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

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ABSTRACT

Objective Autoantibodies against antigens carrying distinct post-translational modifications (PTMs), such as citrulline, homocitrulline or acetylslysine, 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 (CP). 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 AMPA and the ability of different types of PTM-antigens to activate CP-reactive BCRs.

Methods 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 on stimulation with PTM-antigens investigated.

Results 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 on stimulation with various types of PTM-antigens.

Conclusions Our study illustrates that AMPA 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

Key messages

What is already known about this subject?

- Autoantibodies in patients with rheumatoid arthritis (RA) target different post-translational modifications (PTMs), such as citrullination (anti-citrullinated protein antibodies (ACPAs)), homocitrullination/carbamylation (anti-carbamylated protein antibodies (ACarPAs)) and acetylation (anti-acetylated protein antibodies (AAPAs)).

What does this study add?

- ACPA, ACaPA and AAPA-IgG show a broad reactivity to various antigenic backbones and are highly cross-reactive towards at least two different PTMs.
- Citrullinated protein-reactive B-cell receptors show activation not only upon stimulation with citrullinated, but also after contacting carbamylated or acetylated antigens indicating a broad cross-reactive nature on the cellular level. These results indicate that B cells directed against a particular PTM can be activated by other PTM-antigens in inflamed tissues or other sites conceivably involved in the breach of B-cell tolerance.
- ACPAs, ACaPAs and AAPAs cannot be separated into three independent autoantibody classes and should be regarded as anti-modified protein antibodies (AMPAs).

How might this impact on clinical practice or future developments?

- AMPA probably reflects a better serological marker and combinatorial ACPA/ACaPA/AAPA immunoassays could improve RA diagnosis and treatment.
- The data further our understanding of the breach of B-cell tolerance in RA.

RA-diagnosis and included into the EULAR/ACR-criteria for RA classification.¹ ACPAs are present in 50%–70% of patients with RA and are known to recognise 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 recognising other post-translationally modified (PTM)-antigens, such as anti-carbamylated protein antibodies (ACarPAs) and anti-acetylated protein antibodies (AAPAs), were identified.^{11–13} ACPAs are directed against homocitrulline-containing (carbamylated) antigens and present in approximately 45% of patients with RA, while AAPAs target acetylated-lysine residues and are found in 40% of patients with RA.^{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, ACPAs 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-level 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 be relevant for the comprehension of the breach of B-cell tolerance in RA and 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, ACPAs and ACPAs and their cross-reactivity, could help in understanding RA-initiation and could also lead to more refined serological markers for RA-diagnosis. Therefore, we characterised 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 recognising citrullinated antigens are cross-reactive and can be activated by other PTMs.

MATERIALS AND METHODS

Patient and public involvement

Peripheral blood samples from ACPA+ or ACPA+/AAPA+ patients with RA visiting the outpatient clinic of the Rheumatology Department at the Leiden University Medical Center (LUMC) were included in this study. Additional information on patient characteristics is given in the supplementary section (online supplementary table S1).

Protein modification and peptide synthesis

Experimental procedures for protein modification and peptide synthesis are provided in the supplementary section. Peptide sequences and masses are given in online supplementary tables 2–4. Protein masses are provided in online supplementary table S5.

Production of monoclonal anti-modified protein antibody (AMPA)-IgG based on BCR sequences from patients with RA

Eleven ACPA-IgG sequences were isolated from patients with ACPA+RA. Cyclic-citrullinated-peptide 2 (CCP2) and CArgP2 streptavidin-tetramers were used for the isolation of

CP-reactive B cells as previously described.¹⁸ Single sorted cells were cultured on irradiated CD40 L-cells and a cytokine mixture in complex IMDM (Gibco) medium for 10–12 days.¹⁹ RNA isolation, cDNA synthesis, ARTISAN PCR and sequencing were performed as previously described.^{20,21} The same methodology using acetylated-vimentin (HC55) and lysine-vimentin (HC56)²² streptavidin-tetramers was used to isolate two AAPA-IgG sequences. The ACPA-IgG 7E4 sequence was provided by Dr Rispen, Sanquin, The Netherlands.²³ Expression vector cloning, monoclonal antibody (mAb) production and purification procedures are described in the supplementary section.

