavy chains are possible due to specific enzymes, such as RAG proteins, that initiate the process. Further diversity is increased by nucleotide additions by Terminal deoxynucleotidyl transferase (TdT). Adapted from [118].

SHM is also a major mechanism for producing antibody diversity and increasing antibody affinity, which occurs in GC of secondary lymphoid organs [119-121]. SHM is a process that introduces point mutations, mainly nucleotide substitutions, as well as occasional deletions and duplications at a very high rate into the DNA of heavy and light-chain variable-region genes, which may alter the specificity of the encoded antibodies [122-124]. SHM introduces mutations at a rate of ~$10^{-3}$ mutations per base pair per cell division, which is $10^{6}$-fold higher than the spontaneous mutation rate in somatic cells. As a result, following exposure to antigen, these B cells with higher-affinity receptors will be preferentially selected for survival. This differential selection is due to an increase in antibody affinity for an antigen, a process known as affinity maturation. Thus, only B cells with the best fitting receptors are selected for rapid proliferation and further maturation. SHM appears early in the phylogeny of vertebrate immune system and operates in all

studied vertebrates [125]. Although the mechanism of SHM remains unknown, excitement in this field was triggered by the discovery of a requirement for activation-induced cytidine deaminase (AID), which has homology with the RNA-editing cytidine deaminase APOBEC-1 [126, 127].

Class or isotype switching is a deletional DNA recombination process that occurs in mature B cells and consists of the replacement of an expressed heavy-chain constant-region gene (usually $C_\mu$ for IgM) with another one of a different biological function, thus allowing the expression of IgG, IgA or IgE [128]. The deleted sequence is excised in a form of circular DNA [129]. In this way, the antigen binding specificity of the antibody remains unaltered; meanwhile its effector functions are changed. This process is mediated by a recombination event that deletes the DNA between repeated sequences, "switch regions", located upstream of the constant-region genes involved. AID catalyzes the hydrolytic deamination of cytidine to uridine, either as a free nucleotide or in the context of RNA and thus participates in class-switch recombination (CSR) [130, 131]. In fact, AID-deficient mice that are generated by gene-targeted mutation, lack completely the ability to undergo CSR and only form antibodies belonging to IgM isotype [126]. Moreover, in a group of immunodeficient patients with hyper-IgM syndrome, who had impaired CSR, the human gene encoding AID was mutated [127], so that their B cells only produced IgM molecules. AID is expressed specifically in GC-centroblasts [130], however, its exact role remains unknown. Importantly, it was demonstrated that AID deficiency also affects SHM in mice and humans, which supports the idea that AID is involved in both mechanisms, CSR and SHM [126, 132].

## 2.3. Antigen presentation and T cell stimulation

Antigen presentation results from the ability of cells to process and display small antigenic peptides (epitopes) associated with class I or class II MHC molecules and to deliver a costimulatory signal to T cells. Essentially, the T cell subset activated and the immune response consequently initiated depends on the context in which the antigen was first encountered by the APC [133, 134]. Endogenous antigens, processed within the cytoplasm of a cell, such as peptides derived from viruses or bacteria infecting a host cell, or proteins derived from a cancer cell, are associated with class I MHC molecules and

presented to $CD8^+$ T cells. Contrarily, exogenous antigens, internalized by phagocytosis or endocytosis are processed and coupled to class II MHC molecules and presented to $CD4^+$ T cells.

Three cell types are classified as professional APC: dendritic cells (DCs), macrophages and B cells. DCs are considered the most effective of APCs, since they constitutively express a high level of class II MHC molecules and present costimulatory activity [135-137]. In fact, DCs have a central role in the immune system as they regulate both innate and adaptive immunity, central tolerance to self-antigens and peripheral tolerance to environmental antigens and allergens [137-139]. Of interest, DCs activation and maturation may stimulate not only T-cell proinflammatory cytokine production, but also B-cell antibody production. Indeed, it has been demonstrated that DCs can also promote the differentiation and maturation of antibody-producing B cells [140-142].

B cells can also act as major APCs and prior to DCs [143, 144]. Binding of antigen to B cell receptor (BCR) leads to antigen internalization and presentation to T cells, a critical process in the initiation of the humoral immune response [145, 146]. B cells can internalize and present antigen that has been encountered either in soluble or particulate form, as well as when tightly bound to a non-internalizable surface [147]. The dependence of the efficiency of presentation differs for these three forms of antigen and it relies on antigen-BCR affinity [148].

After encountering an antigen, APCs can internalize it by three means: phagocytosis, fluid-phase pinocytosis and receptor-mediated endocytosis. B cells have the ability to present antigen efficiently, since they can find T cells in secondary lymphoid organs shortly after antigen entrance and BCR-mediated endocytosis allows them to concentrate small amounts of specific antigen [149-151]. BCR affinity is directly proportional to the capacity of B cells to present antigen to $CD4^+$ T cells. It has been demonstrated that cells with a BCR of low affinity required ten times more antigen to induce $CD4^+$ T cell proliferation than cells with a BCR of very high affinity, while presentation after uptake by fluid phase pinocytosis needed concentrations about 5000 times higher [147]. Consequently, the high affinity of the BCR for its specific antigen allows its efficient internalization even in the presence of small amounts of antigen [150]. Moreover, BCR signaling up-regulates MHC-II expression and consequently the generation of peptide-MHC II complexes [152, 153],

which enhances the efficiency of antigen presentation. Given the complexity of immune responses and the diversity of antigens relevant to human disease, B cells probably participate as APC in many situations.

### 2.4. B-T cell interaction

For an efficient activation of B cells by soluble protein antigens, an involvement of T cells is required. Binding of an antigen to the BCR does not, by itself, induce an effective competent signal without additional interactions between numerous receptor-ligand pairs on the two cell types.

Antigen capture by membrane immunoglobulin on B cells initiates signaling through the BCR (signal 1) that leads to an upregulation of the expression of class II MHC molecules [154, 155] and costimulatory B7 molecules [156-158]. Antigen is internalized, processed and degraded to peptides that are then presented to the cell surface as MHC-peptide complexes [152, 159]. T cells can recognize these MHC-peptide complexes on B cell membrane. Both T and B cells interact to form a T-B conjugate that ultimately leads to T cell-dependent B cell activation [158, 160, 161]. This interaction stimulates the expression of CD40-L (CD154) on T cells, which is the ligand for CD40, expressed on B cells [162, 163]. CD40, expressed on B-lineage cells (pro-B through plasma cells), belongs to the TNF family of cell-surface proteins and soluble cytokines that regulate cell proliferation. CD40L, expressed on T cells, belongs to the TNF receptor (TNFR) family. Interaction of CD40-L with CD40 on B cells delivers a signal (signal 2) [164-166] to the B cell that, in concert with the signal generated by the BCR crosslinkage, induces the expression of costimulatory molecules of the B7 family [167, 168].

The B7 costimulatory family has two ligands, B7-1 (CD80) and B7-2 (CD86) that can activate or inhibit a T-cell response, depending on the membrane molecule on T cell with which they interact, CD28 or CTLA-4 [169, 170]. CD28 is expressed by both resting and activated T cells and is the predominant receptor for B7 on resting T cells. CTLA-4 is expressed only on activated T cells, but has higher affinity for B7-1 and B7-2 than CD28, and can therefore limit the extent of the response. Upon binding of a B7-costimulatory molecule with CD28, a positive costimulatory signal is generated and the T cell is activated, while signaling through CTLA-4 inhibits the response of the T cell [171].

Therefore, for an efficient T-cell-dependent B-cell activation a B7-CD28 interaction has to occur.

The Inducible Costimulator Molecule (ICOS), a member of the CD28 family of costimulatory molecules expressed by T cells [172], is also a key regulator of humoral immunity and B cell homeostasis and functions. ICOS binds to a B7-like molecule, B7RP-1 [173, 174], expressed constitutively on B cells [175]. A major role for the ICOS/B7RP-1 pathway in the T cell-dependent B cell responses is supported by studies with both $ICOS^{-/-}$ and $B7RP\text{-}1^{-/-}$ mice that have decreased levels of serum antibodies and defective GC formation [176-179].

Once activated, B cells begin to express membrane receptors for various cytokines produced by the interacting T cell. The signals produced by these cytokine-receptor interactions induce a number of intracellular signaling pathways that ultimately result in changes in gene expression that support B cell proliferation and can induce differentiation into plasma and memory cells, class switching and affinity maturation. Moreover, it has been demonstrated that activated B cells by sequential BCR and CD40 stimulation proliferate and secrete TNF, lymphotoxin and IL-6, cytokines that can act not only as autocrine growth and differentiation factors, but also serve to amplify the ongoing immune response [180].

# 3. B cells and rheumatoid arthritis

## 3.1. The immunopathogenic role of T and B cells in rheumatoid arthritis

RA was classicaly considered mainly as a T-cell driven disease. The accumulation of $CD4^+$ T cells in rheumatoid synovium [181-183] suggested that macrophages could be activated by T cells, especially T helper (Th)1, to produce the proinflammatory cytokines TNF and IL-1β [184-186]. In addiction, it has been demonstrated that Th1 cells predominate in the joints of patients with RA [183, 187], while the Th2 subset seems to have a modest contribution for RA pathogenesis as can be appreciated by the low expression level of Th2 cytokines, such as IL-4 and IL-13, in the synovial tissue [181, 188-

190]. Similarly, studies in collagen-induced arthritis (CIA) mice have demonstrated that there is an expansion of $CD4^+$ T-cells bearing the Th1 phenotype [191], mainly tipifyed by IL-12 and IFN-γ [192]. Furthermore, research developed in the SKG mice model, that have a point mutation in the gene encoding ZAP-70, a tyrosine kinase crucial to signal transduction from the T cell receptor (TCR) [193], supported the participation of $CD4^+$ T cells in arthritis onset and progression [194].

Th17 cells [195, 196] have also been associated with RA pathogenesis. In humans, increased levels of IL-17 have been found in RA patients and a positive correlation has been demonstrated with the severity of joint damage [197, 198]. Moreover, the percentage of Th17 cells is significantly increased in RA synovial fluid in comparison with RA or normal peripheral blood [199]. In animal models of arthritis, it has been demonstrated that local expression of IL-17 in mouse joints results in acute inflammation [200], while the incidence and severity of arthritis is markedly attenuated in IL-17–deficient mice [201-203]. Furthermore, it has been shown that transferring Th17 cells to T-cell deficient mice induced arthritis [202].

Interestingly, another subset of T cells classified as $CD4^+CD25^+$, regulatory T cells (Tregs), contrarily to Th1, Th2 and Th17 subpopulations, may possibly hold promise for the treatment of RA and other autoimmune diseases, due to their self-tolerance maintenance and suppressive functions [204, 205]. Indeed, alterations have been found in the phenotype and suppressive effects of Tregs in blood and synovial fluid from RA patients [206-209]. In animal models of arthritis, depletion of Tregs exacerbates the disease and amplifies the cell-mediated and humoral responses [210], whereas adoptive transfer of regulatory T cells is effective in treating arthritis [211].

Despite all the observations that support a role for T cells in RA development, the disappointing results obtained with anti-CD4 therapy in humans [212-214] reinforced the notion that perhaps other parts of the inflammatory puzzle of RA should be studied more carefully to better understand disease pathogenesis [215-217]. Indeed, a crucial point remains unresolved in RA pathogenesis: do T cells activated by one or more autoantigens initiate the disease process, or is the influence of T cells secondary to a synovial-driven inflammatory response originating within the joints?

Although the cause that triggers RA autoimmune process remains unknown, it has been demonstrated that several types of cells from both innate and adaptive immune system actively participate and form complex networks of cell-cell interactions that contribute to the development and chronicity of rheumatoid synovitis and systemic inflammation [9, 11, 141, 218]. Notably, it has been proposed that both synovial and systemic inflammation characteristic of RA are due to the engagement of macrophages expressing Fcgamma-receptors (Fcγ-R), namely FcγRIII, by immune complexes formed by RF of the IgG class in particular [217, 219]. IgG-RF self-associating dimers are small immune complexes that fix complement very poorly [220, 221] and thus are expected to gain significant access to the extravascular space and allow interaction with tissue macrophages, leading not only to synovial, but also to systemic inflammation [219, 222].

The concept that autoantibodies such as RF could have an important role in RA triggering prior to T cell activation [223, 224] set the basis for the acknowledgement of B cell intervention in RA development. In fact, B cells play critical roles in the pathogenesis of RA and several lines of evidence have pointed to B cell function as the critical factor in the development of this disease (Figure 4).

B cells are responsible for the production of autoantibodies such as RF and ACPAs, which contribute to immune complex formation, complement activation and consequent deposition in the joints, thus leading to the inflammatory process characteristic of RA. Furthermore, B cells are also very efficient APC that can activate T cells through the expression of costimulatory molecules. B cells can also function as cytokine and chemokine producing cells that promote leukocyte infiltration in the joints and formation of ectopic lymphoid structures, consequently aggravating angiogenesis and synovial hyperplasia.

The discovery of K/BxN mouse model in 1996 [225] brought new attention to the idea that antibodies to systemic autoantigens might be a principal effector mechanism in initiating the inflammatory process in the joint. Similarly to human RA, this model presents lymphocyte infiltration of the synovium, synoviocyte proliferation, pannus formation, cartilage and bone erosion, polyclonal B cell activation, hypergammaglobulinaemia and autoantibody production.

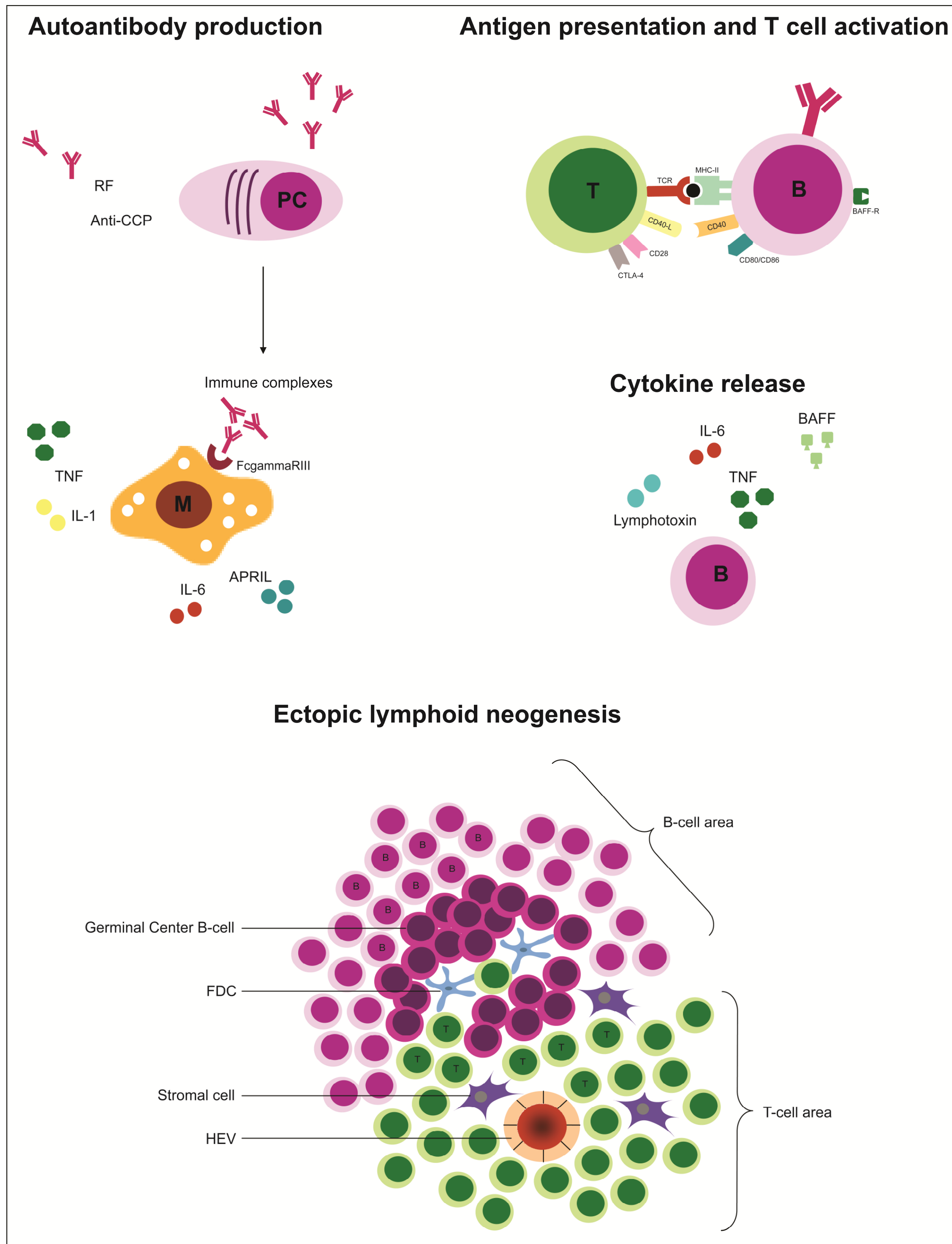


**Figure 4.** *An overview on the roles of B cells in rheumatoid arthritis pathogenesis.*
B cells play several important roles in RA development and progression. B cells produce autoantibodies that form immune complexes that activate Fcgamma-receptors and induce proinflammatory cytokine production; they function as antigen presenting cells and activate T cells, release cytokines upon activation and participate in ectopic lymphoid neogenesis. FDC: follicular dendritic cell, HEV: high endothelial venule.

The K/BxN mouse was originally generated by crossing a TCR transgenic strain (KRN-C57BL/6) with a non-obese diabetic (NOD) mouse. Unexpectedly, the transgenic TCR from F1 offspring recognized a peptide derived from a ubiquitously expressed protein, glucose-6-phosphate isomerase (GPI). The T cell-dependent B cell activation by GPI together with a costimulatory signal via CD40L-CD40 interaction led to the production of arthritogenic anti-GPI autoantibodies.

Furthermore, serum transfer to wild type, B cell-deficient or lymphocyte-deficient mice led to rapid onset of arthritis, which is transient, unlike the persistent arthritis in the K/BxN mouse [226]. Importantly, K/BxN mice devoid of B cells did not develop arthritis [225]. The identification of the target of the pathogenic antibody as GPI demonstrated that antibody to a ubiquitous antigen could lead to joint-specific disease and, therefore, renewed attention to the role of antibodies and immune complexes as a cause of human RA. Consequently, there was a revived interest in B cells as important effector cells in RA, as has been assumed in earlier years following the discovery of RF.

Notably, the success of B cell depletion therapy with RTX [59, 60] and the consequent clinical improvement achieved by patients has brought recent attention to B cells, confirming their unquestionable role in RA development and progression.

## 3.2. B cells produce autoantibodies

Autoantibodies are the serological hallmark of autoimmune diseases and serve as indicators of the break in self-tolerance.

RA is characterized by the presence of several autoantibodies in serum and synovial fluid. The first autoantibody to be documented in RA, RF, was described by Waaler in 1940 [227] and it was later found to be directed to the constant (Fc) region of IgG.

There are several autoantigens recognized by the autoantibodies present in RA that include cartilage components (for instance, anti-type II collagen or anti-CII; anti-human cartilage glycoprotein 39 or anti-gp39) [228]; enzymes (anti-glucose-6 phosphate isomerase or anti-GPI, anti-enolase) [229]; nuclear proteins (anti-RA33) [230, 231]; stress proteins and anti-citrullinated protein antibodies (ACPAs) [232, 233]. Moreover, the spectrum of these self-antigens and immunologically relevant epitopes probably varies during the disease course, and the set of autoantigens in one individual may differ from person to

person [234]. In fact, the composition of the antibody response varies over time, with limited specificities in early RA and a mature response in late disease, in which more epitopes are recognized and more isotypes are used [235, 236].

The clinical significance and pathogenic role of these autoantibodies are, however, largely unknown, except for RF and ACPAs, whose clinical usefulness has been acknowledged due to their good diagnostic sensitivity, specificity, as well as prognostic value.

### 3.2.1. Rheumatoid factor

RFs are autoantibodies that directly bind to the Fc portion of normal human IgG and are locally produced in RA by B cells present in the lymphoid follicles and GC-like structures that develop in the inflamed synovium [237, 238].

RF has been hypothesized to be a pathogenic autoantibody with a key role in the physiopathology of RA [217]. In fact, RFs can be detected in serum and synovial fluid samples from patients and its presence is associated with more aggressive articular disease, a higher prevalence of extra-articular manifestations and increased morbidity and mortality [239, 240]. RFs induce the formation of immune complexes at the sites of synovial inflammation, the activation of complement and leukocyte infiltration by means of the downstream products of complement activation [241].

RFs are found in multiple immunoglobulin isotypes (IgM, IgG, IgA), even if IgM is the RF usually measured in clinical assays, being detected in 60-80% of RA patients [233]. RF has been observed in many other autoimmune diseases, including systemic lupus erythematous (SLE) and Sjögren's syndrome (SS), as well as in non-autoimmune conditions, such as chronic infections and also in healthy individuals. Nevertheless, RFs in RA patients are different from RFs found in healthy individuals. RFs from healthy subjects are of the IgM class, polyreactive and with low affinity [242], whereas RFs from RA patients belong to all classes and exhibit affinity maturation [243]. Indeed, RFs from RA patients derive from a wide range of immunoglobulin germ line genes and show a high frequency of substitution mutations, indicating affinity maturation. Contrarily, although RFs from healthy individuals show evidence of immunoglobulin gene mutation, these tend to be silent [244]. These observations support the concept that RFs are under strict control

in healthy subjects to prevent the emergence of high affinity RF. Furthermore, transient production of low-affinity IgM-RF is regularly induced by immune complexes [245] and polyclonal B-cell activators, such as bacterial lipopolysaccharides and EBV [246, 247].

Interestingly, RF specificity to RA is strongly correlated to high titers of IgM and IgA-RF isotypes [239, 248]. A high titer of IgA-RF is also associated with radiologic erosion, extra-articular manifestations and, consequently, worse outcomes [239, 240, 249]. In addition, high concentrations of IgG RFs within the confines of the joint are associated with the formation of larger, complement-fixing complexes which, together with IgM RF-based complexes, are likely to amplify the inflammatory process [221]. Indeed, the association between high titers of RF and worse prognosis indicate that RF may have an important role in the pathogenesis of RA. However, there are RA patients seronegative for RF who manifest the same disease symptoms as seropositive patients. It is possible that B cells can have other pathogenic functions in RF-negative patients that justify the development of the disease despite the absence of autoantibody production, such as antigen presentation. Another hypothesis is that those patients have a slightly different pathogenesis, which together with different prognosis and different response to treatment should classify them into a different condition.

RF has proven to be, together with ACPAs, the most useful diagnostic marker of RA, having been included in the 1987 ACR classification criteria for RA [4] and in the new ACR/EULAR classification criteria [5, 6]. Furthermore, it is thought that B cells with RF specificity migrate into the synovium of RA patients, therefore presenting a variety of antigens to T cells. This leads to the perpetuation of local inflammatory responses and amplification of RF production in the synovium. Thus, RFs may prolong B-cell survival and hence maintain their own production.

### 3.2.2. Anti-citrullinated protein antibodies

ACPAs are a group of recently described autoantibodies which are found in 70-90% of RA patients and have high disease specificity (90-95%) [250, 251]. They are rarely present in other diseases or in healthy individuals.

ACPAs were first detected in RA in 1998 [232], a result later confirmed by other groups [252, 253]. These autoantibodies recognize peptides or proteins containing citrulline, a non-

standard aminoacid generated by the post-translational modification of arginine by peptidylarginine deiminase (PAD) enzymes [254], in a process known as citrullination. Of interest, citrullination occurs during a variety of biologic processes, including inflammation, apoptosis or keratinization.

ACPAs are locally produced in RA joints, where proteins are citrullinated during the inflammatory process [253]. In fact, several citrullinated proteins can be found in RA synovium such as filaggrin, fibrin, vimentin, nuclear and stress proteins [255]. However, the major citrullinated protein in RA joint was found to be fibrin [256].

Immune complex formation between ACPAs and citrullinated proteins with subsequent complement fixation and joint deposition occurs in RA synovium, which is thought to perpetuate RA synovial inflammation, causing a vicious cycle [257]. In 2000, the first ELISA test to detect ACPA was developed by Schellekens and co-workers [250], with the objective of creating a highly specific diagnostic tool for RA.

An ACPAs-positive disease is associated with an increased joint damage and low remission rates [258]. Evidence from animal models and in vivo data suggest that ACPAs are pathogenic on the basis of induction of arthritis in rodents and additionally due to the presence of immunological responses in ACPAs-positive patients in a citrulline-specific manner [259, 260]. Interestingly, the majority of ACPAs positive patients is also positive for RF [261].

Findings of clinical studies show that patients with RA who are simultaneously positive for RF and ACPAs (autoantibody-positive disease) differ from individuals with so-called autoantibody-negative disease. Indeed, histologically, ACPAs positive patients have more infiltrating lymphocytes in the synovial tissue, whereas those with ACPAs negative RA have more fibrosis and increased thickness of the synovial lining layer [262].

Importantly, ACPAs autoantibodies not only have a better diagnostic value than RF in terms of sensitivity and specificity, but also seem to be better predictors of poor prognostic features such as progressive joint destruction [261]. Moreover, they also help to predict the disease outcome in patients with undifferentiated arthritis [263-265].

In 2007, ACPAs were formally included in the EULAR published guidelines for the diagnosis of early RA [6].

## 3.3. B cells participate in ectopic lymphoid organogenesis

The rheumatoid synovium is a highly vascularized tissue infiltrated by cells from both the innate (macrophages and neutrophils) and adaptive (B and T cells) immune systems [9, 218, 266, 267].

It is possible to distinguish at least three different patterns of synovial infiltration in RA patients: a diffuse rearrangement of B and T cells; aggregates of B and T cells without a follicular microstructure; or synovial lymphocytes arranged in B-cell follicular-like structures with B and T cells arranged around a network of FDC that resemble functional ectopic GCs [266-268]. Interestingly, the patterns of lymphoid infiltrates correlate with clinical disease activity [269, 270] and severity [271].

Furthermore, some chemokines have been identified to contribute to the formation of these ectopic lymphoid structures, namely CXCL13 [272] and CXCL12 [273], which play a key role in the generation of GC as the responsible factors for the recruitment of B and T cells, respectively. Additionally, another cytokine, lymphotoxin (LT)-β, produced by activated T and B cells, also appears to be essential for the formation of primary B cell follicles in rheumatoid synovium [274, 275].

The TNF-family member B cell activating factor (BAFF), an important B cell survival cytokine has also been detected at high levels in rheumatoid synovial fluid [276], suggesting that the local production of BAFF in RA joints might favour the activation of autoreactive B cells with simultaneous ectopic lymphoid tissue formation [277].

Other cytokines are known to have an important role in the recruitment of B cells towards RA synovium and hence contribute to the development and organization of lymphoid structures. In fact, previous studies have documented an increase in RA joints of IL-6 [278], a major B cell chemoattractant [279] and IL-21, a cytokine that directly affects plasma cell differentiation and supports autoantibody production [280].

## 3.4. B cells can function as antigen presenting cells

Autoimmune conditions are the result of disorders in the immune system that erroneously recognizes self-antigens. Indeed, the APC function of B cells can have a central role in autoimmunity.

A B cell is classified as autoreactive when its BCR targets a self-antigen. This contrasts with other APC, whose activation depends on the recognition of molecular patterns from the antigen as foreign. Furthermore, in many cases, autoimmunity arises when antigens that are sequestered from the immune system become accessible and the capacity of B cells to concentrate small amounts of antigen would allow them to initiate a response.

In the case of RA, autoreactive B cells can function as APC and present processed self-antigens to T cells, thus allowing the development of an autoreactive immune response [281, 282]. The presence of B cells in synovial tissue from RA patients implanted in severe combined immunodeficiency (SCID) mice was shown to be necessary for activation of CD4 T cell clones isolated from the same or other HLA-matched patients [283]. Furthermore, in animal models of RA, genetic manipulation to generate mice with B cells unable to secrete antibodies demonstrated that antigen presentation by B cells is critical for disease development, as targeting of the autoantigen to specific B cells resulted in T cell activation and joint inflammation in the absence of autoantibodies [284].

The role of B cells as APC in autoimmunity has also been observed in several other autoimmune conditions such as SLE [285, 286], type 1 diabetes [287, 288] or multiple sclerosis (MS) [289]. Importantly, B cells seem to have a relevant role as APC in the pathogenesis of autoimmunity even in cases where the autoantibody secreting function of B cells is not a major contributor. Therefore, the importance of antigen presentation by B cells can vary in different situations and at different stages of each disease.

## 3.5. T cell activation is B-cell dependent

The activity of B cells as efficient APCs induces the stimulation of T cells within rheumatoid ectopic lymphoid structures [284]. The establishment of B-T cell contacts allows an optimal development of memory $CD4^+$ and $CD8^+$ T cells [290, 291]. Importantly, $RF^+$ B cells are believed to play an important role in antigen presentation. In fact, it has been demonstrated that they can take up antigen-antibody immune complexes through their membrane Ig receptors, which have RF specificity [281]. These autoreactive B cells then process and present peptides from the antigen to T cells, inducing T cell activation and help. Furthermore, previous studies by Takemura and colleagues have demonstrated that T cell response in RA synovitis and ectopic lymphoid organization is B cell dependent [283].

When $CD4^+$ T cells were transferred into SCID mice, the animals developed arthritis but only in the presence of B cells and when B cells where depleted, the disease ceased to occur. Moreover, the production of proinflammatory cytokines by T cells could be disrupted by B cell depletion.

Most of the studies that concern B-T cell interactions in RA involve $CD4^+$ T helper cells. The recent discovery in mice of a new lineage of $CD4^+$ effector T helper cells that selectively produce IL-17, termed Th17 cells, has provided new insights into the immune regulation and pathogenesis of some autoimmune diseases [195].

The role of Th17 cells in the inflammatory process that occurs in collagen-induced arthritis (CIA) mice served as a trigger to investigate the possible involvement of this T cell subset in human RA progression [292]. Of interest, Th17 cells have indeed proven to be associated with RA pathogenesis [197]. In fact, it has been demonstrated that IL-17 induces the secretion of proinflammatory cytokines, the upregulation of RANKL and stimulates the activity of matrix metalloproteases, leading to cartilage destruction and bone resorption [293-295]. In humans, differentiation of Th17 cells is driven by the simultaneous presence of IL-1β and IL-6 [296], also known to affect B cell biology in RA. Recent findings state that signaling pathways regulated by IL-17 contribute to the development of spontaneous GCs in autoimmune BXD2 mice [297] through the activation of autoreactive B cells by NF-κB signaling [298]. Furthermore, it has been demonstrated in SLE patients that IL-17 alone or in combination with BAFF controls the survival and proliferation of human B cells and their differentiation into Ig-secreting cells [299]. Moreover, studies performed in CIA mice model have shown that local BAFF gene targeting inhibited proinflammatory cytokine expression, suppressed the generation of plasma cells and Th17 cells expansion, thus improving joint pathology [300]. Importantly, recent in vitro studies have shown that Th17 cells are also effective B-cell helpers due to their ability to stimulate B-cell differentiation and class switch recombination [301]. Indeed, Th17 cells express B cell chemoattractant CXCL13, which can have important consequences not only in autoimmunity, but also in protective immunity against pathogens [302]. Therefore, these observations reinforce the notion that perhaps the interaction between B and Th17 cells in RA are worthy of further investigations.

# 4. Targeting B cells: present and future

## 4.1. B cell depletion therapy: the case of rituximab

Autoimmune conditions such as RA when not properly diagnosed and treated can have life-threatening consequences. The majority of autoimmune diseases are B-cell dependent particularly due to autoantibody production. These autoreactive B cells can arise in the bone marrow or in the periphery, if the self-tolerance mechanisms fail. Thus, research areas that concern B cell targeted therapies are of great importance to stop or at least to attenuate the effects of diseases with a B cell origin such as autoimmune disorders, or B cell lymphomas.

RA has traditionally been treated with analgesics and NSAIDs to control symptoms and with DMARDs to slow the processes involved in joint destruction. Advances in research into the pathogenic mechanisms underlying RA have led to the development of agents able to tackle the inflammatory process such as anti-cytokine therapies, namely against TNF. Although this approach has been effective, not all patients respond to treatments [303, 304]. Therefore, the contributions made by other cells, particularly B cells, to the immunopathogenesis of RA have become the focus of intense research.

The identification of a surface antigen that is both exclusively and highly expressed on B cells is essential if B cells are to be targeted effectively. CD20 expression is limited to B cells, begins in humans at the early pre-B cell stage and persists until the B cells undergo terminal plasma cell differentiation [305, 306]. Indeed, CD20 is not found on stem cells, pro-B cells, DCs or plasma cells [307, 308]. This specificity is vital when selecting a viable therapeutic target, thus making CD20 an attractive, if not ideal, target.

Rituximab (RTX) is a genetically engineered mouse-human chimeric monoclonal antibody specifically targeting CD20. RTX depletes B cells by inducing cell lysis, which can be mediated by complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity or apoptosis [309, 310]. RTX was first approved as a treatment of relapsed or refractory CD20$^{+}$ B-cell non-Hodgkin's lymphoma (NHL) in 1997 [311, 312]. In this context, RTX has been shown to diminish the number of B cells in the blood and bone

marrow of NHL patients for 9-12 months after just one treatment [313]. Importantly, RTX was generally well tolerated, with a very low incidence of use-associated infections.

In 1998, Edwards and Cambridge published a seminal paper proposing, for the first time, the use of RTX treatment in RA patients [217], which was highly controversial and revolutionary at the time. However, to their credit and facing considerable scepticism, the results observed in a small open-label study conducted by these authors provided the first indication of the therapeutic potential of RTX in RA [59]. In fact, in this study, patients who were non-responsive to DMARD therapy had a clearcut improvement in disease symptoms that was evident even beyond six months after a single RTX treatment. Indeed, circulating B cells were depleted to near undetectable levels and no major adverse events attributable to the treatment were observed. Notably, the work developed by Edwards and Cambridge [59] together with further studies [60, 314] provided robust evidence that B cells play a key role in RA and that selective B-cell depletion using RTX could be beneficial. Importantly, the first randomized, double-blind, placebo-controlled trial using RTX in any autoimmune disease was the phase IIa trial in RA reported by Edwards *et al* in 2004 in the New England Journal of Medicine [60]. These results were also reinforced by a phase IIb clinical trial in 2006 [315].

Despite the overall clinical efficacy of TNF antagonists in RA, a substantial fraction of RA patients do not respond to anti-TNF therapies [316-318]. Of note, the clinical efficacy of RTX was also confirmed in a large study of RA patients refractory to TNF-antagonist therapy [319]. Furthermore, RTX therapy has proven to be successful in other autoimmune conditions, such as SLE [320-322], SS [323-325], or idiopathic thrombocytopenic purpura (ITP) [326, 327].

Despite some controversy, there is evidence supporting a higher efficacy of RTX in patients with ACPA and/or RF in the serum [315, 319, 328-333]. Although RTX treatment has proven to be efficient in RA patients, clinical relapses have been reported to occur [334], which means that re-treatment is routinely necessary. B cell return into circulation antedates clinical relapse by variable periods of time among RTX-treated RA patients. In some patients, relapse is virtually coincident with B cell return, whereas in others relapse occurs months or even years after B cell repopulation. Importantly, disease reappearance is temporally more closely associated with a rise in titers of circulating autoantibodies (RF,

for instance) related to putative pathogenic autoreactive B cells than with global B cell return *per se* [334].

The reasons that might account for clinical relapse can include: a) incomplete B cell depletion in secondary lymphoid organs or bone marrow with rescuing and the survival of some pathogenic B cells that could restore the disease [335-337]; b) activity of long-lived plasma cells residing in the bone marrow or in the synovial tissue, which are not targeted by RTX [60]; c) persistence of autoreactive T cells in RTX-treated patients that could lead to the activation of newly generated pathogenic B cell clones; d) increased BAFF circulating levels observed during B cell depletion therapy with RTX, which could promote a support for surviving or re-emerging pathogenic B cells [338]. Therefore, the continuous research for new B cell targeting agents that directly act on other B cell antigens (for instance, CD19 or CD22) may possibly reveal even more promising results.

The failure of B cell depletion therapy observed in some patients, particularly in RF-negative patients, raises the issue of the existence of two different patterns of RA physiopathology with potential implications for prognosis and treatment [336, 339]. This concept is still in debate due to conflicting results that have been obtained in studies of repeated cycles of RTX treatment [340-342]. Apparently, in nonresponder patients, RTX is not able to break the self-reactive pathogenic B cell clones causing RA. The reasons that may account for this phenomenon are unknown, but suboptimal depletion of B cells, survival of plasma cells or higher numbers of memory B cells before treatment may explain refractoriness to the first RTX course [336, 339].

According to a consensus statement on the use of RTX, treatment with RTX should be repeated (six months after the first infusion) if patients experience a clinical response to the first treatment course and significant disease activity remains or recurs [343]. Some studies have demonstrated that re-treatment is not effective in patients who do not exhibit clinical improvement after the first RTX infusion, which reinforces the hypothesis that such patients represent a different pathogenic subset of RA [341]. Nevertheless, recent studies contradict this hypothesis [340, 342]. Indeed, it has been shown that RA patients who initially fail to exhibit a response to RTX may respond to a second cycle [342]. Importantly, it was demonstrated that re-treating nonresponders prior to full B cell repopulation is a possible strategy to enhance depletion in these patients. Furthermore, the

use of a different B-cell depleting agent or a different initial dose of RTX can present an alternative strategy. Therefore, these observations seem to indicate that the full potential of B cell depletion therapy has not been achieved by current approaches and further studies are required.

## 4.2. The importance of BAFF and BAFF-receptors in B cell survival

BAFF, also known as BLyS, THANK, TALL-1, zTNF-4 or TNFSF13b, is a protein member of the TNF family that has an important role in B cell maturation, homeostasis and survival [344]. BAFF binds to three receptors: B-cell maturation antigen (BCMA), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and BAFF-receptor (BAFF-R or BR3) [345]. The precise mechanisms that control expression of all three BAFF receptors are currently unclear, but studies indicate that the developmental stage, cytokine milieu and the microenvironment play a key role in the regulation of the receptor expression [346]. Nevertheless, BAFF-R is the receptor to which BAFF binds with higher affinity [347].

Studies of BAFF-R-deficient mice demonstrated that BAFF was essential for B cell survival, primarily through the alternative NF-κB pathway [348], and for B cell maturation and immunoglobulin class switching through the classical NF-κB pathway [349]. Moreover, the essential requirement for BAFF in B cell development was shown by the phenotype of BAFF knockout mice, which not only had a severe deficit of mature B cells, but also reduced levels of immunoglobulins [350].

Importantly, evidence supports that BAFF is implicated in the development of autoimmunity. Indeed, BAFF transgenic mice have B cell hyperplasia, increased levels of immunoglobulins and prolonged BAFF overexpression induces autoantibody production [344, 351]. BAFF also plays a key role in promotion and/ or progression of malignant B cells [352], which suggests that BAFF may represent a link between autoimmunity and the increased frequency of B cell neoplasms observed in autoimmune conditions. Conversely, BAFF may also play a role in the autoimmune manifestations observed in such B cell malignancies. It is suggested that excess BAFF may lower the threshold for BCR signaling and maintain survival of pathogenic autoreactive B cells, thus breaking B cell tolerance.

Several autoimmune diseases might be partially triggered by deficient B-cell tolerance associated with the over-production of BAFF that might lead to inappropriate survival of autoreactive B cells.

