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The Role of IgM Anti-acetylated Protein Antibodies and B Cells in the Origin of Antimodified Protein Autoimmunity in Rheumatoid Arthritis

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Objective. Rheumatoid arthritis (RA) is characterized by anti-modified protein antibodies (AMPAs), including anti-citrullinated protein antibodies (ACPA), anti-carbamylated protein antibodies (anti-CarP), and anti-acetylated protein antibodies (AAPA). In contrast to other AMPAs, AAPA IgM is found in healthy individuals, raising questions about its role in early immune responses. We investigated whether AAPA IgM serves as a precursor for other AMPAs, marking the initial breach of tolerance in RA.

Methods. AAPA IgM levels were measured in cord blood and serum of children up to three years old to assess whether it represents a natural (auto)antibody. To evaluate whether AAPA IgM presence precedes other AMPAs, we longitudinally measured AAPA and ACPA IgM in individuals before RA onset. To assess whether AAPA IgM–expressing B cells display a naive phenotype and carry germline-encoded B cell receptors (BCRs), single cells were sorted and sequenced.

Results. AAPA IgM was not detected in early life, but before RA onset, significantly more individuals were AAPA IgM positive (27.3%) compared to ACPA IgM positive (11.7%). Toward disease onset, ACPA IgM positivity increased to 51.2%, whereas AAPA IgM positivity remained stable. Furthermore, AMPAs developed in individuals who were AAPA IgM negative. Regarding B cell characteristics, germline-encoded BCRs were identified among both AAPA- and ACPA-expressing B cells in patients with RA.

Conclusion. AMPA responses in RA do not appear to arise solely from AAPA IgM, as suggested by the lack of association between predisease AMPA IgM positivity and later AMPA evolution. This is further supported by the presence of germline-configured ACPA BCRs, suggesting multiple possible starting points for AMPA responses.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by various autoantibodies. Among these, anti-modified protein antibodies (AMPAs) target post-translationally modified (PTM) proteins and include anti-citrullinated protein antibodies (ACPA), anti-acetylated protein antibodies (AAPA), and anti-carbamylated protein antibodies (Anti-CarP).[1] AMPAs can be detected years before RA onset and are associated with a more severe disease phenotype, suggesting they may contribute to RA pathogenesis.[2–5] As disease onset approaches, these responses typically undergo epitope spreading and isotype switching, suggesting a dynamic and evolving autoreactive B cell response during the preclinical phase of RA.[6,7]

Despite substantial efforts, the cellular origins and antigenic triggers of these autoantibodies remain unclear. Specifically, it is

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unknown whether germline-encoded B cells can evade tolerance mechanisms directly or whether accumulation of somatic hypermutations (SHMs) is required for them to escape regulation and acquire autoreactive properties.[8–10] Autoreactive B cells recognizing distinct PTM peptides have been identified in the peripheral blood of patients with RA, including both single PTM–specific and cross-reactive B cells.[11] This raises the question of whether such B cells arise from multiple independent clones, each specific for a different PTM, or from a single precursor that expands and diversifies. In support of a scenario involving a single precursor, immunization studies in mice have shown that exposure to either an acetylated or a carbamylated protein led to the development of AMPA responses directed against both PTMs.[12] These data indicate that one inciting trigger could lead to a promiscuous antibody response toward different PTMs. Indeed, cross-reactivity has been observed in both monoclonal and polyclonal AMPA IgG responses and is hypothesized to be driven by repeated antigen exposure and SHM.[13,14] However, whether SHM is a prerequisite for the development of promiscuous AMPA responses is debatable because germline-reverted AMPA IgM antibodies have been shown to not only retain anti-PTM reactivity but even exhibit some cross-reactivity.[15] This implies that naive B cells may initiate these responses before acquiring B cell receptor (BCR) mutations. However, to our knowledge, no fully germline AMPA B cells have been identified in patients until now. Determining whether autoreactive B cells arise at the naive stage or after SHM is important as it could provide clues on the timing of tolerance breakdown during B cell development and may guide therapeutic strategies aiming to prevent or halt RA by targeting B cell tolerance mechanisms early in disease development.

