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Abatacept modulates CD80 and CD86 expression and memory formation in human B-cells

Raquel Lorenzetti[a], Iga Janowska[a], Cristian Roberto Smulski[b], Natalie Frede[a], Nadine Henneberger[a], Lea Walter[a], Marei-Theresa Schleyer[a], Janika M. Hüppe[a], Julian Staniek[a], Ulrich Salzer[a], Ana Venhoff[a], Arianna Troilo[a], Reinhard Edmund Voll[a], Nils Venhoff[a], Jens Thiel[a,1], Marta Rizzi[a,*,1]

[a] Department of Rheumatology and Clinical Immunology, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg im Breisgau, Baden-Württemberg, 79106, Germany

[b] Medical Physics Department, Centro Atómico Bariloche and Instituto Balseiro – CONICET, Av. E. Bustillo 9500, San Carlos de Bariloche, 8400, Río Negro, Argentina

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ABSTRACT

Background: Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits T-cell activation and is expressed on T-regulatory cells. Human CTLA-4 deficiency results in severe immune dysregulation. Abatacept (CTLA-4 Ig) is approved for the treatment of rheumatoid arthritis (RA) and its mechanism of action is attributed to effects on T-cells. It is known that CTLA-4 modulates the expression of its ligands CD80 and CD86 on antigen presenting cells (APC) by transendocytosis. As B-cells express CD80/CD86 and function as APC, we hypothesize that B-cells are a direct target of abatacept.

Objectives: To investigate direct effects of abatacept on human B-lymphocytes *in vitro* and in RA patients.

Methods: The effect of abatacept on healthy donor B-cells' phenotype, activation and CD80/CD86 expression was studied *in vitro*. Nine abatacept-treated RA patients were studied. Seven of these were followed up to 24 months, and two up to 12 months only and treatment response, immunoglobulins, ACPA, RF concentrations, B-cell phenotype and ACPA-specific switched memory B-cell frequency were assessed.

Results: B-cell development was unaffected by abatacept. Abatacept treatment resulted in a dose-dependent decrease of CD80/CD86 expression on B-cells *in vitro*, which was due to dynamin-dependent internalization. RA patients treated with abatacept showed a progressive decrease in plasmablasts and serum IgG. While ACPA-titers only moderately declined, the frequency of ACPA-specific switched memory B-cells significantly decreased.

Conclusions: Abatacept directly targets B-cells by reducing CD80/CD86 expression. Impairment of antigen presentation and T-cell activation may result in altered B-cell selection, providing a new therapeutic mechanism and a base for abatacept use in B-cell mediated autoimmunity.

1. Introduction

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is mainly expressed on activated T-cells and regulatory T-cells. CTLA-4 binds to the costimulatory molecules CD80 and CD86, expressed on antigen presenting cells (APC). CTLA-4 outcompetes CD28, because of his higher affinity for CD80/CD86[1], and prevents T-cell costimulation. In humans, CTLA-4 deficiency results in a complex immune dysregulation syndrome comprising hypogammaglobulinemia, granulomatous disease, autoimmunity, enteropathy and extensive organ CD4 T-cell infiltration [2]. In these patients, T regulatory cells fail to control the expression of CD80 and CD86 on APCs [3]. Additionally, patients show a progressive decrease in B-cell numbers, especially class-switched memory cells, probably because of cell exhaustion due to persistent T-cell-dependent stimulation [1,2]. There is an expansion of B-cells with low expression of the complement co-receptor CD21 ($CD21^{Low}$), characterized by reduced responsiveness to BCR-engagement and increased apoptosis [4,5]. $CD21^{Low}$ B-cells are also increased in inflammatory diseases like rheumatoid arthritis (RA) [6]. Autoimmune manifestations are common in CTLA-4 deficiency [2], with autoimmune arthritis

* Corresponding author. Department of Internal Medicine, Clinic for Rheumatology and Clinical Immunology University Medical Center Freiburg, Hugstetter Strasse 55, 79106, Freiburg, Germany.

E-mail address: marta.rizzi@uniklinik-freiburg.de (M. Rizzi).

[1] contributed equally.

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reported in 14% of patients. Importantly, CTLA-4 variants have been associated with increased susceptibility for RA [7] underlining the importance of CTLA-4 in maintenance of immune tolerance. CTLA-4 Ig (abatacept) is successfully used to treat autoimmune diseases and approved for the treatment of RA [8,9]. Abatacept is a recombinant fusion protein of CTLA-4 with the Fc portion of human IgG1 comprised of hinge region, CH2 and CH3 domains modified to prevent complement fixation and antibody-dependent cellular cytotoxicity (ADCC) [8]. By binding to CD80 and CD86, abatacept inhibits the costimulation and activation of T-cells, resulting in downregulation of inflammatory mediators. Abatacept impairs the adhesion and migratory capacity of monocytes [10], the inflammatory activity of synovial macrophages [11] and induces activation of indoleamine 2,3-dioxygenase (IDO) in dendritic cells [12–14]. Little is known about the effect of abatacept on B-lymphocyte which play an important role in the pathophysiology of RA [15,16]. They are recruited to the synovia, secrete pro-inflammatory mediators and present antigens to T-cells. Autoreactive B-cells can produce rheumatoid factor (RF) and anti-citrullinated peptide autoantibodies (ACPA). ACPA emerged as sensitive and specific serological markers of RA, and are associated with more severe disease [15]. ACPA may contribute to pathology promoting osteoclastogenesis, leading to bone erosions [17].

