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Bitter Sweet Symphony: the impact of sugars on autoimmunity

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Citation
Kissel, T. (2022, December 1). *Bitter Sweet Symphony: the impact of sugars on autoimmunity*. Retrieved from https://hdl.handle.net/1887/3492105

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Antibodies and B cells recognizing citrullinated proteins display a broad cross-reactivity towards other post-translational modifications

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Ann Rheum Dis., 2020 Apr;79(4):472-480

Abstract

Autoantibodies against antigens carrying distinct post-translational modifications (PTMs), such as citrulline, homocitrulline or acetyl-lysine, are hallmarks of Rheumatoid Arthritis (RA). The relation between these anti-modified protein antibody (AMPA)-classes is poorly understood as is the ability of different PTM antigens to activate B-cell receptors (BCRs) directed against citrullinated proteins. Insights into the nature of PTMs able to activate such B cells are pivotal to understand the "evolution" of the autoimmune response conceivable underlying the disease. Here, we investigated the cross-reactivity of monoclonal AMPAs and the ability of different types of PTM antigens to activate citrullinated protein (CP)-reactive BCRs.

Therefore, BCR sequences from B cells isolated using citrullinated or acetylated antigens were used to produce monoclonal antibodies (mAb) followed by a detailed analysis of their cross-reactivity towards PTM antigens. Ramos B-cell transfectants expressing CP-reactive IgG BCRs were generated and their activation upon stimulation with PTM antigens investigated.

Most mAbs were highly cross-reactive towards multiple PTMs, while no reactivity was observed to the unmodified controls. B cells carrying CP-reactive BCRs showed activation upon stimulation with various types of PTM antigens.

Our study illustrates that AMPAs exhibit a high cross-reactivity towards at least two PTMs indicating that their recognition pattern is not confined to one type of modification. Furthermore, our data show that CP-reactive B cells are not only activated by citrullinated, but also by carbamylated and/or acetylated antigens. These data are vital for the understanding of the breach of B-cell tolerance against PTM antigens and the possible contribution of these antigens to RA-pathogenesis.

Introduction

Autoreactive B cells and their secreted autoantibodies are important players in many autoimmune diseases and often implicated in disease pathogenesis. Rheumatoid arthritis (RA) is hallmarked by the presence of several autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). The presence of these autoantibody families is routinely tested to aid RA-diagnosis and included into the EULAR/ACR-criteria for RA classification[1]. ACPAs are present in 50-70% of RA patients and are known to recognize multiple citrullinated antigens, such as α-enolase, fibrinogen, filaggrin, vimentin and type II collagen[2-7]. Their recognition profile is generally broad and the serological ACPA response expands closer to disease-onset (epitope spreading) probably reflecting an escalation in the activation of citrullinated protein (CP)-reactive B cells[8-10]. Recently, autoantibodies recognizing other post-translationally modified (PTM) antigens, such as anti-carbamylated protein antibodies (ACarPAs) and anti-acetylated protein antibodies (AAPAs), were identified[11-13]. ACarPAs are directed against homocitrulline-containing (carbamylated) antigens and present in approximately 45% of RA patients, while AAPAs target acetylated-lysine residues and are found in 40% of RA patients[12,13]. So far, it is unclear how these autoantibodies are generated and if their underlying B-cell responses are interrelated. As citrullination targets arginine residues, while carbamylation/acetylation predominantly lysine residues, the "modified" epitopes are, by definition, unrelated as they occur at different positions in the protein backbone and hence are surrounded by different flanking regions. Likewise, although homocitrullination and acetylation are both lysine modifications, they are structurally dissimilar. Consequently, ACPAs, ACarPAs and AAPAs are often considered as three independent autoantibody classes[11]. Nevertheless, these autoantibodies often occur concurrently in RA and cross-reactivity has been reported, both on a polyclonal- and monoclonal-level, within an ELISA setting[13-17]. Hence, it is clearly relevant to understand the (in)dependence of these different autoantibody responses in greater detail and to delineate the possibility that autoreactive B cells expressing a B-cell receptor (BCR) against one particular PTM can be activated by other modifications as well. Such understanding would not only be relevant for the comprehension of the breach of B-cell tolerance in RA, but also to uncover the antigens that could drive the expansion of autoreactive B cells conceivably present in the inflamed joint. Likewise, insights into the relations between AAPAs, ACarPAs and ACPAs and their cross-reactivity, could not only help understanding RA-initiation, it could also lead to more refined serological markers for RA-diagnosis. Therefore, we characterized the properties of monoclonal IgG generated from BCR sequences of citrullinated and acetylated antigen-reactive B cells. Additionally, we generated, for the first time, human B-cell transfectants expressing CP-reactive BCRs to investigate the hypothesis that B cells recognizing citrullinated antigens are cross-reactive and can be activated by other PTMs.

Results

Isolation and successful production of monoclonal ACPA and AAPA IgG from peripheral blood B cells of RA patients

To characterize the reactivity patterns of various AMPA IgG, we produced 14 monoclonal IgG1 antibodies from BCR sequences of single cell sorted B cells from ACPA$^+$ and AAPA$^+$ RA patients. 11 antibodies were obtained from cyclic citrullinated peptide 2 (CCP2)-reactive B cells, one antibody from citrullinated-fibrinogen (7E4) and two antibodies from acetylated-vimentin (HC55)-reactive B cells (Table 1)[18]. All monoclonal antibodies (mAbs) were successfully produced as IgG1 molecules and exhibited the expected apparent molecular weight as determined by SDS-PAGE (Figure 1A). The mAbs were subsequently tested for reactivity towards peptides carrying the same modification as used for the isolation of the B cell from which the mAbs were generated (Figure 1B). All 12 ACPA IgG showed a high reactivity to CCP2 but not to its arginine control variant (CArgP2). Likewise, the AAPA IgG molecules showed acetylated-vimentin (HC55) reactivity, but no reactivity to the unmodified lysine-vimentin peptide (HC56).

Cross-reactivity of ACPA and AAPA IgG towards various PTM antigens

Having verified the successful production of monoclonal PTM antigen-directed IgGs, we next determined their binding characteristics towards various PTM peptides and proteins. We analyzed their reactivity to four linear peptides (fibrinogen α 27-43, fibrinogen β 36-52, vimentin 59-74 and enolase 5-20) and three cyclic peptides (CCP1, CCP2 and CCP4) carrying three different modifications: citrulline (cit), homocitrulline (hcit) and acetyl-lysine (ac). Likewise, reactivity to their arginine (arg), respectively lysine (lys)-containing controls was determined (Table S2, Figure 2 and Figure S5). Noteworthy, none of the mAbs was exclusively reactive towards the PTM that was originally used for the isolation of the autoreactive B cell. In fact, all mAbs showed reactivity towards at least two different PTMs, whereas several mAbs recognized all three PTMs (1F2, D9, 2C4 and 2F5) within the same antigenic backbone (Figures 2, A and B). No binding was observed for the non-modified control peptides indicating PTM antigen-specific reactivity.

To further validate these findings, we next analyzed the cross-reactivity of the mAbs towards modified proteins, using three PTM proteins (fibrinogen, OVA and vinculin) as well as carbamylated FCS (Figures 2, C and D). The results obtained largely confirmed the results of the peptide ELISA studies. We observed no reactivity of the ACPA and AAPA mAbs to the unmodified control proteins, but extensive cross-reactivity to the PTM proteins (Figures 2, C and D). The cross-reactive nature of the antibodies was further confirmed in another experimental setting examining three mAbs in western blot analyses. These antibodies (2G9, 7E4 and 2C4) were selected on the basis of their differential binding patterns in the peptide

and protein ELISAs. The results obtained by western blot indicated that monoclonal AMPA IgG recognize different epitopes within the PTM-fibrinogen α, β and λ chains (Figure 2E). More importantly, 7E4 recognized citrullinated- and acetylated-fibrinogen, as also observed in ELISA. Likewise, in agreement with the ELISA data, 2C4 reacted to all three PTM-variants of fibrinogen, whereas 2G9 mainly reacted to citrullinated-fibrinogen (Figure 2E).

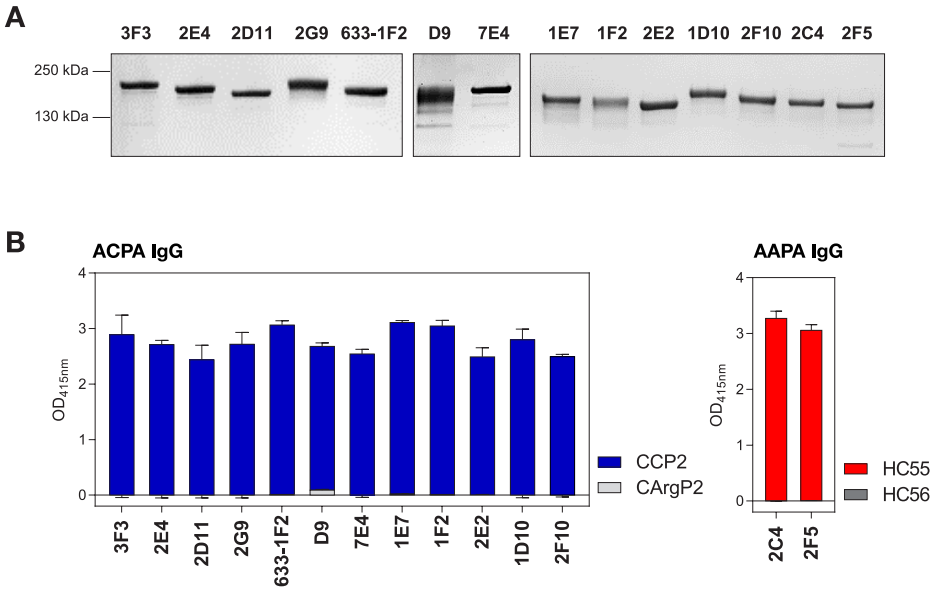

Figure 1. Production of 14 monoclonal AMPA IgG. (**A**) SDS-PAGE of purified monoclonal AMPA IgG using 4-15% gradient protein gels (BioRad). The size was determined using the PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific). Molecular weights are higher than 150 kDa and vary between monoclonals due to the expression of different amounts of N-linked glycans within their V-domains. (**B**) Stacked bar graph of the CCP2/ CArgP2 (patent protected sequences) and acetylated-vimentin (HC55)/ lysine-vimentin (HC56) peptide ELISA of 12 purified monoclonal ACPA IgG and two AAPA IgG respectively. Reactivities were determined by the OD at 415 nm represented on the y-axis. The data represent the mean and standard error of 3 technical replicates.

