Identification and characterisation of anti-citrullinated protein antibody (acpa)-producing b cells in patients with rheumatoid arthritis (ra)

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Chapter 1

General introduction

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Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease characterised by chronic systemic inflammation. In western societies, about 1% of the population is affected, with a higher prevalence in women. The disease is characterised by systemic inflammation of joints leading to cartilage destruction and bone erosions with, as a consequence, disability of the patient.

One of the hallmarks of RA is the presence of autoantibodies. During the course of the last 50 years several autoantibodies have been identified, amongst which rheumatoid factor (RF)[1], anti-citrullinated protein antibodies (ACPA) and anti-carbamylated protein antibodies (aCARP)[2]. Following their identification, many researchers studied the characteristics, specificity and, as most interesting aspect, their (pathogenic) role in RA.

Citrullination and ACPA

In 1964, Nienhuis *et al.* were first to show that RA patient serum contains specific antibodies against keratohyaline granules, which were initially termed antiperinuclear factor (APF) due to their staining pattern on buccal mucosa cells[3]. Independently, the same group of antibodies was described as anti-keratin antibodies (AKA) in 1979[4]. It took until 1998, however, before it was found that the non-classical amino acid citrulline is essential for recognition by this group of antibodies[5]. Citrulline is the result of deimination of the amino acid arginine by the enzyme peptidyl arginine deiminase (PAD) (figure 1.1). Due to this post-translational modification (PTM), the positively charged arginine

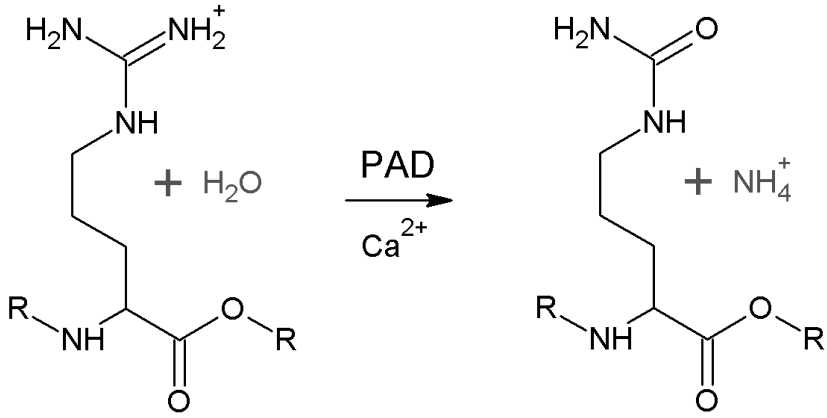

Figure 1.1: Post-translational modification of an arginine to a citrulline.

is converted into a neutral citrulline. Loss of charge can result in structural changes on the protein level, such as differences in protein folding, change of polarity or denaturation. Under physiological conditions, citrullination is linked to various processes in different cell types[6], including apoptosis[7-9] and histone modifications[10-12]. Citrullination of histones can facilitate transcription of genes as the structure of histones becomes less condense, or it can allow extensive chromatin decondensation which facilitates the formation of neutrophil extracellular traps; NETs[12, 13]. It is unknown why tolerance against citrullinated antigens is broken in the majority of patients with RA, but it has been shown that citrullinated proteins can be found in inflamed tissues including joints of patients[14, 15]. Moreover, an intriguing observation is that a polymorphism in the gene encoding the PAD enzyme is by itself a risk factor for ACPA-positive RA[16].

ACPA in RA

Following the identification of citrulline as the essential part of the antigen recognized by both APF and AKA, an ELISA using a synthetic cyclic citrullinated peptide (CCP) was developed, called the anti-CCP1 test. This diagnostic test has a specificity of 98% and a sensitivity of 68% for the diagnosis RA[17]. The commercial CCP2 version of the test for routine testing, available since 2002, made it possible to use ACPA as a biomarker for RA and, in the 2010 criteria used for classification of patients, ACPA status was included[18]. In recent years, many groups studied the role of ACPA in disease pathogenesis, although the precise contribution of ACPA to disease-related processes remains still unclear. However, accumulating clinical as well as experimental data suggest that ACPA indeed contribute to inflammatory processes and chronicity of inflammation in RA.

Concerning the role of ACPA as a biomarker, it is intriguing that ACPA can be detected in serum of individuals with joint pain (arthralgia) before the onset of RA. Arthralgia patients that harbour ACPA have a higher chance of developing RA compared to patients without ACPA[19]. Also, patients with undifferentiated arthritis (UA) have a higher risk for RA development when they are ACPA-positive[20]. Furthermore, patients with RA being ACPA-positive have more severe disease progression[21]. Thus, ACPA are useful diagnostic and predictive biomarkers that identify patients at risk for severe progressive disease.

Pathogenicity of ACPA

As ACPA are a hallmark of RA, many studies tried to identify direct ACPA-mediated biological effector functions. In mice, the induction or development of ACPA is debated[22-24], but infusion of ACPA in mice can exacerbate arthritis[22, 25] suggesting that ACPA can at least enhance inflammation. Similar conclusions could be derived from in vitro studies, in which ACPA could promote inflammation by influencing different processes and cell types of the immune system. For example, ACPA can activate the classical and alternative, but not the lectin pathway of the complement system[26]. Plate-bound immune complexes (IC) of ACPA can activate mast cells, mainly via FcγRIIA[27] and, enhanced by IgM-rheumatoid factor, increase cytokine production by

macrophages[28]. As citrullinated antigens are present in the inflamed joints of patients with RA[29], ACPA-IC could be formed at these sites and contribute to inflammation. Moreover, purified ACPA skew macrophage differentiation to the inflammatory M1 phenotype, resulting in an increased M1/M2 ratio after culture[30]. Also, ACPA-containing IgG, but not control IgG, potentially induces NET formation by neutrophils, thereby creating a vicious circle where NETs contain citrullinated antigens and thereby trigger additional autoantibody formation and inflammation[31]. Furthermore, isolated ACPA-IgG but not control IgG can induce osteoclastogenesis and bone loss[32]. Focussing on signalling pathways, it was shown that ACPA can activate ERK1/2 and JNK signalling which leads to NF-κB activation and TNF-α production[33]. As ACPA have been implicated in the pathogenesis of these processes, it is very important to understand whether these effector functions are ACPA specific and if ACPA have molecular features that are important for the observations described above.

In our department, detailed characterisation of ACPA on the molecular level has been performed for many years. Studies of isotype usage and isotype subclasses showed that ACPA are expressed in most isotypes, i.e. IgG, IgA and IgM. Of interest, patients negative for ACPA-IgM can be positive for ACPA-IgM later in time indicating formation of new ACPA-expressing B cells also during the course of disease[34]. ACPA have a low avidity compared to antibodies against recall antigens and show only avidity maturation before disease onset[35, 36]. Separating ACPA-positive patients in groups according to ACPA avidity revealed that patients with the lowest avidity have more severe joint destruction[37]. In line with avidity maturation, also epitope spreading was shown to happen before the onset of RA while no changes in fine specificity were observed later in the disease course[38]. And, more recently, the majority of ACPA was found to carry N-glycans in the Fab region which modulate the binding avidity to their antigen[39]. All these data indicate that ACPA have different characteristics and dynamics compared to recall antibodies. Very interesting aspects of ACPA research include the questions on why ACPA are already present before disease onset, what makes the ACPA immune response mature just before clinical disease onset as indicated by a rise of titre, change of isotype usage and epitope spreading, and why patients in remission can have persistent high ACPA-IgG titres. Therefore, a better understanding of ACPA and the underlying immune response is required to determine why (a certain group of) ACPA show these features that are presumably related to pathogenic properties. To dissect these issues, the study of citrullinated antigen-specific B cells could give insights in the origins of this autoantigen-specific immune response and could elucidate differences between normal B cells, 'non-pathogenic' early citrullinated antigen-specific B cells, citrullinated antigen-specific B cells in remission and 'pathogenic' citrullinated antigen-specific B cells during active disease. Eventually, such knowledge could identify targets that allow interfering with the development of citrullinated antigen-specific B cells.

B cells

B cells are key players in our adaptive immune system and provide humoral immune responses. B cells are part of the adaptive immune system, recognize a specific antigen on their surface via the B cell receptor (BCR) and can develop into highly specific memory B cells. B cells differ from T cells in antigen recognition as T cells recognize small peptides whereas B cells recognize an antigen in their native form, which can include for example lipids or sugars. Furthermore, upon differentiation to plasmablast/plasmacells (PB/PC), B cells secrete antibodies, which are part of the humoral immune system. In general, B cell development can be divided into four stages[40]: generation, elimination of self-reactive B cells, activation by foreign antigens and differentiation to antibody secreting cells (ASC) and memory B cells.

Generation of B cells starts in the bone marrow where B cells obtain their BCR. The BCR is a Y-shaped molecule comprised of two heavy and two light chains. The upper part of the V part of the Y contains the variable regions and the rest of the molecule is the constant region. The variable region contains a variable (V), a diversity (D; only in Ig heavy chain) and a joining (J) element. For all these elements of the variable region, many potential genes are available providing an enormous number of possible V(D)J combinations, thereby creating a wide range of possible BCRs. To obtain an expressed BCR the DNA needs to be rearranged. In this process, recombination activated genes (RAGs) encode enzymes which will induce cleavage of DNA at specific sites between the selected V(D)J elements. Next, the DNA of the selected V(D)J elements will become adjacent and the DNA will be repaired. As a result, the BCR gene transcript is formed and the in-between parts of the B cell's genome will be lost. Rearrangement of the BCR starts at the heavy chain locus creating a pre-B cell receptor with a surrogate light chain (LC)[41, 42]. Next, the LC is rearranged starting mostly at the kappa locus[43, 44]. When rearrangement does not result in a functional BCR the cell rearranges again until a functional BCR is formed, rescuing it from apoptosis. Finally, cells positively selected due to the expression of a functional BCR enter the stage of immature B cells and continue their development. Although the rearrangements could be completely random, it was shown that this is a tightly regulated process[45]. Immature B cells express BCRs that are often autoreactive. Under normal conditions, these autoreactive B cells will be negatively selected and undergo apoptosis, become anergic or modify (again) their receptor by receptor editing, which alters (mostly) the LC. When a cell changes its LC, the cell transcribes a kappa locus genetically further downstream, switches transcription to the second kappa containing chromosome, or it switches to one of the two lambda containing chromosomes. Choosing for a locus located downstream results in loss of the upstream loci. Thus, each time a B cell undergoes receptor editing the amount of available light chains is reduced, since part of the DNA is lost. As a final result of control checkpoints and receptor editing, mature naïve B cells can enter the periphery.

Mature naïve B cells circulate in the periphery patrolling through secondary lymphoid organs, spleen and lymph nodes, until they die from lack of survival signals or until they encounter their antigen. Upon antigen encounter in

secondary lymphoid organs and a second activation signal[46], they become activated and start proliferating and differentiating, thereby generating memory B cells and/or antibody secreting plasmablasts/plasmacells (PB/PC). Broadly, this second activation signal can be divided into two subgroups of signals: T cell dependent and T cell independent signals. T cell dependent B cell activation is characterised by the formation of germinal centres (GC). Activated T cells provide a stimulatory signal via CD40L-CD40 interaction[47, 48]. GC B cells proliferate extensively while somatic hypermutation (SHM) introduces changes in the recognition region of the BCR, a process termed affinity maturation. B cells with the highest affinity for their cognate antigen are positively selected and some will re-enter SHM resulting in B cells with even higher affinity. Other B cells can undergo class switch recombination (CSR) and become high affinity memory B cells or Ig-producing PB/PC[49]. T cell independent activation and memory formation needs activation of several receptors on one B cell. This can be achieved by combining activation of the BCR and another receptor, like toll like receptors (TLRs) or by crosslinking of several BCRs recognizing lipids and carbohydrates[50, 51]. Memory B cells can more easily be (re-)activated than naïve B cells, but both can differentiate to short-lived antibody secreting plasmablasts (PB) or long-lived plasmacells (PC). To become long-lived PC, they home to the bone marrow (BM)[52, 53] where they can become long-lived PC when they successfully compete for plasma cell survival niches[54].

Outline of this thesis

In many autoimmune diseases including RA, there is excessive production of antibodies that can bind self-antigens. How these self-reactive B cells escape negative selection is not known. To understand this, characterisation of autoreactive B cells is required preferably by analysing these cells in an antigen-specific manner. Since we know that ACPA have different molecular characteristics and dynamics compared to recall antibodies, we hypothesised that also ACPA-expressing B cells could have different characteristics if compared to non-autoreactive B cells. How these cells develop, mature and survive is still largely unknown. The studies presented in this thesis describe the first steps to understand ACPA-expressing B cells in terms of identification, localization, isolation and characterisation.

In **chapter 2**, studies are described addressing the question where ACPA-expressing B cells are located. In contrast to conventional immune responses, ACPA-IgM in serum can appear at later stages of established disease which suggests that ACPA are continuously generated as a result of an ongoing immune response[34]. Therefore, we cultured B cells isolated from peripheral blood of ACPA-positive RA patients. We were able to identify ACPA-expressing B cells in peripheral blood of ACPA-positive RA patients but not in ACPA-negative RA patients or healthy controls. Unstimulated versus stimulated peripheral blood mononuclear cell (PBMC) cultures and cultures after FACS sorting experiments revealed that, next to ACPA-expressing B cells in the memory pool, spontaneously ACPA-secreting PB/PC are present in peripheral blood. These findings support

the hypothesis of an ongoing citrullinated antigen-specific immune response in ACPA-positive RA patients.

As we identified spontaneously ACPA-secreting PB/PC in peripheral blood in the context of the studies presented in chapter 2, we questioned if this ACPA immune response is similar near the site of inflammation, in synovial fluid (SF). To this end, and to understand the role of cells from the synovial compartment in maintaining ACPA B cells, we characterised ACPA-secreting cells from SF in detail. These studies are presented in **chapter 3**. Even though the percentage of B cells in synovial fluid mononuclear cells (SFMC) is lower than in peripheral blood, we identified an enrichment of spontaneous ACPA secretion and ACPA secreting cells in the SF compartment. Furthermore, we found that total SFMC have the potential to spontaneously form a survival niche for long-lasting (auto) antibody secretion.

Studying ACPA B cells on a single cell level could give many insights into the ACPA immune response. Therefore, we also set out to develop an antigen-specific staining for the identification of citrullinated antigen-specific B cells by flow cytometry. As peripheral blood is relatively easy to obtain from patients compared to SF, we focussed on peripheral blood despite the lower number of ACPA B cells (chapter 3). **Chapter 4** describes the successful development of a flow cytometry staining that identifies ACPA-expressing B cells. For the method to be successful, we constructed a human embryonic kidney (HEK) cell line expressing a monoclonal ACPA molecule on the cell membrane and used three streptavidin tetramers in each staining. This combination of tetramers, two containing citrullinated antigens and one an arginine control variant, is needed to identify cells recognizing citrullinated antigens but not the control antigen. This technique was validated by culturing cells isolated by flow cytometry, which yielded high amount of ACPA production in positively sorted wells, whereas higher numbers of negatively sorted cells gave very low or no detectable degree of ACPA production.

Next, we included phenotypic markers CD20 and CD27 to discriminate between the naïve, memory and PB/PC compartment, which allowed calculating the frequency of ACPA-expressing B cells for every subpopulation. This revealed that most ACPA-expressing B cells have a memory phenotype with a median of 1 in every 2600 memory B cells. Using isotype-specific antibodies, we could show that ACPA-expressing B cells in peripheral blood are mostly class-switched memory B cells expressing IgG, but also IgA, IgM and IgD ACPA B cells could be detected.

To better understand the provenance of ACPA-expressing B cells in terms of B cell development, the usage of lambda and kappa light chains was studied in the context of the studies presented in **chapter 5**. In general, B cells start with the expression of a kappa light chain-containing BCR[43, 44]. Prior to entering the periphery the cells need to pass the checkpoint of being non-autoreactive. Although this is a very important checkpoint, this checkpoint in itself is incomplete and some autoreactive cells 'escape' and enter the periphery. We hypothesised that a combination of starting with a kappa LC, more rounds of failing the autoreactivity checkpoint and more additive chance of escaping the checkpoint generates, on average, more lambda usage in autoreactive B cells. If ACPA B cells were generated this way we expected that ACPA would more

frequently carry lambda light chains. To this end, we characterised ACPA-IgG antibodies isolated from serum and SF and studied the usage of lambda light chains by ACPA-expressing B cells. We found a shift in frequency of lambda LC usage, as serum ACPA-IgG and ACPA(-IgG$^+$) B cells more frequently use lambda light chains. This could indicate that ACPA-expressing B cells underwent, on average, more rounds of receptor editing.

As we hypothesised that long-lived plasma cells could be important in (ACPA-positive) RA, we also analysed the expression of CD28 on B cells in RA patients in the context of the studies presented in **chapter 6**. As has been described for mice, expression of CD28 on B cells is involved in the live span of PC[55]. For human B cells, this has not been shown so far, but the expression of CD28 on B cells has been reported. Therefore, we studied the expression of CD28 on the different B cell subsets in peripheral blood (and SF) of RA patients compared to patients with systemic lupus erythematosus (SLE) and healthy controls, as well as on ACPA-expressing B cells. We identified CD28 expression on PB/PC in both patient groups and heathy controls and this was found to be comparable. In PB/PC in SF, this percentage was increased. ACPA-expressing PB also expressed CD28. Yet also here, the expression level was found to be comparable for peripheral blood as well as SF PB/PC.

Finally, **chapter 7** summarizes and discusses the results and describes possible directions for future research.

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Chapter 2

Circulating plasmablasts / plasmacells as a source of anti-citrullinated protein antibodies in patients with rheumatoid arthritis

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Abstract

Objectives: To study the characteristics and phenotype of anti-citrullinated protein antibody (ACPA) specific B cells in peripheral blood of patients with rheumatoid arthritis (RA).

Methods: Peripheral blood B cells from ACPA-positive RA patients were cultured with or without stimulating factors. Following culture, supernatants were assessed for the presence of ACPA-IgG and non-specific total IgG by ELISA.

Results: Following stimulation, ACPA were detectable in up to 100% of culture wells. Of interest, ACPA were also produced spontaneously by unstimulated PBMC. In both cases, the average ACPA titre per culture well correlated with ACPA serum titres. No ACPA production was detectable in B cell cultures from ACPA-negative RA patients or healthy controls. Importantly, FACS-sorting experiments located spontaneous ACPA production to the CD20 negative B cell population corresponding to circulating plasmablasts/cells.

Conclusions: ACPA specific peripheral blood B cells are not confined to the CD20 positive memory pool, as circulating plasmablasts/cells spontaneously producing ACPA are also readily detectable. The latter points to an ongoing B cell immune response against citrullinated proteins and contrasts conventional immune responses against, for example, vaccines, where antigen-specific plasmablasts appear in peripheral blood only shortly after vaccination. These circulating, ACPA specific plasmablasts/cells might represent targets for novel therapeutic interventions.

Introduction

In many autoimmune diseases, including rheumatoid arthritis (RA), autoantibodies are believed to be the causative agent that drives disease pathogenesis. Therefore, novel therapeutic approaches that target autoantibody producing B cells require a thorough understanding of their nature, phenotype and disease specific localization.

Several autoantibodies have been described in RA. Among those, anti-citrullinated protein antibodies (ACPA) exhibit the highest specificity for the disease, predict disease onset and severity, and identify a subgroup of patients eligible for more aggressive treatment.[1-3] Citrullinated antigens are present in the inflamed joints of RA patients, supporting the concept of ACPA specific effects at the site of inflammation.[4] Indeed, recent evidence suggests a role for ACPA in disease pathogenesis.[5-8]

So far, little is known on the characteristics of ACPA producing B cells. A single study detected ACPA production in cultures of synovial fluid mononuclear cells.[9] Another study identified ACPA production by peripheral blood B cells upon stimulation.[10] However, it remained unclear whether ACPA detected in this latter study originated primarily from stimulated, otherwise resting memory B cells, or from circulating, differentiated plasmablasts/cells (PB/PC).

In anti-vaccine immune responses, antigen-specific PB transiently appear in peripheral blood in the week after vaccination. These cells rapidly disappear from the circulation and are thought to home to the bone marrow to become long-lived PC that provide high antibody serum titres for years.[11, 12] Like antibody titres against recall antigens, ACPA serum titres in RA patients are relatively stable and can persist at high titre. Nevertheless, it has been postulated that these high ACPA serum titres in RA patients are due to a persistently active immune response against citrullinated proteins, as suggested by the continuous presence of IgM ACPA in some ACPA-positive patients.[13] If this would be correct, one could predict the presence of ACPA-secreting B cells in peripheral blood. Here, we set out to define ACPA-producing B cells in more detail to increase our understanding of anti-citrullinated protein immunity.

Patients and Methods

Patients and healthy individuals

Peripheral blood and serum samples were obtained from patients with ACPA-positive and ACPA-negative RA visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre, Leiden, The Netherlands. Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent for sample acquisition. Treatment included disease-modifying anti-rheumatic drugs, biological agents, and glucocorticoids. Permission for conduct of the study was obtained from the ethical review board of Leiden University Medical Centre.

Isolation of PBMC, B cells and B cell subsets

Mononuclear cells were isolated from peripheral blood using Ficoll-Paque gradient centrifugation (LUMC pharmacy). CD19-positive B cells were isolated by magnetic bead-based positive selection using Dynabeads and DETACHaBEAD CD19 (Invitrogen) according to the manufacturer's instructions.

For selected experiments, B cells were sorted into subsets on a BD FACS Aria flow cytometer. PBMC were surface stained with CD3 Alexa Fluor 700 (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD20 PerCP (clone L27), and CD27 PECy7 (clone M-T271, all BD Biosciences). Subpopulations were defined as $CD3^-CD14^-CD19^+CD20^-CD27^{++}$ PB/PC, $CD3^-CD14^-CD19^+CD20^+CD27^-$ naïve and $CD3^-CD14^-CD19^+CD20^+CD27^+$ memory B cells (figure 2.3A).[14, 15]

Culture conditions

B cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin/streptomycin (100U/ml), and 2mM Glutamax in 96-wells flat bottom plates at a density of $2*10^4$ cells/well on a layer of irradiated (7000 rad) mouse fibroblast cells stably transfected with human CD40 ligand (CD40L, $5*10^3$ cells/well) in the presence of B cell activating factor (BAFF) (Miltenyi,100ng/ml), IL21 (Invitrogen, 50ng/ml) and anti-IgM $F(ab')_2$-fragments (JacksonImmunoresearch Laboratories, 5µg/ml). Supernatants were harvested after 6-7 days.

To assess spontaneous ACPA production, PBMC were cultured at a density of $2*10^5$ cells/well in IMDM supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100U/ml), and 2mM Glutamax with no additional stimuli. FACS-sorted B cell subsets were cultured under similar conditions, with only variation in cell density ($2*10^4$ cells/well for memory/naïve populations; 1500–8000 cells/well for PB/PC) and culture plate (96-well round-bottom plates). Supernatants were collected at day 6 and 13.

Measurement of IgG-ACPA and total IgG

Serum and culture supernatants were assessed for the presence of IgG-ACPA by ELISA based on reactivity against the CCP2-peptide (Euro Diagnostica). Serum samples were tested at a 1:50 dilution or higher according to the manufacturer's instructions, culture supernatants were tested undiluted. The commercial standard was diluted to assess the lowest concentration at which the standard curve was consistently linear (0.125U/ml (supplementary figure 2.S1)). This value takes into account that the concentration of the commercial standard relates to a 1:50 dilution of serum samples, requiring a division by 50 of the manufacturer's units. A representative set of patients (25%) was tested, and found negative, for reactivity against the arginine containing control peptide.

The presence of total IgG in culture supernatants was assessed by standard ELISA (Bethyl Laboratories).