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 blasticidin resistance to be able to infect them with Moloney murine leukemia virus-based retrovirus particles, was provided by Dr Engels, University Göttingen. The MDL-AID (IGHM, IGHD, IGLC and activation-induced cytidin deaminase (AID)) knockout (KO) variant of the *slc7a1* expressing Ramos cells was generated by Dr He, University Freiburg. All inserts were verified by sequencing. Ramos cell lines were cultured in RPMI1640/GlutaMAX/10%FCS/10mMHEPES medium (Thermo Scientific) with 100 units/mL penicillin/streptomycin (P/S) (Lonza). Retroviral transductions in Ramos cells were performed as previously described.²⁴ 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*.

ELISA, SDS-PAGE and western blot analysis

Experimental procedures used for the analysis of the monoclonal AMPA-IgG (ELISA, SDS-PAGE and western blot) are given in the online supplementary section.

Activation assays of Ramos B cells expressing CP-reactive BCRs

GFP+BCR+ (7E4, 2G9, 2C4) Ramos B-cell lines (1×10^6 cells) were stimulated with C(Arg/Lys/C/Hcit/Ac)P2 streptavidin-tetramers (10 µg/mL)¹⁸ for 5 min at 37°C in stimulation medium (RPMI/GlutaMAX/1%FCS/10mMHEPES/100 units/mL P/S). Additionally, stimulation was performed with unmodified, citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen proteins (50 µg/mL). Afterwards, cells were fixed (Biologend Fixation Buffer, 420801) and permeabilized (TruePhos 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 analysed on a BD LSR-II flow cytometry instrument. Data were analysed with FlowJo_V10.

RESULTS

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

To characterise the reactivity patterns of various AMPA-IgG, we produced 14 monoclonal IgG1 antibodies from BCR sequences of single cell sorted B cells from patients with ACPA+ and AAPA+RA. Eleven antibodies were obtained from CCP2-reactive B cells, one antibody from citrullinated-fibrinogen (7E4) and two antibodies from acetylated-vimentin (HC55)-reactive B cells (table 1).²³ All mAbs were successfully produced as IgG1 molecules and exhibited the expected apparent molecular weight as determined by SDS-PAGE (figure 1A and online supplementary figure S1). 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-IgG and AAPA-IgG towards various PTM-antigens

Having verified the successful production of monoclonal PTM-directed IgGs, we next determined their binding characteristics towards various PTM-peptides and proteins. We analysed 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 (hcrit) and acetyllysine (ac). Likewise, reactivity to their arginine (arg), respectively, lysine (lys)-containing controls was determined (online supplementary table S2, figure 2 and online supplementary 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 recognised all three PTMs (1F2, D9, 2C4 and 2F5) within the same antigenic backbone (figure 2A,B). No binding was observed for the non-modified control peptides indicating PTM-specific reactivity.

To further validate these findings, we next analysed the cross-reactivity of the mAbs towards modified proteins, using three PTM-proteins (fibrinogen, OVA and vinculin) as well as carbamylated-FCS (figure 2C,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 (figure 2C,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 recognise different epitopes within the PTM-fibrinogen α, β and λ chains (figure 2E). More importantly, 7E4 recognised citrullinated-fibrinogen 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).

To substantiate and further characterise the cross-reactive nature of the ACPA-IgG 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/hcrit/Ac)P2

Table 1 Monoclonal AMPA variable region sequences.

