

RESEARCH ARTICLE

B-cell phenotype and IgD-CD27- memory B cells are affected by TNF-inhibitors and tocilizumab treatment in rheumatoid arthritis

Rita A. Moura^{1*}, Cláudia Quaresma^{1☯}, Ana R. Vieira^{1☯}, Maria J. Gonçalves^{1,2}, Joaquim Polido-Pereira^{1,2}, Vasco C. Romão^{1,2}, Nádia Martins², Helena Canhão^{1,2}, João E. Fonseca^{1,2}

1 Rheumatology Research Unit, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal, **2** Rheumatology Department, Centro Hospitalar de Lisboa Norte, EPE, Hospital de Santa Maria, Lisbon Academic Medical Centre, Lisbon, Portugal

☯ These authors contributed equally to this work.

* ritaaguarmoura@gmail.com



OPEN ACCESS

Citation: Moura RA, Quaresma C, Vieira AR, Gonçalves MJ, Polido-Pereira J, Romão VC, et al. (2017) B-cell phenotype and IgD-CD27- memory B cells are affected by TNF-inhibitors and tocilizumab treatment in rheumatoid arthritis. PLoS ONE 12(9): e0182927. <https://doi.org/10.1371/journal.pone.0182927>

Editor: Menno C. van Zelm, Monash University, AUSTRALIA

Received: November 20, 2016

Accepted: July 18, 2017

Published: September 8, 2017

Copyright: © 2017 Moura et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The authors confirm that all data are fully available without restriction. All relevant data are within the paper.

Funding: RAM was funded by Fundação para a Ciência e a Tecnologia (FCT, www.fct.pt) (SFRH/BPD/81936/2011), Portugal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

The use of TNF-inhibitors and/or the IL-6 receptor antagonist, tocilizumab, in rheumatoid arthritis (RA) have pleiotropic effects that also involve circulating B-cells. The main goal of this study was to assess the effect of TNF-inhibitors and tocilizumab on B-cell phenotype and gene expression in RA.

Methods

Blood samples were collected from untreated early RA (ERA) patients, established RA patients under methotrexate treatment, established RA patients before and after treatment with TNF-inhibitors and tocilizumab, and healthy donors. B-cell subpopulations were characterized by flow cytometry and B-cell gene expression was analyzed by real-time PCR on isolated B-cells. Serum levels of BAFF, CXCL13 and sCD23 were determined by ELISA.

Results

The frequency of total CD19+ B cells in circulation was similar between controls and all RA groups, irrespective of treatment, but double negative (DN) IgD-CD27- memory B cells were significantly increased in ERA and established RA when compared to controls. Treatment with TNF-inhibitors and tocilizumab restored the frequency of IgD-CD27- B-cells to normal levels, but did not affect other B cell subpopulations. TACI, CD95, CD5, HLA-DR and TLR9 expression on B-cells significantly increased after treatment with either TNF-inhibitors and/ or tocilizumab, but no significant changes were observed in BAFF-R, BCMA, CD69, CD86, CXCR5, CD23, CD38 and IgM expression on B-cells when comparing baseline with post-treatment follow-ups. Alterations in B-cell gene expression of BAFF-R, TACI, TLR9, FcγRIIB, BCL-2, BLIMP-1 and β2M were found in ERA and established RA patients, but no significant differences were observed after TNF-inhibitors and tocilizumab treatment when

Competing interests: The authors have declared that no competing interests exist.

comparing baseline and follow-ups. Serum levels of CXCL13, sCD23 and BAFF were not significantly affected by treatment with TNF-inhibitors and tocilizumab.

Conclusions

In RA patients, the use of TNF-inhibitors and/ or tocilizumab treatment affects B-cell phenotype and IgD-CD27- memory B cells in circulation, but not B-cell gene expression levels.

Introduction

Rheumatoid arthritis (RA) is a systemic immune mediated inflammatory disease characterized by progressive joint damage. The etiology of RA is unknown, but different effector pathways and cells are involved in the cascade of events leading to the progression and persistence of the disease [1]. B cells have a critical role in the development of RA [2]. In fact, recent studies by our group have demonstrated that very early RA patients (with less than 6 weeks of disease duration) have disturbances in circulating memory B cells [3], increased levels of cytokines and B cell gene expression levels relevant for B cell maturation [4–6], which supports an active role of B cells in RA pathogenesis from early disease onset. This is reinforced by the therapeutic efficacy of rituximab, an anti-CD20 monoclonal antibody that specifically depletes B cells [7]. Other biologic treatments such as tumor necrosis factor (TNF) antagonists and the interleukin (IL)-6 receptor blocking antibody, tocilizumab, have also proven to be clinically effective in RA [8, 9] by interfering with specific cytokine dependent mechanisms. In addition, both TNF and IL-6 can have B cell regulatory effects. For instance, IL-6 plays an important role in plasma cell differentiation [10] and TNF promotes B-cell proliferation and immunoglobulin secretion [11]. Furthermore, it is known that IL-6 supports B-cell recruitment towards RA synovium [12] and TNF influences diverse pathologic processes including joint destruction and synovial hyperplasia [13]. Thus, it is plausible that some of the positive effects of TNF-inhibitors and tocilizumab on RA disease activity might be at least partially mediated by interference with B cells. In fact, preliminary reports have suggested that B cell function and humoral immune responses might be modulated by anti-TNF and tocilizumab treatments in RA [14, 15]. Therefore, the main goal of the present study was to analyze B cell phenotype and gene expression directly related with B cell activation and survival in established RA patients before and after treatment with TNF-inhibitors and tocilizumab in order to assess the effects of these agents on B cell homeostasis.

Materials and methods

Patients

Blood samples were collected from 13 consecutive patients with untreated polyarthritis with less than 1 year of disease duration, who fulfilled the 2010 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) criteria for RA and were classified as early RA (ERA). In addition, blood samples were also collected from established RA patients treated with methotrexate (MTX) (n = 20) and established RA patients before and after treatment with TNF inhibitors (Anti-TNF) (n = 10) or tocilizumab (TCZ) (n = 11). Furthermore, blood samples from 22 healthy donors were also collected and processed for comparison (Table 1). Samples were stored and managed at Biobanco-IMM, the biobank facility from Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa. All patients

Table 1. Clinical characterization of patients.

	Controls (n = 22)	ERA (n = 13)	RA (n = 20)	Anti-TNF (n = 10)		TCZ (n = 11)	
				Before	After	Before	After
Age (years)	48±8	58±14	56±13	56±15		59±15	
Sex (% female)	73	85	85	80		91	
Disease duration (years)	NA	≤ 1	8±5	14±13		10±7	
RF (+) %	ND	69	80	78		64	
ACPA (+) %	ND	62	63	38		82	
CRP (mg/dl)	ND	1.5±2.1	0.5±0.6	1.7±1.6	1.9±2.4	1.9±4.7	0.2±0.4#
ESR (mm/1 st hour)	ND	40±25	22±21	48±27	48±37	23±18	6±5*#&
VAS	NA	40±33	42±35	73±16	57±24	73±18	57±22
DAS28	NA	3.8±2.2	2.9±1.6	5.2±1.1**	4.0±1.5	4.9±1.7	2.9±1.2
Swollen joints	NA	3±4	2±4	6±3**	2±3	5±4	2±2
Tender joints	NA	5±5	3±5	11±9**	5±5	9±6	3±3
Concomitant treatment:							
PDN	NA	0/13	0/20	1/10		2/11	
MTX	NA	0/13	9/20	2/10		1/11	
Cyclosporine	NA	0/13	0/20	1/10		0/11	
MTX+PDN	NA	0/13	11/20	2/10		2/11	
SLZ+PDN	NA	0/13	0/20	2/10		1/11	
LFM+PDN	NA	0/13	0/20	1/10		0/11	
MTX+SLZ	NA	0/13	0/20	1/10		0/11	
MTX+LFM	NA	0/13	0/20	0/10		1/11	
SLZ+HCQ+PDN	NA	0/13	0/20	0/10		1/11	
MTX+SLZ+HCQ+PDN	NA	0/13	0/20	0/10		1/11	
No treatment	NA	13/13	0/20	0/10		2/11	

ACPA—Anti-Citrullinated Protein Antibody; CRP—C-reactive protein; DAS28—Disease Activity Score of 28 joints; ERA—Early Rheumatoid Arthritis; ESR—Erythrocyte Sedimentation Rate; HCQ—Hydroxychloroquine; LFM—Leflunomide; MTX—Methotrexate; PDN—Prednisone; RA—Rheumatoid Arthritis; RF—Rheumatoid Factor; SLZ—Sulfasalazine; VAS—Visual Analogue Scale; NA—not applicable; ND—not determined. Values are represented as mean ± standard deviation.

* $p < 0.05$ in comparison with ERA.

** $p < 0.05$ in comparison with RA.

$p < 0.05$ in comparison with Anti-TNF before.

& $p < 0.05$ in comparison with Anti-TNF after.

<https://doi.org/10.1371/journal.pone.0182927.t001>

included in this study were attending the Rheumatology Department, Hospital de Santa Maria, Lisbon Academic Medical Centre, Portugal. The local ethics committee (Comissão de Ética do Hospital de Santa Maria, Lisbon, Portugal) approved this study and all patients and healthy donors 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 Fortaleza, Brazil (2013).

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from 50 ml heparinized whole blood following density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare, Sweden). Cells were washed twice in 1X phosphate buffered saline (PBS) and cellular viability was estimated with 0.4% Trypan blue (Sigma, USA).

Flow cytometry

To analyze the frequency of B cell subpopulations in the periphery, B cells were classified using the IgD/CD27 classification system that allows the identification of four main B cell subsets (gated in CD19): naïve B cells (IgD+CD27-), pre-switch-memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double-negative (DN, IgD-CD27-) B cells. A second classification system based on IgD/CD38 (gated in CD19) was also used to identify circulating transitional (IgD+CD38++) B cells and plasmablasts (IgD-CD38++). To characterize B cell phenotype, the expression of several cellular markers was analyzed, which included: BAFF-R, TACI and BCMA, the three BAFF receptors on B cells; CD69, CD86 and HLA-DR, activation markers; CXCR5, important for B cell chemotaxis; CD95, also known as Fas receptor (FasR), to analyze Fas-mediated apoptosis; IgM, a component of the B cell receptor (BCR); CD5, a marker of B cell differentiation; and toll-like receptor (TLR)-9, the main TLR expressed by B cells. Immunophenotyping of B cells was performed in PBMC samples (1×10^6 cells/ sample) using matched combinations of anti-human murine monoclonal antibodies (mAbs) conjugated to FITC, phycoerythrin (PE), peridinin chlorophyll protein (PerCP)-Cy5.5, allophycocyanin (APC), PE-Cy7, eFlour 450 and APC-eFlour780. Combinations of anti-CD19 conjugated to PerCP-Cy5.5 or APC, anti-IgD conjugated to PE-Cy7 or FITC, anti-CD27 conjugated to eFlour450 or FITC, anti-CD38 conjugated to APC-eFlour780, anti-BAFF-R conjugated to PE, anti-TACI conjugated to APC, anti-CD86 conjugated to PE, anti-CD69 conjugated to PerCP or APC, anti-IgM conjugated to PE, anti-CD5 conjugated to APC, anti-CXCR5 conjugated to PE, anti-HLA-DR conjugated to APC, anti-CD95 conjugated to APC, anti-BCMA conjugated to PE and anti-TLR9 conjugated to APC were used. All antibodies were purchased from BD Pharmingen (USA), eBioscience (USA) and R&D Systems (United Kingdom). For cell surface stainings, PBMC were incubated with antibodies during 30 minutes, in the dark, at 4°C. For TLR9 intracellular staining, PBMC were fixed during 20 minutes at room temperature with IC Fixation Buffer (eBioscience, USA), permeabilized with 1X Permeabilization Buffer (eBioscience, USA) and stained according to eBioscience intracellular antigen staining protocol. A total of 50.000 cells/ sample gated in CD19+ B cells were acquired with LSR Fortessa (BD). Data were analyzed with FlowJo (TreeStar, Stanford University, California, USA). All samples were acquired on the same day of the staining protocol.

B cell separation

B cells were isolated by positive MACS Separation using CD19 Microbeads and LS Columns (Miltenyi Biotec GmbH, Germany), according to the manufacturer's instructions, using ice cold buffers and reagents to avoid cellular activation. After isolation, B cells were immediately stored at -80°C until further. Purity of isolated B cells was analyzed by flow cytometry using fluorochrome-conjugated CD20 FITC (BD Biosciences, USA) and CD3 APC (eBioscience, USA) antibodies. A total of 20.000 cells/ sample were acquired with LSR Fortessa (BD Biosciences, USA).

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from B cells 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). Total RNA was reverse-transcribed into cDNA using DyNAmo™ cDNA Synthesis Kit for qRT-PCR (Finnzymes, Finland) with Moloney murine leukemia virus (M-MuLV) reverse transcriptase, random hexamers (300 ng/μl) and 2X RT Buffer, according to the

manufacturer’s instructions, performed on Piko Thermal Cycler (Finnzymes, Finland). The cDNA samples were stored at -20°C.

Real-time quantitative polymerase chain reaction

The expression of a group of genes directly related with B cell activation through either BAFF (BAFF-R, TACI, BCMA) or TLRs (TLR7, TLR9, TLR10), chemotaxis (CXCR5), B cell inhibition (FcγRIIB or CD32), apoptosis (BCL-2), class-switch recombination (AID), plasma cell differentiation (BLIMP-1) and cellular activation (β2M) was assessed by real-time quantitative polymerase chain reaction (qPCR) performed on Rotor-Gene 6000 (Corbett Life Science, USA) using SensiMix SYBR No-ROX Kit (Bioline, United Kingdom). The qPCR program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. Genes and primer sequences analyzed in this study are indicated in Table 2. Primers were designed using the National Center for Biotechnology Information (NCBI)/ Primer-BLAST. The 18S ribosomal RNA (18S rRNA) was used as endogenous control in relative quantification using the standard curve method. All data were analyzed with Rotor-Gene 6000 Series Software.

Enzyme-linked immunosorbent assay (ELISA)

B cell activating factor (BAFF) (Bender MedSystems, Austria), B-lymphocyte chemoattractant (BLC) also known as C-X-C motif chemokine 13 (CXCL13) and the soluble form of CD23 (sCD23) (R&D systems, United Kingdom) were quantified in serum samples from all groups

Table 2. Genes and sequences of primers used in quantitative real time PCR.