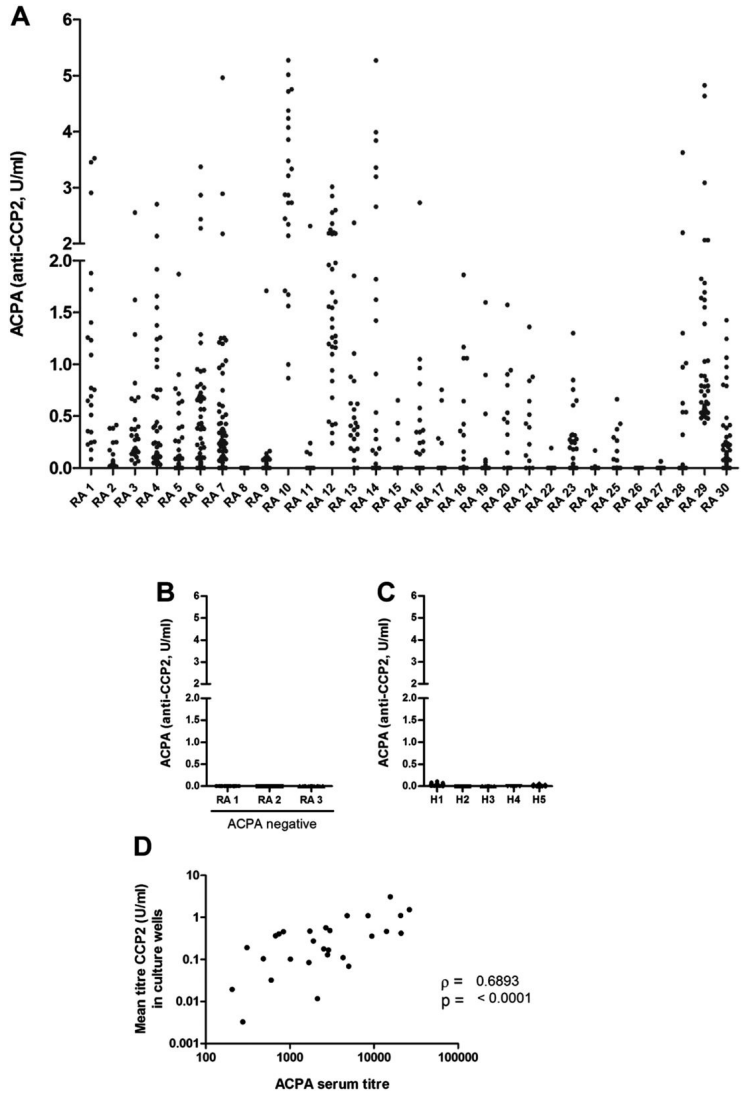

Figure 2.1: ACPA production upon in vitro B cell stimulation. *CD19-positive B cells were isolated and cultured for 6-7 days. Supernatants were assessed for the presence of IgG-ACPA by ELISA. Each dot represents a culture well, results are grouped per patient. Values below linearity of the standard (0.125U/ml) were calculated by extrapolating the standard curve linear through zero (supplementary figure 2.S1). Displayed are results from* ***(A)*** *ACPA-positive RA patients. On average, we obtained 38 (6-170) wells per patient, with a mean ACPA titre of 0.45U/ml (0.0-3.1)) at the end of the cultures,* ***(B)*** *ACPA-negative RA patients, n=3; average of 45 (16-96) wells per experiment,* ***(C)*** *Healthy donors, n=5; average of 51 (32-73) wells per experiment.* ***(D)*** *Shows the correlation between ACPA serum titres and mean ACPA titres produced in culture.*

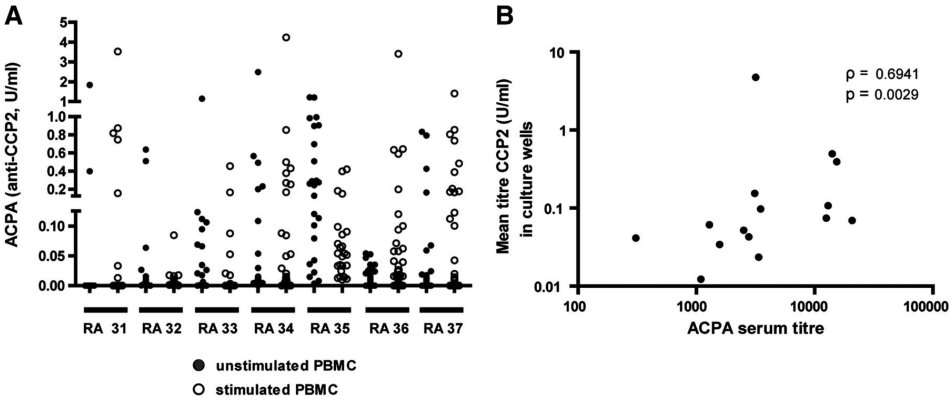

Figure 2.2: Spontaneous versus stimulated ACPA production. ***(A)*** *PBMC of ACPA-positive RA patients were split in two and cultured either with or without stimulation at a density of 2*10^5 cells/well. Supernatants were assessed for the content of IgG-ACPA at day 13 of culture.* ***(B)*** *Shows the correlation between ACPA serum titres and mean ACPA titres spontaneously produced in culture.*

Statistical analysis

Analysis was performed using GraphPad Prism 5.01. Correlations were assessed as non-parametric correlation. p Values <0.05 were considered significant.

Results

ACPA production upon in vitro B cell stimulation

To assess the general presence of ACPA-producing B cells in peripheral blood, we stimulated freshly isolated CD19$^+$ B cells of ACPA-positive RA patients (n=30) as described previously.[16, 17] After 6 to 7 days, ACPA were detected in 80% of the cultures and, in some patients, in up to 100% of the culture wells (figure 2.1A). No ACPA were detected using B cells isolated from healthy individuals and ACPA-negative RA patients (figure 2.1B-C), although total IgG production was comparable (not shown). Of interest, mean ACPA titres obtained in the cultures correlated with ACPA serum titres (figure 2.1D). Based on the number of B cells per culture well, we estimate that, in some patients, at least one in every 2*10^4 B cells can produce ACPA.

Spontaneous ACPA production

If the ACPA-response would result from an ongoing immune response, we reasoned that naïve or memory B cells should be continuously activated resulting in the presence of IgG-ACPA producing PB/PC in peripheral blood. Therefore, we analysed whether B cells spontaneously producing ACPA could be observed. To this end, peripheral blood mononuclear (PBMC) cells were cultured without exogenous stimuli. Interestingly, comparable amounts of ACPA-positive culture wells were detected in most patients when comparing stimulated versus

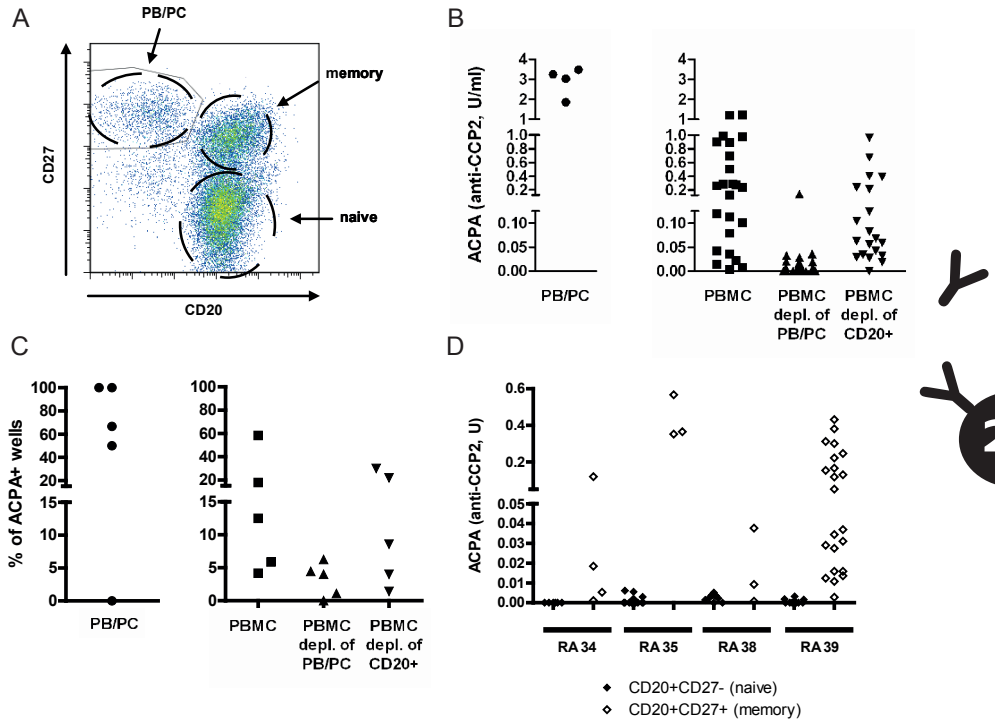

Figure 2.3: Circulating plasmablasts/cells as primary source of ex-vivo ACPA production. *CD19-positive B cell subpopulations were FACS-sorted based on the expression of surface markers CD20 and CD27 into $CD20^{-}CD27^{++}$ plasmablasts/cells (PB/PC), $CD20^{+}CD27^{+}$ memory B cells and $CD20^{+}CD27^{-}$ naïve B cells* ***(A)****. In addition, the markers were used to deplete PBMC of the respective B cell subset. Sorted B cell populations and PBMC depleted of subsets were cultured without stimulation.* ***(B)*** *Representative data of one patient. Each dot corresponds to one culture well.* ***(C)*** *Combined data of 5 patients. Each dot represents one donor. The lowest point of linearity of the standard was used as cut-off for ACPA positivity.* ***(D)*** *Naïve and memory B cells were FACS-sorted as described above for four patients. Both populations were stimulated as described in the Methods section. Supernatants were harvested after 6 days of culture and assessed for ACPA by ELISA. Data are depicted as units corrected for culture volume.*

non-stimulated PBMC per patient (figure 2.2A), whereas non-specific IgG production was strongly increased upon stimulation (up to 100 fold, data not shown). Importantly, also mean titres of spontaneous ex-vivo ACPA production correlated with ACPA serum titres (figure 2.2B). This suggests that ACPA detected in the culture wells originate primarily from circulating PB/PC.

Circulating plasmablasts/cells as primary source of ACPA ex vivo

To confirm the presence of ACPA-producing PB/PC in peripheral blood, and to exclude the possibility of endogenous stimulation of memory B cells by antigen presenting cells within the PBMC population, we next isolated PB/PC by FACS sort (see figure 2.3A for gating strategy). ACPA production by purified PB/PC was compared to ACPA production of total PBMC, PBMC depleted of PB/PC, and to PBMC depleted of the CD20 positive B cell fraction (figure 2.3B-C). In line with our hypothesis, isolated PB/PC readily produced ACPA ex-vivo. The total PBMC fraction and PBMC depleted of CD20-positive cells both showed comparable ACPA production, whereas ACPA production by PBMC depleted of PB/PC was considerably diminished.

ACPA were not produced by unstimulated, FACS sorted memory B cells (not shown), but were detectable upon stimulation. This indicates the additional presence of CD20-positive, ACPA-specific memory cells in the circulation (figure 2.3D). ACPA were hardly detected in wells containing naïve B cells, irrespective of stimulation.

Together, these data highlight the presence of circulating, ACPA-producing PB/PC in peripheral blood of RA patients and indicate that depletion of the CD20-positive B cell compartment does not eliminate these cells.

Discussion

The immune response against citrullinated antigens in RA is incompletely understood. Specifically, characteristics of ACPA-producing B cells have scarcely been studied. Here, we show that ACPA-producing PB/PC circulate in peripheral blood of patients with RA.

In the steady state, most circulating PB/PC in healthy individuals are thought to originate from mucosal immune responses.[11] Upon vaccination, antigen-specific PB appear in the circulation only transiently. Therefore, our findings provide evidence for the recent and continuous activation of ACPA-producing B cells in RA patients. This observation is relevant, as recently activated PB/PC are phenotypically different from fully differentiated, long-lived plasma cells or memory B cells. Indeed, depletion of the CD20-positive B cell compartment did not eliminate the spontaneous ACPA production observed.

Next to circulating PB/PC, we identified ACPA-specific memory B cells that produced ACPA upon stimulation. The latter observation could be taken as a surprise as it is conceivable that citrullinated protein-directed memory B cells are constantly driven to the PB/PC population as a consequence of the persistent and systemic presence of citrullinated antigens. Novel therapeutic approaches aimed at inhibiting ACPA production should thus target the CD20-positive B cell subset, but need to also include the PB/PC compartment.

Finally, we found an association between ACPA production in culture and ACPA serum titres. This suggests that ACPA serum titres originate partly from tissue resident, long-lived plasma cells producing rather stable ACPA "background" titres, and partly from recently generated, circulating plasmablasts/cells. The latter reflect the active part of the immune response and are thus more variable in their contribution to the ACPA serum titre, as suggested by our study.

In line with this, intensive treatment was previously shown to be able to decrease ACPA serum titres by up to 50%.[18-20], suggesting indeed a fluctuation in ACPA titres due to an ongoing immune response.

In conclusion, we here identify ACPA-specific plasmablasts/cells circulating in peripheral blood of ACPA positive RA patients. This population, which is not targeted by CD20 depletion, reflects the active part of the ACPA-specific B cell response and could represent a relevant target for therapeutic intervention.

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Supplementary figures

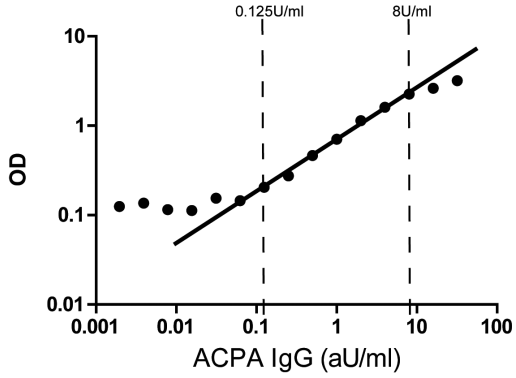

Figure 2.S1: Standard curve determining the linearity of the IgG-ACPA ELISA. *To determine the linearity in the lower range of the IgG-ACPA ELISA the standard was extended using a 1:1 dilution series. The concentration of 0.125U/ml IgG-ACPA was identified as the lowest point that is consistently within the linear part of the standard curve.*

Chapter 3

Synovial fluid mononuclear cells provide an environment for long-term survival of antibody-secreting cells and promote the spontaneous production of anti-citrullinated protein antibodies

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Abstract

Objectives: In rheumatoid arthritis (RA), observations point to a crucial role for (autoreactive) B cells in disease pathogenesis. Here, we studied whether cells from the synovial environment impact on the longevity of autoreactive B cell responses against citrullinated antigens.

Methods: Synovial fluid and peripheral blood mononuclear cells (SFMC/PBMC) were obtained from patients with established RA and assessed for the presence of B cell subpopulations. Cells spontaneously secreting anti-citrullinated protein antibodies (ACPA-IgG) directly ex vivo were detected by antigen-specific ELISpot. SFMC and PBMC were cultured to assess the degree of spontaneous ACPA-IgG secretion. Cells surviving for several weeks were characterized by CFSE-labelling and Ki-67 staining.

Results: Cells spontaneously secreting ACPA-IgG were readily detectable in peripheral blood and synovial fluid (SF) of ACPA-positive RA patients. SFMC showed an up to 200-fold increase in ex vivo ACPA-IgG secretion compared to PBMC despite lower numbers of B cells in SFMC. ELISpot confirmed the presence of spontaneously ACPA-IgG secreting cells, accounting for up to 50% (median 12%) of all IgG-secreting cells in SF. ACPA-IgG secretion was remarkably stable in SFMC cultures, maintained upon depletion of the $CD20^+$ B cell compartment, and detectable for several months. CFSE labelling and Ki-67 staining confirmed the long-term survival of non-dividing plasma cells.

Conclusions: This study demonstrates a high frequency of differentiated, spontaneously ACPA-IgG secreting cells in SF. These cells are supported by SFMC for prolonged survival and autoantibody secretion, demonstrating that the synovial compartment is equipped to function as inflammatory niche for plasma cell survival.

Introduction

Many autoimmune diseases are characterised by the generation of self-reactive B cells leading to the production of pathogenic autoantibodies. Experiments in mice and evidence obtained from human bone marrow transplantation studies suggest that these autoantibodies are primarily produced by autoreactive plasma cells (PCs) residing in specified niches in the bone marrow.[1-3] However, non-dividing PCs that can survive and secrete antibodies for weeks in vitro, have also been detected in healthy human tonsils, spleen, and in the small intestine.[4-6] This has fuelled the hypothesis that also other, non-lymphoid tissues could, especially in the context of chronic autoimmune inflammation, provide an environment for long-term survival of PCs and, indeed, autoreactive PCs have been detected in kidneys of mice with lupus-nephritis.[7, 8] As these cells secrete potentially pathogenic autoantibodies, they could constitute an important if not crucial component of the inflammatory cascade that initiates and perpetuates autoimmune inflammation.

Rheumatoid arthritis (RA) is characterised by the presence of antibodies against citrullinated protein antigens (ACPA). ACPA are found in the majority of patients and represent one of the earliest detectable signs of immune disturbance, frequently predating the onset of clinical symptoms.[9] In established disease, ACPA associate with a severe disease phenotype, as ACPA-positive patients show more erosive joint destruction and extra-articular disease manifestations than ACPA-negative patients.[10] As ACPA are also highly specific for RA, these observations suggest that ACPA and/or ACPA-expressing B cells could be actively involved in initiating, aggravating and/or perpetuating the disease process. Indeed, emerging evidence shows that ACPA can exert effector functions in vitro that could promote in vivo inflammation, such as the activation of complement, mast cells, monocytes, neutrophils and osteoclasts.[11-15]

While these findings support a prominent role for ACPA in disease pathogenesis, little is known about the underlying autoreactive B cell response. So far, ACPA-secreting PCs were shown to reside in synovial tissue and ACPA were successfully cloned from synovial fluid (SF) B cells.[16, 17] Furthermore, enrichment of ACPA in SF has been suggested.[18] However, it remains unknown how and where the generation of ACPA-expressing B cells is initiated and maintained. We recently demonstrated that ACPA-expressing B cells circulate in the peripheral blood of patients with ACPA$^+$ RA.[19, 20] Next to ACPA-expressing memory B cells, we could identify a population of plasmablasts/plasma cells (PBs/PCs) that actively secrete ACPA in this compartment. These cells expressed primarily IgG or IgA, and their frequency was found to correlate with ACPA serum titres. The latter suggests that the circulating PB/PC population reflects an active part of the citrulline-specific B cell response and, thus, could contain valuable information on patient-specific disease activity. Until now, however, it is unknown whether these circulating PBs/PCs home to secondary lymphoid organs to compete for survival niches, or whether the synovial compartment of inflamed joints could be one of the tissues attracting these cells. As PCs can produce excessive amounts of antibodies and

survive in respective niches for years, they could be involved in driving and maintaining disease-specific pathogenic processes. In the current study, we set out to phenotypically and functionally characterise ACPA-secreting PBs/PCs in the synovial compartment. We hypothesised that spontaneously ACPA-secreting PBs/PCs could be frequent in SF, and that the synovial compartment and its cellular composition could provide a microenvironment supporting the long-term survival of (auto)antibody-secreting cells.

Methods

Patients

Peripheral blood, serum and SF samples were obtained on a cross-sectional basis from patients with ACPA-positive RA visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC), Leiden, The Netherlands. Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent for sample acquisition. No selection based on specific treatments was performed, but no patient had previously been treated with rituximab. Permission for conduct of the study was obtained from the ethical review board of LUMC. Additional SF samples were obtained from the Department of Rheumatology at Groene Hart Ziekenhuis, Gouda, The Netherlands, as rest material from arthrocentesis on an anonymous basis.

Cell isolation and culture

Mononuclear cells were isolated from peripheral blood and SF using Ficoll-Paque gradient centrifugation (LUMC pharmacy). Synovial fluid mononuclear cells and peripheral blood mononuclear cells (SFMC and PBMC) were cultured at a density of 2×10^5 cells/well in IMDM (Lonza) supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100U/ml), and 2mM Glutamax with no additional stimuli. Medium was replaced every 7 days. In some experiments, SFMC were depleted for $CD20^+$ B cells by magnetic beads (Miltenyi Biotech) and/or cells were labelled with CFSE prior to culture. Also, for specific experiments, PBMC (2×10^5 cells/well) were cultured on a layer of irradiated (7000 rad) mouse fibroblast cells stably transfected with human CD40 ligand (CD40L, 5×10^3 cells/well) in the presence of BAFF (Miltenyi, 100ng/ml), interleukin (IL)-21 (Invitrogen, 50ng/ml) and anti-IgM $F(ab')_2$-fragments (Jackson Immunoresearch Laboratories, 5µg/ml). This is indicated where appropriate.

Flow cytometry

The following antibodies were used to stain and analyse PBMC and SFMC by flow cytometry: CD3 Pacific Blue (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD20 Alexa Fluor 700 (clone 2H7), CD27 PE-Cy7 (clone M-T271), CD38 PerCP-Cy5.5 (clone HIT2), Ki-67 PE (clone 20Raj1), CD138 FITC (MI15) (all (except CD20 (Biolegend) and Ki-67 (eBioscience) obtained from BD Biosciences) and DAPI (Molecular Probes). Proliferation was assessed by labelling cells with CFSE (Molecular Probes).

Enzyme-Linked ImmunoSpot assay

The frequency of spontaneously (ACPA-)IgG secreting cells was determined using an Enzyme-Linked ImmunoSpot (ELISpot) assay. Briefly, PVDF-based membrane plates (type MSIP) were pre-treated with ethanol and coated overnight with unlabelled antibodies to human IgG (MT91/145, Mabtech). After blocking the plate with medium for at least 2 hours, total mononuclear cells (25×10^3-400×10^3 cells/well) were incubated overnight on the plate directly after ex vivo isolation and without prior stimulation. IgG produced spontaneously during the incubation period was visualised with either anti-IgG-biotin (MT78/145, Mabtech), biotinylated CCP2-peptide or its arginine control variant (CArgP2), followed by incubation with extravidin-AP (Sigma) and finally development with BCIP/NBT (Sigma).

Measurement of ACPA-IgG and total IgG

Serum and culture supernatants were assessed for the presence of ACPA-IgG by ELISA based on reactivity against the CCP2-peptide. The CCP2-peptide was coupled to streptavidin-coated ELISA plates, followed by detection with polyclonal rabbit anti human IgG HRP (DAKO). Serum samples were tested at a 1:50 dilution or higher; culture supernatants were tested 1:10 diluted or undiluted. The standard was diluted to the lowest concentration at which the standard curve was consistently linear, as previously described.[19] Total IgG was assessed by coating ELISA plates with anti-Igλ light chain (clone JDC-12, BD Pharmingen), followed by detection with polyclonal rabbit anti human IgG HRP (DAKO).

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.6.02. Correlations were assessed as non-parametric correlations. p Values <0.05 were considered significant.

Results

Both SFMC and PBMC harbour spontaneously ACPA-IgG-secreting cells

Previously, we demonstrated the presence of spontaneously ACPA-IgG-secreting PBs/PCs in the circulation of patients with ACPA-positive RA. To determine whether these cells would also be present in SF, we isolated paired PBMC and SFMC from individual patients followed by ex vivo culture at equal total cell density without the addition of exogenous stimuli for 7 days followed by the detection of ACPA-IgG in culture supernatants. Analysis by flow cytometry of the total mononuclear cell fraction of these and additional non-paired samples directly after isolation demonstrated that SFMC harboured, relatively, less B cells than PBMC (figure 3.1A, upper panel), whereas the proportion of memory B cells as well as PBs/PCs was comparable in the two compartments (figure 3.1A, lower panel). Despite the lower proportion of B cells, however, we detected enhanced cumulative production of ACPA-IgG in SFMC compared with

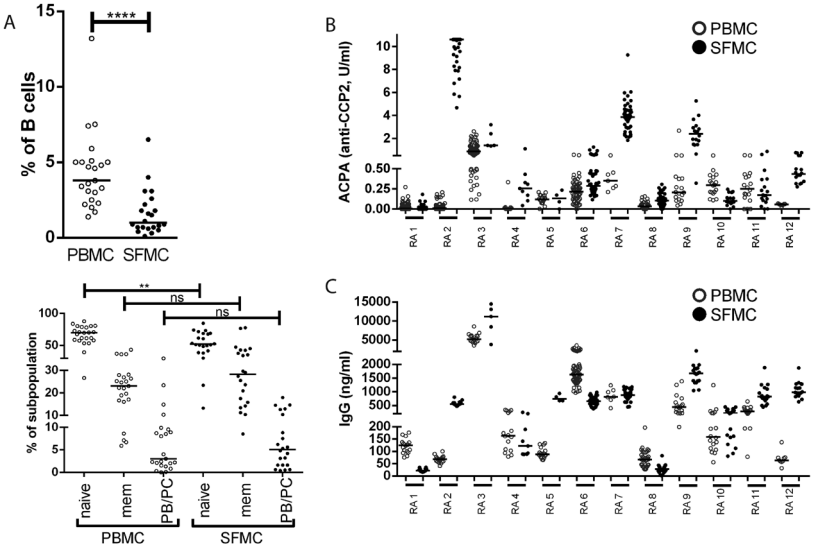

Figure 3.1: Synovial fluid mononuclear cells and peripheral blood mononuclear cells (SFMC and PBMC) harbour spontaneously ACPA-IgG-secreting cells. ***(A)*** *The frequency of B cells in SFMC is lower than in PBMC (upper panel). Although less B cells with a naïve phenotype (defined as CD19$^+$CD20$^+$CD27$^-$) were detected in SFMC, the relative distribution of the memory (CD19$^+$CD20$^+$CD27$^+$) and PB/PC (CD19$^+$CD20$^-$CD27^{++}) compartment was comparable (lower panel). (PBMC n=25; SMFC n=22)* ***(B)*** *Compared to PBMC, SFMC show enhanced spontaneous ACPA-IgG production after 7 days in most donors. Each dot represents a culture well.* ***(C)*** *Total IgG detected in the wells of the cultures shown in* ***(B)****.*

PBMC culture wells at day 7 in most donors (figure 3.1B). This was reflected by an enhanced secretion of total, non-specific IgG in some but not all donors (figure 3.1C).