Tetramer*	AMPA	Patient	IGH-CDR3aatt	(nt) mut			Identity (%) [†]	IGHDT	IGHJ	IGL-CDR3aatt	(nt) mut			Identity (%) [†]	IGLJ
				HC V-gene†	IGHV†	IGHV-gene†					LC V-gene†	IGKV†	IGLV†		
CCP2	3F3	1	CARGTYLPVDESAAFDVW	56	IGHV1-2*02	80.56	IGHD2-8*01	IGHJ3*01	CQQYYEAPYTF	κ	IGKV4-1*01	39	87.54	IGKJ2*01	
CCP2	2E4	1	CARGSFLEPESVPHPW	71	IGHV1-2*02	75.35	IGHD3-3*01	IGHJ3*01	CLQYHAEPYTF	κ	IGKV4-1*01	61	79.46	IGKJ2*01	
CCP2	2D11	2	CARRGGKDNVWGDW	21	IGHV5-51*01	92.71	IGHD3-3*01	IGHJ4*02	CQQYNDWVPVTF	κ	IGKV3-15*01	11	96.06	IGKJ2*01	
CCP2	2G9	2	CVRWGEDRTEGLW	63	IGHV4-34*02	78.60	IGHD2-21*02	IGHJ5*02	CMQRLRPPLTF	κ	IGKV2-40*01	31	89.56	IGKJ4*01	
CCP2	633-1F2	2	CVRGGSLGIFGSGVYW	45	IGHV7-4-1*01	84.72	IGHD3-10*02	IGHJ4*03	CQSYRGGDWL	λ	IGLV6-57*02	47	84.19	IGLJ3*02	
CCP2	D9	3	CARDLSKIFPYYGMDVW	54	IGHV3-30-3*01	81.25	IGHD3-3*01	IGHJ6*02	CHHYGFSPCSF	κ	IGKV3-20*01	26	90.78	IGKJ2*04	
Cit-fibrinogen	7E4 (22)	4	CVRIRGGSSNWLDPW	63	IGHV4-39*01	77.08	IGHD2-15*01	IGHJ5*02	CAAWNGRLSAFVF	λ	IGLV1-51*01	48	83.86	IGLJ1*01	
CCP2	1E7	5	CARGIGLDVICEGFDVW	48	IGHV4-30-4*01	83.45	IGHD3-10*01	IGHJ3*01	CQSFDSGLIF	λ	IGLV6-57*01	30	90.38	IGLJ2*01	
CCP2	1F2	5	CARGFSAEELCYGMDVW	50	IGHV4-30-4*04	82.76	IGHD2-15*01	IGHJ6*02	CQSYVDVSGLVF	λ	IGLV6-57*01	15	95.53	IGLJ2/J3*01	
CCP2	2E2	5	CARLQCSNGLCYLGGDTFDIW	29	IGHV4-34*01	89.82	IGHD2-8*01	IGHJ3*02	CQQYVAVSYSTF	κ	IGKV1-5*01	18	93.55	IGKJ1*01	
CCP2	1D10	5	CARGLGKTSLWGVDAFDVW	55	IGHV4-30-4*08	81.10	IGHD3-16*02	IGHJ3*01	CQQQSSSSSTIF	κ	IGKV1-39*01	43	85.66	IGKJ4*01	
CCP2	2F10	5	CARALGKPLVWGVDSFDVW	38	IGHV4-30-4*01	86.94	IGHD2-15*01	IGHJ3*01	CQQQSNSTLSLTF	κ	IGKV1-39*01	36	87.10	IGKJ4*01	
Ac-vimentin	2C4	6	CATRHHDDIWHGSSVFFDTW	57	IGHV4-39*01	80.76	IGHD3-16*01	IGHJ5*02	CQSADSEGLDILF	λ	IGLV3-25*03	54	80.65	IGLJ2/J3*01	
Ac-vimentin	2F5	6	CATRHYDDIRGRSSVFFETW	53	IGHV4-39*01	81.79	IGHD3-16*01	IGHJ5*02	CQSSDSTGTDILF	λ	IGLV3-25*03	44	84.23	IGLJ2/J3*01	

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

†Determined by IMGT/V-QUEST.

AMPA, anti-modified protein antibody.

