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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
identification and characterisation of acpa-producing b cells in ra

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

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 Pmel restriction sites (GeneArt, Life Technologies). The pcDNA vector encoding the CitFib1.1 light chain (LC), also flanked with Pmel restriction sites, was described previously.[15] Lentiviral vectors encoding the LC and the transmembrane HC were generated by inserting the Pmel 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.
Identification and characterisation of ACPA-producing B cells in RA

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 $5 \times 10^3$) did not yield detectable ACPA levels, except for very low amounts at $5 \times 10^3$ 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 4.2: 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 4.3: 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.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Population</th>
<th>Cells/well (n)</th>
<th>ACPA-IgG (AU/mL)</th>
<th>IgG (µg/mL)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>tetramer +</td>
<td>207</td>
<td>456</td>
<td>15.7</td>
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<tr>
<td></td>
<td>tetramer -</td>
<td>5000</td>
<td>5.6</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>1900</td>
<td>9.3</td>
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<tr>
<td></td>
<td></td>
<td>740</td>
<td>6.0</td>
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<td></td>
<td>370</td>
<td>3.1</td>
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<td></td>
<td>370</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>tetramer +</td>
<td>14</td>
<td>1.6</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>tetramer -</td>
<td>5000</td>
<td>0.158</td>
<td>3.8</td>
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<td></td>
<td></td>
<td>1900</td>
<td>6.9</td>
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<td>740</td>
<td>2.6</td>
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<td></td>
<td>740</td>
<td>4.4</td>
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<tr>
<td>3</td>
<td>tetramer +</td>
<td>317</td>
<td>301</td>
<td>4.0</td>
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<td></td>
<td>tetramer -</td>
<td>5000</td>
<td>0.178</td>
<td>9.3</td>
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<td>370</td>
<td>4.1</td>
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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+;
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 (CD20dim/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.
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.

**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).
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.

**Acknowledgements**

We kindly thank Jan Wouter Drijfhout for synthesising and providing the CCP2 peptide and its arginine control variant.

**References**


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 4.52: Binding profiles of secreted mAb CitFib1.1 and of the surface expressed antibody by HEKACPA_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 HEKACPA_TM cells as tested by FACS. Closed histograms represent unstained HEKACPA_TM cells.

Figure 4.53: Pre-incubating HEKACPA_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 4.54:** 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 4.55:** 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.