Gene	Name	Primer sequences
18S rRNA	18S ribosomal RNA	Fw: 5' -GGAGTATGGTTGCAAAGCTGA-3' Rv: 5' -ATCTGTCAATCCTGTCCGTGT-3'
BAFF-R	BAFF receptor	Fw: 5' -CTGGTCTGGTGGGTCTG-3' Rv: 5' -ACCTTGTCCAGGGGCTCT-3'
TACI	Transmembrane activator and calcium modulator cyclophilin ligand interactor	Fw: 5' -AGGCTCAGAAGCAAGTCCAG-3' Rv: 5' -CCAGGAAGCAGCAGAGGA-3'
BCMA	B cell maturation antigen	Fw: 5' -AGGACGAGTTTAAAAACACAGGA-3' Rv: 5' -TCACAGGTGCATTCTCCAC-3'
CXCR5	C-X-C chemokine receptor 5	Fw: 5' -GGAGCCTCTCAACATAAGACAGT-3' Rv: 5' -ATTTTCCACCAGGGAGGTGTC-3'
AID	Activation-induced cytidine deaminase	Fw: 5' -GGACTTTGGTTATCTTCGCAAT-3' Rv: 5' -GTCGGGCACAGTCGTAGC-3'
BLIMP-1	B lymphocyte-induced maturation protein	Fw: 5' -ACGTGTGGGTACGACCTTG-3' Rv: 5' -CTGCCAATCCCTGAAACCT-3'
β2M	Beta 2-microglobulin	Fw: 5' -CTATCCAGCGTACGCCAAAGATTC-3' Rv: 5' -CTTGCTGAAAGACAAGTCTGAATG-3'
BCL-2	B-cell lymphoma 2	Fw: 5' -TTGACAGAGGATCATGCTGTACTT-3' Rv: 5' -ATCTTTATTTTCATGAGGCAGTT-3'
FcγRIIB (CD32)	Fc-gamma receptor IIB	Fw: 5' -GTGCTATTCTGGCTCCTGTT-3' Rv: 5' -CGTGTGGGTGGGAATGAGAT-3'
TLR7	Toll-like receptor 7	Fw: 5' -CACCTTCATGCTCTGCTCTC-3' Rv: 5' -TCTAGCCCCAAGGAGTTTGGA-3'
TLR9	Toll-like receptor 9	Fw: 5' -GGACCTCGAGTGTGAAGCAT-3' Rv: 5' -TGGAGCTCACAGGGTAGGAA-3'
TLR10	Toll-like receptor 10	Fw: 5' -TCTGCTGAGAGAGTGAAGC-3' Rv: 5' -ACATGTTGGAGCAGTTGGTCA-3'

<https://doi.org/10.1371/journal.pone.0182927.t002>

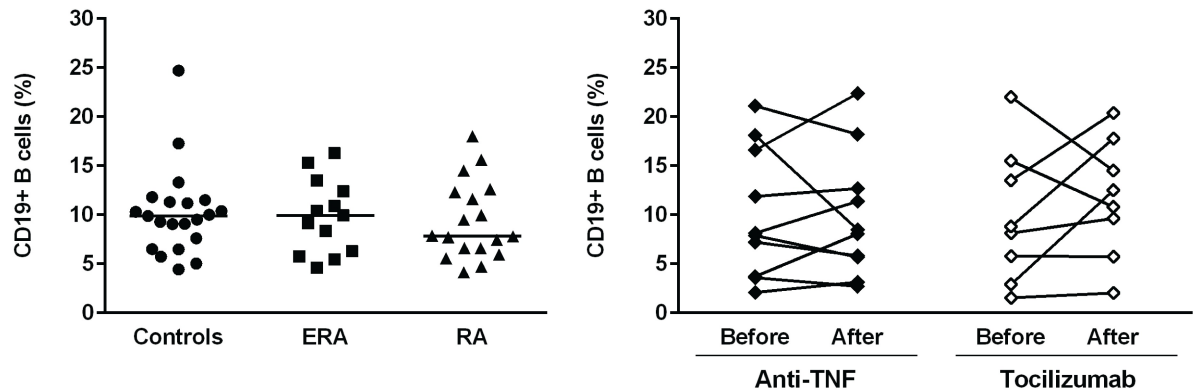


Fig 1. Peripheral blood CD19+ B cell levels are not affected by treatment with TNF-inhibitors and tocilizumab in rheumatoid arthritis. The frequency of total CD19+ B cells was determined by flow cytometry in early RA (ERA) and established RA patients under methotrexate treatment. In addition, the effect of TNF-inhibitors and tocilizumab treatment on circulating B cells was also assessed in established RA patients at baseline and after an average of 8 months of treatment. A group of healthy individuals was also included as controls. Lines represent median values. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g001>

by ELISA according to the manufacturer’s instructions. Samples were analyzed using plate reader Infinite M200 (Tecan, Switzerland).

Statistical analysis

Statistical differences were determined with GraphPad Prism (GraphPad, San Diego, USA). For populations that did not follow a Gaussian distribution, non-parametric tests were used. The Mann-Whitney test was used for comparisons between 2 independent groups. For comparisons between 3 or more groups, the Kruskal-Wallis and Dunn’s multiple comparison tests were used. The Wilcoxon matched pairs test was used for comparisons between 2 paired groups. Correlation analyses were performed using Spearman’s test. Differences were considered statistically significant for $p < 0.05$.

Results

Clinical characterization of patients

A group of untreated polyarthritis patients ($n = 13$), with less than 1 year of disease duration, fulfilling the 2010 ACR/ EULAR criteria for RA, was included in this study and was classified as early RA (ERA). ERA patients had a disease activity score of 28 joints (DAS28) of 3.8 ± 2.2 . A group of established RA patients, under MTX treatment ($n = 20$), with a DAS28 of 2.9 ± 1.6 , was also included. Moreover, established RA patients that had either initiated treatment with TNF-inhibitors ($n = 10$) or tocilizumab ($n = 11$) were evaluated at baseline and after an average follow-up of 8 months of treatment (minimum 3 months). At baseline, RA patients treated with TNF-inhibitors had a DAS28 of 5.2 ± 1.1 and, at post-treatment, 4.0 ± 1.5 . Fifty percent of the patients had initiated etanercept, 40% golimumab and 10% adalimumab. RA patients treated with tocilizumab had at baseline a DAS28 of 4.9 ± 1.7 and, at post-treatment, 2.9 ± 1.2 . All patients were concomitantly being treated with synthetic disease modifying anti-rheumatic drugs (DMARDs). Most patients were rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) seropositive in all studied groups. In addition, a group of age and sex-matched healthy donors ($n = 22$) was included. The clinical information from all patients and data from healthy controls included in this study are indicated in [Table 1](#).

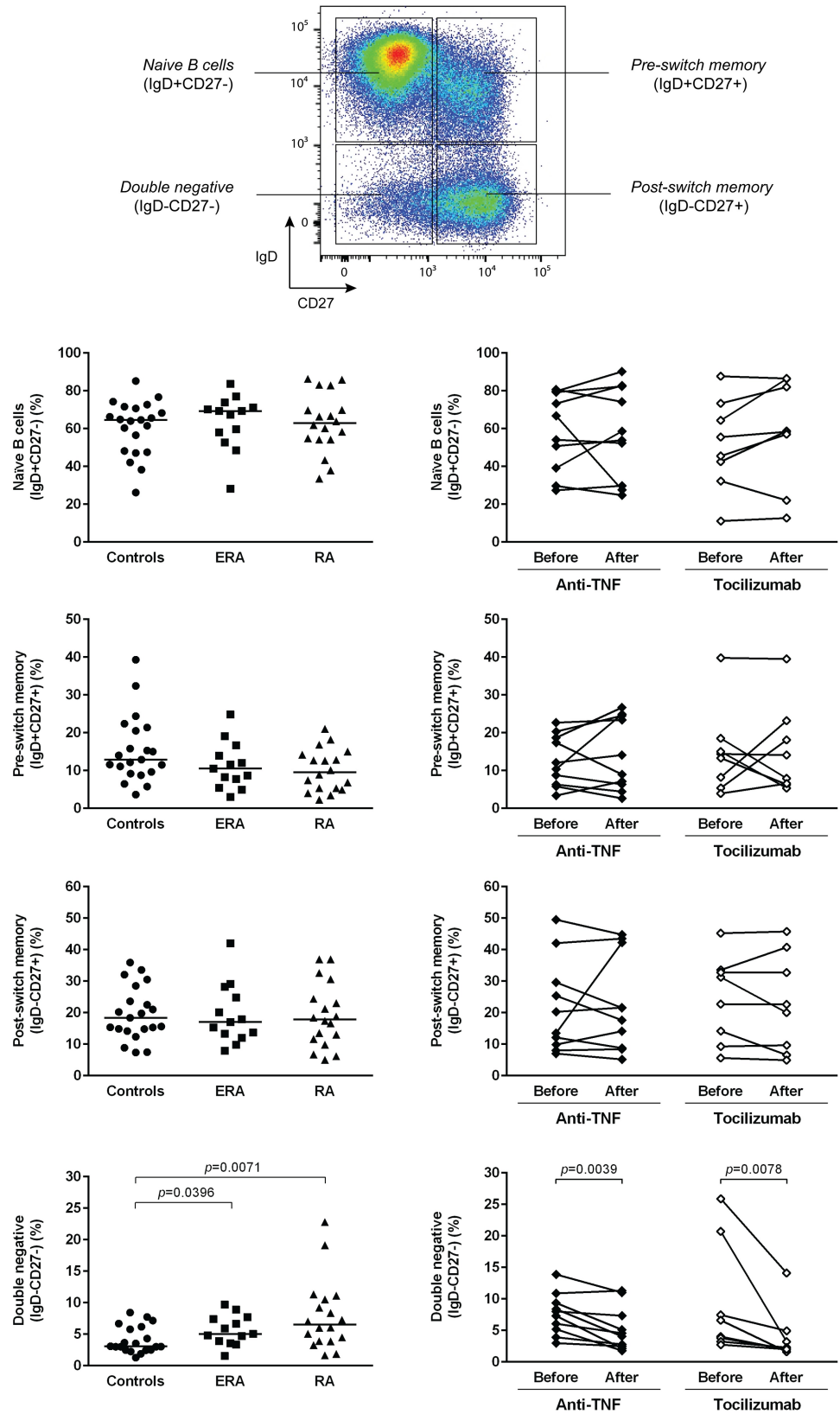


Fig 2. In rheumatoid arthritis, treatment with TNF-inhibitors and tocilizumab restores the frequency of IgD-CD27- memory B cells to normal levels. The frequency of B cell subpopulations based on IgD/ CD27 classification was determined by flow cytometry in early RA (ERA) and established RA patients under methotrexate treatment. In addition, the effect of TNF-inhibitors and tocilizumab treatment on circulating B cell subsets was also assessed in established RA patients at baseline and after an average of 8 months of treatment. A group of healthy individuals was also included as controls. Lines represent median values. Gating strategy for B cell subpopulations (defined in CD19+ B cells) based on IgD/ CD27 classification is shown. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g002>

Early and established RA patients have alterations in memory B cell subpopulations in peripheral blood when compared to healthy individuals

The analysis of the frequency of total CD19+ B cells has revealed no statistically significant differences between ERA, established RA patients and healthy controls. Furthermore, no effect of TNF-inhibitors and tocilizumab treatment was observed when comparing baseline and follow-ups (Fig 1). ERA and established RA patients had similar circulating levels of transitional (IgD+CD38++), naïve (IgD+CD27-), pre-switch memory B cells (IgD+CD27+), post-switch memory B cells (IgD-CD27+) and plasmablasts (IgD-CD38+++ in comparison with controls and no significant differences were observed between the two patient groups (Figs 2 and 3). In addition, both ERA and established RA patients had significantly higher frequencies of DN (IgD-CD27-) B cells when compared to controls (Fig 2). Treatment with TNF-inhibitors and tocilizumab did not significantly affect B cell subpopulations in circulation, except DN (IgD-CD27-) B cells, whose frequencies were significantly lower when comparing baseline and follow-ups (Figs 2 and 3). In fact, both TNF-inhibitors and tocilizumab treatment restored the frequency of DN (IgD-CD27-) B cells to normal values when compared to healthy controls [median (range) of DN (IgD-CD27-) B cells 4.3 (1.8–11.3) % after TNF-inhibitors; 2.2 (1.6–14.1) % after tocilizumab; 3.1 (1.3–8.4) % controls, respectively] (Fig 2). The majority of DN (IgD-CD27-) B cells was class-switched, since an average of 5% of these cells were IgM positive in all groups analyzed (S1 Fig). Furthermore, IgM expression by IgD-CD27- B cells was not significantly affected by treatment with TNF-inhibitors, but decreased after tocilizumab (S1 Fig). Moreover, no significant correlations were found between the frequencies of all B cell subpopulations with age, disease activity score (DAS28), or with clinical parameters, namely erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), swollen and tender joint counts, or disease duration in all studied groups (data not shown).

Treatment with TNF-inhibitors and tocilizumab affects the expression of B cell markers in RA patients

The expression of several cellular markers (frequency, % and median fluorescence intensity, MFI) was analyzed to characterize B cell phenotype in circulation (Figs 4–8). It was observed that BAFF-R, TACI and BCMA expression were similar between all studied groups (Fig 4). Treatment with TNF inhibitors and tocilizumab did not significantly affect the expression of these markers when comparing baseline and follow-ups, except TACI, whose MFI values significantly increased after anti-TNF therapy (Fig 4). The expression of TLR9 was similar between ERA patients and healthy donors, but it was significantly increased in established RA when compared to controls (Fig 4). Furthermore, TLR9 MFI values significantly increased in RA patients after tocilizumab, but not after treatment with TNF-inhibitors (Fig 4). ERA and established RA patients had significantly lower levels of CD5+ B cells in circulation when compared to controls, although no significant differences were detected in CD5 MFI values (Fig 5).

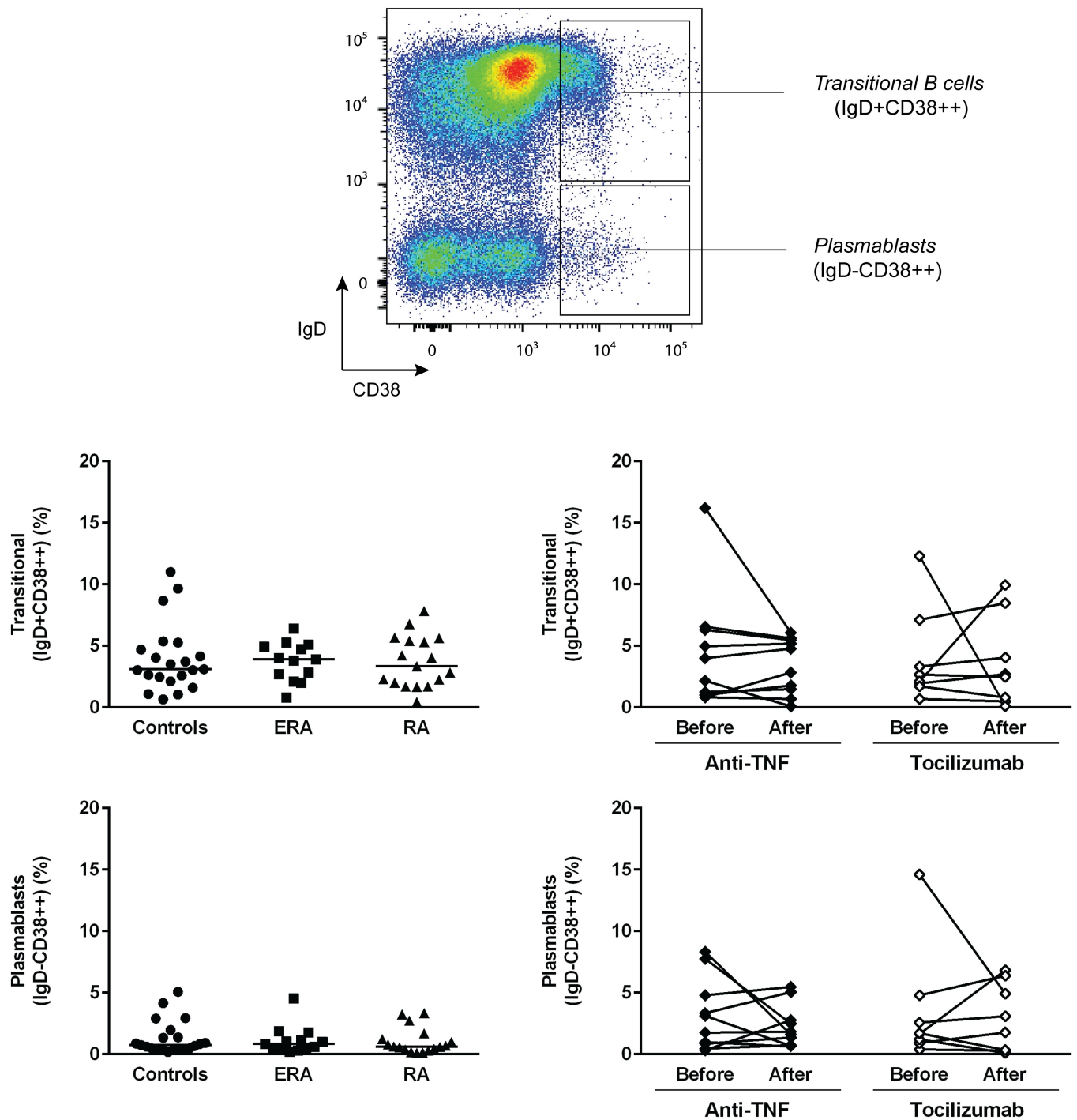


Fig 3. Transitional B cells and plasmablasts in circulation are not affected by treatment with TNF-inhibitors and tocilizumab in rheumatoid arthritis. The frequency of peripheral blood transitional B cells and plasmablasts based on IgD/ CD38 classification was determined by flow cytometry in early RA (ERA) and established RA patients under methotrexate treatment. In addition, the effect of TNF-inhibitors and tocilizumab treatment on these circulating B cell subsets was also assessed in established RA patients at baseline and after an average of 8 months of treatment. A group of healthy individuals was also included as controls. Lines represent median values. Gating strategy for B cell subpopulations (defined in CD19+ B cells) based on IgD/ CD38 classification is shown. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g003>