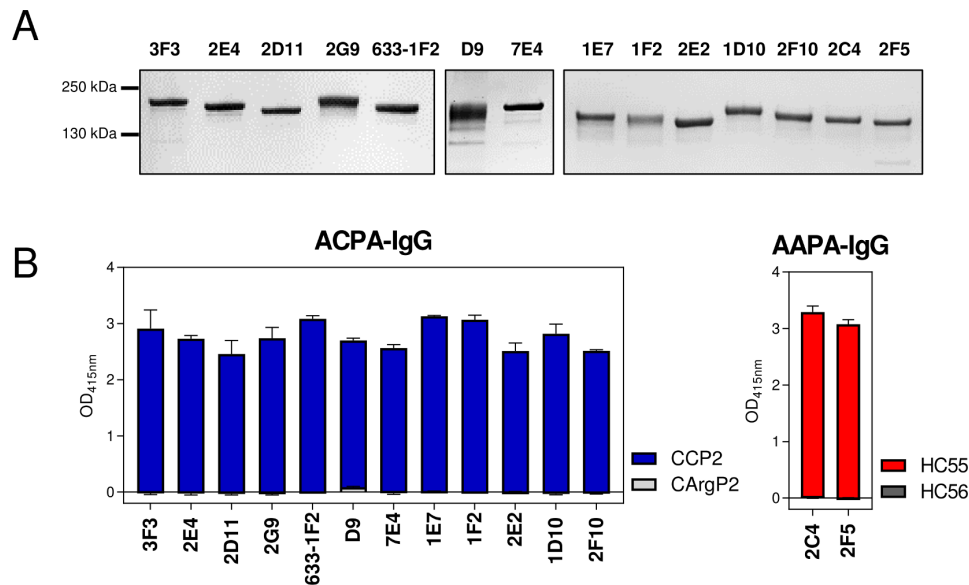


Figure 1 Production of 14 monoclonal AMPA-IgG. (A) SDS-PAGE of purified monoclonal AMPA-IgG using 4%–15% gradient protein gels (BioRad). The size was determined using the PageRuler Plus Prestained Protein Ladder (Thermo Fisher). Molecular weights are higher than 150 kDa and vary between monoclonals due 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 SE of three technical replicates. AAPA, anti-acetylated protein antibody; ACPA, anti-citrullinated protein antibody; AMPA, anti-modified protein antibody.

and C(C/Hcit/Ac)P4 as well as proteins, citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen. The cross-inhibition studies showed that the reactivity of 7E4 to CCP2 and CCP4 could be inhibited by the citrullinated-peptide itself and by its acetylated counterpart, while almost no inhibition could be observed after incubation with CHcitP2/CHcitP4 (figure 3A and online supplementary figure S5). Similarly, reactivity towards citrullinated-fibrinogen 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 (online supplementary figure S2a), binding of 7E4 to citrullinated-fibrinogen and acetylated-fibrinogen could be inhibited after preincubation 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 figure 3A,B, similar findings were made for 2G9 and 2C4 reaffirming the outcome of the reactivity patterns observed by the peptide-/protein-ELISAs (figures 2 and 3A,B).

Altogether, these data indicate that all ACPA and AAPA mAbs analysed cross-react to a varying extent to at least one other PTM and hence should be regarded as anti-modified protein antibodies (AMPA) 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 AMPA 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 patients with RA, 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-chain and light-chain sequences and the gene encoding for AID have been deleted (MDL-AID). This ‘triple KO’ cell line is unable to show BCR-signalling as it lacks an endogenous BCR. Moreover, it cannot modify a transduced BCR as it lacks AID. On 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 (online supplementary figure S3). 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 figure 4 and online supplementary figure S4, 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 ($25.25\% \pm 7.142\%$) and to stimulation with acetyllysine-containing peptides ($22.35\% \pm 7.990\%$). In contrast, the cells did not respond to stimulation with a homocitrulline-containing peptide ($0.9450\% \pm 0.8560\%$) (online supplementary 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-derived and 2G9-derived B cells, showing activation on stimulation with citrullinated peptides (3F3: $28.85\% \pm 2.475\%$; 2G9: $15.00\% \pm 4.950\%$) and with homocitrullinated peptides (3F3: $21.30\% \pm 2.828\%$; 2G9: $14.49\% \pm 6.944\%$). In line with our results obtained by ELISA, these cell lines did not respond to