In humans, BAFF levels are elevated in patients with SLE, RA, SS and other systemic autoimmune diseases [276]. In RA patients, BAFF levels were found to be higher in the synovium than in the corresponding serum, thus suggesting that local production of BAFF in the synovium drives the maturation of autoreactive B cells that exacerbate the inflammatory process [353]. In fact, generalized inflammation and high levels of BAFF may drive continued production of plasma cells producing pathogenic autoantibodies [299, 354]. These observations seem to implicate BAFF in the etiology and progression of autoimmune disease. Furthermore, BAFF receptor system displays an intriguing biological complexity in the sense that two of the receptors, TACI and BCMA, also interact with a related TNF family ligand, known as A proliferation-inducing ligand (APRIL). APRIL not only affects class-switch recombination process [355-357], but also plasma cell differentiation and survival [358-360]. BAFF binds to BAFF-R and TACI with high avidity and to BCMA with lower (humans) or no (mouse) avidity, whereas APRIL binds only to TACI and BCMA [361]. Also, high levels of both BAFF and APRIL, along with their receptors, are observed in the rheumatoid synovium [362], with APRIL being produced by synovial DCs and BAFF by tissue macrophages [363] and synovial fibroblasts. Indeed, this observation could thus explain the maintenance of autoreactive B cells in joints [360]. Interestingly, both cytokines are also produced by the synovial B cells [364, 365].

Additionally, BAFF up-regulates Toll-like receptors (TLRs) expression and, together with IL-6, promotes Ig class-switching and plasma cell differentiation [366]. In fact, plasma cells express TACI and/ or BCMA and their survival can be supported by either BAFF or APRIL that are secreted by multiple cell types within the lymph node or bone marrow microenvironment [367]. Therefore, targeting BAFF, APRIL or their receptors may provide a novel therapeutic approach to autoimmunity.

In animal models of autoimmune disease, BAFF and/ or its receptors' antagonists reduce the disease severity and delay its progression [347, 368, 369]. Clinical trials of a selective antibody to BAFF (belimumab) and with the BAFF/ APRIL inhibitor TACI-Ig (atacicept) are also currently in progress with promising results, at least in SLE [370-372]. The results

observed in clinical trials will shed a light over the perspectives of using BAFF, APRIL or its receptors as potential future B cell targets in RA, either alone or eventually in combination therapy with B cell depleting agents, such as RTX [373, 374]. Therefore, continuous research on B cell biology in RA is fundamental to better understand the mechanisms underlying B cell intervention in RA autoimmunity and how the pathogenic effects of these cells can be abrogated to stop the development of autoimmune conditions.

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# CHAPTER II

## Aims

# AIMS

The main goal of the present work was to analyze the pathogenic role of B cells in the early phase of RA and in relapsing RA patients after B cell depletion therapy.

The specific aims of this study were:

i. To characterize B cell subpopulations in the peripheral blood from very early arthritis patients.
ii. To quantify cytokine, chemokine and autoantibody production in patients' serum.
iii. To study the influence of immunosuppressive treatments (corticosteroids and MTX) on B cells activity and function in very early rheumatoid arthritis patients.
iv. To analyze the expression of a group of B-cell related activation, maturation and survival genes in the initial phase of RA.
v. To characterize B cell subpopulations, BAFF-R expression and serum BAFF levels before and after B cell depletion therapy.

# CHAPTER III

## Results

# RESULTS

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

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**Published in *Rheumatology (Oxford)* 2010; 49(6):1082-1092**

### Abstract

**Objectives:** To characterize circulating B cell subpopulations of arthritis patients with less than 6 weeks of disease duration. **Methods:** Peripheral blood samples were collected from very early untreated polyarthritis patients, with less than 6 weeks of disease duration, for flow cytometric evaluation of B cell subpopulations. Samples from patients that were later diagnosed as rheumatoid arthritis (very early rheumatoid arthritis, 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 methotrexate (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 to 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.

# 1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease of unknown aetiology affecting about 1% of the 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, 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 interleukin 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 rheumatoid factor (RF). RF can interact with FcγRIIIa (CD16) receptors on monocytes and macrophages inducing the production of tumour necrosis factor (TNF) [13]. Moreover, B cell depletion therapy with Rituximab, a monoclonal antibody 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 which 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 very scarce. In addition, the effect of commonly used Disease Modifying Anti-Rheumatic Drugs (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 RA (VERA) and in other very early arthritis (VEA) patients when compared to healthy donors, and to evaluate whether corticosteroids and MTX therapies have an impact on the frequencies of these cell subsets.

# 2. Materials and Methods

## 2.1. Patients

Blood samples were obtained from 46 untreated polyarthritis patients (Rheumatology Department, Hospital de Santa Maria, Lisbon) with less than 6 weeks of disease duration. 22 of these patients later on fulfilled the American College of Rheumatology (ACR) criteria for RA [16]. These patients were classified as VERA patients and further samples were collected 4-6 weeks after starting a low dose of oral prednisone (5-10mg) (time 1) and 4 months after reaching the minimum effective dose of MTX, up to a maximum of 20mg/week, that was needed to reduce the 28 joints disease activity score (DAS28) to less than 3.2 (time 2) [17]. The baseline blood samples from VERA patients were compared with other 24 very early arthritis (VEA) patients and 29 healthy donors used controls. The health assessment questionnaire (HAQ) [18] was applied to all patients and the DAS28 was calculated in all patients that fulfilled the ACR criteria for RA. The local ethics committee 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).

## 2.2. 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 monoclonal antibodies conjugated to fluorescein isothiocyanate (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, USA) or APC (HIB19, eBioscience, 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, Germany) and anti-CD8 APC (MEM-31, Immunotools).

## 2.3. Whole blood staining

Approximately 5 ml of whole blood were collected by venipuncture into tubes containing EDTA. Erythrocytes were lysed with FACS™ Lysing Solution (BD Biosciences, USA) and cells were stained, incubated for 20 minutes at 4ºC, washed and stored in the dark at 4ºC until analyzed 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). Data were analyzed with FlowJo (TreeStar, Stanford University, CA). Absolute cell counts were calculated from differential leukocyte count determined at each time point for all patients.

## 2.4. Peripheral blood mononuclear cells isolation

PBMC were isolated from 20 ml heparinized whole blood following density gradient centrifugation with Percoll™ (Amersham, Sweden). Cellular viability was estimated with Trypan Blue (Sigma, USA). Cells were frozen in 1 ml/ $10^7$ cells RPMI-1640 (GIBCO, UK), 40% fetal calf serum (FCS) (Invitrogen, UK), 10% dimethyl-sulfoxide (DMSO) (Sigma, USA) and stored at -80ºC until further use.

## 2.5. Measurement of autoantibodies

Rheumatoid factors (IgM, IgG and IgA) and anti-cyclic citrullinated peptide (anti-CCP) were determined at baseline in all patients and also at times 1 and 2 for VERA patients. IgM-RF, IgG-RF and IgA-RF were measured in the serum by DRG Rheumatoid Factor ELISA test system kits (DRG International, Inc., USA) according to the manufacturer instructions and samples were processed using a ChemWell® 2910 automated analyzer. Serum levels of anti-CCP were measured by ELIA™ CCP test system (Phadia GmbH, Germany) and samples were analyzed using an ImmunoCAP® 100 instrument.

### 2.6. Statistical analysis

Statistical differences were determined using one-way ANOVA and Bonferroni's multiple comparison tests using GraphPad Prism (GraphPad, San Diego, CA). For populations that did not follow Gaussian distribution, the Kruskal-Wallis nonparametric test was used. Differences were considered statistically significant for $p < 0.05$.

## 3. Results

### 3.1. Disease assessment and autoantibody production

A total of 46 polyarthritis patients with less than 6 weeks of disease duration were evaluated. 22 patients, 18 female and 4 male, 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 RF (IgG) 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 very early arthritis (VEA) subjects, 16 female and 8 male, with a mean age of 44.8±18.5 years (range 19-87 years), patients were later classified as having systemic lupus erythematosus (4 cases), crystal induced arthritis (3 cases), psoriatic arthritis (2 cases), colon adenocarcinoma (1 case), multiple myeloma (1 case), polymyalgia rheumatica (1 case), arthritis associated to HIV infection (1 case), arthritis associated with Crohn's disease (1 case), unremitting undifferentiated arthritis (2 cases), unremitting reactive arthritis (2 cases), 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, 22 female and 7 male, with a mean age of 39.8±13.6 years (range 22-63 years) were also analyzed.

**Table 1.** Characteristics of very early rheumatoid arthritis patients and other very early arthritis patients.

| Clinical parameter | VERA (n=22) | | | VEA (n=24) |
|---|---|---|---|---|
| | Baseline | Visit 1 | Visit 2 | |
| DAS 28 | 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 |
| Leukocyte 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 - Four months after reaching the minimum effective dose of methotrexate; NA – not applicable;

*Differences considered statistically significant for $p$ values < 0.05. All values indicated represent the mean ± standard deviation (SD).

## 3.2. Very early rheumatoid arthritis patients have a reduced memory B cell subpopulation irrespective of therapy

The main B cell memory subsets were analyzed, 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 ($CD19^+IgD^+CD27^-$), pre-switch memory B cells ($CD19^+IgD^+CD27^+$), post-switch memory B cells ($CD19^+IgD^-CD27^+$), total memory B cells ($CD19^+CD27^+$) and plasma cells ($CD19^+CD27^{high}$) from VERA patients were compared to the same populations of healthy donors (Figure 1).

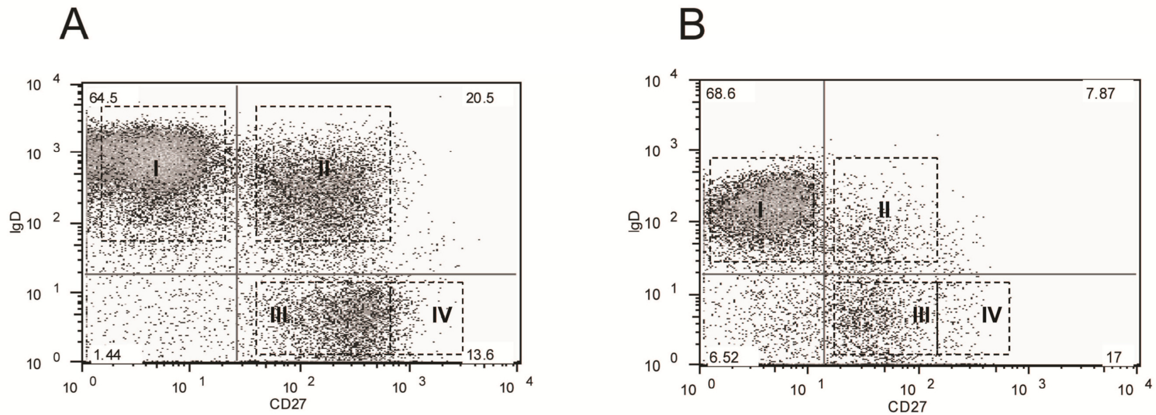


**Figure 1.** *B cell analysis by flow cytometry.*
A region (gate) was defined around total lymphocytes in peripheral blood, depending upon cell size (FSC – forward scatter) and granularity (SSC – side scatter). Total B cells were identified based on the expression of the cell surface marker CD19 and its subpopulations were classified according to IgD and CD27 expression in healthy controls **(A)** and patients **(B)**. Thus, naïve B cells (I) were classified as $CD19^{+}IgD^{+}CD27^{-}$ ; pre-switch memory B cells (II) as $CD19^{+}IgD^{+}CD27^{+}$; post-switch memory B cells (III) as $CD19^{+}IgD^{-}CD27^{+}$ and plasma cells (IV) as $CD19^{+}IgD^{-}CD27^{high}$.

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 (Figure 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 (Figure 2A). Naïve B cells were significantly higher in VERA patients without treatment when compared to controls (Figure 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 (Figure 2B). Both controls and VERA patients had comparable very low levels (less than 3%) of circulating plasma cells (Figure 2C). VERA patients had significantly lower frequencies of pre-switch memory B cells when compared to controls (Figure 3A), irrespective of therapy. Importantly, this observation was also confirmed by a decrease in the absolute numbers of this B cell subpopulation (Figure 3B). In contrast, no statistically significant differences were observed in post-switch memory B cells between VERA patients and controls (Figure 3C). A lower percentage of total memory B cells was

observed in untreated VERA patients as compared to controls (Figure 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 analyzed 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 T ($CD3^+$), $CD4^+$ 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 to controls, or after treatment (*data not shown*).

Although 22 VERA patients were selected for this study, 5 patients were lost for 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 figures.

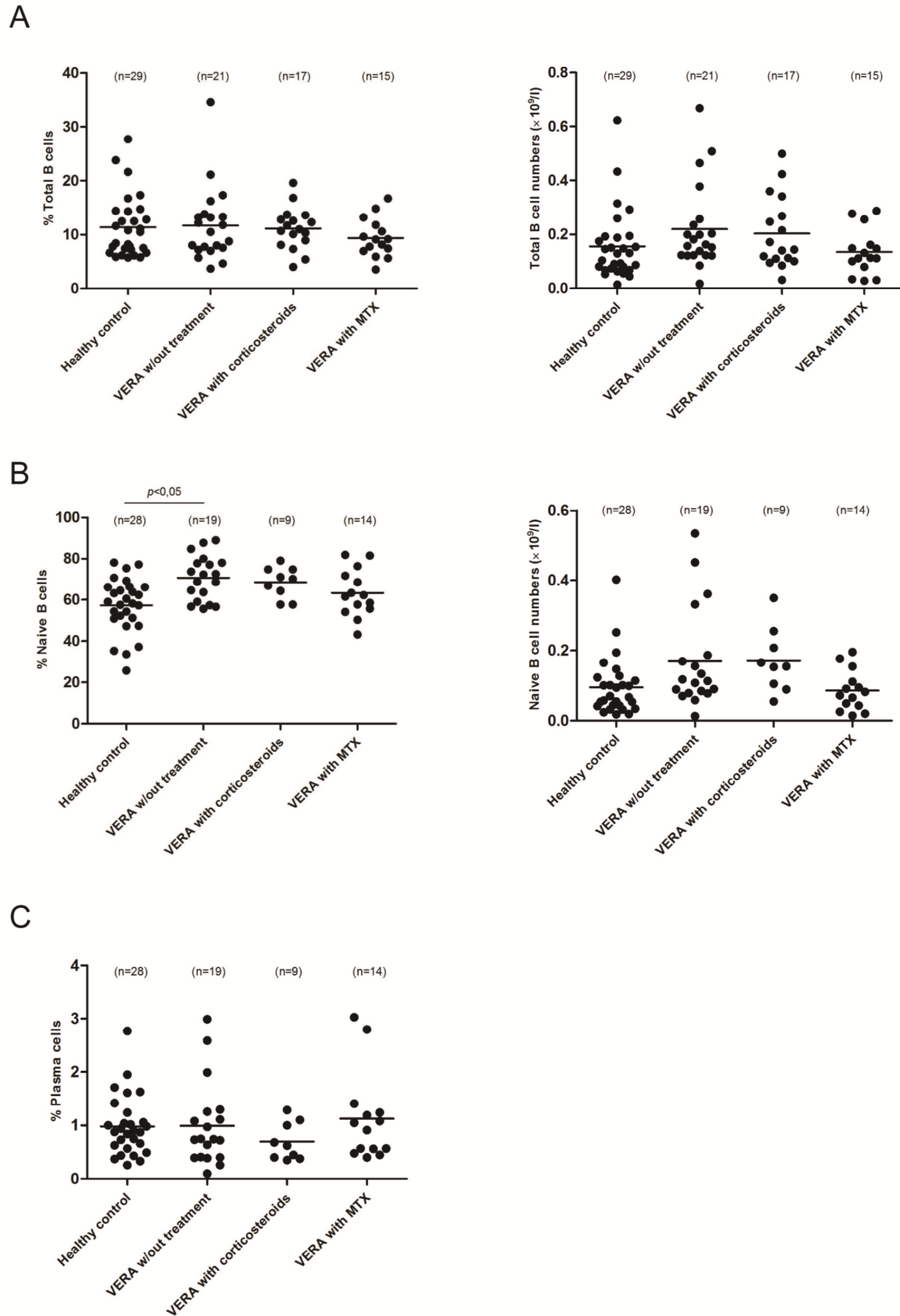


**Figure 2.** *Therapy with corticosteroids and methotrexate does not affect circulating total B cells, naïve B cells and plasma cells of very early rheumatoid arthritis patients.*

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 ($CD19^+CD27^{high}$) **(C)** were gated in $CD19^+$ B cells. Differences are considered statistically significant for $p < 0.05$.

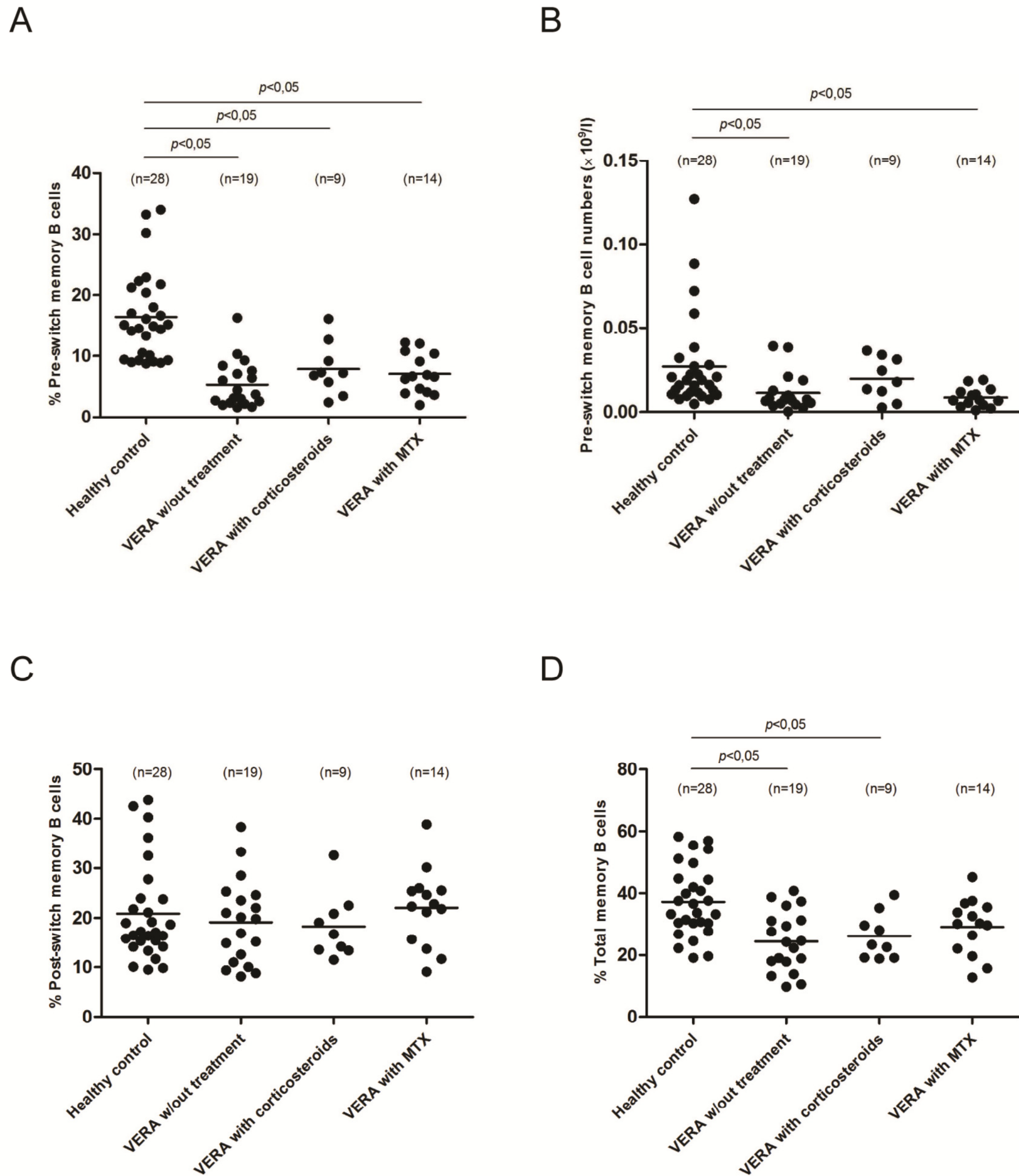


**Figure 3.** *Very early rheumatoid arthritis patients have reduced frequencies and absolute numbers of pre-switch memory B cells.*

Data from flow cytometry analysis of memory B cells from VERA patients without treatment and after therapy with corticosteroids and MTX. All subpopulations were gated in $CD19^+$ B cells. Representation of Pre-switch memory B cells ($CD19^+IgD^+CD27^+$) frequencies **(A)** and absolute numbers **(B)**. Post-switch memory B cells ($CD19^+IgD^-CD27^+$) **(C)** and Total memory B cells ($CD19^+CD27^+$) **(D)** frequencies are represented. Differences are considered statistically significant for $p < 0.05$.

### 3.3. Very early arthritis patients have a diminished pre-switch memory B cell subset at baseline

Other very early polyarthritis patients evaluated with less than 6 weeks of disease duration who later were diagnosed as different 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 (Figure 4A), naïve B cells (Figure 4B) or plasma cells (Figure 4C) when compared to controls. However, similarly 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 to controls (Figure 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 (Figure 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 (Figure 5B). No other statistically significant results were obtained with post-switch memory B cells (Figure 5C), or with $CD4^+$ and $CD8^+$ T cells (*data not shown*).

The sub analysis of the 6 patients that 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*).

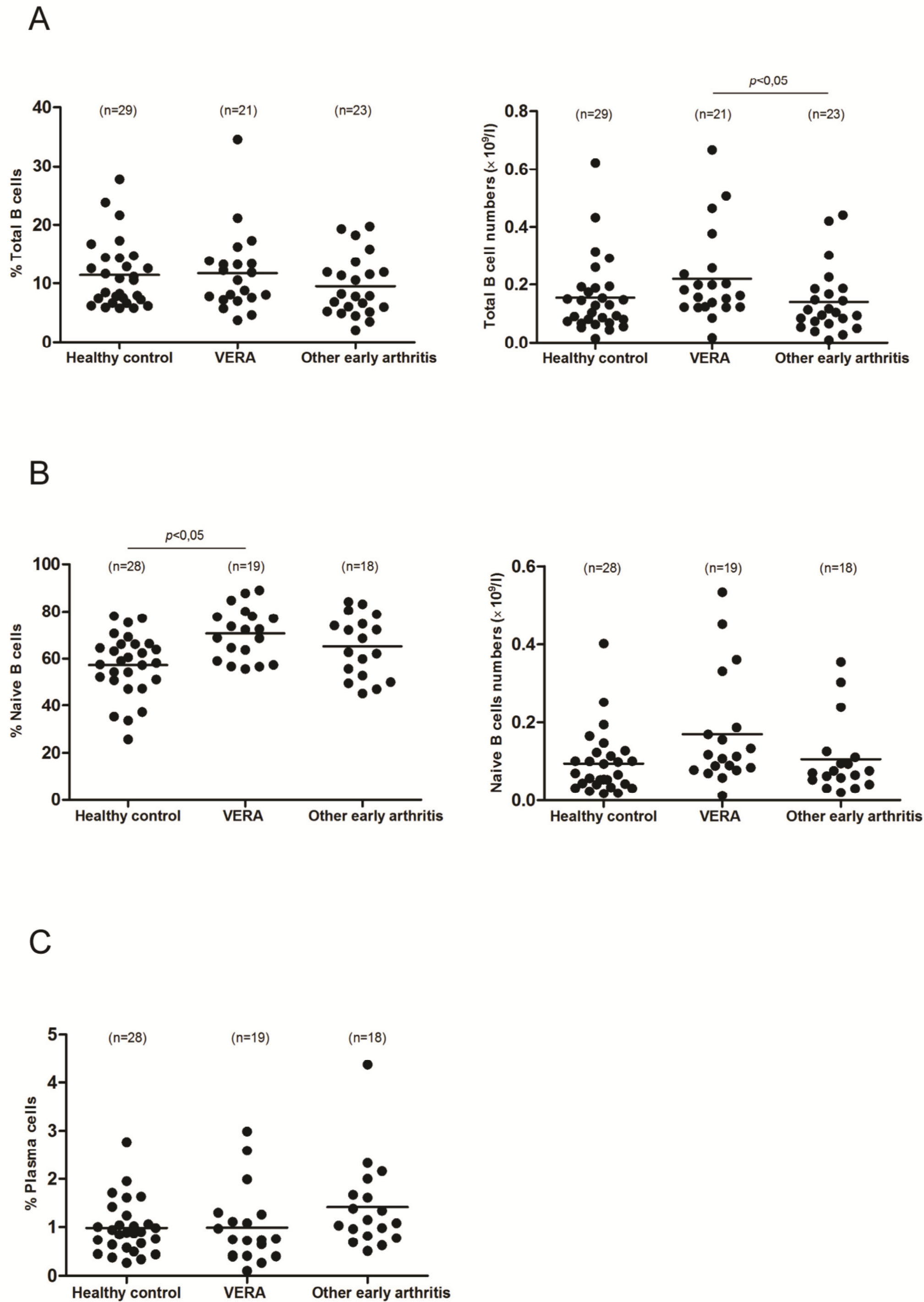


**Figure 4.** *Early arthritis patients do not have any significant difference in circulating total B cells, naïve B cells and plasma cells when compared to healthy controls.*

Data from flow cytometry analysis of peripheral blood B cells from other early arthritis patients at baseline. 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 ($CD19^{+}CD27^{high}$) **(C)** were gated in $CD19^{+}$ B cells. Differences are considered statistically significant for $p < 0.05$.

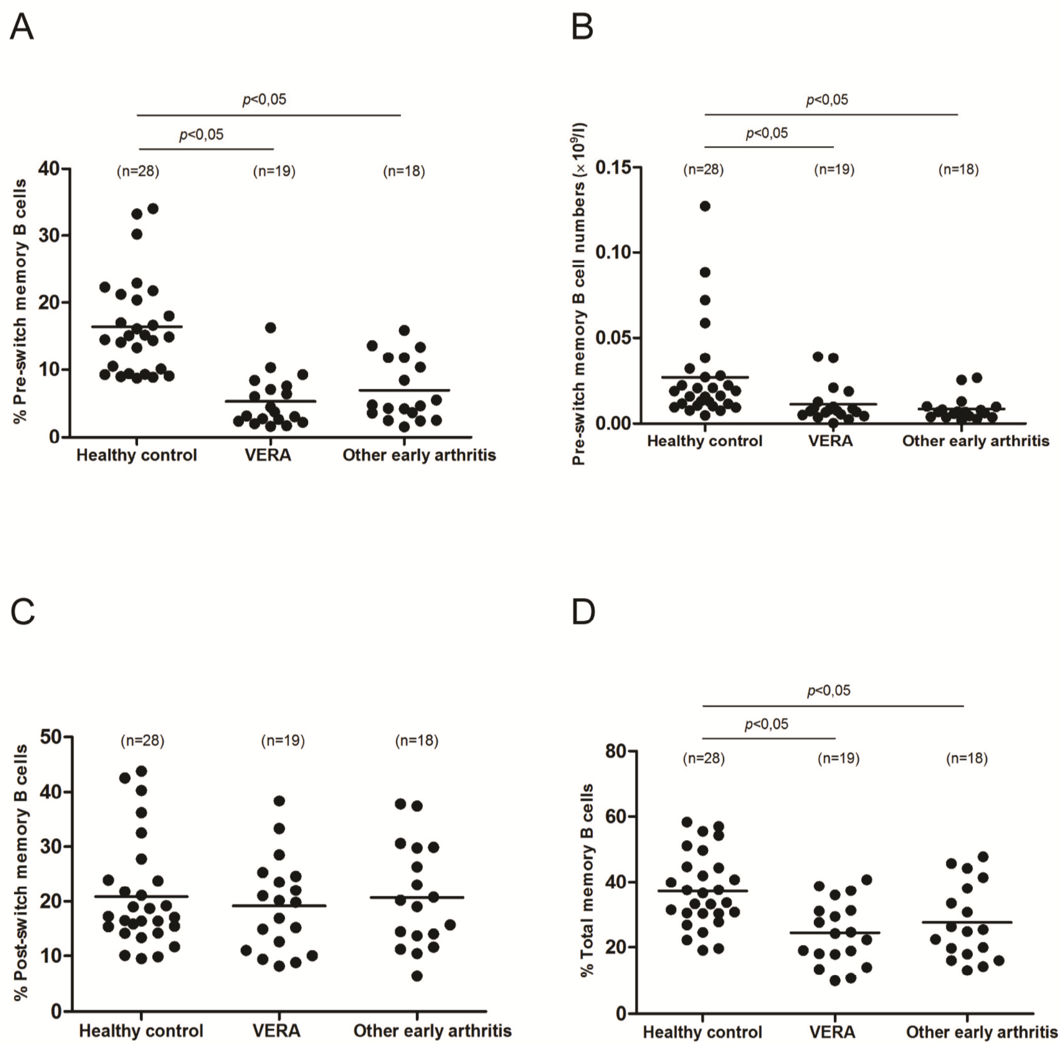


**Figure 5.** *Early arthritis patients have reduced frequencies and absolute numbers of pre-switch memory B cells.*
Data from flow cytometry analysis of memory B cells from other early arthritis patients at baseline. All subpopulations were gated in $CD19^+$ B cells. Representation of Pre-switch memory B cells ($CD19^+IgD^+CD27^+$) frequencies **(A)** and absolute numbers **(B)**. Post-switch memory B cells ($CD19^+IgD^-CD27^+$) **(C)** and Total memory B cells ($CD19^+CD27^+$) **(D)** frequencies are represented. Differences are considered statistically significant for $p < 0.05$.

# 4. 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 to 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. 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 (AID), 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 of IgD+CD27+ memory B cell subset in patients with systemic lupus erythematosus (SLE) [31], primary Sjögren Syndrome (SS) [32-34] or Systemic Sclerosis (SSc) [35]. Also, a reduction in circulating memory B cells was demonstrated in patients with X-linked Hyper-IgM Syndrome [36], Chronic Granulomatosus Disease [37] and HIV infection [38]. Hence, lower levels of circulating memory B cells seem to be a 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 postrecombinational 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 of 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 to 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 to controls. Also, similar results were observed in VEA patients when analyzing 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 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 corticosteroids 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 *et al* [48] it was observed that SLE patients treated with short-term MTX had slightly increased levels of total $CD3^+$, $CD4^+$ and $CD8^+$ T cells, while monocytes and B cells remained stable. However, long-term MTX treatment decreased absolute numbers of both B and T cells. Moreover, a decrease of autoantibody levels accompanied the B cell response to long-term MTX. Nevertheless, several studies by Lacki *et al* [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 of 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 VERA patients.

# 5. 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 to controls. In addition, the short term use of corticosteroids and MTX do not

seem to affect circulating B cell subpopulations in VERA patients. However, since other early arthritis patients, who did not fulfil the ACR criteria for RA, also had a decrease of 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.

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

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**Published in *Rheumatology (Oxford)* 2011; 50(2):278-282**

## Abstract

**Objectives:** B cells play an important role in the perpetuation of rheumatoid arthritis (RA), particularly as autoantibody producing cells. The immune complexes that further develop deposit in the joints and aggravate the inflammatory process. However, B cells 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 less than 6 weeks of disease duration, who latter on evolved into RA (VERA). **Methods:** APRIL, BAFF and IL-21 levels were measured by ELISA in the serum of VERA, other very early arthritis (VEA), established RA patients and controls. Synovial fluid (SF) samples of established RA were also analyzed. **Results:** VERA patients have higher levels of APRIL and BAFF as compared to VEA, established RA and controls. Furthermore, APRIL and BAFF levels are also significantly elevated in RA-SF when compared to 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.

# 1. Introduction

B cells play critical roles in rheumatoid arthritis (RA) pathogenesis. They are the source of rheumatoid factors (RF) and anti-cyclic citrullinated peptide (CCP) autoantibodies, which contribute to immune complex formation in the joints. These cells are also efficient antigen presenting cells and contribute to T cell activation through expression of costimulatory molecules. B cells simultaneously respond and produce chemokines and cytokines that promote leukocyte 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 cells 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 less than 6 weeks of disease duration, when compared to controls [4]. In accordance with the results of another group it is highly likely that this B cell subset migrates towards the synovial membrane, contributing for the onset of the synovitis process [5]. Our hypothesis is that the cytokine environment in early RA favors the recruitment, activation and survival of B cells and herein we tested this concept in a cohort of very early polyarthritis patients.

# 2. Materials and Methods

## 2.1. Patients

Blood samples were collected from 19 untreated very early polyarthritis patients with less than 6 weeks of disease duration, who after a minimum follow-up of 3-4 months fulfilled the 1987 American College of Rheumatology (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 corticosteroids (5-10 mg of prednisone) (Time 1) and 4 months after reaching the minimum effective dose of methotrexate (MTX) (Time 2) up to a maximum of 20 mg/week required to reduce the 28 joints disease activity score (DAS28) to

less than 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 synovial fluid (SF) samples were obtained from MTX treated established RA patients. Of note, SF samples were only collected from established RA, since most VERA patients did not have joint effusions in easily accessible joints (Table 1). The health assessment questionnaire (HAQ) [8] and the DAS28 were applied to all patients. The study was approved by the local ethics committee and all patients signed an informed consent. Patient care was conducted in accordance with the standard clinical practice in the Rheumatology Department, Hospital de Santa Maria, Lisbon and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

## 2.2. Cytokine quantification

A proliferation-inducing ligand (APRIL), B cell activating factor (BAFF) and interleukin (IL)-21 levels were determined by enzyme linked immunosorbent assay (ELISA) (Bender MedSystems GmbH, Vienna, Austria) according to the provider's instructions. Samples were analyzed using plate reader Infinite® M200 (Tecan, Männedorf, Switzerland).

## 2.3. 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 instructions and samples were processed using a ChemWell® 2910 automated analyzer. Serum levels of anti-CCP were measured by ELIA™ CCP test system (Phadia GmbH, Freiburg, Germany) and samples were analyzed using an ImmunoCAP® 100 instrument.

## 2.4. Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis and Mann-Whitney tests using GraphPad Prism (GraphPad, San Diego, CA). Correlation analysis was

performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

# 3. Results

## 3.1. Characterization of patients and disease evaluation

A total of 38 polyarthritis patients with less than 6 weeks of disease duration were consecutively included. VERA patients had a mean age of 59±17 years, 84.2% were female, 42% were RF positive and 32% anti-CCP positive. The baseline DAS28 and HAQ were 6.1±1.8 and 1.4±0.8, respectively. After treatment with corticosteroids and MTX there was a significant reduction of both DAS28 and HAQ values (Table I). VEA patients were classified as having spondyloarthritis (5 cases), systemic lupus erythematosus (4 cases), crystal induced arthritis (2 cases), Sjögren's syndrome (1 case), paraneoplastic polyarthritis related to multiple myeloma (1 case), arthritis associated to HIV infection (1 case), and 5 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 DAS28 and HAQ values to those of VERA patients at baseline (Table 1).

## 3.2. APRIL and BAFF levels are increased in very early rheumatoid arthritis patients at baseline

At baseline, APRIL and BAFF levels were significantly higher in VERA patients as compared to both VEA and controls (Figure 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 both VEA patients and controls, or after therapy with corticosteroids and MTX (*data not shown*). Furthermore, there was no correlation between DAS28, anti-CCP or RF autoantibodies and APRIL and BAFF serum concentrations (*data not shown*).

**Table 1.** Clinical information and cytokine levels in healthy controls, very early rheumatoid arthritis, very early arthritis and rheumatoid arthritis patients.

| | Controls | VERA (n=19) | | | VEA | RA | RA SF |
|---|---|---|---|---|---|---|---|
| | (n=24) | Baseline | Time 1 | Time 2 | (n=19) | (n=12) | (n=15) |
| Age, years | 40±13 | | 50±17 | | 40±13 | 63±10 | 57±10 |
| Sex (females/males) | 17/7 | | 16/3 | | 15/4 | 11/1 | 11/4 |
| Disease duration, years | NA | | < 6 weeks | | < 6 weeks | 8±9 | 9±12 |
| DAS28 | NA | 6.1±1.8 | 4.1±1.6* | 3.1±1.6* | 4.5±1.6* | Baseline:4.3±0.8 [a]<br>Last observation:5.2±1.0 | Baseline:4.7±0.7 [a]<br>Last observation:4.6±1.4 |
| HAQ | NA | 1.4±0.8 | 0.8±0.7* | 0.8±0.7 | 0.8±0.6* | 1.5±1.0 | 1.4±0.8 |
| RF-positive, % | ND | 42 | ND | ND | 0 | 67 | ND |
| Anti-CCP-positive, % | ND | 32 | ND | ND | 0 | 45 | ND |
| IL-21, pg/ml | 261.2±272.6 | 1122.0±3170.0 | 1617.0±3415.0 | 1226.0±1158.0 | 464.7±1178.0 | 246.5±478.7 | 548.3±454.7 |
| APRIL, ng/ml | 6.0±11.3 | 18.8±19.7 | 12.5±8.7 | 23.2±29.9 | 5.9±5.2 | 13.1±27.6 | 26.2±25.8 |
| BAFF, ng/ml | 0.3±0.6 | 0.8±0.6 | 0.5±0.2 | 0.8±0.3 | 0.3±0.4 | 0.2±0.1 | 0.9±0.6 |

All values indicated in table represent mean ± standard deviation. NA - not applicable; ND - not determined; SF – synovial fluid.

[a] The indicated DAS28 value corresponds to the baseline DAS28 3V score due to the unavailability of Visual Analogue Scale (VAS) parameter at baseline in established RA group of patients.

* DAS28 and HAQ values were compared between VERA and VEA patients with reference to VERA baseline values.

Differences are considered statistically significant for $p < 0.05$.

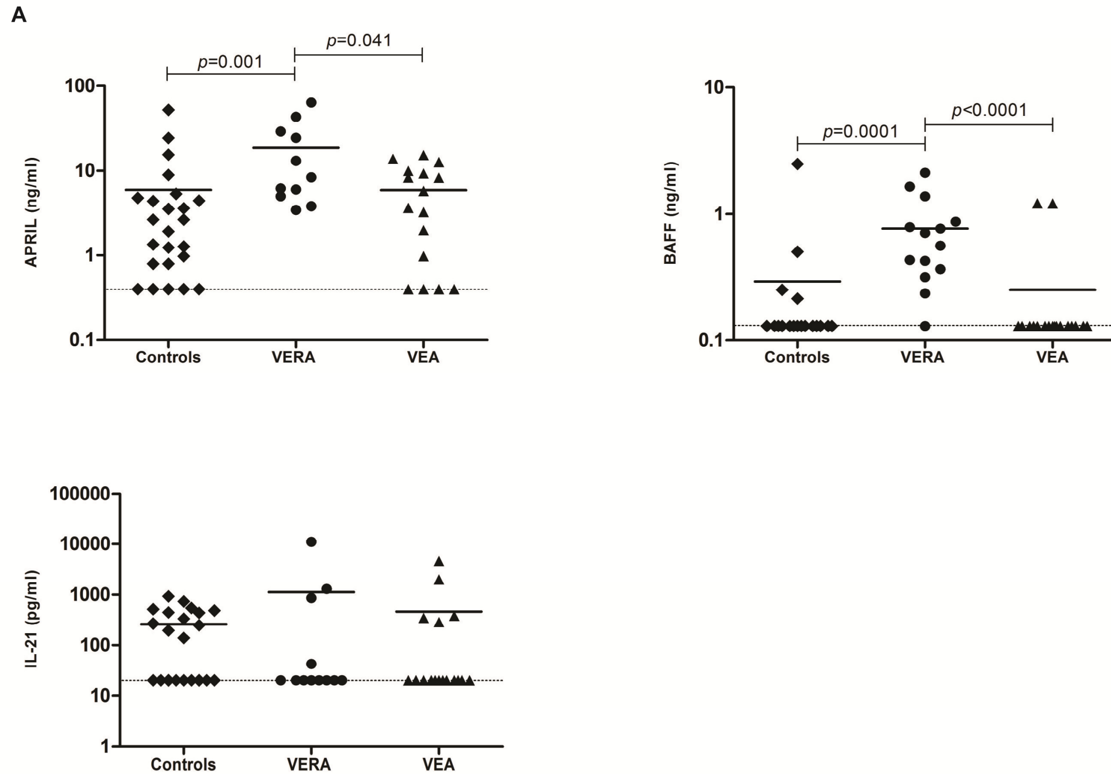


**Figure 1A.** *Very early rheumatoid arthritis patients have higher APRIL and BAFF levels than controls and those with very early arthritis at baseline.*
Serum samples from VEA patients with no therapy and healthy controls were analyzed by ELISA technique. Dotted lines represent the limit of detection for the assay. Differences were considered to be statistically significant at $p < 0.05$.