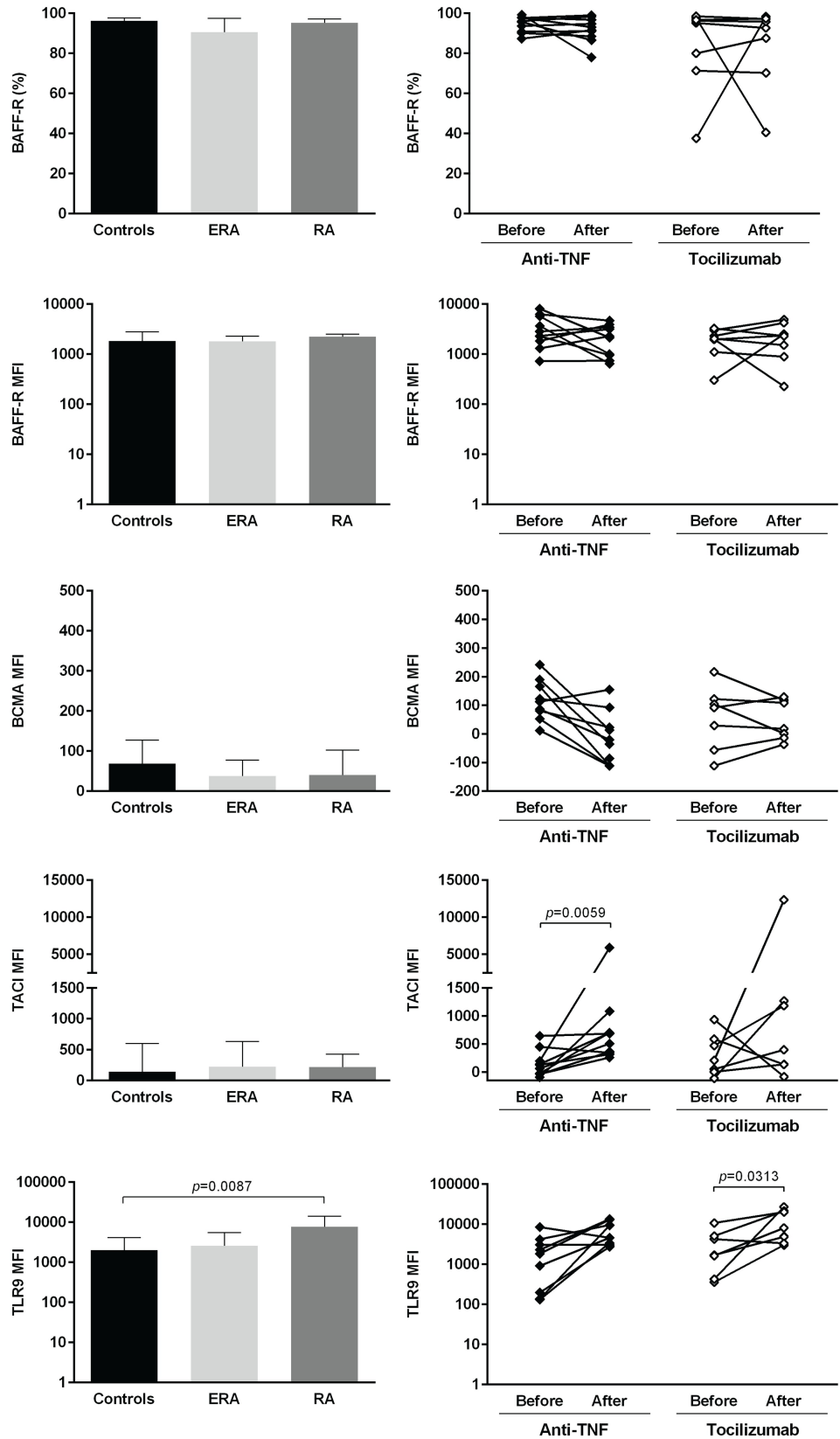


Fig 4. TACI, but not BAFF-R or BCMA, and TLR9 B-cell expression increase after treatment with TNF-inhibitors and tocilizumab in rheumatoid arthritis. The expression of B-cell activating factor receptors (BAFF-R, BCMA, TACI) and toll-like receptor 9 (TLR9) was analyzed on B-cells (frequency and median fluorescence intensity, MFI) to characterize B-cell phenotype in circulation in early RA (ERA) and established RA patients under methotrexate treatment. The effect of TNF-inhibitors and tocilizumab treatment on B-cell markers expression was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Bars represent median values with interquartile range. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g004>

No effect of treatment with TNF-inhibitors and tocilizumab was observed in the frequency of CD5+ B cells, but CD5 MFI significantly increased after TNF-inhibitors, but not tocilizumab treatment, when comparing baseline and follow-ups (Fig 5). The frequency of CD23+ B cells in circulation was similar between ERA, established RA and controls. However, CD23 MFI values were significantly increased in ERA, but not established RA patients, when compared to controls (Fig 5). Although no effect of treatment with TNF-inhibitors and tocilizumab was observed in CD23 MFI values, the frequency of CD23+ B cells significantly increased after tocilizumab, but not TNF-inhibitors treatment, when comparing baseline and follow-ups (Fig 5). In addition, ERA, but not established RA patients, had significantly increased CD38 MFI levels when compared to controls and no effect of treatment with TNF-inhibitors and tocilizumab was detected (Fig 5). Moreover, ERA, but not established RA patients, had significantly increased levels of CD69+ B cells in circulation when compared to controls, but no significant differences were detected in CD69 MFI values (Fig 6). Also, no effect of TNF-inhibitors and tocilizumab treatment was observed when comparing baseline and follow-ups (Fig 6). CD86 expression (both frequency and MFI) was similar between all studied groups and no effect of treatment with TNF-inhibitors and tocilizumab was detected (Fig 6). Although no significant differences were observed in CD95 MFI values between ERA, established RA and controls, CD95 expression significantly increased in RA patients after TNF-inhibitors, but not after tocilizumab treatment (Fig 6). The frequency of HLA-DR+ B cells was similar between all patient groups when compared to controls. Nevertheless, ERA, but not established RA patients, had significantly increased HLA-DR MFI values when compared to healthy individuals (Fig 7). Furthermore, HLA-DR expression (both frequency and MFI) significantly increased in RA patients after treatment with TNF-inhibitors and tocilizumab, when comparing baseline and follow-ups (Fig 7). Additionally, IgM and CXCR5 expression (both frequency and MFI) were similar between all studied groups and no effect of treatment with TNF-inhibitors and tocilizumab was detected (Figs 7 and 8, respectively). No significant correlations were observed between the frequency or MFI values of any of the B cell markers studied with age, DAS28, ESR, CRP, swollen and tender joint counts, or disease duration in all groups analyzed (data not shown).

Changes in B cell gene expression occur in RA patients, but no effect of treatment with TNF-inhibitors and tocilizumab is observed

The expression of a group of genes directly related with B cell activation and survival was analyzed in all studied groups (Fig 9). It was observed that BAFF-R gene expression was significantly higher in established RA patients in comparison with both ERA and controls (Fig 9A). TACI gene expression was significantly higher in established RA when compared to ERA, but no significant differences were found in comparison with controls. No significant differences were observed in BCMA gene expression between ERA, established RA and controls (Fig 9A). TLR9 gene expression levels were significantly higher in established RA when compared not

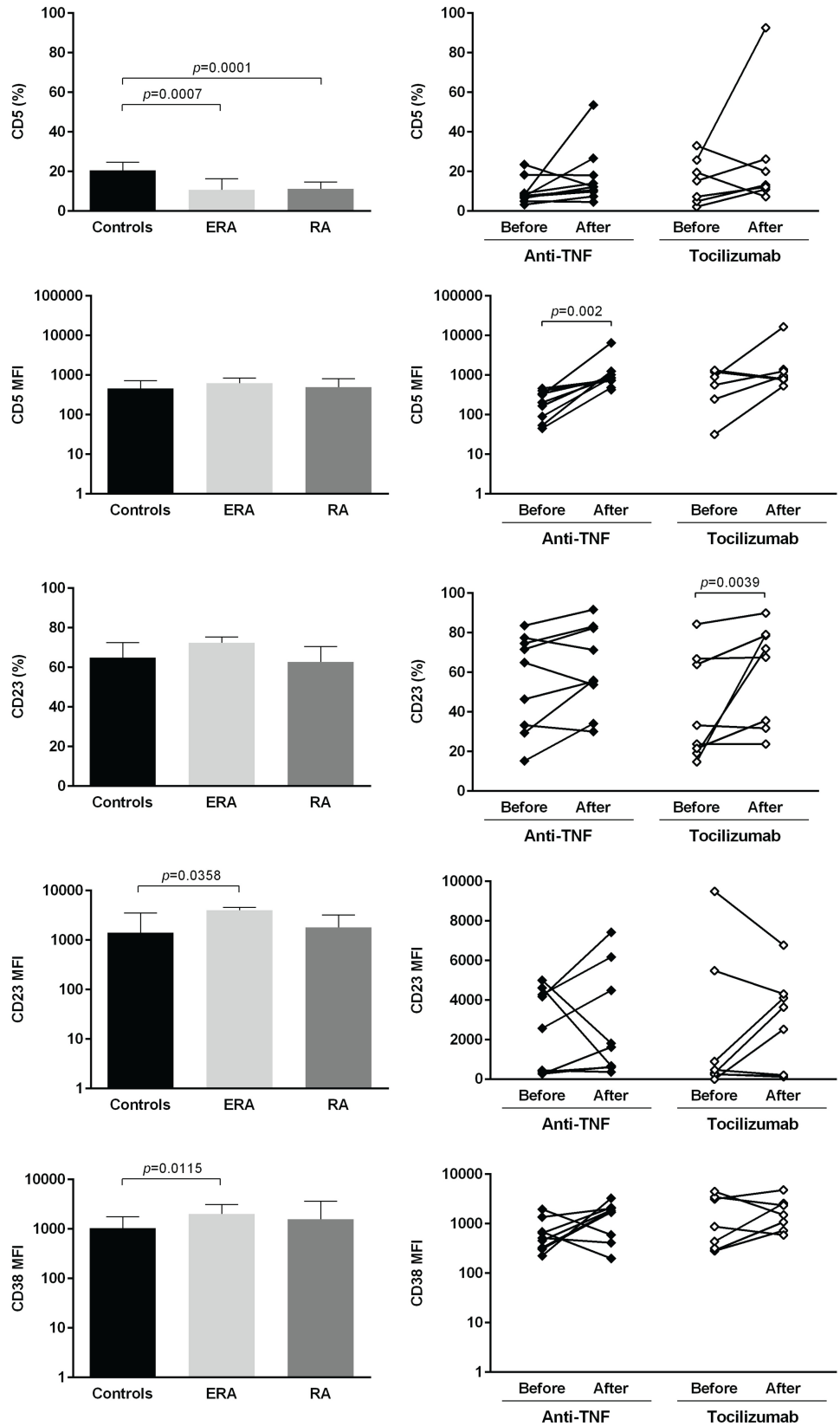


Fig 5. In rheumatoid arthritis, treatment with TNF-inhibitors and tocilizumab does not affect the frequency of CD5+ B cells in circulation, but CD5 MFI increases. The expression of B-cell differentiation (CD5), maturation (CD23) and calcium signaling (CD38) cell markers was analyzed on B-cells (frequency and median fluorescence intensity, MFI) to characterize B-cell phenotype in circulation in early RA (ERA) and established RA patients under methotrexate treatment. The effect of TNF-inhibitors and tocilizumab treatment on B-cell markers expression was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Bars represent median values with interquartile range. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g005>

only to ERA patients, but also to controls. However, no significant differences were detected in TLR7 and TLR10 B cell gene expression levels between ERA patients, established RA patients and controls (Fig 9B). FcγRIIB B cell gene expression levels were significantly higher in established RA when compared to ERA patients, but no significant differences were detected when compared to controls (Fig 9C). BCL-2 gene expression levels were significantly higher in established RA when compared to both ERA patients and controls, and no significant differences were detected in CXCR5 B cell gene expression levels between all groups (Fig 9C). Moreover, although ERA patients had similar BLIMP-1 B cell gene expression levels when compared to controls, the expression of this gene was significantly higher in ERA when compared to established RA patients (Fig 9D). β2M B cell gene expression was significantly higher in established RA patients when compared to controls, but no significant differences were detected in ERA patients in comparison, not only with controls, but also with established RA patients (Fig 9D). Furthermore, AID B cell gene expression levels were similar between all groups analyzed (Fig 9D). Of note, treatment with TNF-inhibitors and tocilizumab did not significantly affect B cell gene expression levels of any of the analyzed genes, when comparing baseline and follow-ups (data not shown). In addition, no significant correlations were found between B cell gene expression levels with age, DAS28, ESR, CRP, swollen and tender joint counts, or disease duration in all groups analyzed (data not shown).

CXCL13 and sCD23 serum levels are increased since early RA onset and do not change after treatment with TNF inhibitors and tocilizumab

The serum levels of CXCL13, a chemokine important for B cell chemotaxis and a ligand for CXCR5; sCD23, a marker of B cell maturation, and BAFF, a cytokine important for B cell survival, were also quantified in this study to analyze the effect of TNF-inhibitors and tocilizumab in the production of serological markers relevant for B cell activation (Fig 10). It was observed that CXCL13 serum levels were significantly higher in ERA and established RA patients when compared to healthy controls, but no significant differences were detected between both patient groups (Fig 10A). Moreover, treatment with TNF-inhibitors and tocilizumab did not affect CXCL13 circulating levels in RA patients when comparing baseline and follow-ups (Fig 10A). In addition, it was found that ERA patients had significantly higher sCD23 serum levels when compared to established RA patients, but no significant differences were observed in comparison with controls (Fig 10B). Furthermore, treatment with TNF-inhibitors and tocilizumab did not affect sCD23 circulating levels in RA patients when comparing baseline and follow-ups (Fig 10B). BAFF serum levels were similar between all groups analyzed and no effect of TNF-inhibitors and tocilizumab treatments was observed (Fig 10C). Of note, no significant correlations were detected between CXCL13, sCD23 and BAFF serum levels with B cell subpopulations in circulation, B cell markers expression, B cell gene expression, or with age, DAS28, ESR, CRP, swollen and tender joint counts, or disease duration in all groups analyzed (data not shown).

