Anti-citrullinated protein antibody B cells in rheumatoid arthritis: from disease-driving suspects to therapeutic targets

Kroos, S.

CHAPTER 2

Identification of a naïve anti-citrullinated protein antibody-expressing B cell

Sanne Kroos[1], Rian Schopmeijer[1], Renee van de Wetering[1], Linda M. Slot[1], René E.M. Toes[1], Hans U. Scherer[1]

[1] Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands

Abstract

The presence of autoantibodies against post-translationally modified proteins is a major hallmark of rheumatoid arthritis (RA). Autoantibodies recognizing citrullinated proteins, also called anti-citrullinated protein antibodies (ACPA), are present in 50-60% of patients with RA. The origin of ACPA-expressing B cells and the factors that determine the development of RA in the presence of such B cells remain incompletely understood. Here, we hypothesized that identifying ACPA-expressing B cells in the naïve ($CD19^+$ $CD20^+$ $CD27^-$ IgD^+ IgM^+) B-cell repertoire provides important insights into the loss of tolerance in RA, as naïve B cells are likely the first responding cells in the initiation of the autoreactive B cell response. To capture naïve autoreactive B cells in RA, we isolated naïve B cells from ACPA-positive RA-patients reacting with streptavidin tetramers linked to biotinylated cyclic citrullinated peptide 2 (CCP2). Subsequently, recombinant IgM monoclonal antibodies were generated using the B-cell receptor (BCR) sequence of isolated B cells. Most of the monoclonal antibodies revealed streptavidin reactivity rather than CCP2 reactivity. However, one monoclonal antibody derived from a naïve, ACPA-expressing B cell exhibited reactivity against CCP2, confirming that patients can carry naïve B cells with germline-encoded autoreactivity towards citrulline. In addition, this germline-encoded autoreactive antibody displayed cross-reactivity to another post-translational modification, homocitrulline. These findings indicate that autoreactivity towards post-translational modifications may be part of the naïve BCR repertoire. Furthermore, these results demonstrate that the breach of tolerance in RA can originate from the activation of B cells with germline-encoded autoreactivity.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of synovial joints. A hallmark of RA is the presence of B cells targeting the post-translational modification (PTM) citrulline [1]. Anti-citrullinated protein antibodies (ACPA), a specific biomarker for RA, are present in 50-60% of patients and are associated with poor disease prognosis [2]. Notably, ACPA can be present years before clinical symptoms emerge [3, 4]. Prior to the transition from asymptomatic ACPA-positive autoimmunity to clinically apparent disease, the ACPA response matures, involving isotype switching, epitope spreading, accumulation of variable domain glycans and rising serum autoantibody levels [4-9]. These findings suggest that the presence of ACPA alone is not sufficient to drive the development of RA. Nonetheless, the clinical efficacy of B cell-depleting therapies, the high specificity of ACPA for RA, and the poor prognosis of ACPA-seropositive patients compared to seronegative RA patients underscore the pivotal role of this autoreactive B-cell response in RA pathogenesis [10-13].

The origin of ACPA-expressing B cells and the factors determining progression to RA in their presence remain incompletely understood. Identifying the antigen responsible for the initial activation of ACPA-expressing B cells is challenging. This difficulty arises because ACPA-expressing B cells often undergo extensive somatic hypermutation, causing considerable divergence from their germline BCR sequences, which are present at the time of initial activation. Adding to this complexity, ACPA frequently exhibit promiscuity to a plethora of citrullinated antigens as well as antigens carrying different post-translational modifications, such as homocitrulline and acetyllysine [14-17]. Likewise, antibodies against homocitrulline and acetyllysine, known as anti-carbamylated protein antibodies (ACarPAs) and anti-acetylated protein antibodies (AAPAs), respectively, can also recognize citrulline [14, 18, 19]. Due to this cross-reactivity, antibodies against PTMs are collectively called anti-modified protein antibodies (AMPAs). Previous studies have demonstrated that IgM AMPA-expressing B cells can be cross-reactive.

These B cells expressed a memory phenotype in concordance with the observation that their BCR had undergone somatic hypermutation (SHM). Nonetheless, these observations suggest that citrullinated, homocitrullinated and/or acetylated antigens may be capable of activating naïve ($CD19^+$ $CD20^+$ $CD27^-$ IgD^+ IgM^+) autoreactive B cells and thereby initiate the initial breach of tolerance [16]. This notion is relevant as a few other autoimmune responses have been indicated to emerge as a consequence of SHM [20, 21]. In line, polyclonal AMPA-IgM and monoclonal, germline-reverted IgM autoantibodies from RA patients have been found to display cross-reactivity. This finding thus indicates that PTM recognition is not necessarily dependent on SHM nor that broad PTM reactivity is a consequence of a high mutation load of the B-cell receptor (BCR) [16]. However, PTM reactivity has not yet been detected in the circulating naïve B-cell repertoire expressing germline-encoded IgM. Conceptually, the identification of such unmutated, naïve, PTM-recognizing B cells in RA patients could provide valuable insights into the triggers that initiate autoreactive B cell response.