Important insights into the initial stages of AMPA development can also be gained by focusing on IgM antibodies, the first isotype produced during humoral immune responses. Recent investigation into AMPAs in serum revealed that although AMPA IgG and IgM levels are generally highly specific for RA, AAPA IgM is a striking exception. Unlike other AMPAs, AAPA IgM is also present in healthy individuals and patients with other forms of arthritis at levels comparable to those in patients with RA, suggesting it may reflect a broader, nonpathogenic immune response.[16] This raises the question whether AAPA IgM represents an early, physiologic stage of the AMPA response, potentially serving as a precursor to other RA-specific autoantibodies.

To investigate this hypothesis, we sought to better understand the origins of AAPA responses both in terms of B cell development and antibody maturation. Using multiple cohorts ranging from birth to the early years of RA, we examined whether AAPA IgM precedes other AMPAs and whether it is required for their consecutive development. Additionally, we sorted AAPA-expressing B cells from blood and sequenced their BCRs to determine whether these B cells exhibit a BCR profile with limited SHM, which could indicate an early stage of B cell activation.[8] Together, our findings aim to provide a more comprehensive understanding of AAPA responses and offer new insight into how AMPA-associated autoimmunity may develop in RA.

METHODS

Study cohorts. AMPA responses were analyzed in serum samples from multiple cohorts spanning early life up to the initial years following RA diagnosis. Informed consent was provided from all individuals and/or guardians before participation in this research.

Cord blood cohorts. Cord blood and paired maternal samples (when available) were obtained from three distinct Leiden University Medical Center (LUMC) cohorts: one consisting of full term fetal and paired maternal samples (cohort 1), a recurrent miscarriage (REMI) cohort including full-term infants and their mothers (cohort 2), and a third cohort comprising of fetal samples from full-term pregnancies only (cohort 3). Mothers had no prior medical history related to rheumatic diseases. Approval was given by the medical-ethical committee in the LUMC (P10.009 for cohorts 1 and 2 and P11.196 for cohort 3).

Young children cohort. Serum samples from children up to three years old were obtained from LUMC biobanks (approval ID BB24.025 and BB24.026), including samples from children with autoimmune conditions (such as celiac disease and inflammatory bowel disease) and neuromuscular disorders.

Presymptomatic and early RA cohort. This cohort included presymptomatic individuals identified through analysis of the Medical Biobank and Maternity cohort registers in Umeå, Sweden. Individuals who were later classified as RA patients, as previously described, after presenting at the Department of Rheumatology at Umeå University Hospital were matched to their earlier biobank donations (Dnr 2013-347-31).[7] These individuals were categorized in two groups: group 1, with samples available only from the presymptomatic phase, and group 2, with longitudinal samples spanning both the presymptomatic and RA phases.

Early Arthritis Clinic cohort. The Early Arthritis Clinic (EAC) cohort from the LUMC includes patients with arthritis with a symptom duration of less than two years.[17] For this study, both patients with RA and patients with other forms of arthritis were selected if longitudinal samples of up to five years follow-up were available. The EAC was approved by the Medical Ethics Committee of the LUMC (B19.008).[17]

Healthy donors. Serum was collected from healthy volunteers participating in the LUMC Voluntary Donor Service (LuVDS), which is coordinated by the biobank facility of the LUMC. Approval was obtained from the institutional review board, and all donors provided consent.

Fresh material. Peripheral blood was obtained from patients with RA recruited from the outpatient clinic of the Department of Rheumatology at the LUMC. All patients met the 2010 ACR/EULAR criteria for RA and provided written informed consent (protocol P17.151). Fresh material from healthy donors (HDs) was either obtained from peripheral blood of LuVDS donors or buffy coats from the Dutch blood bank (Sanquin).