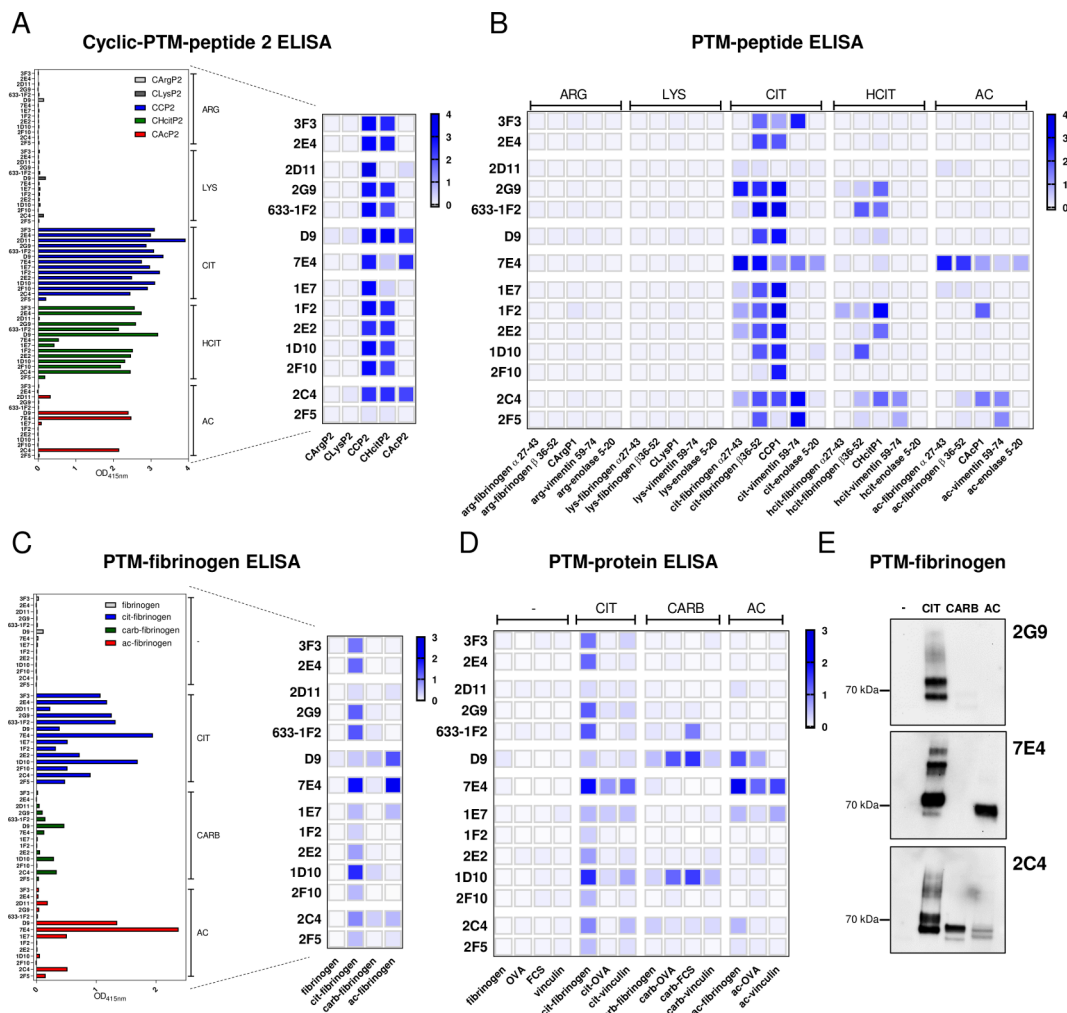


Figure 2 Cross-reactivity of monoclonal AMPA-IgG determined by ELISA and western blot analysis. (A) Bar graph and heatmap of a cyclic-PTM-peptide 2 (C(C/Hcit/AC)P2) ELISA of 14 monoclonal AMPA-IgG. Monoclonal AMPA-IgG reactivity towards the CCP2 (patent protected sequence) peptide in five modifications (citrulline, homocitrulline, acetyllysine, 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 analysed. (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 analysed. Reactivities were determined by the OD at 415 nm represented on the x-axis (bar graphs) or by colour (blue, high OD values, light grey, low OD values) within the heatmaps. Monoclonal AMPA-IgG were tested in a concentration of 10 μ g/mL. 2D11 was analysed in a concentration of 20 μ g/mL within the cyclic-PTM-peptide 2 ELISA. All ELISA experiments were repeated independently 2–3 times. (E) Western blot analysis of monoclonal AMPA-IgG 2G9, 7E4 and 2C4. Binding towards citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen and to the unmodified version (-) was analysed under reducing conditions (separately to the α , β and λ chain). Western blot analysis was repeated three times within independent experiments. AMPA, anti-modified protein antibody; FCS, fetal calf serum; OVA, ovalbumin; PTM, post-translational modification.

acetyllysine-containing peptides (3F3: 0.8250% \pm 0.2470%; 2G9: 0.0000% \pm 0.0000%) (online supplementary table S6, figure 4C). To expand the findings described above to the recognition of protein antigens, we next analysed the ability of the different modified forms of fibrinogen to stimulate the CP-reactive B cells. As shown in figures 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 (online supplementary figure S4). More importantly, and in agreement with the data presented in figure 2B, Ramos B cells transduced with 7E4 responded to citrullinated-fibrinogen and also displayed reactivity towards the acetylated counterpart (online supplementary 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.