Upon activation, B-cells express CD80 and CD86, which can activate T-cells by binding to CD28. Activated T-cells not only express CD28 but also CTLA-4. The interaction between T- and B-cells within the germinal center, is crucial for antibody somatic hypermutation and affinity maturation and for the differentiation of B-cells into either memory cells or plasmacells. CD86 is up-regulated on B-cells upon engagement of the BCR [18], CD40 [19], IL-4R [20] or TLR4 [21]. *In vitro* data show that the short cytoplasmic domain of CD86 can be phosphorylated upon activation [22], and concomitant treatment of murine B-cells with anti-CD86 antibody and stimulation of CD40 or IL-4R can increase the production of IgG1 and IgE [23,24]. In contrast, agonistic anti-CD80 treatment impaired proliferation and IgG secretion of normal B-cells and B-cell lymphomas [25]. Whether binding of abatacept to CD80 and CD86 in B-cells induces intracellular signaling is unknown. While it has been reported that during 6 months of abatacept treatment the distribution of B-cell subpopulations remained stable [26], other reports demonstrated that the clinical efficacy is paralleled by a progressive decrease in B-cell numbers [27]. In summary, even though B-lymphocytes upregulate and express CD80 and CD86, the consequences of binding of abatacept to B-cells has not been studied.

We assessed the direct effect of abatacept on B-cell differentiation using *in vitro* models and investigated the dynamics of abatacept binding to CD80 and CD86 on B-cells. Furthermore, we studied the *in vivo* effect of abatacept on B-lymphocyte subpopulations and on the frequency of ACPA-specific memory B-cells in RA patients.

2. Materials and methods

2.1. Healthy donors and patients

Buffy coats were purchased from the Blood Bank of the University Medical Center Freiburg (approval of the Ethic Committee of the Freiburg University: 147/15). The study included patients with moderate to severe seropositive RA that received abatacept (ORENCIA®; Bristol-Myers Squibb) treatment, and had at least a 12 months follow-up, and provided informed consent. The study was approved by the Ethic Committee of the Freiburg University (507/16) and registered in the German registry of clinical studies (DRKS00012864).

2.2. Cell isolation and cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats or blood by density gradient centrifugation. B-cells were isolated with the EasySep™ Human B-cell Isolation Kit (Stemcell Technologies) following manufacturers' instructions. B-cells were stimulated in U96 well at 30.000 cells per well with CD40L and IL-21 (as described in detail in supplemental material) in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies) supplemented with 10% FCS, insulin, apo-transferrin, non-essential amino acids, glutamine and glutathione as described [28]. Abatacept was dialyzed with a 2.000 MWCO membrane and for specific use was conjugated with either FITC, or APC or biotin (Innova Biosciences Lightning-Link® kit) and used at concentration between 0, 5 and 5000 ng/ml as indicated.

2.3. Flow cytometry

Phenotype of *in vitro* cultivated B-cells or of RA patients PBMCs was determined at indicated time points by flow cytometry (FACS Canto II, BD Biosciences) with the antibodies listed in supplemental table 1. Dead cell exclusion was performed by Zombie NIR/Aqua Fixable Viability Kit (Biolegend). Measurement of phosphorylated protein and of intracellular calcium release upon activation was performed as described in supplemental material.

2.4. Determination of autoantibodies and immunoglobulin concentrations

ACPA antibodies (Euroimmun, Germany) and total immunoglobulin (Ig) isotype concentrations were quantified by ELISA. Briefly, 96-well plates (Nunc Maxisorp) were coated with anti-human Ig mix (Jackson ImmunoResearch) in bicarbonate buffer. Bound Ig were detected with alkaline phosphatase-conjugated anti-human IgM and IgA (Jackson ImmunoResearch), and developed with p-nitrophenyl phosphate (Sigma-Aldrich) in DEA buffer. Ig concentrations were calculated by the interpolation of calibration curves generated by using a Ig standard (N Protein Standard SL; Siemens). RF and C-reactive protein (CRP) were quantified by nephelometry (Dade Behring BN ProSpec Nephelometer, Siemens AG, Germany).

2.5. CD80 and CD86 mRNA expression

Quantitative PCR for CD80 and CD86 in activated B cells treated with 5000 ng/ml of abatacept was performed as described in supplemental material.