In ACPA-positive patients with RA, the majority of the circulating ACPA-expressing B-cell population consists of Ig-class-switched memory ($CD19^+$ $CD20^+$ $CD27^+$) B cells [22, 23]. These memory B cells exhibit an activated, proliferative phenotype

that persists during clinical remission. Due to the capacity of memory B cells to present antigen, secrete pro-inflammatory cytokines and differentiate into migratory, antibody-secreting plasmablasts, these cells are thought to be contributors to disease in RA [22, 24, 25]. Naïve ($CD19^+$ $CD20^+$ $CD27^-$ IgD^+) B cells, on the other hand, have been observed to only form a small fraction of the ACPA-expressing B-cell population in these patients [23]. To date, the role of naïve B cells in the initial development of ACPA-positive RA and their contribution to the generation and persistence of ACPA-expressing memory B cells in established disease, remains unclear. After encountering their antigen and receiving help from $CD4^+$ T cells, naïve, ACPA-positive B cells could give rise to and fuel the memory B-cell compartment, thereby contributing to the chronic autoimmune response observed in RA [14].

Research into primary, autoreactive B cells underlying the ACPA B-cell response, i.e. naïve, ACPA-expressing B cells, is important to increase our understanding of the origin of the ACPA-autoimmune response in RA. Previously, we developed an antigen-specific staining panel for flow cytometry for the acquisition of ACPA-expressing B cells from peripheral blood using CCP2-tetramers [23]. Naïve, antigen-specific IgM-expressing B cells are present in very low frequencies as they have not been expanded as a consequence of antigen-recognition. Therefore, we considered it challenging to identify these cells. Similarly, these cells conceivably display a lower avidity for their antigen compared to high-affinity IgG memory B cells making it challenging to interrogate them and to distinguish “adequately” stained naïve, antigen-specific B cells from background signal. Nonetheless, we set out to obtain additional insight into the processes preceding the development of autoimmunity and clinically apparent disease by exploring the naïve compartment of ACPA-expressing B cells in RA.

Patients and methods

Human material
Peripheral blood (PB) was obtained from two ACPA-positive patients with RA recruited from the outpatient clinic of the department of rheumatology at Leiden University Medical Center (LUMC). RA patients met the 2010 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) criteria for RA. Patients received methotrexate or anti-TNF treatment and had positive CCP2 serum levels as determined by diagnostic testing. Participants provided written informed consent.

Antigen labeling
As previously described, Allophycocyanin (APC) and Brilliant Violet 605 (BV605)-labeled streptavidins were conjugated to biotinylated cyclic citrullinated peptide (CCP)2, whereas the arginine variant, CArgP2, was conjugated to phycoerythrin (PE)-labeled extravidin [8]. To determine the optimal concentrations for identifying antigen-specific B cells, the tetramers were titrated on ACPA-expressing Ramos 3F3 cells and Ramos cells expressing no BCR (MDL AID KO) [9].

PBMC isolation and single-cell sorting
Peripheral blood mononuclear cells (PBMCs) were isolated from 45 mL of blood using Ficoll-Paque PLUS gradient centrifugation (density 1.077 g/mL, Pharmacy) (39). Cells were stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (ThermoFisher), mouse anti-human CD3-PB (clone UCHT1, BD), CD14-PB (clone M5E2, BD), CD19-APC-Cy7 (clone SJ25C1, BD), CD20- AF700 (clone 2H7, BioLegend), CD27-PE-Cy7(clone M-T271, BD), IgG-BV510 (clone X40, BD) and IgD-FITC (clone IA6-2, BD). In addition, patient-derived PBMCs were stained with the antigen-coupled tetramers CCP2-APC, CCP2-BV605 and CArgP2-PE. The BD FACSAria III Cell Sorter at the Flow cytometry Core Facility of the LUMC was used to sort single antigen-specific B cells as well as B-cell populations in bulk. B cells staining positive for both CCP2-carrying tetramers while negative for the CArgP2-coupled control tetramer were considered as ACPA-expressing B cells, although their specificity was yet to be verified.

B cell-receptor sequences
To obtain B cell-receptor (BCR) sequences of single CCP2-reactive B cells isolated from patients with RA (n = 2), cells were directly sorted into lysis buffer containing 0.2% Triton X-100 (ThermoFisher), recombinant RNAse inhibitor (Takara bio), dNTP mix (ThermoFisher) and dT30VN oligo primer. cDNA was synthesized by three-minute incubation of obtained sample mRNA at 72°C, before addition of cDNA mix, which consisted of betaine >99% (Sigma-Aldrich), template switch oligo (TSO) primer (IDT), dithiothreitol (DTT), 5x first strand buffer, SMARTScribe™ Reverse Transcriptase (Takara Bio) and UltraPure™ DNase/RNase-Free Distilled Water (ThermoFisher). Mixture of mRNA and cDNA mix was incubated at 42°C for 90 minutes and underwent 10 cycles of 50°-42°C for two minutes each, followed by a last step at 72°C for fifteen minutes. cDNA was pre-amplified using the KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and ISPCR oligo, as per manufacturer's instructions. Amplified cDNA was purified using Ampure XP Beads (Beckman Coulter) according to the manufacturer's instructions with annealing at 67°C and extension for 6 minutes and a total of 24 cycles. Full-length V(D)J regions were obtained by performing an ARTISAN PCR with using heavy and light chain-specific

primers, SA primer (IDT) and Phusion Flash High-Fidelity PCR master mix (ThermoFisher), as previously described [26]. ARTISAN PCR products were loaded on a 1% agarose gel prepared with 1x TAE buffer and Nancy-520 (Sigma-Aldrich) to select the correct PCR product for sequencing. Only for PCR products with multiple bands, DNA was purified from the gel using NucleoSpin Gel & PCR Clean-up Kit (BIOKÉ) before Sanger sequencing at Macrogen. Sequencing data underwent quality control and was aligned with germline sequences of IMGT/V-Quest (43). Selection of V(D)J sequences from BCRs of single ACPA-B cells was based on quality of both heavy- and light-chain sequencing data, similarity to germline (for IgM), and the position of the B cell in the antigen-specific gate during single-cell sorting.