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

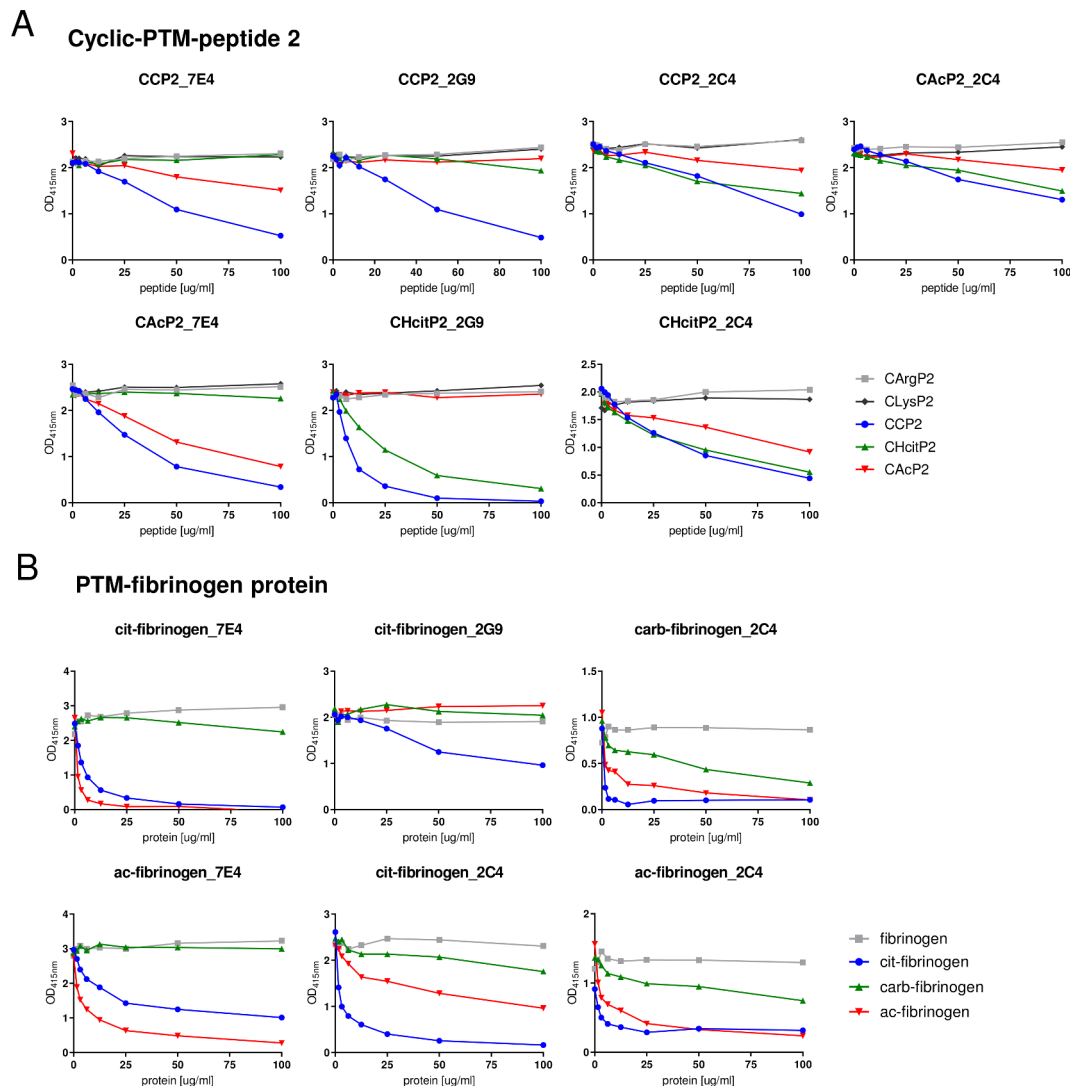


Figure 3 Cross-inhibition studies of monoclonal AMPA-IgG determined by ELISA. (A) Cross-inhibition ELISA with cyclic-PTM-peptide 2 as an inhibitor depicted for 7E4 using CCP2 as well as CACp2-coated plates, for 2G9 using CCP2 and CHcitP2-coated plates and for 2C4 using CCP2, CHcitP2 and CACp2-coated 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 for 7E4 for citrullinated-fibrinogen coated plates, of 2G9 for a citrullinated-fibrinogen coated plate and of 2C4 for citrullinated-fibrinogen coated, carbamylated-fibrinogen coated 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 (online supplementary figure S2). Binding is represented by the OD at 415 nm on the y-axis. Cross-inhibition studies were performed two times within independent experiments. Light grey octagon: arginine; dark grey diamond: lysine; blue circle: citrulline; red triangle: acetyl; green square: homocitrulline/carbamyl. AMPA, anti-modified protein antibody; CCP2, Cyclic-citrullinated-peptide 2; PTM, post-translational modification.

prominent genetic risk factors for RA (the HLA-SE-alleles), it is clear that autoantibody responses present in patients with RA 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, AMPA 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, for example, ACarPAs does not have to be carbamylated, but could be represented by an acetylated protein. We now show that human monoclonal ACPA and AAPA isolated from AMPA positive patients with RA (online supplementary figure S6) are highly cross-reactive towards various PTM-antigens (figure 2). Noteworthy, all ACPA-IgG and AAPA-IgG analysed were able to recognise at least two diverse modifications. This finding has general importance, as it indicates that ACPA, ACarPA and AAPA should be considered as AMPA that are not specific for one type of PTM. Furthermore, our results indicate that besides the affinity of the mAb towards a particular modification also the antigenic backbone and consequently the flanking regions around a modification can contribute