Table 1. Monoclonal AMPA variable region sequences.

Tetramer*	AMPA	Patient	IGH-CDR3aa†	(nt) mut HC V-gene†	IGHV†	Identity [%]†
CCP2	3F3	1	CARGTYLPVDESAAFDVW	56	IGHV1-2*02	80.56
CCP2	2E4	1	CARGSFLERPESVPFHPW	71	IGHV1-2*02	75.35
CCP2	2D11	2	CARRGGKDNVWGDW	21	IGHV5-51*01	92.71
CCP2	2G9	2	CVRWGEDRTEGLW	63	IGHV4-34*02	78.60
CCP2	633-1F2	2	CVRGGSLGIFGGSVGYW	45	IGHV7-4-1*01	84.72
CCP2	D9	3	CARDLSKIFPLYYGMDVW	54	IGHV3-30-3*01	81.25
Cit-fibrinogen	7E4[18]	4	CVRIRGGSSNWLDPW	63	IGHV4-39*01	77.08
CCP2	1E7	5	CARGIGLGDVIICEGFDVW	48	IGHV4-30-4*01	83.45
CCP2	1F2	5	CARGFGSAEELVCYGMDVW	50	IGHV4-30-4*04	82.76
CCP2	2E2	5	CARLQCSNGLCYLGGDTFDIW	29	IGHV4-34*01	89.82
CCP2	1D10	5	CARGLGKTSLWGVDAFDVW	55	IGHV4-30-4*08	81.1
CCP2	2F10	5	CARALGKPLVWGVDSFDVW	38	IGHV4-30-4*01	86.94
Ac-vimentin	2C4	6	CATRHHDDIWGHSSVIFFDTW	57	IGHV4-39*01	80.76
Ac-vimentin	2F5	6	CATRHYDDIRGRSSVIFFETW	53	IGHV4-39*01	81.79

* Tetramer used for single B cell isolation via flow cytometry.

† Determined by IMGT/ V-QUEST.

To substantiate and further characterize the cross-reactive nature of the ACPA and AAPA IgG in a third experimental setting, we performed cross-inhibition studies using 2G9, 7E4 and 2C4 in combination with both modified peptides, C(C/Hcit/Ac)P2 and C(C/Hcit/Ac)P4 as well as proteins, citrullinated-, carbamylated- and acetylated-fibrinogen. The cross-inhibition studies showed that the reactivity of 7E4 to CCP2 and CCP4 could not only be inhibited by the citrullinated peptide itself, but also by its acetylated counterpart, while almost no inhibition could be observed after incubation with CHcitP2/CHcitP4 (Figures 3, A and S4). Similarly, reactivity towards citrullinated- and acetylated-fibrinogen could be inhibited by both the citrullinated version as well as the acetylated version of fibrinogen (Figure 3B). In agreement with titration ELISAs showing some reactivity of 7E4 towards carbamylated-fibrinogen at high concentrations (Figure S1A), binding of 7E4 to citrullinated- and acetylated-fibrinogen could be inhibited after pre-incubation with high amounts of carbamylated-fibrinogen (Figure 3B). Thus, together, these cross-inhibition results show that the mAb reactivity towards one particular PTM can be inhibited by another PTM and thereby confirm the reactivity data obtained by ELISA. Likewise, as depicted in Figures 3, A and B, similar findings were made for 2G9 and 2C4 reaffirming the outcome of the reactivity patterns observed by the peptide/protein ELISAs (Figure 2 and Figure 3, A and B).

IGHD†	IGHJ†	IGL-CDR3aa†	(nt) mut LC V-gene†	LC	IGK/LV†	Identity [%]†	IGLJ†
IGHD2-8*01	IGHJ3*01	CQQYYEAPYTF	39	κ	IGKV4-1*01	87.54	IGKJ2*01
IGHD3-3*01	IGHJ3*01	CLQYHAEPYTF	61	κ	IGKV4-1*01	79.46	IGKJ2*01
IGHD3-3*01	IGHJ4*02	CQQYNDWPVTF	11	κ	IGKV3-15*01	96.06	IGKJ2*01
IGHD2-21*02	IGHJ5*02	CMQRLRFPLTF	31	κ	IGKV2-40*01	89.56	IGKJ4*01
IGHD3-10*02	IGHJ4*03	CQSYYRGDWVL	47	λ	IGLV6-57*02	84.19	IGLJ3*02
IGHD3-3*01	IGHJ6*02	CHHYGFSPCSF	26	κ	IGKV3-20*01	90.78	IGKJ2*04
IGHD2-15*01	IGHJ5*02	CAAWNGRLSAFVF	48	λ	IGLV1-51*01	83.86	IGLJ1*01
IGHD3-10*01	IGHJ3*01	CQSFDSSGLIF	30	λ	IGLV6-57*01	90.38	IGLJ2*01
IGHD2-15*01	IGHJ6*02	CQSYDVSGLVF	15	λ	IGLV6-57*01	95.53	IGLJ2/J3*01
IGHD2-8*01	IGHJ3*02	CQQYVSYSTF	18	κ	IGKV1-5*01	93.55	IGKJ1*01
IGHD3-16*02	IGHJ3*01	CQQSNSSSSITF	43	κ	IGKV1-39*01	85.66	IGKJ4*01
IGHD2-15*01	IGHJ3*01	CQQSNSTLSLTF	36	κ	IGKV1-39*01	87.10	IGKJ4*01
IGHD3-16*01	IGHJ5*02	CQSADSEGLDILF	54	λ	IGLV3-25*03	80.65	IGLJ2/J3*01
IGHD3-16*01	IGHJ5*02	CQSSDSTGEDILF	44	λ	IGLV3-25*03	84.23	IGLJ2/J3*01

Altogether, these data indicate that all ACPA and AAPA mAbs analyzed cross-react to a varying extent to at least one other PTM and hence should be regarded as anti-modified protein antibodies (AMPAs) rather than as antibodies with a single specificity.

Human B cells expressing CP-reactive BCRs are activated upon stimulation with different PTM antigens

The data described above show a high degree of cross-reactivity of AMPAs towards several modifications and hence suggest that also CP-reactive B cells could react to multiple PTMs. To determine whether such B cells can indeed be activated by several PTMs, we next expressed three different IgGs (7E4, 3F3 and 2G9), isolated from CP-reactive B cells of RA patients, in a membrane-bound (mIgG) state on a human reporter B-cell line. To this end, we used the human lymphoma Ramos B-cell line in which the genes encoding the endogenous IgD and IgM heavy- and light-chain sequences and the gene encoding for activation-induced cytidine deaminase (AID) have been deleted, (MDL-AID). This "triple knockout (KO)" cell line is unable to show BCR signaling as it lacks an endogenous BCR. Moreover, it cannot modify a transduced BCR as it lacks AID. Upon transduction Ramos B-cell lines showed GFP and BCR expression, indicating a successful transduction and expression of CP-reactive BCRs. Indeed, binding of the CCP2 antigen, but not of the arginine containing control peptide CArgP2, was observed after incubating the transduced B cells with these antigens (Figure S2).