Monoclonal antibody production

Using the In-Fusion HD Cloning Kit (Takara Bio), codon-optimized V(D)J sequences that were ordered at GeneArt (ThermoFisher), including the original leader sequence, were ligated into a pcDNA3.1(+) expression vector (Invitrogen) containing an IGHM, IGHG, IGKC or IGLC constant region (UniProt). Accordingly, separate vectors were created for heavy- and light-chain variable domains of isolated naïve IgM and memory IgG CCP2-reactive B cells. Ligated expression vectors were transformed into Stellar Competent cells (Takara Bio) after the which PureYield™ Plasmid Miniprep System (Promega) was used to purify the amplified plasmid DNA. Constructs of DNA were transiently transfected in Freestyle HEK293-F cells (Gibco) using 293-Fectin (Invitrogen) and Opti-MEM® (Gibco). Help vectors with SV40 large T antigen, hp21 and hp27 were included to enhance expression of the plasmid in Freestyle HEK293-F cells (44). For the production of IgM monoclonal ACPA, vectors carrying a J-chain (UniProt) were co-transfected to enable stable IgM-pentamer formation after protein production. Freestyle HEK293-F cells were cultured in FreestyleTM 293 expression medium (Gibco) at 37°C, 5% CO_2 while continuously shaking. Culture supernatants were obtained 5 to 6 days post-transfection and filtered through a 0.45 µm pore size filter (Corning).

CCP2 and CArgP2 ELISAs

For the detection of ACPA in supernatants of transfected Freestyle HEK293-F cell cultures, pre-coated streptavidin plates (Microcoat, 65001) received 1 µg/ml of biotinylated-CCP2 or -CArgP2 peptides diluted in PBS/0.1%BSA for 1 hour at RT. After washing three times with PBS/0.05%Tween, culture supernatants diluted in PBS/1%BSA/0.05%Tween were added to the wells for 1 hour at 37°C. Sample dilution factors are indicated in the respective figures. Goat anti-human IgM (Millipore, AP114P) was incubated for 1 hour at 37°C, respectively. Finally, plates were developed using ABTS (Sigma-Aldrich) and H_2O_2. Optical density (OD) was measured at 415nm using SpectraMax i3x Microplate Reader (Molecular Devices).

Streptavidin and extravidin ELISAs

To determine whether the produced IgM-monoclonal antibodies (mAbs) were reactive to streptavidin, Nunc Maxisorp 96-well plates (ThermoFisher, 430341) were coated overnight at 4°C with 1 µg/ml of streptavidin or extravidin diluted in coating buffer (0.1 M Na2CO3 + 0.1 M NaHCO3 in MQ). Plates were washed three times with PBS/0.05% Tween and incubated for 1 hour at RT with PBS/0.1%BSA or 1 µg/ml of biotinylated-CCP2 or -CArgP2 peptides diluted in PBS/0.1%BSA as coating control. After washing three times with PBS/0.05%Tween, culture supernatants diluted in PBS/1%BSA/0.05%Tween were added to the wells for 1 hour at 37°C.

Dilution factors of samples are indicated in the respective figures. To detect streptavidin-reactive IgM-mAbs, 0.1 µg/ml of goat anti-human IgM (Millipore, AP114P) was added to the wells for 1 hour at 37°C. Plate development and readouts were carried out as described above.

Homocitrulline and acetyllysine ELISAs

To evaluate whether IgM-mAbs recognized peptides with other post-translational modifications, pre-coated streptavidin plates (Microcoat, 65001) were incubated for 1 hour at RT with 1 µg/ml of biotinylated peptides containing either citrulline (CCP4), arginine (CArgP4), homocitrulline (CHcitP4), acetyllysine (CAcetylP4) or lysine (CLysP4) residues diluted in PBS/0.1%BSA. Washing and sample incubation was performed similarly as described for the detection of CCP2 binding. For the detection of cross-reactive IgM-monoclonal antibodies, plates were incubated with 0.1 µg/ml of goat anti-human IgM (Millipore, AP114P) for 1 hour at 37°C. Plate development and readouts were performed as described above.

Results

Antigen-specific isolation reveals unmutated, antigen-inexperienced, naïve (CD27⁻ IgD⁺) B cells

To obtain citrullinated-antigen directed BCRs, we used CCP2- and CArgP2-tetramers to isolate single-B cells binding to citrulline and not to its native control variant arginine (CCP2⁺ CArgP2⁻). In line with previously published data, the majority of CCP2-isolated B cells exhibited a switched memory B cell (MBC) phenotype (CD19⁺ CD27⁺ IgG⁺) [23]. However, several CCP2-isolated B cells resembled a naïve phenotype based on flow cytometry markers (CD19⁺ CD27⁻ IgD⁺) (Figure 1). All CCP2-tetramer reactive cells were isolated and lysed for subsequent cDNA production and BCR sequencing. Out of 336 isolated cells derived from two patients, seven sequences from the CD19⁺ CD27⁻ IgD⁺ population were selected for mAb production