Enzyme-linked immunosorbent assays. *Total Ig enzyme-linked immunosorbent assays.* Total IgM in serum was measured in plates coated with anti-human IgM Fc, followed by blocking with phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 50 mM Tris. Serum diluted in PBS, 1% BSA, 0.05% Tween, and 50 mM Tris was then added, and after incubation with a goat anti-human IgM–horseradish peroxidase (HRP)–conjugated antibody, samples were visualized using ABTS at 415 nm. Total IgM levels were determined using a commercially available normal human serum (Merck Millipore) as reference standard with a stock concentration of 0.1 mg/mL IgM. Serial dilutions were prepared to generate a standard curve, and IgM concentrations in samples were calculated by interpolation of optical density (OD) values. For total Ig measurements in culture supernatants, plates were coated with a goat anti-human IgG +IgM+IgA heavy and light chain antibody, and samples were detected with a goat anti-human IgG+IgM+IgA heavy and light chain-HRP–conjugated antibody. All antibodies were purchased from Bethyl Laboratories, unless stated otherwise.

Anti–double stranded DNA IgM enzyme-linked immunosorbent assays. Plates (Invitrogen) were coated with UltraPure Salmon Sperm DNA Solution (Thermo Fisher Scientific) and blocked with PBS, 1% BSA, and 50 mM Tris. After washing with PBS and 0.5% Tween, serum samples were added and incubated for one hour at room temperature. Bound antibodies were detected using HRP-conjugated goat anti-human IgM (Millipore) and visualized with ABTS substrate. Antibody levels were quantified in arbitrary units per milliliter (AU/mL) by interpolation against a standard curve generated from pooled human sera. Background signals were subtracted from all samples, and samples with ODs above the upper limit of detection (defined by the linear range of the standard curve) were assigned a maximum quantifiable value.

AMPA Ig enzyme-linked immunosorbent assays. Serum samples, monoclonal antibodies (mAbs), or supernatants from bulk peripheral blood mononuclear cells (PBMCs) and B cell cultures were assessed for AMPA levels as previously described.[12,13,16] Assay details are provided in the Supplemental Methods.

AMPA Ig cross-linking enzyme-linked immunosorbent assay. For the supernatants from single B cell sorts in which AMPA enzyme-linked immunosorbent assays (ELISAs) were inconclusive due to low OD values (<0.5; Supplemental Figure S1), we employed a cross-linking ELISA to enhance the sensitivity of AMPA Ig detection. Samples were preincubated with goat anti-human IgG+IgM+IgA heavy and light chain–HRP antibody (Bethyl Laboratories) for one hour to facilitate immune complex formation. This preincubation step promotes the formation of multivalent primary-to-secondary antibody complexes, thus increasing functional avidity for plate-bound antigen and amplifying the detection signal. The immune complexes were subsequently applied to streptavidin-coated plates (Invitrogen) that had been preloaded with either CAcP2/4, CHcitP2/4, CCP2/4, or the unmodified CLysP2/4 and CArgP2/4 peptides. Cyclic modified peptides of second and fourth generation have been previously shown to yield similar results, indicating that they can be used interchangeably.[18] Purified AMPA mAbs 7E4 and 2G9 were used as positive controls, whereas a purified mAb against tetanus toxoid was used as a negative control.[13] Detection of bound AMPA and secondary antibody immune complexes was conducted using the ABTS substrate at 415 nm. The OD values obtained from the unmodified peptide were subtracted from those of the modified peptide to calculate the Delta (Δ) OD value.

Calculation of cut-off and data analysis. Serum AMPA positivity was determined by calculating the average AU/mL plus two times the SD of the anti-PTM–specific signal from 30 LuVDS donors. In the case of the commercial anti-CCP2 enzyme immunoassay, the recommended cut-off of 25 AU/mL was used.[7] Data analysis was performed using GraphPad Prism and R Studio, with linear mixed model analysis conducted in R Studio.

Human sample processing and analysis. Details of human sample processing, single-cell sorting, cell cultures, and BCR sequencing analyses are described in the Supplemental Methods.