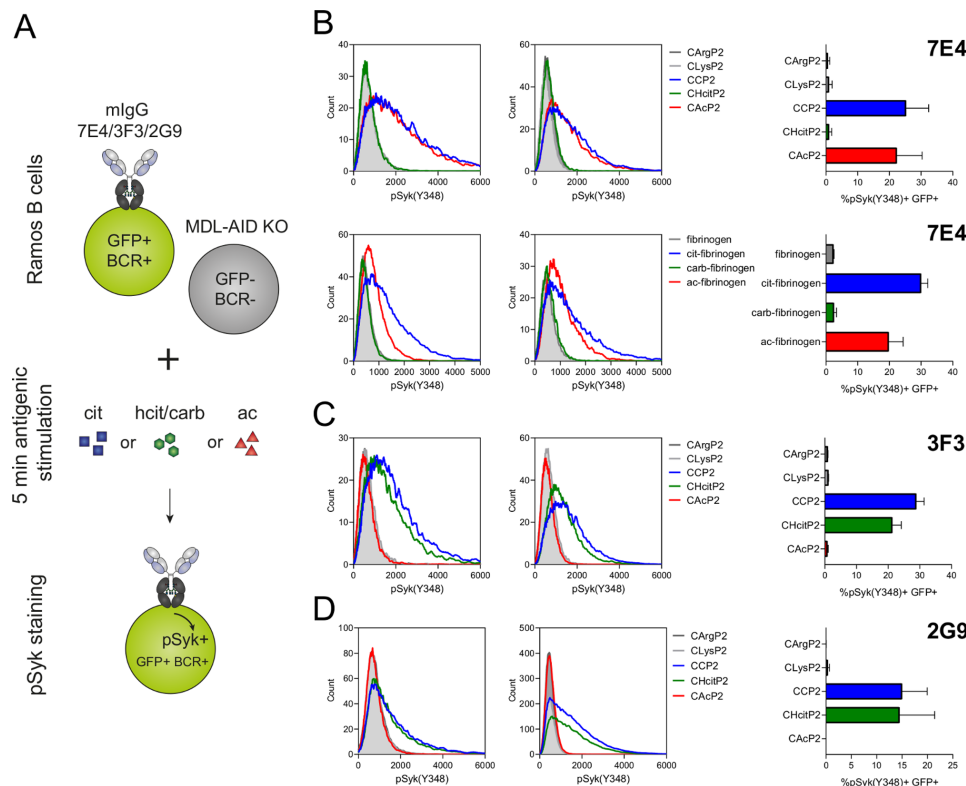


Figure 4 B-cell receptor signalling (pSyk expression) of CP-reactive BCR+GFP+ Ramos B-cell transfectants after stimulation with PTM-antigens. (A) Schematic depiction of the experimental activation assay design. GFP+mlgG-BCR+ Ramos B-cell transfectants and the untransfected GFP-BCR- control MDL-AID KO 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 mlgG 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 mlgG 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 mlgG Ramos B cells after stimulation with cyclic-PTM-peptide 2. The C-PTM-P2 sequences are patent protected. All activation assays were repeated 2–3 times within independent experiments. CArgP2: dark grey; CLysP2: light grey; CCP2: blue; CHcitP2: green; CAcP2: red. Unmodified fibrinogen: light grey; cit-fibrinogen: blue; carb-fibrinogen: green; ac-fibrinogen: red. AAPA, anti-acetylated protein antibody; ACarPA, anti-carbamylated protein antibody; ACPA, anti-citrullinated protein antibody; AID, activation-induced cytidin deaminase; BCR, B-cell receptor; CP, citrullinated proteins; GFP, green fluorescent protein; KO, knockout; PTM, post-translational modification.

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 (figure 3 and online supplementary figure S2). 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 with carb-fibrinogen, as this pattern is not consistent across different antibodies analysed. Nonetheless, it is clear that additional analyses are required to elucidate the potential contribution of flanking regions to the reactivity of AMPA 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 on stimulation.