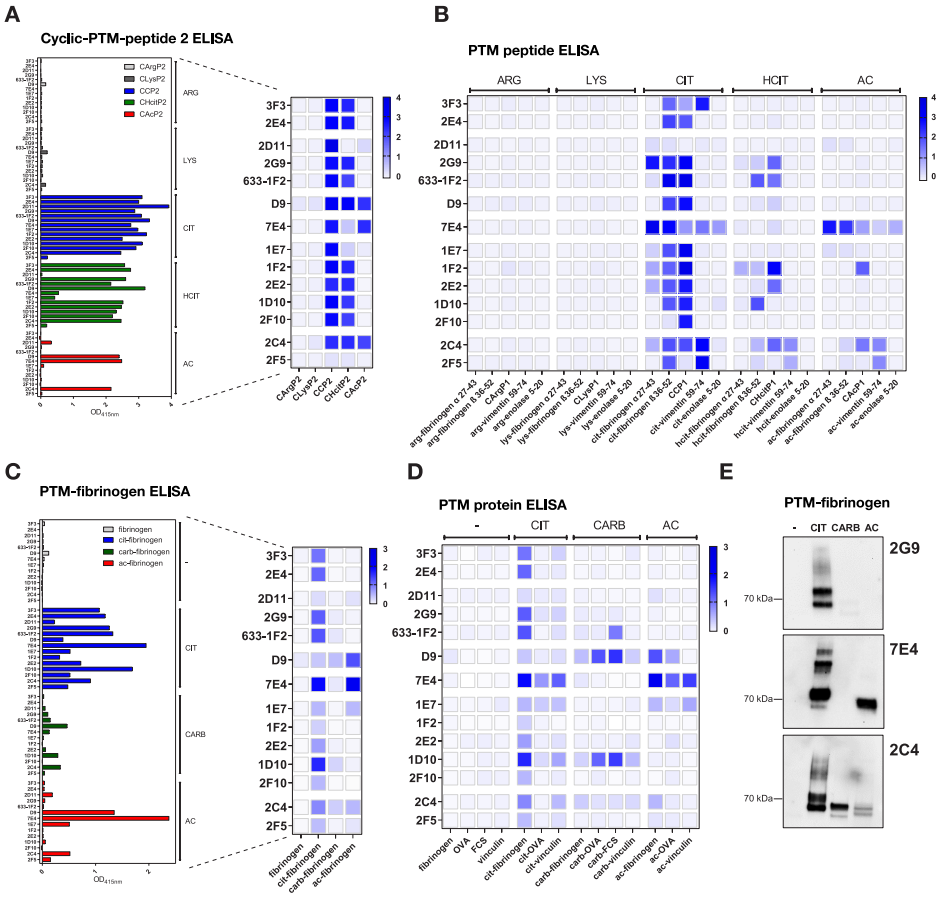

Figure 2. Cross-reactivity of monoclonal AMPA IgG determined by ELISA and western blot analysis. (**A**) Bar graph and heatmap of a cyclic-PTM-peptide 2 (C(C/Hcit/Ac)P2) ELISA of 14 monoclonal AMPA IgG. Monoclonal AMPA IgG reactivity towards the CCP2 (patent protected sequence) peptide in five modifications (citrulline, homocitrulline, acetyl-lysine, arginine, lysine) was tested. (**B**) Heatmap of PTM peptide ELISAs of 14 monoclonal AMPA IgG. Monoclonal reactivity to four linear PTM peptides (fibrinogen α 27-43, fibrinogen β 36-52, vimentin 59-74 and enolase 5-20) and the CCP1 peptide in five modifications (arg, lys, cit, hcit, ac) was analyzed. (**C**) Bar graph and heatmap of PTM-fibrinogen ELISA of 14 monoclonal AMPA IgG. Monoclonal AMPA IgG reactivity to the fibrinogen protein in four different versions (unmodified, cit, carb and ac) was tested. (**D**) Heatmap of PTM protein ELISAs of 14 monoclonal AMPA IgG. Monoclonal reactivity to fibrinogen, OVA and vinculin proteins in four different modifications (unmodified, cit, carb and ac) as well as to carb-FCS and unmodified FCS was analyzed. Reactivities were determined by the OD at 415 nm represented on the x-axis (bar graphs) or by color (blue, high OD values, light gray, low OD values) within the heatmaps. Monoclonal AMPA IgG were tested in a concentration of 10 μg/ml. 2D11 was analyzed in a concentration of 20 μg/ml within the cyclic-PTM-peptide 2 ELISA. All ELISA experiments were repeated independently 2 to 3 times. (**E**) Western blot analysis of monoclonal AMPA IgG 2G9, 7E4 and 2C4. Binding towards citrullinated-, carbamylated- and acetylated-fibrinogen and to the unmodified version (-) was analyzed under reducing conditions (separately to the α, β and λ chain). Western blot analysis was repeated 3 times within independent experiments.

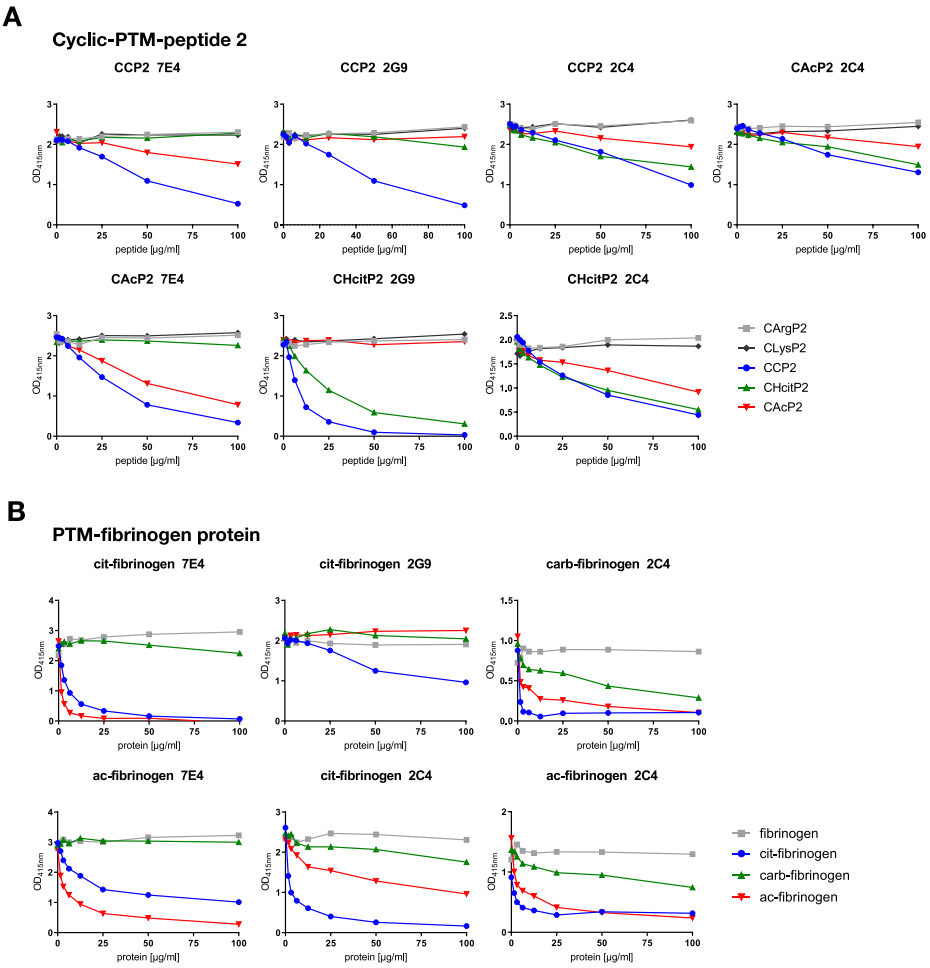

Figure 3. Cross-inhibition studies of monoclonal AMPA IgG determined by ELISA. (**A**) Cross-inhibition ELISA with cyclic-PTM-peptide 2 as an inhibitor depicted for 7E4 using CCP2- as well as CAcP2-coated plates, for 2G9 using CCP2- and CHcitP2-coated plates and for 2C4 using CCP2-, CHcitP2- and CAcP2-coated ELISA plates. Cross-inhibition was performed with increasing concentrations of the C-PTM-P2 peptide in all three modifications (cit, hcit and ac) and with the negative control peptides CArgP2 and CLysP2. The C-PTM-P2 peptide sequences are patent protected. (**B**) PTM-fibrinogen cross-inhibition ELISA curves of 7E4 for citrullinated- and acetylated-fibrinogen coated plates, of 2G9 for a citrullinated-fibrinogen coated plate and of 2C4 for citrullinated-, carbamylated- and acetylated-fibrinogen coated plates. Cross-inhibition was performed with increasing concentrations of all four different versions of fibrinogen (unmodified, cit, carb and ac). Monoclonals were tested in concentrations that bound within the linear range of the respective peptide or protein titration ELISA (Figure S1). Binding is represented by the OD at 415 nm on the y-axis. Cross-inhibition studies were performed 2-times within independent experiments. Light gray octagon: arginine, dark gray diamond: lysine, blue circle: citrulline, red triangle: acetyl, green square: homocitrulline/carbamyl.

Next, we used the cells to study BCR activation via phosphorylation of intracellular Syk (pSyk) 5 min after stimulation with different PTM antigens. The non-transduced MDL-AID KO cell line (BCR^-GFP^-) was taken along as a negative/gating control. As depicted in Figures 4 and S3, Syk was phosphorylated after stimulating the 7E4, 3F3 and 2G9 Ramos B-cell transfectants with the respective PTM antigen. To quantify B-cell activation, the percentage of $pSyk^+GFP^+$ cells was determined. 7E4 mIgG carrying B cells readily reacted to stimulation with citrullinated peptides (25.25 ± 7.142%) and to stimulation with acetyl-lysine-containing peptides (22.35 ± 7.990%). In contrast, the cells did not respond to stimulation with a homocitrulline-containing peptide (0.9450 ± 0.8560%) (Table S6, Figure 4B). These data indicate that the results obtained in the "non-functional assays" described above translate to the functional activation of 7E4 CP-reactive B cells. More importantly, these results also show that such B cells respond to several PTMs. Similar results were obtained in the activation assays using 3F3- and 2G9-derived B cells, showing not only activation upon stimulation with citrullinated peptides (3F3: 28.85 ± 2.475%; 2G9: 15.00 ± 4.950%), but also with homocitrullinated peptides (3F3: 21.30 ± 2.828%; 2G9: 14.49 ± 6.944%). In line with our results obtained by ELISA these cell lines did not respond to acetyl-lysine-containing peptides (3F3: 0.8250 ± 0.2470%; 2G9: 0.0000 ± 0.0000%) (Table S6, Figure 4, C and D). To expand the findings described above to the recognition of protein antigens, we next analyzed the ability of the different modified forms of fibrinogen to stimulate the CP-reactive B cells. As shown in Figure 2B, 3F3 and 2G9 bind solely to citrullinated-fibrinogen in ELISA. In agreement, Ramos cells transduced with these IgG sequences displayed only reactivity to this modification (Figure S3). More importantly, and in agreement with the data presented in Figure 2B, Ramos B cells transduced with 7E4 not only responded to citrullinated-fibrinogen, but also displayed reactivity towards the acetylated counterpart (Table S7, Figure 4B), indicating that CP-reactive B-cells can respond to several PTM proteins. Together, these data show that autoreactive B cells expressing a BCR directed against one type of modification can also be activated by other PTMs.