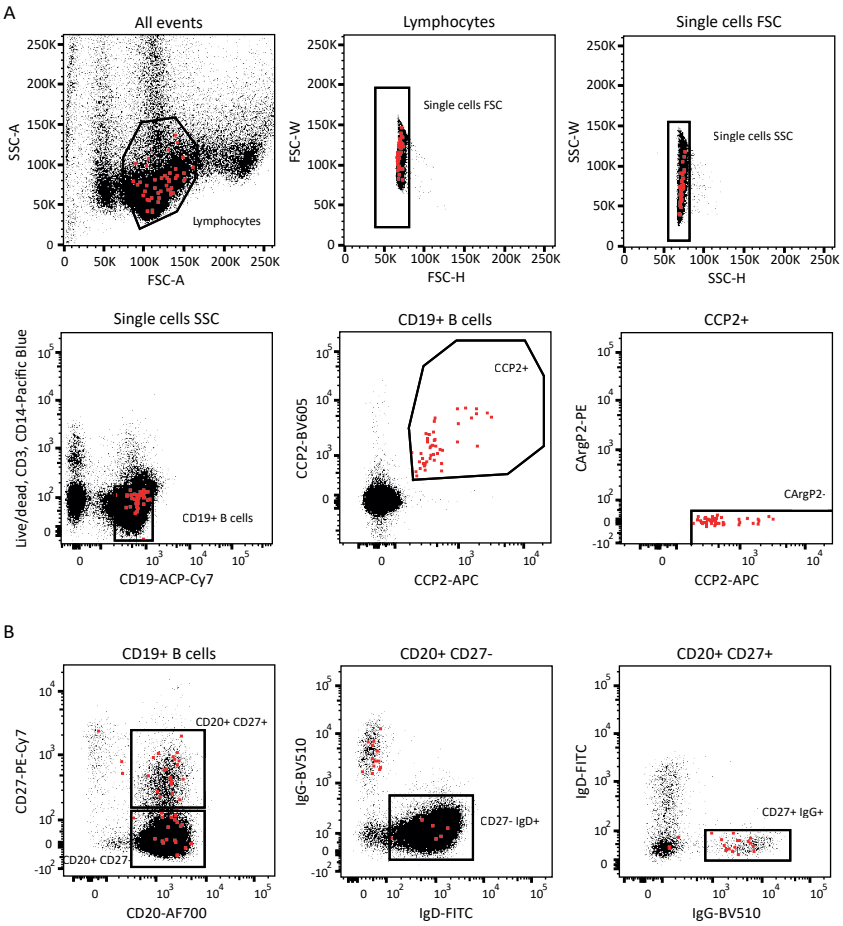

Figure 1. Gating strategy applied to isolate CCP2-tetramer reactive B cells from PBMCs of patients with RA. A. Antigen-specific gating strategy: single, living CD19⁺ CCP2⁺ and CArgP2⁻ B cells are considered ACPA-expressing B cells. B. Back-gating of antigen-specific cells for several B-cell markers. Text above each dot plot indicates the population visualized in the plot. Red dots indicate isolated antigen-specific cells. Plots depict gating from one of the two patients.

and verification of reactivity, of which five harboured no heavy- and light chain mutations, indicating that these were derived from *bona-fide*, antigen-inexperienced, naïve B cells (Table 1).

CCP2 reactivity of mAbs derived from a naïve, CCP2-isolated B cell

To verify CCP2 reactivity of the naïve isolated B cells, we obtained the BCR sequences and used a selection for subsequent monoclonal IgM antibody production. We selected sequences from five cells harbouring 0-mutations in the immunoglobulin heavy chain variable (IGHV) region and 0-mutations in the immunoglobulin lambda chain variable (IGLV) region or immunoglobulin kappa chain variable (IGKV) region. As non-naïve controls, we selected sequences from two cells harbouring some IGHV and/or IGLV mutations (Table 1). The selected sequences were cloned and the expression vectors were used for transient transfection into HEK293-F cells. The reactivity of the mAbs in the resulting supernatants was tested in ELISA by incubation on ELISA plates coated with streptavidin and biotinylated CCP2 peptide or biotinylated CArgP2 peptide as a control. Clear binding curves were observed in the CCP2 ELISA indicating binding of the produced antibodies. Notably, for six out of seven mAbs produced, similar binding signals were observed in the CArgP2 ELISA indicating that these mAbs were not specifically reactive to CCP2. However, one mAb demonstrated a binding signal in the CCP2 ELISA and not in the CArgP2 ELISA, indicating citrulline-directed reactivity (mAb 1 in Figure 2).