The frequency of ACPA-IgG-secreting cells is greatly increased in SF

The data described above suggest a higher frequency of ACPA-secreting PBs/PCs in SF. To assess whether indeed more antibody secreting cells specific for citrullinated antigens are present in SFMC as compared with PBMC, we developed an ELISpot assay to detect and enumerate ACPA-secreting cells directly upon ex vivo isolation. Importantly, cells were directly seeded and did not receive exogenous stimulation. In this system, we readily observed spontaneously IgG-secreting cells in SFMC plated at a 1×10^5 cells/well density, but hardly any IgG-positive spots in PBMC-containing wells. PBMC plated at a higher cell density (4×10^5 cells/well) did show IgG-spots, but the frequency of citrulline-specific spots was still far lower than observed for SFMC-containing cultures (figure 3.2A). In fact, the abundant presence of citrulline-specific spots for SFMC allowed us to estimate the frequency of ACPA-IgG per total IgG

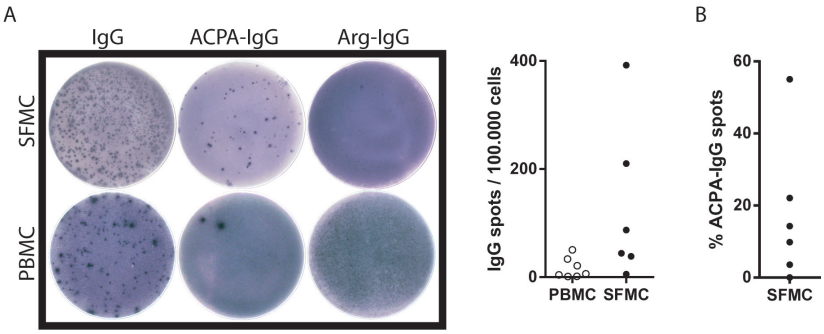

Figure 3.2: Enhanced frequency of anti-citrullinated protein antibodies (ACPA)-IgG-secreting cells in synovial fluid mononuclear cells (SFMC).
(A) *Representative ELISpot for total IgG, CCP2-reactive IgG and the control variant (IgG reactive with the arginine variant of CCP2) of a representative SFMC and PBMC sample (left). The right panel summarizes the quantity of IgG spots per 100.000 cells for PBMC and SFMC. Each dot is one donor.* ***(B)*** *Percentage ACPA-IgG specific spots of total IgG spots in the SFMC population. The overnight spontaneous production of IgG by PBMC and, as a consequence, the frequency of ACPA-IgG specific spots in PBMC samples was too low for a comparable analysis.*

secreting cells (figure 3.2B). Despite considerable variation between donors (median: 12%; IQR: 2.7-30%), we found that up to 50% of all IgG-secreting cells derived from SF had produced ACPA-IgG during overnight incubation. Importantly, hardly any spots reactive with the arginine control variant were detected and, if present, they were subtracted from the analysis. Together, these data indicate that a remarkably high frequency of differentiated, spontaneously ACPA-IgG secreting PBs/PCs is present in the SF of patients with ACPA^{+} RA.

SFMC promote the long-term survival of ACPA-IgG secreting PBs/PCs

PCs can survive in specified niches in the bone marrow for many years. In these niches, PCs are non-dividing and critically depend on direct contact with surrounding stromal cells.[3] Upon isolation, however, PCs rapidly die in in vitro cultures unless they are seeded on a layer of tissue-derived stromal cells.[21] Indeed, both bone marrow derived stromal cells and fibroblast-like synoviocytes (FLS) derived from RA or osteoarthritis synovium can prolong in vitro PC survival by several days.[22-27] It is unclear, however, whether PCs can only survive in specialised tissues or whether prolonged survival can also be observed in other compartments. Given our observation of a high frequency of autoreactive PBs/PCs in SF, we investigated whether the cellular and molecular composition of SF could provide a microenvironment favouring survival of (autoreactive) PCs. To this end, we seeded total SFMC and, when possible, paired PBMC (SFMC n=12, of which n=5 paired) and cultured this heterogeneous population without external stimuli for several weeks. Medium was refreshed completely every week and supernatants were assessed for the presence of ACPA-IgG. We hypothesised to find proliferation and differentiation of pre-existing PB into PC within the

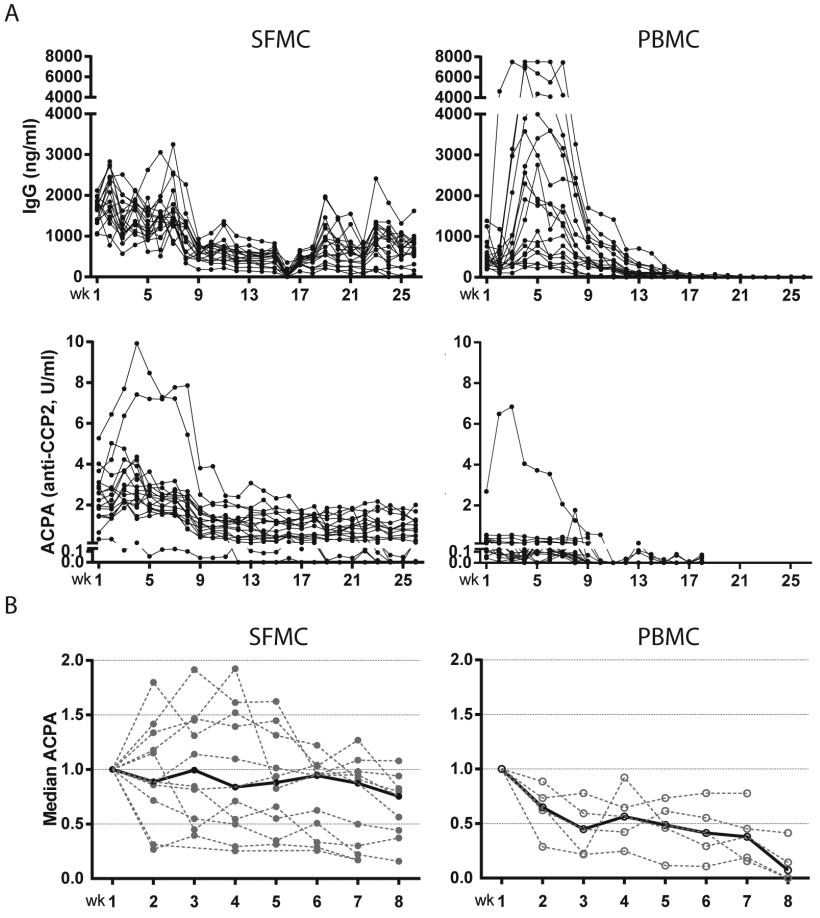

Figure 3.3: Spontaneous anti-citrullinated protein antibodies (ACPA)-IgG production is maintained in peripheral blood mononuclear cells (PBMC) for several weeks and in synovial fluid mononuclear cells (SFMC) for several months. ***(A)*** *Weekly spontaneous total IgG and ACPA-IgG production of one representative donor (paired sample); each dot represents one well; lines connect individual wells at different time points, thereby depicting the course of (ACPA-)IgG production over time.* ***(B)*** *Spontaneous ACPA-IgG production during a course of 8 weeks in SFMC and PBMC. Dotted lines represent median ACPA-IgG production of all culture wells of individual donors, the solid line represents the median of all donors. To allow for comparison between donors, the median ACPA-IgG production in wells at week 1 from each donor was set to 1. After week 8, ACPA-IgG production by PBMC was below the detection level in almost all donors.*

first days of culture followed by either survival or rapid cell-death. Interestingly, we observed spontaneous production of ACPA-IgG for several weeks in both PBMC and SFMC cultures (figure 3.3A). This secretion of ACPA-IgG was maintained even if the $CD20^+$ B cell compartment had been depleted prior to

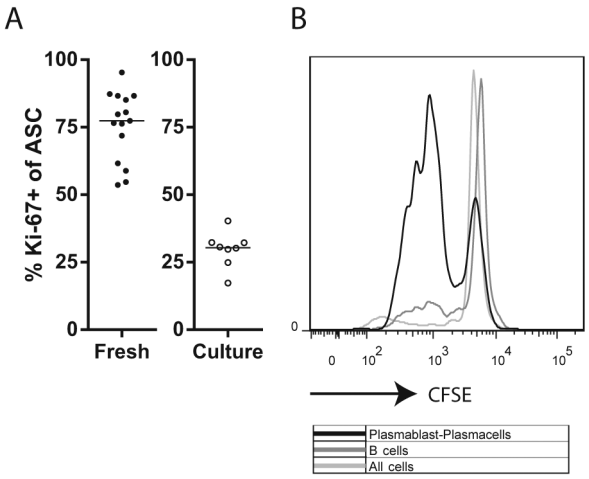

Figure 3.4: Antibody secreting cells (ASC) in culture show a rapid decline in Ki-67 expression and a non-dividing PC population. (A) *Ki-67 expression by $CD19^{+}CD20^{-}CD27^{++}$ PB/PC upon isolation and after two weeks of culture. Every dot is one donor* ***(B)*** *CFSE labelling shows that part of the cells that have a PB/PC phenotype after three weeks of culture have not divided.*

culture (figure 3.S2 and 3.S3). While ACPA production declined in cultured PBMC towards week 8, SFMC cultures, maintained continuous production that was, in some donors, remarkably stable for as long as 6 months (figure 3.3A-B). ELISpot performed at several time points during these cultures confirmed the presence of actively IgG secreting cells in the culture wells (not shown). As hypothesised, Ki-67 staining revealed a rapid decline of Ki-67 expression in the $CD19^{+}CD27^{++}CD20^{-}$ PB/PC compartment within the first 2 weeks of culture, suggesting differentiation of PBs towards non-dividing PCs (figure 3.4A). At later time-points, the frequency of Ki-67^{+} cells in this compartment was low while almost all cells expressed CD138 (not shown), supporting the notion that the surviving cell population was indeed bona-fide PCs. Finally, CFSE labelling directly upon isolation was performed and cells were analysed for dilution of the dye after several weeks in culture (figure 3.4B and 3.S1). Again, we observed that a fraction of cells with a PC phenotype had recently proliferated. However, we also observed a population with this phenotype that had not divided, supporting the notion that these PCs had already been present at the time of isolation and survived during weeks in culture.

Part of the IgG production detected could have originated from memory B cells differentiating to PCs upon autologous stimulation by bystander cells in the SF. To assess whether also memory B cells would differentiate into PCs by autologous stimulation in our PBMC cultures, and if so, to which degree, we determined the presence of anti-tetanus toxoid (TT) IgG in the supernatants as a proxy for memory B cell activation. This was performed in donors with protective serum IgG titres of anti-TT in which we expected the presence of

TT-reactive memory B cells. Indeed, we observed anti-TT IgG in supernatants of stimulated PBMC but not in non-stimulated cultures of the same donors (not shown). Although this does not fully exclude that part of the IgG produced in our cultures originates from autologous stimulation of memory B cells, it suggests that memory B cell proliferation/differentiation had no major contribution to the overall IgG production in the unstimulated cultures.

Together, these data provide evidence that SFMC of patients with ACPA-positive RA contain the cellular prerequisite to form a microenvironment in which both pre-existing PCs and PBs differentiating to PCs can survive for extended periods of time.

Discussion

We previously identified spontaneously ACPA-secreting PBs/PCs in peripheral blood of patients with ACPA-positive RA.[19] We suggested that this fraction of circulating B cells could reflect an active part of the citrulline-specific immune response that is constantly regenerated from the memory compartment. We now demonstrate that a remarkably high frequency of these cells is also present in SF. In addition, by comparing unstimulated cultures of PBMC and SFMC, we show that SFMC can provide a microenvironment supporting prolonged survival of ACPA-secreting PCs, and thus, could favour prolonged production of potentially pathogenic autoantibodies and/or B cell populations.

The provenance of ACPA-expressing B cells in SF is unknown. Although overall B cell counts were lower in SFMC as compared with PBMC, the frequency of spontaneously ACPA-IgG-secreting cells in this compartment was remarkably increased. Of note, we determined this frequency by antigen-specific ELISpot assessing over-night ACPA-IgG production by unstimulated cells, thus detecting only those cells that had already differentiated to antibody-secreting cells in vivo prior to isolation. The relatively high frequency of these ACPA-IgG-secreting PBs/PCs could be the result of directed migration of PBs from peripheral blood, specific retention, local generation and/or proliferation or prolonged survival due to the local microenvironment. Studies that directly compare the BCR repertoire of ACPA-expressing B cells and/or ACPA-secreting PBs/PCs in peripheral blood and SF in individual patients are missing, but high BAFF and TACI expression in RA synovitis, increased transcripts of CXCL13 and signs of local clonal B cell expansion suggest that ACPA-secreting PBs/PCs could be generated locally.[16, 28-31] Direct evidence for this assumption, however, is lacking. Importantly, we recently developed a technique to identify ACPA-expressing B cells and ACPA-secreting PBs/PCs by flow cytometry, which will allow to further investigate this particular question.[20]

Irrespective of the place of generation, however, ACPA-secreting PCs secrete autoantibodies for which an increasing number of potentially pathogenic effector functions have been described. Here, we show that ACPA-IgG-secreting PCs can survive in total SFMC cultures for more than 6 months without stimulation. We observed fluctuating quantities of ACPA-IgG in the first weeks of culture, which likely reflect differentiation and proliferation of preformed PBs. However, the weekly ACPA-IgG production at later time-points was

remarkably stable. Analysis by flow cytometry confirmed the presence of a non-dividing $CD19^{+}CD27^{++}CD20^{-}Ki\text{-}67^{-}CD138^{+}$ cell population in these cultures, consistent with the phenotype of terminally differentiated PCs. Although we cannot exclude that this long-term survival and microenvironment only forms in vitro, our data suggest that APCA-IgG-secreting PCs in SF could potentially be long-lived also in vivo. Therefore, not only inflamed tissues and SFMC and, to a lesser extent, even PBMC have the ability to generate conditions in which (autoreactive) PCs are able to survive for extended periods of time.

In the present study, we have not addressed the molecular and cellular components of the SFMC-derived survival niche, but it will be important to decipher the underlying mechanisms of PC survival in order to develop targeted interventions. PCs in bone marrow survival niches depend on contact with reticular stromal cells that produce CXCL12.[32] Also other cells, including eosinophils,[33] basophils,[34] monocytes/DCs[35, 36] and osteoclasts,[37] have been found to provide important PC survival signals. Next to CXCL12, IL-6 and APRIL,[26, 32, 38] and the interaction of CD80/86 on monocytes/DCs with CD28 on PCs are critical for bone marrow PC survival. Different lymphatic and non-lymphatic tissues can also provide such signals, however, especially under inflammatory conditions. 'Inflammatory niches' have been identified in humans and mice in several diseases, including SLE[8, 39] and allergic rhinitis.[40] Also here, cell-contact and factors derived from stromal cells, especially synovium-derived FLS in RA, were found to be critical for PC differentiation and survival.[41] FLS express several relevant factors, such as VCAM-1,[24, 42] BAFF[43] and IL-15.[22, 23] The expression of Bcl-x_L induced in B cells upon interaction with FLS also supports PC longevity.[25] Interestingly, our experiments indicate that also the SF compartment harbours all components needed to create a PC survival niche. In fact, this capacity was maintained in the cellular fraction of SF for months in vitro, while IgG production in PBMC cultures terminated much earlier. This could be due to some degree of (epigenetic) imprinting in the non B cell fraction of SFMC, as has previously been suggested for FLS, and could play an important role in chronicity of synovial inflammation.[44] Further studies need to be performed to identify cell type(s) and soluble factors responsible for this observation. In fact, targeting the crucial components of this survival niche could be an alternative way of targeting long-lived PCs and thereby decrease persistent ACPA production in patients with RA.

In conclusion, SFMC can create an environment that promotes survival of ACPA-IgG secreting PCs and, consequently, secretion of ACPA-IgG for months. Our study sheds light on the characteristics, distribution and dynamics of the citrulline-specific immune response in RA. Our finding of a high frequency of differentiated, ACPA-secreting PBs/PCs in SF, and the observation that SF can create an ideal environment for these cells to become long-lived, support the notion that SF and its components are active contributors to chronicity of synovial inflammation. Further studies using these spontaneously generated niches could provide more detailed knowledge on how ACPA-secreting PCs survive in vivo and identify potential targets that could shorten the lifespan of ACPA-secreting PCs.

Acknowledgements

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Supplementary figures

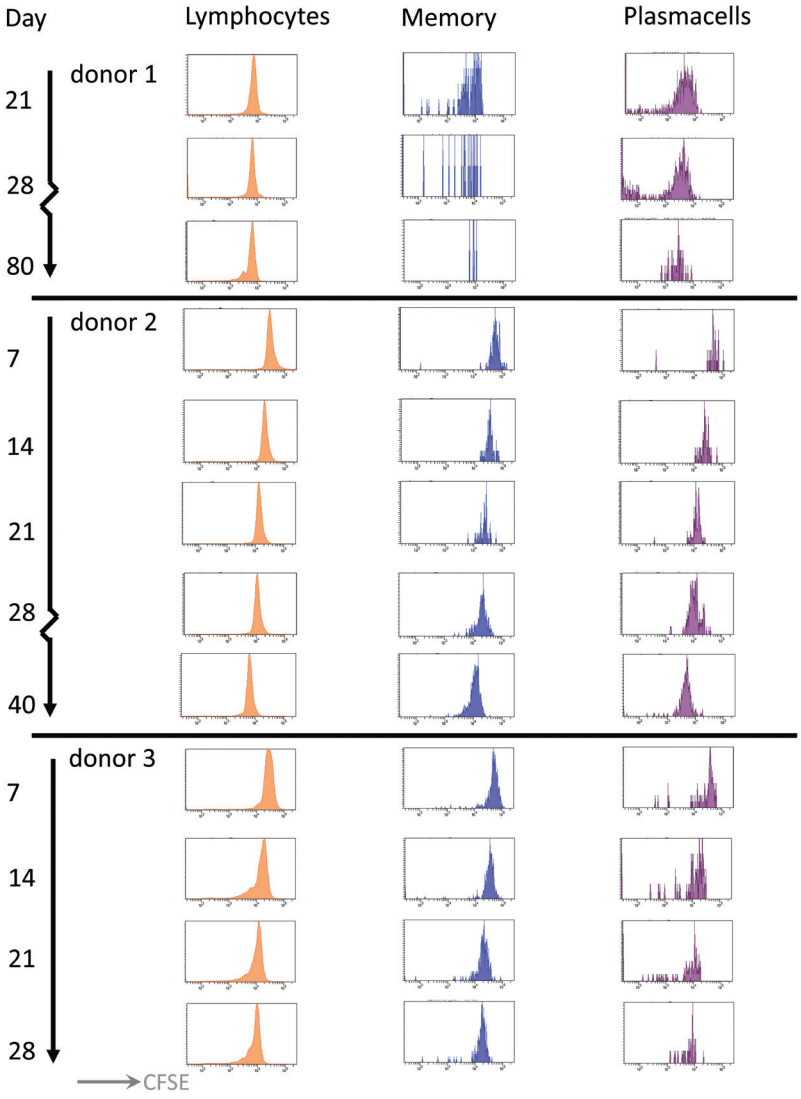

Figure 3.S1: Long-term persistence of a non-dividing plasma cell population in SFMC in vitro cultures. *Dilution of CFSE by lymphocytes and B cell subsets of three SFMC donors at different time points of culture. Despite decreasing signal strength and low numbers of cells, a fraction of cells with a plasma cell phenotype ($CD19^{+}CD27^{++}CD38^{+}CD138^{+}$) did not show dilution of CFSE even after weeks of culture.*

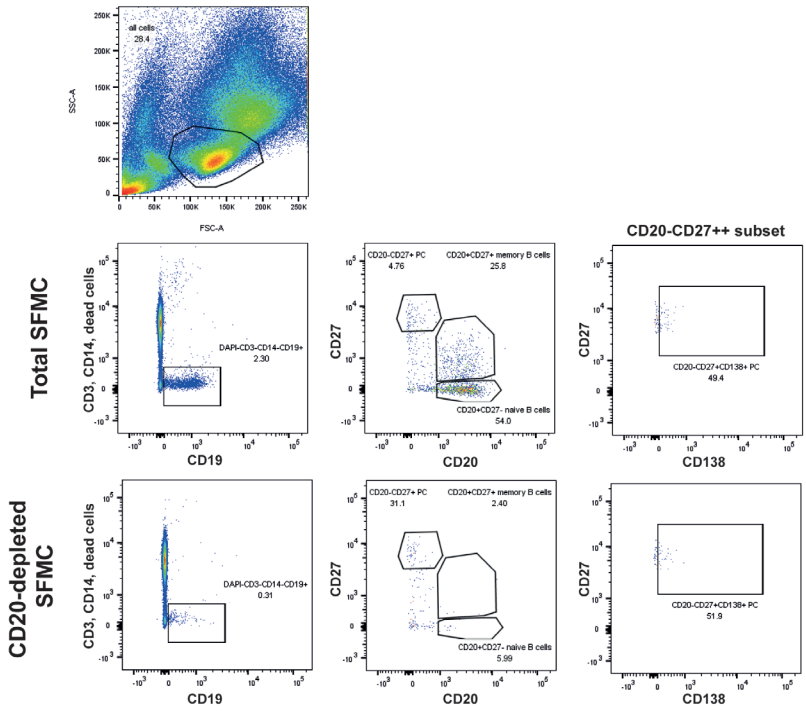

Figure 3.S2: Depletion of CD20⁺ B cells from synovial fluid mononuclear cells. *Depicted is one representative experiment of five. Upon depletion of CD20⁺ B cells, a fraction of CD19⁺CD20⁻CD27⁺⁺ cells remained, of which ~50% expressed CD138 (lower right panel). Gates are set based on the respective isotype control.*

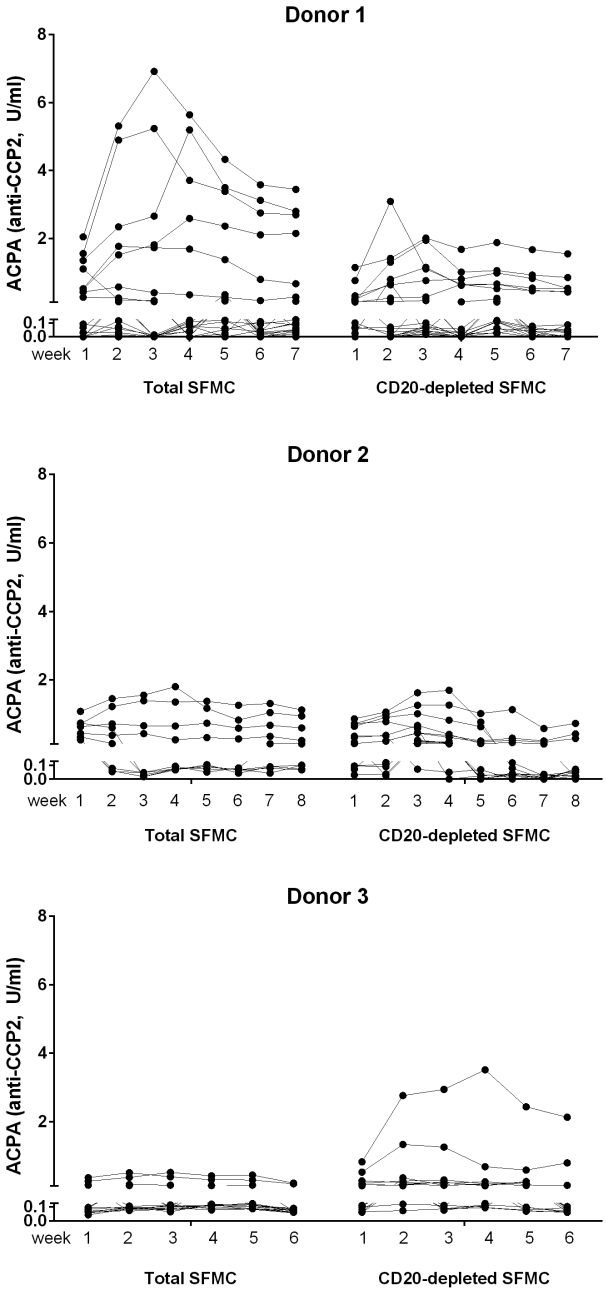

Figure 3.S3: Spontaneous production of ACPA-IgG in cultures of total SFMC and of SFMC depleted of CD20⁺ B cells as measured by ELISA. *Data are depicted for three donors, each dot represents one culture well.*

Chapter 4

Identification and characterisation of citrullinated antigen-specific B cells in peripheral blood of patients with rheumatoid arthritis

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Abstract

Objectives: Immunity to citrullinated antigens is a hallmark of rheumatoid arthritis (RA). We set out to elucidate its biology by identifying and characterising citrullinated antigen-specific B cells in peripheral blood of patients with RA.