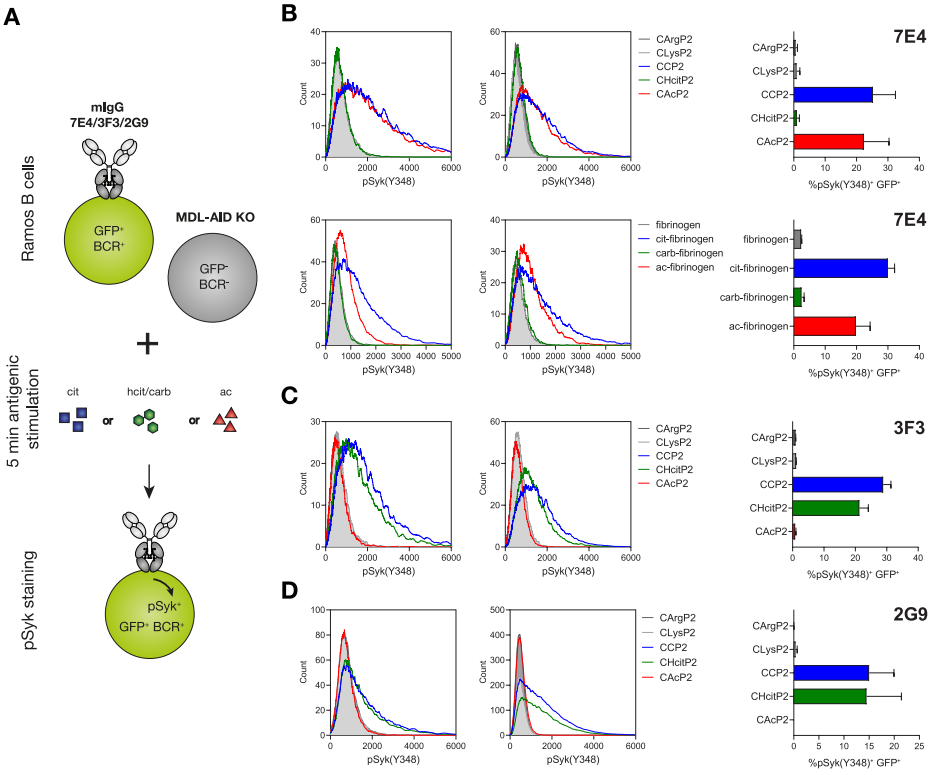

Figure 4. B-cell receptor signaling (pSyk expression) of CP-reactive BCR$^+$GFP$^+$ Ramos B-cell transfectants after stimulation with PTM antigens. (**A**) Schematic depiction of the experimental activation assay design. GFP$^+$mIgG$^-$BCR$^+$ Ramos B-cell transfectants and the untransfected GFP$^-$BCR$^-$ control MDL-AID KO cell line were stimulated for 5 min with PTM antigens. BCR activation was determined as the proportion/percentage of GFP$^+$pSyk(Y348)$^+$ B cells. Stimulation with cit-antigens leads to an "ACPA" response (blue), hcit/carb-activation results in an "ACarPA" response (green) and ac-antigen activation leads to an "AAPA" response (red). (**B**) Histograms of two biological replicates and a bar graph (n = 2) showing the percentage of pSyk(Y348)$^+$GFP$^+$ 7E4 mIgG Ramos B-cells after stimulation with cyclic-PTM-peptide 2 and PTM-fibrinogen (**C**) Histograms of two biological replicates and a bar graph (n = 2) showing the percentage of pSyk(Y348)$^+$GFP$^+$ 3F3 mIgG Ramos B cells after stimulation with cyclic-PTM-peptide 2 (**D**) Histograms of two biological replicates and a bar graph (n=2) showing the percentage of pSyk(Y348)$^+$GFP$^+$ 2G9 mIgG Ramos B cells after stimulation with cyclic-PTM-peptide 2. The C-PTM-P2 peptide sequences are patent protected. All activation assays were repeated 2 to 3 times within independent experiments. CArgP2 = dark gray, CLysP2 = light gray, CCP2 = blue, CHcitP2 = green, CAcP2 = red. Unmodified fibrinogen = light gray, cit-fibrinogen = blue, carb-fibrinogen = green, ac-fibrinogen = red.

Discussion

Insights into the dynamics of autoimmune responses are vital to understand the breach of tolerance to self-antigens and the "evolution" of the autoimmune response conceivably underlying the disease. Even though the ACPA response is considered as the dominating AMPA-response linked to the most prominent genetic risk factors for RA (the HLA-SE alleles), it is clear that autoantibody responses present in RA patients extend towards several modifications, such as acetylation and/or carbamylation. AMPA-responses are currently considered to consist of different autoantibody classes that are largely distinct in origin and development. Nonetheless, AMPAs also display a certain degree of cross-reactivity and often occur concurrently in individual patients. Recently, we made the crucial observation that vaccinating mice with an acetylated protein leads to the formation of autoantibodies against carbamylated proteins, indicating that different AMPA-responses can evolve from the exposure to only one type of modification. These data provide a conceptual framework for the simultaneous presence of different AMPA-responses in RA by showing that the inciting antigen responsible for the induction of e.g. ACarPAs does not have to be carbamylated, but could be represented by an acetylated protein. We now show that human monoclonal ACPAs and AAPAs isolated from AMPA-positive RA patients (Figure S5) are highly cross-reactive towards various PTM antigens (Figure 2). Noteworthy, all ACPA and AAPA IgG analyzed were able to recognize at least two diverse modifications. This finding has general importance, as it indicates that ACPAs, ACarPAs and AAPAs should be considered as AMPAs that are not specific for one type of PTM. Furthermore, our results indicate that not only the affinity of the mAb towards a particular modification, but also the antigenic backbone and consequently the flanking regions around a modification can contribute to the reactivity-pattern of AMPA IgG. Depending on the antigen tested (CCP2 peptide or fibrinogen protein), and thus the flanking amino acids around a modification, the AMPAs showed a higher reactivity towards one or another PTM as detected in titration and cross-inhibition ELISAs (Figures 3 and S1). We consider it unlikely that these observations can solely be explained by the number of modifications per protein, which likely differ per PTM generated and might explain the higher mAb reactivity to carb-FCS compared to carb-fibrinogen, as this pattern is not consistent across different antibodies analyzed. Nonetheless, it is clear that additional analyses are required to elucidate the potential contribution of flanking regions to the reactivity of AMPAs towards PTMs.

Most importantly, our data show that B-cell lines transfected with a BCR derived from one type of defined "ACPA" can not only be activated by citrullinated, but also by other PTM antigens. For these studies, we implemented a unique and novel tool by expressing different CP-reactive IgG as BCRs in human Ramos B cells, an accepted model cell line to study BCR responses upon stimulation[19]. This enabled us to study human autoreactive B-cell responses on the cellular level. Our observations support the notion that B cells expressing a BCR against citrullinated antigens

could be activated by other, non-citrulline containing PTM antigens. Conceptually, these results are highly relevant to further understand and define the antigens that could be recognized in inflamed joints or at other locations in the body (mucosal tissues) which could be involved in the induction of autoimmunity. Likewise, these findings point to the possibility that a first encounter with a particular PTM can not only initiate an AMPA-response, but also determines the direction of it, conceivably dictating a progression towards "ACPA-, ACarPA- or AAPA-dominated" B-cell responses. It is tempting to hypothesize that subsequent antigenic contacts, with different PTM antigens, could (re)direct the B-cell response towards other modifications, or reinforce the original direction of the AMPA-response. In this way, the concurrent presence of multiple AMPA-reactivities, as observed in many RA patients, can be explained, and the observation that in other patients the response can be dominated by one AMPA-response towards e.g. citrullinated, carbamylated or acetylated proteins. It would be interesting to investigate the extent of cross-reactivity in different disease stages, ranging from health to arthralgia, undifferentiated arthritis and RA within future studies. Here, we suggest that AMPA B-cell responses should be considered dynamic responses without a "fixed" categorization into different AMPA-classes. We speculate that the inciting and subsequent encounters with particular PTM antigens define the course of the autoreactive B-cell responses, resulting in the heterogeneous reactivity-pattern observed in patients with RA (Figure 5).

Thus, our data disclose a strong relationship and high cross-reactivity between various autoantibodies and their B cells in RA patients, explaining the concurrent presence of ACPA-, ACarPA- and AAPA-responses. These findings are important to further our understanding of the breach of B-cell tolerance in RA and to unmask the antigens recognized in inflamed tissues.

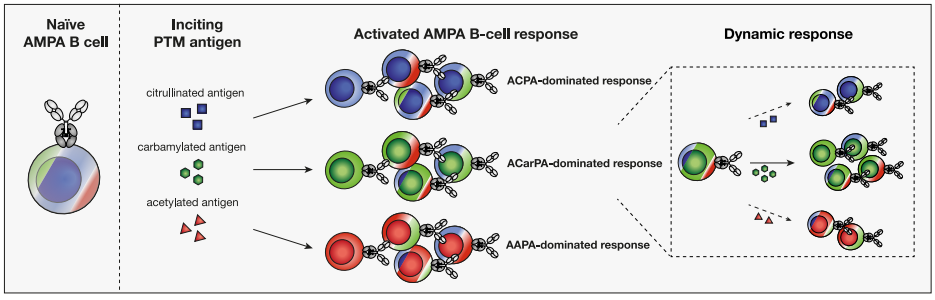

Figure 5. Schematic depiction of an hypothesis proposing the course of autoreactive AMPA B-cell responses. Naïve B cells expressing BCRs directed against PTM antigens display reactivity towards citrullinated (blue), carbamylated (green) or acetylated (red) antigens. The inciting trigger could represent either a citrullinated, carbamylated or acetylated antigen. Dependent on this initial priming, the B cells are directed towards an "ACPA-, ACarPA- or AAPA-dominated" B-cell response. Upon subsequent encounter of other PTMs, the AMPA-response can be (re)directed towards another AMPA-class (dynamic response) or the original direction of the AMPA-response can be reinforced (outgrowth of e.g. "ACarPA-dominated" B-cell responses).