2.6. Generation of BJAB CD80^{-} or CD86-deficient cell lines

Human Burkitt lymphoma B (BJAB) cell line deficient for CD80 (CD80 KO) or CD86 (CD86 KO) or both (CD80/CD86 KO) were generated as described in supplemental material.

2.7. CD86 and CD80 receptor internalization

BJAB wild type (WT), CD80 KO and CD86 KO cells were treated with abatacept (10 μg/ml) for 2 h and CD86 and CD80 expression assessed by flow cytometry prior removal of bound abatacept by acidic elution. When indicated, cells were pre-incubated with Dynasore, a dynamin inhibitor (25 μg/ml, Sigma-Aldrich) [29] for 30 min, followed by incubation with biotinylated abatacept (10 μg/ml) at 37 °C for two or 16 h. Staining with streptavidin-FITC (DAKO) revealed the amount of CTLA-4 Ig that remained on the surface. For microscopy, BJAB WT cells were seeded on polylysine (0.5 μg/ml) coated Nunc™ Lab-Tek™ II Chamber Slide™. Subsequently, cells were treated with 1 or 5 μg/ml of APC- or FITC-labeled CTLA-4 Ig at 37 °C or 4 °C for indicated time, with or without dynamin inhibitor pre-treatment. Cells were then fixed with 4% paraformaldehyde. DAPI staining allowed nuclei localization. Data acquisition was performed with confocal laser scanning microscopy (CLSM, Leica TCS SP2 AOBS, Germany) with a 63× water immersion objective (HCX PL APO/bd. BL 63.0 × 1.2 W, Leica, Germany). Fluorescence intensity was measured in membrane and cytoplasmic areas delineated using the freehand tool of the ImageJ software.

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2.8. Analysis of ACPA specific IgG memory B-cells frequency

PBMCs from patients treated with abatacept were stained to assess the frequency of IgG positive memory cells, then seeded at scalar cell densities (1×10^5, 3×10^4 or 1×10^4 cells/well in 96 well plates) and stimulated with CpG ODN (Apara Bioscience) (0,5 μM) and IL-2 (1000 IU/ml) as described [30]. After nine days supernatants were screened for ACPA IgG antibodies (Orgentec Diagnostika). Supernatants were considered positive when the OD was above the mean of background wells plus three times the standard deviation. The frequency of the ACPA IgG^+ per 10^6 IgG^+ cells was calculated by the ratio between the number of positive clones and the total IgG^+ cells plated, multiplied by 10^6.

2.9. Statistical analysis

Statistical analysis was done using GraphPad Prism 5 (GraphPad Software, La Jolla, CA), using Wilcoxon analysis and Student's t-test. Data are reported in median or mean as detailed in each graph.

3. Results

3.1. Normal in vitro B-cell development in the presence of abatacept

The direct effect of abatacept on human B-cell development was studied *in vitro*. The addition of abatacept to the T-dependent stimulus (CD40L and IL-21) did not change the proportion of switched memory cells (Fig. 1A, figure S1A,B) and plasmablasts (Fig. 1A, figure S1C) obtained from primary human B-cells. Also the absolute numbers of cells were unchanged (Figure S1D), indicating that abatacept did not induce toxicity or impair cell proliferation. Moreover, IgM and IgA secretion in the supernatant of culture was similar in all conditions (Fig. 1B), IgG secretion could not be tested as abatacept, present in culture, has a human IgG1 tail.

3.2. Reduction in surface expression of CD86 and CD80 on human B-cells in the presence of abatacept

Ex vivo isolated B-cells progressively upregulated CD86 and CD80 after CD40L and IL-21 activation with a peak of expression at day 2 (Fig. 2A). Binding of abatacept to CD80 and CD86 hinders their detection by flow cytometry. Hence, to study the absolute expression levels of CD80 and CD86, we performed acidic elution of bound abatacept prior cell staining (figure S2A,B). The expression of CD80 was significantly lower compared to controls in presence of 50 ng/ml of abatacept (Fig. 2A). CD86 expression was decreased by abatacept treatment, although not significantly (Fig. 2A), reflecting the higher affinity of abatacept for CD80 compared to CD86. The decreased expression of CD80 and CD86 was not due to reduced transcription (Fig. 2B), pointing to a regulation at the protein level. To study the fate of abatacept bound to its ligand on the B-cell surface, we incubated the human BJAB-cell line that expresses high levels of both CD80 and CD86 (figure S2C) with fluorescently-labeled abatacept. At 4 °C abatacept remained on the cell membrane (Fig. 2C and D), as physiological processes are strongly decelerated. However, incubation at 37 °C resulted in internalization of abatacept (Fig. 2C and D). The quantification of this process, calculating the ratio between membrane-localized and cytoplasmic fluorescent abatacept, revealed that a significant amount of abatacept was internalized at 37 °C (Fig. 2E). Binding and internalization were specific to the interaction between abatacept and its ligands, as a BJAB-cell line deficient for both CD80 and CD86 was devoid of any fluorescent signal (figure S2C-E). To assess the relative contribution of CD80 and CD86 to the internalization of abatacept we generated BJAB-cell lines deficient for either CD80 (CD80 KO) or CD86 (CD86 KO) (figure S2C). Both CD80 KO and CD86 KO cells were still able to bind abatacept (figure S2D). Overnight incubation with abatacept at 37 °C, followed by acidic elution, resulted in significant reduction of CD80 expression both in wild type and in CD86 KO cell lines (Fig. 2F). Similarly to primary cells, the expression of CD86 remained unchanged in the wild type cells, but was significantly reduced in the CD80 KO cells (Fig. 2G). These data indicate that abatacept can bind to both CD80 and CD86 and induce their internalization. Vesicular internalization is dependent on clathrin and the formation of vesicles. In BJAB-cells incubated with fluorescently-labeled abatacept, the formation of vesicles was inhibited using a dynamin inhibitor. Confocal microscopy studies showed that the inhibition of dynamin resulted in the permanence of abatacept on the cell surface (Fig. 2H). In a complementary approach we incubated BJAB-cells with biotin-conjugated abatacept. Upon overnight incubation in the presence or absence of a dynamin inhibitor, the amount of abatacept bound to the cell surface was assessed by streptavidin-FITC staining. In line with our microscopic analysis, dynamin inhibition resulted in permanence of abatacept on the cell surface (Fig. 2I).