Methods: Differentially labelled streptavidin and extravidin tetramers were conjugated to biotinylated CCP2 or control antigens and used in flow cytometry to identify citrullinated antigen-specific B cells in peripheral blood. Tetramer-positive and tetramer-negative B cells were isolated by FACS followed by in vitro culture and analysis of culture supernatants for the presence of antibodies against citrullinated protein antigens (ACPA) by ELISA. Cells were phenotypically characterised by flow cytometry.

Results: By combining differentially labelled CCP2 tetramers we successfully separated citrullinated antigen-specific B cells from non-specific background signals. Isolated tetramer-positive B cells, but not tetramer-negative cells, produced large amounts of ACPA upon in vitro stimulation. Phenotypic analyses revealed that citrullinated antigen-specific B cells displayed markers of class-switched memory B cells and plasmablasts, whereas only few cells displayed a naïve phenotype. The frequency of tetramer-positive cells was high (up to 1/500 memory B cells with a median of 1/12,500 total B cells) and correlated with ACPA serum titres and spontaneous ACPA production in culture.

Conclusions: We developed a technology to identify and isolate citrullinated antigen-specific B cells from peripheral blood of patients with RA. Most cells have a memory phenotype, express IgA or IgG and are present in relatively high frequencies. These data pave the path for a direct and detailed molecular characterisation of ACPA-expressing B cells and could lead to the identification of novel therapeutic targets.

Introduction

Rheumatoid arthritis (RA) is characterised by the presence of antibodies against citrullinated protein antigens (ACPA) in the majority of patients. ACPA are highly disease specific biomarkers that associate with destruction of joints, the pathological hallmark of RA.[1] Importantly, ACPA presence pre-disease prognosticates RA development, suggesting involvement of ACPA and/or ACPA-producing B cells in relevant disease-initiating processes. Experimental data support this notion as infusion of ACPA in mice exacerbates arthritis.[2] Moreover, ACPA can trigger a variety of inflammatory processes in vitro such as osteoclast activation[3], netosis by neutrophils[4], activation of complement pathways[5], and cytokine production by macrophages and mast cells.[6, 7] Likewise, variants in genes encoding peptidyl arginine deiminases, the enzymes that generate citrullinated antigens, are risk factors for RA.[8] Together, these observations suggest that ACPA and/or the citrullinated protein-specific B cell response have a central role in RA pathogenesis. Therefore, there is great interest to better understand the precise cellular origin of this response.

While ACPA have intensively been studied, little is known about the citrullinated antigen-specific B cell response. We have gathered evidence that this immune response is remarkably different from "conventional" B cell responses such as those generated upon, for example, infection or vaccination. For example, the pool of polyclonal ACPA is of remarkably low avidity and shows considerable cross-reactivity for different citrullinated proteins, whereas protective immune responses mostly generate high-affinity antibodies.[9, 10] Also, in contrast to protective immune responses where the presence of circulating, antigen-specific plasmablasts is restricted to a short time-window following antigenic triggering, ACPA-producing B cells seem to be constantly regenerated.[11, 12] Finally, the vast majority of ACPA-IgG molecules carries variable domain N-glycans.[13] As N-glycans require a specific amino acid consensus sequence in the protein backbone (Asn-X-Ser/Thr, where X is not a proline) that is absent from most germline encoded heavy chain (HC) and light chain (LC) sequences, this observation suggests that citrullinated antigen-reactive B cells undergo putative selection processes that favour survival of B cells harbouring N-glycosylation sites in their BCR.

Despite these atypical features, it is likely that citrullinated antigen-specific B cells mature in germinal centres (GC). Variable regions of monoclonal ACPA (mACPA) from both peripheral blood and synovial fluid (SF) show a high degree of somatic hypermutation.[14, 15] This indicates that the originating B cells received T cell help, a hallmark of the GC reaction.[10, 16] In fact, as "back-mutation" of variable regions to germline-encoded sequences leads to loss of citrulline-reactivity in these antibodies, it seems that classical GC T cell help is crucial for citrulline-reactivity to develop.[14]

Advances in the field and a more detailed characterisation of the citrullinated antigen-specific B cell response have been hampered, however, as it has proven notoriously difficult to identify these cells. These difficulties could be due to low frequency and the low affinity of ACPA. Using in vitro cultures of B cells of patient with RA, we demonstrated the presence of ACPA-producing B cells

in peripheral blood and SF ([11] and unpublished observations), and others demonstrated their presence in synovial tissue.[17-19] All of these studies, however, used ACPA as a proxy for ACPA-producing B cells, but none could study the B cell response itself.

Here, we show the identification and isolation of autoreactive B cells specific for citrullinated antigens using multicolour antigen-specific tetramer staining and flow cytometry. This technique offers unprecedented possibilities for phenotypic and functional characterisation of citrullinated antigen-specific B cells directly ex vivo and, thus, allows detailed studies of the citrullinated antigen-specific immune response.

Methods

Patients and healthy individuals

Peripheral blood and serum samples were obtained from ACPA-positive and ACPA-negative RA patients visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC). Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent. Treatment included disease-modifying anti-rheumatic drugs, biological agents, and glucocorticoids. Permission for conduct of the study was obtained from the ethical review board of LUMC.

Generation of HEK 293T cells expressing surface ACPA (HEK^{ACPA_TM})

To obtain cells expressing cell-surface mACPA, a synthetic gene was generated coding for the natural heavy chain (HC) amino acid sequence of mAb CitFib1.1[15] fused to the C-terminus of the transmembrane and cytoplasmic region (Q367-A445) of membrane-bound human IgG (Ref. GenBank BAA11363.1), including a linker. The gene was optimised for expression in human cells and flanked by PmeI restriction sites (GeneArt, Life Technologies). The pcDNA vector encoding the CitFib1.1 light chain (LC), also flanked with PmeI restriction sites, was described previously.[15] Lentiviral vectors encoding the LC and the transmembrane HC were generated by inserting the PmeI digested cDNAs into the EcoRV restriction sites of pRRL-CMV-bc-Puro and pRRL-CMV-bc-GFP, respectively. DNA constructs were validated by restriction analysis and sequencing. Third generation self-inactivating lentiviral vectors were produced as described.[20] Lentiviral vectors were quantified by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corporation). The infectious titre was derived from p24 concentrations assuming that 1ng p24 corresponds to 2500 infectious particles. HEK 293T cells were maintained in DMEM + 8% FCS + penicillin/streptomycin. The LC-expressing lentivirus was added to fresh medium supplemented with 8 μg/mL polybrene (Sigma); cells were incubated overnight. Three days post transduction cell cultures were treated with puromycin (1μg/mL). Stable transductants were subsequently transduced with the transmembrane HC-expressing lentivirus. Double-positive cells expressing both GFP and surface LC were sorted by FACS. LC expression

was detected using a biotinylated anti-lambda LC antibody (clone JDC-12, BD Pharmingen) followed by fluorescent staining with streptavidin-phycoerythrin (PE, eBiosciences). The binding profiles of both the secreted mACPA and the surface expressed ACPA were analysed and found to be comparable (see supplementary figure 4.S2).

Tetramers and flow cytometry

To construct antigen-carrying tetramers, biotinylated CCP2-peptide or its arginine control variant (CArgP2) were coupled to Allophycocyanin (APC) or BrilliantViolet 605 (BV605)-labelled streptavidin or Phycoerythrin (PE)-labelled extravidin molecules (Life Technologies, Sigma, BioLegend), as previously described.[21] In brief, 12.5µL biotinylated peptide (850µM) was incubated with 37,5µL labelled streptavidin or extravidin molecules (7µM) overnight at 4°C followed by removal of unbound peptide. Of note, the CCP2 antigen detects, despite its synthetic nature, natural ACPA.[22] PBMCs were isolated from 40 ml of peripheral blood by Ficoll-Paque gradient centrifugation and subsequently stained with CD3 Pacific Blue (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD20 Alexa Fluor 700 (clone 2H7, Biolegend), CD27 PE-Cy7 (clone M-T271), IgD FITC (clone IA6-2, all (except CD20) BD Biosciences) and DAPI. In addition, cells were stained with CCP2-coupled APC-tetramers and BV605-tetramers, and with a PE-labelled arginine control (CArgP2) tetramer. CD19-positive B cells were considered citrulline-specific if they stained double positive for both CCP2-containing tetramers but not for the arginine control variant. For selected experiments, citrullinated antigen-specific B cells were sorted on a BD FACS Aria flow cytometer.

Cell culture

B cells sorted by FACS were cultured in IMDM supplemented with 10% heat-inactivated FCS, penicillin/streptomycin (100U/ml), and 2mM Glutamax in 96-wells flat bottom plates on a layer of irradiated (7000 rad) mouse fibroblasts stably transfected with human CD40 ligand (CD40L, $5*10^3$ cells/well) in the presence of BAFF (Miltenyi, 100ng/mL), IL-21 (Invitrogen, 50ng/mL) and anti-IgM $F(ab')_2$-fragments (JacksonImmunoresearch Laboratories, 5µg/mL). Supernatants were harvested at day 14.

To assess for spontaneous ACPA production, PBMCs were cultured at a density of $2*10^5$ cells/well in IMDM (10% heat-inactivated FCS, penicillin/streptomycin (100U/mL), 2mM Glutamax) without additional stimuli. Supernatants were harvested at day 7.

Measurement of ACPA-IgG and total IgG

Serum and culture supernatants were assessed for the presence of ACPA-IgG by ELISA based on reactivity against the biotinylated CCP2-peptide or, where indicated, the arginine control variant. The CCP2-peptide was coupled to streptavidin-coated ELISA plates, followed by detection with polyclonal rabbit anti human IgG HRP (DAKO). Serum samples were tested at a 1:50 dilution

or higher; culture supernatants were tested 1:10 diluted or undiluted. The standard was diluted to the lowest concentration at which the standard curve was consistently linear.[11] Total IgG was assessed by ELISA (Bethyl Laboratories).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.02. Correlations were assessed as non-parametric correlations. p Values <0.05 were considered significant.

Results

Generation of HEK 293T cells expressing cell-surface ACPA

The reliable identification of rare, antigen-specific B cell populations in the circulation using antigen-carrying tetramers is challenging and specificity needs to be controlled at several stages.[23, 24] Next to "stickiness" of tetramers to cell surfaces, B cells can either specifically recognise the fluorophore or the bacterial streptavidin/extravidin molecule, or in the case of ACPA, cross-react with the arginine variant of the antigen. As these events could lead to false positive fluorescent signals, we first wished to control for these possibilities. Therefore, we generated a "positive control cell" expressing cell-surface mACPA (HEK^{ACPA_TM}, figure 4.1A). The mAb used for this purpose was initially selected based on recognition of citrullinated fibrinogen but also reacts with the CCP2 peptide.[15] Indeed, HEK^{ACPA_TM} but not non-transfected control cells stained positive for CCP2 tetramers (see figure 4.1B supplementary figure 4.S3), demonstrating that antigen-carrying tetramers can, in principle, specifically identify ACPA expressed on cell-surfaces.

Titrations of CCP2 and CArgP2 tetramers with different labels (APC, BV605 or PE) on HEK^{ACPA_TM} cells were subsequently used to determine optimal concentrations for further experiments (not shown).

Identification and validation of citrullinated antigen-specific B cells by multicolour antigenic tetramers

Given the positive staining results using HEK^{ACPA_TM} cells, we next applied the CCP2 tetramers to PBMCs of patients with $ACPA^+$ RA. Indeed, CCP2 tetramers identified positively stained B cell populations also in PBMCs (see supplementary figure 4.S1A). However, similar populations were also present in the CArgP2 tetramer and "streptavidin/extravidin-only" controls, making it impossible to discern specific from non-specific signals. Therefore, we combined differentially labelled CCP2 tetramers (APC/BV605) in one staining and also added an arginine control tetramer harbouring a third label (PE). This approach revealed a $CD19^+$ B cell population double positive for the two CCP2 tetramers but negative for the CArgP2 control, indicating that the combined staining procedure allowed for the detection of ACPA-expressing B cells (see figures 4.2 supplementary figure 4.S5). Separate control experiments were performed using combinations of three CArgP2 tetramers (APC/BV605/PE) or the three corresponding "empty" streptavidin/extravidin molecules (see supplementary

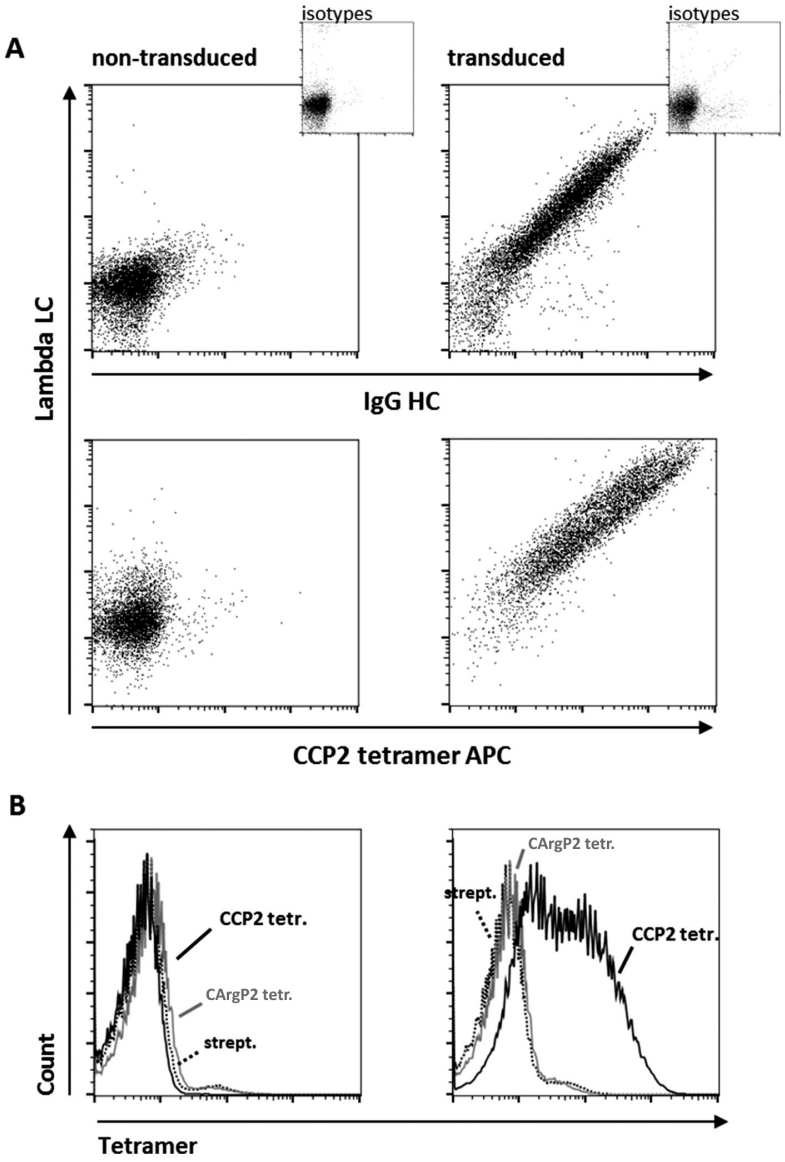

Figure 4.1: HEK 293T cells expressing cell surface monoclonal antibodies against citrullinated protein antigens (ACPA) (HEK^{ACPA_TM}). (A) *HEK 293T cells transduced with lentiviruses encoding the heavy chain and light chain of a monoclonal ACPA-IgG molecule modified by the addition of an IgG transmembrane sequence. Double staining for heavy and light chain (LC) (lambda) confirms cell surface expression of the antibody. Double staining with CCP2-tetramer (APC) and anti-lambda LC shows antigen-dependent binding.* ***(B)*** *Control staining with tetramers containing CArgP2, the arginine control variant of CCP2, or labelled streptavidin alone confirms specificity for citrulline.*

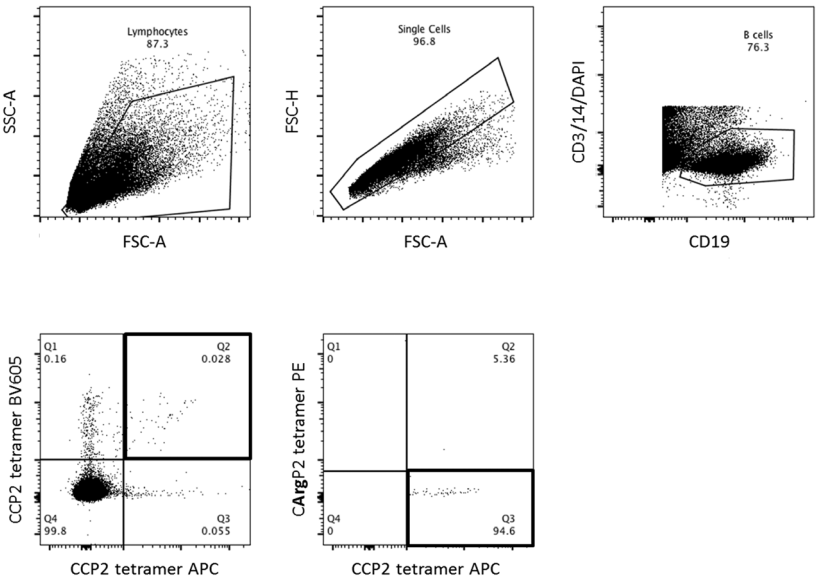

Figure 4.2: Gating strategy for the identification of citrullinated antigen-specific B cells. *CD19⁺ live B cells were considered specific for citrullinated antigens if they stained positive for both CCP2 tetramers and negative for the arginine (CArgP2) control variant. The figure depicts one representative staining of PBMCs obtained from a patient with ACPA⁺ RA. See also supplementary figure S1 for additional control stainings.*

figure 4.S1B). Eventually, B cells identified by two CCP2 tetramers were only considered specific if such cells were absent from the control stainings (see supplementary figure 4.S1B). Importantly, the staining signal of the population double positive for the two CCP2 tetramers (APC/BV605) could completely be blocked by pre-incubation with PE-labelled CCP2 tetramers, but not with the arginine control variant (CArgP; see supplementary figure 4.S4).

To confirm/validate that cells with this phenotype indeed represent citrullinated antigen-specific B cells, we isolated the respective population by FACS from PBMCs of three patients with ACPA⁺ RA. In doing so, we obtained 207, 14 and 317 tetramer-reactive cells from the three donors, respectively. In addition, tetramer-negative B cells were sorted. Upon isolation, cells were directly cultured in vitro with stimulants known to induce differentiation of B cells towards Ig-secreting plasmablasts. After 14 days, we could detect ACPA-IgG by ELISA in culture supernatants from tetramer-reactive cells of all donors (figure 4.3 and table 4.1). Importantly, culture of an equal or higher number of tetramer negative cells (up to $5*10^3$) did not yield detectable ACPA levels, except for very low amounts at $5*10^3$ cells/well in two of the three donors (table 4.1). Total IgG was detectable in both ACPA-positive and ACPA-negative wells, and no reactivity was detected against the arginine control peptide (not shown).

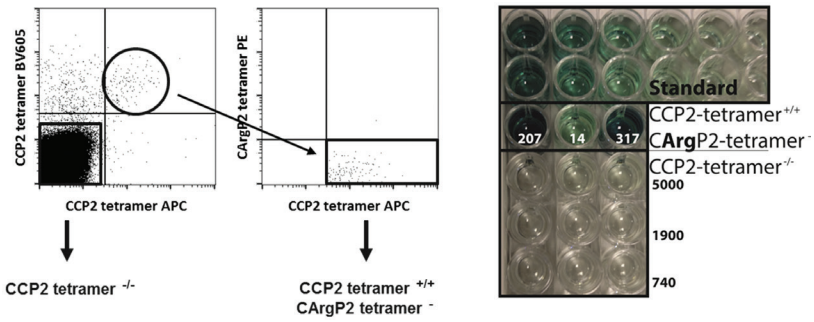

Figure 4.3: Isolation and culture confirm specificity for citrullinated antigens. *CD19⁺ B cells staining double positive for two differentially labelled CCP2-tetramers (BV605 and APC) and negative for the arginine control tetramer (PE) were isolated by FACS (n=3). Cells staining negative for both CCP2 tetramers were also isolated. Cells obtained (donor 1: 207 cells, donor 2: 14 cells, donor 3: 317 cells) were cultured in vitro for 14 days in the presence of CD40L-transfected fibroblasts, anti-IgM, IL-21 and BAFF. A titration of tetramer-negative cells was cultured under equal conditions. The presence of ACPA in culture supernatants was evaluated by CCP2-ELISA (right panel). See table 1 for absolute values obtained by ELISA.*

Table 4.1: *In vitro ACPA production by isolated, tetramer-positive B cells.*

Donor	Population	Cells/well (n)	ACPA-IgG (AU/mL)	IgG (µg/mL)
1	tetramer +	207	456	15.7
	tetramer -	5000		5.6
		1900		9.3
		740		6.0
		370		3.1
		370		1.7
2	tetramer +	14	1.6	0.3
	tetramer -	5000	0.158	3.8
		1900		6.9
		740		2.6
		740		4.4
3	tetramer +	317	301	4.0
	tetramer -	5000	0.178	9.3
		1900		11.9
		740		11.0
		370		3.0
		370		4.1

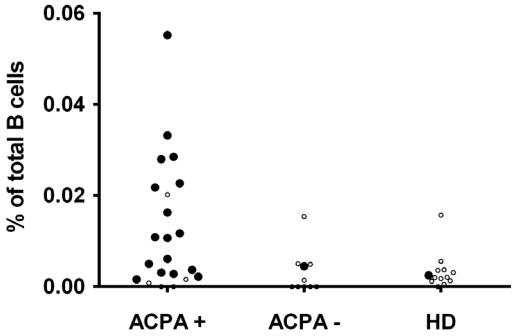

Figure 4.4: The frequency of citrullinated antigen-specific B cells in peripheral blood. *Dots depict the frequency of CD19⁺ B cells staining positive for both CCP2 tetramers and negative for the arginine (CArgP2) control variant. Samples with open circles depict donors in which a comparable population in the control staining using either three arginine tetramers or three "empty" streptavidin/extravidin molecules was observed. As these cases do not allow definite conclusions as to the specificity of the CCP2 staining, they were excluded from further analyses but depicted here for illustration. Of interest, such non-specific staining patterns in the control stainings were primarily observed in patients with ACPA- RA and healthy donors (HD). ACPA⁺ RA: n=22, ACPA⁻ RA: n=10; HD: n=13.*

These data show that the cell population identified represents B cells capable of producing ACPA upon stimulation, thus confirming specificity of the staining.