Materials and Methods

Patient and public involvement – Peripheral blood samples from ACPA$^+$ or ACPA$^+$/AAPA$^+$ RA patients visiting the outpatient clinic of the Rheumatology Department at the Leiden University Medical Center (LUMC) were included in this study. RA patients included in this study were selected on their high anti-CCP2 positivity (ACPA$^+$, >340 U/ml) based on in-house CCP2 ELISAs (see ELISA section). Patient 6, used for the monoclonal AAPA isolation, was selected based on AAPA-positivity determined by an acetylated-vimentin (HC55) ELISA (Orgentec)[20]. All patients fulfilled the EULAR/ACR 2010-criteria for classification of RA and provided written informed consent prior to inclusion[1]. None of the patients was previously treated with B-cell depletion therapies. Ethical permission was obtained from the institutional review board. Further clinical patient characteristics, including AMPA status, are given in Table S1.

Protein modification and mass spectrometry (MS) analysis – Vinculin, fibrinogen, and ovalbumin (OVA, Sigma Aldrich) protein modification (citrullination, carbamylation and acetylation) as well as FCS carbamylation was performed as previously described[13,16]. Mass spectrometry (MS) of PTM-fibrinogen was performed as previously described[16]. Briefly, in-gel tryptic digestion of reduced PTM-fibrinogen (native, citrullinated, acetylated, carbamylated) was performed. Tryptic peptides were extracted from the gel, lyophilized, dissolved and analyzed by on-line C18 nano HPLC MS/MS with a system consisting of an Easy nLC 1200 gradient HPLC system (Thermo, Bremen, Germany), and an orbitrap Fusion LUMOS mass spectrometer (Thermo). In a post-analysis process, the raw data were first converted to peak lists using Proteome Discoverer version 2.2 (Thermo Electron), and then submitted to the Uniprot Homo sapiens canonical database (67911 entries), using Mascot v. 2.2.07 (www.matrixscience.com) for protein identification. Mascot searches were with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Up to two missed cleavages were allowed. The following modifications were set for the searches: oxidation (on M), carbamylation (on K and on peptide N-terminus), citrullination (on R), acetylation (on K and on peptide N-terminus) and deamidation (on N-terminus) were set as a variable modification; carbamidomethyl (on C) was set as a fixed modification. An FDR threshold of 0.01 was initially set. All spectra of the post-translationally modified peptides were manually evaluated for proper identification. In addition, all citrullinated peptides identified after PAD treatment were specifically cross checked in the other post-translationally modified samples as well as in the native control to exclude cross-contamination. The results are listed in Table S5. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[21] partner repository with the dataset identifier PXD017127.

Peptide synthesis and integrity identification – Four linear N-terminal biotinylated peptides, fibrinogen α 27-42, fibrinogen β 36-52, vimentin 59-74 and enolase 5-20, and three cyclic N-terminal biotinylated peptides, C(C/Hcit/Ac)P1, C(C/Hcit/Ac)P2 (patent EP2071335) and C(C/Hcit/Ac)P4, were synthesized in five different modifications including the altered amino acid residue(s) at the same positions within the peptide sequence (citrulline, arginine, homocitrulline, acetyl-lysine, lysine)[10,22]. The CCP4 peptides were cyclized as previously described[23]. The patent protected C(C/Hcit/Ac)P2 peptides were provided by J. W. Drijfhout (LUMC, The Netherlands). The HC55 and HC56 peptides, used for the isolation of AAPA IgG sequences, were synthesized and provided by H. Bang (Orgentec, Germany)[20]. The known peptide sequences are listed in Table S2. The integrity of the synthesized peptides after purification was examined by ultra-performance liquid chromatography (UPLC) on a Acquity instrument (Waters) and the exact mass measured via MS on a Microflex instrument (Bruker) and crosschecked with the calculated masses (Tables S3 and S4).

Production of monoclonal AMPA IgG based on BCR sequences from RA patients – 11 ACPA IgG sequences were isolated from ACPA$^+$ RA patients. CCP2- and CArgP2-streptavidin tetramers were used for the isolation of CP-reactive B cells as previously described[24]. Single sorted cells were cultured on irradiated CD40L-cells and a cytokine mixture in complex IMDM (Gibco) medium for 10 to 12 days[25]. RNA isolation, cDNA synthesis, ARTISAN PCR and sequencing were performed as previously described[26,27]. The same methodology using acetylated-vimentin-(HC55) and lysine-vimentin-(HC56) streptavidin tetramers was used to isolate two AAPA IgG sequences. The ACPA IgG 7E4 sequence was provided by T. Rispens (Sanquin, The Netherlands)[18]. Codon optimized AMPA heavy chain (HC) and light chain (LC) V gene sequences, including 5'-BamHI and 3'-XhoI restriction sites, the Kozak sequence, and the IGHV1-18*01 leader sequence, were designed, ordered from GeneArt (Life Technologies) and ligated into a pcDNA3.1 (+) expression vector (Invitrogen) carrying the IGHG1 or the IGLC3/IGKC constant regions (UniProt) respectively flanking 3'-XhoI site. The ACPA IgG HC and LC containing vectors were transfected into Freestyle™ 293-F cells (Gibco). Cells were cultured in Freestyle™ 293 expression medium (Gibco) at 37 °C, 8% CO2 on a shaking platform. Transfection was performed in Opti-MEM® (Gibco) together with the expression enhancing plasmids, encoding the large T antigen of the SV40 virus (GeneArt) and the cell cycle inhibitors p21 and p27 (Invivogen), as well as 293-Fectin™ (Invitrogen) as previously described[28]. Glycoengineering was performed by adding D-galactose substrate to the medium before transfection (Sigma Aldrich, G0750-5G). Further, to generate complex-type N-glycans on the antibody V-domains, recombinant AMPA IgG were co-expressed with 1% β-1,4-*N*-acetylglucosaminyltransferase III (GnTIII), 2.5% α2,6-sialyltransferase 1 (ST6galT) and 1% β-1,4-Galactosyltransferase 1 (B4GalT1). The supernatants were harvested 5 to 6 days post-transfection.

Protein G affinity purification and size exclusion chromatography of AMPA IgG – The IgG1 antibodies were purified using a 1ml HiTrap® Protein G HP affinity column (GE Healthcare) according to the manufacturer's instructions. Size exclusion chromatography (SEC) was performed for buffer exchange and for the exclusion of single HCs or LCs using a 53 ml HiPrep™ 26/10 Desalting column (GE Healthcare) according the manufacturer's instructions. Monoclonal antibodies (mAbs) were concentrated with Amicon® Ultra-15 50 K filter devices (Merck) to a final concentration of 1 mg/ml and used for further experiments.

Generation of human Ramos B-cell transfectants expressing CP-reactive IgG BCRs – 7E4, 2G9 and 3F3 ACPA IgG1 HC and LC containing single vector constructs were created with the In-Fusion HD Cloning Kit (Clontech) using the pMIG-IRES-GFP-2AP vector as a backbone including the IGHG1 transmembrane domain. The lymphoma Ramos cell line expressing the murine cationic amino-acid transporter 1 (*slc7a1*) under blasticidine resistance to be able to infect them with Moloney Murine Leukemia Virus (MoMLV)-based retrovirus particles, was provided by Dr. Engels (University Göttingen, Germany). The MDL-AID (IGHM, IGHD, IGLC and activation-induced cytidin deaminase, AID) knock-out (KO) variant of the *slc7a1* expressing Ramos cells was generated by Dr. He (University Freiburg, Germany). All inserts were verified by sequencing. Ramos cell lines were cultured in RPMI1640/GlutaMAX™/10% FCS/10 mM Hepes medium (Thermo Fisher Scientific) with 100 units/ml penicillin/streptomycin (P/S) (Lonza). Retroviral transductions in Ramos cells were performed as previously described[29]. Briefly, Phoenix-ECO (ATCC CRL-3212™) cells were transfected with PolyJet DNA transfection reagent following the manufacturer's instructions (SignaGen Laboratories). Retrovirus containing supernatants were collected 72 hours after transfection and used for the transduction into MDL-AID KO Ramos cells carrying *slc7a1*.

Enzyme-linked immunosorbent assays (ELISA) – Briefly, 1 μg/ml biotinylated C-PTM-P2 and 10 μg/ml biotinylated PTM peptides were coupled to pre-coated streptavidin plates (Microcoat, #65001) via incubation at RT for 1 hour. 10 μg mAbs were added in PBS/1%BSA/0.05% Tween (PBT) (Sigma Aldrich) and samples incubated for 1 hour at 37 °C. Protein ELISAs were performed as previously described[13]. Briefly, 10 μg/ml PTM protein was directly coupled to Nunc Maxisorp plates (Thermo Fisher Scientific) and incubated overnight on ice at 4 °C. Following blocking with PBS/2% BSA for 6 hours, 10 μg of the monoclonal was added in PBT and incubated overnight on ice at 4 °C. Concentrations used for titration ELISAs are mentioned within the legend to the figure. Monoclonal AMPA IgG binding was detected using an HRP-conjugated rabbit-anti-human IgG secondary antibody (DAKO, P0214). ELISA read-out was performed using ABTS and H2O2. Reactivity to PTM peptides and proteins was determined based on the unmodified protein or the arginine or lysine version of the peptide. For cross-inhibition studies, mAbs were pre-incubated

with increasing concentrations (0 to 100 μg/ml) of either unmodified, citrullinated-, carbamylated- or acetylated-fibrinogen protein, C(Arg/Lys/C/Hcit/Ac)P2 or C(Arg/Lys/C/Hcit/Ac)P4 peptides for 1 hour at RT. The antibody-antigen complexes were added to PTM-fibrinogen, cyclic-PTM-peptide 2 or cyclic-PTM-peptide 4 coated plates respectively and the ELISA read-out was performed as described above.