## 3.3. Very early rheumatoid arthritis patients have higher APRIL and BAFF levels in comparison to established rheumatoid arthritis patients

In order to compare early RA with the chronic phase, we also analyzed 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*).

## 3.4. Established rheumatoid arthritis synovial fluid has increased levels of APRIL, BAFF and IL-21

To verify if 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 (Figure 1B).

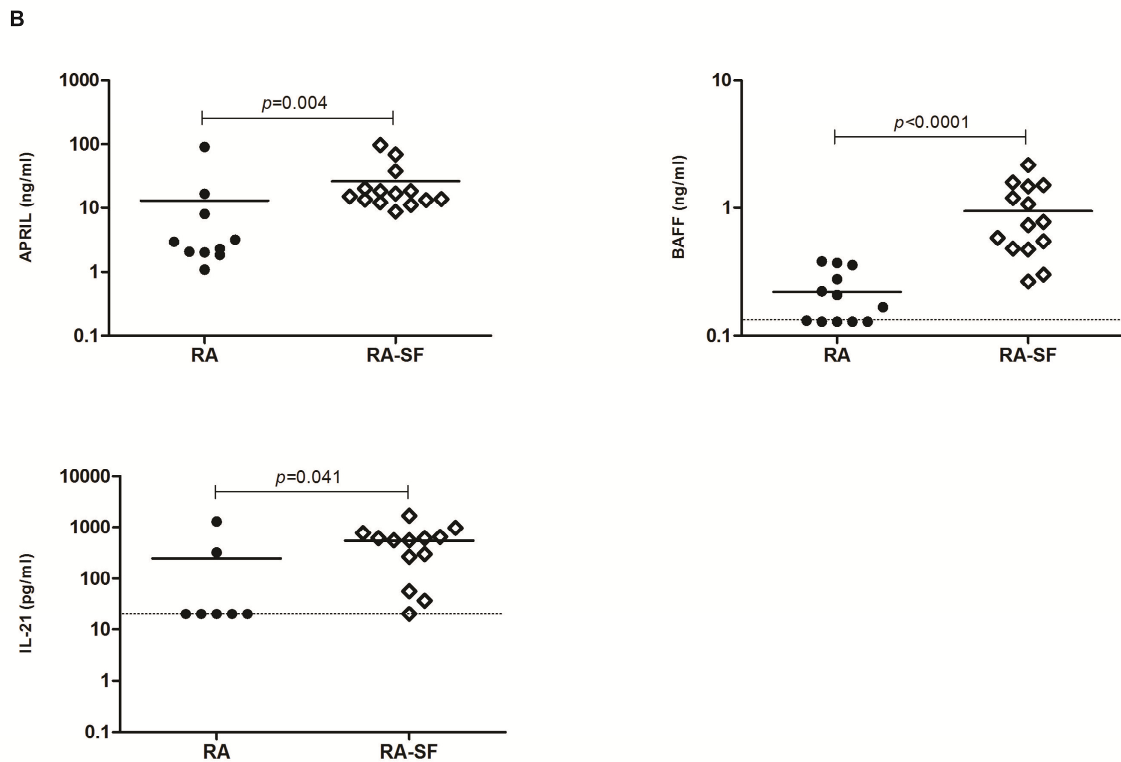


**Figure 1B.** *Established rheumatoid arthritis patients have increased levels of APRIL, BAFF and IL-21 locally in the joints.*
Serum and synovial fluid (SF) samples from established RA patients were analyzed by ELISA technique. Dotted lines represent the limit of detection for the assay. Differences were considered statistically significant at $p < 0.05$.

# 4. Discussion

In the present work, a cytokine pattern favoring B cell activation is observed in RA patients with less than 6 weeks of disease duration, when compared with other causes of very early arthritis (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 other group referring a migration of this B cell subset towards the synovial membrane [5]. In fact, in established RA, ectopic germinal center-like structures develop in the inflamed synovial tissue that support survival of B cells and autoantibody production [9]. Therefore, we also analyzed 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 to VEA and controls. Interestingly, APRIL was also increased in VERA patients' serum as compared to established RA and its levels were even higher in RA-SF suggesting a local upregulation in the synovium. APRIL affects not only 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 synovial fluid levels of APRIL has been demonstrated in RA patients [15, 16]. BAFF, similarly 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, IL-21 increased levels in RA-SF support local plasma cell differentiation and autoantibody production [18]. Additionally, therapy with neither corticosteroids 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 corticosteroids and this might have influenced the serum levels of APRIL and BAFF. The effect of low dose corticosteroids and MTX on cytokine production in RA patients is still controversial [19, 20]. So, an absence of effect of short-term therapy with corticosteroids and MTX on the cytokines analyzed was not entirely unexpected in VERA, but an effect on chronic-treated patients might in fact occur.

## 5. Conclusions

In conclusion, we have shown increased APRIL and BAFF levels in very early RA and in RA-SF, which could hypothetically support the maintenance, expansion, activation and survival of autoreactive B cells since 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.

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# Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis

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**Published in *Arthritis Research and Therapy* 2010; 12(5):R196**

## Abstract

**Introduction:** Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by sustained synovitis. Recently, several studies have proposed neutrophils and Th17 cells as key players in the onset and perpetuation of this disease. The main goal of this work was to determine whether cytokines driving neutrophil and Th17 activation are dysregulated in very early rheumatoid arthritis patients with less than 6 weeks of disease duration and before treatment (VERA). **Methods:** Cytokines related to neutrophil and Th17 activation were quantified in the serum of VERA and established RA patients and compared with other very early arthritis (VEA) and healthy controls. Synovial fluid (SF) from RA and osteoarthritis (OA) patients was also analyzed. **Results:** VERA patients had increased serum levels of cytokines promoting Th17 polarization (IL-1β and IL-6), as well as IL-8 and Th17-derived cytokines (IL-17A and IL-22) known to induce neutrophil-mediated inflammation. In established RA this pattern is more evident within the SF. Early treatment with MTX or corticosteroids led to clinical improvement but without an impact on the cytokine pattern. **Conclusions:** VERA patients already display increased levels of

cytokines related with Th17 polarization and neutrophil recruitment and activation, a dysregulation also found in SF of established RA. Thus, our data suggest that a cytokine-milieu favoring Th17 and neutrophil activity is an early event in RA pathogenesis.

# 1. Introduction

Rheumatoid arthritis (RA) is the most common chronic autoimmune disease, affecting approximately 1% of the population worldwide. This disease comprises a syndrome of pain, stiffness and symmetrical synovitis which leads to joint destruction, functional disability and substantial comorbidity due to involvement of multiple organs and systems. The migration of leukocytes towards the synovium is crucial for the establishment of a chronic inflammatory process in RA [1-3]. This multi-regulated mechanism involves interactions with endothelial cells through cell adhesion molecules and complex cytokine and chemokine pathways.

Neutrophils specifically play an important role in the onset and perpetuation of RA, not only as interleukin producing cells, but also for being responsible for the release of high amounts of reactive oxygen species (ROS) and destructive enzymes, such as metalloproteases, contributing to joint erosions [4]. Neutrophils are among the first leukocytes to arrive at sites of inflammation. In fact, these cells are the most abundant in the synovial fluid of patients with active RA and previous results from our group showed that the synovial tissue is heavily infiltrated by neutrophils in the first weeks of RA onset [5]. Interestingly, in animal models of arthritis, neutrophil depletion prevented joint inflammation if neutrophil depleting antibodies were given before the induction of arthritis. Moreover, when the depleting antibody was given very early after the induction of arthritis complete abrogation of the inflammatory symptoms was also achieved [6].

T helper (Th)-17 cells have also been proposed to have a relevant role in the early phase of RA through the production of interleukin (IL)-17 [7, 8]. This cytokine promotes the recruitment and survival of neutrophils, induces the secretion of proinflammatory cytokines, the upregulation of RANK ligand (RANKL) and stimulates the activity of matrix metalloproteases, leading to cartilage catabolism and bone resorption [9, 10]. The recruitment, activation and effector function of Th17 cells and neutrophils are driven by a

network of cytokines and chemokines, secreted by multiple cellular sources. In established RA, it has been reported that IL-1β, IL-6, IL-8, IL-17 and tumor necrosis factor (TNF) are elevated in the serum and this correlates with a higher disease activity [11-13]. Nevertheless, our knowledge on the influence of the cytokine network on RA onset remains scarce. The characterization of the cytokine profile at this stage, where the transition from an acute to a chronic inflammatory phase occurs, may lead to the identification of early key players, with potential implications for early treatment strategies.

Thus, the main goal of our work was to determine whether cytokines driving neutrophil and Th17 cell activation and proinflammatory function were already present in very early RA (with less than 6 weeks of disease duration) and how this early cytokine environment differs from established RA. We also evaluated whether the introduction of low dose corticosteroids and methotrexate (MTX) therapy had any influence on the cytokine profile observed at that early stage of the disease.

We found that cytokines related with Th17 polarization and neutrophil recruitment and activation were elevated in early RA and that the conventional therapeutic options, although able to control clinical manifestations of the disease, were ineffective in reversing this underlying proinflammatory drive.

## 2. Materials and Methods

### 2.1. Patients

Blood samples were obtained from 38 consecutive untreated polyarthritis patients with less than 6 weeks of disease duration. Some of these patients (19) after a minimum follow up of 3 months fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [14]. These patients were classified as Very Early Rheumatoid Arthritis (VERA) patients and further samples were collected 4-6 weeks after starting a low dose of oral corticosteroids (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, that was required to reduce the 28 joints disease activity score (DAS28) to less than 3.2 [15]. The remaining early arthritis patients (19), who did not evolve into RA, were classified as very early polyarthritis (VEA). Baseline blood samples from VERA and VEA patients were compared

with 27 healthy donors used as controls. Additionally, 12 blood and 15 SF samples were obtained from established RA patients. SF samples were also collected from 10 OA patients (Rheumatology Department, Hospital de Santa Maria, Lisbon) (Table 1). Due to the clinical characteristics of the very early arthritis patients, SF in easily accessible joints was not available in VERA and VEA patients and thus SF was not analyzed in these groups of patients. The health assessment questionnaire (HAQ) [16] and DAS28 were applied to all patients. The study was approved by the local ethics committee and all patients signed an informed consent. Patient care was conducted in accordance with the standard clinical practice and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

## 2.2. Cytokine quantification

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IL-22, IL-23 and interferon-gamma (IFN-γ) levels were measured in the serum and SF by FlowCytomix assay kit (FC) (Bender MedSystems, Vienna, Austria) according to the manufacturer instructions. Standard curves for each cytokine were generated by using reference cytokine concentrations supplied by the manufacturer. Samples were acquired with a FACS Calibur (BD Biosciences, San Jose, CA, USA). Raw data of the FC bead assay were analyzed by FlowCytomixPro2.2 software (Bender MedSystems).

## 2.3. Measurement of autoantibodies

Rheumatoid factor (RF)-IgM was determined in all patients by IMTEC Autoimmune Diagnostics ELISA kit (Human GmbH, Wiesbaden, Germany) according to the manufacturer instructions and samples were processed using a ChemWell® 2910 automated analyzer (GMI, Ramsey, Minnesota, USA). Serum levels of anti-cyclic citrullinated peptide (anti-CCP) were measured by ELIA™ CCP test system (Phadia GmbH, Freiburg, Germany) and samples were analyzed using an ImmunoCAP® 100 instrument (Phadia GmbH).

## 2.4. Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis, Mann-Whitney and Wilcoxon Signed-Rank tests using GraphPad Prism (GraphPad, San Diego, CA). Correlation analysis was performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

# 3. Results

## 3.1. Characterization of patients and disease evaluation

A total of 38 polyarthritis patients with less than 6 weeks of disease duration were evaluated. Nineteen patients fulfilled the 1987 ACR criteria for RA after a minimum follow-up of 3 months and were classified as VERA patients. The mean age of the VERA patients was 59±17 years-old, 84% were female, 42% were RF positive and 32% anti-CCP positive, the initial DAS28 was of 6.1±1.8 and the initial HAQ was of 1.4±0.8. After treatment with a low dose of prednisone and MTX there was a significant reduction of both DAS28 and HAQ values (Table 1).

The group of VEA patients included 19 patients, who evolved into several different diagnosis: spondyloarthritis (5 cases), systemic lupus erythematosus (4 cases), crystal induced arthritis (2 cases), Sjögren's syndrome (1 case), paraneoplastic polyarthritis related to multiple myeloma (1 case), arthritis associated to HIV infection (1 case). Five patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited form of polyarthritis. The mean age of the VEA patients was 40±13 years-old, 79% were female, all patients were RF and anti-CCP negative, the initial DAS28 was of 4.5±1.6 and the initial HAQ was of 0.8±0.6. Both DAS28 and HAQ values were significantly lower than those of VERA patients at baseline (Table 1). These early polyarthritis patients represent a subset of a larger cohort previously described by our group [17].

Furthermore, blood samples were collected from 12 established RA patients, with a mean age of 60±10 years old, 92% were female, 67% were RF positive and 45% anti-CCP positive (Table 1). Additionally, SF samples were also collected from 12 established RA patients, with a mean age of 57±10 years old and 73% were female (Table 1). The

established RA group of patients had a DAS28 and a HAQ mean scores similar to VERA baseline values.

**Table 1.** Clinical information about healthy controls and patients with very early rheumatoid arthritis, very early arthritis, rheumatoid arthritis, or osteoarthritis.

| | Controls | VERA (n=19) | | | VEA | RA | RA SF | OA SF |
|---|---|---|---|---|---|---|---|---|
| | (n=24) | Baseline | Time 1 | Time 2 | (n=19) | (n=12) | (n=15) | (n=10) |
| Age in years, mean ± SD | 40±13 | | 50±17 | | 40±13 | 63±10 | 57±10 | 67±13 |
| Sex, female/male | 17/7 | | 16/3 | | 15/4 | 11/1 | 11/4 | 5/5 |
| DAS28, mean ± SD | NA | 6.1±1.8 | 4.1±1.6* | 3.1±1.6* | 4.5±1.6* | 5.2±1.0 | 4.6±1.4 | NA |
| HAQ, mean ± SD | NA | 1.4±0.8 | 0.8±0.7* | 0.8±0.7 | 0.8±0.6* | 1.5±1.0 | 1.4±0.8 | NA |
| RF-positive, % | ND | 42 | ND | ND | 0 | 67 | ND | ND |
| Anti-CCP-positive, % | ND | 32 | ND | ND | 0 | 45 | ND | ND |

NA – not applicable; ND – not determined.
SF – synovial fluid

*DAS28 and HAQ values were compared between VERA and VEA patients with reference to VERA baseline values.

Differences were considered statistically significant for $p$ values < 0.05.

## 3.2. IL-8 is increased in very early rheumatoid arthritis patients and locally in the joints of established rheumatoid arthritis patients

Given the proposed role of neutrophils in the pathogenesis of RA [18, 19], we quantified the major neutrophil chemoattractant, IL-8, in the serum of VERA patients. At baseline, VERA patients had significantly higher levels of IL-8 when compared to both VEA and healthy controls (Figure 1A). After 2-4 weeks of low-dose corticosteroids and after 4 months of MTX therapy there were no significant changes in the levels of circulating IL-8 (*data not shown*). Interestingly, VERA patients also had significantly higher circulating levels of IL-8 in comparison with serum from established RA (Figure 1A). Neutrophils accumulate locally in the joints of RA patients [20]. Thus, we quantified the concentration of IL-8 in the SF of RA patients and compared to SF from OA patients. We found

significant higher levels of IL-8 in the SF of RA patients in comparison to OA SF (Figure 1B).

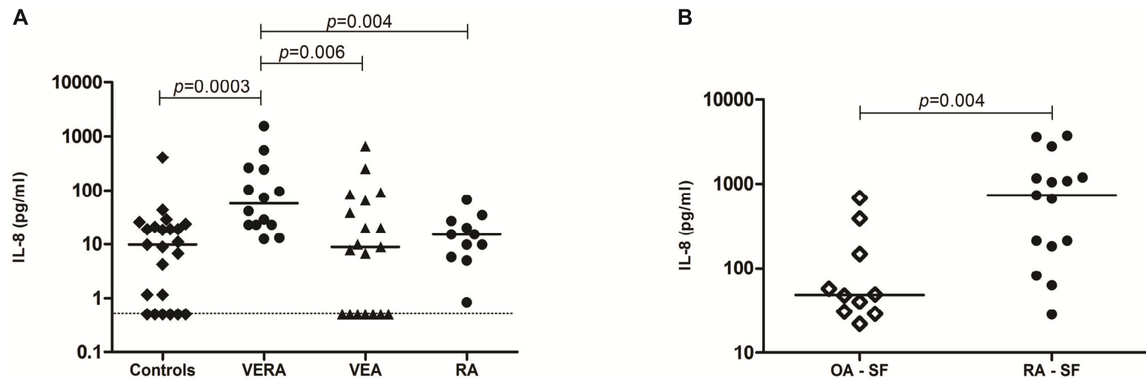


**Figure 1.** *IL-8 is increased in the serum of very early rheumatoid arthritis patients and in the synovial fluid of established rheumatoid arthritis***.**
**(A)** The serum concentration of IL-8 was measured in VERA and VEA patients as well as healthy controls and patients with established RA. Serum concentration of IL-8 was increased in VERA patients, compared with any other group. Dotted line represents the limit of detection for the assay. **(B)** The concentration of IL-8 was measured in the synovial fluid (SF) collected from patients with established RA and from a control group with OA. We found a significant increase of IL-8 in RA-SF. Differences were considered statistically significant for $p$ values < 0.05 according to the Mann-Whitney test.

## 3.3. IL-17 levels are dysregulated in both very early rheumatoid arthritis and established rheumatoid arthritis patients

Previous studies from our group showed that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21]. Therefore, we analyzed IL-17A levels in these patients, since it has already been described that this cytokine is important for the survival of neutrophils [22]. Moreover, IL-17A is a signature cytokine of Th17 cells, a subset proposed to have a key role in RA pathogenesis [9, 23]. We found that VERA patients had significantly higher levels of IL-17A when compared to healthy controls, but not with VEA patients (Figure 2A). Furthermore, in our previous work we found no difference in the frequency and absolute numbers of $CD4^+$ and $CD8^+$ T cell subpopulations in the peripheral blood of these patients, when analyzed by flow cytometry [17].

Regarding the effects of early therapy, we found that neither corticosteroids nor MTX affected the level of IL-17A (*data not shown*). Moreover, IL-17A was also significantly

increased locally within the joints of established RA patients in comparison with control SF from OA patients (Figure 2B).

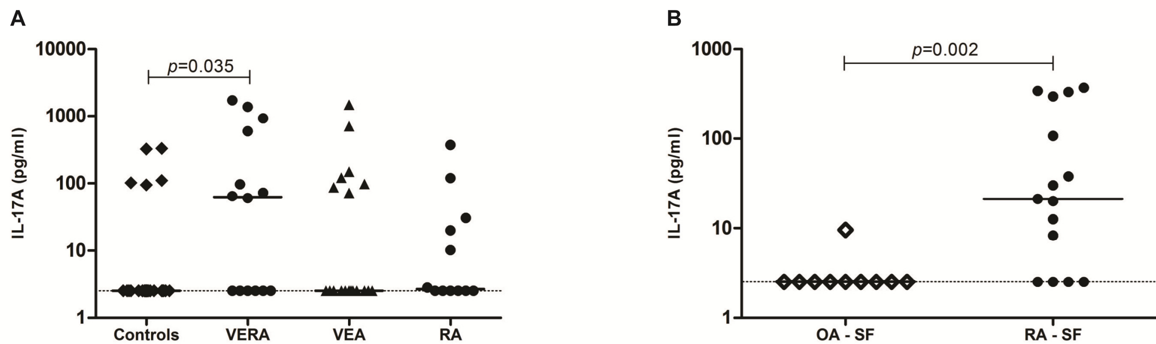


**Figure 2.** *Very early rheumatoid arthritis patients and synovial fluid of established rheumatoid arthritis display increased levels of IL-17A.*
**(A)** The serum concentration of IL-17A was measured in VERA and VEA patients as well as healthy controls and patients with established RA. The serum concentration of IL-17A was increased in VERA patients compared with healthy controls. **(B)** The concentration of IL-17A was measured in the synovial fluid (SF) collected from patients with established RA and from a control group with OA. In the SF of RA patients, we observed a significant increase of IL-17A. Dotted lines represent the limit of detection for the assay. Differences were considered statistically significant for $p$ values $< 0.05$ according to the Mann-Whitney test.

## 3.4. Rheumatoid arthritis has a Th17- cytokine pattern since the very first weeks of onset

Having found that IL-17A was elevated in VERA patients, we decided to quantify a panel of cytokines known to be associated with Th17 polarization. At baseline, VERA patients had significantly higher levels of IL-1β and IL-22 in comparison with both VEA and healthy controls. In addition, we also found that VERA patients have significantly higher IL-6 levels than healthy controls (Figure 3). Furthermore, the significantly higher circulating levels of IL-6 and IL-22 were maintained in established RA (Figure 3).

Locally, within the joints of RA patients, the synovial fluid displayed elevated levels of IL-1β and IL-6 in comparison with OA SF (Figure 4 and Table 2). Moreover, no significant differences could be observed for IL-23 in circulation or locally in the joints (*data not shown*).

We have also studied cytokines associated with the function of Th2 (IL-4, IL-10) and Th1 (IL-2, IL-12 (p70) and INFγ) cells. However, no statistically significant differences could be observed for any of these cytokines (*data not shown*).

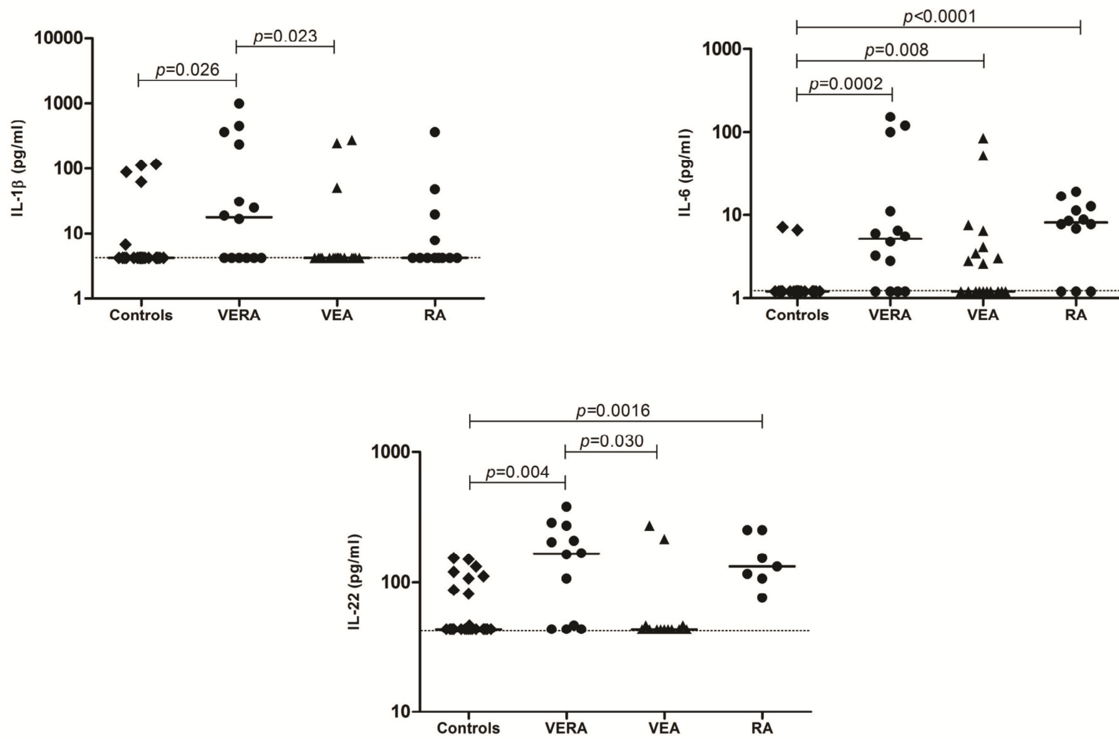


**Figure 3.** *Cytokines related to Th17 polarization are increased in the serum of very early rheumatoid arthritis patients.*
The serum concentrations of IL-1β, IL-6 and IL-22 were measured in VERA and VEA patients as well as healthy controls and patients with established RA. All three cytokines were increased in VERA patients compared with healthy controls. IL-6 was equally elevated in all groups of patients with an inflammatory disease, while the other two cytokines were increased only in VERA (IL-1β), or in VERA and RA patients (IL-22). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for $p$ values < 0.05 according to the Mann-Whitney test.

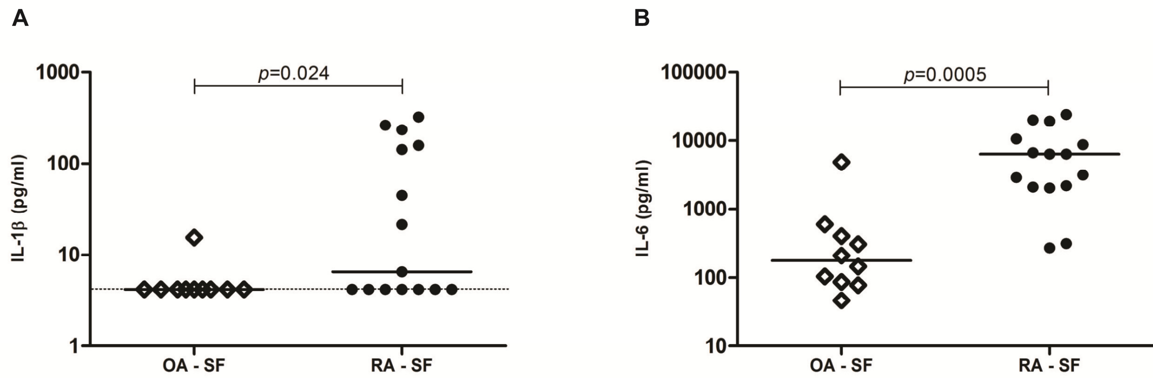


**Figure 4.** *Cytokines related to Th17 polarization are increased in the synovial fluid of established rheumatoid arthritis.*
The concentrations of IL-1β and IL-6 were markedly increased in the synovial fluid (SF) collected from patients with established RA, when compared with OA. Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for $p$ values $< 0.05$ according to the Mann-Whitney test.

**Table 2.** Cytokine levels in healthy controls, very early rheumatoid arthritis, very early arthritis, established rheumatoid arthritis and osteoarthritis patients.

| Cytokine (pg/ml) | Controls | VERA | VEA | RA | RA SF | OA SF |
|---|---|---|---|---|---|---|
| **IL-1β** | 4.2 (4.2-116.8) | 17.7 (4.2-99.7) | 4.2 (4.2-272.7) | 4.2 (4.2-360.3) | 6.5 (4.2-322.1) | 4.2 (4.2-15.5) |
| **IL-6** | 1.2 (1.2-7.2) | 5.2 (1.2-153.2) | 1.2 (1.2-84.7) | 8.2 (1.2-19.7) | 6361.0 (272.7-24135.0) | 177.6 (46.6-4881.0) |
| **IL-8** | 9.9 (0.5-407.7) | 57.6 (12.5-1546.0) | 8.9 (0.5-665.2) | 15.2 (0.8-67.5) | 735.7 (28.3-3717.0) | 48.2 (22.0-680.0) |
| **IL-17A** | 2.5 (2.5-333.7) | 62.0 (2.5-1714.0) | 2.5 (2.5-1477.0) | 2.6 (2.5-375.6) | 21.1 (2.5-369.1) | 2.5 (2.5-9.5) |
| **IL-22** | 43.3 (43.3-153.4) | 165.3 (43.3-380.6) | 43.3 (43.3-270.5) | 131.7 (75.8-250.7) | 153.4 (75.8-336.0) | 151.7 (92.4-235.3) |

Values are represented as median (range).
SF – synovial fluid

# 4. Discussion

Several studies have previously demonstrated that neutrophils play an important role in the onset of RA [21]. This hypothesis is supported by data from animal models [24]. In fact, neutrophils are the most abundant leukocytes in the synovial fluid of patients with active RA and, in early RA, these cells show significantly lower levels of apoptosis when compared to patients with other persistent forms of arthritis or with arthritis that have a self limited disease course [25]. Additionally, previous results from our group demonstrated that there is also a delay in the apoptosis of circulating neutrophils in VERA patients [21] and that these cells heavily infiltrate the synovial tissue during RA onset [5].

In the present study, we demonstrate that a neutrophil- and Th17-driving cytokine pattern is present in untreated VERA patients with less than 6 weeks of disease duration. We consider this observation of interest because the knowledge concerning the immune mechanisms associated with the onset of RA is still elusive. In fact, the majority of early RA studies include patients with 3 to 12 months of disease duration or even more. In accordance with an early participation of neutrophils in RA, our results revealed that VERA patients have increased levels of IL-8 when compared to both VEA and healthy controls, which could explain the pre-activated state of circulating neutrophils [18] and their recruitment towards the synovial fluid since the very first weeks of RA onset.

In addition, Th17 cells are known to be important for the promotion of neutrophil-mediated inflammation by producing IL-17A, a cytokine known to indirectly activate neutrophil chemotaxis and extend their survival [10, 22]. We found a high serum concentration of IL-17A in VERA patients, as well as locally within the joints of patients with established RA. This might indicate that there is an activation of Th17 cells from a very early phase of the disease, that can promote neutrophil participation in RA pathogenesis [22]. However, we found no evidence for changes in the frequency of T cell subsets in the peripheral blood of VERA patients [17]. This observation is not unexpected, given the relatively small representation of antigen-specific T cells in the circulating pool, being the activated T cells driving the pathology more likely found within the tissues [26]. In a study performed by Kokkonen and colleagues [27] the levels of several cytokines and chemokines were analyzed in blood samples from a group of individuals 3.3 years before

RA onset ("pre-patients") and compared with healthy donors and RA patients with 7.7±3.6 months of disease duration. An interesting finding was that IL-17 was present at its highest concentration in pre-patients and the level of this cytokine was lower in RA patients. This is in accordance with our own results, since we observed an increased level of IL-17 in RA patients with less than 6 weeks of disease duration, while in established RA patients the levels were not significantly different to healthy controls. Remarkably, the IL-17 median concentration observed in our established RA cohort (2.6 pg/ml) was even lower than that of RA patients from the work of Kokkonen and colleagues (6.0 pg/ml). Thus, this observation reinforces the role of IL-17 in the initial phase of RA and, as the pathogenesis progresses to a chronic stage, other factors are subsequently brought into action in the peripheral blood. Unlike Kokkonen and colleagues, we have not detected differences in Th1 and Th2-related cytokines between both VERA and established RA patients in comparison with controls. These discrepancies might be related with the different methodologies used.

Additionally, the elevated levels of IL-1β observed in VERA patients can stimulate endothelial cells, T and B cells and fibroblasts in the joints to produce IL-6 and IL-8. But importantly, IL-1β together with IL-6, both found to be increased in VERA patients, are known to promote the differentiation of Th17 cells, which in turn secrete IL-17A and IL-22 [28, 29], two cytokines that were elevated in VERA patients and have an essential function in the pathogenesis of autoimmune diseases [29].

Currently, the treatment of choice for RA at the time of presentation is MTX. Interestingly, in spite of clinical improvement (DAS28 reduced from 6.1±1.8 to 3.1±1.6), neither therapy with low-dose corticosteroids nor combined therapy with low-dose corticosteroids and MTX corrected the dysregulated cytokine pattern observed in VERA patients. In fact, low-dose corticosteroids and MTX have unclear effects on RA cytokine network. For instance, corticosteroids fail to reduce serum levels of IL-1β and IL-8 [30] and MTX does not alter serum IL-1β concentration when compared to pre-treatment levels [31, 32]. Our results suggest that the conditions contributing to Th17 cells and neutrophil-mediated inflammation, thus driving early pathogenesis, are not modified with early treatment with low-dose corticosteroids and MTX.

The elevated IL-1β, IL-6, IL-8 and IL-17A levels observed in the SF of RA patients confirm a local role for these cytokines in the maintenance of synovitis. Moreover, IL-6 can support a continuous recruitment of autoreactive B cells towards the synovium [33, 34], contributing to an exacerbation of the inflammatory process due to the production of autoantibodies and immune complexes.

## 5. Conclusions

Taken together, our data reinforce the potential relevance of therapies targeting IL-1β [35, 36] and IL-6 [37, 38] in early RA. In addition the data also establish IL-8 and IL-17A as other potential therapeutic targets at an early stage of the disease. Finally, we found that MTX and corticosteroids, although effective in reducing disease activity in VERA patients, do not appear to correct underlying cytokine dysregulation driving the Th17/neutrophil mediated inflammation.

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# BAFF and TACI gene expression are increased in untreated very early rheumatoid arthritis patients

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**Submitted for publication in May 2011.**

## Abstract

**Background:** B cells play several important roles in Rheumatoid Arthritis (RA). Given the beneficial effect of B cell depletion therapy in RA as well as the observed alterations in B cell subpopulations in this disease, we evaluated whether changes in the expression of genes related with B cell survival and activation were already present in untreated very

early RA (VERA) patients (<6 weeks of disease duration). **Methodology/Principal Findings:** The expression of a group of B-cell related activation and survival genes was quantified in peripheral blood mononuclear cells (PBMC) from VERA patients by real-time PCR and compared with untreated early RA (<1 year, ERA), established treated RA patients (RA) and other untreated early arthritis conditions (EA). Serum BAFF was quantified by ELISA. BAFF gene expression and serum levels were highest in VERA patients. The expression of BAFF-R increased with disease progression, while TACI was elevated since the first weeks of RA onset. Pax5 gene expression was also increased in all RA stages. CXCR5 was only elevated in established RA. No differences were observed in BCMA, AID, Blimp-1 and Bcl-2 expression. **Conclusions:** Disturbances in the expression of B-cell related activation and survival genes, particularly BAFF and TACI, occur since the onset of RA and precede changes in BAFF-R. These alterations can potentiate the development of autoreactive B cells since the first weeks of RA onset.

## 1. Introduction

Rheumatoid Arthritis (RA) is a chronic autoimmune disease that mainly targets the joints and affects approximately 1% of the population worldwide [1, 2]. B cells play several critical roles in RA pathogenesis. Previous studies by our group have demonstrated that very early RA patients have disturbances in peripheral blood memory B cells [3] and increased circulating B-cell related cytokines [4]. Furthermore, it has also been documented that genes regulating and affecting cellular processes such as proliferation, apoptosis, cytokine networks and autoimmunity were differently expressed in RA B-cells [5, 6]. These observations set the grounds for hypothesizing that B-cell biology is dysregulated since early RA development and consequently contributes to the induction and perpetuation of a pathogenic humoral immune response. Importantly, gene expression profiling of peripheral blood cells in different stages of RA progression may be useful not only to provide insights into pathogenesis, but also to identify and allow the future development of clinically useful biomarkers [7-10]. Therefore, the main goal of this work was to analyze a group of genes related with B cell survival, homeostasis and activation in very early RA patients and compare with different stages of RA progression.

## 2. Materials and Methods

### 2.1. Patients

Blood samples were collected from 8 consecutive untreated polyarthritis patients (Rheumatology Department, Hospital de Santa Maria, Lisbon) with less than 6 weeks of disease duration who, after a minimum follow-up of 3 months, fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA and were classified as Very Early Rheumatoid Arthritis (VERA) patients. Additionally, blood samples were also obtained from 7 untreated early polyarthritis patients with less than 1 year of disease duration, but more than 6 weeks of disease duration, who, after a minimum follow-up of 3 months, fulfilled the 1987 ACR criteria for RA and were classified as early RA (ERA), and 10 untreated early polyarthritis patients with less than 1 year of disease duration who did not evolve into RA and were classified as early arthritis (EA) (Rheumatology Department, Hospital de Santa Maria, Lisbon and Hospital da Luz, Lisbon). Furthermore, blood samples from 14 established RA patients treated with methotrexate (MTX) (Rheumatology Department, Hospital de Santa Maria, Lisbon) were also collected for comparison. Blood samples from VERA, ERA, RA and EA patients were compared with 16 healthy donors used as controls. The local ethics committee (Comissão de Ética do Hospital de Santa Maria, Lisbon, Portugal) 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 Seoul (2008).

### 2.2. Measurement of autoantibodies and cytokine quantification

Rheumatoid Factor (RF)-IgM was determined in all patients by IMTEC Autoimmune Diagnostics ELISA kit (Human GmbH, Germany) according to the manufacturer instructions and samples were processed using a ChemWell® 2910 automated analyzer. Serum levels of anti-cyclic citrullinated peptide (anti-CCP) were measured by ELIA™ CCP test system (Phadia GmbH, Germany) and samples were analyzed using an ImmunoCAP® 100 instrument. B cell activating factor (BAFF) levels were quantified by enzyme linked immunosorbent assay (ELISA) (Bender MedSystems, Austria) according to

the provider's instructions and samples were analyzed using plate reader Infinite® M200 (Tecan, Switzerland).

## 2.3. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml heparinized whole blood following density gradient centrifugation with Percoll (Amersham, Sweden). Cellular counts were estimated with Trypan Blue (Sigma-Aldrich, USA). Cells were frozen in 1 ml/ $10^7$ cells RPMI-1640 (GIBCO, UK), 40% fetal calf serum (FCS) (Invitrogen, UK), 10% dimethyl-sulfoxide (DMSO) (Sigma-Aldrich, USA) and stored at -80ºC until further use.

## 2.4. RNA extraction and cDNA synthesis

Total RNA from frozen PBMC was extracted using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions and treatment with RNase-free DNase Set (Qiagen, Germany) was performed to avoid contamination of genomic DNA. RNA concentration and purity were determined with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). RNA integrity of samples was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Pico Assay (Agilent Technologies, Germany) and by observing distinct 28S and 18S ribosomal bands on an agarose gel. Total RNA was reverse-transcribed into cDNA using DyNAmo™ cDNA Synthesis Kit for qRT-PCR (Finnzymes, Finland) with random hexamers (300 ng/µl), according to the manufacturer's instructions, performed on Piko Thermal Cycler (Finnzymes, Finland). The cDNA samples were stored at -20ºC.

## 2.5. Quantitative Real Time PCR analysis

The quantitative real time polymerase chain reaction (qPCR) was performed on Rotor-Gene™ 6000 (Corbett Life Science, USA) using SensiMix™ SYBR No-ROX Kit (Bioline, UK). The qPCR programme consisted of an initial denaturation step at 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15s, 60ºC for 15s and 72ºC for 15s. Genes and primer sequences analyzed in this study are indicated in Table 1. Primers were designed using the Universal ProbeLibrary Assay Design Center from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) and ProbeFinder software version 2.45. All the qPCR

products were analyzed by electrophoresis in 1% agarose gels. The 18S rRNA was used as endogenous control in relative gene quantification using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) [11]. All data were analyzed with Rotor-Gene 6000 Series Software version 1.7.87.

### 2.6. Statistical analysis

Statistical differences were determined using non-parametric Mann-Whitney and Kruskal-Wallis tests for comparisons between two and three or more groups, respectively, using GraphPad Prism (GraphPad, San Diego, CA). Correlation analysis was performed using Spearman's test. Differences were considered statistically significant for $p < 0.05$.

## 3. Results

### 3.1. Characterization of patients and disease evaluation

RA patients in three different clinical stages were included in this study. Untreated VERA patients (n=8) with less than 6 weeks of disease duration had a mean age of 59±15 years-old, 75% were female, 63% were RF positive and 43% anti-CCP positive. The baseline 28 joints disease activity score (DAS28) was of 7.0±0.7. These VERA patients represent a subset of a larger cohort previously described by our group [3]. The group of untreated ERA patients (n=7) had a mean disease duration of 0.6±0.3 years, a mean age of 53±5 years-old, 87% were female, 83% were RF positive, 57% were anti-CCP positive and the mean DAS28 score was of 5.4±1.4. Established RA patients (n=14) had an average disease duration of 13.2±12.3 years, were mostly under MTX monotherapy (n=12) and two patients were receiving MTX and sulphasalazine (SLZ) simultaneously. The mean age of these established RA patients was 61±12 years-old, 79% were female, 71% were RF positive, 64% anti-CCP positive and the mean DAS28 score was of 5.1±1.2 (Table 2).