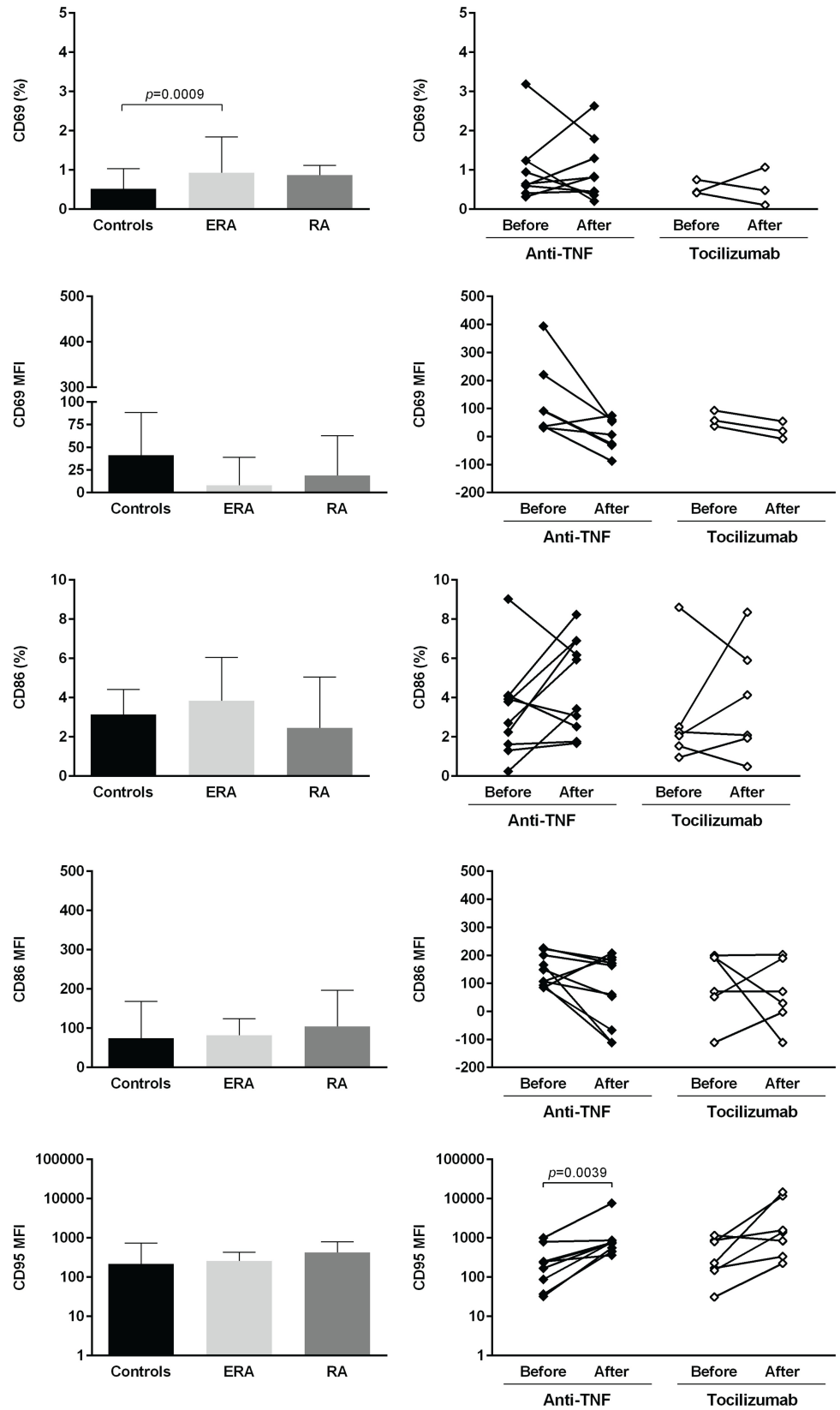


Fig 6. CD95, but not CD69 or CD86 B-cell expression increase after treatment with TNF-inhibitors and tocilizumab in rheumatoid arthritis. The expression of activation (CD69, CD86) and apoptosis (CD95) cell markers was analyzed on B-cells (frequency and median fluorescence intensity, MFI) to characterize B-cell phenotype in circulation in early RA (ERA) and established RA patients under methotrexate treatment. The effect of TNF-inhibitors and tocilizumab treatment on B-cell markers expression was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Bars represent median values with interquartile range. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g006>

Biologic therapy affects blood memory B cells and B cell phenotype of rheumatoid arthritis patients irrespective of response to treatment

In order to understand if response to biologic therapy affected the results observed in circulating B cell subpopulations, B cell phenotype and B cell gene expression, we have analyzed RA patients before and after treatment with TNF-inhibitors and/ or tocilizumab based on whether the patients presented a low to moderate disease activity ($\text{DAS28} < 3.2$) or high disease activity ($\text{DAS28} > 3.2$) after treatment (Figs 11–14). Similarly to the results observed in Fig 1, we found that the frequency of total CD19+ B cells was not significantly affected after biologic therapy, irrespective of response to treatment. Nevertheless, the frequency of IgD-CD27- memory B cells was significantly decreased after biologic therapy not only in RA patients with low to moderate disease activity, but also in patients with high disease activity after treatment (Fig 11). Furthermore, no significant differences were observed in the frequencies of transitional, naïve B cells, pre-switch memory, post-switch memory and plasmablasts in RA patients after biologic therapy, irrespective of response to treatment (Fig 11). B cell phenotype analysis has shown that no significant differences were detected in BAFF-R (both frequency and MFI) and BCMA expression after biologic therapy irrespective of response to treatment, but TACI expression significantly increased in RA patients after biologic therapy who had a low to moderate, but not high, disease activity after treatment (Fig 12). No significant differences were observed in CD5+ B cells, but CD5 MFI values were significantly increased after biologic therapy, irrespective of response to treatment (Fig 13). The frequency of CD23+ B cells was significantly higher in RA patients after biologic therapy who had a low to moderate, but not high, disease activity after treatment, but no significant differences were observed in CD23 MFI values (Fig 13). CD38 MFI, CD69 and CD86 (both frequency and MFI) were not significantly different between RA patients after biologic therapy, irrespective of response to treatment (Fig 13). CD95 expression significantly increased in RA patients after biologic therapy not only in patients with low to moderate, but also high disease activity after treatment (Fig 13). HLA-DR expression (both frequency and MFI) significantly increased in RA patients after biologic therapy, irrespective of response to treatment (Fig 14), but no significant differences were observed in CXCR5 and IgM expression. TLR9 MFI was significantly increased in RA patients after biologic therapy who had a low to moderate, but not high disease activity after treatment (Fig 14). In addition, no significant differences were found in B cell gene expression and serum levels of CXCL13, sCD23 and BAFF in RA patients after biologic therapy, irrespective of response to treatment (data not shown).

Discussion

In this study, the effect of TNF-inhibitors and tocilizumab treatments on peripheral blood B cell phenotype and B cell gene expression was analyzed in patients with RA. We found that untreated early RA and established RA patients have alterations in memory B cell

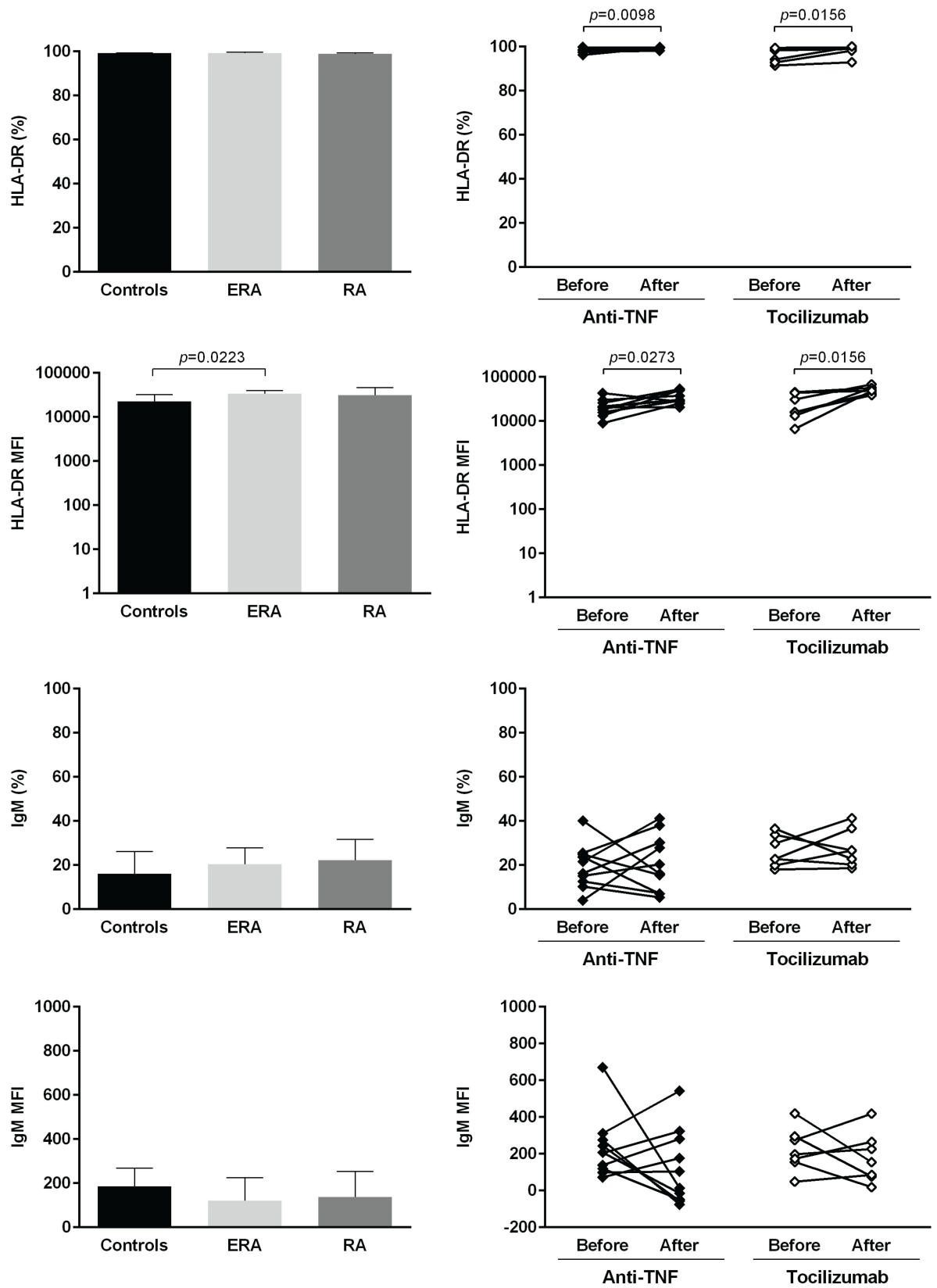


Fig 7. The frequency of HLA-DR+ B cells and HLA-DR MFI increase after treatment with TNF-inhibitors and tocilizumab in rheumatoid arthritis. The expression of cell markers related to antigen presentation (HLA-DR) and B-cell receptor (IgM) was analyzed on B-cells (frequency and median fluorescence intensity, MFI) to characterize B-cell phenotype in circulation in early RA (ERA) and established RA patients under methotrexate treatment. The effect of TNF-inhibitors and tocilizumab treatment on B-cell markers expression was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Bars represent median values with interquartile range. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g007>

subpopulations, particularly in DN (IgD-CD27-) memory B cells, and treatment with either TNF antagonists or tocilizumab restored the frequency of this B cell subset to normal levels. Furthermore, changes in the expression of some B cell markers such as TACI, TLR9, CD5, CD95 and HLA-DR, were also detected after treatment with TNF-inhibitors and/ or tocilizumab. No significant alterations were found in B cell gene expression, CXCL13, sCD23 and BAFF serum levels after treatment with TNF-inhibitors or tocilizumab.

Previous studies have demonstrated that alterations in B cell subpopulations, particularly memory B cell subsets, occur in RA patients, not only in peripheral blood, but also locally in the joints [3, 16, 17]. In fact, our group has described for the first time that untreated very early RA patients with less than 6 weeks of disease duration have significantly decreased circulating levels of pre-switch memory B cells (IgD+CD27+) [3], which can be related with B cell trafficking in the tissues and/ or with infiltration of the synovial membrane [16]. In this study, we found that untreated early RA patients (<1 year of disease duration, ERA) and established RA patients (MTX treated and before initiating anti-TNF or tocilizumab treatment) had significantly increased levels of DN (IgD-CD27-) B cells when compared to controls, which has also been recently described by others [18, 19]. Indeed, increased levels of IgD-CD27- memory B cells have been found in other autoimmune diseases such as systemic lupus erythematosus [20, 21] and multiple sclerosis [22]. Although the exact function of this B cell subset is not entirely understood, it is known that it belongs to the memory B cell compartment due to the high levels of somatic hypermutation [23–25]. Moreover, it has been suggested that these cells might contribute to inflammation by induction of T cell responses and the production of proinflammatory cytokines [22]. Also, since most of IgD-CD27- B cells were class-switched, it is possible that these B cells are linked with the production of higher affinity antibodies relevant in inflammation [26, 27]. Elevated numbers of DN memory B cells have also been related to aging [28, 29], but no correlation was found between the frequency of this B cell subpopulation and age in all studied groups, nor with inflammation parameters such as DAS28, ESR or CRP values. We found that treatment with TNF-inhibitors and tocilizumab restored the frequency of DN (IgD-CD27-) B cells to normal levels, which is in accordance with previous studies [16, 30–32], although there are some contradictory results [16, 33–35]. The disparities found between studies could be related to the use of different anti-TNF agents, disease activity, cohort size and previous treatments administered to RA patients. Previous works have demonstrated that TNF-inhibitors affect memory B cell subpopulations not only in RA, but also in other inflammatory diseases [16, 19, 31, 36–39]. In fact, circulating naive B cells and class-switched memory B cells were found to be normally present in peripheral blood of patients with Crohn's disease, whereas IgM+ memory B cell numbers were reduced. Nonetheless, a restoration of IgM+ memory B cell pool occurred after treatment with infliximab [31, 36, 38]. Also, it has been shown that in patients with sarcoidosis, the increased blood levels of CD27-IgA+ memory B cells are normalized after treatment with infliximab [37]. Furthermore, it has been recently demonstrated that patients with Behçet's disease have significantly lower memory B cell numbers in peripheral blood, particularly CD27+IgA+ B cells, when compared to controls, but treatment with adalimumab restored to normal levels blood B cell numbers [39]. Of note, it

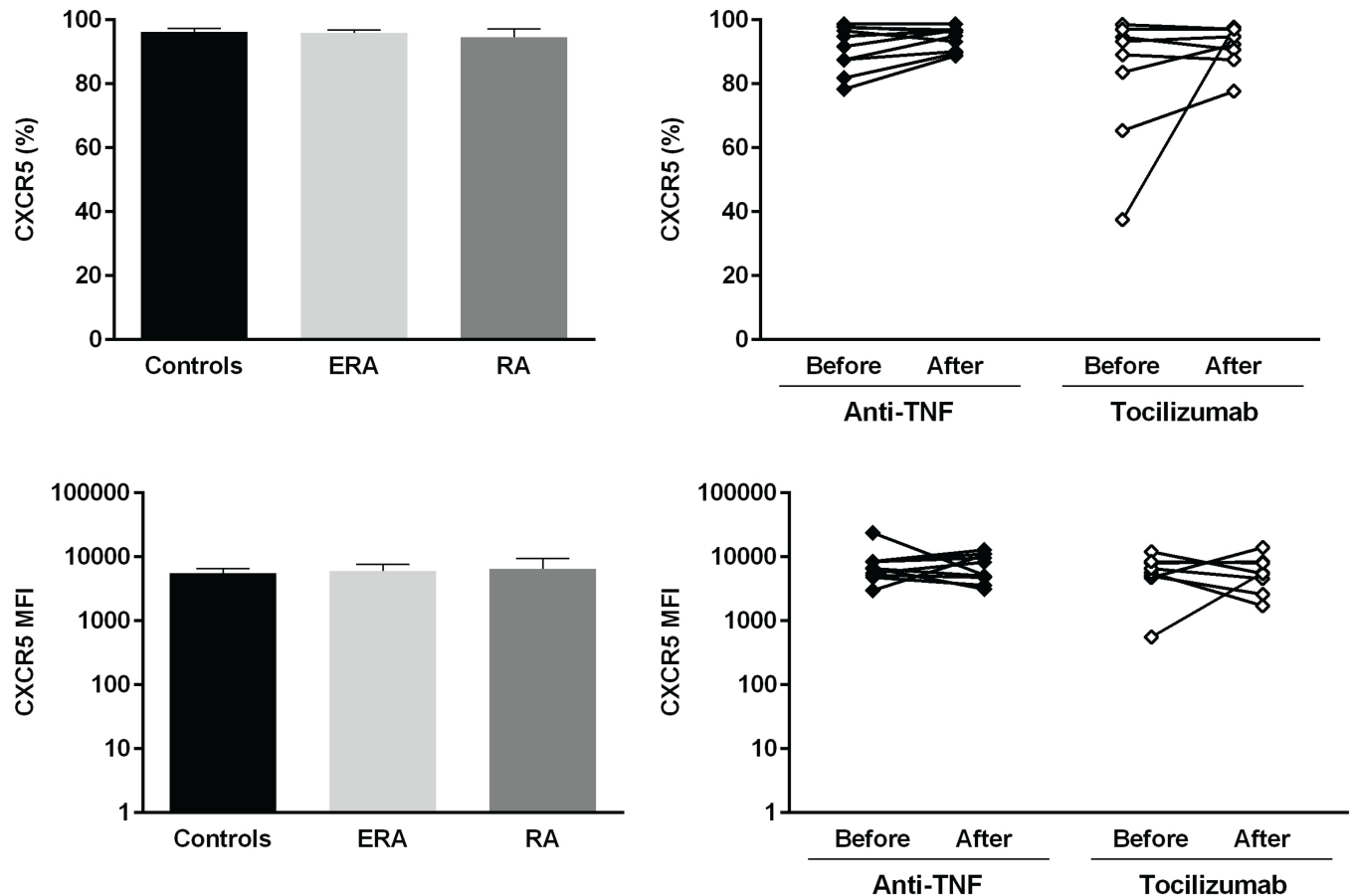


Fig 8. Treatment with TNF-inhibitors and tocilizumab does not affect the circulating levels of CXCR5+ B cells or CXCR5 MFI in rheumatoid arthritis. The expression of chemokine receptor CXCR5 was analyzed on B-cells (frequency and median fluorescence intensity, MFI) to characterize B-cell phenotype in circulation in early RA (ERA) and established RA patients under methotrexate treatment. The effect of TNF-inhibitors and tocilizumab treatment on B-cell markers expression was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Bars represent median values with interquartile range. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g008>