SDS-PAGE and western blot analysis – For SDS-PAGE, 1.5 μg of each monoclonal AMPA IgG in PBS was diluted in 4× Laemmli buffer (Bio Rad) and incubated for 5 min at 95 °C. 10 μl sample and 3 μl PageRuler™ Plus Prestained Protein Ladder (Thermo Fisher Scientific) were loaded on 4-15% SDS-polyacrylamide gels (Bio Rad). Western blot analysis was performed as previously described[13]. In brief, PTM-fibrinogen proteins (fibrinogen, cit-, carb- and ac-fibrinogen) were subjected to 4-15% SDS-polyacrylamide gels (Bio Rad). Subsequently immunoblotting was performed on a Nitrocellulose membrane (Bio Rad). Blots were incubated in blocking buffer (3% skim milk powder/PBS/0.05% tween) for 1 hour at RT. Following washing with PBS/0.05% tween (PT), the blots were incubated with 1 to 3 μg/ml monoclonal AMPA IgG diluted in 5 ml blocking buffer at 4 °C overnight. After washing with PT, blots were incubated for 1 hour at RT with 5 ml HRP-conjugated rabbit-anti-human IgG secondary antibody (DAKO, P0214), diluted 1:5000 in blocking buffer. Blots were washed and bound antibodies visualized using enhanced chemiluminescence (GE Healthcare, RPN2109).

Activation assays of Ramos B cells expressing CP-reactive BCRs – GFP^+BCR^+ (7E4, 2G9, 2C4) Ramos B-cell lines ($1x10^6$ cells) were stimulated with C(Arg/Lys/C/Hcit/Ac)P2-streptavidin tetramers (10 μg/ml)[24] for 5 min at 37 °C in stimulation medium (RPMI/GlutaMAX™/1% FCS/10 mM Hepes/100 units/ml P/S). Additionally, stimulation was performed with unmodified, citrullinated-, carbamylated- and acetylated-fibrinogen proteins (50 μg/ml). Afterwards, cells were fixed (Biolegend Fixation Buffer, 420801) and permeabilized (True-Phos™ Perm Buffer, 425401). After washing, cells were stained with mouse anti-human pSyk(Y348)-PE mAb (moch1ct, eBioscience™) diluted 1:20 in PBS/0.5% BSA/0.02% NaN3. The rate of pSyk expression in Ramos cells was calculated as the percentage and proportion of $pSyk^+GFP^+$ double positive cells. Gating was based on the MDL-AID KO control cell line stimulated with the citrullinated antigen and on isotype control staining's using mouse IgG1 kappa isotype control-PE mAb (P3.6.2.8.1, eBioscience™). Stained cells were analyzed on a BD LSR-II flow cytometry instrument. Data were analyzed with FlowJo_V10.

Acknowledgments

The authors would like to thank Natasja Dolezal (LUMC, Leiden, The Netherlands) for synthesizing the PTM peptides and Dr. Theo Rispens (Sanquin, Amsterdam, The Netherlands) for providing the 7E4 ACPA IgG BCR sequence.

Funding

This work was supported by ReumaNederland 17-1-402 (to R.E.M.T.), the IMI-funded project RTCure 777357 (to T.W.J.H.), ZonMw TOP 91214031 (to R.E.M.T.), Target-to-B LSHM18055-SGF (to R.E.M.T.), NWO-ZonMW clinical fellowship 90714509 (to H.U.S.), NWO-ZonMW VENI grant 91617107 (to H.U.S.), ZonMW Enabling Technology Hotels grant 435002030 (to H.U.S.), Dutch Arthritis Foundation 15-2-402 and 18-1-205 (to H.U.S.), Excellence Initiative of the German Federal and State Governments EXC 294 (to M.R.), DFG through TRR130-P02 (to M.R.), RO1 grant A031503 (to M.R.) and by the Investment Grant NWO Medium 91116004, which is (partially) financed by ZonMw. (to P.A.v.V.)

Conflict of interest

No.

Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Conceptualization: T.K., S.R., T.W.J.H., H.U.S., M.R., and R.E.M.T. Methodology: T.K., S.R., L.M.S., M.C., C.M.W., R.D.V. and G.M.C.J. Software: T.K., J.W.D., H.B., G.M.C.J. and P.A.v.V. Investigation: T.K., S.R., L.M.S., M.C., C.M.W., R.D.V., G.S.-R., J.C.K., A.S.B.K., E.W.N.L., J.W.D., H.B., K.M.B., G.M.C.J., P.A.v.V. Visualization: T.K. Supervision: M.R., T.W.J.H., H.U.S., and R.E.M.T. Writing—original draft: T.K. and R.E.M.T. Writing—review and editing: S.R., L.M.S., M.C., C.M.W., R.D.V., G.S.-R., J.C.K., A.S.B.K., E.W.N.L., J.W.D., H.B., K.M.B., G.M.C.J., P.A.v.V., T.W.J.H., H.U.S. and M.R.

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Supplemental Tables

Table S1. Clinical patient characteristics.

Patient	Age	Sex [m/f]	Ethnicity	Disease stage	Disease characteristics
1	64	f	Asian (Thai)	established (13 years)	erosive, destructive RA
2	57	f	Suriname	established (7 years)	non-erosive RA
3	57	f	Caucasian	established (6 years)	non-erosive RA
5	66	m	Caucasian	established (36 years)	erosive RA
6	53	f	Caucasian	established (6 years)	erosive RA

* based on CCP2 and CCP4 reactivity

† based on CAcP2 and CAcP4 reactivity

‡ based on CHcitP2 and CHcitP4 reactivity

Table S2. PTM peptide sequences.

Peptide	aa-sequence	Number of epitopes
HC55/HC56 vimentin[20]	Ttds-O-Ttds-GRVYAT-(ac)K-SSAVR/ Ttds-O-Ttds-GRVYAT-K-SSAVR	1
CCP1 (cyclic)[10]	HQCHQESTXGRSRGRCGRSGSZO	1
CCP2 (cyclic)	Patent EP2071335	unknown
CCP4 (cyclic)	HQFRFXGNleSRAACZO	1
fibrinogen α 27-43 (linear)[22]	FLAEGGGVXGPRVVERHZO	1
fibrinogen β 36-52 (linear)[22]	NEEGFFSAXGHRPLDKKZO	1
vimentin 59-74 (linear)[22]	VYATXSSAVXLXSSVPZO	3
enolase 5-20 (linear)[22]	KIHAXEIFDSXGNPTVZO	2

X = arginine, lysine, citrulline, homocitrulline, acetyl-lysine.

Z = 6-aminohexanoic acid

O = lys (biotine)-amide

Ttds-linker = 1,13-diamino-4,7,10-trioxatridecane succinimic acid linker

Nle= norleucine

Table S3. Cyclic PTM peptide masses. The calculated versus the measured monoisotopic masses of C-PTM-P1 and average masses of C-PTM-P4.

Peptide	MH+, monoisotopic [*m/z*] calculated masses	MH+, monoisotopic [*m/z*] measured masses
CArgP1	2806.3	2806.6
CLysP1	2778.3	2778.5
CCP1	2807.3	2807.7
CHcitP1	2821.3	2821.6
CAcP1	2820.3	2820.5
Peptide	**MH+, average [*m/z*] calculated masses**	**MH+, average [*m/z*] measured masses**
CArgP4	2056.5	2055.9
CLysP4	2028.5	2028.5
CCP4	2057.5	2056.8
CHcitP4	2071.5	2071.7
CAcP4	2070.5	2070.2

Swollen joint count	Treatment at time of sampling	ACPA IgG status*	AAPA IgG status†	ACarPA IgG status‡	RF status
0	methotrexate, hydroxychloroquine	positive	positive	positive	positive
8	methotrexate	positive	positive	positive	positive
0	methotrexate, hydroxychloroquine	positive	positive	positive	negative
0	methotrexate	positive	positive	positive	positive
0	none	positive	positive	positive	positive

Table S4. Linear PTM peptide masses. The calculated versus the measured average masses of four linear PTM peptides.

Peptide	MH+, average [*m/z*] calculated masses	MH+, average [*m/z*] measured masses
arg-fibrinogen β 36-52	2455.8	2456.0
lys-fibrinogen β 36-52	2427.8	2429.3
cit-fibrinogen β 36-52	2456.8	2456.8
hcit-fibrinogen β 36-52	2470.8	2470.3
ac-fibrinogen β 36-52	2469.9	2469.7
arg-fibrinogen α 27-43	2303.7	2303.2
lys-fibrinogen α 27-43	2275.7	2275.8
cit-fibrinogen α 27-43	2304.7	2304.7
hcit-fibrinogen α 27-43	2318.7	2318.2
ac-fibrinogen α 27-43	2317.8	2317.1
arg-vimentin 59-74	2216.7	2216.7
lys-vimentin 59-74	2132.6	2131.7
cit-vimentin 59-74	2219.6	2219.1
hcit-vimentin 59-74	2261.7	2261.4
ac-vimentin 59-74	2258.9	2258.0
arg-enloase 5-20	2307.7	2308.2
lys-enloase 5-20	2251.7	2251.0
cit-enloase 5-20	2309.7	2310.4
hcit-enloase 5-20	2337.8	2336.6
ac-enloase 5-20	2335.9	2335.0

Table S5. Citrullinated peptides detected via MS in all four PTM-fibrinogen samples (native, citrullinated, acetylated and carbamylated).