**Table 1.** Genes and sequences of primers used in quantitative real time PCR.

| Gene | Name | Primer sequences |
|---|---|---|
| BAFF | B cell activating factor | Fw: 5'-GAGAAGCTGCCAGCAGGA-3'<br>Rv: 5'-GGAGCTGGTGGTTCAAAGATT-3' |
| BAFF-R | BAFF-receptor | Fw: 5'-CTGGTCCTGGTGGGTCTG-3'<br>Rv: 5'-ACCTTGTCCAGGGGCTCT-3' |
| TACI | Transmembrane activator and calcium modulator cyclophilin ligand interactor | Fw: 5'-AGGCTCAGAAGCAAGTCCAG-3'<br>Rv: 5'-CCAGGAAGCAGCAGAGGA-3' |
| BCMA | B cell maturation antigen | Fw: 5'-AGGACGAGTTTAAAAACACAGGA-3'<br>Rv: 5'-TCACAGGTGCATTCTTCCAC-3' |
| AID | Activation-induced cytidine deaminase | Fw: 5'-GGACTTTGGTTATCTTCGCAAT-3'<br>Rv: 5'-GTCGGGCACAGTCGTAGC-3' |
| CXCR5 | Chemokine (C-X-C motif) receptor 5 | Fw: 5'-GCCATGAACTACCCGCTAAC-3'<br>Rv: 5'-TCTGTCCAGTTCCCAGAACA-3' |
| Blimp-1 | B lymphocyte-induced maturation protein | Fw: 5'-ACGTGTGGGTACGACCTTG-3'<br>Rv: 5'-CTGCCAATCCCTGAAACCT-3' |
| Pax5 | Paired box 5 | Fw: 5'-GCCAAAATCCCACCATGT-3'<br>Rv: 5'-GTGGCTGCTGTACTTTTGTCC-3' |
| β2m | β2-microglobulin | Fw: 5'-CTATCCAGCGTACGCCAAAGATTC-3'<br>Rv: 5'-CTTGCTGAAAGACAAGTCTGAATG-3' |
| Bcl-2 | B-cell lymphoma 2 | Fw: 5'-TTGACAGAGGATCATGCTGTACTT-3'<br>Rv: 5'- ATCTTTATTTCATGAGGCACGTT-3' |
| 18S rRNA | 18S ribosomal RNA | Fw: 5'-GGAGTATGGTTGCAAAGCTGA-3'<br>Rv: 5´- ATCTGTCAATCCTGTCCGTGT-3' |

The EA group included 10 patients, who evolved into several different diagnoses: systemic lupus erythematosus (3 cases), spondyloarthritis (2 cases), arthritis associated to dermatomyositis (1 case), arthritis associated to HIV infection (1 case), unremitting undifferentiated arthritis (2 cases) and one patient entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and was thus

classified as presenting a self-limited form of polyarthritis. EA patients had mean disease duration of 0.4±0.6 years, a mean age of 39±15 years-old, 91% were female, all patients were RF and anti-CCP negative and the initial DAS28 was of 5.0±1.3 (Table 2).

**Table 2.** Clinical information of early arthritis, very early rheumatoid arthritis, early rheumatoid arthritis and established rheumatoid arthritis patients.

| | **Controls (n=16)** | **EA (n=10)** | **VERA (n=8)** | **ERA (n=7)** | **RA (n=14)** |
|---|---|---|---|---|---|
| Age (years) | 50±11 | 39±15 | 59±15 | 53±5 | 61±12 |
| Sex (% female) | 69 | 91 | 75 | 87 | 79 |
| Disease duration (years) | NA | 0.4±0.6 | < 6 weeks | 0.6±0.3 | 13.2±12.3 |
| CRP (mg/dl) | ND | 1.4±0.9 | 2.5±2.5 | 2.7±1.7 | 1.8±1.8 |
| ESR (mm/1$^{st}$ hour) | ND | 39.9±30.2 | 49.4±37.0 | 59.2±45.1 | 26.1±13.8 |
| DAS28 | NA | 5.0±1.3* | 7.0±0.7 | 5.4±1.4 | 5.1±1.2* |
| RF-positive (%) | ND | 0 | 63 | 83 | 71 |
| Anti-CCP-positive (%) | ND | 0 | 43 | 57 | 64 |

VERA - Very Early Rheumatoid Arthritis; ERA - Early Rheumatoid Arthritis; RA - Rheumatoid Arthritis; EA - Early Arthritis; CRP - C-reactive protein; ESR - Erythrocyte Sedimentation Rate; DAS28 – Disease Activity Score; RF – Rheumatoid Factor; Anti-CCP – anti-cyclic citrullinated peptide; NA – not applicable; ND – not determined.

Values are represented as mean ± standard deviation.
*Differences were considered statistically significant for $p$ values $< 0.05$ in comparison with VERA patients.

### 3.2. Peripheral blood mononuclear cells from very early rheumatoid arthritis patients have increased expression of BAFF, TACI and β2m while established rheumatoid arthritis patients have a higher BAFF-R and CXCR5 expression

A group of genes related with B cell homeostasis and survival (BAFF, BAFF-R, TACI, BCMA), class-switching (AID), chemotaxis (CXCR5), plasma cell differentiation (Pax5 and Blimp-1), immune system activation (β2m) and apoptosis (Bcl-2) was analyzed in three independent groups of RA patients representing different stages of disease progression: less than 6 weeks (VERA), less than a year (ERA) and established RA patients with mean disease duration of 13 years. Results were compared with EA patients and healthy controls. Disturbances in gene expression levels of BAFF receptors were found in both early and established RA patients in comparison with healthy controls. BAFF-R expression was elevated in ERA and RA patients in comparison with controls, while no significant differences were observed in VERA and EA (Figure 1A). Importantly, established RA patients had higher BAFF-R levels when compared to VERA, which suggests that BAFF-R expression increases with disease progression. Furthermore, TACI gene expression levels were significantly increased in VERA, ERA and RA patients in comparison with controls, while no differences were detected in EA (Figure 1B). BCMA levels were similar in all patients when compared to controls and no significant differences were detected (*data not shown*). Interestingly, BAFF expression was highest in VERA patients in comparison with controls, RA and EA (Figure 1C). Importantly, this early increase in BAFF mRNA expression observed in VERA was not observed in EA patients, thus suggesting a possible specific event of RA pathogenesis. Contrarily, CXCR5 expression was higher in RA patients not only in comparison with controls, but also with VERA patients (Figure 1D). Additionally, Pax5 gene mRNA levels were raised in all RA patients in comparison with controls (Figure 1E). Of note, EA patients had a significant lower expression of Pax5 in comparison with established RA. The expression levels of β2m were significantly elevated in VERA, ERA and EA patients in comparison with controls and established RA (Figure 1F). Interestingly, established RA patients had significantly reduced β2m levels when compared to controls. Moreover, no significant differences were observed in AID, Blimp-1 and Bcl-2 expression (*data not shown*).

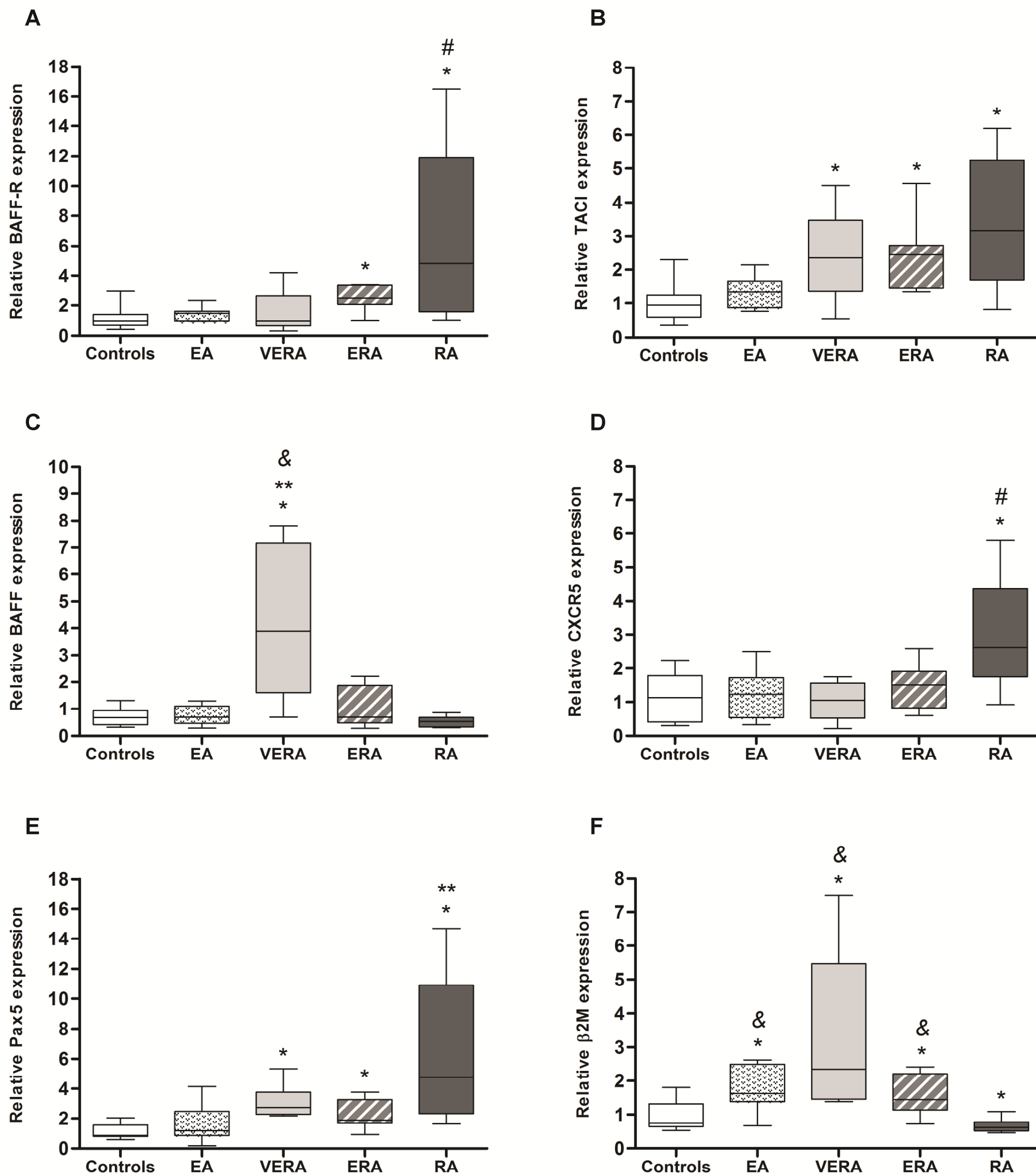


**Figure 1.** *Very early rheumatoid arthritis patients have increased BAFF, TACI and β2m mRNA levels in comparison with controls and established rheumatoid arthritis have higher BAFF-R and CXCR5 expression.* Relative gene expression from peripheral blood mononuclear cells (PBMC) was determined by real-time PCR in VERA, ERA, RA, EA patients and controls. The relative mRNA quantities ($2^{-\Delta\Delta Ct}$) of **(A)** BAFF-R, **(B)** TACI, **(C)** BAFF, **(D)** CXCR5, **(E)** Pax5 and **(F)** β2m are indicated. Differences were considered statistically significant for $p<0.05$ in comparison with *Controls, **EA, #VERA, ##ERA and &RA.

## 3.3. Correlation analysis between gene expression, serum BAFF levels and clinical data

Since there were alterations in BAFF-receptors relative gene expression not only in early RA, but also in established RA patients, BAFF serum levels were quantified in all groups of patients to analyze the possible existence of a correlation between the cytokine production and its receptors mRNA expression. VERA patients had the highest BAFF serum levels in comparison with controls, ERA, RA and EA patients (Figure 2), but no correlation could be observed with any of the BAFF-receptors expression (*data not shown*). Furthermore, no correlation was found between BAFF serum levels with age, DAS28, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), swollen and tender joint counts, or with any of the genes analyzed for all patients (*data not shown*).

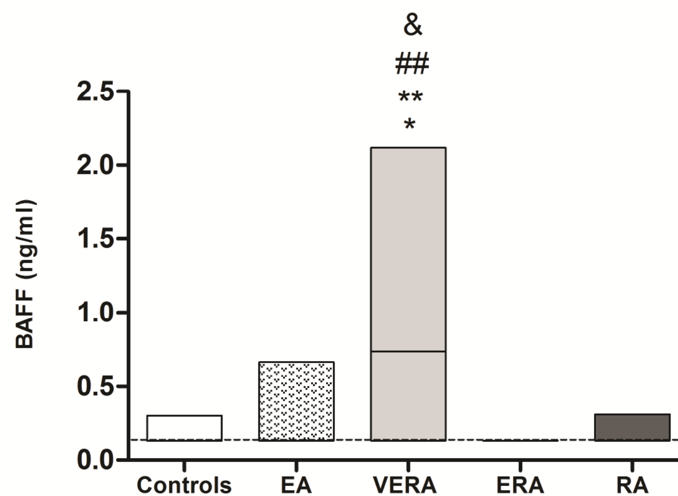


**Figure 2.** *BAFF serum levels are raised in very early rheumatoid arthritis patients when compared to early rheumatoid arthritis, established rheumatoid arthritis, early arthritis patients and controls.*
Serum BAFF levels were determined by ELISA in all patients' samples and controls. Represented are the median values and intervals. Dashed line represents the limit of detection for the assay. Differences were considered statistically significant for $p<0.05$ in comparison with *Controls, **EA, #VERA, ##ERA and &RA.

## 4. Discussion

Differences in expression of BAFF, BAFF-receptors and genes related with B-cell and immune system activation were found between early and established RA patients (Figure 3). BAFF is a fundamental B cell survival factor essential for B cell proliferation and activation [12]. BAFF binds to three receptors: BAFF-R, BCMA and TACI. The precise mechanisms that control expression of all three BAFF receptors are currently unclear, but studies indicate that the developmental stage, cytokine milieu and the microenvironment play a key role in regulation of receptor expression [13]. Interestingly, disturbances in BAFF-receptors expression occur in several pathologies [14-18], including RA, where it has been demonstrated an increase in both BAFF and BAFF-R mRNA expression in RA synovial tissue [19].

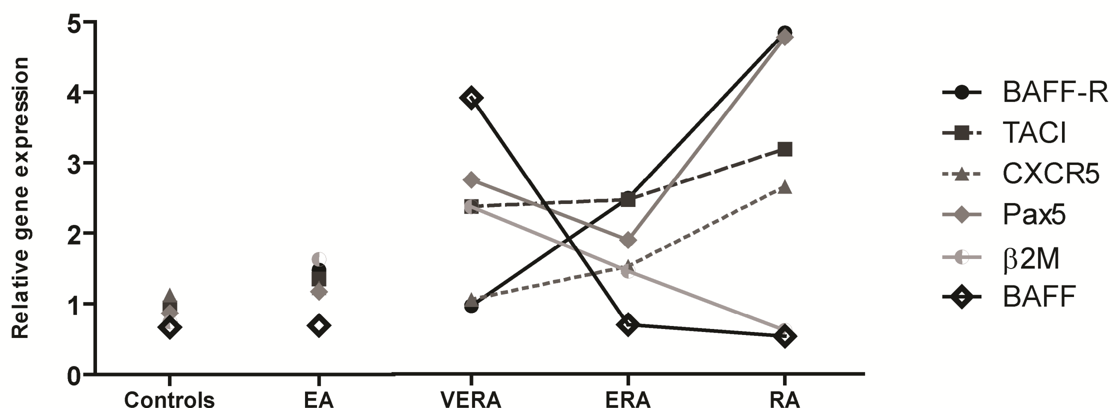


**Figure 3.** *Comparison of relative gene expression between very early rheumatoid arthritis, early rheumatoid arthritis, established rheumatoid arthritis, early arthritis patients and controls.*
Represented are the median values of BAFF-R, TACI, CXCR5, Pax5, β2m and BAFF relative mRNA quantity ($2^{-\Delta\Delta Ct}$) for each group analyzed.

We have found that BAFF-R was significantly increased in later stages of RA (both ERA and RA patients) and TACI expression was elevated in all RA patients since the first weeks of RA development. These observations suggest that disturbances in TACI expression occur since very early RA onset and BAFF-R expression increases with RA

progression (Figure 3), which can contribute for the development of autoreactive B cells and consequent disease aggravation. Furthermore, BAFF serum levels and mRNA expression were highest in VERA patients. Studies with knockout mice have demonstrated that B-cell proliferation depends upon either BAFF-R or TACI signaling [20]. Excess BAFF may lower the threshold for B cell receptor (BCR) signaling and maintain survival of pathogenic autoreactive B cells, thus breaking B cell tolerance. Signaling through BAFF-R can result not only in survival enhancement [21, 22], but also in B cell maturation [23], while TACI triggering can stimulate isotype switching and plasma cells differentiation [24]. It has been demonstrated that TACI provides positive signals driving T-independent B cell response [25] and survival of activated B cells and plasmablasts [24, 26], but also delivers negative signals suppressing B cell activation [27]. In fact, TACI functional activity is ambiguous: while $TACI^{-/-}$ mice predominantly develop autoimmunity and lymphoproliferation [28], TACI deficiency in humans primarily manifests itself as an antibody deficiency syndrome [29]. It is interesting to consider the hypothesis that during the first weeks of RA onset, the elevated BAFF serum levels could negatively regulate BAFF-R expression on B cells and, simultaneously, up-regulate TACI. The induction of TACI expression on activated B cells could serve as a danger signal, reflecting the need to avoid the potential harmful effects of prolonged activation of B cells through BAFF-BAFF-R interaction [21, 30]. With disease progression, signaling through BAFF-R eventually surpasses TACI, thus allowing autoreactive B cells to persist. Indeed, the higher affinity of BAFF, present at lower concentrations at later RA stages, towards BAFF-R instead of TACI expressed by B cells more prone to be autoreactive supports this notion. Also, TACI binds A proliferation-inducing ligand (APRIL) and BAFF equally with high affinity [31] and serves as the only receptor for BAFF/ APRIL heterotrimers [32]. Previous results from our group demonstrated that APRIL levels are increased not only in serum from VERA patients, but also in synovial fluid from established RA [4]. Since class-switch recombination (CSR) process depends on APRIL signaling via TACI [33], the maintenance of high levels of TACI expression in VERA, ERA and RA patients could contribute for the development of CSR and autoantibody formation by autoreactive B cells. Nevertheless, TACI role is still not clear and further studies on TACI function are required. Of note, contrarily to untreated VERA patients, in established RA the hypothesis that MTX therapy

influences serum BAFF levels and inversely affects BAFF-receptors gene expression cannot be excluded. Pax5 gene codifies a transcription factor, also known as B-cell-specific activation protein (BSAP), which is essential for B cell commitment as well as for B cell development, and continuous expression of Pax5 is required throughout the B cell lineage to maintain the functional identity of B cells [34]. Therefore, it was not surprising that Pax5 expression was increased in all RA patients. Importantly, Pax5 gene expression was higher in RA patients in comparison with EA. This observation, together with the absence of differences in BAFF, BAFF-R and TACI expression in EA patients as compared to controls reinforces the specific role of B cells in RA progression when compared to other forms of arthritis. Furthermore, the increased levels of β2m observed in VERA, ERA and EA patients indicate early immune system activation not only in RA diagnosis, but also in other forms of arthritis. Of note, increased serum and synovial fluid β2m have been found in RA [35, 36]. The reduced expression of β2m mRNA in established RA patients could be due to treatment with immunosuppressive agents, such as MTX, that downregulate immune responses. Moreover, no differences were observed in AID levels in our study. Since CSR and somatic hypermutation (SHM), both processes that require the action of AID [37], occur during germinal-center (GC) responses in organized lymphoid tissues, we believe that AID expression in early and established RA is higher in B cells infiltrating not only RA synovium [38], but also secondary lymphoid organs [39] than in circulating B cells. Additionally, CXCR5 and also its ligand, CXCL13, involved in B cell chemotaxis, are upregulated in RA synovium [40], suggesting a local role in B cell chemotaxis towards synovial membrane. Indeed, the increased CXCR5 levels observed in RA patients in comparison with controls and VERA reveals that this effect in circulation is stronger in later RA stages. Blimp-1 genetic variants have recently been associated with RA risk [41]. Blimp-1 is expressed in antibody-secreting cells from human and mouse, but is absent from earlier stages of B cell ontogeny [42]. Thus, the absence of differences in circulating Blimp-1 levels is due to the fact that only a minority of plasma cells is present in circulation and the expression of this gene initiates during terminal differentiation of plasma cells [43] during GC reactions. Additionally, the absence of variations in Bcl-2 expression in RA patients in comparison with controls suggests that this gene does not play a significant role in RA pathogenesis [44]. In summary, disturbances in the expression of B cell related

survival and activation genes occur since the very early phase of RA. Increased BAFF serum and mRNA levels can contribute for the persistence of autoreactive B cells and, together with TACI elevated expression, reinforce the importance of an earlier introduction of therapies targeting not only B cells directly (anti-CD20), but also BAFF-receptors, or its ligands [45].

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# B-cell-activating factor receptor expression on naïve and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B-cell depletion therapy

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**Published in *Annals of Rheumatic Diseases* 2010; 69(12):2181-2188**

## Abstract

**Objectives:** To examine the expression of B-cell activating factor receptor (BAFF-R) on naïve CD27− and memory CD27+ B cells in normal individuals and patients with rheumatoid arthritis (RA) undergoing B-cell depletion therapy with rituximab. **Patients and Methods:** BAFF-R expression on B-cell subsets was determined in normal controls (NC, n=11), active patients with RA pre-rituximab (pre-RX, n=15), relapsing patients either concordant for B-cell repopulation (C-R, n=13) or discordant, with relapse more than 3 months after repopulation (D-R, n=11) and patients in remission over 3 months post-repopulation (discordant non-relapsing (D-NR), n=5). Serum BAFF was measured by ELISA and analyzed using Mann–Whitney test. **Results:** There was no significant difference between NC, pre-RX and D-NR patients in % BAFF-R-positive B cells or mean fluorescence intensity (MFI) in naïve and memory B cells. Relapsing patients had significantly lower MFI and % BAFF-R-positive cells in both naïve and memory compartments from NC and pre-RX (C-R and D-R, $p<0.01$). BAFF levels in pre-RX patients were within the normal range and did not correlate with BAFF-R expression in any

patient group. D-NR patients had relatively lower proportions of pre and post-switch CD27+ B cells than pre-RX patients (D-NR versus pre-RX, $p<0.05$ for both) and also lower numbers of post-switch B cells than D-R patients (D-NR versus D-R, $p<0.05$). **Conclusion:** BAFF-R expression was significantly reduced on both naïve and memory B cells in patients at relapse, regardless of the relationship with B-cell repopulation or serum BAFF levels. Re-establishment of active disease was also associated with an increase in class-switch recombination. Factors responsible for lower levels of BAFF-R may relate to altered thresholds for autoreactive B-cell generation at relapse in patients with RA.

# 1. Introduction

B-cell-activating factor (BAFF), also known as B lymphocyte stimulator (BLyS), THANK, TALL-1, zTNF-4 or TNFSF13b, is a protein member of the tumour necrosis factor (TNF) family that plays an important role in B-cell maturation, homeostasis and survival [1]. BAFF binds to three receptors on B cells: TACI, BCMA and BAFF-R (or BR3), with BAFF-R being the only ligand that exclusively binds BAFF [2].

Studies of BAFF-R-deficient mice demonstrated that BAFF was essential for B-cell survival, primarily through the alternative nuclear factor κ light-chain enhancer of activated B cells pathway [3], and for B-cell maturation and immunoglobulin (Ig) class switching through the classic nuclear factor κ B pathway [4]. B cells from these animals develop normally up to the IgM+ transitional stage but do not complete their maturation in the spleen [5]. The numbers of memory and marginal zone cells and of serum Ig are severely reduced (> 90%) as were responses to both T-dependent and T-independent stimuli [2].

Human B cells are less dependent on BAFF-R signaling for maturation than murine B cells. In the few reported cases of common variable immunodeficiency related to BAFF-R deficiency in humans, circulating BAFF levels were greatly elevated and peripheral B-cell numbers were reduced (fourfold lower). The condition was of late onset and was not associated with severe infections [6]. Although mature but not transitional B cells were reduced in these individuals, class-switched memory B cells were detectable. Correspondingly, vaccination studies showed that T-dependent responses appeared to be

much less dependent on BAFF-R signaling than T-independent responses. As in BAFF-R–/– mice, IgA responses in these patients were not compromised.

One of the suggested roles for BAFF, based largely on animal experiments, is that increased levels will lower thresholds for the survival of autoreactive B-cell clones, and decrease competition within germinal center environments [7, 8]. In humans, elevated levels of serum BAFF have been associated with both the presence and activity of several autoimmune diseases (Sjögren's syndrome, systemic lupus erythematosus (SLE) and idiopathic thrombocytopenia) [9-15]. In patients with rheumatoid arthritis (RA), the relationship is less clear, with conflicting reports as to whether levels of circulating BAFF are significantly elevated and if there is a relationship with disease activity or titers of autoantibodies [14, 16, 17].

Following treatment of RA patients with rituximab, serum BAFF levels have been shown to rise, reflecting the loss of the majority of peripheral B cells. The correlation of BAFF levels with peripheral B-cell counts is not, however, perfect and it has been suggested that BAFF levels form a more accurate indicator of total B-cell mass [18]. Chronically raised BAFF levels have been suggested to exert a negative feedback on BAFF-R expression [19].

One of the key initial findings of the clinical response to rituximab was that B-cell return was not always associated with relapse, and that disease could remain in remission for months or even years following B-cell repopulation [20]. In patients with RA, rises in autoantibodies, particularly rheumatoid factor (RF), seem to herald relapse following regardless of whether clinical relapse has been concordant or discordant with B-cell return [21]. Serum BAFF levels rose rapidly after rituximab and then fell with rising peripheral B-cell numbers, although the relationship was not always clear. There was also some evidence for an increased uptake of BAFF if relapse occurred concurrently [17, 21].

The re-establishment of clinical disease following B-cell depletion therapy relies on engaging proinflammatory pathways, which are quiescent in the absence of circulating B cells and, therefore, provides a unique opportunity to study the re-emergence of the disease process. As a result of its importance in B-cell survival, particularly for new emigrant and naïve B cells, we hypothesized that the balance between BAFF-R expression and soluble BAFF may influence the relationship between returning B-cell populations and the

resumption of autoimmunity. With this in mind, we measured serum BAFF levels and BAFF-R expression on B-cell populations to determine whether there was any relationship between serum BAFF levels and BAFF-R on naïve and memory B cells at different key time points after rituximab therapy.

## 2. Materials and Methods

### 2.1. Patients

Blood samples were obtained from 11 normal controls (NC) and 44 consecutive patients with a diagnosis of RA who fulfilled the American College of Rheumatology criteria for RA [22]. The patients were all attending the Department of Rheumatology at University College London Hospital and were treated with rituximab, on the basis of clinical need. Patients received 2-weekly infusions of 1 g rituximab preceded by 100 mg intravenous methylprednisolone. The study was approved by the hospital ethics committee and all patients gave informed consent before entering the study. The normal range for cluster of differentiation 19+ (CD19+) B cells used by the local pathology laboratory was 0.03–$0.40\times10^9$/l. Levels less than $0.005\times10^9$/l were defined as undetectable.

### 2.2. Detection of B cells

Depletion of B cells in the peripheral blood was deemed to have occurred when CD19+ B cells were undetectable. In all patients, total peripheral blood B-cell depletion was achieved for at least 2 months. B-cell return (B-cell repopulation) was defined as when B cells were again detectable in the peripheral blood (i.e., when the CD19+ cell count was $\geq0.005\times10^9$/l).

### 2.3. Clinical relapse

Clinical relapse after rituximab was based on any return of symptoms of RA plus a rise in C-reactive protein following an original fall of at least 50% in C-reactive during the previous course of B-cell depletion therapy. Patients at clinical relapse were classified as

concordant (relapse ≤ 3 months after repopulation in peripheral blood) or discordant (relapse > 3 months after repopulation in peripheral blood).

## 2.4. Peripheral blood mononuclear cells isolation and staining

Peripheral blood mononuclear cells (PBMC) were freshly isolated from 10 ml heparinized whole blood following density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and stained on the same day of collection. Cells were washed and cellular viability was estimated with Trypan blue (Sigma, St. Louis, USA). PBMC ($1\times10^6$/sample) were incubated with appropriate conjugated antibodies for 15 minutes at room temperature in the dark and then washed. Erythrocytes were lysed with BD PharmLyse (BD, Biosciences, San Diego, USA) buffer according to the manufacturer's instructions and cells were then fixed with paraformaldehyde 2% for 5 minutes at room temperature. PBMC were stored in the dark at 4°C until analyzed by flow cytometry. A total of 300.000 cells/sample gated in total lymphocytes were acquired with FACSCalibur (BD). Data were analyzed with FlowJo (TreeStar, Stanford University, California, USA). Absolute cell counts were calculated from the differential leukocyte count determined at each time point for all patients.

## 2.5. Phenotypic analysis

Immunophenotyping of B and T cells in PBMC samples was performed using matched combinations of anti-human murine monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein cyanin (PerCP-Cy5.5), or allophycocyanin (APC). For B-cell analysis, combinations of anti-CD19 conjugated to PerCP-Cy5.5 or APC (clone HIB19), anti-IgD FITC (clone IA6-2) and anti-CD27 PE (clone O323) were used to define naïve (CD19+IgD+CD27−), pre-switch memory (CD19+IgD+CD27+), post-switch memory (CD19+IgD−CD27+) and IgD−CD27− B cells. The expression of BAFF-R was analyzed using combinations of anti-CD19 PerCP-Cy5.5, anti-CD27 PE and anti-BAFF-R FITC (11C1, mouse IgG1, clone 8A7) and the mean fluorescence intensity (MFI) of BAFF-R was determined for naïve (CD19+CD27−) and memory B cells (CD19+CD27+). All antibodies used were purchased from BD Biosciences and eBioscience (San Diego, USA).

### 2.6. Measurement of BAFF

BAFF levels were quantified in serum samples from healthy controls and from patients at the various time points, using the Human Quantikine BAFF/BLyS immunoassay ELISA kit (R&D Systems, United Kingdom). The mean ± SD for normal sera (n=36) were given as 1.17±0.28 ng/ml (range 0.67–2.45 ng/ml).

### 2.7. Statistical analysis

Statistical differences were determined using the non-parametric Mann–Whitney test using GraphPad Prism (GraphPad, San Diego, California, USA). Differences were considered statistically significant for $p<0.05$. Linear regression was used to correlate BAFF levels and BAFF-R expression.

## 3. Results

### 3.1. Patient cohorts

The mean age was 42 years (range 33–81) and the mean disease duration was 15 years (range 2–20). All patients had erosive disease. Two patients were seronegative for both RF and anti-citrullinated protein antibodies before treatment. The cohorts of consecutive patients with RA included 15 patients with active disease who were studied pre-rituximab (pre-RX) and 29 patients who had already received one or more courses of rituximab. In consecutive patients presenting at clinic, blood for FACS analysis was taken from all relapsing patients. CD19 counts were subsequently examined and patients assigned to concordant relapsing (C-R) or discordant relapsing (D-R) groups. If the patients had already repopulated according to the laboratory notes, but had remained well for more than 3 months after the date of repopulation, they were assigned to the discordant non-relapsing (D-NR) group. Five groups were therefore described for analyses: (1) NC (n=11); (2) patients with active RA pre-RX (n=15); (3) C-R (n=13); (4) D-R (n=11); (5) D-NR (n=5), taking into account repopulation and relapse after rituximab as described (Table 1).

**Table 1.** Serum BAFF levels and percentage of BAFF-R expression on CD19+ B cells before and after B cell depletion therapy.

| | BAFF (ng/ml) | BAFF-R expression (%) | |
|---|---|---|---|
| | | Naïve B cells CD19+CD27- | Memory B cells CD19+CD27+ |
| NC (n=11) | 0.88 (0.68-1.03) | 98.4 (95.7-99.8) | 88.3 (77.3-95.8) |
| Pre-RX (n=15) | 1.09 (0.69-1.89) | 97.8 (71.7-99.7) | 87.3 (69.3-97.1) |
| C-R (n=13) | 3.03** # (1.31-7.09) | 90.3** (58.6-98.6) | 54.8**** # # (23.4-82.1) |
| D-R (n=11) | 2.55** # (1.02-7.46) | 85.4*** # (57.7-97.6) | 55.8 **** # # # (27.4-77.6) |
| D-NR (n=5) | 2.25* (1.11-4.13) | 98.3 (88-99.7) | 79.3 (64.1-96.9) |

NC: normal controls; Pre-RX: pre-rituximab; C-R, concordant clinically relapsing; D-R, discordant clinically relapsing; D-NR, discordant clinically non-relapsing.

All values indicated in table represent median (range).

*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ compared with NC (Mann-Whitney).

#$p<0.01$, # #$p<0.001$, # # #$p<0.0001$ compared with pre-RX.

## 3.2. Serum BAFF levels in normal controls and in rheumatoid arthritis patients

Median BAFF levels in the 16 patients with RA tested before rituximab were within the normal range (Table 1). As previously described, BAFF levels rose following rituximab [22]. Levels were found to remain raised at relapse in both patient groups (C-R and D-R, $p<0.01$, compared with both NC and patients pre-RX) and to a lesser extent in patients who had repopulated but not relapsed (D-NR, $p<0.05$) when compared with NC only (Table 1), but no statistically significant differences in BAFF levels were observed between C-R, D-R and D-NR patients.

## 3.3. Percentage of naïve and memory B cells expressing BAFF-R

The percentages of CD19+ B cells expressing BAFF-R in CD27+ and CD27− subpopulations in patients and NC are represented in Table 1. Significant differences were seen between the percentage expression of BAFF-R on naïve and memory B cells in relapsing patients (C-R and D-R groups) compared with NC, and for the memory B-cell population, levels in relapsing patients were also significantly reduced compared with pre-RX values. In the D-R group, decreased expression on naïve B cells was also significant compared with pre-RX values. In patients whose B cells had returned but had not yet relapsed (D-NR), no such decreases were found between the percentages of BAFF-R expressing naïve or memory populations compared with NC or with the pre-RX group. Furthermore, when the percentages of BAFF-R expressing naïve and memory B cells in patients who were relapsing (both C-R and D-R) were compared with D-NR patients, a significantly lower percentage expression was found in both naïve and memory populations (naïve D-NR versus C-R and D-R, $p<0.05$ and $p<0.01$, respectively; memory D-NR versus C-R or D-R, $p<0.01$ for both). The low expression of BAFF-R was not caused by contamination of the CD19+ gate with plasmablasts (which express low levels of BAFF-R) as they were gated out of the analysis.

## 3.4. Mean fluorescence intensity of BAFF-R expression on naïve and memory B cells

Figure 1 shows representative dot plots and histograms of BAFF-R expression on CD19+ B cells (panel A), PBMC differentiated into naïve and memory B cells on the basis of CD27 expression (panel B) and histograms of the MFI of BAFF-R on naïve (grey line) and memory (dark line) B cells (panel C). BAFF-R expression appeared to be decreased in patients who were discordant for relapse after B-cell return, both when remaining well or relapsing, in the examples shown. These individuals also had a tendency for a higher proportion of naïve to memory B cells (panels A and B). A left shift in MFI of BAFF-R expression was also observed in all naïve and memory populations following rituximab compared with those of NC (panel C). In order to explore these initial observations, the MFI of BAFF-R expression on naïve and memory CD19+ B cells pre-RX were compared with those of B cells from NC (Figure 2). Naïve B cells from both groups of patients at

relapse (C-R and D-R) expressed significantly lower levels of BAFF-R when compared with NC (MFI; C-R 58.8 and D-R 53.7 versus NC 85.5). Memory B cells from both cohorts of relapsing patients also showed significantly lower expression of BAFF-R than NC (MFI; C-R 56.5 and D-R 46.7 versus NC 78.0). Furthermore, C-R patients showed a tendency to lower MFI levels than pre-RX patients in both naïve (58.8 versus 70.4, $p$=0.06) and memory B-cell populations (56.5 versus 61.2, $p$=0.07). We also observed a tendency towards lower BAFF-R MFI in D-R patients when compared with pre-RX levels in memory B cells, although again this did not reach statistical significance (46.7 versus 61.2, $p$=0.07). Of interest, no significant differences were found in the MFI of BAFF-R expression on either naïve or memory B-cell populations in the D-NR patient group when compared with NC or RA patients before treatment.

### 3.5. Relationship between BAFF levels and BAFF-R mean fluorescence intensity in naïve and memory B cells

Our initial observation was that BAFF-R expression was significantly lower on B cells from patients undergoing relapse than on B cells from patients whose B cells had returned but in whom disease was not yet re-established (Figure 2). We therefore examined whether there was any relationship between circulating BAFF levels and expression (MFI) of BAFF-R (Figure 3). Although no statistically significant correlations were found, we could distinguish different patterns between pre-RX, C-R, D-R and D-NR patients. When patients were relapsing (C-R and D-R), BAFF-R MFI was consistently lower, irrespective of serum BAFF levels. Nevertheless, in patients who had repopulated but remained well (D-NR), there was a tendency for a negative correlation between circulating BAFF and BAFF-R MFI more evident in memory B cells ($r^2$=0.88, $p$=0.06) (Figure 3). However, samples from only four patients were available for analysis in this group.

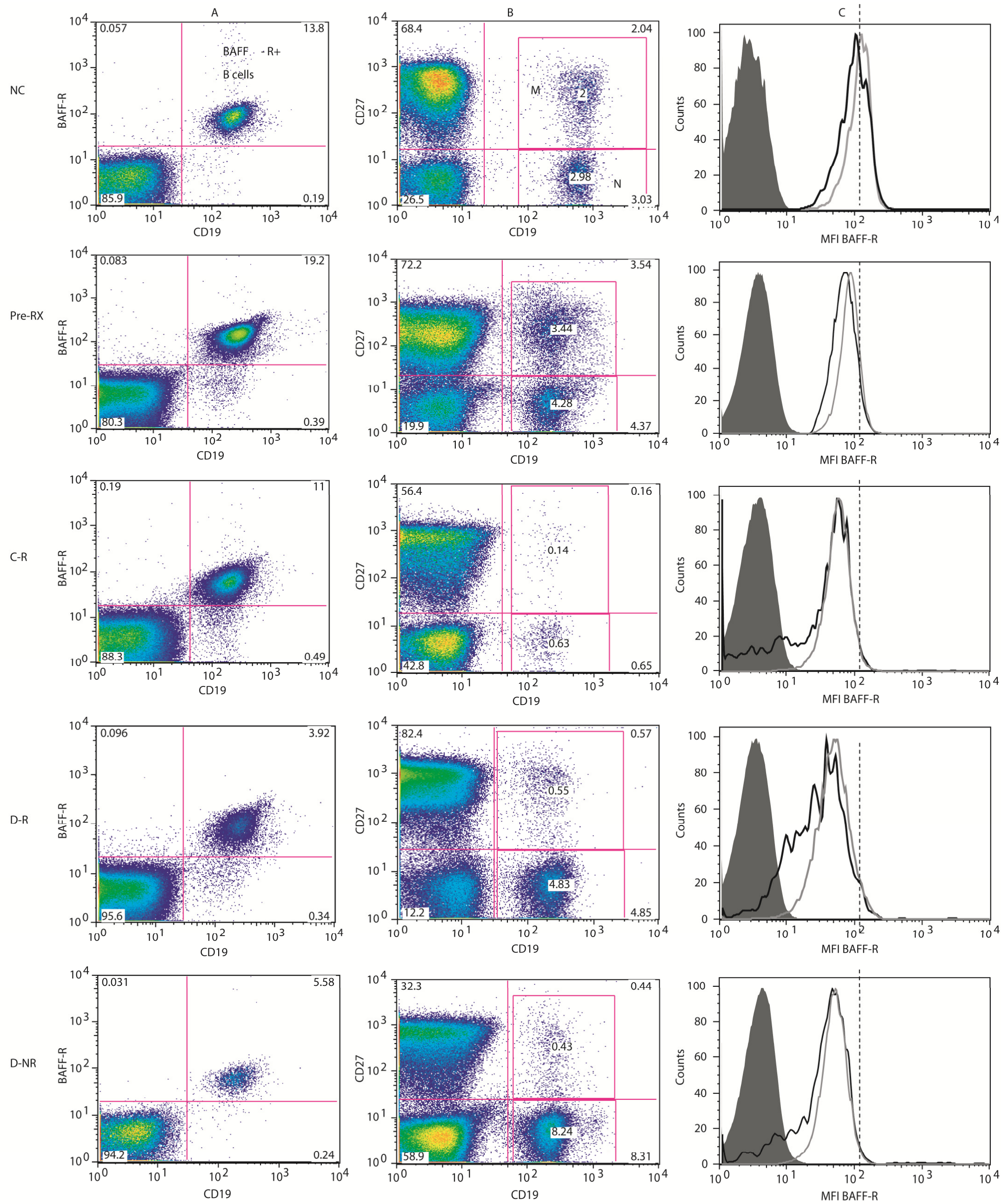


**Figure 1.** *Characterization of BAFF-R expression in normal controls and patients with rheumatoid arthritis before and after B-cell depletion therapy.*

The expression of BAFF-R on CD19+ B cells in the lymphocyte gate in peripheral blood mononuclear cells is shown in panel A. In panel B, combinations of CD27 and CD19 were used to differentiate memory (M; CD19+CD27+) and naïve (N; CD19+CD27−) B cells. Numbers in each quadrant represent percentages of each subpopulation in individual experiments. The histograms in panel C show representative experiments in which the mean fluorescence intensity (MFI) of BAFF-R expression for CD27+ (dark line) and CD27− (grey line) are shown. Dotted lines depict the MFI of BAFF-R expression on the naïve B-cell (CD27−) population from normal controls (NC) to enable comparison with other histograms in each example. Pre-RX: pre-rituximab; C-R: concordant clinically relapsing; D-R: discordant clinically relapsing; D-NR: discordant clinically non-relapsing.