has been previously shown that established RA patients have significantly lower levels of peripheral blood pre-switch IgD+CD27+ memory B cells when compared to healthy individuals, but treatment with infliximab restored the frequency of this B cell subpopulation to normal levels [16]. In the present study, the TNF-inhibitors analyzed were adalimumab (1 case), etanercept (5 cases) and golimumab (4 cases), which restored the frequency of IgD-CD27- memory B cells in RA patients to normal levels. Nevertheless, no significant differences were found in B cell subpopulations when TNF-inhibitors (etanercept and golimumab) were independently analyzed and compared (data not shown). Also, no significant differences were detected in B cell phenotype, B cell gene expression or serum levels of BAFF, CXCL13 and sCD23 before and after treatment with either golimumab or etanercept (data not shown). This is an important limitation of our study, since the low number of patients included of each anti-TNF treatment does not allow a robust statistical analysis of the individual TNF-inhibitors. Future studies with a higher number of RA patients included after treatment with TNF-inhibitors will be necessary to reinforce the conclusions regarding the effect of individual TNF-inhibitors on B cells. Tocilizumab has also been shown to induce a reduction in the frequency of memory B

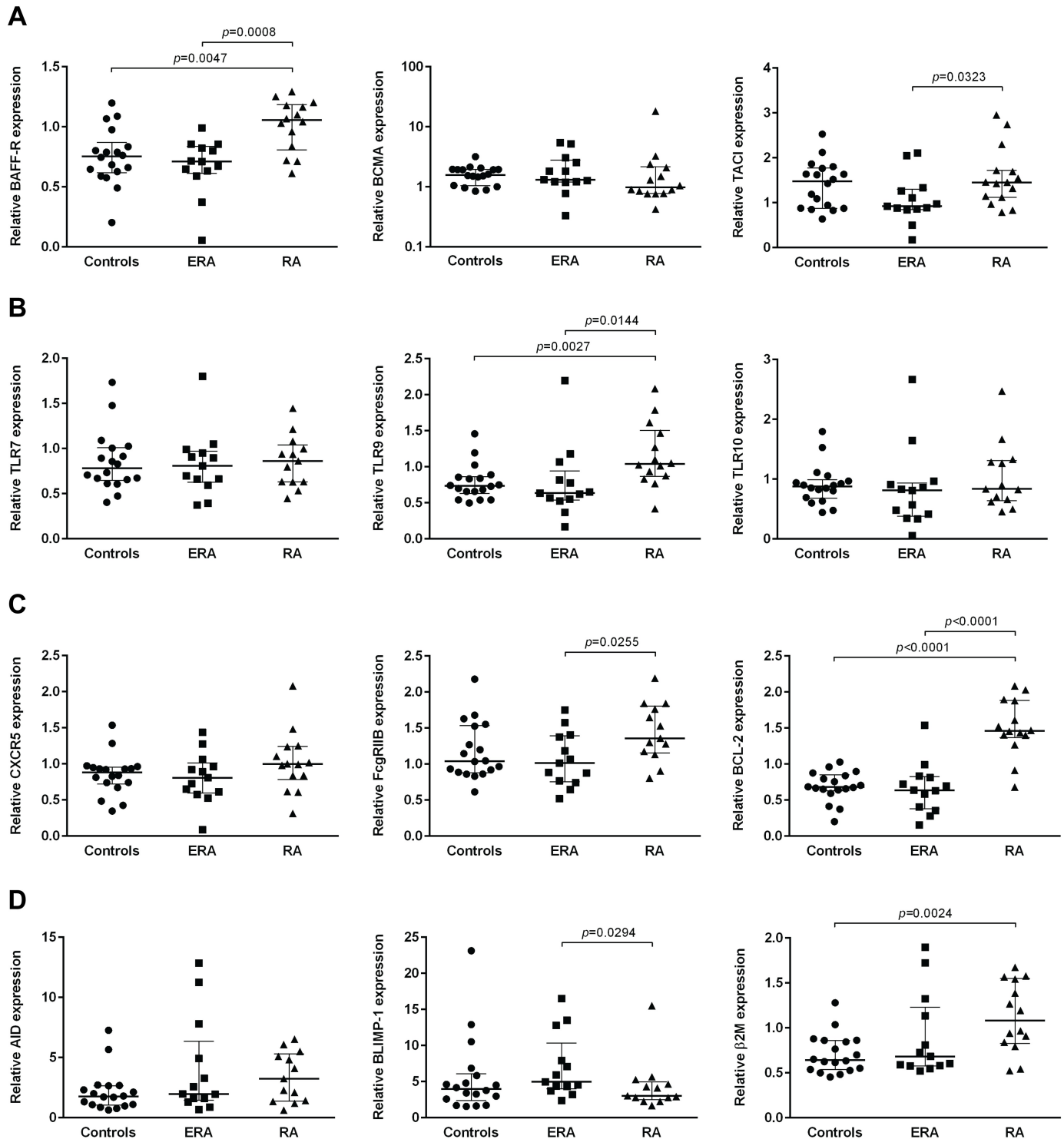


Fig 9. Changes in B-cell gene expression levels occur in rheumatoid arthritis, but no effect of TNF-inhibitors and tocilizumab treatment is observed. The expression of a group of genes related with B-cell activation through B-cell activating factor receptors (BAFF-R, BCMA, TAC1) (A); toll-like receptors (TLR7, TLR9, TLR10) (B); chemotaxis (CXCR5), inhibition (FcgRIIB), apoptosis (BCL-2) (C); class-switching (AID), plasma cell differentiation (BLIMP-1) and cellular activation ($\beta 2M$) (D) was analyzed on isolated B-cells by real time PCR in early RA (ERA) and established RA. The effect of TNF-inhibitors and tocilizumab treatment on B-cell gene expression was assessed in established RA patients at baseline and after an average of 8 months of treatment. Lines represent median values with interquartile range. Differences were considered statistically significant for $p<0.05$ (non-parametric Mann-Whitney test).

<https://doi.org/10.1371/journal.pone.0182927.g009>

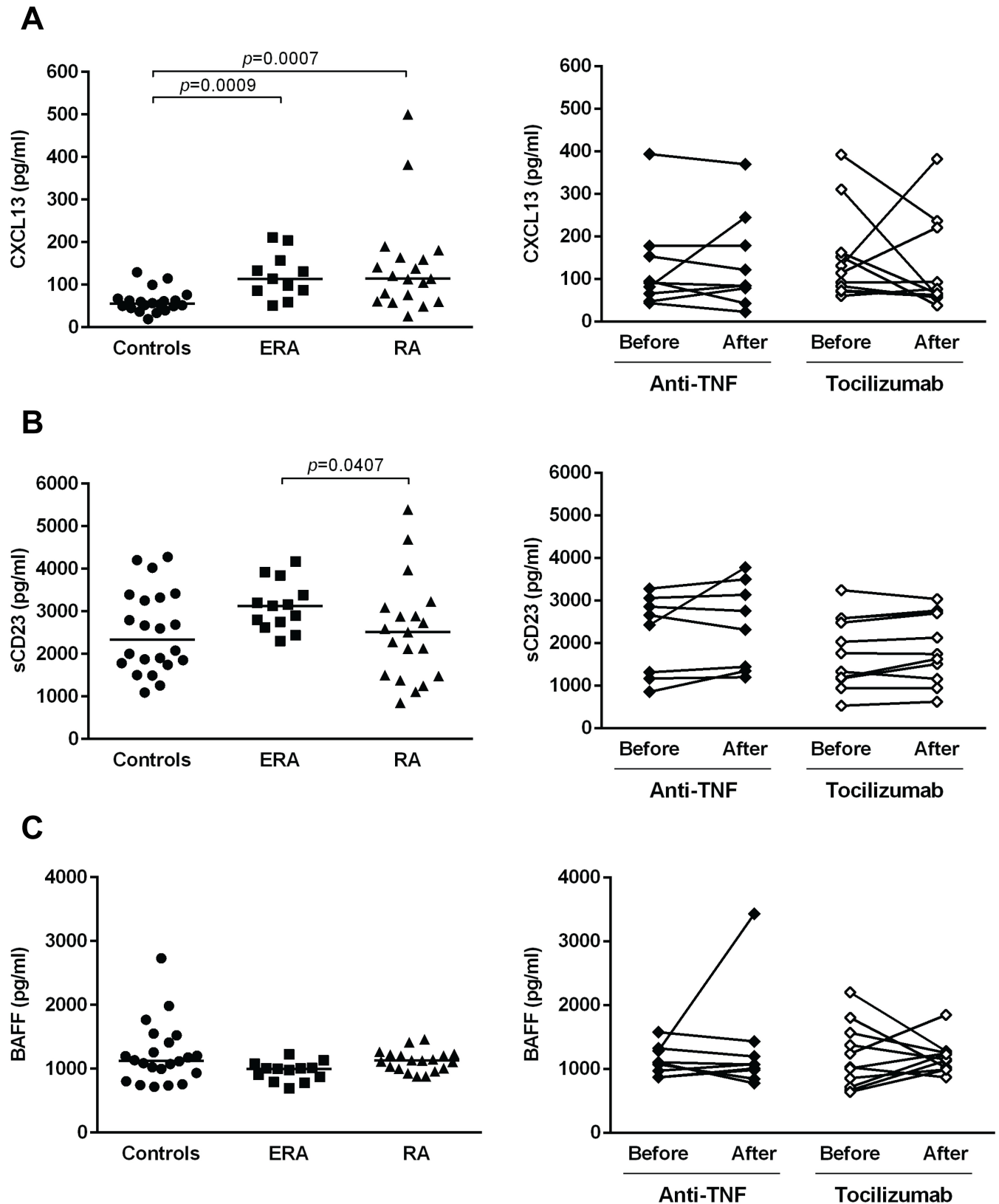


Fig 10. CXCL13 and sCD23 serum levels are increased since early rheumatoid arthritis onset and do not change after treatment. The serum levels of C-X-C motif chemokine 13 (CXCL13) (A), soluble CD23 (sCD23) (B) and B cell activating factor (BAFF) (C) were quantified in early

RA (ERA) and established RA patients under methotrexate treatment by ELISA. The effect of TNF-inhibitors and tocilizumab treatment in the production of these serological markers was also assessed in established RA patients at baseline and after an average of 8 months of treatment. Lines represent median values. Differences were considered statistically significant for $p < 0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used.

<https://doi.org/10.1371/journal.pone.0182927.g010>

cell subpopulations in RA [40, 41], namely IgA+ and IgG+ B cells [40], which supports our results. Interestingly, it has been suggested that the frequency of DN (IgD-CD27-) B cells in RA might serve as a baseline predictor of subsequent response to tocilizumab treatment [18]. Possibly, the restoration of DN memory B cells to normal circulating levels in RA patients after treatment with either TNF-inhibitors or tocilizumab reflects a state of chronic B cell hyperactivity dependent on TNF and IL-6 [10, 11], which is inhibited by treatment. Of note, recent studies have demonstrated that microRNA-155, an important regulator of B cell activation, is highly expressed in peripheral blood B cells, particularly in IgD-CD27- memory B cells, in ACPA+ patients [27], which supports an enhanced activation of this particular B cell subset in RA. The normalization of IgD-CD27- B cells in peripheral blood after treatment with TNF-inhibitors and tocilizumab might suggest a relevant role of these B cells in RA pathogenesis either through T cell activation, cytokine release and/ or antibody production. Further studies are necessary in larger cohorts of patients to clarify the role of this B cell subpopulation in RA, particularly comparing seropositive and seronegative RA.

B cell phenotype analysis of ERA patients suggests an early B cell activation due to the increased frequencies of CD69+ B cells in circulation when compared to controls. Furthermore, the higher CD23 and CD38 MFI values observed in ERA patients reinforce an early B cell maturation and triggering, also supported by the increased serum levels of sCD23 detected in ERA [42, 43]. In addition, the higher CXCL13 (ligand for CXCR5 and a B cell chemotactic factor) serum levels observed not only in ERA, but also in established RA patients when compared to healthy individuals, support an active recruitment of B cells towards inflammatory sites since early RA onset [44][45]. Nevertheless, follicular T helper cells also express CXCR5 and respond to its ligand, CXCL13 [46]. Furthermore, it has been demonstrated that Th17 cells, known to contribute to RA pathogenesis, are an important source of CXCL13 and interact with B cells, triggering autoantibody production [47]. Therefore, the hypothesis that the increased serum levels of CXCL13 detected since early RA onset affect not only B cells, but also T cells, cannot be excluded. Thus, the recruitment and activation of CXCR5+ B and T cells, stimulated by CXCL13, towards joints and secondary lymphoid organs contribute to an exacerbation of the inflammatory process in RA [46, 48, 49]. Moreover, similarly to previous studies, alterations in the frequency of CD5+ B cells were detected in both ERA and established RA patients when compared to controls [50, 51]. CD5+ B cells are known to be associated with bone resorption through IL-6 production, a cytokine that supports osteoclast differentiation [52]. It is possible that the reduced circulating levels of CD5+ B cells are due to a recruitment of these cells towards the synovial tissue, where they might contribute to bone erosions.