Class switched memory formation requires T-cell help. To address the impact of reduced B cell CD80 and CD86 expression on T-cell activation, we performed mixed lymphocyte reaction (MLR) using non-HLA matched B- and CD4 T-cells. B-cells were activated with CD40L in presence or absence of abatacept, and then used to stimulate CD4 T-cells from a second donor (figure S3 A,B). T-cell activation was impaired when B-cells were pre-incubated with abatacept (figure S3 C-E), as indicated by the significantly higher proportion of non-proliferating T-cells (division 1) compared to control. As expected, T-cell proliferation was even more impaired when abatacept was maintained in the MLR culture (Figure S3 B,D,F). Hence the reduction of costimulatory molecules on B-cells may results in inefficient T-cell activation. This may become relevant in a competitive setting like the germinal center where B-cells need T-cell help to be selected.

3.3. Abatacept fails to induce signaling in activated human B-cells

We sought to investigate signaling events in B-cells upon CD80 and CD86 engagement by abatacept. CD80 and CD86 positive B-cells

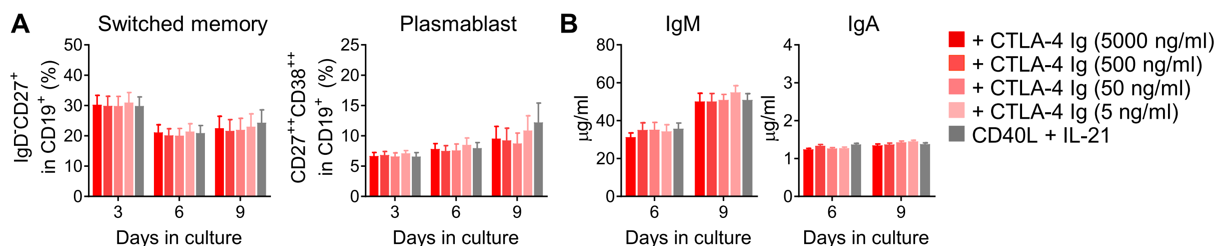

Fig. 1. Normal B-cell development *in vitro* in presence of abatacept. Isolated human B-cells were stimulated with CD40L + IL-21 in presence or absence of abatacept (CTLA-4 Ig) at indicated concentrations and B-cell subpopulations were analyzed after 3, 6 and 9 days by flow cytometry. **A** Bars indicate the percentage of IgD^-CD27^+ switched memory, or the $CD27^{++}CD38^{++}$ plasmablast cells within the live/CD19 gate. **B** Concentration of IgM and IgA in supernatant measured by ELISA. Data are represented as mean ± SEM of 4 independent experiments, each in triplicate. Analyzed with two-tailed paired Student's *t*-test.