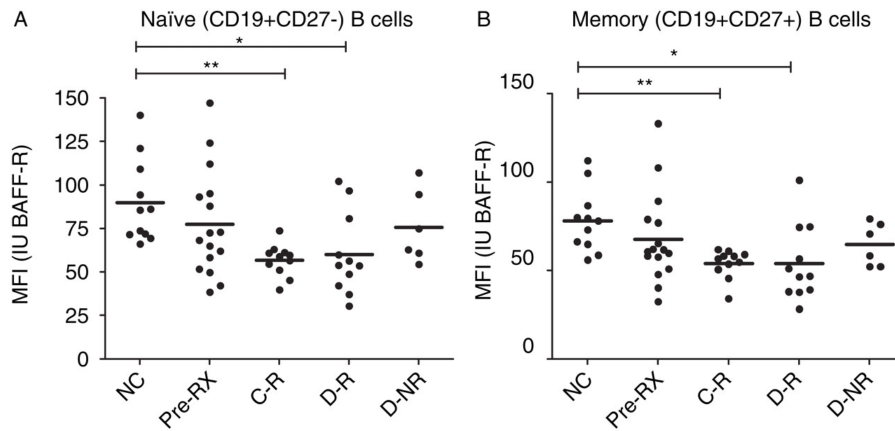


**Figure 2.** *The mean fluorescence intensity of BAFF-R expression on B cells is reduced in rheumatoid arthritis patients at clinical relapse.*

Peripheral blood mononuclear cells were gated on the CD19+ B-cell population and the mean fluorescence intensity (MFI) of BAFF-R expression determined on CD27- naïve **(A)** and CD27+ memory **(B)** B-cell subpopulations. Normal controls (NC) and patient groups were compared using the Mann–Whitney test and statistical significance indicated (*p<0.01, **p<0.001). Pre-RX: pre-rituximab; C-R: concordant clinically relapsing; D-R: discordant clinically relapsing; D-NR: discordant clinically non-relapsing.

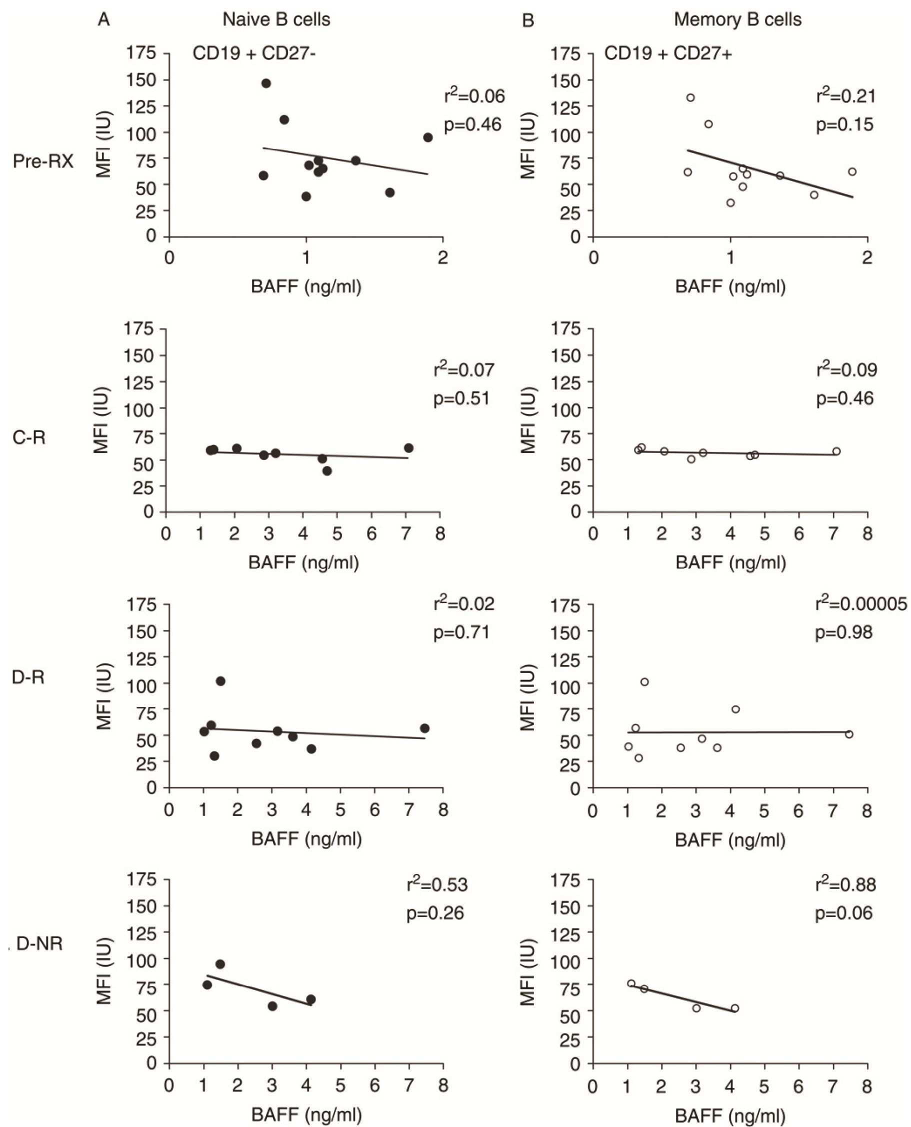


**Figure 3.** *Relationship between circulating BAFF levels and BAFF-R expression.*
The values for the mean fluorescence intensity (MFI) of BAFF-R expression on CD27- naïve **(A)** and CD27+ memory **(B)** B-cell subpopulations in individual patients were plotted against BAFF levels (ng/ml) that were measured in serum stored at the same time point (upper limit of normal range 2.45 ng/ml). Regression analysis was performed and significance values determined for each curve. Pre-RX: pre-rituximab; C-R: concordant clinically relapsing; D-R, discordant clinically relapsing; D-NR: discordant clinically non-relapsing.

## 3.6. B-cell subpopulations in peripheral blood associated with different patterns of response to rituximab

It has previously been suggested that patients with higher numbers of circulating memory/post-switch B cells, either before treatment induction or at relapse were more likely to have a shorter clinical response to rituximab [23, 24]. We showed here that memory B cells from relapsing patients in particular tended to express lower levels of BAFF-R (MFI) and that this was so over a wide range of circulating BAFF levels. We therefore examined whether there was any relationship between subpopulations of B cells in patients with different patterns of clinical response/relapse (Figure 4). Circulating B-cell (CD19+) subpopulations were defined according to IgD and CD27 expression and classified as naïve (IgD+CD27−), pre-switch memory (IgD+CD27+), post-switch memory (IgD−CD27+) and double-negative (IgD−CD27−) B cells. The median (range) of total numbers of CD19+ B cells in each patient group were for pre-RX: 175 (58–472)×$10^6$/l, C-R: 42 (15–192)×$10^6$/l, D-R: 61 (13–166)×$10^6$/l, D-NR: 71 (21–328)×$10^6$/l. The median percentage and interquartile range of each subpopulation are shown for NC and patient groups in Figure 4. The distribution of naïve to memory B cells in humans is approximately 60:40. The B-cell subpopulation distribution of B cells from patients with RA pretreatment was broadly similar to NC, except for a slightly increased proportion of double-negative B cells (7.3% versus 3.9%, $p$=0.04). Following rituximab, repopulation of the periphery begins with naïve B cells, which mature at a greater or lesser rate into pre and post-switch CD27+ B cells. Full restoration of memory cell populations may not be achieved for years after treatment. As expected, the relative ratio of naïve to memory B cells was increased at repopulation, mirroring ontogeny, but the relative percentage of naïve cells was only significantly higher in the non-relapsing patients compared with NC ($p$<0.05). The pre-switch memory B-cell population was significantly decreased in all patients post-rituximab (C-R 4.2%, $p$=0.0004; D-R 4.9%, $p$=0.002 and D-NR 4.7%, $p$=0.03) when compared with NC (12.2%). In patients who relapsed at B-cell return, post-switch memory B cells had already reached levels approximating those of NC (C-R 12.4% versus NC 19.6%, $p$=0.28), but remained reduced in the D-NR group when compared with NC (6.6% versus 19.6%, $p$=0.03), pre-RX (6.6% versus 18.1%, $p$=0.02) and D-R (6.6% versus 17.9%, $p$=0.05).

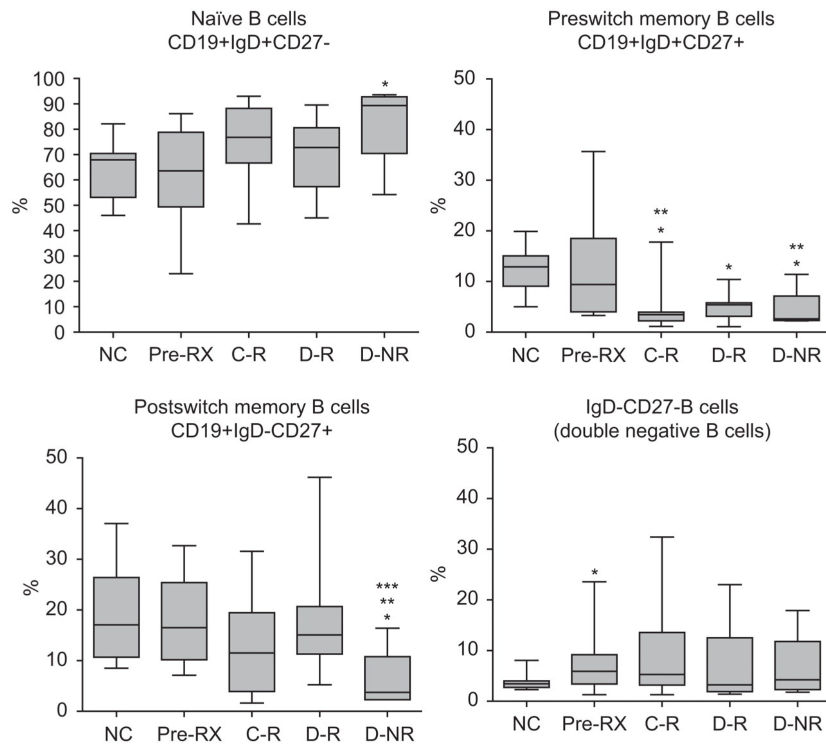


**Figure 4.** *Characterization of circulating B cell subpopulations in normal controls and patients with rheumatoid arthritis before and after B cell depletion therapy.*
Peripheral blood mononuclear cells were gated on CD19 to characterize B-cell subpopulations further on the basis of IgD and CD27 expression into naïve, pre and post-switch memory and double negative B cells. Significance levels, using the Mann–Whitney test, for comparisons between the different groups are depicted as: *$p<0.05$ versus normal controls (NC), **$p<0.05$ versus pre-rituximab (pre-RX), ***$p<0.05$ discordant clinically non-relapsing (D-NR) versus discordant clinically relapsing (D-R). C-R: concordant clinically relapsing.

We also used regression analysis to determine whether serum BAFF levels were related to the percentage of particular B-cell subsets. This produced mostly negative results, although higher BAFF levels were statistically associated with numbers of pre-switch memory B cells in patients who were relapsing more than 3 months after B-cell repopulation (D-R, $p=0.02$, *data not shown*).

### 3.7. Relationship of BAFF-R expression and B-cell phenotypes with months between repopulation and relapse

It is also possible that differences in levels of BAFF-R and B-cell phenotype distribution in patients discordant for repopulation and relapse (D-R and D-NR cohorts) were related at least partly to differences in the time of sample collection following repopulation. We therefore compared months between repopulation and relapse in the two cohorts and found them not to be significantly different (months between repopulation and sample collection in the D-NR cohort range 4–46, median=13; D-R cohort range 5–24, median=7; $p$=0.594, Mann–Whitney, *data not shown*).

## 4. Discussion

Using the 11C1 monoclonal antibody, which recognizes occupied and unoccupied BAFF-R on human cells [25], we found that BAFF-R levels on CD27+ and CD27− B cell subpopulations in RA patients before rituximab were similar to those of B cells from NC, although there was a tendency towards lower values especially in memory B-cell populations (MFI, $p$=0.098, Table 1 and Figure 2). We also report that BAFF-R expression was significantly reduced on B cells from patients with RA when they were undergoing relapse following rituximab. Low BAFF-R levels were not due to an increase in circulating plasmablasts (*data not shown*), which have been associated with clinical relapse [26]. Significantly, reduced BAFF-R expression was found in both naïve and memory subpopulations and on B cells from patients who relapsed when B cells returned, as well as in those patients who relapsed more than 3 months after B-cell return. In addition, as all patients in the pre-RX group had active RA and were studied before the induction of B-cell depletion therapy for resistant disease the results also imply that reduced BAFF-R expression was not necessarily a consequence of inflammation and active disease, but was more likely related to the re-establishment of disease.

The bioactive trimeric form of soluble BAFF is produced by a variety of cell types, namely myeloid and dendritic cells, neutrophils and some stromal cells [27]. In humans, BAFF signaling through BAFF-R does not appear to be directly involved in B-cell maturation or in most T-cell-independent responses [6]. BAFF-R is expressed on human B

cells from the transitional stage onwards and is then lost from memory B cells as they differentiate into Ig-secreting plasma cells [28]. Stimulation through Toll-like receptor pathways (CpG, IL-2, IL-15) is more efficient than T-dependent pathways (CD40L, IL-2, IL-10) at downregulating BAFF-R expression on memory B-cell populations [7, 28]. It was also found that stimulation of naïve cells through either pathway had no effect on BAFF-R expression, although positive responses of naïve human cells to either stimulus are poor [28].

Investigations of BAFF-R expression on B cells from patients with rheumatic diseases are limited and to some extent report contrasting findings and none describe patients with RA or the effect of rituximab. Carter *et al* [25] showed that normal levels of BAFF-R were present on B cells in patients with SLE, but were of reduced availability due to occupancy by soluble BAFF. Low levels of BAFF-R expression and a negative correlation between BAFF-R expression and higher disease activity in patients with SLE have been described [19]. In patients with Sjögren's syndrome, BAFF-R expression was decreased on both naïve and memory B cells, with the lowest levels found in patients with extraglandular involvement [19]. Analysis of BAFF-R messenger RNA from B cells from these patients suggested that the lower expression was due to a post-translational modification and not to any reduction in BAFF-R mRNA. This could be explained if decreased BAFF-R expression reflected the recent binding of bioactive BAFF to BAFF-R followed by internalization or shedding [25]. In turn, this could be due to the chronic exposure of B cells to constantly high circulating levels of BAFF, which are often found in these patients. Carter *et al* [25] showed that the receptor/cytokine complex was not internalized. Loss of the receptor following ligation by BAFF was, however, confirmed by Sellam *et al* [19], who showed that overnight, but not short-term, culture with physiologically encountered concentrations of BAFF reduced BAFF-R levels on B cells from normal donors.

Our experience here and in earlier studies [17] was that BAFF levels were not significantly raised in patients with active RA. However, following rituximab, BAFF levels rise rapidly and remain raised for variable periods of time. In our serial studies [17], we found that in individual patients, BAFF levels fell more sharply in patients in whom relapse was associated with B-cell return, than in those patients who relapsed months after B-cell return. This may have reflected increased BAFF binding to BAFF-R or TACI following B-

cell receptor (BCR) engagement. We could not confirm these results here as individual patients were not studied serially over the course of their cycle of treatment. In the present study, serum BAFF remained raised to similar levels in sera from patient cohorts discordant or concordant with respect to B-cell return and relapse. Within each group of patients, however, there was no correlation between BAFF levels and BAFF-R expression (Figure 3) suggesting that reduced expression was not solely due to chronically raised BAFF levels, although this possibility could not be excluded with certainty.

When levels of expression of BAFF-R on naïve and memory populations in pre-RX patients and controls were compared, CD27+ memory B-cell values tended to be lower, although this did not reach statistical significance ($p$=0.07, *data not shown*). The main finding of the study was that BAFF-R levels were significantly reduced on both naïve and memory B cells from relapsing, compared with non-relapsing, patients and pre-RX patients. This suggested that the reduction in BAFF-R levels may have been initiated in naïve cells, which then carried the “low” BAFF-R phenotype into the mature memory B-cell stage. In-vitro experiments by others have shown that BAFF could modulate BAFF-R expression in a dose-dependent manner [25]. As we found no correlation between raised BAFF levels and reduced BAFF-R expression and low BAFF-R expression on naïve populations as well as memory B cells associated with the re-establishment of disease, we suggest that another mechanism may be responsible.

Patients with RA have been shown to possess a number of genetic polymorphisms linked to tolerance checkpoints in the bone marrow or the periphery [29]. Evidence of altered thresholds for BCR signaling [30] have been described in patients with RA and suggested to contribute to the generation of autoreactive cells. There is also accumulating evidence that pre-B/immature B cells from patients with RA are pre-activated before they exit from the bone marrow [31]. It was recently shown that B cells at the pre-B/immature naïve stage in the bone marrow of patients with RA had evidence of increased TLR9 protein expression and mRNA compared with osteoarthritis patients and also expressed the activation marker CD86 [32]. Disease-induced changes such as the formation of germinal center-like structures, cytokines such as TNF, or increased levels of bacterial DNA/RNA in the bone marrow environment in patients with RA may disrupt the environment in which pre-B cells are developing [33]. Reduced ability to express receptors for the pro-survival

cytokine BAFF on transitional and naïve B cells after exiting the bone marrow may result as part of the unusual cytokine environment in which the B cells developed.

Once in the circulation, BCR specificity becomes a crucial determinant of B-cell survival and function. The expression of BCR specific for citrullinated proteins and increased numbers of RF-specific B cells have been described by Samuels *et al* [31] within early (CD10+IgM+) populations exiting the bone marrow and also in mature naïve B cells (CD10−IgM+) in RA patients compared with normal individuals. The conclusion is therefore that alterations of tolerance checkpoints both within the bone marrow and in the periphery may be occurring in patients with RA. Within the polyreactive pool exiting the bone marrow, BCR specificities with promiscuous potential such as those possessed by the relatively high proportion of RF B cells that can obtain help from T cells with specificity for a wide range of processed peptides [34], may have a survival advantage that could override the disadvantages of low BAFF-R expression/signaling in a genetically predisposed individual. We have shown that BAFF levels do not appear to be limiting in relapsing patients, but lower levels of BAFF-R expression on newly generated B cells may reduce survival potential and favour "expanded" cohorts of B cells able more easily to obtain productive, but not necessarily antigen-specific, T-cell help.

As well as being an effective treatment for many patients with RA, rituximab gives us an opportunity to study elements important for relapse through our ability to study the re-establishment of disease. Following clearance of rituximab from the bone marrow, newly generated B cells expand into an environment that has retained a variable number of resistant B cells, a mature T-cell compartment and long-lived plasma cells. First, we know that B-cell return to the periphery is mandatory for relapse [20]. Second, the gap between B-cell return and relapse may reflect the relationship between the re-expansion of surviving memory B-cell clones under the influence of newly generated B cells. In both scenarios, maturation into Ig-secreting cells is often coincident with relapse, as shown by rises in autoantibodies [17, 35] and also by the reported association with circulating plasmablasts [23]. Our finding of a lower proportion of post-switched memory B cells in non-relapsing, repopulated patients compared with relapsing patients supports the view that B-cell class-switch recombination is associated with relapse. The depletion of $CD27^{high}$ memory B cells has previously been associated with clinical response to rituximab [23, 36] and low

circulating numbers of IgD−CD27+ memory B cells associated with low clinical activity indices and a longer clinical response, as reported by our group and others [20, 37]. The parallel finding that BAFF-R levels in the non-relapsing repopulated patients were similar to those of pre-RX patients support our hypothesis that the signals required for BAFF-R downregulation are intimately linked to the re-establishment of disease. We also found that a population of double-negative (IgD−CD27−) B cells was significantly raised in patients with RA compared with controls, but their proportions did not change following rituximab. These cells may be similar to a previously described memory B-cell population in patients with SLE by Wei *et al* [38], although others have found that only approximately 50% of this double-negative B-cell population bore markers of mutation and class switch [39].

Although no statistically significant reduction in BAFF-R expression was present in RA patients before treatment, the ranges of percentage expression and MFI were wide (Table 1, Figure 2). It is possible that a proportion of B cells with low BAFF-R levels are being generated throughout the clinical course of RA, but that the numbers of detectable BAFF-R$^{low}$ B cells may vary.

B-cell survival and differentiation is the result of a complex interplay between survival factors, in particular the BAFF/APRIL system in concert with the specificity and strength of signal through the BCR [40]. From this study, we have shown that low levels of BAFF-R expression are related to the re-expansion of possibly pathogenic species of B cells associated with relapse. The re-establishment of active disease in patients with RA after rituximab may therefore involve preferential survival properties of autoreactive B cells, due to the unusual properties of their BCR and the ability also to overcome possible consequences of lower levels of BAFF-R signaling [27, 31].

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# CHAPTER IV

## Discussion

# DISCUSSION

## The role of B cells in the very early phase of rheumatoid arthritis

Several studies have documented the importance of B cells in RA pathogenesis through diverse mechanisms. In fact, B cells produce autoantibodies such as RF and ACPAs, which can form immune complexes and deposit in the joints, causing inflammation; present antigens and activate T cells; release cytokines and participate in ectopic lymphoid neogenesis. The discovery that B cell depletion therapy with RTX was effective in RA patients reinforced the key position of these cells in this autoimmune disease and placed B cells in a central stage of research. Nevertheless, the majority of the research in this field has been focused in established RA patients, while the knowledge associated with the immune mechanisms involved and the role of B cells in RA onset is still very scarce. In fact, even the so called "early RA" studies have included patients with three to 12 months of disease duration that in many aspects already correspond to an established immunobiology. Therefore, the major novelty of the present study was the analysis of the first few weeks of RA onset. Indeed, a cohort of untreated polyarthritis patients with less than six weeks of disease duration was followed up and a full characterization of peripheral blood B cell subpopulations, serum cytokine and chemokine environment and B-cell gene expression profile analysis were performed. The follow-up of these patients allowed the identification of a subset that evolved into RA, which was classified as very early RA (VERA). The remaining patients either had self-limited forms of arthritis or evolved into other chronic inflammatory joint diseases. Clinical and laboratorial evaluations of VERA patients were done at baseline and after short-term treatment with corticosteroids and MTX.

Interestingly, it was found that VERA patients have disturbances in circulating memory B cells, namely a decrease in pre-switch memory ($IgD^{+}CD27^{+}$) subpopulation; have increased serum levels of cytokines related with B cell recruitment and activation; and have

alterations in the expression of genes related with B cell homeostasis, development and survival. Importantly, these observations support an ongoing role of B cells in RA pathogenesis since the first weeks of RA onset. Furthermore, it was observed that short-term therapy with corticosteroids and MTX, although effective in clinical improvement, did not seem to affect peripheral B cells or cytokine content in circulation.

# Characterization of peripheral blood B cell subpopulations

An interesting and surprising finding of the present work was the observation that VERA patients displayed disturbances in circulating memory B cells, particularly lower numbers of pre-switch memory B cells ($IgD^+CD27^+$). Importantly, this constituted the first evidence of an early involvement of B cells in RA physiopathology. Therefore, understanding the role of this B cell subpopulation in RA pathogenesis might help to better comprehend the mechanisms involved in RA progression.

In humans, up to 40% of peripheral blood B cells express CD27 and have hypermutated variable regions in their Ig receptors. These cells are usually considered to be memory B cells generated in GCs during immune responses to T-cell dependent antigens [1, 2]. During GC reactions, B cells proliferate and undergo repeated cycles of SHM, with antigen-selected clones eventually leaving the GC to become memory B cells [3]. In humans, GCs reactions and the development of both memory B cell subsets (pre-switch $IgD^+CD27^+$ and post-switch $IgD^-CD27^+$) begin soon after birth [4]. Indeed, blood $IgM^+IgD^+CD27^+$ cells are present and mutated in infants with less than two years of age, suggesting that these cells are generated and diversified at early stages of life [5, 6]. Nevertheless, there is still considerable debate and controversy concerning the exact function and phenotype of $IgD^+CD27^+$ B cells in humans. In fact, it has not yet been clearly established whether the $IgD^+CD27^+$ memory subpopulation only participates in T cell-independent immune responses, due to the fact that this population can also express heavily mutated Ig genes [7]. Some studies have suggested that $IgD^+CD27^+$ cells differ from classical memory cells and are instead splenic marginal zone (SMZ) B cells involved in T-

cell independent responses. Contrarily to the equivalent B cell population in mice, these cells recirculate and diversify their Ig receptors by hypermutation in a GC-independent pathway [5, 8]. The presence of T-independent antigens in the mature environment of the SMZ is assumed to trigger the effector function of $IgM^{+}IgD^{+}CD27^{+}$ cells, the differentiation into IgM-secreting plasma cells or, after isotype switch, the secretion of mutated antibodies [9-11]. Furthermore, the dependence of these cells on the splenic microenvironment is highlighted by their reduced numbers in splenectomized patients and the existence of a correlation between this specific subset and protective immunity against pneumococcal infections [12, 13].

The knowledge concerning pre-swich memory $IgD^{+}CD27^{+}$ B cells' ability to somatically hypermutate their Ig genes is also controversial. Recently, it has been shown that no significant AID expression is detected in the splenic $IgM^{+}IgD^{+}CD27^{+}$ fraction in young infants (less than two years old) [6]. B cells displaying an $IgM^{+}IgD^{+}CD27^{low}$ phenotype are present in the SMZ and, despite numerous antigenic challenges provided by standard childhood vaccinations, the overall complexity of the heavy chain variable ($V_H$) gene rearrangement repertoire of $IgM^{+}IgD^{+}CD27^{+}$ cells in blood and spleen was similar to that in naïve B cells, with no sign of antigen activation or clonal expansion [6]. Nevertheless, other studies seem to contradict this notion [1, 14, 15]. Indeed, it has been demonstrated in a study of healthy adult population that $IgM^{+}IgD^{+}CD27^{+}$ B cells resemble class-switched and IgM-only memory cells in terms of cell phenotype, and comprise 15% of circulating B cells [1]. Importantly, it was shown that in addition to IgM-only B cells, $IgM^{+}IgD^{+}CD27^{+}$ cells represent a further population of IgM-bearing B cells in the peripheral blood that expresses somatically mutated $V_H$ genes. $IgD^{+}CD27^{+}$ B cells also comprise a small subpopulation of $IgM^{-}IgD^{+}CD27^{+}$ (IgD-only cells) (less than 1%) expressing a highly mutated $V_H$ region genes [1, 16, 17]. Of note, it has been shown that in a transgenic mouse model, marginal zone B cells can also be recruited for T-cell dependent responses, forming GCs that subsequently undergo SHM and affinity maturation [18]. Another possible explanation for the generation of $IgD^{+}CD27^{+}$ cells involves an early branching point in GC differentiation, before the onset of isotype switching [19, 20], which accounts for the lower mutation frequency generally observed in these cells than for class-switched $IgD^{-}CD27^{+}$ B cells from the same individual [5, 21].

In VERA patients, the hypothesis that pre-switch memory $IgD^{+}CD27^{+}$ cells are able to differentiate into plasmablasts and produce RF cannot be excluded. In fact, $IgD^{+}CD27^{+}$ B cells present several features that suggest a role in autoimmunity [22-24]. Previous reports had already mentioned a decrease in $IgD^{+}CD27^{+}$ memory B cells in patients with SLE [25], SS [26, 27], or systemic sclerosis [28]. Interestingly, their number was associated with RA relapse during B cell reconstitution in RTX treated patients [29, 30]. Moreover, similarly to post-switch memory $IgD^{-}CD27^{+}$ B cells, they may also differentiate into plasmablasts and produce isotype-switched antibodies [22, 31-35].

It has been demonstrated in a transgenic mouse model for RF that the activation of splenic autoreactive B cells, as well as hypermutation, occurred in the T zone-red pulp border rather than in the GC [36-38]. Importantly, this observation contradicted the idea that GCs were the only site in the immune system that supported and sustained high rates of SHM [39]. Notably, it has been observed that not only autoreactive B cell responses often occur extrafollicularly, generating short-lived plasmablasts [40], but also that T cells are not required for initiation of RF responses and TLR signals are essential [38].

The decrease in circulating levels of pre-switch memory B cells ($IgD^{+}CD27^{+}$) in VERA patients can possibly be explained by a recruitment of this B cell subpopulation towards the synovial membrane, where it accumulates and contributes to the main inflammatory process characteristic of RA. Indeed, it is known that rheumatoid synovial membrane is infiltrated by B and plasma cells [41, 42]. In addition, a migration of this B cell subset to secondary lymphoid organs, such as spleen or lymph nodes, cannot be excluded. Importantly, it has been demonstrated that established RA patients have also a significant lower frequency and lower absolute numbers of peripheral blood pre-switch memory $IgD^{+}CD27^{+}$ B cells, an effect that was abrogated by anti-TNF therapy [43]. Notably, it was found that both pre-switch $IgD^{+}CD27^{+}$ and post-switch $IgD^{-}CD27^{+}$ memory B cells accumulate in the synovial membrane of RA patients [43, 44], which reinforces the proposed hypothesis and the pathogenic role of memory B cell subpopulations in RA progression.

In the first weeks of RA onset, pre-switch memory B cells can be responsible for the production of high affinity IgM antibodies and, eventually, isotype-switched antibodies, that accumulate locally in the joints. These antibodies can contribute to immune complexes

formation and help to induce the triggering of an inflammatory response in the rheumatoid synovium, leading to the activation of both innate and adaptive immune systems.

Furthermore, short-term therapy with corticosteroids and MTX, despite improved clinical symptoms in VERA patients as indicated by a decrease in DAS28, was not able to restore pre-switch memory $IgD^+CD27^+$ B cells to normal levels. Long-term therapy either with corticosteroids or MTX affects B cell numbers, suggesting that the absence of an effect on these cells that we have observed is justified by the short term exposure [45, 46].

The beneficial effects of B cell depletion therapy with RTX in RA patients have also brought a new attention to the role of memory B cells in RA pathogenesis and might help to elucidate the relevance of $IgD^+CD27^+$ B cells in RA progression. It is known that B cell depletion is not definitive and B cell repopulation usually occurs 6-9 months after RTX infusions. During B cell reconstitution, newly generated immature B cells are the first to predominate early in the blood, while memory B cells tend to be profoundly and continuously depleted [47, 48]. However, it has been demonstrated that high numbers of overall memory B cells ($CD19^+CD27^+$) and enhanced C-reactive protein (CRP) levels before the initiation of RTX treatment are predictive of an earlier relapse [30, 47-49]. Interestingly, patients with lower numbers of $IgD^+CD27^+$ memory B cells at the beginning of peripheral B cell repopulation had a much more favourable clinical response. It was shown that nonresponder patients had higher absolute numbers of $IgD^+CD27^+$ in comparison with responders, at B cell return [30]. Therefore, the extent of memory B cell repletion, particularly $IgD^+CD27^+$ cells, seems to be a key factor in influencing the pathophysiology of RA.

Since a high number of memory B cells before therapy may be a less favourable biomarker for early relapse, an effective depletion of this B cell subset might be essential to achieve longer and persistently effective clinical results. However, the occurrence of clinical relapses is a concern and implies re-treatment of patients, indicating that a single cycle of B cell depletion seems to be insufficient to reestablish self-tolerance by eliminating autoreactive memory B cell clones.

Very early arthritis (VEA) patients, who evolved into other conditions than RA, also had a decrease in $IgD^+CD27^+$ B cells in peripheral blood. Of interest, a reduction in circulating memory B cells has been shown in patients with X-linked hyper-IgM syndrome [22],

chronic granulomatous disease [50] and human immunodeficiency virus (HIV) [51]. Hence, lower levels of circulating memory B cells seem to be linked with chronic inflammation rather than an exclusive feature of autoimmune conditions. Nevertheless, other factors such as environment or genetic predisposition, can influence and contribute to the development and outcome of autoimmune diseases in patients who already display disturbances in circulating memory B cells [52-55], such as the case of our very early polyarthritis patients cohort that have evolved into RA.

# Cytokine environment and cellular interactions

Since VERA patients display disturbances in circulating B cell subpopulations, an extensive analysis of the cytokine enviroment that could potentially contribute to this effect was performed. Importantly, it was found that VERA patients have increased levels of cytokines related to B cell recruitment (IL-6), maturation and survival (APRIL, BAFF), thus reinforcing the notion of an early activation of B cells during RA onset.

Several types of cells from both innate and adaptive immune system have an intervention in RA pathogenesis [41, 56-61], as illustrated in figure 1. Macrophages, neutrophils, fibroblasts, B and T cells, osteoclasts, are able to interact with each other and lead to RA progression, mainly through the production and secretion of cytokines, chemokines and adhesion molecules that facilitate cellular recruitment and infiltration of joints, thus triggering a chronic inflammatory response [56, 62-64]. Therefore, the serum panel of cytokines selected for testing in VERA patients aimed at encompassing all the major known RA pathogenesis players. Of interest, it was observed that in addition to a B cell activation pattern, VERA patients have also a cytokine profile that supports neutrophil recruitment (IL-8) and Th17 cells polarization (IL-1β and IL-6) and activation (IL-17A and IL-22), that are ultimately able to assist the autoreactive B cells in RA progression. Furthermore, the analysis of SF samples from established RA patients, illustrating the inflammatory process that occurs locally in the joints, also revealed a similar pattern of cytokines, thus supporting the pathogenic role not only of the B cells, but also of the neutrophils and Th17 cells in RA development and chronicity.

Neutrophils are the most abundant leukocytes in the SF of patients with active RA and it has been observed that these cells can heavily infiltrate the synovial tissue in the first days of RA onset [61]. Moreover, previous results from our group demonstrated that there is a delay in the apoptosis of circulating neutrophils in VERA patients [65]. The elevated serum IL-8 levels in VERA patients when compared with both VEA and healthy controls, could explain the preactivated state of circulating neutrophils and their recruitment towards the SF in the very first weeks of RA onset [66]. Interestingly, once activated, neutrophils are able to produce high levels of BAFF [67], also found to be increased in VERA patients' serum and established RA SF.

BAFF is a fundamental B-cell survival factor essential for B cell proliferation and activation [68]. In fact, increased serum BAFF levels have been associated with several autoimmune diseases such as SLE [69-72], SS [73, 74] and ITP [75]. In RA patients, several studies have demonstrated that BAFF is elevated in serum [69, 76-78], although there are findings that also show no differences in comparison to healthy controls [79, 80]. Nevertheless, similarly to what was observed in the established RA cohort included in this study, BAFF levels in RA SF are higher than their corresponding serum levels [81], which is consistent with local production of BAFF in inflamed joints [82].

In VERA patients, activated neutrophils could release high quantities of BAFF that stimulate autoreactive B cells, while in established RA this effect is more localized to the joints. Once stimulated with an activation and survival factor like BAFF, the B cells that are prone to be autoreactive can produce higher amounts of autoantibodies, such as RF or ACPAs, and function as more efficient APCs to activate T cells [76, 83, 84]. The immune complexes that further deposit in the joints activate Fcγ-receptors, such as FcγRIIIa, expressed by macrophages [63, 85, 86], which are consequently triggered and further increase the inflammatory process with the production of proinflammatory cytokines [62, 63, 87], such as TNF or IL-1β, that were also found to be increased not only in VERA, but also in established RA SF.

APRIL was also increased in VERA patients' serum as compared with VEA and established RA, and its levels were even higher in RA SF, suggesting a local upregulation in the rheumatoid synovium. APRIL is expressed by macrophages [88, 89], dendritic cells [90] and fibroblast-like synoviocytes [91] and, like BAFF, is an important cytokine

involved in B cell survival. APRIL affects not only the class-switch recombination process [92-94], but also plasma cell differentiation and survival [95, 96], which could explain the maintenance of autoreactive B cells in RA joints [97]. Importantly, a highly positive association between the infiltration of plasma cells and SF levels of APRIL has been demonstrated in RA patients [97, 98]. Therefore, in VERA patients, the activated macrophages and possibly fibroblasts infiltrating the synovial membrane can be responsible for APRIL production, which induces the differentiation of plasma cells and stimulates isotype-switching. Furthermore, the increased concentrations of APRIL detected locally in RA joints can be associated with the observed accumulation of both pre-switch memory $IgD^{+}CD27^{+}$ and post-switch memory $IgD^{-}CD27^{+}$ B cells in the synovial tissue as previously observed [43, 44]. It is possible that both APRIL and BAFF help to maintain the survival of autoreactive memory B cells in the joints. Moreover, APRIL could induce the occurrence of SHM process within joints, namely in areas of ectopic lymphoid neogenesis, and increase affinity maturation.

Additionally, the observed increased IL-21 levels in SF from the established RA cohort also support local plasma cell differentiation and autoantibody production [99], that is consistent with the infiltration of plasma cells in the synovial membrane [97, 100].

In VERA patients, the detection of elevated concentrations of Th17-derived cytokines, namely IL-17A and IL-22, suggests that these cells might also have a role to play in RA pathogenesis since the first weeks of RA onset [101, 102]. Indeed, Th17 cells have been implicated in RA pathogenesis through the production of IL-17 [103-105] and increased levels of this cytokine are correlated with more severe joint damage [106]. Of note, IL-1β and IL-6, both found to be increased in VERA patients, are known to promote the differentiation of Th17 cells [107, 108], which in turn secrete IL-17A and IL-22 [109, 110]. Interestingly, IL-17A is a cytokine that promotes the recruitment and survival of neutrophils [111, 112], induces the secretion of proinflammatory cytokines (such as IL-1β, IL-6, IL-8 and TNF) [62, 113], upregulation of RANKL [104] and stimulates the activity of matrix metalloproteases, thus leading to cartilage catabolism and bone resorption [114-117].