Treatment with TNF-inhibitors and tocilizumab influenced some B cell surface markers. While no significant changes were observed in BAFF-R (both frequency and MFI values) or BCMA, TACI MFI increased after anti-TNF therapy. TACI plays an important role in humoral immunity [53], but its functional activity can be ambiguous as it can not only provide positive signals driving T-independent B cell responses and survival of activated B cells [54, 55], but also delivers negative signals suppressing B cell activation [56]. The increased TACI and CD95 MFI values detected after treatment with TNF-inhibitors might suggest an inhibition of B cell activation [56, 57]. Recent reports indicate that TNF-inhibitors modulate Fas-mediated apoptosis in RA [13, 58] and it has been demonstrated that anti-TNF treatment in

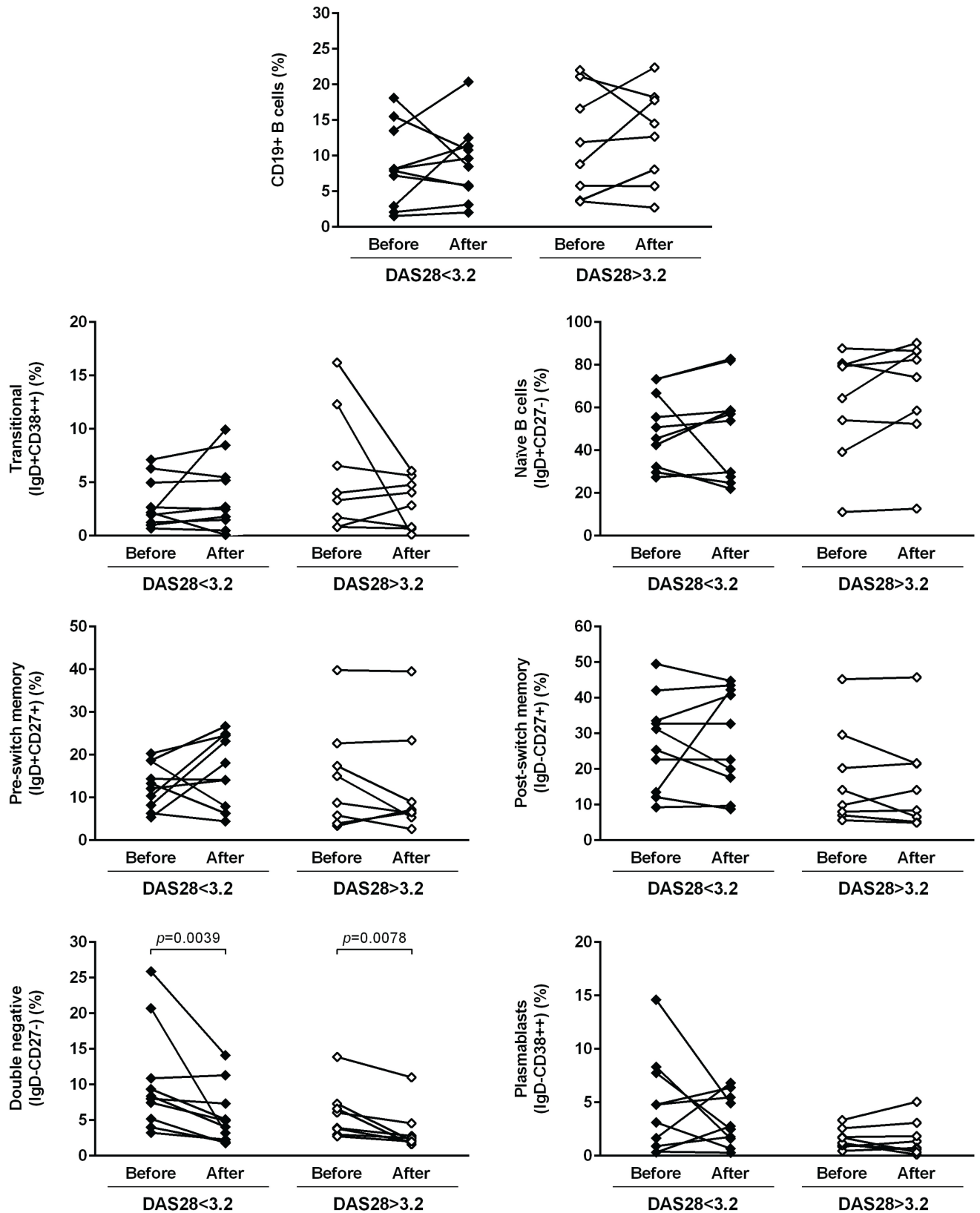


Fig 11. Biologic therapy affects peripheral blood memory B cells in rheumatoid arthritis patients irrespective of response to treatment. The frequency of peripheral blood B cell subpopulations was analyzed by flow cytometry in established RA patients before and after an average of 8 months of biologic therapy with TNF-inhibitors and/ or tocilizumab, based on whether the patients presented a low to moderate disease activity (DAS28<3.2) or high disease activity (DAS28>3.2) after treatment. Differences were considered statistically significant for $p < 0.05$ (Wilcoxon signed-rank test).

<https://doi.org/10.1371/journal.pone.0182927.g011>

RA inhibits B cell function by disrupting germinal centre reactions [14, 33] and decreasing T-cell dependent humoral responses [14]. B cell trafficking into inflamed tissues in RA is regulated by TNF and can be modulated not only by TNF-inhibitors [16, 59, 60], but also by tocilizumab treatment [61]. In this study, higher frequencies of HLA-DR+ B cells and HLA-DR MFI values were observed after treatment with both TNF-inhibitors and tocilizumab. It is possible that by inhibiting B cell recruitment and ameliorating cellular infiltration at inflammatory sites, treatment of RA patients with either TNF-inhibitors or tocilizumab leads to B cell recirculation through blood and lymphatic systems. These activated B cells have an altered phenotype, with changes in B cell activation markers such as HLA-DR, TLR9 and/ or chemokine receptors [14, 16, 30, 32, 57, 62], as observed in this study, which can be related with modifications on B cell triggering mechanisms potentiated by anti-TNF and/ or tocilizumab treatment.

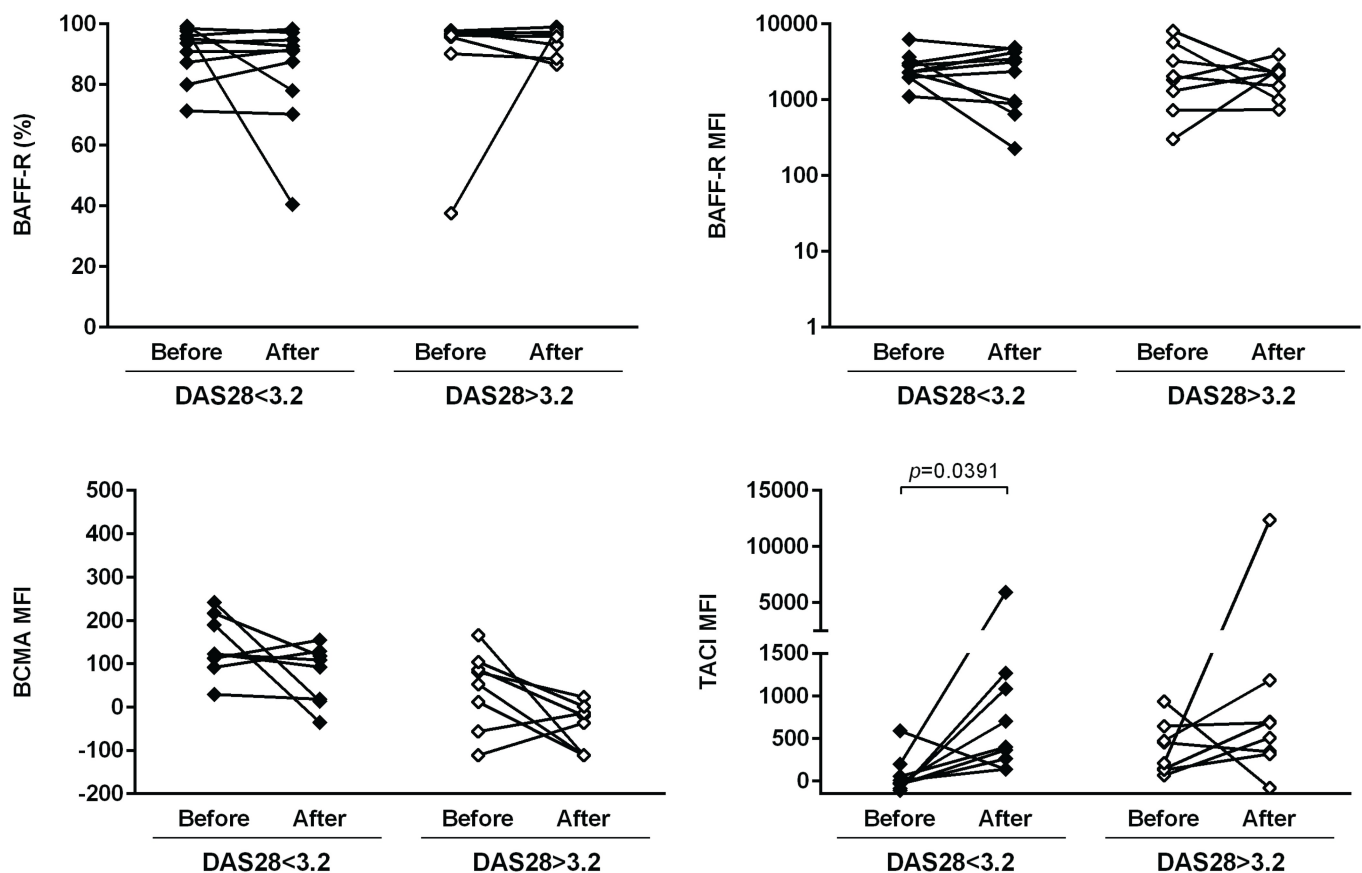


Fig 12. Rheumatoid arthritis patients with low to moderate disease activity after biologic therapy have increased TACI, but not BAFF-R or BCMA B-cell expression. The expression of B-cell activating factor receptors (BAFF-R, BCMA, TACI) was analyzed by flow cytometry (frequency and median fluorescence intensity, MFI) in established RA patients before and after an average of 8 months of biologic therapy with TNF-inhibitors and/ or tocilizumab, based on whether the patients presented a low to moderate disease activity (DAS28<3.2) or high disease activity (DAS28>3.2) after treatment. Differences were considered statistically significant for $p < 0.05$ (Wilcoxon signed-rank test).

<https://doi.org/10.1371/journal.pone.0182927.g012>

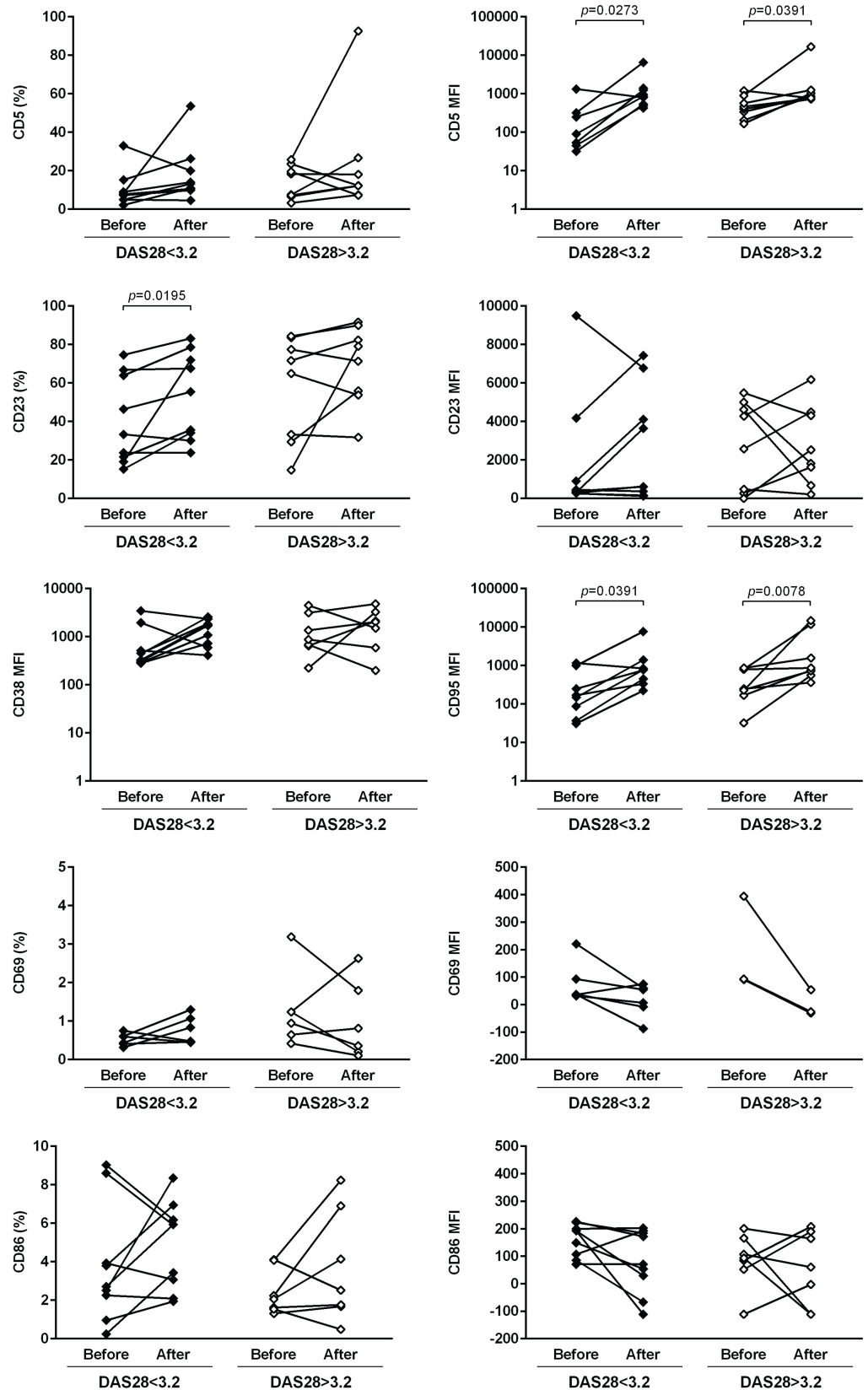


Fig 13. CD5 and CD95 B-cell expression increase in rheumatoid arthritis patients after biologic therapy irrespective of response to treatment. The expression of cell markers (CD5, CD23, CD38, CD69, CD86 and CD95) was analyzed on B-cells (frequency and median fluorescence intensity, MFI) by flow cytometry in established RA patients before and after an average of 8 months of biologic therapy with TNF-inhibitors and/or tocilizumab, based on whether the patients presented a low to moderate disease activity (DAS28<3.2) or high disease activity (DAS28>3.2) after treatment. Differences were considered statistically significant for $p<0.05$ (Wilcoxon signed-rank test).

<https://doi.org/10.1371/journal.pone.0182927.g013>

Furthermore, it was also found that the frequency of B cell subpopulations and B cell phenotype in established RA patients are affected by biologic therapy, irrespective of response to treatment. These results support the absence of correlation between circulating B cell levels, B cell phenotype and disease activity.

Our results also suggest that B cell gene expression in RA is not significantly affected by treatment with either TNF-inhibitors or tocilizumab, but alterations occur in ERA and established RA patients treated with MTX. The significantly increased BAFF-R, TACI and TLR9 B cell gene expression levels observed in established RA patients under MTX treatment when compared to ERA and controls support a relevant role of BAFF and T-cell independent mechanisms in B cell activation, particularly at later stages of RA development [4, 6, 63, 64]. Nevertheless, no significant differences were found at a protein level for BAFF-R and TACI MFI values, except for TLR9, whose expression was significantly increased in established RA when compared to controls. Thus, we cannot exclude the hypothesis that MTX treatment affects B cells at the gene, but not at the protein level, at least in the case of some cellular markers such as BAFF-R and TACI. Furthermore, BCL-2 gene expression levels were significantly increased in established RA, suggesting an inhibition of B cell apoptosis [65], which can be due to immunosuppressive treatment [66]. Similarly, the elevated FcγRIIB gene expression levels observed in established RA, when compared to ERA, might be related with suppression of B cell responses during MTX therapy [67]. Nonetheless, the higher β2M gene expression levels found in established RA support an increased B cell activation in chronic patients when compared to healthy individuals [68]. BLIMP-1, important for plasma cell differentiation [69], had B cell gene expression levels significantly increased in ERA patients in comparison with established RA under MTX treatment, which supports an activation of antibody secreting cells since early RA onset.

Overall, total CD19+ B cells were analyzed in this study and differences were only found in protein expression on B cells in some of the markers studied. Since alterations were detected in B cell subpopulations, particularly in IgD-CD27- memory B cells, after treatment with TNF-inhibitors and tocilizumab, future studies concerning an independent analysis of all B cell subsets (frequency and absolute numbers) would be fundamental. In fact, an analysis based on absolute numbers would be particularly relevant and more accurate to confirm any changes observed in circulating B cell subpopulations detected with relative numbers.

Conclusions

To sum up, in RA, treatment with either TNF-inhibitors or tocilizumab affects B cell phenotype and the frequency of memory B cell subpopulations in peripheral blood, particularly DN (IgD-CD27-) B cells, but not B cell gene expression or serum levels of CXCL13, sCD23 and BAFF, when comparing baseline with post-treatment follow up. Overall, our results may suggest that TNF-inhibitors and tocilizumab inhibit B cell trafficking towards inflammatory sites, thus supporting activated B cell recirculation from tissues through blood and lymphatic systems.