Peptide sequences	Citrulline position within the peptide	Protein subunit
MELERPGGNEITRGGSTSYGTGSETESPR	13	alpha
GDFSSANNRDNTYNR	9	alpha
GDFSSANNRDNTYNRVSEDLR	9.15	alpha
GPRVVERHQSACKDSDWPFCSDEDWNYK	3.7	alpha
DNTYNRVSEDLR	6	alpha
VTSGSTTTTRRSCSK	10.1	alpha
SCRGSCSRALAR	3. 8	alpha
GSCSRALAR	5	alpha
TFPGFFSPMLGEFVSETESRGSESGIFTNTK	20	alpha
SSSYSKQFTSSTSYNRGDSTFESK	16	alpha
QFTSSTSYNRGDSTFESK	10	alpha
GPRVVERHQSACK	3.7	alpha
EVVTSEDGSDCPEAMDLGTLSGIGTLDGFRHR	30	alpha
VTSGSTTTTRRSCSKTVTK	10.11	alpha
SSSYSKQFTSSTSYNRGDSTFESKSYK	16	alpha
LVTSKGDKELRTGK	11	alpha
DRQHLPLIK	2	alpha
ESSSHHPGIAEFPSRGK	15	alpha
EEAPSLRPAPPPISGGGYRARPAK	19	beta
EEAPSLRPAPPPISGGGYRARPAK	7.19	beta

n.d.= no citrullination detected

Table S6. %pSyk(Y348)$^+$GFP$^+$ and #pSyk(Y348)$^+$GFP$^+$ CP-reactive mIgG expressing B cells after 5min stimulation with C-PTM-P2 (10 µg/ml).

ACPA BCR	#pSyk(Y348)$^+$GFP$^+$ B cells									
	CArgP2		CLysP2		CCP2		CHcitP2		CAcP2	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
3F3	339	138.6	445.5	129.4	9253	1105	6953	1250	260.5	38.89
7E4	300	271.5	312.5	323.1	8491	2065	334	275.8	7469	2286
2G9	230	66.47	177.5	53.03	5273	814.6	3576	347.9	213.5	51.62

n = 2

Table S7. %pSyk(Y348)$^+$GFP$^+$ and #pSyk(Y348)$^+$GFP$^+$ CP-reactive mIgG expressing B cells after 5min stimulation with PTM-fibrinogen (50 µg/ml).

ACPA BCR	#pSyk(Y348)$^+$GFP$^+$ B cells							
	fibrinogen		cit-fibrinogen		carb-fibrinogen		ac-fibrinogen	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
3F3	305.5	9.192	2598	1776	391.5	65.76	443.5	183.1
7E4	691	80.61	9698	929.8	725.5	194.5	6288	1311
2G9	380.3	188.6	791.3	363.6	512.5	454.3	563.5	512.5

n = 2 to 3

Abundance of citrullinated peptides in PTM-fibrinogen samples			
native	**citrullinated**	**acetylated**	**carbamylated**
3.90E+06	1.00E+07	3.50E+06	1.10E+07
2.60E+07	2.20E+08	6.60E+05	6.30E+05
n.d.	8.30E+07	n.d.	n.d.
n.d.	2.50E+06	n.d.	n.d.
n.d.	3.00E+07	n.d.	n.d.
n.d.	1.20E+07	n.d.	n.d.
n.d.	1.70E+06	n.d.	n.d.
n.d.	1.10E+05	n.d.	n.d.
n.d.	3.00E+06	n.d.	n.d.
n.d.	2.40E+07	n.d.	n.d.
n.d.	5.50E+06	n.d.	n.d.
n.d.	2.70E+07	n.d.	n.d.
n.d.	1.40E+06	n.d.	n.d.
n.d.	1.60E+07	n.d.	n.d.
n.d.	1.20E+06	n.d.	n.d.
n.d.	2.70E+07	n.d.	n.d.
n.d.	1.90E+06	n.d.	n.d.
n.d.	1.30E+08	n.d.	n.d.
n.d.	2.20E+07	n.d.	n.d.
n.d.	6.20E+07	n.d.	n.d.

%pSyk(Y348)$^+$GFP$^+$ B cells									
CArgP2		**CLysP2**		**CCP2**		**CHcitP2**		**CAcP2**	
Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
0.965	0.049	1.145	0.021	28.85	2.475	21.3	2.828	0.825	0.247
0.62	0.537	0.915	1.011	25.25	7.142	0.945	0.856	22.35	7.99
0.01	0.013	0.355	0.332	15	4.95	14.49	6.944	0	0

%pSyk(Y348)$^+$GFP$^+$ B cells							
fibrinogen		**cit-fibrinogen**		**carb-fibrinogen**		**ac-fibrinogen**	
Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
1.01	0.028	8.285	5.678	1.285	0.148	1.41	0.58
2.35	0.17	30	2.121	2.495	0.799	19.8	4.25
1.218	0.573	2.558	0.829	1.433	0.754	1.335	0.456

Supplemental Figures

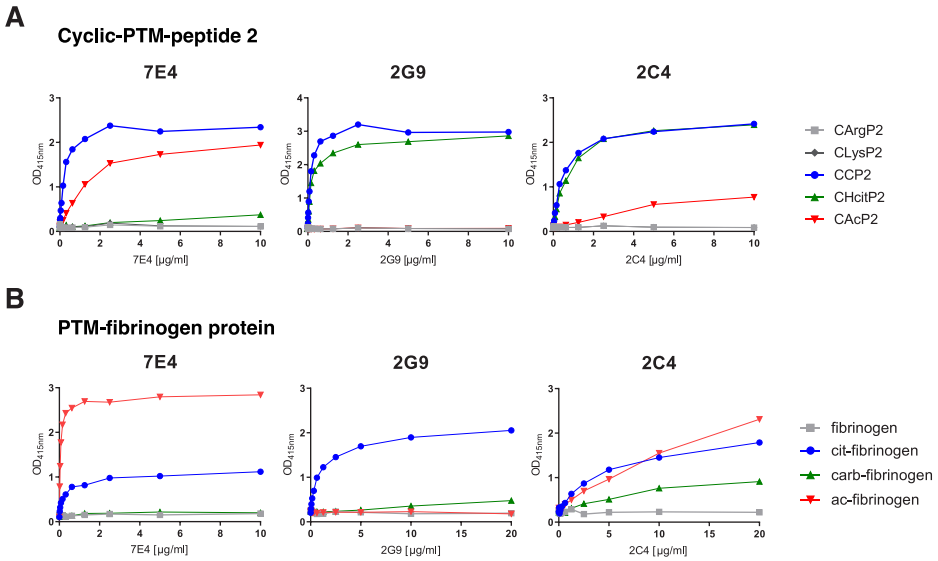

Figure S1. Titration ELISA curves of monoclonal AMPA IgG 7E4, 2G9 and 2C4. (**A**) Cyclic-PTM-peptide 2 (patent protected sequence) titration ELISA curves of the monoclonal AMPA IgG 7E4, 2G9 and 2C4 (0 to 10 µg/ml). (**B**) PTM-fibrinogen titration ELISA curves of the monoclonal AMPA IgG 7E4 (0 to 10 µg/ml), 2G9 and 2C4 (0 to 20 µg/ml).

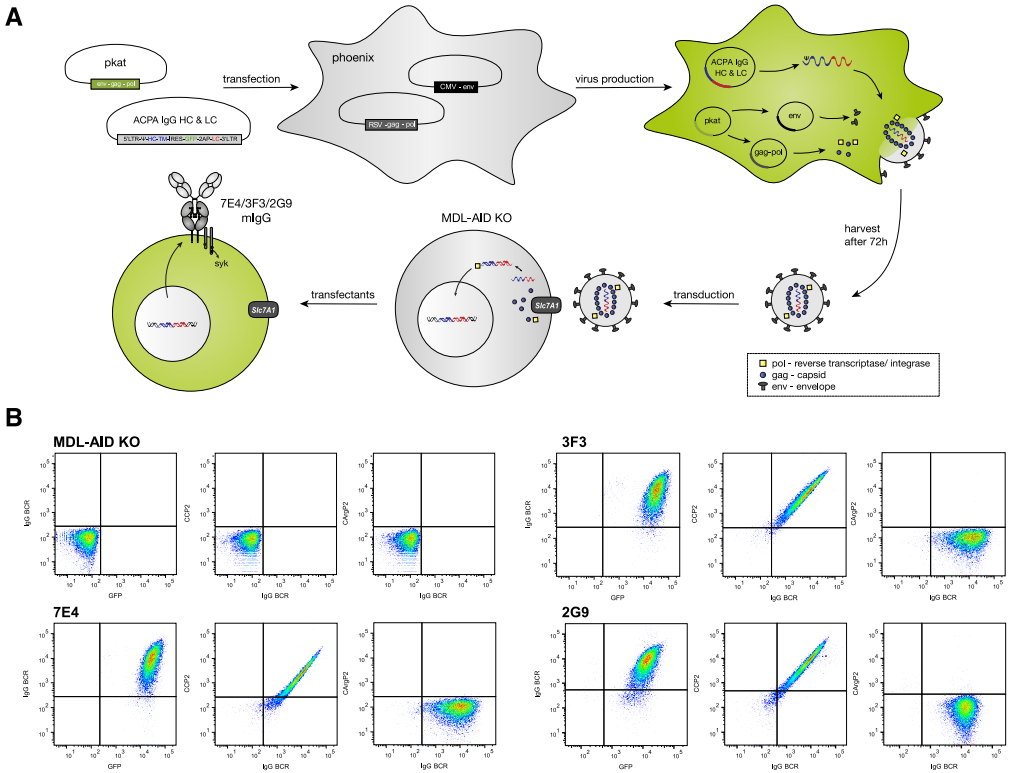

Figure S2. MDL-AID KO Ramos B-cell transfectants expressing CP-reactive BCRs. (**A**) Schematic depiction of the generation of human Ramos B-cell transfectants expressing CP-reactive BCRs. Phoenix-ECO cells were transfected with the help vector pkat and a single vector including the ACPA IgG HC and LC sequences as well as the selection marker GFP. Retroviruses produced by the phoenix cells were collected after 72 hours and transduced into MDL-AID KO human Ramos B-cell lines via the *slc7a1* receptor. B-cell transfectants express the CP-reactive IgG 7E4, 3F3 and 2G9 as membrane-bound BCR (mIgG) and the selection marker GFP (green). (**B**) Dot plot chromatograms showing GFP, mIgG-BCR expression and binding towards CCP2 and CArgP2 of the untransduced MDL-AID KO control cell line and the Ramos B-cell transfectants expressing 7E4, 3F3 and 2G9.