Tetramer-positive B cells of three patients with ACPA⁺ rheumatoid arthritis were sorted by flow cytometry as depicted in figure 4.4 followed by in vitro culture and analysis of the culture supernatants at day 14 for the presence of ACPA-IgG or total IgG by ELISA.

The frequency and phenotype of citrullinated antigen-specific B cells in peripheral blood

Having established/validated the staining procedure in functional experiments, we next wished to use this technique on a larger set of patients (n=22 ACPA⁺ RA, n=10 ACPA⁻ RA) and healthy donors (HD) (n=13) to determine the presence/frequency of ACPA-expressing B cells in RA. We found that up to 0.05% of CD19⁺ B cells derived from patients with ACPA⁺ RA displayed the double CCP2 tetramer-positive/CArgP2 tetramer-negative phenotype (median 0.008%, IQR 0.002-0.022%; figure 4.4). Hardly any such cells were identified in ACPA⁻ patients and HD.

Given the ubiquitous presence of citrullinated antigens in vivo, it could be hypothesised that citrullinated antigen-specific memory B cells hardly exist, as these would constantly be stimulated to differentiate to the plasmablast stage. Therefore, we wished to determine whether ACPA-expressing memory B cells are present and, if so, in which frequencies. Combining our tetramer staining with B cell surface markers, we found a relatively high number of citrullinated antigen-specific B cells (up to 1 in 500) exhibiting a memory phenotype (CD20⁺CD27⁺;

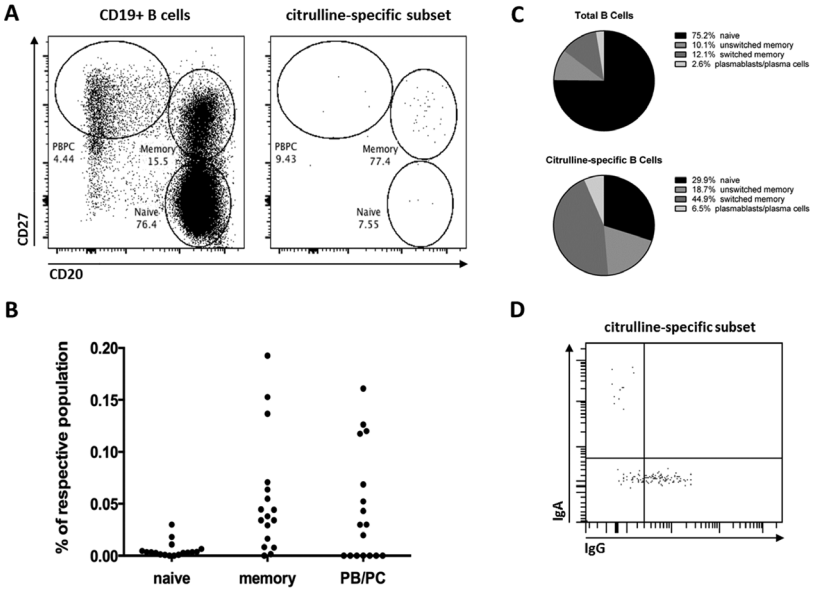

Figure 4.5: Phenotype of citrullinated antigen-specific B cells. (A) *CD19⁺ B cells were divided into subpopulations (naïve B cells, memory B cells, plasmablasts/plasmacells (PB/PC)) based on the expression of CD20 and CD27. Backgating of citrulline-specific B cells shows that the majority of these cells have a memory phenotype.* ***(B)*** *Subpopulation analysis in n=17 patients with ACPA⁺ RA.* ***(C)*** *The size of the respective subpopulations in the total B cell compartment and in the citrullinated antigen-specific B cell compartment based on the 17 patients depicted in (B). Unswitched memory B cells were defined as CD20⁺CD27⁺IgD⁺, and switched B cells were defined as CD20⁺CD27⁺IgD⁻.* ***(D)*** *The majority of citrullinated antigen-specific B cells express IgG or IgA isotypes. Depicted is one representative experiment of six.*

median 0.038%, IQR 0.012-0.067%; figure 4.5). In fact, the majority of memory B cells were class-switched, IgD^- B cells expressing either IgG or IgA isotypes. Also, we identified citrullinated antigen-specific plasmablasts ($CD20^{dim/-}CD27^{++}$; median 0.029%, IQR 0-0.092%), in line with our previous work.[11] This population also expressed IgG or IgA isotypes. Finally, we noted in some donors very few $CD20^+CD27^-$ B cells reactive with the CCP2-antigen. Together, these data indicate that most cells phenotypically correspond to antigen-experienced, class-switched post-GC B cells.

The frequency of citrullinated antigen-specific B cells correlates with spontaneous ACPA production and ACPA serum levels

Finally, we investigated whether the frequency of citrullinated antigen-specific B cells obtained by flow cytometry would correlate with the in vitro capacity of the cells to spontaneously produce ACPA. To this end, we cultured PBMC without stimulation and analysed supernatants for the presence of ACPA-IgG.

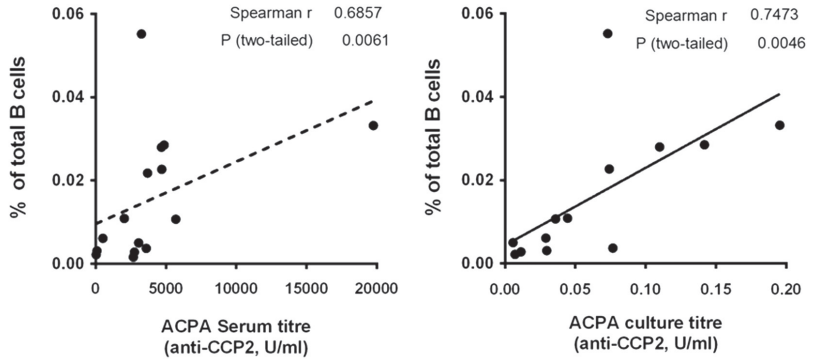

Figure 4.6: *The frequency of citrullinated antigen-specific CD19⁺ B cells correlates with ACPA serum titre (left panel) and the spontaneous production of ACPA-IgG in vitro (right panel). The correlation in the left panel is still observed upon removal of the datapoint with the highest ACPA serum titre (r=0.6357; p=0.0128).*

Indeed, the frequency of citrullinated antigen-specific B cells in peripheral blood directly correlated with spontaneous production of ACPA-IgG in vitro (figure 4.6). This was the case for both the memory B cell fraction and the plasmablast fraction, but not for the frequency of B cells with a naïve phenotype (not shown). Finally, the frequency of citrullinated antigen-specific B cells in the circulation showed a correlation with ACPA serum levels. Together, this suggests that the frequency of circulating, citrullinated-antigen specific B cells as identified by our staining technique reflects the dynamics of the in-vivo citrulline-specific immune response.

Discussion

There is great interest in understanding the biology of the citrulline-specific immune response in RA due to its early, pre-disease development, its strong association with disease risk and parameters of disease activity and severity, and the therapeutic efficacy of B cell depletion. The present study is, to the best of our knowledge, the first example of the direct ex vivo identification, isolation and characterisation of citrullinated antigen-specific B cells from patients with RA. Using flow cytometry and a multicolour tetramer staining approach, we succeeded in separating citrulline-specific from non-specific/ irrelevant "background" signals. Specificity of the staining was confirmed, as sorted tetramer-positive B cells produced detectable levels of ACPA-IgG upon stimulation, despite their very low number (as little as 14). Although we cannot formally claim that the cell surface staining approach presented here identifies all citrullinated antigen-specific B cells in the circulation, or that all cells identified as double-tetramer positive are indeed citrullinated antigen-specific, our data point to a very strong enrichment of these cells. Crucial to this enrichment is the use of two tetramers carrying different fluorophores, the addition of arginine control tetramers, and the generation of a positive control cell to evidence the specific recognition of the tetramers by cell surface ACPA in flow cytometry.

The observation that the frequency of citrullinated antigen-specific B cells as determined by tetramer staining correlates with ACPA serum levels and with the capacity of PBMCs to spontaneously produce ACPA in culture further supports the notion that the double-tetramer positive cell population is a very close approximation of the citrullinated antigen-specific B cell population.

Our analyses show a remarkable frequency of class-switched, IgG-expressing or IgA-expressing memory B cells in the citrullinated antigen-specific B cell pool. This frequency is estimated to exceed previously reported frequencies of circulating tetanus-specific B cells.[25] Phenotypically, the cells detected here could correspond to GC-derived memory B cells. This is interesting, as the requirements for the development of memory B cells in the GC reaction and the role of the antigen in this process have been intensively studied and debated.[16, 26, 27] GC-derived memory B cells are antigen-experienced, affinity-matured and affinity-selected cells thought to reside in a resting state.[16] It has initially been postulated that such a quiescent state would require separation of the BCR from its specific antigen, which occurs, for example, upon resolution of an immune response, as has also been demonstrated for T-cell responses directed against persistent viruses.[28-30] Therefore, one could expect that citrullinated antigen-specific B cells would constantly differentiate towards antibody secreting cells due to ubiquitous availability of citrullinated antigens, resulting in the absence of specific memory B cells. However, post-switch memory B cells accumulate in peripheral blood of patients with RA, and evidence of their clonal expansion has been detected in rheumatoid synovium.[31, 32] Moreover, circulating autoreactive memory B cells have been detected in SLE based on the expression of the 9G4 idiotype.[33] Finally, recent data from murine studies suggest that GC B cells require the presence of T follicular helper (T_{FH}) cells, which themselves critically depend on antigenic stimulation.[34, 35] In fact, continuous antigenic stimulation was found to support the persistence of T_{FH} cells and, at high dose, favoured the development of autoreactive B cell memory.[35] Thus, it is plausible that chronic availability of auto-antigens supports rather than inhibits the formation of also human memory B cells, which is indeed supported by our findings. Whether chronic availability of auto-antigens also impairs affinity maturation, as has been observed for the polyclonal ACPA response[9], and whether citrullinated antigen-specific B cells are indeed GC-derived or generated at extrafollicular sites, are interesting questions for future study.

We also detected relevant numbers of citrullinated antigen-specific plasmablasts. This is in line with our previous work employing B cell cultures, which, however, only allowed a rough approximation of their frequency.[11] The factors determining the size of this compartment are unclear, but it could reflect a dynamic part of the citrullinated-antigen specific immune response that is fuelled by the memory pool. In fact, it is intriguing that the circulating memory compartment correlates with ACPA serum titres. Partially, these titres originate from tissue-resident plasmablasts/plasmacells in bone marrow and/or inflamed tissues, but our observation suggests that the circulating memory compartment could also contribute to the degree of ACPA secretion, most likely via the formation of plasmablasts/plasmacells. Also, plasmablasts migrate and it

is tempting to speculate that they could be involved in spreading of the immune response to different joints. More extensive studies will be needed to determine their homing characteristics, proliferative capacity and longevity.

Together, we here provide the first, detailed antigen-specific, multiple tetramer-based ex vivo identification, characterisation and isolation of citrulline-reactive B cells from patients with RA. Our results demonstrate a remarkable expansion of the memory compartment, give first insights into developmental features of the citrulline-specific immune response, and pave the path for a dissection of citrullinated antigen-specific B cells at the molecular level as well as in response to (B cell-targeted) therapies.

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Supplementary figures

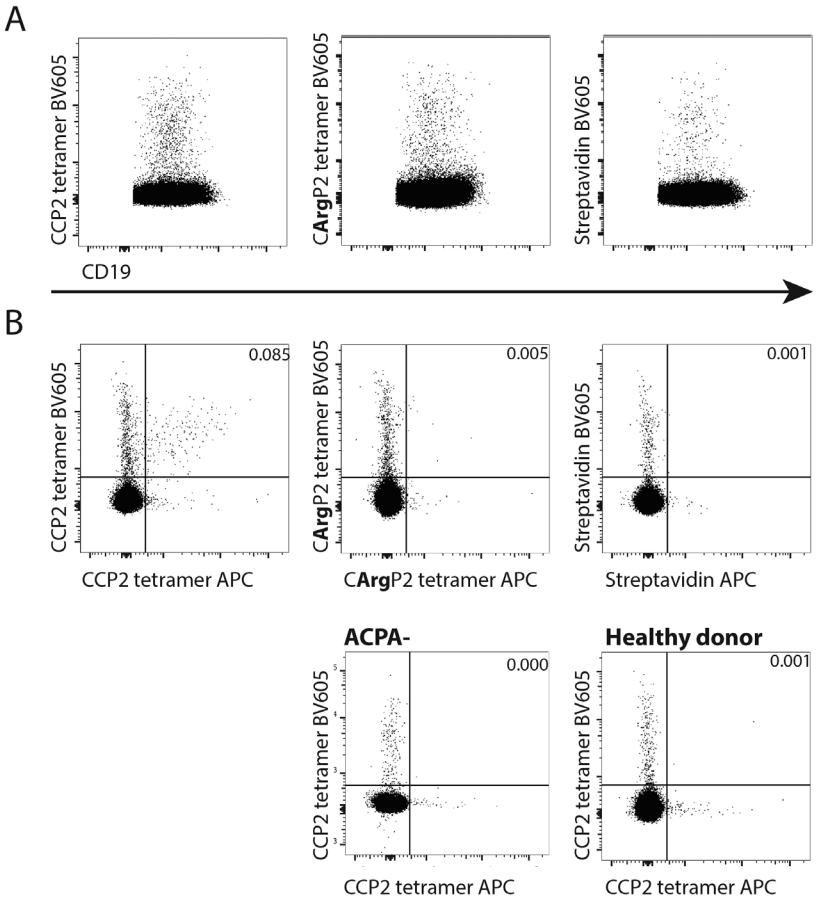

Figure 4.S1: Relevance of using a multicolour tetramer staining approach for the identification of citrullinated antigen-specific B cells. ***(A)*** *Single tetramer staining of CD19⁺ B cells obtained from a patient with ACPA⁺ RA using BV605 labelled CCP2 tetramers, CArgP2 tetramers or streptavidin alone. Staining patterns in samples stained with CArgP2 tetramers or streptavidin alone make it impossible to discern specific from non-specific signals. Similar stainings were observed with APC- and PE-labelled tetramers.* ***(B)*** *Effect of adding a second CCP2 tetramer with another fluorescent label (APC). Patients with ACPA⁺ RA show a clear double positive population, which is absent in patients with ACPA⁻ RA and healthy controls. Also, no double positive population could be detected in the ACPA⁺ sample when applying differentially labelled CArgP2 tetramers or "empty" streptavidin/extravidin molecules.*

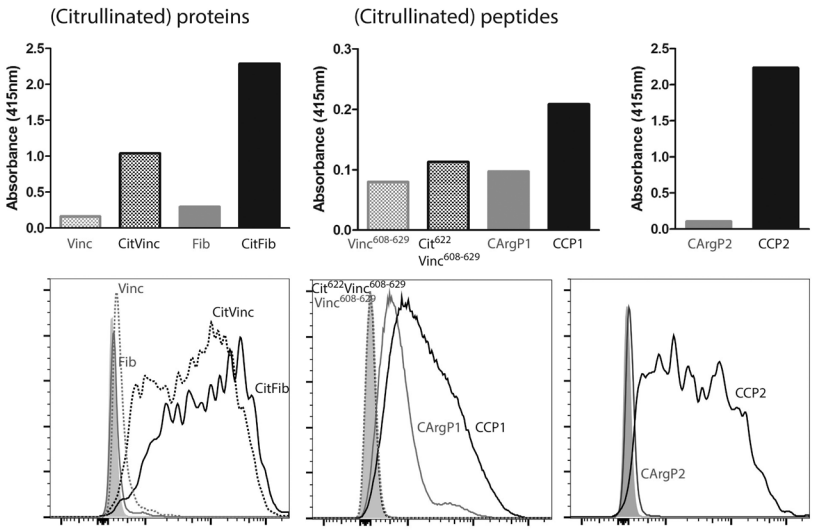

Figure 4.S2: *Binding profiles of secreted mAb CitFib1.1 and of the surface expressed antibody by HEK^{ACPA_TM} cells are similar. The upper row of panels shows specificity of mAB CitFib1.1 as tested by ELISA; the lower row of panels shows the corresponding binding of HEK^{ACPA_TM} cells as tested by FACS. Closed histograms represent unstained HEK^{ACPA_TM} cells.*

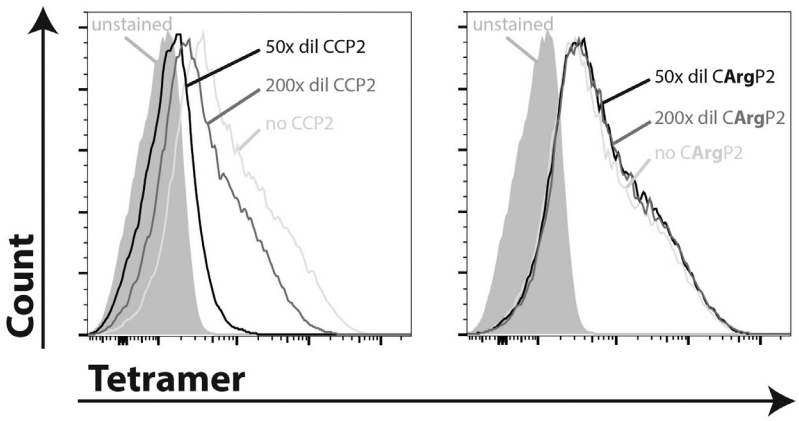

Figure 4.S3: *Pre-incubating HEK^{ACPA_TM} cells with CCP2 tetramer-PE inhibits the staining with the CCP2 tetramer-APC (left panel). No inhibitory effect is observed upon pre-incubation with the CArgP2 tetramer-PE control (right panel). Closed histograms represent unstained cells.*

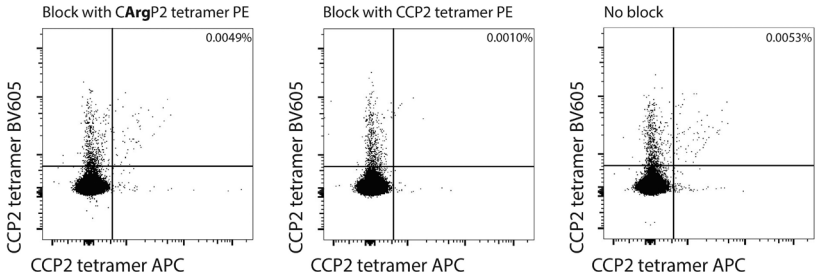

Figure 4.S4: *Pre-incubation of PBMC with PE-labelled CCP2 tetramers (middle panel) but not with the arginine control variant (CArgP2, left panel) inhibits binding of CCP2 tetramer-BV605 and CCP2 tetramer-APC.*

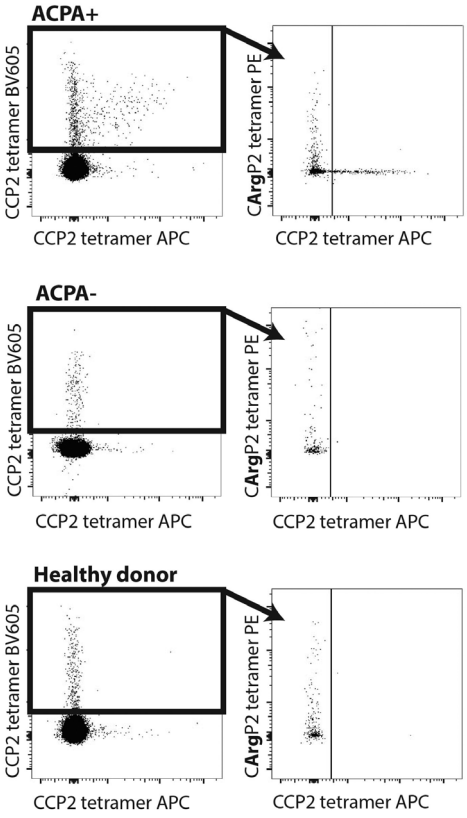

Figure 4.S5: *Non citrulline-specific background signal of CArgP2 tetramer-PE on B cells. Gating on all CCP2 tetramer-BV605 positive B cells (left column) and subsequently plotting the CArgP2 tetramer-PE signal versus the CCP2 tetramer-APC signal shows non citrulline-specific background staining in the PE channel, but no cells that are positive for all three tetramers.*

Chapter 5

Enhanced use of lambda light chains by citrullinated antigen-specific B cells: a sign of extensive receptor editing to avoid auto-reactivity?

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Manuscript in preparation

Abstract

Background: Despite considerable efforts in recent years to understand mechanisms of B cell tolerance, it remains unclear for most auto-immune diseases how autoreactive B cells arise and which (potentially defective) mechanisms govern their maturation. Secondary immunoglobulin light chain (LC) gene rearrangement in early B cell development represents an important mechanism of central tolerance aimed at eliminating autoreactive B cells. This mechanism, which depends on the activity of recombination activated genes (RAG) enzymes, has recently been shown to be operational also in antigen-activated mature B cells in the context of synovial inflammation, potentially to avoid auto-reactivity in the periphery.

Aim: To obtain insight into the characteristics and the development of human autoreactive B cells in the context of rheumatoid arthritis (RA), we studied the expression of functional kappa (κ) and lambda (λ) LC by citrullinated antigen-specific B cells and the LC usage of secreted anti-citrullinated protein antibodies (ACPA) in serum and synovial fluid of affected patients.

Methods: ACPA were isolated from serum and synovial fluid (SF) of ACPA-positive RA patients. Quantities of κ and λ LC and of total IgG of isolated ACPA or serum/SF depleted of ACPA were determined by ELISA. Peripheral blood from ACPA-positive RA patients was analysed by flow cytometry for the presence of kappa LC-expressing B cells in combination with specificity of the B cell receptor for citrullinated antigens.

Results: The κ-to-λ LC ratio of ACPA-IgG isolated from peripheral blood and SF was lower than the κ-to-λ LC ratio of ACPA-depleted total IgG. ACPA-IgG,λ was identified in a median percentage of 42% (IQR 33-54%) of total ACPA-IgG. ACPA were identified in a median frequency of 1 in 500 IgG,λ molecules (IQR 255-888), whereas the calculated frequency in the IgG,κ fraction was 1 in 560 (IQR 340-1200). Flow cytometry revealed reduced expression of functional kappa LC within the citrullinated antigen-specific B cell subset, corresponding to an enhanced frequency of lambda LC-positive B cells in this subset (median fraction lambda LC of total B cells: 0.42 (IQR 0.39-0.46); ACPA: 0.60 (IQR 0.55-0.79)). In patients before and three months after treatment with tocilizumab, we observed a decrease of ACPA-IgG in the total IgG,λ fraction but not in the total IgG,κ fraction.

Conclusions: The citrullinated antigen-specific B cell response more frequently generates lambda LC expressing B cells and secreted antibodies as compared to conventional immune responses. This could be the result of additional rounds of B cell receptor editing before entering the circulation or of secondary rearrangements in, for example, synovial tissue. This points to possible differences in the development of these autoreactive B cells and could help to unravel how autoreactive B cells arise.

Introduction

Anti-citrullinated protein antibodies (ACPA) are autoantibodies found in the majority of patients with rheumatoid arthritis (RA). Presence of ACPA associates with a severe disease phenotype[1] and ACPA can be found before clinical disease onset in some but not all patients[2]. ACPA are highly specific biomarkers for RA and various in vitro studies suggest that ACPA could promote in vivo inflammation by, for example, activating complement, mast cells, monocytes, neutrophils and osteoclasts[3-7].