Table 1. Characteristics of the selected BCR sequences from single CCP2-reactive B cells isolated from patients with RA.

mAb	1	2	3	4	5	6	7
Reactivity	CCP2	Streptavidin	Streptavidin	Streptavidin	Streptavidin	Streptavidin	Streptavidin
Heavy chain	μ	μ	μ	μ	μ	μ	μ
IGHV	V7-4-1	V3-23	V4-39	V4-39	V3-23	V3-53	V2-5
IGHD	D6-19	D3-22	D4-17	D5-12	D6-19	D4-17	D3-22
IGHJ	J4	J5	J5	J4	J4	J4	J2
IGHV mutations (nt)	0	0	0	0	0	6	0
IGH-CDR3 aa	CAREVAVAEGGTEVGDYW	CAKLDYDSSAKTGSDPW	CARHVTGDGDYSNWFDPW	CACETRVATPFDYW	CAKDPPGGWYRGPFDYW	CARELQRFFDYW	CAHSSPLHYYDSSGHFDLW
Light chain	κ	λ	κ	κ	κ	κ	κ
IGLV	V2-28	V3-10	V1-39	V1-NL1	V3-20	V3-20	V1-5
IGLJ	J1	J3	J1	J1	J1	J2	J1
IGLV mutations (nt)	0	0	0	0	0	2	2
IGL-CDR3 aa	CMQALQTPWTF	CYSTDSSGNHRVF	CQQSYSTPWTF	CQQYYSTPWTF	CQQYGSSPQTF	CQQYGSSPPYTF	CQQYNSYSPWTF

mAbs not specifically reactive to CCP2 are streptavidin reactive

Since B cells showing reactivity to the arginine variant of CCP2 should have been excluded by the sorting procedure, we hypothesized that mAbs 2-7 may in fact be streptavidin directed. This notion was supported by the fact that the staining procedure used in this experiment contained biotinylated CCP2 peptides coupled to streptavidin-APC or streptavidin-BV605, whereas the control peptide CArgP2 was coupled to extravidin-PE. This way, background reactivity to the peptide

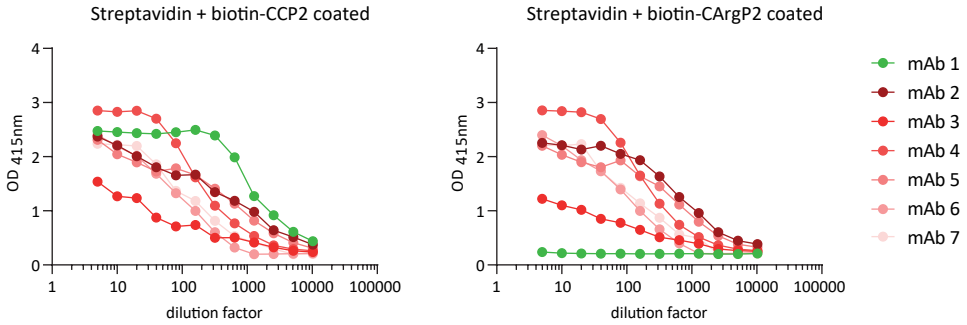

Figure 2. mAb1 harbours anti-citrulline reactivity. Supernatants of transfected HEK293-F cells were analysed for the presence of IgM mAbs reactive towards CCP2, while lacking reactivity towards CArgP2. The detection of reactivity is indicated by optical density at 415 nm (OD_{415nm}). The *x*-axis depicts the sample dilution factor. mAb1 shows citrulline reactivity while lacking arginine reactivity, whereas mAbs2-7 show reactivity to both antigens.

backbone is excluded whereas possible streptavidin reactivity is not due to differences between extravidin and streptavidin. To test this hypothesis, we coated ELISA plates with streptavidin or extravidin and tested binding of the mAbs. These experiments revealed binding signals for mAbs 2-7 in the streptavidin coated ELISA. In contrast, mAb 1, the antibody reactive to CCP2, but not its arginine control peptide, showed no binding (Figure 3A). Additionally, extravidin binding was not observed for any of the antibodies (Figure 3A, right) while extravidin coating could be confirmed by adding biotinylated CCP2 (Figure 3B, left). Additionally, supernatant from untransfected HEK293 cells demonstrated no binding in all coating conditions (Figure 3B, right).

The ACPA mAb derived from a naïve, CCP2-isolated B cell harbours cross-reactivity towards homocitrulline

ACPA cross-reactivity to other post-translational modifications (PTMs) such as homocitrulline or acetyllysine has previously been described in both ACPA IgG as well as ACPA IgM [15, 16]. After confirming citrulline reactivity of mAb 1, we sought to investigate whether this reactivity could extend to other PTMs. To this end, we used the citrullinated, homocitrullinated and acetylated peptides CCP4, CHcitP4 and CAcetylP4 respectively. mAb 1 demonstrated a binding signal to both CCP4 as well as CHcitP4. Additionally, native control variants with arginine and lysine amino acids (CArgP4 and CLysP4) were tested and revealed no binding signal. Altogether, these observations indicate cross-reactivity of the germline-encoded mAb 1 to citrulline and homocitrulline.