Fig. 2. Decrease of surface CD80 and CD86 upon treatment with abatacept. B-cells were stimulated with CD40L + IL-21 in presence of CTLA-4 Ig: **A** Mean fluorescence intensity (MFI) of CD80 and CD86 by flow cytometry (mean ± SEM, 5 independent experiments, Two-tailed unpaired Student's *t*-test; $*p < 0.01$). **B** qPCR of CD80 and CD86 mRNA of CD40L + IL-21 ± CTLA-4 Ig stimulated B cells (mean ± SD, each dot represents one individual experiment). **C** Confocal microscopy of BJAB WT cells incubated at 4 °C or at 37 °C for 2 h with CTLA-4 Ig APC. **D** Quantification of fluorescence intensity (A.U., arbitrary units) along the yellow line in **C** in cells incubated at 4 °C or 37 °C, (M = plasma membrane, C = cytoplasm). **E** Ratio of cytoplasm/membrane fluorescence intensity of CTLA-4 Ig APC internalization in BJAB WT. Each dot represent a different point within a cell. Two-tailed unpaired Student's *t*-test ($****p < 0.0001$). **F/G** BJAB WT or CD86 or CD80 knock out (KO) cells were incubated with CTLA-4 Ig. Acidic elution was performed prior staining for CD80 and CD86. Each dot represent an independent experiment, lines are mean ± SEM. Two-tailed paired Student's *t*-test ($*p < 0.05$). **H** Confocal microscopy of BJAB WT incubated for 3 or 6 h with FITC-labeled CTLA-4 Ig (green) in presence or absence of dynamin-inhibitor. DAPI staining in blue. **I** BJAB-cells incubated with or without dynamin inhibitor, overnight at 37 °C in presence of CTLA-4 Ig biotin. Surface CTLA-4 Ig was stained with SA-FITC and analyzed by flow cytometry. Two independent experiments, performed in triplicate. Lines are mean ± SEM. Two-tailed paired Student's *t*-test ($****p < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

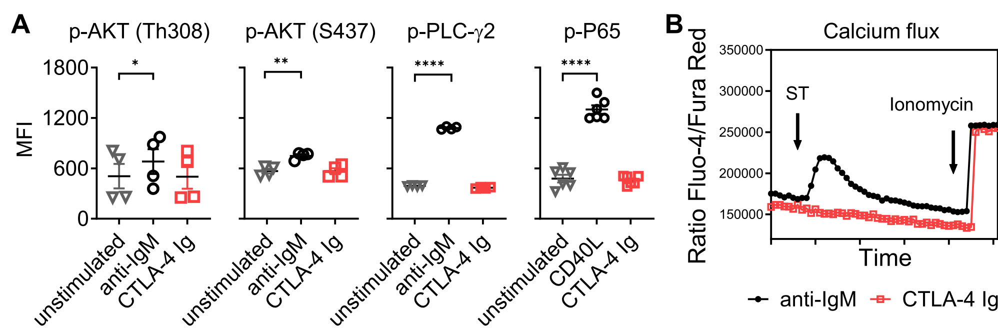

Fig. 3. Abatacept does not induce PLC-γ2, Akt or NF-kB phosphorylation. **A** Activated B-cells were stimulated with CTLA-4 Ig (0.5 μg/ml) or adequate positive control. Each dot represents an independent experiment. Lines represent mean ± SEM. Two-tailed paired Student's *t*-test. **B** Induction of calcium flux in CD40L-activated B-cells after stimulation with CTLA-4 Ig (10 μg/ml), or anti-IgM (10 μg/ml).