RESULTS

Emergence of an AAPA IgM response is not an early life event. Because AAPA IgM has been previously detected in healthy individuals and in patients with various forms of arthritis at levels similar to those found in patients with RA, we wondered whether AAPA IgM might be part of natural antibody responses.[16] Natural antibodies are present in the body without prior exposure to a specific pathogen or antigen, and they are even found in newborns.[19–21] To explore this hypothesis, we examined three different cohorts of cord blood sera, two of which also included paired maternal sera. We detected AAPA IgM in maternal samples (in line with earlier findings in HDs) but not in cord blood (Figure 1A–1C).[16] However, total IgM levels in cord blood were markedly lower than in maternal serum, which likely limited our ability to detect AAPA IgM (Supplemental Figure S2A–S2C). As a positive control, we also measured anti–double-stranded DNA (dsDNA) IgM, a previously described natural antibody,[21] which was likewise barely detectable in cord blood, further supporting

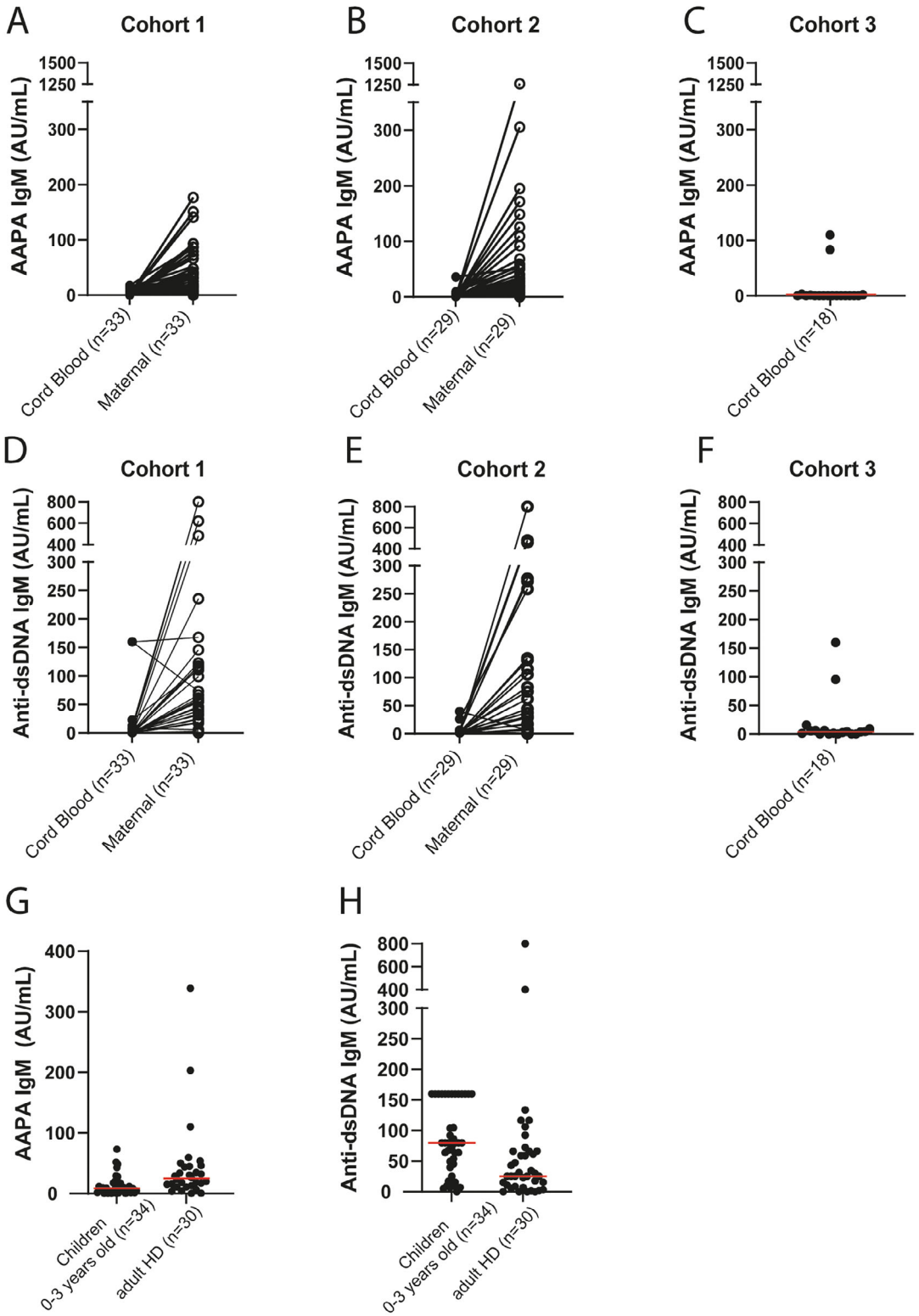

Figure 1. AAPA IgM levels in early life. Serum measurements of AAPA IgM in (A) cord blood and paired maternal samples from full-term pregnancies (cohort 1), (B) cord blood and paired maternal samples from a recurrent miscarriage cohort (cohort 2), and (C) additional cord blood samples from full-term pregnancies (cohort 3). Serum measurements of anti-dsDNA IgM in (D) cohort 1, (E) cohort 2, and (F) cohort 3. (G) AAPA IgM and (H) anti-dsDNA IgM levels in samples from children aged up to three years old and adult HDs as controls. To account for lower total IgM levels in cord blood, these samples were diluted 1:10, whereas maternal samples were diluted 1:50. Dilution factors were incorporated during interpolation of optical density values into AU/mL. Levels are shown as AU/mL, and the median AAPA IgM or anti-dsDNA IgM value for nonpaired samples is shown in red. AAPA, anti-acetylated protein antibody; AU/mL, arbitrary unit per mL; dsDNA, double stranded DNA; HD, healthy donor.

the interpretation that low total IgM levels limit detection of antigen-specific IgM in neonatal samples (Figure 1D–1F).