Considering the cytokine pattern observed in VERA patients, it is therefore tempting to consider that a pathogenic relationship exists between B cells, neutrophils, macrophages

and Th17 cells since the first weeks of RA onset. After an initial unknown triggering event that induces RA development, neutrophils are attracted to the joints, mediated by an IL-8 chemotactic gradient [66] and, once activated, can release high amounts of BAFF [67], which consequently stimulate autoreactive B cells function and survival [68, 69, 76]. The activated macrophages and fibroblasts are also able to produce APRIL, that induces plasma cell differentiation and CSR [92-96]. Simultaneoulsy, proinflammatory cytokines such as IL-1β and IL-6 are released and drive Th17 cells polarization. Activated Th17 cells produce high levels of IL-17A and IL-22, and are able to extend neutrophil survival [111, 112], help B cells in autoantibody production [118-120] and stimulate osteoclastogenesis, thus leading to cartilage and bone destruction [104, 116, 117]. Importantly, the increased IL-6 levels generated not only in VERA patients, but also in established RA serum and SF samples, support a continuous recruitment of autoreactive B cells toward the synovium [121, 122] during RA progression, thus contributing to an exacerbation of the inflammatory process due to the production of autoantibodies and immune complexes. Furthermore, in established RA patients, the increased levels of IL-21, mainly produced by $CD4^+$ T cells, including Th17 cells [123-125], not only induce plasma cell differentiation, but also potently enhance Th17 proliferation in RA [126].

Interestingly, cytokines associated with the function of Th1 (IL-2, IL-12 (p70), and INF-γ) and Th2 (IL-4 and IL-10) cells were not significantly elevated in VERA patients. Therefore, Th17-cell polarization and activation seems to be the most relevant T-cell related occurence in very early RA patients.

The establishment of a pathogenic interaction between Th17 and B cells since early RA onset is also reinforced by the fact that B cell chemoattractant CXCL13 is preferentially expressed by Th17 cells [127]. CXCL13 is essential for B cell migration towards RA joints and B cell follicular organization [64] and recent findings have correlated CXCL13 expression with RA synovitis [128] and identified this chemokine as a novel serological marker predictive of RA severity [129]. Importantly, these observations suggest that B cell and Th17-related cytokine targeted therapies could be beneficial to RA patients since the first weeks of RA onset. Indeed, therapies such as atacicept or belimumab, that target B-cell related survival cytokines APRIL and BAFF, are being evaluated for RA treatment [130-135]. Additionally, tocilizumab, an IL-6 receptor inhibiting agent, was recently approved

for RA treatment and is highly effective in established RA [136, 137]. Similarly, neutralizing antibodies to IL-17 are being tested for treating established RA patients [138].

# B-cell related gene expression analysis

Some studies have highlighted the association between RA gene expression profile and disease severity, thus providing valuable insights into RA pathogenesis [139-142]. Importantly, it has been documented that genes regulating and affecting cellular processes such as proliferation, apoptosis, cytokine networks and autoimmunity were differently expressed in RA B cells [143-145]. Interestingly, previous studies have shown that among the highest induced genes in human established RA were several B-cell associated genes, including immunoglobulins, B-cell markers such as CD20, and cytokines and chemokines that directly act on B cells, such as BAFF and CXCL13 [145]. These observations, together with our previous results, prompt us to hypothesize that B-cell biology is already dysregulated in RA onset, setting the grounds for the induction and perpetuation of a pathogenic humoral immune response. Therefore, the expression of a group of genes related to B cell homeostasis, maturation and survival was evaluated in VERA patients and compared to different stages of RA progression: untreated early RA patients with less than one year of disease duration (ERA) and established RA patients. Furthermore, other untreated early arthritis patients (EA) that did not evolve to RA and had less than one year of disease duration were also included in this study.

It was found that BAFF gene expression, similarly to BAFF serum levels, was highest in VERA patients. The expression of BAFF-R significantly increased with disease progression, while TACI was elevated since the first weeks of RA onset; Pax5 gene expression was also increased in all RA stages; CXCR5 was only elevated in established RA and no differences were observed in BCMA, AID, Blimp-1 and Bcl-2 expression. Importantly, these alterations can potentiate the development of autoreactive B cells since the first weeks of RA onset.

The disturbances that were found in BAFF/ BAFF-receptors system in VERA patients were extremely interesting and appealing for a B cell intervention treatment in early RA. BAFF binds to three receptors expressed by B cells: BAFF-R, BCMA and TACI, with

BAFF-R being the only one that exclusively binds BAFF and to which BAFF has higher affinity [146]. Although the precise mechanisms that control expression of all three BAFF receptors are currently unclear, studies indicate that the developmental stage, cytokine milieu and the microenvironment play a key role in regulating the receptor expression [147].

Studies with knockout mice have demonstrated that B-cell proliferation depends on either BAFF-R or TACI signaling [96]. Excess BAFF may lower the threshold for BCR signaling and maintain survival of pathogenic autoreactive B cells, thus breaking B cell tolerance. Signaling through BAFF-R can result not only in survival enhancement [148, 149], but also in B cell maturation [150], while TACI triggering can stimulate isotype switching and plasma cells differentiation [151]. Nevertheless, TACI functional activity is ambiguous. In fact, it has been demonstrated that TACI not only provides positive signals driving T-independent B cell response [152] and survival of activated B cells and plasmablasts [151, 153], but also delivers negative signals suppressing B cell activation [154]. Moreover, while $TACI^{-/-}$ mice predominantly develop autoimmunity and lymphoproliferation [155], TACI deficiency in humans primarily manifests itself as an antibody deficiency syndrome. Mutations in TACI were associated with common variable immunodeficiency (CVID) [156] and it has been shown that antibody responses after vaccination with pneumovax were selectively impaired in TACI-deficient humans [157]. Furthermore, it has been demonstrated that TLR7 and TLR9 triggering strongly upregulated TACI expression, which suggests that autoimmunity might also be correlated with reciprocal induction and activation of TACI and TLRs [158-160]. Of note, it has been shown that TLR9 can directly affect activation and proliferation of bone marrow B cells from RA patients and, thus, influence the pathogenesis of RA [161].

Considering the gene expression pattern observed in VERA patients, it is possible to hypothesize that the elevated BAFF serum levels negatively regulate BAFF-R expression on B cells and, simultaneously, upregulate TACI. The induction of TACI expression on activated B cells could serve as a danger signal, reflecting the need to avoid the potential harmful effects of prolonged activation of B cells through BAFF-BAFF-R interaction [148, 162]. With disease progression, signaling through BAFF-R eventually surpasses TACI, thus allowing autoreactive B cells to persist. Interestingly, an increase in both BAFF and

BAFF-R mRNA expression was found in RA synovial tissue [145, 163]. The higher affinity of serum BAFF, present at lower concentrations at later RA stages, towards BAFF-R instead of TACI expressed by B cells which are more prone to be autoreactive supports this notion. Importantly, TACI binds APRIL and BAFF equally with high affinity [164] and serves as the only receptor for BAFF/ APRIL heterotrimers [165]. Thus, since CSR depends on APRIL signaling via TACI [92], the high levels of TACI expression and increased APRIL serum concentrations observed in VERA patients could contribute to the development of CSR and autoantibody formation by autoreactive B cells since the first weeks of RA onset. Moreover, the maintenance of elevated levels of TACI expression in ERA and established RA patients and the increased concentrations of APRIL found in RA SF support the notion of an activation of CSR during RA progression.

Previous studies have also demonstrated that BAFF increases CXCL13-dependent chemotaxis of memory B cells through BAFF-R triggering [166]. Therefore, increased BAFF serum and mRNA levels since the first weeks of RA onset could support the migration of pre-switch memory $IgD^{+}CD27^{+}$ B cells towards the synovial membrane, thus justifying the decrease of this B-cell subpopulation in circulation not only in VERA patients, but also in established RA [43]. In established RA patients, this effect is probably higher in joints than in peripheral blood, since BAFF levels are increased in RA SF.

The observation that Pax5 gene expression was elevated in all RA stages also reinforces the role of B cells in RA pathogenesis. Indeed, Pax5 has been referred to as the guardian of B cell identity and function [167, 168]. Pax5 gene codifies a transcription factor, also known as B-cell-specific activation protein (BSAP), which is essential for B cell commitment as well as for B cell development, and continuous expression of Pax5 is required throughout the B cell lineage to maintain the functional identity of B cells [169]. Pax5 is essential for many aspects of B lymphopoiesis including the initial commitment to the lineage, immunoglobulin rearrangement, pre-BCR signaling and maintaining cell identity in mature B cells [168, 170]. Importantly, Pax5 not only regulates B cell fate by concurrently activating the expression of B cell-specific genes, such as CD19, but also represses non-B cell lineages [167, 171-174]. In fact, dysregulated or reduced Pax5 activity has also been implicated in human disease, such as in CVID patients [172] and B-cell neoplasms [175].

Pax5 gene expression was significantly higher in RA patients in comparison with other early forms of arthritis. Importantly, this observation, together with the absence of differences in BAFF, BAFF-R and TACI expression in these patients in comparison to controls reinforces the specific role of B cells in RA progression when compared to other inflammatory joint diseases.

Silencing of Pax5 gene by upregulation of B-lymphocyte-induced maturation protein-1 (Blimp-1) is essential for terminal differentiation of B cells to plasma cells [176, 177]. In an animal model of SLE, a reduction in Pax5 gene expression with a simultaneous upregulation of Blimp-1 mRNA and IL-21 serum levels was shown [178], which is in accordance with previous observations of increased plasma cell frequencies in blood from SLE patients [25, 179, 180]. Nevertheless, the absence of differences in IL-21 serum levels in VERA patients in comparison to healthy controls supports the findings of normal absolute numbers and frequencies of circulating plasma cells in VERA patients. Indeed, since the concentration of IL-21 was only significantly elevated in RA SF and plasma cells are known to infiltrate RA synovial membrane [181], this cytokine should be inducing Blimp-1 upregulation and decreasing Pax5 gene expression locally in the joints, thus allowing the differentiation of plasma cells [99, 182].

CXCR5, a receptor involved in B cell chemotaxis, is upregulated in RA synovium [183], suggesting a local role in B cell chemotaxis towards synovial membrane. The increased CXCR5 levels observed in established RA patients in comparison to controls and VERA patients reveals that this effect is stronger in later RA stages. Nevertheless, the expression of CXCR5 is not restricted to B cells. $CD4^{+}$ T cells present in B cell follicles, named follicular helper T (TFH) cells, which have been established as a T cell subset specialized for providing help to B cells in GCs [184, 185] also express CXCR5 and migrate towards B cell follicles in response to a CXCL13 gradient [186-189]. Therefore, the hypothesis that the increased CXCR5 gene expression in established RA patients is due not only to B cells, but also to circulating TFH cells cannot be excluded.

Interestingly, $CD4^{+}CXCR5^{+}$ T cells can be subdivided in Th1, Th2 and Th17 subsets in human blood and it has been recently demonstrated that both $CXCR5^{+}$ Th2 and $CXCR5^{+}$ Th17 cells, but not $CXCR5^{+}$ Th1 cells, are able to help B cells in plasma cell differentiation and isotype-switching through the production of IL-10 and IL-21 [188-190]. These

observations also reinforce the proposed interaction between Th17 and B cells in RA pathogenesis.

The expression of β2-microglobulin (β2m), a component of MHC class I molecules [191, 192], was also found to be significantly increased in VERA, ERA and EA patients. This observation indicates early immune system activation not only at RA onset, but also in other forms of arthritis. The immune system triggering since the first weeks of RA onset is supported by the increased serum and synovial fluid β2m levels that have been found in RA [78, 193].

## B cell depletion therapy and characterization of the newly emergent circulating B cell subpopulations during B cell reconstitution

In the present work there was also the opportunity to study an established cohort of RA patients under B cell depletion therapy with RTX. The main advantages of analyzing patients that initiate RTX therapy rely on the concept that B cell depletion may restore the immune system, eliminate autoreactive B cell clones and reestablish tolerance. Therefore, it is extremely important to study and characterize the newly emergent circulating B cell subpopulations during B cell reconstitution, in order to better understand the pathogenic mechanisms that might be associated with B cells and thus explain their involvement in RA progression. Indeed, the relapse of disease activity following B cell depletion therapy is based on altered mechanisms that are quiescent during the absence of circulating B cells and, therefore, provides a unique opportunity to study the re-emergence of the disease process.

A phenotypic characterization of peripheral blood B cell subpopulations was performed in established active RA patients before initiating RTX treatment (pre-RTX), in clinically relapsing RA patients either concordant for B cell repopulation (C-R), or discordant, with relapse more than three months after repopulation (D-R) and in RA patients who were in remission over three months after B cell repopulation (discordant non-relapsing (D-NR)). Similarly to previous studies [47, 48], during B cell repopulation and irrespective of clinical

relapse, the majority of circulating B cells had a naïve $CD19^{+}IgD^{+}CD27^{-}$ phenotype and, as expected, there was a lower frequency of memory $CD27^{+}$ memory B cells in circulation. In particular, the frequencies of pre-switch memory $CD19^{+}IgD^{+}CD27^{+}$ were decreased after RTX therapy in comparison to pre-RTX patients, which is consistent with a favourable clinical response to RTX treatment [30, 49].

Interestingly, patients in remission (D-NR) had lower frequencies of post-switch memory $IgD^{-}CD27^{+}$ B cells in comparison with relapsing patients, thus suggesting that an activation of class-switch recombination process is related to the re-establishment of an active disease. Importantly, these results support previous studies that documented an association between lower circulating numbers of post-switch memory $IgD^{-}CD27^{+}$ B cells with a longer clinical response [47, 48]. These observations also reinforce the intervention of memory B cells in the development and pathogenesis of RA, thus supporting the need to achieve efficient memory B cell depletion during RTX treatment.

Furthermore, considering the importance of BAFF for B cell survival and the previously discussed results observed in VERA patients, it was hypothesized that the balance between BAFF-R expression and soluble BAFF could influence the relationship between returning B-cell subpopulations and clinical relapse. Therefore, the mean fluorescence intensity (MFI) and surface BAFF-R expression were analyzed in circulating B cell subsets and serum BAFF levels were quantified in RA patients before and after RTX treatment.

BAFF-R gene expression analysis in VERA, ERA and established RA patients revealed that the expression of this receptor significantly increased with disease progression in comparison to healthy controls. However, no differences could be observed in BAFF-R expression in the cohort of RA patients before RTX treatment when comparing with controls. The reasons that may account for these differences in the two independent established RA cohorts could rely on the different therapeutic agents that were administered to the patients. In fact, while the established RA patients enrolled in the BAFF-R gene expression study were under MTX therapy, the majority of the RA patients before initiating RTX treatment had failed anti-TNF therapies. Importantly, previous studies have demonstrated that a good clinical response to TNF antagonist therapy in patients with RA is associated with a significant decline in serum BAFF protein levels [77], although no correlation has been established with the expression of BAFF-receptors. Moreover,

treatment with TNF inhibitors induces phenotypical and functional alterations in peripheral B cells, such as an increase in CD20 expression [194]. Thus, the possibility that anti-TNF therapy alters surface expression of BAFF-receptors, namely BAFF-R, on B cells cannot be excluded.

After RTX treatment, a decrease in BAFF-R expression (MFI and frequencies) in both naïve and memory B cells of relapsing patients (C-R and D-R) was found in comparison to healthy controls and pre-RTX patients, while no differences were detected in patients in remission (D-NR). Particularly, it was observed that memory B cells from relapsing patients tended to express lower levels of BAFF-R than naïve B cells. Indeed, it is known that BAFF-R is expressed on human B cells from the transitional stage onwards and is lost from memory B cells as they differentiate into Ig-secreting plasma cells [195]. Nevertheless, the reduction in BAFF-R expression after RTX treatment particularly on memory B cells was not due to an increase in circulating plasmablasts (*data not shown*), which are known to be associated with clinical relapse [29].

BAFF serum levels were within normal range in pre-RTX patients and significantly increased after RTX treatment in all patients groups, as previously described [79], thus reflecting the loss of the majority of circulating B cells. Of note, chronically raised BAFF levels have been suggested to exert a negative feedback on BAFF-R expression [196]. However, no correlation was found between BAFF-R expression and BAFF serum levels within each group of patients. Therefore, the reduced BAFF-R expression must be related with disease re-establishment. Indeed, since BAFF-R expression in patients in remission (D-NR) was similar to those before starting RTX (pre-RTX), the signals required for BAFF-R downregulation must be closely linked to clinical relapse.

It has been suggested that BAFF levels form a more accurate indicator of total B-cell mass [197]. This notion implies that serum BAFF levels are raised when there is a diminution in peripheral blood B cell subpopulations and return to normal values during B cell reconstitution. In fact, a decline in serum BAFF levels has been associated with the reemergence of B cells in peripheral blood [79]. It was demonstrated that BAFF levels fell more sharply in patients in whom clinical relapse was associated with B-cell return, than in those who relapsed months after B cell reconstitution [79]. It is interesting to speculate that, similarly to RTX treated RA patients who experience a transient B cell depletion, the

reduction observed in the circulating levels of pre-switch memory IgD$^+$CD27$^+$ B cell subpopulation in VERA patients could also support the increased BAFF serum levels observed in these patients.

After B cell depletion therapy, newly generated B cells expand into an environment that has retained a variable number of resistant B cells, a mature T-cell compartment and long-lived plasma cells. It has been demonstrated that transitional B cells exit the bone marrow already expressing BAFF-R, but their BAFF-R expression levels are lower than mature B cells [198], thus suggesting that further B cell maturation yields an increase in BAFF-R expression. Furthermore, evidence supporting the existence of a pre-activated state of immature B cells before they exit the bone marrow in RA patients [161] are consistent with the early impairment in B cell tolerance observed in RA patients [199].

Altered thresholds for BCR signaling have also been described in RA and other autoimmune diseases that can contribute to the generation of autoreactive B cells [200-202]. Interestingly, it has been previously demonstrated that BCR and BAFF signaling occur through independent mechanisms that can directly affect the development of autoimmunity [203]. In fact, in vitro studies have shown that the engagement of each BAFF-receptor (BAFF-R, TACI, or BCMA) blocked BCR-induced cell death in B cell lines [203]. Moreover, the relevance of IgM BCR-mediated signals for the induction of arthritis in CIA mouse models is supported not only by the high numbers of IgM-expressing B cells and IgM-producing plasma cells that are present in arthritic joints, but also for the deficiency in RF production observed in IgM$^{-/-}$ mice [200]. Importantly, these observations reinforce the notion that most of the B cells that migrate into joints have not undergone class switching and, consequently, predominant CSR should be occuring locally in the joints during the inflammatory response, which is also consistent with the data observed in VERA patients.

B cell stimulation through TLRs can also be a mechanism directly related with autoimmunity and eventually involved in clinical relapse. TLR9, which is constitutively expressed by B cells, recognizes unmethylated microbial CpG DNA, which, in turn, is similar to certain CpG DNA motifs within the mammalian genome [204-206]. Interestingly, hypomethylated genomic CpG DNA has been found in RA and SLE patients, supporting the involvement of TLR9 signaling and the development of autoimmunity [207-

210]. Previous studies support that dual engagement of BCR and TLR9 may activate autoreactive B cells that recognize endogenous CpG DNA released from apoptotic cells and CpG DNA-IgG immune complexes in RA joints, and induce their development into RF-producing B cells [38, 211-213]. Importantly, stimulation through TLRs, namely TLR9, is more efficient than T-dependent pathways at downregulating BAFF-R expression on memory B-cell populations [195, 214]. CpG DNA motifs have also been shown to amplify autoreactive IgG production by upregulating BAFF [215]. Therefore, it is possible that in relapsing patients B cells are stimulated by CpG DNA-immune complexes, released during B cell apoptosis that occurs due to B cell depletion therapy with RTX. This could affect the expression and triggering of other B cell receptors, such as BAFF-receptors or BCR.

Future studies regarding the analysis of TLRs, the remaining BAFF-receptors (TACI and BCMA) and in vitro studies with BAFF/APRIL heterotrimers are interesting proposals to be addressed in patients undergoing B cell depletion therapy with RTX and their possible relationship with clinical relapse.

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# CHAPTER V

## Conclusions

# CONCLUSIONS

The study of B cells since the first weeks of RA onset supports an early intervention of these cells in RA progression and has revealed that: 1) disturbances occur in peripheral B cell subpopulations consistent with the migration of memory B cells towards synovial membrane, where the main inflammatory process develops; 2) cytokines related with B cell recruitment, activation and survival are increased in circulation in VERA patients and reinforce the important interaction of B cells with other cellular components of the immune system, such as Th17 cells; 3) early short-term treatment with corticosteroids or MTX leads to clinical improvement, but does not seem to have an impact on either circulating B cell subpopulations, or in the cytokine pattern of VERA patients; 4) a gene expression profile supporting B cell maturation, activation and survival is displayed in the first weeks of RA onset, particularly in comparison with other forms of arthritis.

In conclusion, the results of this study suggest that an earlier introduction of B-cell directed therapies, such as B cell depletion (anti-CD19, anti-CD22) or indirect B-cell targeted therapies affecting B cell receptors or its ligands (such as atacicept or belimumab), might be of beneficial clinical use to induce early remission. Furthermore, studies concerning chronic RA patients under B cell depletion therapy with RTX suggest that class-switch recombination process and alterations in the expression of BAFF-receptors are associated with clinical relapse. These observations are extremely important and need to be addressed in further investigations to better understand the exact role of B cells in RA development and disease re-establishment, particularly during relapse.

# List of publications

In agreement with the Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented and discussed in this thesis were published or submitted for publication in the following scientific peer-reviewed journals:

1) Rita A. Moura, Pamela Weinmann, Patrícia A. Pereira, Joana Caetano-Lopes, Helena Canhão, Elsa Sousa, Ana F. Mourão, Ana M. Rodrigues, Mário V. Queiroz, Maria M. Souto-Carneiro, Luís Graça, João E. Fonseca. Alterations on peripheral blood B cell subpopulations in very early arthritis patients. *Rheumatology (Oxford)* 2010; 49(6):1082-1092

2) Rita A. Moura*, Rita Cascão*, Inês Perpétuo, Helena Canhão, Elsa Vieira-Sousa, Ana F. Mourão, Ana M. Rodrigues, Joaquim Polido-Pereira, Mário Viana Queiroz, Henrique S. Rosário, Maria M. Souto-Carneiro, Luis Graca, João E. Fonseca. Cytokine pattern in very early rheumatoid arthritis favours B cell activation and survival. *Rheumatology (Oxford)* 2011; 50(2):278-282
* R.A. Moura and R. Cascão contributed equally to this work.

3) Rita Cascão*, Rita A. Moura*, Inês Perpétuo, Helena Canhão, Elsa Vieira-Sousa, Ana F. Mourão, Ana M. Rodrigues, Joaquim Polido-Pereira, Mário Viana Queiroz, Henrique S. Rosário, Maria M. Souto-Carneiro, Luis Graca, João E. Fonseca. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. *Arthritis Research and Therapy* 2010; 12(5):R196
* R. Cascão and R.A. Moura contributed equally to this work.

4) Inmaculada de La Torre*, Rita A. Moura*, Maria J. Leandro, Jonathan Edwards, Geraldine Cambridge. B-cell-activating factor receptor expression on naïve and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B cell depletion therapy. *Annals of Rheumatic Diseases* 2010; 69(12):2181-2188
* I. de La Torre and R.A. Moura contributed equally to this work.

5) Rita A. Moura, Helena Canhão, Joaquim Polido-Pereira, Ana M. Rodrigues, Márcio Navalho, Ana F. Mourão, Catarina Resende, Raquel Campanilho-Marques, João Madruga Dias, José Alberto Pereira da Silva, Luis Graca, João E. Fonseca. BAFF and TACI gene expression are increased in untreated very early rheumatoid arthritis patients. Submitted for publication in May 2011.

RHEUMATOLOGY

Rheumatology 2010;49:1082–1092
doi:10.1093/rheumatology/keq029
Advance Access publication 7 March 2010

Original article

# 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.

CLINICAL SCIENCE

## 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

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Submitted 31 July 2009; revised version accepted 14 January 2010.

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production of autoantibodies [10–12], such as RF. RF can interact with Fc$\gamma$RIIIa (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 RA (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 tetraacetic 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 FlowJo (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 Percoll (Amersham, Stockholm, Sweden). Cellular viability was estimated with Trypan Blue (Sigma, St. Louis, USA). Cells were frozen in 1 ml/$10^7$ cells RPMI-1640 (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 all 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 ELIA 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, 22 females and 7 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 ($CD19^+IgD^+CD27^-$), pre-switch memory B cells ($CD19^+IgD^+CD27^+$), post-switch memory B cells ($CD19^+IgD^-CD27^+$), total memory B cells ($CD19^+CD27^+$) and plasma cells ($CD19^+CD27^{high}$) 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).

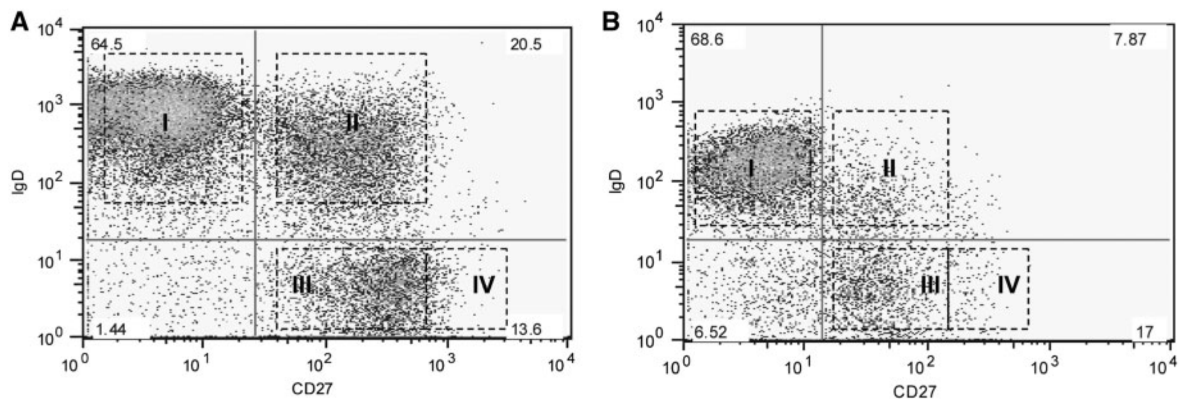
We also investigated differences between VERA patients and controls in circulating T cells, namely total

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

| Clinical parameter | VERA ($n = 22$) Baseline | VERA ($n = 22$) Visit 1 | VERA ($n = 22$) 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.d.). NA: not applicable.

**Fig. 1** B-cell analysis by flow cytometry. A region (gate) was defined around total lymphocytes in peripheral blood, depending upon cell size (forward scatter) and granularity (side scatter). Total B cells were identified based on the expression of the cell surface marker CD19, and its subpopulations were classified according to IgD and CD27 expression in healthy controls (**A**) and patients (**B**). Thus, naïve B cells (I) were classified as $CD19^+IgD^+CD27^-$; pre-switch memory B cells (II) as $CD19^+IgD^+CD27^+$; post-switch memory B cells (III) as $CD19^+IgD^-CD27^+$; and plasma cells (IV) as $CD19^+IgD^-CD27^{high}$.




($CD3^+$), $CD4^+$ 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.

**Fig. 2** 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 ($CD19^+CD27^{high}$) (**C**) were gated in $CD19^+$ B cells. Differences are considered statistically significant for $P < 0.05$.

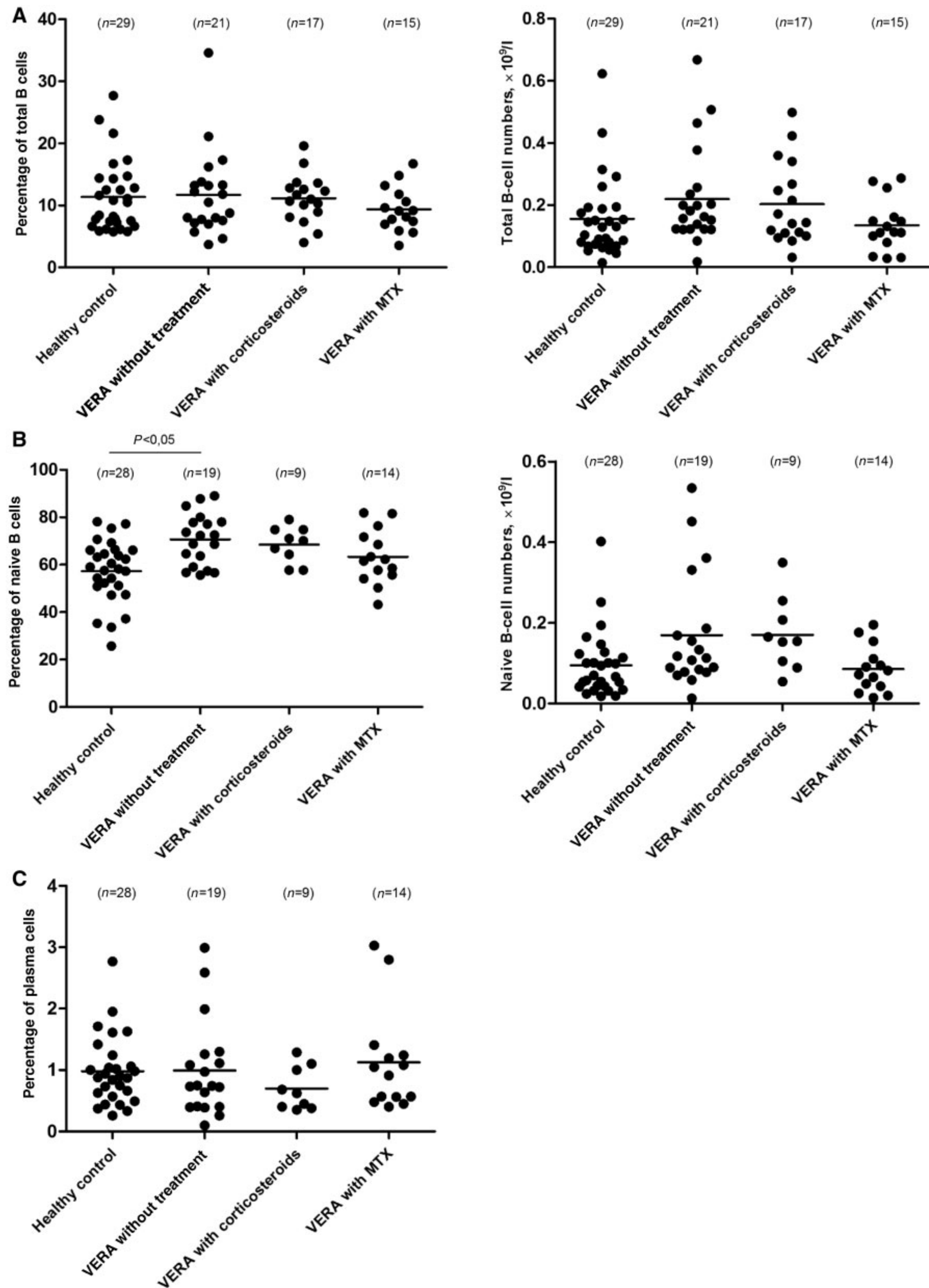
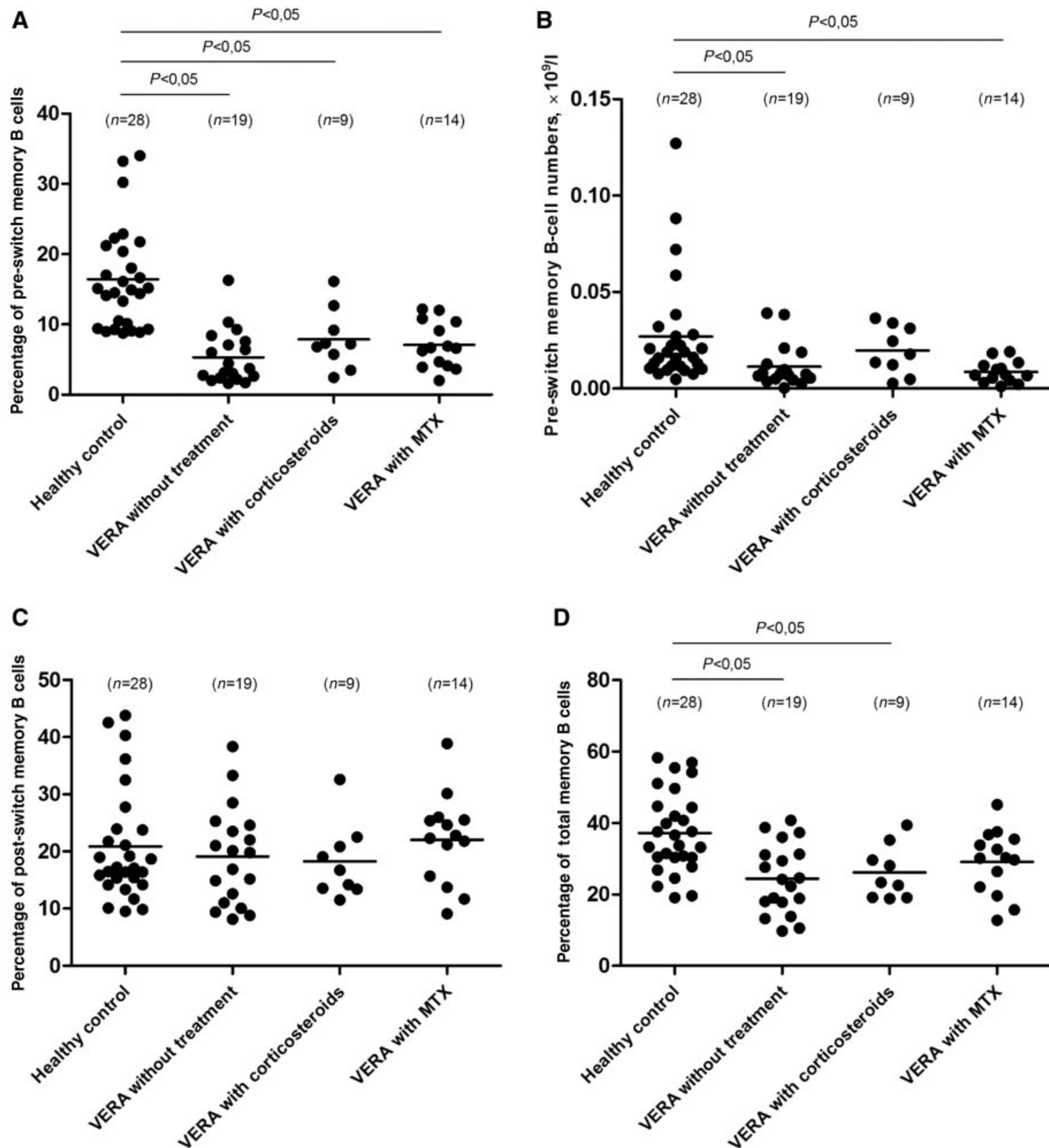


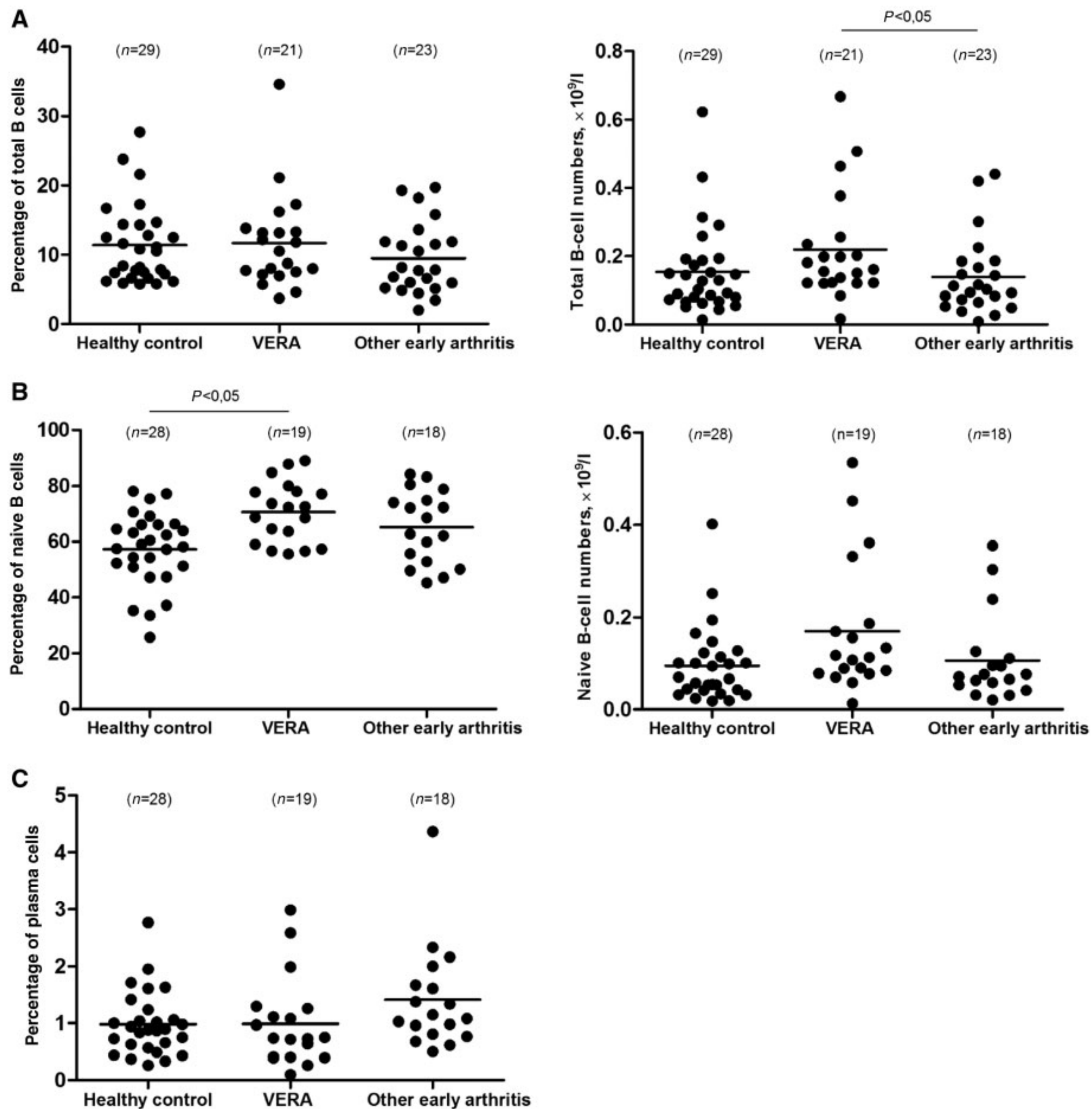
FIG. 3 VERA patients have reduced frequencies and absolute numbers of pre-switch memory B cells. Data from flow cytometry analysis of memory B cells from VERA patients without treatment and after therapy with corticosteroids and MTX. All subpopulations were gated in $CD19^+$ B cells. Representation of pre-switch memory B cells ($CD19^+IgD^+CD27^+$) frequencies (**A**) and absolute numbers (**B**). Post-switch memory B cells ($CD19^+IgD^-CD27^+$) (**C**) and total memory B cells ($CD19^+CD27^+$) (**D**) frequencies are represented. Differences are considered statistically significant for $P < 0.05$.




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].