Functional B cell receptors (BCR) consist of two heavy and two light chains (LC). LC contains a variable (V) and a joining (J) element and are encoded on two different loci: Ig kappa (κ) and lambda (λ). In total, the genes available allow for the expression of 320 different possible LC (40 Vκ x 5 Jκ + 30 Vλ x 4 Jλ[8-11]). Rearrangement of the first BCR during early B cell development results in most cases in a kappa LC-containing receptor[12, 13]. This BCR is often autoreactive. B cells expressing autoreactive receptors will be negatively selected before entering the periphery by either undergoing apoptosis, becoming anergic or by being allowed to modify their BCR by receptor editing. B cell receptor editing results in transcription of a different LC. This new LC can be further downstream on the same chromosome by deleting the locus of the previous LC, and when applicable in between loci, or the B cell can switch to the other kappa containing chromosome or switch to one of the lambda containing chromosomes. As a consequence, the chance of expressing a lambda LC increases with increased rounds of B cell receptor editing. Thus, a decreased κ-to-λ LC ratio could suggest that B cells underwent multiple rounds of B cell receptor editing before gaining their current specificity in an attempt to avoid auto-reactivity. However, the fate of autoreactive B cells is also determined by additional factors (such as the signalling strength through the BCR, affinity for the antigen, co-stimulatory factors, amongst others). Thus, every time a modified BCR is tested for auto-reactivity there is a chance that it escapes negative selection. This implies that a decreased κ-to-λ LC ratio could also be a sign that autoreactive B cells, upon altering their receptor (which could continue for several rounds), finally 'escape' and enter the periphery.

Several studies in mice and humans indicate that B cells can undergo secondary rearrangements in germinal centres and germinal centre like structures in synovium of RA patients[14-16]. To initiate Ig V-gene secondary rearrangements, recombination activating genes (RAG)-1 and RAG-2 need to be upregulated in B cells[17]. RAG-1 and RAG-2 binds recombination signal sequences (RSS) in between V domains and cleaves the DNA and holds it in place during receptor editing, finally resulting in a different BCR gene. Expression of RAG are confined to the developmental stage and is not found in peripheral blood B cells, but has been described in synovium of RA patients. Co-culture of fibroblast like synoviocytes (FLS) isolated from synovial tissue (ST) of RA patients and B cells result in the up-regulation of RAG genes and, as the B cell can thereby undergo additional rounds of B cell receptor editing, in increased usage of the lambda light chain. To obtain re-expression of RAG enzymes, the presence of BAFF and IL-6 is required[18]. Therefore inhibiting the IL-6

pathway by, for example, treating RA patients with the aIL-6R monoclonal antibody tocilizumab, could prevent the up-regulation of RAG genes in B cells in ST and thereby alter the κ-to-λ ratio in serum. As the ACPA immune response is an ongoing immune response, it could specifically result in less ACPA,λ after a few months of treatment.

The expression of κ- or λ LC has been described to be important in antibody specificities in different diseases. In systemic lupus erythematosus (SLE) it was found that λ LC using antibodies have a higher relative activity to hydrolyse human myelin basic protein (hMBP) compared to κ LC using antibodies[19]. In autoimmune pulmonary alveolar proteinosis (aPAP), the total concentration of GM-CSF autoantibodies does not correlate with disease severity but a lower κ-to-λ LC ratio does[20] suggesting that the LC usage might be associated with disease severity. Therefore a differential LC expression in ACPA could result in different effector functions of ACPA or be associated with a more severe disease phenotype. Although in RA the κ-to-λ LC ratio of total IgG has been compared to healthy donors previously[21], to our knowledge no study analysed the κ-to-λ LC ratio in RA for specific autoantibodies. As a second step, we questioned if ACPA-IgG, λ could have specific impact in RA patients which could be suggested if ACPA,λ level or κ-to-λ LC ratio would correlate with disease severity.

In the studies presented in this manuscript, we characterised the ACPA B cell response to obtain insight in how citrullinated antigen-specific B cells develop, using the κ-to-λ LC ratio in peripheral blood and synovial fluid as a proxy. We identified an increased λ LC usage in ACPA-IgG and by ACPA expressing B cells. Tocilizumab treatment, inhibiting IL6 signalling, resulted in a nonsignificant relative ACPA-IgG,λ decrease compared to total IgG,λ. Our data indicate that citrullinated antigen-specific B cells could be the result of additional rounds of B cell receptor editing which could indicate a difference in B cell development in these autoreactive B cells.

Methods

Patients

Peripheral blood, serum and synovial fluid samples were obtained on a cross-sectional basis from patients with ACPA-positive RA visiting the outpatient clinic of the Department of Rheumatology at Leiden University Medical Centre (LUMC), Leiden, The Netherlands. Patients fulfilled the 1987 criteria for RA at the time of diagnosis and gave written informed consent for sample acquisition. No selection based on specific treatments was performed. Peripheral blood samples were obtained from patients before their first tocilizumab treatment and three months thereafter. Permission for conduct of the study was obtained from the ethical review board of LUMC.

ACPA purification using CCP2-coated beads

ACPA and serum or synovial fluid (SF) depleted of ACPA were purified from serum and SF of patients with RA by antigen affinity chromatography using CCP2-coated beads, as previously described[22]. In short CCP2-coated beads

were generated by coupling biotinylated CCP2 (0.5 mg) to 5 ml of Pierce NeutrAvidin Plus UltraLink slurry resin (Thermo Scientific). After washing, the beads were loaded into a 96-well filter plate and 1:5 diluted serum or SF samples were incubated by shaking for 2h. The flow-through was obtained by centrifugation and one washing step, containing serum depleted of ACPA. ACPA were eluted from the resin by washing with 0.1M formic acid and neutralization with 2M Tris buffer.

Measurement of IgG-kappa, IgG-lambda, total IgG and serum ACPA-IgG titres

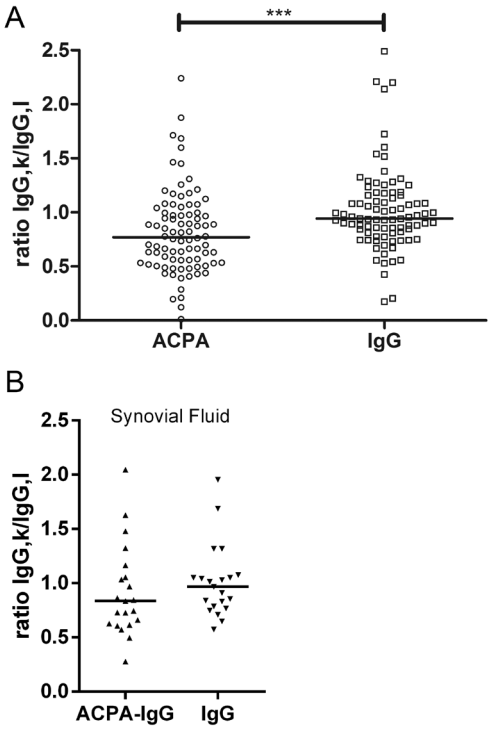
IgG light chain (LC) usage was assessed by coating ELISA plates with anti Igλ LC (clone JDC-12, BD Pharmingen) or anti Igκ LC (clone G20-193, BD pharmingen) monoclonal antibodies, followed by incubation with isolated ACPA or residual serum and then detected with polyclonal rabbit anti human IgG HRP (DAKO). The presence of total IgG was assessed by standard IgG ELISA (Bethyl Laboratories). To compare ELISA results, human reference serum (Bethyl Laboratories) was used as standard in all ELISA experiments. For analysis, arbitrary units of κ and λ were chosen relative to the amount of κ or λ in the aforementioned reference serum. Thereby the κ-to-λ ratio is compared to the ratio in this reference serum were the ratio 1.00 is equal to the ratio in the standard. ACPA-IgG titres in serum of tocilizumab treated patients were based on reactivity towards the CCP2-peptide. The CCP2-peptide was coupled to streptavidin-coated ELISA plates, followed by detection with polyclonal rabbit anti human IgG HRP (DAKO). Serum samples were tested at a 1:50 dilution or higher.

Flow cytometry

Streptavidin tetramer constructs were used to identify ACPA-expressing B cells as previously described[23]. PBMC were isolated from 40 ml of peripheral blood by Ficoll-Paque gradient centrifugation and stained with Fixable Violet (405nm) Dead Cell Stain Kit (Life technologies), CD3 Pacific Blue (clone UCHT1), CD14 Pacific Blue (clone M5E2), CD19 APC-Cy7 (clone SJ25C1), CD27 PE-Cy7 (clone M-T271), IgG-BV510 (clone G18-14), IgA-FITC (DAKO), IgM-PerCP Cy5.5 (clone G20-127), IgD-PE-CF594 (clone IA6-2), Igκ light chain-Alexa Fluor 700 (clone G20-193, (all except IgA) BD Biosciences), CCP2-coupled APC- and BV605-tetramers, and with a PE-labelled arginine control (CArgP2) tetramer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.02. Correlations were assessed as non-parametric paired correlations. p Values <0.05 were considered significant.



	Kappa	Lambda
ACPA	58%	42%
IgG	65%	35%

*Figure 5.1: ACPA-IgG have a decreased κ-to-λ LC ratio compared to IgG depleted of ACPA-IgG. **(A)** Paired ratios of ACPA-IgG and IgG depleted of ACPA in serum. Ratio of 1 corresponds to normal κ-to-λ LC 66%-to-33% ratio. Median κ-to-λ LC ratio of IgG 65% κ and 35% λ and ACPA-IgG 58% κ and 42% λ. (n=87 serum samples) (Median ratio IgG 0.94, IQR 0.81-1.16; Median ratio ACPA-IgG 0.76, IQR 0.53-1.00) **(B)** As (A) but ACPA-IgG and IgG isolated from synovial fluid. Median κ-to-λ LC ratio of IgG 66% κ and 34% λ and ACPA-IgG 61% κ and 39% λ (n=21). Data was assessed as non-parametric paired samples.*

Results

κ-to-λ light chain ratio of ACPA-IgG is decreased compared to total IgG

To obtain insight into the citrullinated antigen-specific immune response we studied if the κ and λ light chain (LC) usage of ACPA deviates from the κ and λ LC usage of total IgG. To this end, we isolated ACPA from serum and measured κ and λ IgG and calculated the κ-to-λ LC IgG ratio of isolated ACPA and residual serum (IgG depleted of ACPA). As was described previously[21], we confirmed that RA patients have a normal[21, 24] IgG 2-to-1 κ-to-λ ratio (figure 5.1A). Interestingly, we found that the κ-to-λ LC ratio of ACPA-IgG is significantly decreased to 0.76 (IQR 0.53-1.00) corresponding to a median of 1.4-to-1 κ-to-λ LC (58% κ and 42% λ). No correlation was observed between this ratio and ACPA-IgG serum tires (data not shown).

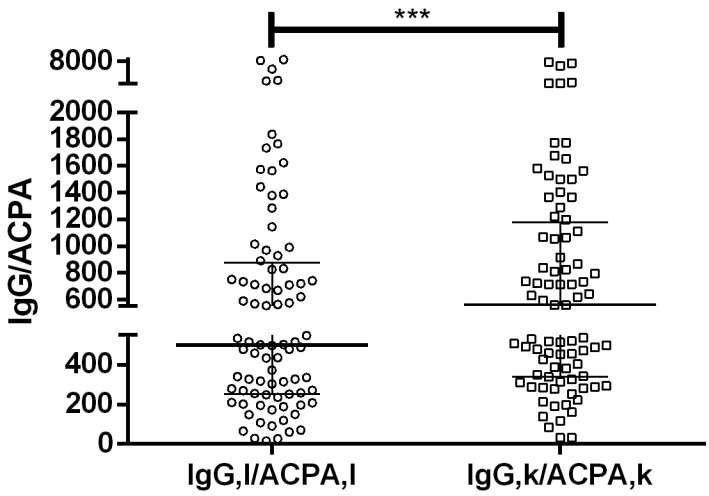


Figure 5.2: Median frequency of ACPA for lambda and kappa LC IgG's. *Left a median of 1 in 500 IgG,λ was identified as ACPA (IQR 255-888) and right a median of 1 in 560 IgG,κ was identified as ACPA (IQR 340-1200). Data was assessed as non-parametric paired samples.*

As it has been described that fibroblast like synoviocytes from synovial tissue (ST) can provide the signals required to up-regulate RAG genes in B cells and thereby can promote κ-to-λ LC switching in vitro[18], we questioned if the relative frequency of ACPA-IgG,λ LC in synovial fluid would be increased. Again, we observed a decreased κ-to-λ ratio in ACPA-IgG but did not find a different ratio compared to serum (figure 5.1B). This was also the case if only paired samples were analysed (data not shown).

Calculating the frequency of ACPA-IgG,λ in IgG,λ or ACPA-IgG,κ in IgG,κ could give additional insights into our data. This to see if the changes in ratio are more due to a decrease in kappa LC or an increase in lambda LC-using ACPA when analysing at different time points or when correlating kappa or lambda LC-using ACPA to disease activity. The identified frequency was 1 in 500 IgG,λ as ACPA (IQR 255-888) and 1 in 560 IgG,κ as ACPA (IQR 340-1200) (figure 5.2). We did not find a correlation between the amount of ACPA-IgG,λ, ACPA-IgG,κ or κ-to-λ LC ratio and disease severity (data not shown).

Distribution of λ and κ light chain usage in ACPA-expressing B cells

Antibodies in serum are secreted by antibody secreting cells. Although we have previously identified such cells for the citrullinated antigen-specific repertoire in peripheral blood[25], it is unknown whether ACPA-expressing cells in the periphery also show a shifted expression of λ LC. To answer this question, we combined our previously described antigen specific staining[23] with a staining for LC. Since λ LC was not but κ LC was available coupled to a fluorochrome which could be implemented in the previous described antigen specific staining we used κ LC as a proxy. In the left part of figure 5.3A we presented a different

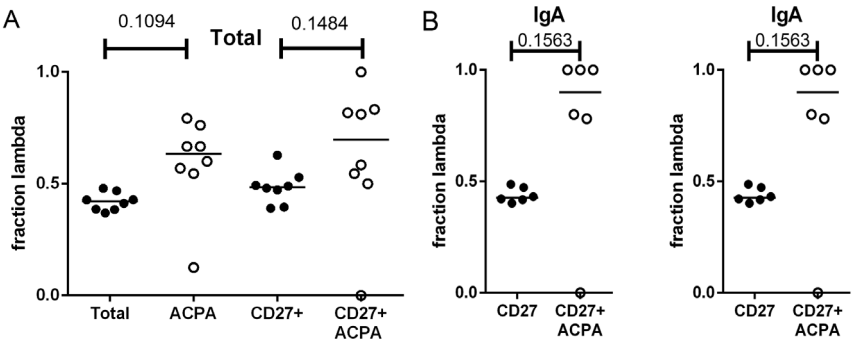


Figure 5.3: ACPA expressing B cell have higher frequency of lambda light chain usage compared to all B cells. (A) *Fraction of cells using lambda LC comparing the total B cells (median: 0.42; IQR: 0.39-0.46) to ACPA-expressing B cells (median 0.63; IQR 0.55-0.74); comparing the CD27⁺ B cells (median: 0.48; IQR: 0.42-0.52) to the CD27⁺ ACPA-expressing B cells (median: 0.70; IQR 0.51-0.83). Each dot is one donor; n=8.* ***(B)*** *Comparing total CD27⁺IgG⁺ B cells (median: 0.43; IQR 0.39-0.51) to ACPA-expressing CD27⁺IgG⁺ B cells (median 0.73; IQR 0.51-0.95) (n=8) and total CD27⁺IgA⁺ B cells (median: 0.43; IQR 0.41-0.48) to ACPA-expressing CD27⁺IgA⁺ B cells (median 0.90; IQR 0.59-1.00) (n=6). Data was assessed as non-parametric paired samples.*

distribution of κ expressing B cells in the citrullinated antigen-specific as compared to the total B cell population. To exclude the possibility that this is driven by a higher percentage of CD27⁺ ACPA-expressing B cells, we also analysed the data by only evaluating CD27⁺ B cells (right part figure 5.3A). This identified a median of 70% of CD27⁺ ACPA-expressing B cells expressing λ LC. Both CD27⁺ IgG⁺ and CD27⁺ IgA⁺ ACPA-expressing B cells showed this preference for λ LC expression, with medians of 73% and 90%, respectively (figure 5.3B).

Effect of tocilizumab treatment on Serum ACPA-IgG and IgG

Given that IL-6 is required for the re-expression of RAG enzymes when B cells undergo secondary rearrangements, we hypothesised that inhibition of IL-6 signalling during treatment with tocilizumab could result in less newly expressed ACPA-IgG carrying λ LC. Our previous data demonstrated that the citrullinated antigen-specific immune response is continuously generating new ACPA B cells, making it likely that a decline in ACPA-IgG,λ could be observed in tocilizumab-treated patients. Therefore, we calculated the ratio of ACPA-IgG prior to compared to three months after first tocilizumab treatment in six patients (figure 5.4A). Although the changes observed were not significant, most patients showed a trend towards lower ratios for ACPA-IgG while the same ratios tended to increase for total IgG.

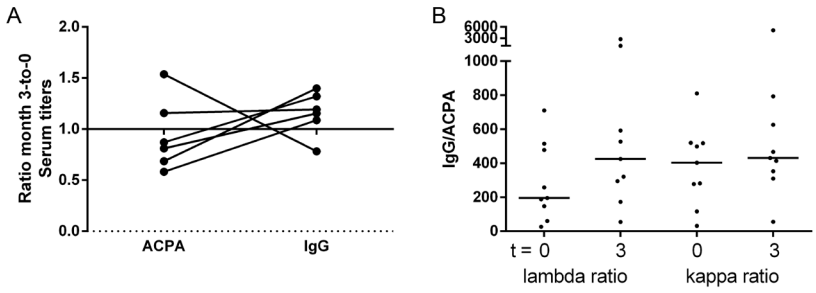


Figure 5.4: Ratio IgG,λ-to-ACPA-IgG,λ and IgG,κ-to-ACPA-IgG,κ before and three months after first tocilizumab treatment. (A) *Ratio of serum ACPA titres and total IgG titres three months after first tocilizumab treatment. Each dot is one patient; n=6.* ***(B)*** *IgG,λ-to-ACPA-IgG,λ in serum of tocilizumab treated patients increased (non-significant) within three months of treatment (t=0 before first treatment: median 196, IQR 104-498; t=3 three months after: median 425, IQR 234-797) were the IgG,κ-to-ACPA-IgG,κ is unchanged (t=0: 403, IQR 197-519; t=3: 431, IQR 331-710). K and λ LC measured in isolated ACPA or serum depleted of ACPA. Each dot is one patient; n=9. Data was assessed as non-parametric paired samples.*

To test if tocilizumab treatment specifically inhibited the active immune response resulting in less formation of ACPA,λ LC expressing B cells and therefore lower ACPA-IgG,λ serum titres, we isolated ACPA from these sera and measured ACPA-IgG and total IgG κ and λ and calculated as in figure 5.2 the frequency of IgG/ACPA (figure 5.4B). Although no significant differences were observed in these nine patients, the median of the IgG/ACPA lambda ratio before treatment was lower than after three months indicating a relative decrease of ACPA-IgG,λ to total IgG,λ in serum, whereas the relative ACPA-IgG,κ to total IgG,κ remained unchanged.

Discussion

In this manuscript, we describe that ACPA-IgG isolated from peripheral blood of ACPA positive RA patients contains a higher frequency of lambda LC compared to total IgG of the same patients. In SF, the ACPA-IgG lambda LC usage was also increased and similar to the frequency found in peripheral blood. Although a decreased κ-to-λ LC ratio in other diseases has been linked to disease severity, we could not find such a correlation for ACPA-IgG, nor did we find a correlation with ACPA-IgG serum titres. We also show that ACPA-expressing B cells more frequently express λ LC as compared to non citrullinated antigen-specific B cells. In patients treated with tocilizumab, we observed a trend towards decreased ACPA-IgG titres and increased IgG titres within three months of treatment. By analysing lambda and kappa LC IgG separately, we observed a relative decrease in ACPA-IgG,λ compared to ACPA-IgG,κ. These data suggest that tocilizumab treatment alters the IgG/ACPA-IgG lambda LC usage, and that part of ACPA-IgG that express lambda LC could be generated upon secondary rearrangements since this is (partially) blocked by treatment.

We observed that serum ACPA-IgG molecules more frequently contain lambda LC compared to total IgG. Even more interesting, not only the κ-to-λ LC distribution of serum antibodies but also the λ LC usage on ACPA-expressing switched memory B cells was higher compared to the total switched memory B cell population. This indicates that citrullinated antigen-specific B cells underwent more rounds of B cell receptor editing. In terms of regular B cell biology this could be the result of two different processes. First it could be a failure in the checkpoint deleting autoantibody-expressing B cells before they enter the periphery, where the autoreactive citrullinated antigen recognizing B cells do not go into apoptosis or undergo additional rounds of B cell receptor editing. Alternatively, secondary rearrangements and tolerance breakdown occurs in ST of RA patients and thereby results in autoreactive antibodies with an increased lambda usage.

As secondary rearrangements in ST are known to occur and lead to enhanced lambda usage[18] and as it was shown that B cells in ST are clonally related[15], we questioned whether lambda LC usage of ACPA-IgG in SF is increased. Although we found a similar difference comparing the κ-to-λ LC ratio of ACPA-IgG to total IgG in synovial fluid compared to peripheral blood, we did not observe more increased frequency of lambda LC usage in the SF. It could be possible that diffusion of antibodies secreted in SF or peripheral blood to the other compartment is quite quick, therefore no differences can be measured. Therefore, in future experiments antigen specific kappa LC staining on synovial fluid B cells or synovial tissue could give more insight into the question whether the higher ACPA,λ frequency could be due to secondary rearrangements in ST of RA patients.

Although in autoimmune pulmonary alveolar proteinosis (aPAP)[20] it was found that a decreased κ-to-λ LC ratio correlates with disease severity, for ACPA we did not observe a correlation. This with the note that ACPA is polyclonal and we did not look into fine specificities. Furthermore it would be interesting to see if the ACPA κ-to-λ LC ratio is already decreased in individuals before disease onset or decreases further depending on disease duration.

To see if tocilizumab can inhibit the development ACPA-IgG,λ B cells we studied the ACPA-IgG and total IgG in serum of patients before and three months after their first tocilizumab treatment. We observed a trend towards less ACPA-IgG,λ compared to total IgG,λ upon treatment. ACPA reflect an active immune response and we have demonstrated the presence of citrullinated antigen-specific plasmablasts/-cells in peripheral blood previously[25]. As the general life span of plasmablasts is short, and formation of new ACPA-IgG,λ secreting cells is, conceivably, inhibited by blocking IL-6 signalling, these data could suggest that short lived ACPA secreting plasmablasts died and less ACPA-IgG,λ expressing B cells were newly generated in these patients. This results in decreasing ACPA-IgG,λ serum titres as IgG gets cleared over time and less

new ACPA-IgG,λ will be secreted. Since no differences were observed in these patients for ACPA-IgG,κ our data indicate that generation of ACPA-IgG,κ secreting B cells is not dependent on IL-6 signalling. It would be interesting to see how IL-6 blockade is modulating the ACPA immune response in patients. Further studies should be done to investigate if IL-6 blockade is indeed directly involved in inhibiting secondary rearrangements resulting in less ACPA,λ and if this effect is long term and ACPA,λ specific.

In conclusion, ACPA-IgG more frequently contain lambda LC. Turning this observation around: altering the LC of an antibody increases the chance that a lambda LC is used and statistically also increases the cumulative chance that an autoreactive cell has escaped negative selection although the specificity is (still) autoreactive. If these autoreactive B cells are specific for citrullinated antigens this would result in an increased λ LC usage of citrullinated antigen-specific B cells. This citrullinated antigen specificity can be gained early in B cell development in the bone marrow but could develop upon secondary rearrangements in local germinal centre like structures in RA patients. In this manuscript, we gained more insight into the biology of citrullinated antigen-specific B cells, demonstrating that these cells show an increased lambda LC usage. This could indicate active B cell receptor editing which could be related to their existence in the periphery as a result of defective negative selection. Further studies are needed to better elucidate the development of these cells.