²⁵ 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 recognised 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 initiate an AMPA-response and determine the direction of it, conceivably dictating a progression towards ‘ACPA-dominated’, ‘ACarPA-dominated’ or ‘AAPA-dominated’ B-cell responses. It is tempting to hypothesise 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 patients with RA, can be explained, and the observation that in other patients the response can be dominated by one AMPA-response towards, for example, citrullinated, carbamylated or acetylated proteins. It would be interesting to investigate the extent of cross-reactivity in

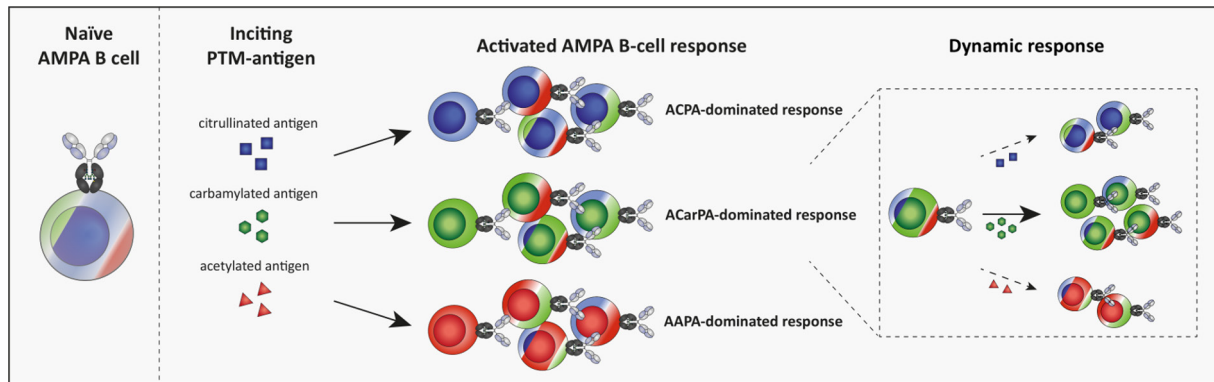


Figure 5 Schematic depiction of an hypothesis proposing the course of autoreactive AMPA B-cell responses. Naïve B cells expressing BCRs directed against PTM-antigens display reactivity towards citrullinated (blue), carbamylated (green) or acetylated (red) antigens. The inciting trigger could represent either a citrullinated, carbamylated or acetylated antigen. Dependent on this initial priming, the B cells are directed towards an 'ACPA-dominated', 'ACarPA-dominated' or 'AAPA-dominated' B-cell response. On 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). AAPA, anti-acetylated protein antibody; ACarPA, anti-carbamylated protein antibody; ACPA, anti-citrullinated protein antibody; BCR, B-cell receptor; PTM, post-translational modification.

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' categorisation 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 RA (figure 5).

Thus, our data disclose a strong relationship and high cross-reactivity between various autoantibodies and their B cells in patients with RA, 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 recognised in inflamed tissues.

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