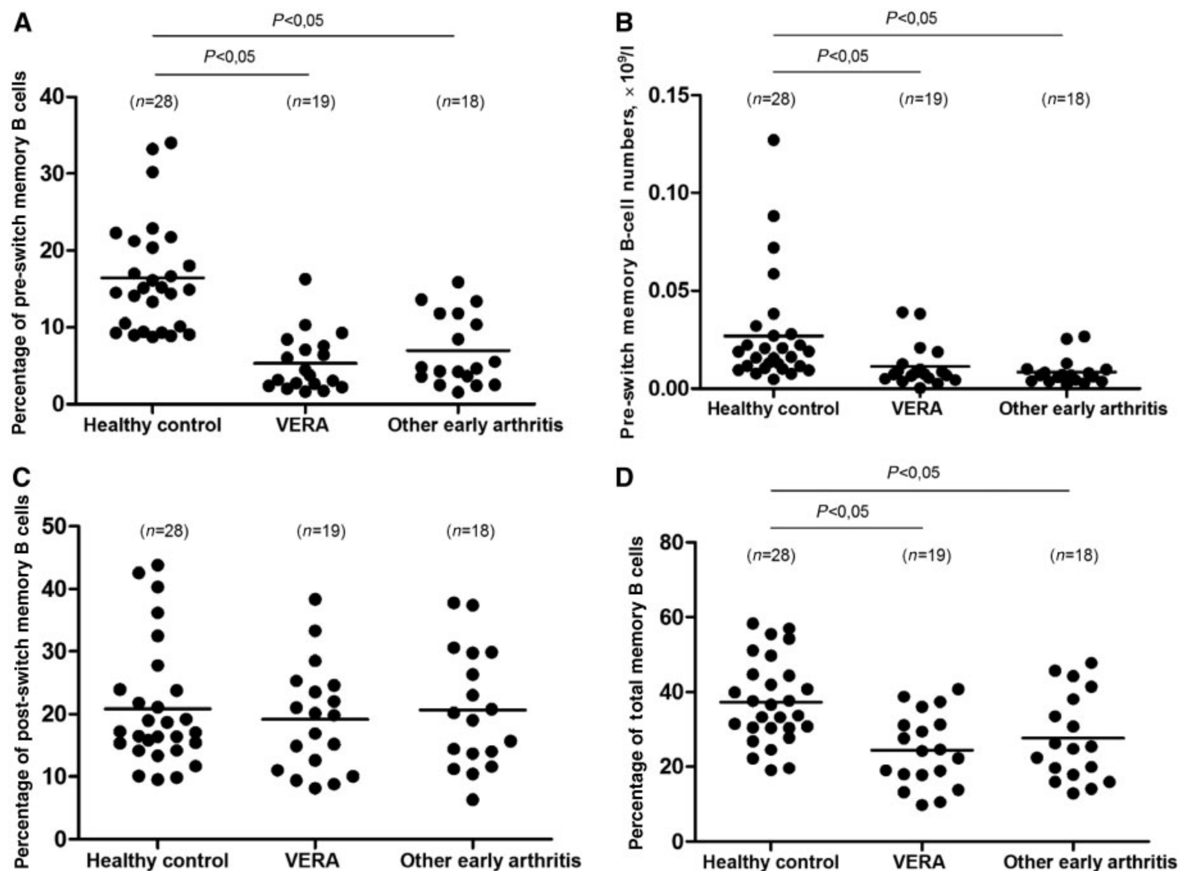
CD27 is now an important marker for analysis of B-cell differentiation in diseases characterized by disturbances in B-cell development. In fact, distinct types of abnormal

**Fig. 4** Early arthritis patients do not have any significant difference in circulating total B cells, naïve B cells or plasma cells when compared with healthy controls. Data from flow cytometry analysis of peripheral blood B cells from other early arthritis patients at baseline. 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 ($CD19^+CD27^{high}$) (**C**) were gated in $CD19^+$ B cells. Differences are considered statistically significant for $P < 0.05$.




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

FIG. 5 Early arthritis patients have reduced frequencies and absolute numbers of pre-switch memory B cells. Data from flow cytometry analysis of memory B cells from other early arthritis patients at baseline. All subpopulations were gated in $CD19^+$ B cells. Representation of pre-switch memory B cells ($CD19^+IgD^+CD27^+$) frequencies (**A**) and absolute numbers (**B**). Post-switch memory B cells ($CD19^+IgD^-CD27^+$) (**C**) and total memory B cells ($CD19^+CD27^+$) (**D**) frequencies are represented. Differences are considered statistically significant for $P < 0.05$.




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 fulfil 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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RHEUMATOLOGY

Rheumatology 2011;50:278–282
doi:10.1093/rheumatology/keq338
Advance Access publication 2 November 2010

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.

BASIC SCIENCE

## 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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Submitted 18 June 2010; revised version accepted 3 September 2010.

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process [5]. 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. Of note, SF samples were only collected from established RA, since most VERA patients did not have joint effusions in easily accessible joints (Table 1). The HAQ [8] and the DAS-28 were applied to all patients. The study was approved by the local ethics committee (Comissão de Ética do Hospital de Santa Maria, Lisbon, Portugal) and all patients gave informed consent. Patient care was conducted in accordance with the standard clinical practice in the Rheumatology Department, Hospital de Santa Maria, Lisbon, Portugal and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

### 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 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 and samples were processed using a ChemWell 2910 automated analyser. 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

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

| | Controls ($n = 24$) | VERA ($n = 19$) | | | VEA ($n = 19$) | RA ($n = 12$) | RA SF ($n = 15$) |
|---|---|---|---|---|---|---|---|
| | | Baseline | Time 1 | Time 2 | | | |
| Age, years | 40 (13) | | 50 (17) | | 40 (13) | 63 (10) | 57 (10) |
| Sex (females/males) | 17/7 | | 16/3 | | 15/4 | 11/1 | 11/4 |
| Disease duration, years | NA | | <6 weeks | | <6 weeks | 8 (9) | 9 (12) |
| DAS-28 | NA | 6.1 (1.8) | 4.1 (1.6)* | 3.1 (1.6)* | 4.5 (1.6)* | Baseline: 4.3 (0.8)[a] Last observation: 5.2 (1.0) | Baseline: 4.7 (0.7)[a] Last observation: 4.6 (1.4) |
| HAQ | NA | 1.4 (0.8) | 0.8 (0.7)* | 0.8 (0.7) | 0.8 (0.6)* | 1.5 (1.0) | 1.4 (0.8) |
| RF positive, % | ND | 42 | ND | ND | 0 | 67 | ND |
| Anti-CCP positive, % | ND | 32 | ND | ND | 0 | 45 | ND |
| IL-21, pg/ml | 261.2 (272.6) | 1122.0 (3170.0) | 1617.0 (3415.0) | 1226.0 (1158.0) | 464.7 (1178.0) | 246.5 (478.7) | 548.3 (454.7) |
| APRIL, ng/ml | 6.0 (11.3) | 18.8 (19.7) | 12.5 (8.7) | 23.2 (29.9) | 5.9 (5.2) | 13.1 (27.6) | 26.2 (25.8) |
| BAFF, ng/ml | 0.3 (0.6) | 0.8 (0.6) | 0.5 (0.2) | 0.8 (0.3) | 0.3 (0.4) | 0.2 (0.1) | 0.9 (0.6) |

All values indicated in table represent mean (S.D.). [a]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).

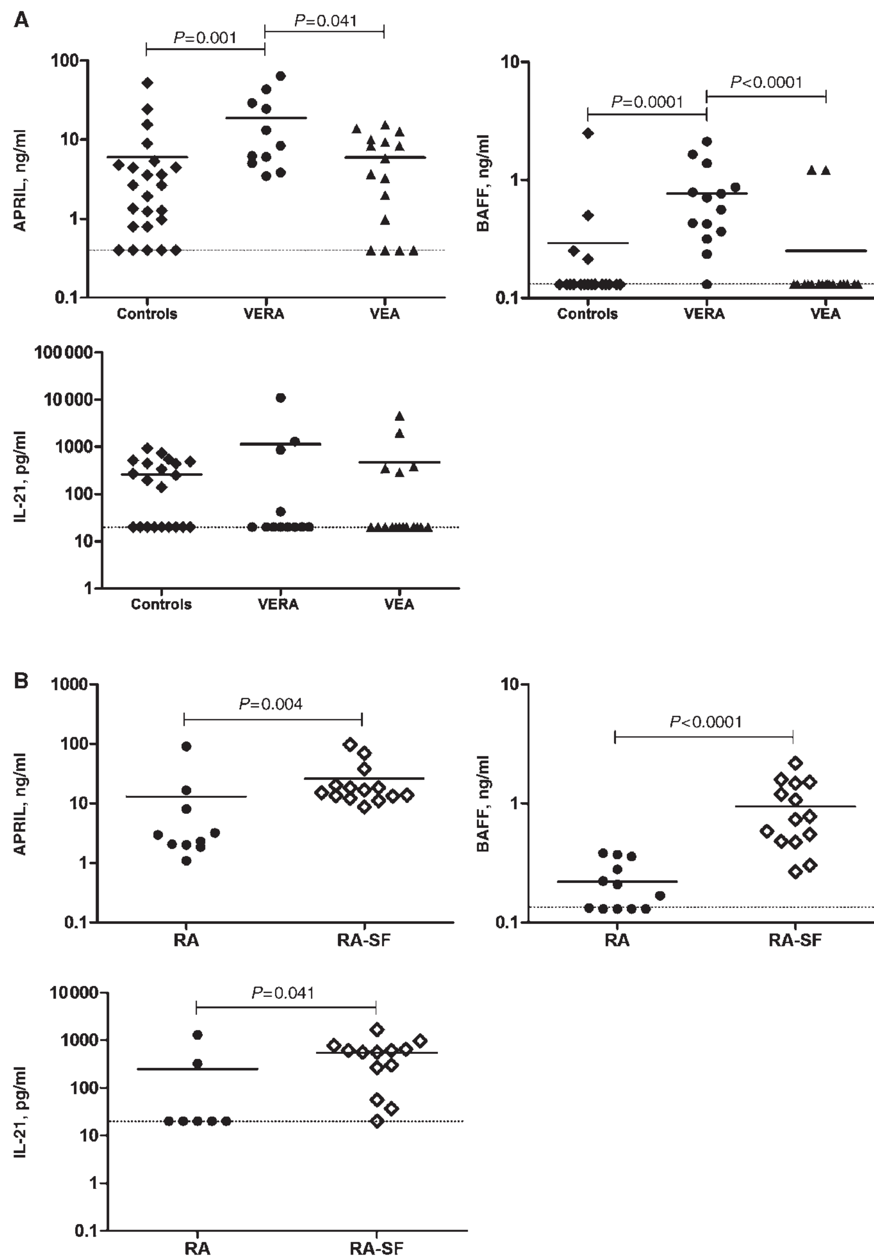
### Established RA-SF has increased levels of APRIL, BAFF and IL-21

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.

**Fig. 1** VERA patients have higher APRIL and BAFF levels than controls and those with VEA at baseline (**A**). Serum samples from VEA patients with no therapy and healthy controls were analysed by ELISA technique. Differences were considered to be statistically significant at $P < 0.05$. Established RA patients have increased levels of APRIL, BAFF and IL-21 locally in the joints (**B**). Serum and SF samples from established RA patients were analysed by ELISA technique. Dotted lines represent the limit of detection for the assay. Differences were considered to be statistically significant at $P < 0.05$.




## 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

The authors would like to acknowledge Bruno Vidal for technical assistance.

*Funding*: This work was supported by a grant from Sociedade Portuguesa de Reumatologia/Schering-Plough 2005. R.A.M. and R.C. were funded by Fundação para a Ciência e a Tecnologia (FCT) SFRH/BD/30247/2006 and SFRH/BD/40513/2007, respectively. M.M.S.-C. was funded by Marie Curie Intra-European Fellowship PERG-2008-239422 and an EULAR Young Investigator Award.

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

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Cascão et al. Arthritis Research & Therapy 2010, 12:R196
http://arthritis-research.com/content/12/5/R196

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RESEARCH ARTICLE

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# Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis

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**Abstract**

**Introduction:** Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by sustained synovitis. Recently, several studies have proposed neutrophils and Th17 cells as key players in the onset and perpetuation of this disease. The main goal of this work was to determine whether cytokines driving neutrophil and Th17 activation are dysregulated in very early rheumatoid arthritis patients with less than 6 weeks of disease duration and before treatment (VERA).

**Methods:** Cytokines related to neutrophil and Th17 activation were quantified in the serum of VERA and established RA patients and compared with other very early arthritis (VEA) and healthy controls. Synovial fluid (SF) from RA and osteoarthritis (OA) patients was also analyzed.

**Results:** VERA patients had increased serum levels of cytokines promoting Th17 polarization (IL-1β and IL-6), as well as IL-8 and Th17-derived cytokines (IL-17A and IL-22) known to induce neutrophil-mediated inflammation. In established RA this pattern is more evident within the SF. Early treatment with methotrexate or corticosteroids led to clinical improvement but without an impact on the cytokine pattern.

**Conclusions:** VERA patients already display increased levels of cytokines related with Th17 polarization and neutrophil recruitment and activation, a dysregulation also found in SF of established RA. 0 Thus, our data suggest that a cytokine-milieu favoring Th17 and neutrophil activity is an early event in RA pathogenesis.

## Introduction

Rheumatoid arthritis (RA), the most common chronic autoimmune disease, affects approximately 1% of the population worldwide. This disease comprises a syndrome of pain, stiffness, and symmetrical synovitis which leads to joint destruction, functional disability, and substantial comorbidity due to the involvement of multiple organs and systems. The migration of leukocytes toward the synovium is crucial for the establishment of a chronic inflammatory process in RA [1-3]. This multi-regulated mechanism involves interactions with endothelial cells through cell adhesion molecules and complex cytokine and chemokine pathways.

Neutrophils specifically play an important role in the onset and perpetuation of RA, not only as interleukin (IL)-producing cells but also as cells responsible for the release of high amounts of reactive oxygen species and destructive enzymes, such as metalloproteases, contributing to joint erosions [4]. Neutrophils are among the first leukocytes to arrive at sites of inflammation. In fact, these cells are the most abundant in the synovial fluid (SF) of patients with active RA, and previous results from our group showed that the synovial tissue is heavily infiltrated by neutrophils in the first weeks of RA onset [5]. Interestingly, in animal models of arthritis, neutrophil depletion prevented joint inflammation if neutrophil-depleting antibodies were given before the

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induction of arthritis. Moreover, when the depleting antibody was given very early after the induction of arthritis, complete abrogation of the inflammatory symptoms was achieved [6].

T helper 17 (Th17) cells have also been proposed to have a relevant role in the early phase of RA through the production of IL-17 [7,8]. This cytokine promotes the recruitment and survival of neutrophils, induces the secretion of proinflammatory cytokines and the upregulation of RANKL (receptor activator of nuclear factor-kappa B ligand), and stimulates the activity of matrix metalloproteases, leading to cartilage catabolism and bone resorption [9,10]. The recruitment, activation, and effector function of Th17 cells and neutrophils are driven by a network of cytokines and chemokines secreted by multiple cellular sources. In established RA, it has been reported that IL-1β, IL-6, IL-8, IL-17, and tumor necrosis factor are elevated in the serum and this correlates with a higher disease activity [11-13]. Nevertheless, our knowledge of the influence of the cytokine network on RA onset remains limited. The characterization of the cytokine profile at this stage, where the transition from an acute to a chronic inflammatory phase occurs, may lead to the identification of early key players, with potential implications for early treatment strategies.

Thus, the main goal of our work was to determine whether cytokines driving neutrophil and Th17 cell activation and proinflammatory function were already present in very early RA (with less than 6 weeks of disease duration) and how this early cytokine environment differs from established RA. We also evaluated whether the introduction of low-dose corticosteroids and methotrexate (MTX) therapy had any influence on the cytokine profile observed at that early stage of the disease. We found that cytokines related to Th17 polarization and neutrophil recruitment and activation were elevated in early RA and that the conventional therapeutic options, though able to control clinical manifestations of the disease, were ineffective in reversing this underlying proinflammatory drive.

## Materials and methods

### Patients

Blood samples were obtained from 38 consecutive untreated polyarthritis patients with less than 6 weeks of disease duration. Some of these patients (19), after a minimum follow-up of 3 months, fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [14]. These patients were classified as very early rheumatoid arthritis (VERA) patients, and further samples were collected 4 to 6 weeks after starting a low dose of oral corticosteroids (5 to 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) that was required to reduce the disease activity score using 28 joint counts (DAS28) to less than 3.2 [15]. The remaining early arthritis patients (19), who did not develop RA, were classified as very early arthritis (VEA). Baseline blood samples from VERA and VEA patients were compared with 27 healthy donors used as controls. Additionally, 12 blood and 15 SF samples were obtained from patients with established RA. SF samples were also collected from 10 patients with osteoarthritis (OA) (Rheumatology Department, Hospital de Santa Maria, Lisbon, Portugal) (Table 1). Owing to the clinical characteristics of the VEA patients, SF in easily accessible joints was not available in VERA and VEA patients and thus SF was not analyzed in these groups of patients. The health assessment questionnaire (HAQ) [16] and DAS28 were applied to all patients. The study was approved by the local ethics committee, and all patients signed an informed consent form. Patient care was conducted in accordance with standard clinical practice, and the study was performed in accordance with the Declaration of Helsinki as amended in Edinburgh (2000).

### Cytokine quantification

IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), IL-17A, IL-22, IL-23, and interferon-gamma levels were measured in the serum and SF by FlowCytomix assay kit (Bender MedSystems, Vienna, Austria) in accordance with the instructions of the manufacturer. Standard curves for each cytokine were generated by using reference cytokine concentrations supplied by the manufacturer. Samples were acquired with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Raw data of the flow cytometry bead assay were analyzed by FlowCytomix Pro 2.2 software (Bender MedSystems).

### Measurement of autoantibodies

Rheumatoid factor (RF)-IgM was determined in all patients by means of an IMTEC Autoimmune Diagnostics ELISA [enzyme-linked immunosorbent assay] kit (Human GmbH, Wiesbaden, Germany) in accordance with instructions of the manufacturer, and samples were processed using a ChemWell 2910 automated analyzer (GMI, Ramsey, Minnesota, USA). Serum levels of anti-cyclic citrullinated peptide (anti-CCP) were measured by ELIA™ CCP test system (Phadia GmbH, Freiburg, Germany), and samples were analyzed with an ImmunoCAP 100 instrument (Phadia GmbH).

### Statistical analysis

Statistical differences were determined with non-parametric Kruskal-Wallis, Mann-Whitney, and Wilcoxon signed-rank tests and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Correlation analysis was performed with the Spearman test.

**Table 1 Clinical information about healthy controls and patients with VERA, VEA, RA, or OA**

| | Controls (*n* = 24) | VERA (*n* = 19) | | | VEA (*n* = 19) | RA (*n* = 12) | RA SF (*n* = 15) | OA SF (*n* = 10) |
|---|---|---|---|---|---|---|---|---|
| | | Baseline | Time 1 | Time 2 | | | | |
| Age in years, mean ± SD | 40 ± 13 | | 50 ± 17 | | 40 ± 13 | 63 ± 10 | 57 ± 10 | 67 ± 13 |
| Sex, female/male | 17/7 | | 16/3 | | 15/4 | 11/1 | 11/4 | 5/5 |
| DAS28, mean ± SD | NA | 6.1 ± 1.8 | 4.1 ± 1.6[a] | 3.1 ± 1.6[a] | 4.5 ± 1.6[a] | 5.2 ± 1.0 | 4.6 ± 1.4 | NA |
| HAQ, mean ± SD | NA | 1.4 ± 0.8 | 0.8 ± 0.7[a] | 0.8 ± 0.7 | 0.8 ± 0.6[a] | 1.5 ± 1.0 | 1.4 ± 0.8 | NA |
| RF-positive, % | ND | 42 | ND | ND | 0 | 67 | ND | ND |
| Anti-CCP-positive, % | ND | 32 | ND | ND | 0 | 45 | ND | ND |

[a]Disease activity score using 28 joint counts (DAS28) and health assessment questionnaire (HAQ) values were compared between very early rheumatoid arthritis (VERA) and very early arthritis (VEA) patients with reference to VERA baseline values. Differences were considered statistically significant for *P* values of less than 0.05. anti-CCP, anti-cyclic citrullinated peptide; IL, interleukin; MTX, methotrexate; NA, not applicable; ND, not determined; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor; SD, standard deviation; SF, synovial fluid.

Differences were considered statistically significant for *P* values of less than 0.05.

## Results

### Characterization of patients and disease evaluation

A total of 38 polyarthritis patients with less than 6 weeks of disease duration were evaluated. Nineteen patients fulfilled the 1987 ACR criteria for RA after a minimum follow-up of 3 months and were classified as VERA patients. The mean age of the VERA patients was 59 ± 17 years, 84% were female, 42% were RF-positive and 32% anti-CCP-positive, the initial DAS28 was 6.1 ± 1.8, and the initial HAQ was 1.4 ± 0.8. After treatment with low doses of prednisone and MTX, there was a significant reduction of both DAS28 and HAQ values (Table 1). The group of VEA patients included 19 patients, and 14 of them later had one of the following diagnoses: spondylarthritis (5 cases), systemic lupus erythematosus (4 cases), crystal induced arthritis (2 cases), Sjögren syndrome (1 case), paraneoplastic polyarthritis related to multiple myeloma (1 case), and arthritis associated with HIV infection (1 case). Five patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited form of polyarthritis. The mean age of the VEA patients was 40 ± 13 years, 79% were female, all patients were RF-negative and anti-CCP-negative, the initial DAS28 was 4.5 ± 1.6, and the initial HAQ was 0.8 ± 0.6. Both DAS28 and HAQ values were significantly lower than those of VERA patients at baseline (Table 1). These early polyarthritis patients represent a subset of a larger cohort previously described by our group [17].

Furthermore, blood samples were collected from 12 patients with established RA; mean age was 60 ± 10 years, 92% were female, and 67% were RF-positive and 45% anti-CCP-positive (Table 1). Additionally, SF samples were collected from 12 patients with established RA; mean age was 57 ± 10 years, and 73% were female (Table 1). The established RA group of patients had a DAS28 and a HAQ mean scores similar to VERA baseline values.

### IL-8 is increased in VERA patients and locally in the joints of patients with established RA

Given the proposed role of neutrophils in the pathogenesis of RA [18,19], we quantified the major neutrophil chemoattractant, IL-8, in the serum of VERA patients. At baseline, VERA patients had significantly higher levels of IL-8 when compared with both VEA and healthy controls (Figure 1a). After 2 to 4 weeks of low-dose corticosteroids and after 4 months of MTX therapy, there were no significant changes in the levels of circulating IL-8 (data not shown). Interestingly, VERA patients also had significantly higher circulating levels of IL-8 in comparison with serum from established RA (Figure 1a). Neutrophils accumulate locally in the joints of patients with RA [20]. Thus, we quantified the concentration of IL-8 in the SF of patients with RA and compared the concentration with that of SF from patients with OA. We found significantly higher levels of IL-8 in the SF of patients with RA in comparison with OA SF (Figure 1b).

### IL-17 levels are dysregulated in both VERA patients and patients with established RA

Previous studies from our group showed that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21]. Therefore, we analyzed IL-17A levels in these patients since it has already been described that this cytokine is important for the survival of neutrophils [22]. Moreover, IL-17A is a signature cytokine of Th17 cells, a subset proposed to have a key role in RA pathogenesis [9,23]. We found that VERA patients had significantly higher levels of IL-17A when compared with healthy controls, but not with VEA patients (Figure 2a). Furthermore, in our previous work, we found no difference in the frequency and absolute

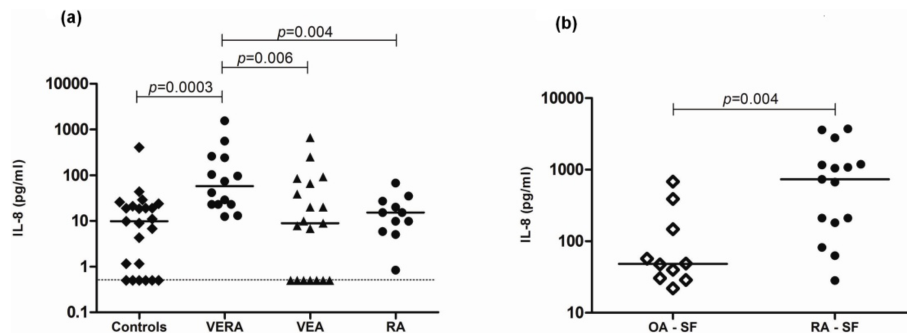


**Figure 1 Interleukin-8 (IL-8) is increased in the serum of very early rheumatoid arthritis (VERA) patients and in synovial fluid (SF) of established rheumatoid arthritis (RA)**. **(a)** The serum concentration of IL-8 was measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. The serum concentration of IL-8 was increased in VERA patients compared with any other group. Dotted line represents the limit of detection for the assay. **(b)** The concentration of IL-8 was measured in the SF collected from patients with established RA and from a control group with osteoarthritis (OA). We found a significant increase of IL-8 in RA-SF. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

numbers of $CD4^+$ and $CD8^+$ T-cell subpopulations in the peripheral blood of these patients when analyzed by flow cytometry [17].

Regarding the effects of early therapy, we found that neither corticosteroids nor MTX affected the level of IL-17A (data not shown). Moreover, IL-17A was significantly increased locally within the joints of patients with established RA in comparison with control SF from patients with OA (Figure 2b).

### RA has a Th17-cytokine pattern since the very first weeks of onset

Having found that IL-17A was elevated in VERA patients, we decided to quantify a panel of cytokines known to be associated with Th17 polarization. At baseline, VERA patients had significantly higher levels of IL-1β and IL-22 in comparison with both VEA and healthy controls. In addition, we found that VERA patients have significantly higher IL-6 levels than healthy controls (Figure 3). Furthermore, the significantly higher circulating levels of IL-6 and IL-22 were maintained in established RA (Figure 3).

Locally, within the joints of patients with RA, the SF displayed elevated levels of IL-1β and IL-6 in comparison with OA SF (Figure 4 and Table 2). Moreover, no significant differences could be observed for IL-23 in circulation or locally in the joints (data not shown). We have also studied cytokines associated with the function of Th2 (IL-4 and IL-10) and Th1 (IL-2, IL-12 (p70), and INFγ) cells. However, no statistically significant differences could be observed for any of these cytokines (data not shown).

## Discussion

Several studies have previously demonstrated that neutrophils play an important role in the onset of RA [21].

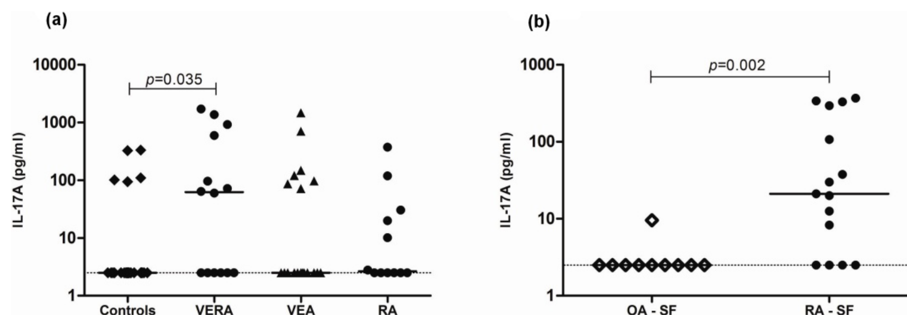


**Figure 2 Very early rheumatoid arthritis (VERA) patients and synovial fluid (SF) of established rheumatoid arthritis (RA) display increased levels of interleukin-17A (IL-17A)**. **(a)** The serum concentration of IL-17A was measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. The serum concentration of IL-17A was increased in VERA patients compared with healthy controls. **(b)** The concentration of IL-17A was measured in the SF collected from patients with established RA and from a control group with osteoarthritis (OA). In the SF of patients with RA, we observed a significant increase of IL-17A. Dotted lines represent the limit of detection for the assay. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

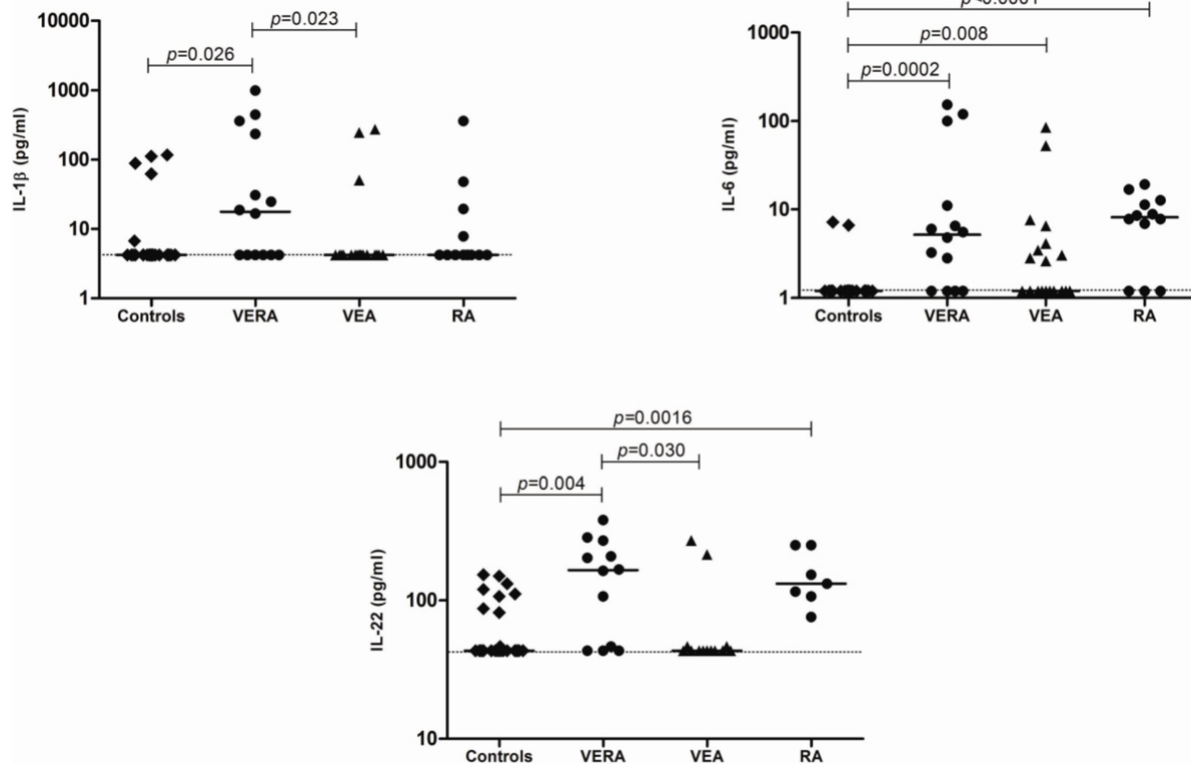


**Figure 3 Cytokines related to T helper 17 (Th17) polarization are increased in the serum of very early rheumatoid arthritis (VERA) patients and synovial fluid of established rheumatoid arthritis (RA)**. The serum concentrations of interleukin (IL)-1β, IL-6, and IL-22 were measured in VERA and very early arthritis (VEA) patients as well as healthy controls and patients with established RA. All three cytokines were increased in VERA patients compared with healthy controls. IL-6 was equally elevated in all groups of patients with an inflammatory disease, whereas the other two cytokines were increased only in VERA (IL-1β) or in VERA and RA patients (IL-22). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

This hypothesis is supported by data from animal models [24]. In fact, neutrophils are the most abundant leukocytes in the SF of patients with active RA, and in early RA, these cells show significantly lower levels of apoptosis when compared with patients with other persistent forms of arthritis or with arthritis that has a self-limited disease course [25]. Additionally, previous results from our group demonstrated that there is a delay in the apoptosis of circulating neutrophils in VERA patients [21] and that these cells heavily infiltrate the synovial tissue during RA onset [5].

In the present study, we demonstrate that a neutrophil- and Th17-driving cytokine pattern is present in untreated VERA patients with less than 6 weeks of

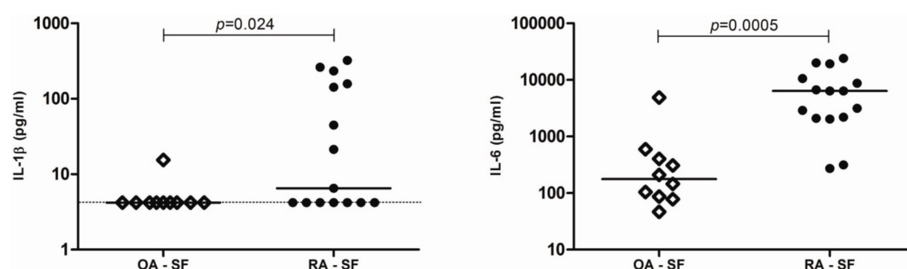


**Figure 4 Cytokines related to T helper 17 (Th17) polarization are increased in the synovial fluid (SF) of established rheumatoid arthritis (RA)**. The concentrations of interleukin (IL)-1β and IL-6 were markedly increased in the SF collected from patients with established RA when compared with osteoarthritis (OA). Dotted lines represent the limit of detection for the assays. Differences were considered statistically significant for *P* values of less than 0.05 according to the Mann-Whitney test.

**Table 2 Cytokine levels in healthy controls and patients with VERA, VEA, established RA, or OA**

| Cytokine, pg/mL | Controls | VERA | VEA | RA | RA SF | OA SF |
|---|---|---|---|---|---|---|
| IL-1β | 4.2 (4.2-116.8) | 17.7 (4.2-99.7) | 4.2 (4.2-272.7) | 4.2 (4.2-360.3) | 6.5 (4.2-322.1) | 4.2 (4.2-15.5) |
| IL-6 | 1.2 (1.2-7.2) | 5.2 (1.2-153.2) | 1.2 (1.2-84.7) | 8.2 (1.2-19.7) | 6361.0 (272.7-24,135.0) | 177.6 (46.6-4,881.0) |
| IL-8 | 9.9 (0.5-407.7) | 57.6 (12.5-1,546.0) | 8.9 (0.5-665.2) | 15.2 (0.8-67.5) | 735.7 (28.3-3,717.0) | 48.2 (22.0-680.0) |
| IL-17A | 2.5 (2.5-333.7) | 62.0 (2.5-1,714.0) | 2.5 (2.5-1,477.0) | 2.6 (2.5-375.6) | 21.1 (2.5-369.1) | 2.5 (2.5-9.5) |
| IL-22 | 43.3 (43.3-153.4) | 165.3 (43.3-380.6) | 43.3 (43.3-270.5) | 131.7 (75.8-250.7) | 153.4 (75.8-336.0) | 151.7 (92.4-235.3) |

Values are presented as median (range). IL, interleukin; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fluid; VEA, very early arthritis; VERA, very early rheumatoid arthritis.

disease duration. We consider this observation of interest because the knowledge concerning the immune mechanisms associated with the onset of RA is still elusive. In fact, the majority of early RA studies include patients with 3 to 12 months of disease duration or even more. In accordance with an early participation of neutrophils in RA, our results revealed that VERA patients have increased levels of IL-8 when compared with both VEA and healthy controls, and this could explain the preactivated state of circulating neutrophils [18] and their recruitment toward the SF from the very first weeks of RA onset.

In addition, Th17 cells are known to be important for the promotion of neutrophil-mediated inflammation by producing IL-17A, a cytokine known to indirectly activate neutrophil chemotaxis and extend their survival [10,22]. We found a high serum concentration of IL-17A in VERA patients as well as locally within the joints of patients with established RA. This might indicate that an activation of Th17 cells from a very early phase of the disease can promote neutrophil participation in RA pathogenesis [22]. However, we found no evidence for changes in the frequency of T-cell subsets in the peripheral blood of VERA patients [17]. This observation is not unexpected; the relatively small representation of antigen-specific T cells in the circulating pool are the activated T cells that drive the pathology more likely found within the tissues [26]. In a study performed by Kokkonen and colleagues [27], the levels of several cytokines and chemokines were analyzed in blood samples from a group of individuals 3.3 years before RA onset ('pre-patients') and compared with healthy donors and RA patients with 7.7 ± 3.6 months of disease duration. An interesting finding was that IL-17 was present at its highest concentration in pre-patients and the level of this cytokine was lower in patients with RA. This is in accordance with our own results; we observed an increased level of IL-17 in RA patients with less than 6 weeks of disease duration, whereas in patients with established RA, the levels were not significantly different from those of healthy controls. Remarkably, the IL-17 median concentration observed in our established RA cohort (2.6 pg/mL) was even lower than that of RA patients from the work of Kokkonen and colleagues [27] (6.0 pg/mL). Thus, this observation reinforces the role of IL-17 in the initial phase of RA, and as the pathogenesis progresses to a chronic stage, other factors are subsequently brought into action in the peripheral blood. Unlike Kokkonen and colleagues, we have not detected differences in Th1- and Th2-related cytokines between both VERA and patients with established RA in comparison with controls. These discrepancies might be related to the different methodologies used.

Additionally, the elevated levels of IL-1β observed in VERA patients can stimulate endothelial cells, T and B cells, and fibroblasts in the joints to produce IL-6 and IL-8. But importantly, IL-1β and IL-6, both found to be increased in VERA patients, are known to promote the differentiation of Th17 cells, which in turn secrete IL-17A and IL-22 [28,29], two cytokines that were elevated in VERA patients and have an essential function in the pathogenesis of autoimmune diseases [29].

Currently, the treatment of choice for RA at the time of presentation is MTX. Interestingly, in spite of clinical improvement (DAS28 reduced from 6.1 ± 1.8 to 3.1 ± 1.6), neither therapy with low-dose corticosteroids nor combined therapy with low-dose corticosteroids and MTX corrected the dysregulated cytokine pattern observed in VERA patients. In fact, low-dose corticosteroids and MTX have unclear effects on the RA cytokine network. For instance, corticosteroids fail to reduce serum levels of IL-1β and IL-8 [30] and MTX does not alter serum IL-1β concentration when compared with pre-treatment levels [31,32]. Our results suggest that the conditions contributing to Th17 cells and neutrophil-mediated inflammation, thus driving early pathogenesis, are not modified with early treatment with low-dose corticosteroids and MTX.

The elevated IL-1β, IL-6, IL-8, and IL-17A levels observed in the SF of patients with RA confirm a local

role for these cytokines in the maintenance of synovitis. Moreover, IL-6 can support a continuous recruitment of autoreactive B cells toward the synovium [33,34], contributing to an exacerbation of the inflammatory process because of the production of autoantibodies and immune complexes.

## Conclusions

Taken together, our data reinforce the potential relevance of therapies targeting IL-1β [35,36] and IL-6 [37,38] in early RA. In addition, the data establish IL-8 and IL-17A as other potential therapeutic targets at an early stage of the disease. Finally, we found that MTX and corticosteroids, though effective in reducing disease activity in VERA patients, do not appear to correct underlying cytokine dysregulation driving the Th17/neutrophil-mediated inflammation.

### Abbreviations

ACR: American College of Rheumatology; anti-CCP: anti-cyclic citrullinated peptide; DAS28: disease activity score using 28 joint counts; HAQ: health assessment questionnaire; IL: interleukin; MTX: methotrexate; OA: osteoarthritis; RA: rheumatoid arthritis; RF: rheumatoid factor; SF: synovial fluid; Th17: T helper 17; VEA: very early arthritis; VERA: very early rheumatoid arthritis.

### Acknowledgements

The authors would like to acknowledge the technical assistance of Bruno Vidal. This work was supported by a grant from Sociedade Portuguesa de Reumatologia/Schering-Plough 2005. RAM and RC were funded by Fundação para a Ciência e a Tecnologia (FCT) SFRH/BD/30247/2006 and SFRH/BD/40513/2007, respectively. MMS-C was funded by Marie Curie Intra-European Fellowship PERG-2008-239422 and a EULAR Young Investigator Award.

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### Authors' contributions

RC and RAM equally performed all of the laboratorial work, data collection, and statistical analysis and wrote the paper. IP contributed to some of the laboratory experiments. HC, ES, AFM, AMR, and JP-P were responsible for the selection, follow-up, and medical care of patients enrolled in this study and helped review the paper. MVQ participated as the head of the Rheumatology Department of Hospital de Santa Maria, which approved the study and patients' management. HSR and MMS-C made a substantial intellectual contribution to the present work and revised it critically. LG and JEF, as senior authors, conceived of the study, participated in its design and coordination, and contributed important intellectual input to the draft of the manuscript. All authors read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

Received: 7 June 2010 Revised: 9 September 2010
Accepted: 20 October 2010 Published: 20 October 2010

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doi:10.1186/ar3168
**Cite this article as:** Cascão *et al.*: Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. *Arthritis Research & Therapy* 2010 **12**:R196.

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# B-cell-activating factor receptor expression on naive and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B-cell depletion therapy

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Accepted 25 May 2010
Published Online First 25 June 2010

## ABSTRACT

**Objectives** To examine the expression of B-cell-activating factor receptor (BAFF-R) on naive CD27− and memory CD27+ B cells in normal individuals and patients with rheumatoid arthritis (RA) undergoing B-cell depletion therapy with rituximab.

**Patients and Methods** BAFF-R expression on B-cell subsets was determined in normal controls (NC; n=11), active patients with RA pre-rituximab (pre-RX; n=15), relapsing patients either concordant for B-cell repopulation (C-R, n=13) or discordant, with relapse more than 3 months after repopulation (D-R, n=11) and patients in remission over 3 months postrepopulation (discordant non-relapsing (D-NR), n=5). Serum BAFF was measured by ELISA and analysed using Mann–Whitney.