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Chapter 6

CD28 is expressed by synovial fluid B cells in rheumatoid arthritis

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Abstract

Objectives: Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of the synovial membrane of joints and the presence of autoantibodies produced by plasmablasts and plasma cells. Recently, expression of CD28 by long-lived plasma cells was found to promote survival and continued antibody production in mice. Considering the central role of B cells, plasma cells and autoantibodies in RA combined with the clinical efficacy of inhibiting CD28-triggering with CTLA4-Ig (Abatacept), we investigated the expression of CD28 by plasmablasts/-cells in peripheral blood of RA patients, systemic lupus erythematosus (SLE) patients and healthy controls. Likewise, we investigated the expression of CD28 by synovial fluid plasmablasts/-cells specific for citrullinated antigens.

Methods: B cells and plasmablasts/-cells obtained from freshly isolated peripheral blood and synovial fluid mononuclear cells (PBMC/SFMC) of RA patients were stained for CD28-expression by flow cytometry. PBMC from SLE patients and healthy donors were included as controls. The expression of CD28 by ACPA-expressing plasmablasts/-cells derived from SFMC of RA patients was determined by antigen-specific tetramer staining.

Results: CD28 was expressed on a subset of both B cells and plasmablasts/-cells from peripheral blood and was comparable in RA patients, healthy controls and SLE patients. CD28 was expressed at significantly higher levels on $CD20^+$ B cells derived from SFMC compared to B cells from PBMC. There was a trend towards higher expression of CD28 on synovial plasmablasts/-cells. In addition, CD28 was also expressed by ACPA-expressing plasmablasts/-cells derived from synovial fluid of RA patients.

Conclusions: CD28 is expressed on a subset of B cells, plasmablasts/-cells and ACPA-expressing B cells of RA patients. CD28-expressing B cells and plasmablasts/-cells are present in higher frequencies in the synovial compartment.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by inflammation of the synovial membrane of joints, leading to severe cartilage damage, bone erosion and disability if left untreated. RA affects approximately 1 % of the population[1] and is often characterised by the presence of autoantibodies. Anti-citrullinated protein antibodies (ACPA) are among the best-described autoantibodies in RA. ACPA are highly disease-specific and their presence associates with disease severity and worse prognosis[2-4]. ACPA have been implicated in disease pathogenesis[5-7] and are enriched in the joints of RA patients as compared to peripheral blood. ACPA-secreting plasmablasts/-cells have been found in both peripheral blood and in the synovial compartment[8-10].

Upon activation, naïve or memory B cells can differentiate into antibody-secreting plasmablasts and plasma cells. Based on their lifespan, plasma cells can be divided into two subsets; short lived plasma cells (SLPC) and long lived plasma cells (LLPC)[11]. Short-lived plasma cells are generated shortly after exposure to an antigen and have a relatively limited lifetime. In contrast, LLPC can persist for a much longer time period, sometimes lifelong, and provide long lasting humoral protection[12-14]. Most LLPC reside in survival niches in the bone marrow[15].

CD28 has been known for a long time as a prototypic co-stimulatory molecule for T cell activation. In conjunction with TCR activation, CD28 triggering leads to enhanced T cell activation, function and survival[16, 17]. The expression of CD28, however, is not confined to T cells, as plasma cells can also express this marker[18-20]. In fact, CD28-expression by plasma cells is regulated by the transcription factor Paired Box (Pax)5[21]. Pax5 represses CD28-expression in B cells, while loss of active Pax5 in plasma cells leads to the induction of CD28-expression. Recently, compelling evidence has been provided for the involvement of CD28 in long-term survival and function of plasma cells in mice. CD28 was found to function as an intrinsic factor that confers the capacity to LLPC to survive and maintain durable antibody production via interacting with its ligand CD80/CD86 expressed by dendritic cells (DC)[19]. Deficiency in CD28-expression resulted in a considerable decrease in LLPC numbers and antibody levels. In addition, CD28 promoted the up-regulation of B lymphocyte-induced maturation protein-1 (Blimp-1), a regulator of plasma cell differentiation[22]. Although CD28 was expressed on both LLPC and SLPC, it only mediated the survival of LLPC. This differential capacity for survival between LLPC and SLPC was shown to depend on downstream signalling of the CD28 Vav motif, which occurred only in LLPC and not in SLPC[22].

Abatacept, a fusion protein consisting of an extracellular domain of human cytotoxic T lymphocytes associated antigen 4 (CTLA-4) and a part of the human IgG Fc region, binds with high affinity to the co-stimulatory molecules CD80 and CD86. Abatacept is used to treat RA patients, and it is widely believed that its effectiveness is attributed to the ability to prevent T cell activation by blocking the binding of CD80/CD86 to CD28 expressed by T cells. The observation that autoantibodies persist upon treatment with B cell targeting therapies suggests continuous production of autoantibodies, potentially by LLPC. Indeed, we have

recently shown that the synovial compartment in RA is equipped to function as an inflammatory niche that promotes survival of ACPA-producing plasma cells[23]. As CD28 is also expressed by plasma cells, we questioned whether Abatacept could potentially also inhibit CD28-triggering of autoantibody producing B cells. Therefore we investigated the expression of CD28 on B cells, plasmablasts/-cells and ACPA-expressing plasmablasts in RA patients.

Materials and methods

Patients

Peripheral blood (n=19) and synovial fluid (SF, n=28) samples were obtained from ACPA-positive RA patients visiting the outpatient clinic of the Department of Rheumatology at the Leiden University Medical Center, the Netherlands. Patients were diagnosed with RA according to the 1987 classification criteria. Peripheral blood from SLE patients (n=10) and healthy donors (HD, n=11) was obtained for control. Written informed consent was obtained from all donors.

Cell isolation and flow cytometric analysis

PBMC were isolated from blood of RA patients, SLE patients and HD using Ficoll Plaque gradient centrifugation (LUMC Pharmacy). SF was obtained from inflamed knee joints of RA patients and first centrifuged to separate the cells from the fluid. Subsequently, SFMC were isolated using ficoll gradient centrifugation. Cells were stained with the following antibodies; CD3 Pacific Blue (UCHT1) or CD3 Alexa Fluor 700 (UCHT1), CD14 Pacific Blue (M5E2), CD19 APC-Cy7 (Sj25C1), CD20 PerCP (L27), CD27 PE-Cy7 (M-T271), CD28 PE (L293) or CD28 PE-CF594 (CD28.2) from BD Biosciences and CD20 Alexa Fluor 700 (2H7) from Biolegend. Dead cells were excluded with the use of 4',6-diamidino-2-phenulindole (DAPI; Molecular Probes). ACPA-expressing B cells were identified with a combination of differentially labelled CCP2 tetramers and a control tetramer, as described previously[24]. All samples were measured on a BD Fortessa or a BD LSRII cell analyser (BD Biosciences) and analysed using BD FACSDIVA software (BD Biosciences) and FlowJo version 7.6.5 (Tree Star Inc).

Absolute numbers of cells in peripheral blood were calculated based on the number of B cells from a pre-set volume of whole blood per pre-set number of beads using flow cytometry.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software Inc.). Percentages and absolute numbers of $CD19^+$ B cells, plasmablasts/-cells and CD28-expressing plasmablasts/-cells were compared using One Way ANOVA. The different cell populations in SFMC and PBMC were compared using Mann Whitney U test. p Values <0.05 were considered significant.

Results

Circulating plasmablasts in peripheral blood of RA patients, SLE patients and healthy donors express CD28

CD28 can function as survival molecule for LLPC[22]. As CTLA-4Ig (Abatacept) blocks the binding of CD28 to CD80/CD86 and has beneficial therapeutic effects in a subgroup of RA patients, we investigated whether antibody secreting cells of RA patients express CD28, and whether this CD28-expression is altered compared to SLE patients or HD. To this end, PBMC isolated from RA patients, SLE patients and HD were stained with antibodies against CD19, CD20, CD27, CD3, CD14 and CD28 and subjected to flow cytometry. Following exclusion of doublets, B cells were visualised by gating on the $CD3^-CD14^-DAPI^-CD19^+$ cell population. B cell subsets were discriminated based on the expression of CD20 and CD27, and plasmablasts/-cells were defined as $CD27^{++}CD20^-$ cells. (figure 6.1A). The percentage of $CD19^+$ B cells in peripheral blood of RA patients was comparable to the percentages detected in SLE patients and HD (figure 6.1B). In line with this finding, the absolute numbers of $CD19^+$ B cells, as determined by number of cells per ml of blood, in RA compared to SLE patients and HD were comparable (figure 6.1C). Likewise, the percentage and absolute number of plasmablasts/-cells was not significantly different between the different groups (figure 6.1D-E), although we did observe a trend towards an enhanced frequency of plasmablasts/-cells in SLE patients, in line with previous reports[25, 26]. Interestingly, we observed that approximately 5% of plasmablasts/-cells in RA peripheral blood expressed CD28. This expression was comparable to the CD28-expression on plasmablasts/-cells of SLE patients and HD (figure 6.1F-G). Together, these results show that a fraction of plasmablast/-cells of RA patients express CD28, although to a comparable extent compared to SLE patients and HD.

CD28-expression on B cells and plasmablasts/-cells from synovial fluid of RA patients

As phenotype and presence of immune cells in peripheral blood is not necessarily representative for cells present at the site of inflammation, we subsequently investigated the expression of CD28 on B cells and plasmablasts/-cells present in SF of inflamed joints of RA patients. CD28-expression was determined on the total $CD3^-CD14^-DAPI^-CD19^+$ B cell population, the $CD27^{++}CD20^-$ plasmablast/-cell population and the $CD20^+$ B cell population (figure 6.2A). In total, SFMC of 28 RA patients were compared to PBMC of 9 RA patients. The percentage of $CD19^+$ B cells in peripheral blood was significantly higher compared to SF (figure 6.2B). However, the percentage of $CD19^+$ B cells and of $CD20^+$ B cells that expressed CD28 was increased in SFMC compared to PBMC (figure 6.2C and E). Furthermore, there was a trend towards an increased percentage of CD28-expressing plasmablasts/-cells in SF but this did not reach statistical significance when compared to CD28-expressing plasmablasts/-cells from peripheral blood (figure 6.2D). These findings show that B cells and plasmablasts/-cells expressing CD28 are enriched at the site of inflammation in a part of RA patients.

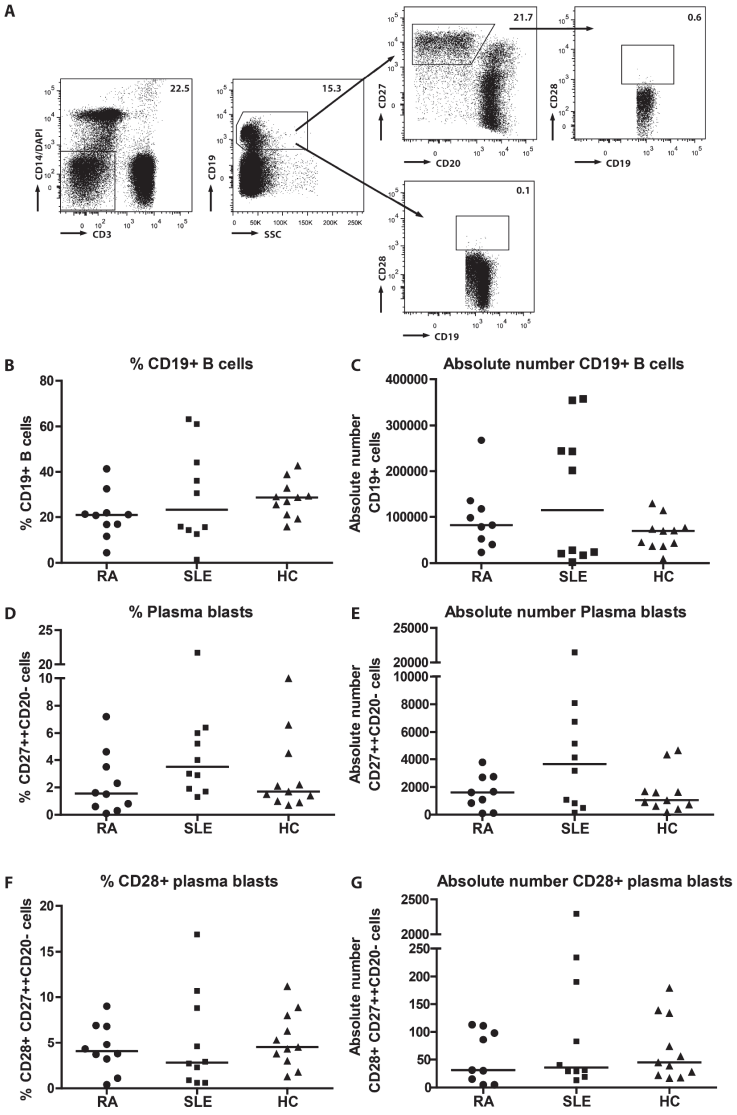

Figure 6.1: CD28 is expressed on plasmablasts in peripheral blood of RA patients, SLE patients and healthy donors. *PBMC were isolated from peripheral blood of RA patients (n=10), SLE patients (n=10) and healthy donors (HD) (n=11) and analysed for CD28 expression by flow cytometry.* ***(A)*** *$CD19^+$ B cells were determined by gating on the $CD3^-CD14^-DAPI^-CD19^+$ cell population and further subdivided in subsets based on the expression of CD20 and CD27. Plasmablasts were defined as $CD27^{++}CD20^-$ cells.* ***(B)*** *Summary of the percentage and absolute number* ***(C)*** *of $CD19^+$ B cells.* ***(D)*** *Percentage and absolute number* ***(E)*** *of plasmablasts for the different groups.* ***(F)*** *Summary of the percentage and absolute number* ***(G)*** *of plasmablasts expressing CD28. Absolute numbers are provided as number of cells per ml of blood. Lines represent median values.*

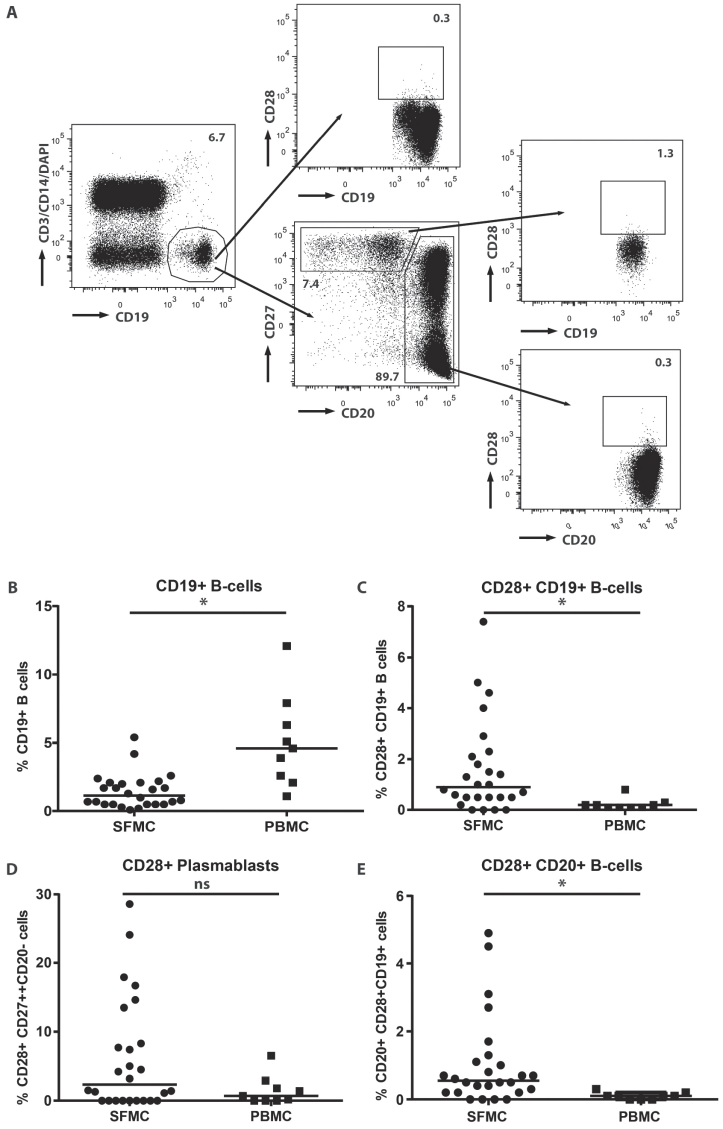

Figure 6.2: CD28 is highly expressed on CD20⁺ B cells in synovial fluid of RA patients. *SFMC were isolated from synovial fluid obtained from inflamed knee joints of RA patients and analysed for CD28-expression by B cells using flow cytometry. In total, SFMC of 28 RA patients were analysed and compared to PBMC of 9 RA patients.* ***(A)*** *CD28-expression on total B cells and on B cell subsets was analysed as in figure 6.1.* ***(B)*** *Identical gating strategies were used to determine the CD19⁺ B cells in SFMC and PBMC, and the summary is depicted.* ***(C)*** *Expression of CD28 by CD19⁺ B cells (as depicted in (B)).* ***(D)*** *Expression of CD28 on plasma blasts (CD27⁺⁺CD20⁻ cells) within SFMC and PBMC.* ***(E)*** *Expression of CD28 by CD20⁺ B cells (naive and memory B cells combined). Lines represent median values. * p<0.05, ns = not significant.*

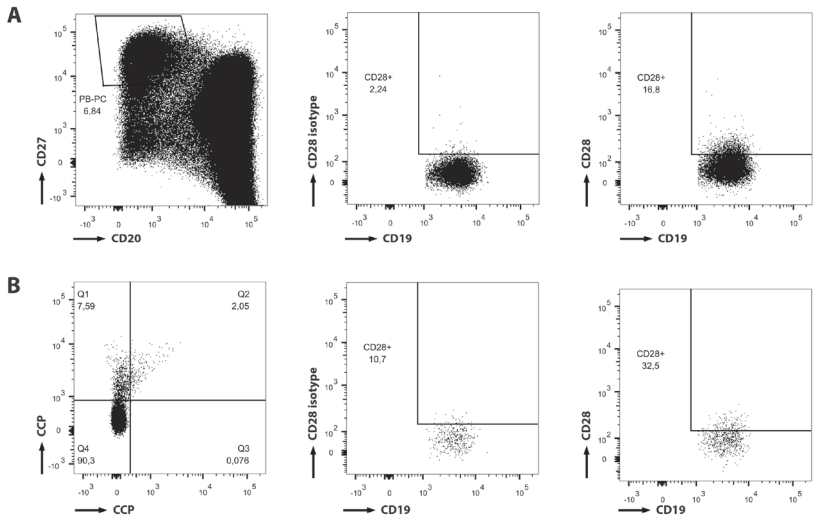

Figure 6.3: CD28 is expressed by ACPA⁺ B cells in synovial fluid. *CD28-expression was determined on ACPA-expressing plasmablasts/-cells in SFMC using flow cytometry.* ***(A)*** *CD19⁺ B cells were gated as depicted in figure 6.1A followed by identification of plasmablasts/-cells (left). Cut-off for CD28-expression (right) was set on the isotype control (middle).* ***(B)*** *ACPA-expressing plasmablasts/-cells were defined by a double staining for citrullinated antigen specific tetramers (left) in combination with negativity for a control tetramer.*

CD28 is expressed by ACPA-expressing plasmablasts/-cells

ACPA-secreting cells are present in the inflamed joints of patients with RA[9, 10]. The finding that a fraction of synovial B cells and plasmablasts/-cells displayed high expression of CD28 raised the question whether ACPA-expressing plasmablasts/-cells derived from the site of inflammation exhibit higher CD28-expression in comparison to total plasmablasts/-cells. To address this question, SFMC from RA patients were stained for the presence of citrullinated antigen-specific B cells that were identified using a combination of streptavidin tetramers as described previously[24]. Subsequently, CD28-expression was assessed and compared to the total plasmablast/-cell population (figure 6.3). ACPA-expressing plasmablasts/-cells were found to express CD28 although the percentage of CD28-expressing ACPA-expressing cells was comparable to the total population of CD28-expressing plasmablasts (figure 6.3A-B). These results show that CD28-expression can be expressed by ACPA-expressing plasmablasts/-cells.

Discussion

This report shows, for the first time, that CD28-expressing B cells and plasmablasts/-cells are present in peripheral blood of patients with RA and SLE. In peripheral blood, CD28 was expressed at comparable levels by plasmablasts/-cells of RA patients, SLE patients and healthy controls. Importantly, the

proportion of $CD28^+$ B cells was significantly higher in SF as compared to peripheral blood. Likewise, we found that ACPA-expressing plasmablasts/-cells present in the SF of patients with RA can express CD28, but that the level of CD28-expression is similar in ACPA-expressing plasmablasts/-cells compared to total plasmablasts/-cells. Hence, CD28-expression is present, but not enhanced on (autoantigen-specific) plasmablast/-cells in RA patients.

We observed a large variation in the proportion of CD28-expressing B cells and plasmablasts/-cells, especially in SF. Here, 0 to up to 30% of plasmablasts/-cells were found to express CD28. The reason for this variation is not known, nor is it known why CD28-expressing B cells are more prominent in the synovial compartment. The latter could be related to the notion that CD28-expression by B cells could be activation-dependent, as suggested by others[18]. Also, activated B cells are more prominently present in the inflamed synovium[27, 28].

The reason why B cells start to express CD28 and the functional consequences of CD28-expression by human B cells are unknown. However, it is conceivable, based on murine studies, that CD28-expression could be involved in the survival and longevity of antibody-secreting cells[19, 22]. Recently, it has been demonstrated that CD28 on LLPCs has a crucial role in maintaining antibody production and survival. Although CD28 is expressed on both LLPCs and SLPCs, it promotes survival in only LLPCs. Likewise, CD28 has been shown to be involved in the survival of multiple myeloma cells within the bone marrow[29, 30] and of Epstein-Barr Virus (EBV)-positive B cells. EBV-positive B cells were protected against Fas-induced apoptosis following triggering of CD28[31]. As also a proportion of ACPA-expressing B cells express CD28, it is tempting to speculate that CD28-signalling is involved in the longevity of these autoantibody-producing B cells, a notion that should be verified in future studies.

Although long-lived plasma cells reside in the bone marrow, it is possible that these cells are also present in (inflamed) tissues[32-35]. Hence, it is possible that CD28-expressing B cells from the inflamed joint of RA patients are part of the body's LLPC population and that they contribute to the chronicity of disease. Lack of enrichment of CD28-expression on ACPA-expressing plasmablasts/-cells when compared to total plasmablasts/-cells does not exclude that CD28 is involved in maintaining their survival, as CD28 has been shown to be expressed by both SLPC as well as LLPC, while it has a survival effect only on LLPC[19]. Hence, CD28-expression on total and autoantigen-specific plasmablasts/-cells could be comparable, but only autoantigen-specific plasmablasts/-cells might receive the survival signal, leading to extended survival and higher antibody production. In addition, it is possible that these cells are susceptible to treatment with CTLA-Ig (Abatacept). CTLA-4Ig leads to disruption of the interaction between CD28 and its ligands CD80/CD86 and is used to treat patients with RA. Intriguingly, Abatacept treatment results in a decrease in ACPA titres and in the percentage of memory B cells in patients with RA[36]. It is still not fully clear how Abatacept exerts its beneficial effects in RA, although its effects on the prevention of T cell activation are widely accepted. Other modes of action, however, might also be at play. For example, we have recently shown in a pre-clinical model of disease, that Abatacept treatment inhibits disease activity in

the absence of $CD4^+$ T cells[37]. These results suggest that the beneficiary effects are also mediated through a $CD4^+$ T cell independent mechanisms, possibly by inhibiting B cell responses. As a considerable proportion of B cells in SF, depending on the donor analysed, can express CD28, it will be important to elucidate whether Abatacept can inhibit CD28-triggering of (autoantibody-producing) B cells in RA. Together, it is tempting to speculate that the beneficial effects of Abatacept treatment may in part be the result of a direct effect of Abatacept on plasmablasts/-cells.

In summary, a small fraction of B cells in peripheral blood of RA patients expresses CD28. The proportion of B cells that express CD28 is higher on B cells in SF. And finally, APCA-expressing B cells in this compartment can also express this marker.