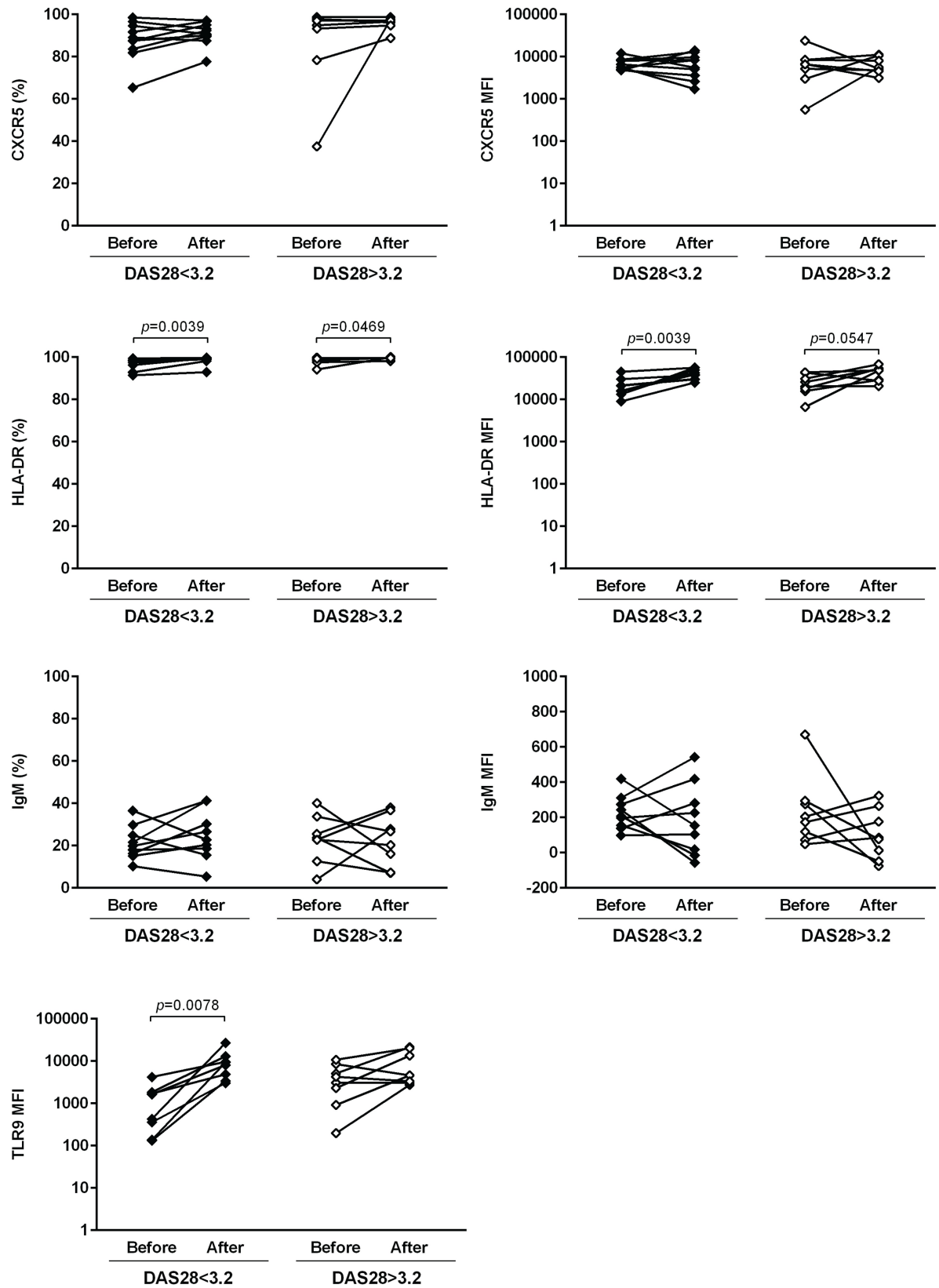


Fig 14. Biologic therapy affects not only the frequency of HLA-DR+ B cells in circulation, but also HLA-DR MFI in rheumatoid arthritis patients irrespective of response to treatment. The expression of cell markers (CXCR5, HLA-DR, IgM and TLR9) was analyzed on B-cells (frequency and median fluorescence intensity, MFI) by flow cytometry in established RA patients before and after an average of 8 months of biologic therapy with TNF-inhibitors and/ or tocilizumab, based on whether the patients presented a low to moderate disease activity (DAS28<3.2) or high disease activity (DAS28>3.2) after treatment. Differences were considered statistically significant for $p<0.05$ (Wilcoxon signed-rank test).

<https://doi.org/10.1371/journal.pone.0182927.g014>

Supporting information

S1 Fig. IgD-CD27- B cells are mainly class-switched and express low IgM levels. The frequency of IgD-CD27- B cells expressing IgM was determined by flow cytometry in early RA (ERA) and established RA patients under methotrexate treatment. In addition, the effect of TNF-inhibitors and tocilizumab treatment on IgM expression by IgD-CD27- B cells was also assessed in established RA patients at baseline and after an average of 8 months of treatment. A group of healthy individuals was also included as controls. Lines represent median values. Differences were considered statistically significant for $p<0.05$. Non-parametric Mann-Whitney test was used for comparisons between 2 independent groups. For paired samples (before and after treatment), the Wilcoxon signed-rank test was used. (TIF)

Acknowledgments

The authors would like to acknowledge Biobank facility from Instituto de Medicina Molecular and Lisbon Academic Medical Center, Portugal, for the assistance in the collection of biological samples from healthy and patient donors. The authors also especially acknowledge all the patients and healthy volunteers that participated in this study.

Author Contributions

Conceptualization: Rita A. Moura, João E. Fonseca.

Data curation: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, Maria J. Gonçalves, Joaquim Polido-Pereira, Vasco C. Romão, Nádia Martins, Helena Canhão, João E. Fonseca.

Formal analysis: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, Helena Canhão, João E. Fonseca.

Funding acquisition: Rita A. Moura, João E. Fonseca.

Investigation: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, Maria J. Gonçalves, Joaquim Polido-Pereira, Vasco C. Romão, Nádia Martins, Helena Canhão, João E. Fonseca.

Methodology: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, João E. Fonseca.

Project administration: Rita A. Moura, João E. Fonseca.

Resources: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, Maria J. Gonçalves, Joaquim Polido-Pereira, Vasco C. Romão, Nádia Martins, Helena Canhão, João E. Fonseca.

Supervision: Rita A. Moura, João E. Fonseca.

Validation: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, João E. Fonseca.

Visualization: Rita A. Moura, Cláudia Quaresma, João E. Fonseca.

Writing – original draft: Rita A. Moura, João E. Fonseca.

Writing – review & editing: Rita A. Moura, Cláudia Quaresma, Ana R. Vieira, Maria J. Gonçalves, Joaquim Polido-Pereira, Vasco C. Romão, Nádia Martins, Helena Canhão, João E. Fonseca.

References

1. Klareskog L, Catrina AI, Paget S. Rheumatoid arthritis. *Lancet*. 2009; 373(9664):659–72. Epub 2009/01/23. [https://doi.org/10.1016/S0140-6736\(09\)60008-8](https://doi.org/10.1016/S0140-6736(09)60008-8) PMID: 19157532.
2. Moura RA, Graca L, Fonseca JE. To B or not to B the conductor of rheumatoid arthritis orchestra. *Clinical reviews in allergy & immunology*. 2012; 43(3):281–91. Epub 2012/06/22. <https://doi.org/10.1007/s12016-012-8318-y> PMID: 22717935.
3. Moura RA, Weinmann P, Pereira PA, Caetano-Lopes J, Canhao H, Sousa E, et al. Alterations on peripheral blood B-cell subpopulations in very early arthritis patients. *Rheumatology (Oxford)*. 2010; 49(6):1082–92. <https://doi.org/10.1093/rheumatology/keq029> PMID: 20211867.
4. Moura RA, Cascao R, Perpetuo I, Canhao H, Vieira-Sousa E, Mourao AF, et al. Cytokine pattern in very early rheumatoid arthritis favours B-cell activation and survival. *Rheumatology (Oxford)*. 2011; 50(2):278–82. <https://doi.org/10.1093/rheumatology/keq338> PMID: 21047805.
5. Cascao R, Moura RA, Perpetuo I, Canhao H, Vieira-Sousa E, Mourao AF, et al. Identification of a cytokine network sustaining neutrophil and Th17 activation in untreated early rheumatoid arthritis. *Arthritis Res Ther*. 2010; 12(5):R196. Epub 2010/10/22. <https://doi.org/10.1186/ar3168> PMID: 20961415; PubMed Central PMCID: PMC2991033.
6. Moura RA, Canhao H, Polido-Pereira J, Rodrigues AM, Navalho M, Mourao AF, et al. BAFF and TACI gene expression are increased in patients with untreated very early rheumatoid arthritis. *The Journal of rheumatology*. 2013; 40(8):1293–302. Epub 2013/06/19. <https://doi.org/10.3899/jrheum.121110> PMID: 23772083.
7. Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, et al. Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med*. 2004; 350(25):2572–81. <https://doi.org/10.1056/NEJMoa032534> PMID: 15201414.
8. Romao VC, Santos MJ, Polido-Pereira J, Duarte C, Nero P, Miguel C, et al. Comparative Effectiveness of Tocilizumab and TNF Inhibitors in Rheumatoid Arthritis Patients: Data from the Rheumatic Diseases Portuguese Register, Reuma.pt. *BioMed research international*. 2015; 2015:279890. Epub 2015/05/23. <https://doi.org/10.1155/2015/279890> PMID: 26000286; PubMed Central PMCID: PMC4427085.
9. Canhao H, Rodrigues AM, Mourao AF, Martins F, Santos MJ, Canas-Silva J, et al. Comparative effectiveness and predictors of response to tumour necrosis factor inhibitor therapies in rheumatoid arthritis. *Rheumatology (Oxford)*. 2012; 51(11):2020–6. Epub 2012/07/31. <https://doi.org/10.1093/rheumatology/kes184> PMID: 22843791; PubMed Central PMCID: PMC3475979.
10. Jourdan M, Cren M, Robert N, Bollere K, Fest T, Duperray C, et al. IL-6 supports the generation of human long-lived plasma cells in combination with either APRIL or stromal cell-soluble factors. *Leukemia*. 2014; 28(8):1647–56. Epub 2014/02/08. <https://doi.org/10.1038/leu.2014.61> PMID: 24504026.
11. Boussiotis VA, Nadler LM, Strominger JL, Goldfeld AE. Tumor necrosis factor alpha is an autocrine growth factor for normal human B cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1994; 91(15):7007–11. Epub 1994/07/19. PMID: 7518925; PubMed Central PMCID: PMC44327.
12. Uson J, Balsa A, Pascual-Salcedo D, Cabezas JA, Gonzalez-Tarrio JM, Martin-Mola E, et al. Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. *The Journal of rheumatology*. 1997; 24(11):2069–75. Epub 1998/02/07. PMID: 9375862.
13. Hong S, Kim EJ, Lee EJ, San Koo B, Min Ahn S, Bae SH, et al. TNF-alpha confers resistance to Fas-mediated apoptosis in rheumatoid arthritis through the induction of soluble Fas. *Life sciences*. 2015; 122:37–41. Epub 2014/12/23. <https://doi.org/10.1016/j.lfs.2014.12.008> PMID: 25529148.
14. Salinas GF, De Rycke L, Barendregt B, Paramarta JE, Hreggvidstottir H, Cantaert T, et al. Anti-TNF treatment blocks the induction of T cell-dependent humoral responses. *Ann Rheum Dis*. 2013; 72(6):1037–43. Epub 2012/09/13. <https://doi.org/10.1136/annrheumdis-2011-201270> PMID: 22968102.
15. Carbone G, Wilson A, Diehl SA, Bunn J, Cooper SM, Rincon M. Interleukin-6 receptor blockade selectively reduces IL-21 production by CD4 T cells and IgG4 autoantibodies in rheumatoid arthritis. *International journal of biological sciences*. 2013; 9(3):279–88. Epub 2013/03/16. <https://doi.org/10.7150/ijbs.5996> PMID: 23493630; PubMed Central PMCID: PMC3596713.
16. Souto-Carneiro MM, Mahadevan V, Takada K, Fritsch-Stork R, Nanki T, Brown M, et al. Alterations in peripheral blood memory B cells in patients with active rheumatoid arthritis are dependent on the action of tumour necrosis factor. *Arthritis Res Ther*. 2009; 11(3):R84. <https://doi.org/10.1186/ar2718> PMID: 19500335.

17. Michelutti A, Gremese E, Morassi F, Petricca L, Arena V, Tolusso B, et al. B-cell subsets in the joint compartments of seropositive and seronegative rheumatoid arthritis (RA) and No-RA arthritides express memory markers and ZAP70 and characterize the aggregate pattern irrespectively of the auto-antibody status. *Mol Med*. 2011; 17(9–10):901–9. Epub 2011/05/25. <https://doi.org/10.2119/molmed.2011.00034> PMID: 21607290; PubMed Central PMCID: PMC3188870.
18. Mahmood Z, Muhammad K, Schmalzing M, Roll P, Dorner T, Tony HP. CD27-IgD- memory B cells are modulated by in vivo interleukin-6 receptor (IL-6R) blockade in rheumatoid arthritis. *Arthritis Res Ther*. 2015; 17:61. Epub 2015/04/19. <https://doi.org/10.1186/s13075-015-0580-y> PMID: 25888920; PubMed Central PMCID: PMC4415279.
19. Nakayamada S, Kubo S, Yoshikawa M, Miyazaki Y, Yunoue N, Iwata S, et al. Differential effects of biological DMARDs on peripheral immune cell phenotypes in patients with rheumatoid arthritis. *Rheumatology (Oxford)*. 2017. <https://doi.org/10.1093/rheumatology/kex012> PMID: 28371836.
20. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. *J Immunol*. 2007; 178(10):6624–33. PMID: 17475894.
21. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, et al. Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis and rheumatism*. 2008; 58(6):1762–73. <https://doi.org/10.1002/art.23498> PMID: 18512812.
22. Claes N, Fraussen J, Vanheusden M, Hellings N, Stinissen P, Van Wijmeersch B, et al. Age-Associated B Cells with Proinflammatory Characteristics Are Expanded in a Proportion of Multiple Sclerosis Patients. *J Immunol*. 2016; 197(12):4576–83. <https://doi.org/10.4049/jimmunol.1502448> PMID: 27837111.
23. Fecteau JF, Cote G, Neron S. A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. *J Immunol*. 2006; 177(6):3728–36. Epub 2006/09/05. PMID: 16951333.
24. Wu YC, Kipling D, Dunn-Walters DK. The relationship between CD27 negative and positive B cell populations in human peripheral blood. *Front Immunol*. 2011; 2:81. <https://doi.org/10.3389/fimmu.2011.00081> PMID: 22566870; PubMed Central PMCID: PMC3341955.
25. Berkowska MA, Heeringa JJ, Hajdarbegovic E, van der Burg M, Thio HB, van Hagen PM, et al. Human IgE(+) B cells are derived from T cell-dependent and T cell-independent pathways. *J Allergy Clin Immunol*. 2014; 134(3):688–97 e6. <https://doi.org/10.1016/j.jaci.2014.03.036> PMID: 24835500.
26. Wu YC, Kipling D, Leong HS, Martin V, Ademokun AA, Dunn-Walters DK. High-throughput immunoglobulin repertoire analysis distinguishes between human IgM memory and switched memory B-cell populations. *Blood*. 2010; 116(7):1070–8. <https://doi.org/10.1182/blood-2010-03-275859> PMID: 20457872; PubMed Central PMCID: PMC3341955.
27. Alivernini S, Kurowska-Stolarska M, Tolusso B, Benvenuto R, Elmesmari A, Canestri S, et al. Micro-RNA-155 influences B-cell function through PU.1 in rheumatoid arthritis. *Nat Commun*. 2016; 7:12970. <https://doi.org/10.1038/ncomms12970> PMID: 27671860.
28. Colonna-Romano G, Bulati M, Aquino A, Pellicano M, Vitello S, Lio D, et al. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev*. 2009; 130(10):681–90. <https://doi.org/10.1016/j.mad.2009.08.003> PMID: 19698733.
29. Bulati M, Buffa S, Martorana A, Gervasi F, Camarda C, Azzarello DM, et al. Double negative (IgG+IgD-CD27-) B cells are increased in a cohort of moderate-severe Alzheimer's disease patients and show a pro-inflammatory trafficking receptor phenotype. *J Alzheimers Dis*. 2015; 44(4):1241–51. <https://doi.org/10.3233/JAD-142412> PMID: 25408215.
30. Roll P, Muhammad K, Schumann M, Kleinert S, Tony HP. RF positivity has substantial influence on the peripheral memory B-cell compartment and its modulation by TNF inhibition. *Scandinavian journal of rheumatology*. 2012; 41(3):180–5. Epub 2012/03/10. <https://doi.org/10.3109/03009742.2011.645056> PMID: 22400711.
31. Li Z, Vermeire S, Bullens D, Ferrante M, Van Steen K, Noman M, et al. Anti-Tumor Necrosis Factor Therapy Restores Peripheral Blood B-cell Subsets and CD40 Expression in Inflammatory Bowel Diseases. *Inflammatory bowel diseases*. 2015. Epub 2015/09/19. <https://doi.org/10.1097/MIB.0000000000000554> PMID: 26383913.
32. Conigliaro P, Triggianese P, Perricone C, Chimenti MS, Di Muzio G, Ballanti E, et al. Restoration of peripheral blood natural killer and B cell levels in patients affected by rheumatoid and psoriatic arthritis during etanercept treatment. *Clinical and experimental immunology*. 2014; 177(1):234–43. Epub 2014/03/29. <https://doi.org/10.1111/cei.12335> PMID: 24666401; PubMed Central PMCID: PMC4089172.
33. Anolik JH, Ravikumar R, Barnard J, Owen T, Almudevar A, Milner EC, et al. Cutting edge: anti-tumor necrosis factor therapy in rheumatoid arthritis inhibits memory B lymphocytes via effects on lymphoid