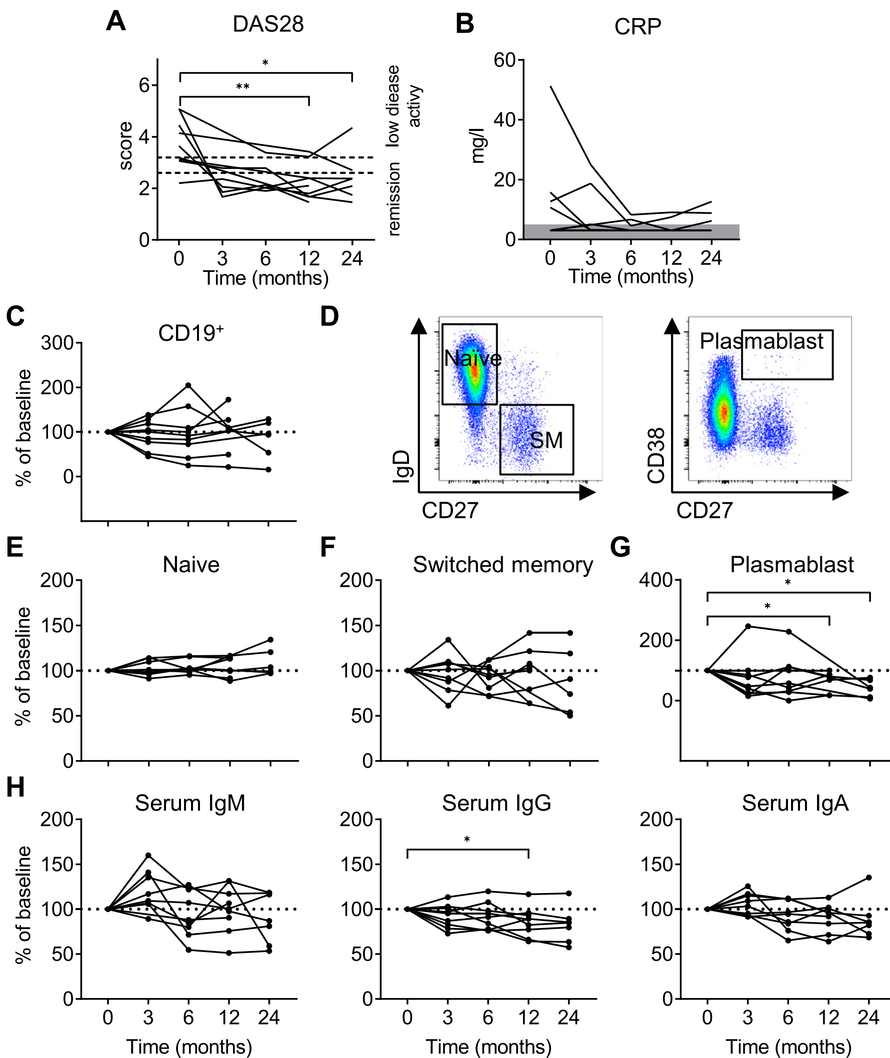

Fig. 4. Clinical response, reduction in plasmablasts and serum IgG in abatacept treated RA patients. Nine abatacept-treated RA patients were studied. Seven of these were followed up to 24 months, and two up to 12 months only. **A** DAS28. Disease remission: < 2,6; low disease activity:2,6-3,2. Wilcoxon matched-pairs signed rank test (*$p < 0.05$; **$p < 0.01$) **B** CRP levels. Shaded grey: normal range. **C** Frequency of CD19$^+$ B-cells by flow cytometry in PBMCs. Data are represented as percentage of baseline values. **D** Gating strategy for B-cell subpopulations based on IgD/CD27 and CD27/38 expression. Effect of abatacept on circulating B-cell differentiation subsets: **E** naïve cells, **F** switched memory cells, **G** plasmablasts, **N** serum immunoglobulins (IgM, IgG and IgA) shown in percentage of baseline. Wilcoxon matched-pairs signed rank test (*$p < 0.05$), only significant differences are indicated.

phosphorylated Akt and PLCγ2 (Fig. 3A) upon B-cell receptor activation, but not in response to abatacept. Similarly, CD40L stimulation induced NF-κB activation, with phosphorylation of P65, while abatacept was unable to directly activate the NF-κB signaling pathway (Fig. 3A). Accordingly, we were not able to detect calcium release within B-cells in response to abatacept (Fig. 3B). Hence, in primary human B-cells we were not able to detect the published reverse signaling of CD80 and CD86 in response to abatacept.

3.4. Abatacept treatment in vivo results in clinical response, reduced plasmablast counts and serum IgG

We assessed the effect of abatacept on disease activity, inflammation markers and autoantibodies in nine RA patients. Of these 9 patients, seven were followed for 24 months, and the remaining two for 12 months only (Fig. 4, Table 1). All patients responded to treatment with reduction in DAS28 (Fig. 4A) and CRP concentrations (Fig. 4B). Leucocytes and lymphocytes numbers did not significantly decrease (figure S3A). At six months, in four out of nine patients we observed a reduction in B-cell numbers compared to baseline (Fig. 4C). We analyzed the changes in distribution of B-cell subpopulations. Naïve B-cells remained stable over time (Fig. 4D and E). Switched memory cells were only slightly reduced especially after 24 months of treatment, albeit not significantly (Fig. 4D,F). Plasmablasts showed a significant decrease after 12 and 24 months of treatment (Fig. 4D,G). The proportion of plasma blasts in blood did not correlate with DAS28 or CRP levels

Table 1
Baseline characteristics of rheumatoid arthritis patients treated with abatacept.

Patients' characteristics (n = 9)	
Age, mean (range) in years	61 (43–73)
Sex, no. (%)	
Female	5 (56)
Male	4 (44)
Smoker, no. (%)	2 (22)
BMI, mean (range) in kg/m^2	28 (21.2–40.8)
Disease duration, mean (range) in months	37 (8–120)
DAS28, mean (S.D.)	4 (0.99)
CRP, mean (S.D.) in mg/l	12 (15.59)
RF positive, no. (%) in IU/ml	7 (78)
ACPA positive, no. (%)	9 (100)
Prednisone, no. (%)	7 (78)
Prednisone dose, mean (range) in mg/day at baseline	6.5 (2.5–10)
Methotrexate, no. (%)	9 (100)
Methotrexate dose, mean (range) in mg/week at baseline	12 (10–15)

AbbreviationsBMI = body mass index; CRP = C-reactive protein; ACPA = anti-citrullinated peptide antibodies; RF = rheumatoid factor; DAS28 = disease activity score in 28 joints, S.D. = standard deviation.