**Results** There was no significant difference between NC, pre-RX and D-NR patients in %BAFF-R-positive B cells or mean fluorescence intensity (MFI) in naive and memory B cells. Relapsing patients had significantly lower MFI and %BAFF-R-positive cells in both naive and memory compartments from NC and pre-RX (C-R and D-R; $p<0.01$). BAFF levels in pre-RX patients were within the normal range and did not correlate with BAFF-R expression in any patient group. D-NR patients had relatively lower proportions of pre and postswitch CD27+ B cells than pre-RX patients (D-NR vs pre-RX; $p<0.05$ for both) and also lower numbers of postswitch B cells than D-R patients (D-NR vs D-R, $p<0.05$).

**Conclusion** BAFF-R expression was significantly reduced on both naive and memory B cells in patients at relapse, regardless of the relationship with B-cell repopulation or serum BAFF levels. Re-establishment of active disease was also associated with an increase in class-switch recombination. Factors responsible for lower levels of BAFF-R may relate to altered thresholds for autoreactive B-cell generation at relapse in patients with RA.

B-cell-activating factor (BAFF), also known as B lymphocyte stimulator (BlyS), THANK, TALL-1, zTNF-4 or TNFSF13b, is a protein member of the tumour necrosis factor (TNF) family that plays an important role in B-cell maturation, homeostasis and survival.[1] BAFF binds to three receptors on B cells: TACI, BCMA and BAFF-R (or BR3), with BAFF-R being the only ligand that exclusively binds BAFF.[2]

Studies of BAFF-R-deficient mice demonstrated that BAFF was essential for B-cell survival, primarily through the alternative nuclear factor κ light-chain enhancer of activated B cells pathway,[3] and for B-cell maturation and immunoglobulin (Ig) class switching through the classic nuclear factor κ B pathway.[4] B cells from these animals develop normally up to the IgM+ transitional stage but do not complete their maturation in the spleen.[5] The numbers of memory and marginal zone cells and of serum Ig are severely reduced (>90%) as were responses to both T-dependent and T-independent stimuli.[2]

Human B cells are less dependent on BAFF-R signalling for maturation than murine B cells. In the few reported cases of common variable immunodeficiency related to BAFF-R deficiency in humans, circulating BAFF levels were greatly elevated and peripheral B-cell numbers were reduced (fourfold lower). The condition was of late onset and was not associated with severe infections.[6] Although mature but not transitional B cells were reduced in these individuals, class-switched memory B cells were detectable. Correspondingly, vaccination studies showed that T-dependent responses appeared to be much less dependent on BAFF-R signalling than T-independent responses. As in BAFF-R$^{-/-}$ mice, IgA responses in these patients were not compromised.

One of the suggested roles for BAFF, based largely on animal experiments, is that increased levels will lower thresholds for the survival of autoreactive B-cell clones, and decrease competition within germinal centre environments.[7 8] In humans, elevated levels of serum BAFF have been associated with both the presence and activity of several autoimmune diseases (Sjogrens syndrome, systemic lupus erythematosus (SLE) and idiopathic thrombocytopaenia).[9–15] In patients with rheumatoid arthritis (RA), the relationship is less clear, with conflicting reports as to whether levels of circulating BAFF are significantly elevated and if there is a relationship with disease activity or titres of autoantibodies.[14 16 17]

Following treatment of RA patients with rituximab, serum BAFF levels have been shown to rise, reflecting the loss of the majority of peripheral B cells. The correlation of BAFF levels with peripheral B-cell counts is not, however, perfect and it has been suggested that BAFF levels form a more accurate indicator of total B-cell mass.[18] Chronically raised BAFF levels have been suggested to exert a negative feedback on BAFF-R expression.[19]

One of the key initial findings of the clinical response to rituximab was that B-cell return was not always associated with relapse, and that disease could remain in remission for months or even years following B-cell repopulation.[20] In patients with RA, rises in autoantibodies, particularly rheumatoid factor (RhF), seem to herald relapse following regardless of whether clinical relapse has been concordant or discordant with B-cell return.[21] Serum BAFF levels rose rapidly after rituximab, and then fell with rising peripheral B-cell numbers, although the relationship was not always clear. There was also some evidence for an increased uptake of BAFF if relapse occurred concurrently.[17 21]

The re-establishment of clinical disease following B-cell depletion therapy relies on engaging pro-inflammatory pathways, which are quiescent in the absence of circulating B cells, and therefore provides a unique opportunity to study the re-emergence of the disease process. As a result of its importance in B-cell survival, particularly for new emigrant and naive B cells, we hypothesised that the balance between BAFF-R expression and soluble BAFF may influence the relationship between returning B-cell populations and the resumption of autoimmunity. With this in mind, we measured serum BAFF levels and BAFF-R expression on B-cell populations to determine whether there was any relationship between serum BAFF levels and BAFF-R on naive and memory B cells at different key time points after rituximab therapy.

## MATERIALS AND METHODS

### Patients

Blood samples were obtained from 11 normal controls (NC) and 44 consecutive patients with a diagnosis of RA who fulfilled the American College of Rheumatology criteria for RA[22] The patients were all attending the Department of Rheumatology at University College London Hospital and were treated with rituximab, on the basis of clinical need. Patients received 2-weekly infusions of 1 g rituximab preceded by 100 mg intravenous methylprednisolone. The study was approved by the hospital ethics committee and all patients gave informed consent before entering the study. The normal range for cluster of differentiation 19+ (CD19+) B cells used by the local pathology laboratory was 0.03–0.40×10$^{9}$/l. Levels less than 0.005×10$^{9}$/l were defined as undetectable.

### Detection of B cells

Depletion of B cells in the peripheral blood was deemed to have occurred when CD19+ B cells were undetectable. In all patients, total peripheral blood B-cell depletion was achieved for at least 2 months. B-cell return (B-cell repopulation) was defined as when B cells were again detectable in the peripheral blood (ie, when the CD19+ cell count was ≥0.005×10$^{9}$/l).

### Clinical relapse

Clinical relapse after rituximab was based on any return of symptoms of RA plus a rise in C-reactive protein following an original fall of at least 50% in C-reactive during the previous course of B-cell depletion therapy. Patients at clinical relapse were classified as concordant (relapse ≤3 months after repopulation in peripheral blood) or discordant (relapse >3 months after repopulation in peripheral blood).

### Peripheral blood mononuclear cells isolation and staining

Peripheral blood mononuclear cells (PBMC) were freshly isolated from 10 ml heparinised whole blood following density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and stained on the same day of collection. Cells were washed and cellular viability was estimated with Trypan blue (Sigma, St Louis, USA). PBMC (1×10$^{6}$/sample) were incubated with appropriate conjugated antibodies for 15 min at room temperature in the dark and then washed. Erythrocytes were lysed with BD PharmLyse (BD, Biosciences, San Diego, USA) buffer according to the manufacturer's instructions and cells were then fixed with paraformaldehyde 2% for 5 min at room temperature. PBMC were stored in the dark at 4°C until analysed by flow cytometry. A total of 300 000 cells/sample gated in total lymphocytes were acquired with FACSCaliBur (BD). Data were analysed with FlowJo (TreeStar, Stanford University, California, USA). Absolute cell counts were calculated from the differential leucocyte count determined at each time point for all patients.

### Phenotypic analysis

Immunophenotyping of B and T cells in PBMC samples was performed using matched combinations of antihuman murine monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein cyanin (PerCP-Cy5.5), or allophycocyanin (APC). For B-cell analysis, combinations of anti-CD19 conjugated to PerCP-Cy5.5 or APC (clone HIB19), anti-IgD FITC (clone IA6-2) and anti-CD27 PE (clone O323) were used to define naive (CD19+IgD+CD27−), preswitch memory (CD19+IgD+CD27+), postswitch memory (CD19+IgD−CD27+) and IgD−CD27− B cells.

The expression of BAFF-R was analysed using combinations of anti-CD19 PerCP-Cy5.5, anti-CD27 PE and anti-BAFF-R FITC (11C1; mouse IgG1, clone 8A7) and the mean fluorescence intensity (MFI) of BAFF-R was determined for naive (CD19+CD27−) and memory B cells (CD19+CD27+). All antibodies used were purchased from BD Biosciences and eBioscience (San Diego, USA)

### Measurement of BAFF

BAFF levels were quantified in serum samples from healthy controls and from patients at the various time points, using the Human Quantikine BAFF/BLyS immunoassay ELISA kit. The mean±SD for normal sera (n=36) were given as 1.17±0.28 ng/ml (range 0.67–2.45 ng/ml).

### Statistical analysis

Statistical differences were determined using the non-parametric Mann–Whitney test using GraphPad Prism (GraphPad, San Diego, California, USA). Differences were considered statistically significant for $p<0.05$. Linear regression was used to correlate BAFF levels and BAFF-R expression.

## RESULTS

### Patient cohorts

The mean age was 42 years (range 33–81) and the mean disease duration was 15 years (range 2–20). All patients had erosive disease. Two patients were seronegative for both RhF and anticitrullinated protein antibodies before treatment. The cohorts of consecutive patients with RA included 15 patients with active disease who were studied pre-rituximab (pre-RX) and 29 patients who had already received one or more courses of rituximab. In consecutive patients presenting at clinic, blood for FACS analysis was taken from all relapsing patients. CD19 counts were subsequently examined and patients assigned to concordant relapsing (C-R) or discordant relapsing (D-R) groups. If the patients had already repopulated according to the laboratory notes, but had remained well for more than 3 months after the date of repopulation, they were assigned to the discordant non-relapsing

(D-NR) group. Five groups were therefore described for analyses: (1) NC (n=11); (2) patients with active RA pre-RX (n=15); (3) C-R (n=13); (4) D-R (n=11); (5) D-NR (n=5), taking into account repopulation and relapse after rituximab as described (table 1).

**Serum BAFF levels in NC and in RA patients**

Median BAFF levels in the 16 patients with RA tested before rituximab were within the normal range (table 1). As previously described, BAFF levels rose following rituximab.[22] Levels were found to remain raised at relapse in both patient groups (C-R and D-R; p<0.01, compared with both NC and patients pre-RX) and to a lesser extent in patients who had repopulated but not relapsed (D-NR; p<0.05) when compared with NC only (table 1), but no statistically significant differences in BAFF levels were observed between C-R, D-R and D-NR patients.

**Percentage of naive and memory B cells expressing BAFF-R**

The percentages of CD19+ B cells expressing BAFF-R in CD27+ and CD27− subpopulations in patients and NC are represented in table 1. Significant differences were seen between the percentage expression of BAFF-R on naive and memory B cells in relapsing patients (C-R and D-R groups) compared with NC, and for the memory B-cell population, levels in relapsing patients were also significantly reduced compared with pre-RX values. In the D-R group, decreased expression on naive B cells was also significant compared with pre-RX values. In patients whose B cells had returned but had not yet relapsed (D-NR), no such decreases were found between the percentages of BAFF-R expressing naive or memory populations compared with NC or with the pre-RX group. Furthermore, when the percentages of BAFF-R expressing naive and memory B cells in patients who were relapsing (both C-R and D-R) were compared with D-NR patients, a significantly lower percentage expression was found in both naive and memory populations (naive D-NR vs C-R and D-R; p<0.05 and p<0.01, respectively; memory D-NR vs C-R or D-R, p<0.01 for both). The low expression of BAFF-R was not caused by contamination of the CD19+ gate with plasmablasts (which express low levels of BAFF-R) as they were gated out of the analysis.

**MFI of BAFF-R expression on naïve and memory B cells**

Figure 1 shows representative dot plots and histograms of BAFF-R expression on CD19+ B cells (panel A), PBMC differentiated into naive and memory B cells on the basis of CD27 expression (panel B) and histograms of the MFI of BAFF-R on naive (dashed lines) and memory (dotted lines) B cells (panel C). BAFF-R expression appeared to be decreased in patients who were discordant for relapse after B-cell return, both when remaining well or relapsing, in the examples shown. These individuals also had a tendency for a higher proportion of naive to memory B cells (panels A and B). A left shift in MFI of BAFF-R expression was also observed in all naive and memory populations following rituximab compared with those of NC (panel C). In order to explore these initial observations, the MFI of BAFF-R expression on naive and memory CD19+ B cells pre-RX were compared with those of B cells from NC (figure 2). Naive B cells from both groups of patients at relapse (C-R and D-R) expressed significantly lower levels of BAFF-R when compared with NC (MFI; C-R 58.8 and D-R 53.7 vs NC, 85.5). Memory B cells from both cohorts of relapsing patients also showed significantly lower expression of BAFF-R than NC (MFI; C-R 56.5 and D-R 46.7 vs NC, 78.0). Furthermore, C-R patients showed a tendency to lower MFI levels than pre-RX patients in both naive (58.8 vs 70.4, p=0.06) and memory B-cell populations (56.5 vs 61.2; p=0.07). We also observed a tendency towards lower BAFF-R MFI in D-R patients when compared with pre-RX levels in memory B cells, although again, this did not reach statistical significance (46.7 vs 61.2; p=0.07). Of interest, no significant differences were found in the MFI of BAFF-R expression on either naive or memory B-cell populations in the D-NR patient group when compared with NC or RA patients before treatment.

**Relationship between BAFF levels and BAFF-R MFI in naive and memory B cells**

Our initial observation was that BAFF-R expression was significantly lower on B cells from patients undergoing relapse than on B cells from patients whose B cells had returned but in whom disease was not yet re-established (figure 2). We therefore examined whether there was any relationship between circulating BAFF levels and expression (MFI) of BAFF-R (figure 3). Although no statistically significant correlations were found, we could distinguish different patterns between pre-RX, C-R, D-R and D-NR patients. When patients were relapsing (C-R and D-R), BAFF-R MFI was consistently lower, irrespective of serum BAFF levels. Nevertheless, in patients who had repopulated but remained well (D-NR), there was a tendency for a negative correlation between circulating BAFF and BAFF-R MFI more evident in memory B cells ($r^2$=0.88; p=0.06) (figure 3). However, samples from only four patients were available for analysis in this group.

**B-cell subpopulations in peripheral blood associated with different patterns of response to rituximab**

It has previously been suggested that patients with higher numbers of circulating memory/postswitch B cells, either before treatment induction or at relapse were more likely to have a shorter clinical response to rituximab.[23 24] We showed here that memory B cells from relapsing patients in particular tended to express lower levels of BAFF-R (MFI), and that this was so over a wide range of circulating BAFF levels. We therefore examined whether there was any relationship between subpopulations of B cells in patients with different patterns of clinical response/relapse (figure 4). Circulating B-cell (CD19+) subpopulations were defined according to IgD and

**Table 1** Serum BAFF levels and BAFF-R expression on CD19+ B cells

| | BAFF (ng/ml) median (range) | Naive B cells CD19+ CD27− (%) median (range) | Memory B cells CD19+ CD27+ (%) median (range) |
|---|---|---|---|
| NC (n:11) | 0.88 (0.68–1.03) | 98.4 (95.7–99.8) | 88.3 (77.3–95.8) |
| Pre-RX (n:15) | 1.09 (0.69–1.89) | 97.8 (71.7–99.7) | 87.3 (69.3–97.1) |
| C-R (n:13) | 3.03**# (1.31–7.09) | 90.3** (58.6–98.6) | 54.8****## (23.4–82.1) |
| D-R (n:11) | 2.55**# (1.02–7.46) | 85.4***# (57.7–97.6) | 55.8****### (27.4–77.6) |
| D-NR (n:5) | 2.25* (1.11–4.13) | 98.3 (88–99.7) | 79.3 (64.1–96.9) |

*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 compared with normal controls (NC) (Mann–Whitney).
#p<0.01; ##p<0.001; ###p<0.0001 compared with pre-B-cell depletion therapy.
BAFF, B cell-activating factor; C-R, concordant clinically relapsing; D-NR, discordant clinically non-relapsing; D-R, discordant clinically relapsing; pre-RX, prerituximab.

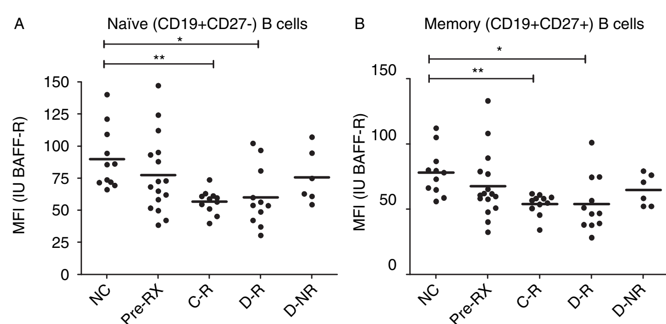


**Figure 1** Peripheral blood mononuclear cells were gated on the CD19+ B-cell population and the mean fluorescence intensity (MFI) of B-cell-activating factor receptor (BAFF-R) expression determined on CD27-naive (A) and CD27+ memory (B) B-cell subpopulations. Normal controls (NC) and patient groups were compared using the Mann–Whitney U test and statistical significance indicated (*p<0.01; **p<0.001). C-R, concordant clinically relapsing; D-NR, discordant clinically non-relapsing; D-R, discordant clinically relapsing; pre-RX, prerituximab.

CD27 expression and classified as naive (IgD+CD27−), preswitch memory (IgD+CD27+), postswitch memory (IgD−CD27+) and double-negative (IgD−CD27−) B cells. The median and range of total numbers of CD19+ B cells in each patient group were for pre-RX 175 (58–472)$\times 10^6$/l, C-R 42 (15–192)$\times 10^6$/l, D-R 61 (13–166)$\times 10^6$/l, D-NR71 (21–328)$\times 10^6$/l. The median percentage and interquartile range of each subpopulation are shown for NC and patient groups in figure 4. The distribution of naive to memory B cells in humans is approximately 60:40. The B-cell subpopulation distribution of B cells from patients with RA pretreatment was broadly similar to NC, except for a slightly increased proportion of double-negative B cells (7.3% vs 3.9%; p=0.04). Following rituximab, repopulation of the periphery begins with naive B cells, which mature at a greater or lesser rate into pre and postswitch CD27+ B cells. Full restoration of memory cell populations may not be achieved for years after treatment. As expected, the relative ratio of naive to memory B cells was increased at repopulation, mirroring ontogeny, but the relative percentage of naive cells was only significantly higher in the non-relapsing patients compared with NC (p<0.05). The preswitch memory B-cell population was significantly decreased in all patients postrituximab (C-R 4.2%, p=0.0004; D-R 4.9%, p=0.002 and D-NR 4.7%, p=0.03) when compared with NC (12.2%). In patients who relapsed at B-cell return, postswitch memory B cells had already reached levels approximating those of NC (C-R 12.4% vs NC 19.6%, p=0.28), but remained reduced in the D-NR group when compared with NC (6.6% vs 19.6% p=0.03), pre-RX (6.6% vs 18.1%, p=0.02) and D-R (6.6% vs 17.9%, p=0.05).

We also used regression analysis to determine whether serum BAFF levels were related to the percentage of particular B-cell subsets. This produced mostly negative results, although higher BAFF levels were statistically associated with numbers of preswitch memory B cells in patients who were relapsing more than 3 months after B-cell repopulation (D-R; p=0.02; Mann–Whitney; data not shown).

### Relationship of BAFF-R expression and B-cell phenotypes with months between repopulation and relapse

It is also possible that differences in levels of BAFF-R and B-cell phenotype distribution in patients discordant for repopulation and relapse (D-R and D-NR cohorts) were related at least partly to differences in the time of sample collection following repopulation. We therefore compared months between repopulation and relapse in the two cohorts and found them not to be significantly different (months between repopulation and sample collection in the D-NR cohort range 4–46, median=13; D-R cohort range 5–24, median=7; Mann–Whitney rank sum p=0.594).

## DISCUSSION

Using the 11C1 monoclonal antibody, which recognises occupied and unoccupied BAFF-R on human cells,[25] we found that BAFF-R levels on CD27+ and CD27− B-cell subpopulations in RA patients before rituximab were similar to those of B cells from NC, although there was a tendency towards lower values especially in memory B-cell populations (MFI; p=0.098; table 1 and figure 2). We also report that BAFF-R expression was significantly reduced on B cells from patients with RA when they were undergoing relapse following rituximab. Low BAFF-R levels were not due to an increase in circulating plasmablasts (data not shown), which have been associated with clinical relapse.[26] Significantly, reduced BAFF-R expression was found in both naive and memory subpopulations and on B cells from patients who relapsed when B cells returned, as well as in those patients who relapsed more than 3 months after B-cell return. In addition, as all patients in the pre-RX group had active RA and were studied before the induction of B-cell depletion therapy for resistant disease the results also imply that reduced BAFF-R expression was not necessarily a consequence of inflammation and active disease, but was more likely related to the re-establishment of disease.

The bioactive trimeric form of soluble BAFF is produced by a variety of cell types, namely myeloid and dendritic cells, neutrophils and some stromal cells.[27] In humans, BAFF signalling through BAFF-R does not appear to be directly involved in B-cell maturation or in most T-cell-independent responses.[6] BAFF-R is expressed on human B cells from the transitional stage onwards, and is then lost from memory B cells as they differentiate into Ig-secreting plasma cells.[28] Stimulation through Toll-like receptor (CpG IL2,15) pathways is more efficient than T-dependent pathways (CD40L,IL2,10) at downregulating BAFF-R expression on memory B-cell populations.[7 28] It was also found that stimulation of naive cells through either pathway had no effect on BAFF-R expression, although positive responses of naive human cells to either stimulus are poor.[28]

Investigations of BAFF-R expression on B cells from patients with rheumatic diseases are limited and to some extent report contrasting findings, and none describe patients with RA or the effect of rituximab. Carter *et al*[25] showed that normal levels of BAFF-R were present on B cells in patients with SLE but were of reduced availability due to occupancy by soluble BAFF. Low levels of BAFF-R expression and a negative correlation between BAFF-R expression and higher disease activity in patients with SLE have been described.[19] In patients with Sjogrens syndrome, BAFF-R expression was decreased on both naive and memory B cells, with the lowest levels found in patients with extraglandular involvement.[19] Analysis of BAFF-R messenger RNA from B cells from these patients suggested that the lower expression was due to a post-translational modification and not to any reduction in BAFF-R mRNA. This could be explained if decreased BAFF-R expression reflected the recent binding of bioactive BAFF to BAFF-R followed by internalisation or shedding.[25] In turn, this could be due to the chronic exposure of B cells to constantly high circulating levels of BAFF, which are often found in these patients. Carter *et al*[25] showed that the receptor/cytokine complex was not internalised. Loss of the receptor following ligation

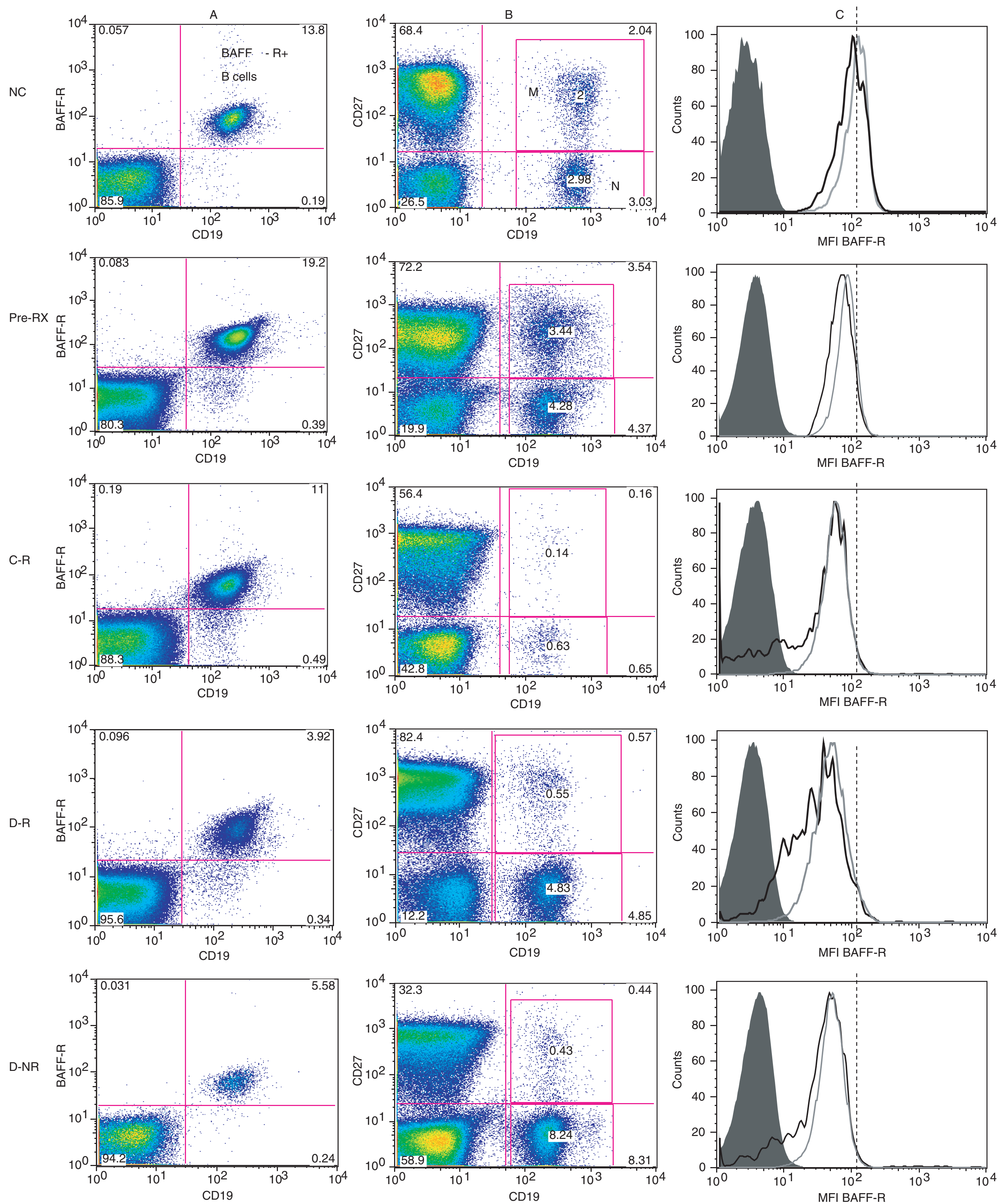


**Figure 2** Characterisation of B-cell-activating factor receptor (BAFF-R) expression and B-cell phenotypes in normal controls (NC) and patients with rheumatoid arthritis before B-cell depletion therapy (pre-RX), relapsing patients concordant (C-R) or discordant (D-R) with B-cell return and patients who had repopulated in the periphery but who remained well (D-NR). The expression of BAFF-R on CD19+ B cells in the lymphocyte gate in peripheral blood mononuclear cells are shown in panel A. In panel B, combinations of CD27 and CD19 were used to differentiate memory (M; CD19+ CD27+) and naive (N; CD19+ CD27−) B cells. Numbers in each quadrant represent percentages of each subpopulation in individual experiments. The histograms in panel C show representative experiments in which the mean fluorescence intensity (MFI) of BAFF-R expression for CD27+ (dotted lines) and CD27− (dotted lines) are shown. Dotted lines depict the MFI of BAFF-R expression on the naive B-cell (CD27−) population from the normal individual to enable comparison with other histograms in each example.

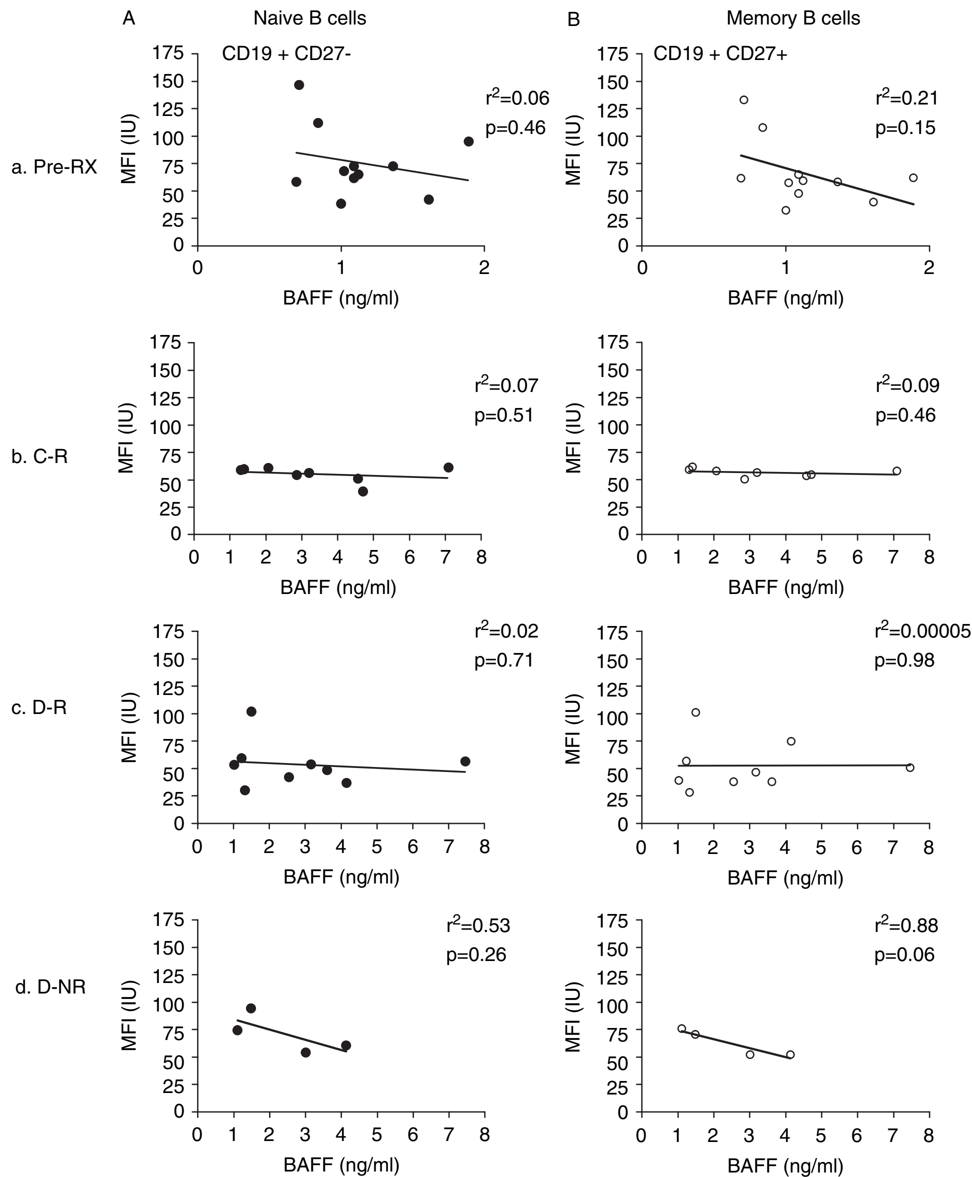


**Figure 3** The values for the mean fluorescence intensity (MFI) of B cell-activating factor receptor (BAFF-R) expression in individual patients were plotted against BAFF levels that were measured in serum stored at the same time point (upper limit of normal range 2.45 ng/ml). Regression analysis was performed and significance values determined for each curve. C-R, concordant clinically relapsing; D-NR, discordant clinically non-relapsing; D-R, discordant clinically relapsing; NC, normal controls; pre-RX, prerituximab.

by BAFF was, however, confirmed by Sellam *et al*,[19] who showed that overnight, but not short-term, culture with physiologically encountered concentrations of BAFF reduced BAFF-R levels on B cells from normal donors.

Our experience here and in earlier studies[17] was that BAFF levels were not significantly raised in patients with active RA. However, following rituximab, BAFF levels rise rapidly and remain raised for variable periods of time. In our serial studies,[17] we found that in individual patients, BAFF levels fell more sharply in patients in whom relapse was associated with B-cell return, than in those patients who relapsed months after B-cell return. This may have reflected increased BAFF binding to BAFF-R or TACI following B-cell receptor (BCR) engagement. We could not confirm these results here as individual patients were not studied serially over the course of their cycle of treatment. In the present study, serum BAFF remained raised to similar levels in sera from patient cohorts discordant or concordant with respect to B-cell return and relapse. Within each group of patients, however, there was no correlation between BAFF levels and BAFF-R expression (figure 3) suggesting that reduced expression was not solely due to chronically raised BAFF levels, although this possibility could not be excluded with certainty.

When levels of expression of BAFF-R on naive and memory populations in pre-RX patients and controls were compared, CD27+ memory B-cell values tended to be lower, although this did not reach statistical significance (p=0.07; data not shown). The main finding of the study was that BAFF-R levels were significantly reduced on both naive and memory B cells from relapsing, compared with non-relapsing, patients and pre-RX patients. This suggested that the reduction in BAFF-R levels may have been initiated in naive cells, which then carried the 'low' BAFF-R phenotype into the mature memory B-cell stage. In-vitro experiments by others have shown that BAFF could modulate BAFF-R expression in a dose-dependent manner.[25] As we found no correlation between raised BAFF levels

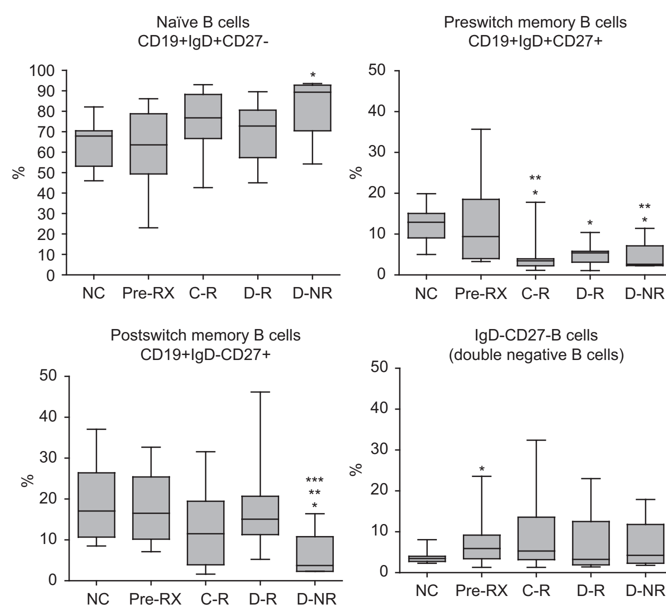


**Figure 4** Peripheral blood mononuclear cells were gated on CD19 to characterise B-cell subpopulations further on the basis of IgD and CD27 expression into naive, pre and postswitch memory and double-negative B cells. Significance levels, using the Mann–Whitney U test, for comparisons between the different groups are depicted as *$p<0.05$ versus normal controls (NC), **$p<0.05$ versus prerituximab (pre-RX), ***$p<0.05$ discordant clinically non-relapsing (D-NR) versus discordant clinically relapsing (D-R). C-R, concordant clinically relapsing.

and reduced BAFF-R expression and low BAFF-R expression on naive populations as well as memory B cells associated with the re-establishment of disease, we suggest that another mechanism may be responsible.

Patients with RA have been shown to possess a number of genetic polymorphisms linked to tolerance checkpoints in the bone marrow or the periphery.[29] Evidence of altered thresholds for BCR signalling[30] has been described in patients with RA and suggested to contribute to the generation of autoreactive cells. There is also accumulating evidence that pre-B/immature B cells from patients with RA are pre-activated before they exit from the bone marrow.[31] It was recently shown that B cells at the pre-B/immature naive stage in the bone marrow of patients with RA had evidence of increased TLR9 protein expression and mRNA compared with osteoarthritis patients and also expressed the activation marker CD86.[32] Disease-induced changes, such as the formation of germinal centre-like structures, cytokines such as TNFα, or increased levels of bacterial DNA/RNA in the bone marrow environment in patients with RA may disrupt the environment in which pre-B cells are developing.[33] Reduced ability to express receptors for the pro-survival cytokine BAFF on transitional and naive B cells after exiting the bone marrow may result as part of the unusual cytokine environment in which the B cells developed.

Once in the circulation, BCR specificity becomes a crucial determinant of B-cell survival and function. The expression of BCR specific for citrullinated proteins and increased numbers of RhF-specific B cells have been described by Samuels *et al*[31] within early (CD10+IgM+) populations exiting the bone marrow and also in mature naive B cells (CD10−IgM+) in RA patients compared with normal individuals. The conclusion is therefore that alterations of tolerance checkpoints both within the bone marrow and in the periphery may be occurring in patients with RA. Within the polyreactive pool exiting the bone marrow, BCR specificities with promiscuous potential, such as those possessed by the relatively high proportion of RhF B cells that can obtain help from T cells with specificity for a wide range of processed peptides,[34] may have a survival advantage that could override the disadvantages of low BAFF-R expression/signalling in a genetically predisposed individual. We have shown that BAFF levels do not appear to be limiting in relapsing patients, but lower levels of BAFF-R expression on newly generated B cells may reduce survival potential and favour 'expanded' cohorts of B cells able more easily to obtain productive, but not necessarily antigen-specific, T-cell help.

As well as being an effective treatment for many patients with RA, rituximab gives us an opportunity to study elements important for relapse through our ability to study the re-establishment of disease. Following clearance of rituximab from the bone marrow, newly generated B cells expand into an environment that has retained a variable number of resistant B cells, a mature T-cell compartment and long-lived plasma cells. First, we know that B-cell return to the periphery is mandatory for relapse.[20] Second, the gap between B-cell return and relapse may reflect the relationship between the re-expansion of surviving memory B-cell clones under the influence of newly generated B cells. In both scenarios, maturation into Ig-secreting cells is often coincident with relapse, as shown by rises in autoantibodies,[17 35] and also by the reported association with circulating plasmablasts.[23] Our finding of a lower proportion of postswitched memory B cells in non-relapsing, repopulated patients compared with relapsing patients supports the view that B-cell class-switch recombination is associated with relapse. The depletion of CD27$^{hi}$ memory B cells has previously been associated with clinical response to rituximab[23 36] and low circulating numbers of IgD−CD27+ MB cells associated with low clinical activity indices and a longer clinical response, as reported by our group and others.[20 37] The parallel finding that BAFF-R levels in the non-relapsing repopulated patients were similar to those of pre-RX patients support our hypothesis that the signals required for BAFF-R downregulation are intimately linked to the re-establishment of disease. We also found that a population of double-negative (IgD−CD27−) B cells was significantly raised in patients with RA compared with controls, but their proportions did not change following rituximab. These cells may be similar to a previously described memory B-cell population in patients with SLE by Wei *et al*,[38] although others have found that only approximately 50% of this double-negative B-cell population bore markers of mutation and class switch.[39]

Although no statistically significant reduction in BAFF-R expression was present in RA patients before treatment, the ranges of percentage expression and MFI were wide (table 1, figure 2). It is possible that a proportion of B cells with low BAFF-R levels is being generated throughout the clinical course of RA, but that the numbers of detectable BAFF-R$^{lo}$ B cells may vary.

B-cell survival and differentiation is the result of a complex interplay between survival factors, in particular the BAFF/APRIL system in concert with the specificity and strength of signal through the BCR.[40] From this study, we have shown that low levels of BAFF-R expression are related to the re-expansion of possibly pathogenic species of B cells associated with relapse. The re-establishment of active disease in patients with RA after rituximab may therefore involve preferential survival properties of autoreactive B cells, due to the unusual properties of their BCR and the ability also to overcome possible consequences of lower levels of BAFF-R signalling.[27 31]

**Funding** The authors wish to thank the Worshipful Company of Grocers of the City of London for their generous support. RAM was supported by a grant from Fundação para a Ciência ea Tecnologia SFRH/BD/30247/2006 and IDLTO by the Alonso Martin Escudero Foundation.

**Competing interests** None.

**Ethics approval** This study was conducted with the approval of the University College Hospitals London Ethics Committee.

**Patient consent** Obtained.

**Contributors** The authors would like to nominate IDLTO as first author, equal with RAM. Both contributed equally to the preparation of the manuscript but IDLTO also collected the clinical data.

**Provenance and peer review** Not commissioned; externally peer reviewed.

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# B-cell-activating factor receptor expression on naive and memory B cells: relationship with relapse in patients with rheumatoid arthritis following B-cell depletion therapy

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*Ann Rheum Dis* 2010 69: 2181-2188 originally published online June 25, 2010
doi: 10.1136/ard.2010.131326

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