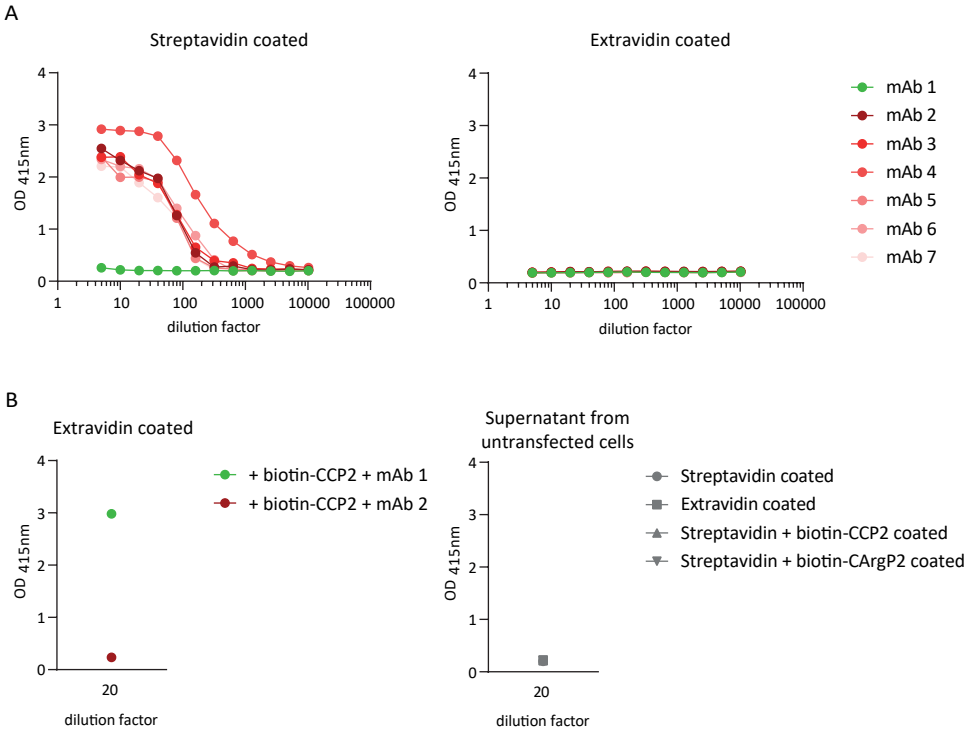

Figure 3. mAbs 2-7 are streptavidin reactive. A. Supernatants of transfected HEK293-F cells analysed for the presence of IgM mAbs reactive towards streptavidin (left) or extravidin (right). B. As a coating control, extravidin-coated wells were incubated with biotinylated CCP2 peptide preceding mAb incubation (left). Additionally, supernatants derived from untransfected cells were tested as a negative control (right). The detection of reactivities is indicated by optical density at 415 nm ($OD_{415_{nm}}$). The x-axis depicts the sample dilution factor.

Discussion

In the current study, we aimed to identify and isolate naïve, CCP2-reactive B cells and verify their reactivity to various PTMs. Production of mAbs from patient-derived BCR sequences allows for the identification of a naïve B cell expressing BCRs in germline configuration that can be analyzed for reactivity against citrullinated antigens. Although we identified only one naïve citrullinated-antigen directed B cell, this finding provides first evidence that B cells capable of recognizing citrullinated antigens can be present in the naïve, germline-encoded repertoire of patients with RA. Notably, the IGHV gene of this naïve B cell used for the production of IgM mAb 1 is identical to the IGHV of the previously validated IgM monoclonal ACPA 2D5, which was obtained from a different patient with RA. While such recurrence of a variant of IGHV might play a role in the initial recognition of citrullinated antigens, further confirmation is clearly required. An important difference between the two ACPA-IgM monoclonals is that the sequence of 2D5 was originally isolated from an IgM memory B cell and harbored several SHM. Although 2D5 was still reactive to citrulline after conversion to germline [16], mAb 1 is truly germline and isolated from a B cell with a naïve phenotype, indicating that the latter cell population can, in principle, harbor citrulline-directed BCRs.

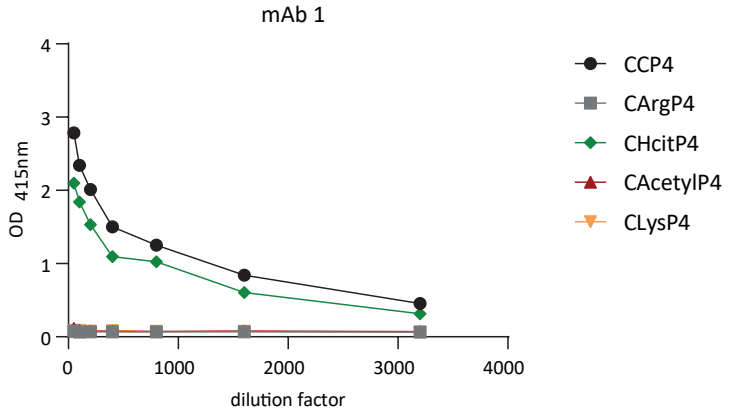

Figure 4. mAb 1 exhibits cross-reactivity towards homocitrulline. Supernatants of transfected HEK293-F cells were analysed for the presence of IgM mAbs reactive towards peptides with post-translational modifications: cyclic citrullinated peptide 4 (CCP4) and negative control variant cyclic arginine peptide 4 (CArgP4), cyclic homocitrullinated peptide 4 (CHcitP4) and cyclic acetylated peptide 4 (CAcetylP4) with negative control variant cyclic lysine peptide 4 (CLysP4). The detection of reactivity is indicated by optical density at 415 nm (OD_{415nm}). The *x*-axis depicts the sample dilution factor. mAb 1 shows reactivity to CCP4 and CHcitP4, but lacks reactivity to CAcetylP4 and negative control variants.

Nonetheless, the previous observation that germline-reverted 2D5 exhibits cross-reactivity towards different PTMs was supported by showing that germline mAb 1 also displays PTM cross-reactivity [16].