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Chapter 7

Summary and discussion

Summary and discussion

This thesis describes the characterisation of citrullinated antigen-specific B cells in patients with rheumatoid arthritis (RA). These cells express and secrete anti-citrullinated protein antibodies (ACPA), which represent the most specific autoantibody system in RA. We started out by identifying ACPA-producing B cells in peripheral blood. FACS sort followed by culture showed that these cells are not confined to the memory B cell subpopulation, as circulating plasmablasts/cells were found that spontaneously produced ACPA in culture (chapter 2). Further characterisation showed an increased frequency of ACPA-secreting plasma cells in synovial fluid (SF), i.e. at the site of inflammation, which harbours the capacity to form a survival niche in culture that promotes sustained (auto-)antibody production (chapter 3). Next, ACPA-expressing B cells were identified directly after isolation using a streptavidin tetramer-based flow cytometry staining technique developed in chapter 4. Furthermore, we provided evidence for altered development of ACPA-producing B cells, as these cells were found to express lambda light chains (LC) more frequently than non-autoreactive B cells (chapter 5). Finally, we investigated CD28 expression on ACPA-expressing B cells and total B cells in RA patients as relevant molecule for an enhanced live span of long-lived plasma cells (chapter 6).

The presence of ACPA in patients with RA is associated with a severe disease phenotype[1]. To better understand the underlying immune response against citrullinated antigens, characterisation of ACPA-expressing B cells is required. So far, studies showed that ACPA are different compared to antibodies against recall antigens since ACPA, for instance, have low avidity and show only limited avidity maturation[2, 3]. Furthermore, several studies show that ACPA can promote inflammatory responses[4-10]. In addition, it was found that ACPA-IgG are more extensively glycosylated in their Fab region compared to other IgG[11]. Together, these data suggest that ACPA-producing B cells could be different in molecular properties compared to other B cells. Therefore, this thesis focuses on the characterisation of ACPA-expressing B cells in patients with RA. We hypothesise that a better understanding of the phenotype and functional characteristics of these cells can lead to the development/identification of new therapeutic targets to prevent development or to eliminate these cells, which could lead to a less severe disease.

A major challenge hampering studies on ACPA-expressing B cells was, prior to the start of this thesis, the absence of a method to directly identify and visualize these cells on a single cell level. Therefore, we set out to develop an appropriate method to achieve this aim. Previous data from another research group had shown that ACPA production was detectable upon culture of mononuclear cells from SF but not from peripheral blood[12]. Despite this observation, we

first focused our research on peripheral blood, as peripheral blood is easier to obtain than SF, and as the detectability of ACPA depends on the assay used. As described in **chapter 2**, we isolated the total CD19-expressing B cell population from peripheral blood mononuclear cells (PBMC) and cultured it under broad stimulating conditions. We could indeed measure ACPA production by B cells isolated from peripheral blood of RA patients but were unable to detect this production if B cells were isolated from ACPA-negative RA patients or healthy donors. In parallel, another group[13] also provided data indicating the presence of ACPA producing B cells in peripheral blood. We extended our research to discriminate between subpopulations of B cells based on the surface expression of CD20 and CD27 to define naïve B cells, memory B cells or plasmablast/-cells (PB/PC). Upon stimulation, we could detect ACPA-production by memory B cells and, in addition, could detect spontaneous production of ACPA by circulating PB/PC. These data supported the hypothesis that the citrullinated antigen-specific immune response is an ongoing, continuously active immune response, which would be different from, for example, a resting memory B cell response against recall antigens.

Since ACPA titres are increased at the site of inflammation compared to peripheral blood[14], one could hypothesise that the frequency of autoreactive B cells is also higher. As we were successful in detecting ACPA in culture indicative of the presence of ACPA-secreting B cells in peripheral blood, we therefore investigated the presence of these B cells in SF. B cells had previously been isolated from synovial tissue (ST) followed by cloning of the BCR. This approach had revealed a substantial frequency of citrullinated antigen-specific B cells in this compartment[15, 16]. Applying our culture technique developed in chapter 2, we indeed identified an increased frequency of spontaneously ACPA-secreting cells in the synovial fluid (**chapter 3**). It remained unclear, however, whether this high frequency is due to migration, retention, local generation or prolonged survival of ACPA-secreting B cells in the synovial fluid compartment. We hypothesised that the inflammatory environment could be crucial for sustained ACPA production and, indeed, found that cells present in synovial fluid can spontaneously generate a niche in culture in which antibody secreting cells from the SF secrete (auto)reactive antibodies for months. Further studies of these niches and the prolonged life span of antibody secreting cells could give relevant insight into therapeutic targets that shorten the life span of (autoreactive) B cells in the synovial compartment.

So far, our studies had demonstrated the presence of ACPA-expressing B cells and their secretory capacity, but the culture system used did not allow for detailed characterisation of ACPA-expressing B cells on the single cell level. As data on ACPA indicate that ACPA differ on the molecular level from other immunoglobulins, we hypothesised that the development of ACPA-expressing B cells could be different compared to most B cells and thereby result in differences in phenotypic and functional characteristics. Therefore, we focused in subsequent work on the development of an antigen specific staining for this autoreactive B cell population, as analysis of single cells could reveal detailed differences between ACPA-expressing B cells and the general

B cell population in individual patients. Difficulties hampering the successful identification of citrullinated antigen-specific B cells so far had been a lack of a positive control, a high background of the flow cytometry staining using control proteins/peptides, a suspected low frequency of these cells and the low affinity of their BCR. In **chapter 4**, we combined the availability of a sequence of a monoclonal ACPA[17] with a triple streptavidin tetramer staining approach to successfully identify citrullinated antigen-specific B cells. Using this technique, we found that most ACPA-expressing B cells in the periphery have a switched (mostly IgG) memory phenotype which could correspond to (resting) GC-derived memory B cells. We could also identify ACPA-expressing PB/PC, which could reflect a dynamic population, as well as some ACPA-expressing B cells with a seemingly naïve phenotype. The frequency of ACPA-expressing B cells correlated with ACPA serum titres and we could detect ACPA-expressing B cells in all isotypes, in-line with available data from studies of serum ACPA[18]. Our technique could be very valuable for further studies of ACPA-expressing B cells, as flow cytometry staining including markers of interest could be used to identify altered characteristics of ACPA B cells. Furthermore, FACS sorting experiments isolating ACPA-expressing B cells can allow the study of functional differences. Culturing ACPA-expressing B cells versus B cells with other specificities can be performed using different stimuli or (potential) therapeutics. Differences in response *e.g.* survival or Ig secretion could help to characterise functional differences. Both type of experiments help to understand the citrullinated antigen-specific immune response and could lead towards new therapeutic targets for ACPA (B cell) specific therapies. BCR repertoire analysis or mRNA sequencing could be used to further characterise the cells. This characterisation could lead to a better understanding of ACPA B cells, for instance, in terms of their development and/or activity. Furthermore, the CCP2 (cyclic citrullinated peptide) antigen in our tetramers could be exchanged for another peptide which is known to be recognized by (autoreactive) B cells. Performing this adjustment could give insight into other (autoreactive) B cells and their development and or characteristics.

During B cell development, the (specificity of a) BCR is tested during several checkpoints for auto-reactivity. As autoreactive, ACPA-expressing B cells exist in the periphery of RA patients, we asked the question on how these cells form and/or escape selection checkpoints. During early B cell development, BCR make mostly use of kappa light chains (LC)[19, 20]. As B cells undergo rounds of B cell receptor editing, the chance of using a lambda LC increases. To investigate if formation of citrullinated antigen-specific B cells could be the result of B cells undergoing additional rounds of B cell receptor editing, potentially as a sign of selection pressure, we studied the kappa and lambda LC usage of citrullinated antigen-reactive BCR (**chapter 5**). BCR editing can occur in the bone marrow before 'escaping' negative selection or in germinal centre-like structures in RA patients, where the inflammatory environment makes it possible for B cells to alter their BCR. We studied the lambda LC usage of ACPA-IgG compared to total IgG showing an increased percentage of lambda LC usage in ACPA-IgG serum antibodies. We also observed an increased percentage of lambda LC

positive, ACPA-expressing CD27$^+$ B cells. Of interest, we found that inhibition of interleukin (IL)-6 signalling in patients upon tocilizumab (aIL-6R) treatment reduced specifically the amount of ACPA-IgG using lambda LC within the first months. As the IL-6 signalling pathway is responsible for the (re-)expression of RAG enzymes required for BCR secondary rearrangements in synovial fluid/tissue[21], this indicates that interference with this pathway could prevent the development of part of lambda LC-positive ACPA resulting in lower ACPA titres and suggests that part of the ACPA-IgG,λ could have been formed upon secondary rearrangements in synovial fluid/tissue.

Recent data from murine studies show that the expression of CD28 on plasma cells promotes survival[22]. At the same time, inhibiting the interaction of CD28 with CD80/86 in RA patients treated with Abatacept (CTLA4-Ig) lowers ACPA serum titres and percentages of switched memory B cells[23]. Therefore, we set out to investigate if and to what extend CD28 is expressed by B cells of RA patients. We compared CD28 expression on peripheral B cell subsets of RA- and systemic lupus erythematosus (SLE)-patients, and of healthy controls (**chapter 6**). We could identify CD28-expressing B cells in comparable frequencies in the three groups. Interestingly, we found a higher frequency of CD28 expression on CD20$^+$ B cells in SF obtained from RA patients and a trend towards a higher expression on PB/PC. Using the antigen-specific staining described in chapter 4, we detected CD28 expression, in similar frequencies, on ACPA-expressing plasmablasts. Whether these CD28-expressing, ACPA-expressing plasmablasts are the cells that survive in long-lived cultures, like in chapter 3, or in the circulation of RA patients is an interesting question and could be studied in future experiments, thereby providing insight into the survival of long-lived citrulline specific plasmacells in patients with RA.

Together, this thesis solves part of the puzzle concerning the characterisation of ACPA B cells and provides insights into the biology of the ACPA immune response. In the first, part we demonstrate the presence of ACPA-expressing B cells in different B cell subpopulations and estimate their frequency in peripheral blood as well as in synovial fluid. These data confirms presents of ACPA-expressing memory B cells and ACPA-secreting cells in peripheral blood. Presence of the latter are a sign of an active immune response. Analysis of lambda light chain usage hints towards a difference of the cells, giving insights into a maybe non-conventional development and thereby to a possible target for intervention. Single cell identification is possible since we succeed in the setup of an antigen-specific staining. This technique will be a very valuable tool for future studies allowing to dissect the characteristics and the study of the ACPA immune response. Together, this stepwise characterisation of ACPA B cells gives more insight into these autoreactive B cells. This thesis provides tools to further characterise these cells which will hopefully lead to better characterisation of B

cells and the underlying immune response. More understanding of autoreactive B cells could then provide better targets for therapeutic interventions. The latter could benefit patients with ACPA-positive RA or maybe even more general, patients with other unwanted autoreactive B cell responses.

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Chapter 8

Nederlandse samenvatting

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Reumatoïde artritis (RA) is een chronische ziekte waarbij gewrichten ontstoken zijn en permanente schade oplopen. Ongeveer één op de 100 mensen lijdt in Nederland aan deze ziekte. Een belangrijk kenmerk van RA is de aanwezigheid van antilichamen die het lichaam 'aanvallen': auto-antilichamen. Er zijn verschillende groepen auto-antilichamen beschreven in RA patiënten waaronder de zogenaamde ACPA (anti-citrullinated protein antibodies), aanwezig in ongeveer twee op de drie patiënten. Deze antilichamen herkennen eiwitten en peptiden die gecitrullineerd zijn. Citrullinatie is een post translationele modificatie waarbij het aminozuur arginine wordt veranderd in een citrulline. Dit proces vindt plaats in iedereen, de antilichamen die dit herkennen zijn specifiek voor patiënten.

Patiënten die ACPA in hun serum hebben ervaren gemiddeld een ander ziektebeeld dan ACPA-negatieve patiënten. Mensen die alleen nog maar pijn klachten hebben (artralgie) en tevens ACPA positief zijn hebben een grotere kans om uiteindelijk RA te krijgen. Daarnaast hebben patiënten met ACPA over het algemeen een ernstiger ziektebeeld, vergeleken met ACPA-negatieve patiënten. Vele verschillende onderzoeksgroepen hebben onderzoek gedaan naar deze specifieke antilichamen waarbij de vraag centraal staat waarom ze samengaan met een ander ziektebeeld. Deze onderzoeken hebben zowel moleculaire als functionele verschillen in kaart gebracht tussen ACPA en andere antilichamen. Gebaseerd hierop vermoeden wij dat de B-cellen die ACPA produceren anders zouden kunnen zijn dan niet auto-reactieve B-cellen. In dit proefschrift worden een aantal verschillende aspecten van het in kaart brengen van deze ACPA-producerende B-cellen beschreven. De beschreven studies in dit proefschrift dragen bij aan een beter beeld van deze auto-reactieve afweerreactie wat uiteindelijk mogelijke nieuwe behandelingen genereert.

B-cellen bestaan uit verschillende subpopulaties die ieder een eigen rol vervullen binnen het immuun systeem. De ontwikkeling van een B-cel begint in het beenmerg waar de cel zijn receptor en daarmee zijn specificiteit verkrijgt. Een B-cel moet meerdere controles passeren voordat de cel in het bloed komt. Als eerste moet er een functionele receptor zijn om positief geselecteerd te kunnen worden. Vervolgens mag deze receptor niet reageren op lichaamseigen structuren (niet auto-reactief zijn). Als dit wel zo is kan de receptor aangepast worden, een proces dat B-cel receptor-editing heet. De B-cel komt (indien goedgekeurd) vervolgens in de circulatie als naïeve B-cel. Naïeve B-cellen kunnen worden geactiveerd door binding van het antigeen aan de receptor waardoor de cel gaat delen en differentiëren. Hierbij ontstaan de antilichaam-producerende cellen, de plasmablasten en plasmacellen, en de B-geheugencelpopulatie.

Om ACPA-producerende B-cellen te kunnen karakteriseren, moet men allereerst weten waar ze zich bevinden. Bij een normale immuunreactie, zoals optreedt na bijvoorbeeld vaccinatie, zijn er in de periode na blootstelling veel antilichaam-producerende cellen in het bloed aanwezig. Op de lange termijn blijft in het bloed een populatie B-geheugencellen en in het beenmerg een kleine populatie lang levende plasmacellen over. In **hoofdstuk 2** wordt beschreven hoe we B-cellen uit het bloed van RA patiënten isoleren en vervolgens kweken waarbij de antilichaamproductie gestimuleerd wordt. Na afloop kunnen we in de supernatanten een deel van de wellen ACPA productie meten. Bij het kweken van cellen van ACPA-negatieve patiënten of gezonde donoren vinden we geen ACPA productie. Op basis van expressie van CD20 en CD27 discrimineren we tussen naïeve B-cellen, B-geheugencellen en plasmablasten/-cellen. Met behulp van flowcytrometrie kunnen cellen geïsoleerd worden waarbij bijvoorbeeld alleen een subpopulatie B-cellen geïsoleerd wordt of juist alle cellen worden behouden behalve een specifieke subpopulatie. Hierdoor wordt duidelijk dat in de B-geheugencelpopulatie en in de plasmablast/-cel populatie cellen zitten die ACPA kunnen produceren. Tevens vinden we spontane productie door plasmablasten/-cellen wat betekend dat de immuunreactie een actieve reactie is. Dit suggereert dat in patiënten continu nieuwe B-cellen geactiveerd worden. Met als gevolg dat plasmablasten/-cellen bezig zijn met productie en secretie van ACPA antilichamen in het bloed. Omdat we benieuwd waren naar deze cellen op de plaats van ontsteking zijn we het synoviaal vocht van RA patiënten gaan bestuderen. In **hoofdstuk 3** laten we zien dat in de celpopulatie in dit vocht relatief meer spontaan ACPA-producerende cellen zitten dan in het bloed. Daarnaast blijkt dat als de totale celpopulatie uit het vocht gekweekt wordt zonder stimulatie dit langere antilichaamproductie tot gevolg heeft in vergelijking met cellen geïsoleerd uit bloed. De cellen uit het synoviaal vocht vormen een soort niche waarin de (auto-reactieve) antilichamen spontaan geproduceerd kunnen worden gedurende een periode van soms meer dan een half jaar.

Om ACPA-producerende B-cellen beter in kaart te brengen is het van belang om op het niveau van een enkele cel naar deze B-cellen te kunnen kijken. Om dit te bereiken is een techniek nodig om deze cellen specifiek aan te kleuren. In **hoofdstuk 4** hebben we hiervoor een flowcytrometrie kleuring ontwikkeld. Om B-cellen specifiek aan te kleuren zijn een goede positieve controle, een ACPA monoklonaal, en een drie-tetrameer kleuring essentieel. Twee tetrameren zijn gekoppeld aan het CCP2 (cyclic citrullinated peptide). De dubbel-positieve populatie bevat de B-cellen die ACPA tot expressie brengen. De enkel positieve cellen zijn of aspecifieke gekleurd of die B-cellen die het fluorchroom van een van de tetrameren zelf herkennen. De derde tetrameer is gekoppeld aan het CArgP2 peptide, het controle peptide zonder citrullines. Dit zorgt voor een extra bevestiging van de specificiteit van de dubbel positieve cellen. Door deze kleuring te combineren met verschillende andere markers, zoals bijvoorbeeld CD20 en CD27, kunnen we de B-cel die ACPA tot expressie brengt karakteriseren. Hieruit blijkt dat de meeste ACPA B-cellen geswitchte (meestal IgG positieve) B-geheugencellen zijn maar ook vinden we B-cellen met een naïef en plasmablast/-cel fenotype. Interessant is dat onze bevindingen van

het fenotype overeenkomen met de in literatuur beschreven isotypes (IgG, IgA, IgM) voor ACPA in serum. Wanneer we in één bloed monster cellen en serum beide bekijken vinden we dat de frequentie van deze cellen met de hoeveelheid ACPA in serum overeenkomt. Deze kleuring kan ook gecombineerd worden met andere markers die tot expressie kunnen worden gebracht onder bepaalde omstandigheden. Op deze manier kunnen we verder onderzoeken of, en zo ja hoe, de B-cellen die ACPA tot expressie brengen anders zijn dan B-cellen met een andere specificiteit. Dit zal helpen om de ACPA B-cel immuunreactie beter in kaart te brengen.

In **hoofdstuk 5** en **6** kijken we verder naar verschillen met andere B-cellen. De B-cel receptor van de ACPA B-cel, die de specificiteit van de B-cel bepaalt, herkent het gecitrullineerde eiwit/peptide. Aangezien de specificiteit anders is dan andere B-cellen zijn we gaan kijken of deze B-cel receptor verschillend is ten opzichte van andere B-cel receptoren. De B-cel receptor bestaat uit twee lichte ketens en twee zware ketens. Deze lichte ketens kunnen kappa of lambda zijn waarbij een B-cel in de meeste gevallen begint met een kappa lichte keten. Indien een B-cel in het beenmerg auto-reactief is moet de receptor veranderen door middel van receptor editing om te kunnen overleven. Tevens is beschreven dat in germinal center-achtige structuren in RA patiënten B-cellen van receptor kunnen veranderen. In beide gevallen kan een B-cel een andere lichte keten tot expressie gaan brengen. Hierbij worden stukken uit het DNA geknipt waardoor het aantal mogelijke nieuwe receptoren afneemt elke keer dat de lichte keten veranderd wordt. Het frequenter gebruik van lambda lichte keten kan wiskundig gezien te maken hebben met het hoger aantal rondes B-cel receptor-editing. Tevens is er in een andere ziekte een correlatie beschreven tussen de ratio kappa/lambda en autoantilichamen. Om een idee te krijgen of ACPA B-cellen op een andere manier ontstaan, en bijvoorbeeld komt doordat een cel meer B-cel receptor-editing ondergaat, zijn we gaan kijken naar de verhouding tussen het gebruik van kappa en lambda in de ACPA antilichamen en ACPA B-cellen. De resultaten van deze studies zijn beschreven in **hoofdstuk 5**. We vinden een frequenter gebruik van lambda in zowel de ACPA-IgG antilichamen in het serum, de ACPA-IgG antilichamen in het synoviaal vocht en de B-cellen die ACPA tot expressie brengen in het bloed. Deze verschuiving in frequentie kan een indicatie zijn dat de ACPA B-cellen bijvoorbeeld in germinal center-achtige structuren hun specificiteit krijgen. Meer onderzoek zal gedaan moeten worden naar de scheefgroei van deze specificiteit. Daarnaast hebben we ook gekeken naar de veranderingen in het bloed van patiënten die worden behandeld met tocilizumab. Tocilizumab is een monoklonaal antilichaam dat interleukine (IL)-6 signalering blokkeert. IL-6 is onder andere essentieel voor het aanpassen van de B-cel receptor in germinal center-achtige structuren in RA patiënten. Als we de ACPA antilichamen in patiënten voor, en drie maanden na, de eerste behandeling met tocilizumab vergelijken zien we dat de frequentie lambda lichte keken bevattende ACPA-IgG in verhouding tot alle lambda lichte keten bevattende IgG afneemt terwijl dit niet het geval is voor de kappa bevattende ACPA-IgG. Dit zou mogelijk verklaard kunnen worden door de gedachte dat er minder of geen nieuwe plasmablasten gevormd worden die een ACPA-IgG

met een lambda lichte keten produceren. Verder onderzoek zal gedaan moeten worden om te bepalen of dit komt doordat in de ontwikkeling van de B-cellen de IL-6 essentieel is om een ACPA lambda lichte keten bevattende B-cel te vormen.

Een andere B-cel marker waarin we geïnteresseerd zijn is CD28. In muizen is beschreven dat CD28 op lang levende plasmacellen een positief effect heeft op de levensduur van de plasmacel. Daarnaast is bekend dat als je RA patiënten behandeld met abatacept (CTLA4-Ig), dat signalering van CD28 inhibeert, er effect is op de ACPA serum titer. De titer vermindert net als het percentage geswitchte B-geheugencellen. Daarom hebben wij gekeken of CD28 op de B-cellen uit het bloed en uit het synoviaal vocht van RA patiënten tot expressie wordt gebracht en of de cellen die ACPA tot expressie brengen in een hogere frequentie CD28 positief zijn. De resultaten van deze studies staan beschreven in **hoofdstuk 6**. CD28 blijkt op plasmablasten/-cellen in het bloed van RA patiënten aanwezig te zijn. Tevens vinden we expressie op plasmablasten/-cellen van systemic lupus erythematosus (SLE) patiënten en gezonde donoren en in alle drie de groepen in vergelijkbare frequentie. Plasmablasten/-cellen uit in het synoviaal vocht van RA patiënten hebben een hogere frequentie CD28 positieve cellen. Ook zijn een deel van de plasmablasten/-cellen die ACPA tot expressie brengen tevens CD28 positief. Toekomstige experimenten zullen moeten uitwijzen of dit nu de cellen zijn die langer overleven.

Samenvattend bevat dit proefschrift verschillende hoofdstukken die bijdragen aan de karakterisatie van de B-cellen die de gecitrullineerde eiwitten herkennen en ACPA produceren. Voor vervolgonderzoek is een grote stap gezet doordat de cellen nu specifiek kunnen worden gekleurd en daarmee als enkele cel kunnen worden bestudeerd. Dit proefschrift draagt daarmee bij aan het in kaart brengen van de totale ACPA immuunreactie in patiënten met RA. Vervolg studies naar deze B-cellen zullen leiden tot het volledig in kaart brengen van deze B-cellen. Hopelijk kunnen verschillen met andere B-cellen nieuwe targets suggereren die gebruikt kunnen worden voor toekomstige therapieën waarbij deze autoreactieve B-cellen specifiek uit de patiënten kunnen worden verwijderd.

Curriculum vitae

Priscilla Francis Kerkman was born on the 13th of July 1987 in Naarden. She grew up in Huizen, were she completed her secondary education at SG Huizermaat in 2005. Directly after finishing, she moved to Leiden to study Life Science and Technology at Leiden University and Technical University of Delft. During her bachelor program she obtained her propaedeutic exam cum laude and received a beta scholarship after completing her bachelor. She continued her studies with the master program of the study. Her first master internship was at Galapagos BV in Leiden where she worked on production and validation of viral constructs. The second internship took place in the Leiden University Medical Center at the Rheumatology department where she started working on B cells in rheumatoid arthritis. After graduation in 2011 she continued working on this project as a PhD student. Currently, Priscilla is working as a postdoctoral researcher at Umeå University in Sweden at the department of clinical microbiology.

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Synovial fluid mononuclear cells provide an environment for long-term survival of antibody secreting cells and promote the spontaneous production of anti-citrullinated protein antibodies

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