(Figure S4B), even though all parameters decreased compared to the respective baseline upon treatment. We did not find significant differences in the frequency of IgM-, IgG- and IgA-positive cells (figure S4C-E). IgG1 and IgG3 expressing B-cells decreased in most of the abatacept-treated patients (figure S4F,G). We observed a relative increase of IgG4

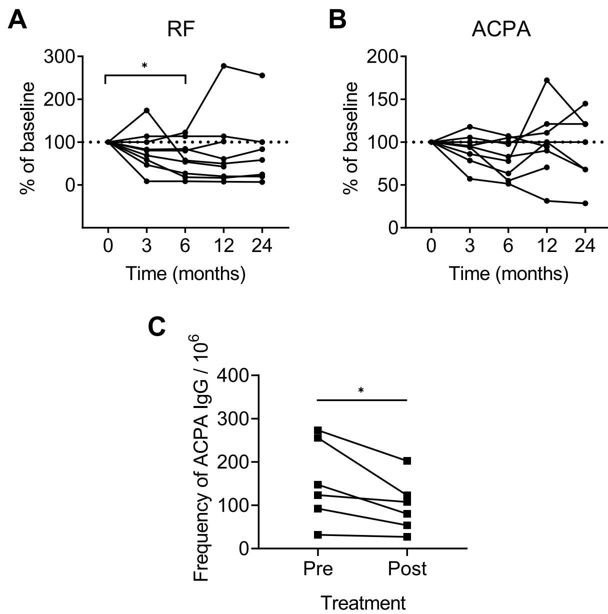

Fig. 5. Abatacept treatment results in reduction of autoantibodies and of frequency of ACPA-specific switched memory B-cells. **A** Changes in serum rheumatoid factor (RF) in percentage of baseline. **B** Changes in serum anti-citrullinated peptide antibody (ACPA) shown in percentage of baseline. Each line represents one patient. Wilcoxon matched-pairs signed rank test (*$p < 0.05$). **C** Frequency of IgG^+ memory cells specific for citrullinated peptides was analyzed by limiting dilution assays before and after (6 or 12-months) abatacept treatment. Each line represents one patient. Wilcoxon matched-pairs signed rank test (*$p < 0.05$).

memory cells in five out of nine patients after 6 months of abatacept treatment (figure S4H). The significant reduction in plasmablasts numbers at 12-months was accompanied by a significant reduction in serum IgG compared to baseline (Fig. 4H), IgA was only slightly reduced at 12 and 24 months in most patients, and serum IgM was reduced in four out of nine patients at 12 months and in four out of seven at 24 months (Fig. 4H).

3.5. Abatacept treatment results in reduced frequency of self-reactive ACPA-specific memory cells

Six months of abatacept treatment resulted in significant reduction of serum titers of RF (Fig. 5A), and in reduction of ACPA titers in six out of nine patients (Fig. 5B). Two patients seroconverted to ACPA-negative status. As described above, plasmablast counts were significantly reduced after 12- and 24-months of treatment. Plasmablast and plasma cells are not the only source of autoantibodies, as activation of memory cells constitute a supply of antibody secreting cells. Hence, we studied the variation in frequency of ACPA-specific memory B-cells in response to abatacept treatment. Stimulation via TLR9 induces Ig secretion from memory cells, without efficiently inducing class switch [30]. Hence, by stimulating patients PBMCs with CpG and looking at specificity of the IgG in the supernatant, we were able to calculate the frequency of ACPA-positive memory B-cells on the total of IgG-positive cells present in the samples. Before treatment ACPA-positive memory B-cells had a mean frequency of 153 in 10^6 IgG memory B-cells (mean ± SD: 153 ± 94.08, range: 31.56–272.22). The frequency of ACPA-positive memory B-cells dropped to 99 per 10^6 IgG memory B-cells (mean ± SD: 98.92 ± 61.46, range: 26.93–202.22) after abatacept treatment (Fig. 5C). Hence, abatacept reduced the frequency of ACPA-specific memory cells.

4. Discussion

CTLA-4 is an essential molecule in the control of APC function and cell activation. Upon activation B-cells acquire professional APC function and express CD80 and CD86. Studying the direct impact of abatacept on B-cell function, we found that abatacept mediated the internalization of CD80 and CD86, thereby impairing their ability to costimulate T-cells. This may impact the formation and maintenance of self-reactive memory cells and short-lived plasma cells, as we found a decrease in ACPA-specific memory B-cell in RA patients treated with abatacept.

Abatacept directly affect the function of B-cells by reducing expression of CD80 and CD86. The down modulation of CD80 and CD86 is exploited by viruses to evade the immune response. The HIV viral protein Nef is expressed in HIV-infected macrophages and dendritic cells and induces reduced expression of CD80 and CD86 by intracellular retention resulting in impaired naïve T cell activation [31]. We found that upon binding to abatacept, CD80 and CD86 are internalized in vesicles, in a dynamin-dependent fashion. While it has been described that CD86 is stored in vesicles in the cytoplasm of human DCs [32] and monocytes [33], allowing the rapid surface expression of CD86 upon activation, the internalization of CD80 and CD86 has never been described in B-cells. It has been shown in DCs that CD86 associates with the E3 ubiquitin ligase MARCH1 and is rapidly internalized, leading to lysosome-dependent degradation [34], thus regulating APC-function of DCs. In B-cells, it is not clear if CD86 is degraded upon internalization. However, mRNA levels of CD80 and CD86 were not changed over three days of culture in presence of abatacept, and the total amount of protein as tested by Western blot was stable (not shown). In murine B-cells [35] and DCs [36] engagement of CD86 elicits intracellular signaling. We did not detect activation of NF-kB or Akt signaling pathways upon engagement of CD80 or CD86 neither with abatacept nor with previously described agonistic antibodies (not shown) [35,37]. Different experimental setting or model system may explain these results.