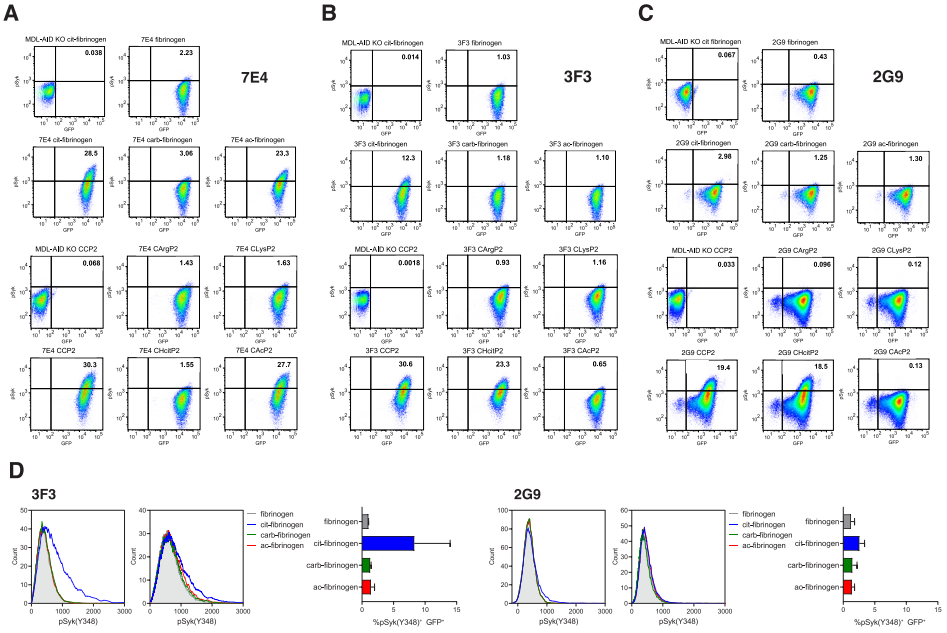

Figure S3. B-cell receptor signaling (pSyk expression) of CP-reactive BCR^{+}GFP^{+} Ramos B-cell transfectants after stimulation with PTM antigens. Dot plot chromatograms showing the percentage of pSyk(Y348)$^{+}$GFP^{+} (**A**) 7E4 mIgG Ramos cells (**B**) 3F3 mIgG Ramos cells and (**C**) 2G9 mIgG Ramos cells after stimulation with C-PTM-P2 (patent protected sequence) and PTM-fibrinogen. MDL-AID KO cell lines stimulated with CCP2 and cit-fibrinogen were used as a negative gating control in every experiment. (**D**) Histograms of two biological replicates and a bar graph (n = 2) showing the percentage of pSyk(Y348)$^{+}$GFP^{+} 3F3 and 2G9 mIgG Ramos cells stimulated with PTM-fibrinogen (cit, carb and ac). Fibrinogen: light gray; cit-fibrinogen: blue; hcit-fibrinogen: green; ac-fibrinogen: red.

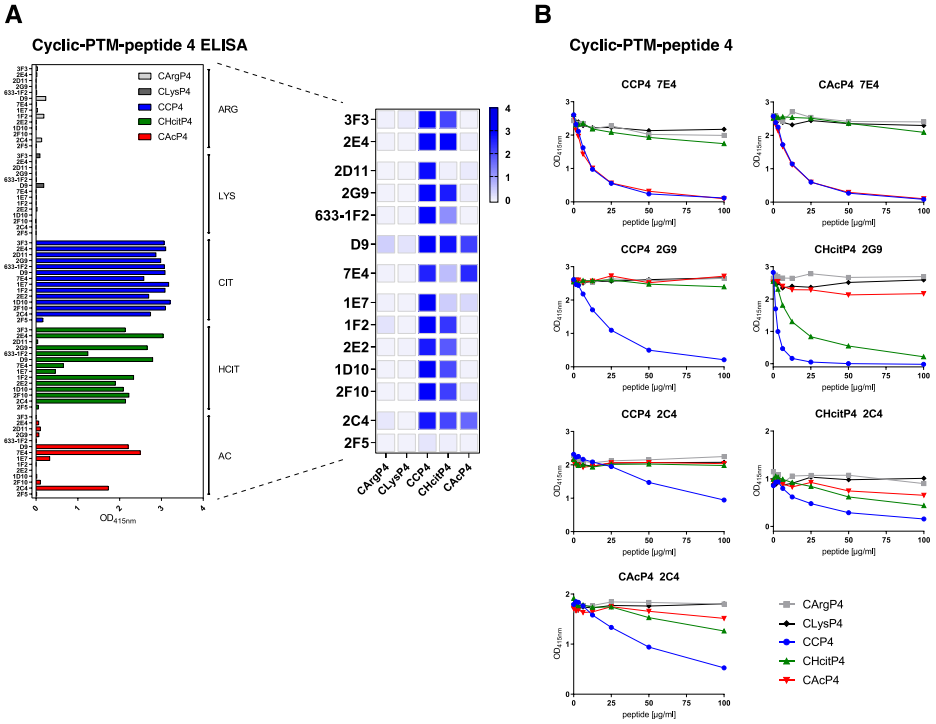

Figure S4. Cross-reactivity of monoclonal AMPA IgG towards C-PTM-P4 and cross-inhibition studies determined by ELISA. (**A**) Bar graph and heatmap of a cyclic-PTM-peptide 4 (C(C/Hcit/Ac)P4) ELISA of 14 monoclonal AMPA IgG. Monoclonal AMPA IgG reactivity towards the CCP4 peptide in five modifications (citrulline, homocitrulline, acetyl-lysine, arginine, lysine) was tested. (**B**) Cross-inhibition ELISA with C-PTM-P4 as an inhibitor depicted for 7E4 using CCP4 and CAcP4-coated ELISA plates and 2G9 using CCP4 and CHcitP4-coated ELISA plates. Cross-inhibition was performed with increasing concentrations of C-PTM-P4 in all three modifications (cit, hcit and ac) and with the negative control peptides CArgP4 and CLysP4.

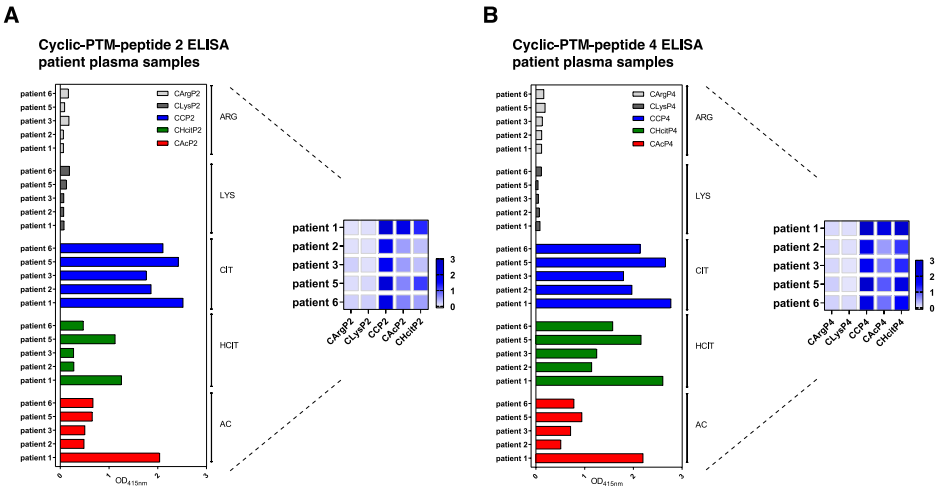

Figure S5. Cross-reactivity of selected RA patient plasma samples used for the mAb sequence isolation based on cyclic-PTM-peptide 2 and cyclic-PTM-peptide 4 ELISAs. (**A**) Bar graph and heatmap of a cyclic-PTM-peptide 2 (patent protected sequence) ELISA of five patients (1 to 3, 5 and 6) used for the mAb sequence isolation (Table 1). (**B**) Bar graph and heatmap of a cyclic-PTM-peptide 4 ELISA of five patients (1 to 3, 5 and 6) used for the mAb sequence isolation (Table 1). Shown are the RA patient plasma sample reactivities towards both peptides in five modifications (arg, lys, cit, hcit and ac) depicted as OD values at 415 nm.