In RA, cross-reactivity of ACPA towards various PTMs has especially been observed for antibodies with the IgG isotype with extensive somatic hypermutation (SHM) [15, 27, 28]. This observation has led to the hypothesis that SHM plays a key role in enabling the cross-reactive properties of ACPA-IgG [29]. Supporting this idea, studies have shown that reverting ACPA-IgG sequences to their predicted germline configurations results in the loss of citrulline reactivity, suggesting that SHM may be responsible for acquiring autoreactivity against citrullinated antigens [29, 30]. However, caution must be taken when interpreting these results since predicting correct germline configurations may be impeded by the extensive SHM undergone by ACPA. In fact, the identification of a naïve, germline-encoded IgM capable of recognizing citrullinated antigens and cross-reacting with other PTMs suggests that reactivity against citrulline as well as PTM cross-reactivity do not necessarily rely on SHM.

It is tempting to speculate that higher numbers of naïve, germline-encoded ACPA-expressing B cells could be detectable in healthy individuals compared to patients with RA. In RA patients, the availability of antigens, the presence of antigen-specific T-cell help, and a pro-inflammatory environment could provide appropriate conditions for a naïve, ACPA-expressing B cell to become activated, to undergo isotype switching and to acquire mutations [18]. In contrast, naïve ACPA-expressing B cells in healthy individuals would be less likely to simultaneously encounter these stimulatory factors, hence increasing the likelihood that naïve, germline-encoded ACPA-expressing B cells remain inactivated and persist in their naïve state. Recent findings indicate that, in a mouse model for arthritis, ACPA may play an anti-inflammatory role during the early stages of arthritis. This suggests a potential

protective function for naïve, germline-encoded APCA-expressing B cells in RA patients as their activation and maturation may be required for the contribution to a protective immune response [31].

The current antigen-specific staining approach successfully identified a naïve B cell expressing germline-encoded ACPA. Nonetheless, the majority of naïve B cells isolated using CCP2-tetramers were, in fact, specific to streptavidin. As this method for isolating CCP2-reactive B cells was originally developed and optimized for memory B cells, nonspecific, naïve B cells were not specifically studied until now. In earlier studies [23], culturing single-sorted B cells and testing the resulting supernatants using streptavidin-CCP2 and streptavidin-CArgP2 ELISAs effectively ruled out streptavidin reactivity. However, when sorted B cells are directly lysed for subsequent BCR analyses, their reactivity cannot directly be verified, potentially leading to false positives from streptavidin-reactive B cells. Furthermore, false positive, streptavidin-reactive B cells could not be distinguished from ACPA-expressing B cells based on their position within flow cytometry gates. To discriminate these groups of different specificities in the future, revision of the staining panel is therefore required. One could consider conjugation of all biotinylated peptides to streptavidin, including the negative control antigen. This adjustment would allow for the exclusion of streptavidin-reactive B cells, enhancing the precision of isolating naïve ACPA-expressing B cells.

In conclusion, we here identified one naïve B cell expressing a germline-encoded ACPA BCR in a patient with RA, providing first indications that reactivity towards citrulline can be part of the naïve BCR repertoire. This finding suggests that an ACPA response, conceptually, could originate from the activation of B cells with germline-encoded autoreactivity. However, further investigation is needed to determine the prevalence of these naïve, germline-encoded ACPA-expressing B cells in both RA patients and healthy individuals. Additionally, it needs to be elucidated whether these cells become activated, mature and develop into effector memory B cells. After all, the presence of naïve, germline-encoded ACPA-expressing B cells does not necessarily imply that these cells serve as a source of class-switched ACPA-expressing memory B cells. Longitudinal blood studies in RA patients and healthy individuals could reveal whether these naïve ACPA-expressing B cells become activated and fuel the memory B-cell compartment. Understanding the dynamics of naïve, autoreactive B cells – when and where they appear, and their involvement at different disease stages – will shed more light on their role in disease development and progression. Such knowledge could pave the way for novel therapeutic strategies targeting autoreactive B cells in RA.

Author contributions

SK and HUS: conceptualization. SK and RS: monoclonal antibody production and ELISAs. RvdW: antigen-specific B-cell isolation and subsequent workup to BCR sequencing. LS, REMT and HUS: guidance in experimental design. SK, RS, REMT and HUS: drafting of manuscript.