- germinal centers and follicular dendritic cell networks. *J Immunol.* 2008; 180(2):688–92. Epub 2008/01/08. PMID: [18178805](https://pubmed.ncbi.nlm.nih.gov/18178805/).
34. Fekete A, Soos L, Szekanez Z, Szabo Z, Szodoray P, Barath S, et al. Disturbances in B- and T-cell homeostasis in rheumatoid arthritis: suggested relationships with antigen-driven immune responses. *Journal of autoimmunity.* 2007; 29(2–3):154–63. Epub 2007/09/11. <https://doi.org/10.1016/j.jaut.2007.07.002> PMID: [17826949](https://pubmed.ncbi.nlm.nih.gov/17826949/).
 35. Karampetsou MP, Andonopoulos AP, Liossis SN. Treatment with TNFalpha blockers induces phenotypical and functional aberrations in peripheral B cells. *Clin Immunol.* 2011; 140(1):8–17. Epub 2011/02/22. <https://doi.org/10.1016/j.clim.2011.01.012> PMID: [21334982](https://pubmed.ncbi.nlm.nih.gov/21334982/).
 36. Di Sabatino A, Rosado MM, Cazzola P, Biancheri P, Tinozzi FP, Laera MR, et al. Splenic function and IgM-memory B cells in Crohn's disease patients treated with infliximab. *Inflammatory bowel diseases.* 2008; 14(5):591–6. <https://doi.org/10.1002/ibd.20374> PMID: [18240280](https://pubmed.ncbi.nlm.nih.gov/18240280/).
 37. Kamphuis LS, van Zelm MC, Lam KH, Rimmelzwaan GF, Baarsma GS, Dik WA, et al. Perigranuloma localization and abnormal maturation of B cells: emerging key players in sarcoidosis? *Am J Respir Crit Care Med.* 2013; 187(4):406–16. <https://doi.org/10.1164/rccm.201206-1024OC> PMID: [23239158](https://pubmed.ncbi.nlm.nih.gov/23239158/).
 38. Timmermans WM, van Laar JA, van der Houwen TB, Kamphuis LS, Bartol SJ, Lam KH, et al. B-Cell Dysregulation in Crohn's Disease Is Partially Restored with Infliximab Therapy. *PloS one.* 2016; 11(7):e0160103. <https://doi.org/10.1371/journal.pone.0160103> PMID: [27468085](https://pubmed.ncbi.nlm.nih.gov/27468085/); PubMed Central PMCID: [PMC4965034](https://pubmed.ncbi.nlm.nih.gov/pmc/PMC4965034/).
 39. van der Houwen TB, van Hagen PM, Timmermans WM, Bartol SJ, Lam KH, Kappen JH, et al. Chronic signs of memory B cell activation in patients with Behcet's disease are partially restored by anti-tumour necrosis factor treatment. *Rheumatology (Oxford).* 2017; 56(1):134–44. <https://doi.org/10.1093/rheumatology/kew366> PMID: [27744360](https://pubmed.ncbi.nlm.nih.gov/27744360/).
 40. Roll P, Muhammad K, Schumann M, Kleinert S, Einsele H, Dorner T, et al. In vivo effects of the anti-interleukin-6 receptor inhibitor tocilizumab on the B cell compartment. *Arthritis and rheumatism.* 2011; 63(5):1255–64. Epub 2011/02/10. <https://doi.org/10.1002/art.30242> PMID: [21305508](https://pubmed.ncbi.nlm.nih.gov/21305508/).
 41. Kikuchi J, Hashizume M, Kaneko Y, Yoshimoto K, Nishina N, Takeuchi T. Peripheral blood CD4(+) CD25(+)CD127(low) regulatory T cells are significantly increased by tocilizumab treatment in patients with rheumatoid arthritis: increase in regulatory T cells correlates with clinical response. *Arthritis Res Ther.* 2015; 17:10. Epub 2015/01/22. <https://doi.org/10.1186/s13075-015-0526-4> PMID: [25604867](https://pubmed.ncbi.nlm.nih.gov/25604867/); PubMed Central PMCID: [PMC4332922](https://pubmed.ncbi.nlm.nih.gov/pmc/PMC4332922/).
 42. Chomarot P, Briolay J, Banchereau J, Miossec P. Increased production of soluble CD23 in rheumatoid arthritis, and its regulation by interleukin-4. *Arthritis and rheumatism.* 1993; 36(2):234–42. Epub 1993/02/01. PMID: [8431213](https://pubmed.ncbi.nlm.nih.gov/8431213/).
 43. Cambridge G, Perry HC, Nogueira L, Serre G, Parsons HM, De La Torre I, et al. The effect of B-cell depletion therapy on serological evidence of B-cell and plasmablast activation in patients with rheumatoid arthritis over multiple cycles of rituximab treatment. *Journal of autoimmunity.* 2014; 50:67–76. Epub 2013/12/25. <https://doi.org/10.1016/j.jaut.2013.12.002> PMID: [24365380](https://pubmed.ncbi.nlm.nih.gov/24365380/).
 44. Schmutz C, Hulme A, Burman A, Salmon M, Ashton B, Buckley C, et al. Chemokine receptors in the rheumatoid synovium: upregulation of CXCR5. *Arthritis Res Ther.* 2005; 7(2):R217–29. <https://doi.org/10.1186/ar1475> PMID: [15743468](https://pubmed.ncbi.nlm.nih.gov/15743468/).
 45. Jones JD, Hamilton BJ, Challener GJ, de Brum-Fernandes AJ, Cossette P, Liang P, et al. Serum C-X-C motif chemokine 13 is elevated in early and established rheumatoid arthritis and correlates with rheumatoid factor levels. *Arthritis Res Ther.* 2014; 16(2):R103. Epub 2014/04/29. <https://doi.org/10.1186/ar4552> PMID: [24766912](https://pubmed.ncbi.nlm.nih.gov/24766912/); PubMed Central PMCID: [PMC4060390](https://pubmed.ncbi.nlm.nih.gov/pmc/PMC4060390/).
 46. Manzo A, Vitolo B, Humby F, Caporali R, Jarrossay D, Dell'accio F, et al. Mature antigen-experienced T helper cells synthesize and secrete the B cell chemoattractant CXCL13 in the inflammatory environment of the rheumatoid joint. *Arthritis and rheumatism.* 2008; 58(11):3377–87. <https://doi.org/10.1002/art.23966> PMID: [18975336](https://pubmed.ncbi.nlm.nih.gov/18975336/).
 47. Takagi R, Higashi T, Hashimoto K, Nakano K, Mizuno Y, Okazaki Y, et al. B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. *J Immunol.* 2008; 181(1):186–9. PMID: [18566383](https://pubmed.ncbi.nlm.nih.gov/18566383/).
 48. Bugatti S, Manzo A, Benaglio F, Klersy C, Vitolo B, Todoerti M, et al. Serum levels of CXCL13 are associated with ultrasonographic synovitis and predict power Doppler persistence in early rheumatoid arthritis treated with non-biological disease-modifying anti-rheumatic drugs. *Arthritis Res Ther.* 2012; 14(1):R34. <https://doi.org/10.1186/ar3742> PMID: [22336440](https://pubmed.ncbi.nlm.nih.gov/22336440/); PubMed Central PMCID: [PMC3392832](https://pubmed.ncbi.nlm.nih.gov/pmc/PMC3392832/).
 49. Kobayashi S, Murata K, Shibuya H, Morita M, Ishikawa M, Furu M, et al. A distinct human CD4+ T cell subset that secretes CXCL13 in rheumatoid synovium. *Arthritis and rheumatism.* 2013; 65(12):3063–72. <https://doi.org/10.1002/art.38173> PMID: [24022618](https://pubmed.ncbi.nlm.nih.gov/24022618/).

50. Loza E, Tinture T, Sanchez-Ibarrola A. CD5 and CD23 expression on B cells in peripheral blood and synovial fluid of rheumatoid arthritis patients: relationship with interleukin-4, soluble CD23 and tumour necrosis factor alpha levels. *Rheumatology (Oxford)*. 1999; 38(4):325–8. PMID: [10378709](#).
51. Cantaert T, Doorenspleet ME, Francosalinas G, Paramarta JE, Klarenbeek PL, Tiersma Y, et al. Increased numbers of CD5+ B lymphocytes with a regulatory phenotype in spondylarthritis. *Arthritis and rheumatism*. 2012; 64(6):1859–68. <https://doi.org/10.1002/art.34364> PMID: [22231660](#).
52. Engelmann R, Wang N, Kneitz C, Muller-Hilke B. Bone resorption correlates with the frequency of CD5 (+) B cells in the blood of patients with rheumatoid arthritis. *Rheumatology (Oxford)*. 2015; 54(3):545–53. <https://doi.org/10.1093/rheumatology/keu351> PMID: [25193807](#).
53. Kanswal S, Katsenelson N, Selvapandiyan A, Bram RJ, Akkoyunlu M. Deficient TACI expression on B lymphocytes of newborn mice leads to defective Ig secretion in response to BAFF or APRIL. *J Immunol*. 2008; 181(2):976–90. PMID: [18606649](#).
54. von Bulow GU, van Deursen JM, Bram RJ. Regulation of the T-independent humoral response by TACI. *Immunity*. 2001; 14(5):573–82. PMID: [11371359](#).
55. Castigli E, Wilson SA, Elkhali A, Ozcan E, Garibyan L, Geha RS. Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation. *J Allergy Clin Immunol*. 2007; 120(4):885–91. <https://doi.org/10.1016/j.jaci.2007.06.012> PMID: [17689597](#); PubMed Central PMCID: [PMCPMC2121612](#).
56. Sakurai D, Kanno Y, Hase H, Kojima H, Okumura K, Kobata T. TACI attenuates antibody production costimulated by BAFF-R and CD40. *European journal of immunology*. 2007; 37(1):110–8. <https://doi.org/10.1002/eji.200636623> PMID: [17154264](#).
57. Catalan D, Aravena O, Sabugo F, Wurmman P, Soto L, Kalergis AM, et al. B cells from rheumatoid arthritis patients show important alterations in the expression of CD86 and FcγRIIb, which are modulated by anti-tumor necrosis factor therapy. *Arthritis Res Ther*. 2010; 12(2):R68. Epub 2010/04/20. <https://doi.org/10.1186/ar2985> PMID: [20398308](#); PubMed Central PMCID: [PMC2888223](#).
58. Rapetti L, Chavele KM, Evans CM, Ehrenstein MR. B cell resistance to Fas-mediated apoptosis contributes to their ineffective control by regulatory T cells in rheumatoid arthritis. *Ann Rheum Dis*. 2015; 74(1):294–302. Epub 2013/11/20. <https://doi.org/10.1136/annrheumdis-2013-204049> PMID: [24249811](#).
59. Smeets TJ, Kraan MC, van Loon ME, Tak PP. Tumor necrosis factor alpha blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial tissue. *Arthritis and rheumatism*. 2003; 48(8):2155–62. Epub 2003/08/09. <https://doi.org/10.1002/art.11098> PMID: [12905468](#).
60. Canete JD, Celis R, Moll C, Izquierdo E, Marsal S, Sanmarti R, et al. Clinical significance of synovial lymphoid neogenesis and its reversal after anti-tumour necrosis factor alpha therapy in rheumatoid arthritis. *Ann Rheum Dis*. 2009; 68(5):751–6. Epub 2008/05/23. <https://doi.org/10.1136/ard.2008.089284> PMID: [18495732](#).
61. Kanbe K, Chen Q, Nakamura A, Hobo K. Inhibition of MAP kinase in synovium by treatment with tocilizumab in rheumatoid arthritis. *Clinical rheumatology*. 2011; 30(11):1407–13. Epub 2011/09/13. <https://doi.org/10.1007/s10067-011-1833-z> PMID: [21909699](#).
62. Snir A, Kessel A, Haj T, Rosner I, Slobodin G, Toubi E. Anti-IL-6 receptor antibody (tocilizumab): a B cell targeting therapy. *Clinical and experimental rheumatology*. 2011; 29(4):697–700. Epub 2011/08/05. PMID: [21813064](#).
63. Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, Scott ML, et al. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. *J Immunol*. 2004; 173(2):807–17. PMID: [15240667](#).
64. Vasquez C, Franco MA, Angel J. Rapid Proliferation and Differentiation of a Subset of Circulating IgM Memory B Cells to a CpG/Cytokine Stimulus In Vitro. *PloS one*. 2015; 10(10):e0139718. Epub 2015/10/07. <https://doi.org/10.1371/journal.pone.0139718> PMID: [26439739](#); PubMed Central PMCID: [PMC4595470](#).
65. Yang J, Zhao S, Yang X, Zhang H, Zheng P, Wu H. Inhibition of B-cell apoptosis is mediated through increased expression of Bcl-2 in patients with rheumatoid arthritis. *International journal of rheumatic diseases*. 2015. Epub 2015/07/16. <https://doi.org/10.1111/1756-185X.12706> PMID: [26176566](#).
66. Parker A, Izmailova ES, Narang J, Badola S, Le T, Roubenoff R, et al. Peripheral blood expression of nuclear factor-kappaB-regulated genes is associated with rheumatoid arthritis disease activity and responds differentially to anti-tumor necrosis factor-alpha versus methotrexate. *The Journal of rheumatology*. 2007; 34(9):1817–22. Epub 2007/08/19. PMID: [17696278](#).
67. Magnusson SE, Engstrom M, Jacob U, Ulfgren AK, Kleinau S. High synovial expression of the inhibitory FcγRIIb in rheumatoid arthritis. *Arthritis Res Ther*. 2007; 9(3):R51. Epub 2007/05/25. <https://doi.org/10.1186/ar2206> PMID: [17521421](#); PubMed Central PMCID: [PMC2206344](#).

68. Gottenberg JE, Miceli-Richard C, Ducot B, Goupille P, Combe B, Mariette X. Markers of B-lymphocyte activation are elevated in patients with early rheumatoid arthritis and correlated with disease activity in the ESPOIR cohort. *Arthritis Res Ther*. 2009; 11(4):R114. <https://doi.org/10.1186/ar2773> PMID: [19627580](https://pubmed.ncbi.nlm.nih.gov/19627580/).
69. Angelin-Duclos C, Cattoretti G, Lin KI, Calame K. Commitment of B lymphocytes to a plasma cell fate is associated with Blimp-1 expression in vivo. *J Immunol*. 2000; 165(10):5462–71. Epub 2000/11/09. PMID: [11067898](https://pubmed.ncbi.nlm.nih.gov/11067898/).