Genetic CTLA-4 variants associate with an increased susceptibility for RA [7] and autoimmune manifestations including inflammatory arthritis are a prominent feature of human CTLA-4 deficiency [2]. CTLA-4 deficient patients present with lymphoproliferation, autoimmune cytopenia and lymphocytic organ infiltration, associated with progressive loss of B-cells and antibodies [38]. Indeed, CTLA-4-deficient mice show spontaneous T-follicular helper cell differentiation with development of autoantibodies [39]. These data point to the interaction between CTLA-4 and CD80/CD86 as an important regulator of the germinal center reaction and output. Abatacept did not intrinsically inhibit B cell development into plasmablasts *in vitro*, excluding an influence of potential CD80/CD86 retrograde signaling into B cells. On the contrary the number of plasmablast and ACPA-specific memory cells significantly decreased *in vivo* indicating that the interaction with T cells is important for this effect. In fact, loss of CTLA-4 on T follicular regulatory cells results in mice in defective suppression of antigen specific antibody responses [40]. During the germinal center reaction, activation and survival cues are essential in driving B-cell selection. T follicular helper cells are the main source of these signals, including CD40L and IL-21 [41]. B-cells compete for that help and therefore, the efficiency of the interaction between B- and T-cells, in terms of antigen presentation and expression of costimulatory molecules, will be essential for their selection. We observed that the reduction in CD80 and CD86 expression on B-cells may hinder T-cell proliferation in MLR setting. Hence, by reducing the expression of CD80 and CD86, abatacept may influence the interaction between B-cells and T-cells, hindering B-cell selection. In line with this hypothesis, abatacept treatment in patients with primary Sjogren's syndrome affects germinal center formation in parotid glands, which is dependent on co-stimulation of activated T follicular helper cells, and consequently local formation of (autoreactive) memory B-cells [42]. Additionally, in RA treated with abatacept T-dependent vaccination resulted in reduced specific IgG

titers, with only a minor reduction in total IgG [43,44].

We found a significant reduction of serum IgG, RF and ACPA, and a tendency to a reduced IgA. Several clinical studies showed after three to six months of abatacept treatment reduced titers of IgA, a tendency in reduction in IgM and IgG, as well as a reduction in autoantibodies compared to baseline [45,46]. These changes correlated with a drop in disease activity. The number of total B-cells dropped compared to baseline, as well as the memory B-cell count [27]. The window of observation in our study is extended to 12 and 24 months, identifying more stable changes in the B-cells compartment. We observed a reduction in plasma cells. Additionally, we found a specific effect on the frequency of self-reactive memory cells. An effect on memory B cell generation was observed in mouse models of hearth transplant rejection. Here, treatment with abatacept during the first week after transplant abrogated the generation of the allograft specific memory B-cell [47].

Abatacept is successfully used to treat immune dysregulation in CTLA-4 insufficiency [48–50]. In these patients abatacept restored Treg-cell functionality and clinically, reduced diarrhea, resolved autoimmune cytopenia and detection of anti-red blood cells antibodies became negative after 8 months of abatacept treatment [49]. The disappearance of the autoantibodies observed in CTLA-4-insufficiency treated with abatacept may recall the reduction in ACPA-specific memory cells and the reduction in plasmacells observed in RA patients. However, abatacept treatment may at the same time impair new memory cell formation leading to exacerbated immunodeficiency, rendering the patients more susceptible to infections. Indeed, long term studies with careful monitoring of both, complex autoimmune diseases such as RA and genetic defects leading to immune dysregulation, will be necessary to understand the impact of the abatacept-driven impairment on the APC function of B cells and their selection in the memory compartment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.04.016.

Conflicts of Interest

R. E. Voll has received consultancy fees from Roche, Celgene, Novartis, Bristol-Myers Squibb, Pfizer, Janssen, Sanofi. N. Venhoff has received research support from Medac GmbH, Pfizer (Aspire Program), and Novartis and has received consultancy fees from Roche. J. Thiel has received research support from Bristol-Myers Squibb, Medac GmbH, Pfizer (Aspire Program), and Novartis and has received consultancy fees from Roche, Bristol-Myers Squibb. U. Salzer has received travel support from Celgene and CSL Behring. M. Rizzi has received research support from Pfizer (Aspire Program), Bristol-Myers Squibb, Novartis, has received lecture fees from Roche, has received consultancy fees from Novartis, Celgene. The rest of the authors declare that they have no relevant conflicts of interest.

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