References

1. Scherer, H.U., D. van der Woude, and R.E.M. Toes, From risk to chronicity: evolution of autoreactive B cell and antibody responses in rheumatoid arthritis. *Nat Rev Rheumatol*, 2022. 18(7): p. 371-383.
2. van der Helm-van Mil, A.H., *et al.*, Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Res Ther*, 2005. 7(5): p. R949-58.
3. Rantapää-Dahlqvist, S., *et al.*, Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum*, 2003. 48(10): p. 2741-9.
4. Nielen, M.M., *et al.*, Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum*, 2004. 50(2): p. 380-6.
5. Kokkonen, H., *et al.*, Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. *Arthritis Res Ther*, 2011. 3.
6. Kissel, T., *et al.*, On the presence of HLA-SE alleles and ACPA-IgG variable domain glycosylation in the phase preceding the development of rheumatoid arthritis. *Ann Rheum Dis*, 2019. 78(12): p. 1616-1620.
7. van de Stadt, L.A., *et al.*, Development of the anti-citrullinated protein antibody repertoire prior to the onset of rheumatoid arthritis. *Arthritis Rheum*, 2011. 63(11): p. 3226-33.
8. van der Woude, D., *et al.*, Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis. *Ann Rheum Dis*, 2010. 69(8): p. 1554-61.
9. Hafkenscheid, L., *et al.*, N-Linked Glycans in the Variable Domain of IgG Anti-Citrullinated Protein Antibodies Predict the Development of Rheumatoid Arthritis. *Arthritis Rheumatol*, 2019. 71(10): p. 1626-1633.
10. Edwards, J.C., *et al.*, Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med*, 2004. 350(25): p. 2572-81.
11. Chatzidionysiou, K., *et al.*, Highest clinical effectiveness of rituximab in autoantibody-positive patients with rheumatoid arthritis and in those for whom no more than one previous TNF antagonist has failed: pooled data from 10 European registries. *Ann Rheum Dis*, 2011. 70(9): p. 1575-80.
12. Burgers, L.E., *et al.*, Differences in the symptomatic phase preceding ACPA-positive and ACPA-negative RA: a longitudinal study in arthralgia during progression to clinical arthritis. *Ann Rheum Dis*, 2017. 76(10): p. 1751-1754.
13. Ten Brinck, R.M., *et al.*, The risk of individual autoantibodies, autoantibody combinations and levels for arthritis development in clinically suspect arthralgia. *Rheumatology (Oxford)*, 2017. 56(12): p. 2145-2153.
14. Scherer, H.U., D. Van Der Woude, and R.E.M. Toes, From risk to chronicity: evolution of autoreactive B cell and antibody responses in rheumatoid arthritis. *Nat Rev Rheumatol*, 2022. 18(7): p. 371-383.
15. Kissel, T., *et al.*, Antibodies and B cells recognising citrullinated proteins display a broad cross-reactivity towards other post-translational modifications. *Ann Rheum Dis*, 2020. 79(4): p. 472-480.
16. Reijm, S., *et al.*, Cross-reactivity of IgM anti-modified protein antibodies in rheumatoid arthritis despite limited mutational load. *Arthritis Res Ther*, 2021. 23(1): p. 230.
17. Sahlström, P., *et al.*, Different Hierarchies of Anti-Modified Protein Autoantibody Reactivities in Rheumatoid Arthritis. *Arthritis Rheumatol*, 2020. 72(10): p. 1643-1657.
18. Scherer, H.U., T. Häupl, and G.R. Burmester, The etiology of rheumatoid arthritis. *J Autoimm*, 2020. 110: p. 102400.
19. Sokolova, M.V., G. Schett, and U. Steffen, Autoantibodies in Rheumatoid Arthritis: Historical Background and Novel Findings. *Clin Rev Allergy Immunol*, 2022. 63(2): p. 138-151.
20. Di Zenzo, G., *et al.*, Pemphigus autoantibodies generated through somatic mutations target the desmoglein-3 cis-interface. *J Clin Invest*, 2012. 122(10): p. 3781-90.
21. Piccoli, L., *et al.*, Neutralization and clearance of GM-CSF by autoantibodies in pulmonary alveolar proteinosis. *Nat Commun*, 2015. 6: p. 7375.
22. Pelzek, A.J., *et al.*, Persistence of Disease-Associated Anti-Citrullinated Protein Antibody-

Expressing Memory B Cells in Rheumatoid Arthritis in Clinical Remission. *Arthritis Rheumatol*, 2017. 69(6): p. 1176-1186.
23. Kerkman, P.F., *et al.*, Identification and characterisation of citrullinated antigen-specific B cells in peripheral blood of patients with rheumatoid arthritis. *Ann Rheum Dis*, 2016. 75(6): p. 1170-6.
24. Kristyanto, H., *et al.*, Persistently activated, proliferative memory autoreactive B cells promote inflammation in rheumatoid arthritis. *Sci Transl Med*, 2020. 12(570).
25. Adlowitz, D.G., *et al.*, Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response. *PLoS One*, 2015. 10(6): p. e0128269.
26. Slot, L.M., *et al.*, Light chain skewing in autoantibodies and B-cell receptors of the citrullinated antigen-binding B-cell response in rheumatoid arthritis. *PLoS One*, 2021. 16(3): p. e0247847.
27. Elliott, S.E., *et al.*, Affinity Maturation Drives Epitope Spreading and Generation of Proinflammatory Anti-Citrullinated Protein Antibodies in Rheumatoid Arthritis. *Arthritis Rheumatol*, 2018. 70(12): p. 1946-1958.
28. Vergroesen, R.D., *et al.*, B-cell receptor sequencing of anti-citrullinated protein antibody (ACPA) IgG-expressing B cells indicates a selective advantage for the introduction of N-glycosylation sites during somatic hypermutation. *Ann Rheum Dis*, 2018. 77.
29. Kongpachith, S., *et al.*, Affinity Maturation of the Anti-Citrullinated Protein Antibody Paratope Drives Epitope Spreading and Polyreactivity in Rheumatoid Arthritis. *Arthritis Rheumatol*, 2019. 71(4): p. 507-517.
30. Steen, J., *et al.*, Recognition of Amino Acid Motifs, Rather Than Specific Proteins, by Human Plasma Cell-Derived Monoclonal Antibodies to Posttranslationally Modified Proteins in Rheumatoid Arthritis. *Arthritis Rheumatol*, 2019. 71(2): p. 196-209.
31. Raposo, B., *et al.*, Divergent and dominant anti-inflammatory effects of patient-derived anticitrullinated protein antibodies (ACPA) in arthritis development. *Ann Rheum Dis*, 2023. 82.