Because other studies have shown that total IgM levels and subsequently natural antibody IgM levels rise within the first years of life, we sought to investigate whether AAPA IgM could be detected within the first years after birth.[22,23] Serum from children up to three years old revealed very low AAPA IgM signals (Figure 1G), despite total IgM levels that were comparable to healthy adults (Supplemental Figure S2D). In contrast, anti-dsDNA IgM was readily detectable in many of the children's samples, often at levels higher than those observed in healthy adult controls (Figure 1H). Based on these findings, we conclude that emergence of AAPA IgM does not appear to be an early life event, as has been described for other natural antibodies, but likely occurs later in life.

AAPA IgM is present before RA symptom onset but is not required for a broader AMPA response. Because it still remains unclear when AAPA IgM first emerges, we sought to determine how long before RA onset this response becomes detectable and how its serological profile evolves over time. To address this, we analyzed longitudinal sera from individuals in a presymptomatic RA cohort, stratifying them into two groups based on sample availability. Group 1 included individuals with two time points during the asymptomatic preclinical phase (some of whom later developed RA), whereas group 2 included individuals with samples extending up to the time of RA symptom onset. Our first aim was to investigate if AAPA IgM appears earlier than ACPA IgM because such an analysis could support the hypothesis that AAPA IgM represents a starting point for subsequent AMPA responses. At the earliest available time point (T1), AAPA IgM antibodies were indeed significantly more prevalent (27.3%) than ACPA IgM antibodies (11.7%) (Figure 2A), with AAPA IgM being detectable up to 30 years before symptom onset (Supplemental Figure S3A). Whereas the proportion of patients positive for AAPA IgM remained unchanged with RA onset (27.9%), ACPA IgM positivity increased to 51.2% upon symptom onset (Figure 2A).

To further evaluate how AMPA responses evolve toward symptom onset, we next examined the breadth of AMPA reactivity over time in this cohort. Specifically, we aimed to evaluate whether AAPA responses begin to include IgG before or upon RA onset and whether the presence of AAPA IgM is required for the emergence of other AMPAs. As expected, the overall AMPA recognition profile diversified in the preclinical phase, with the broadest reactivity profiles observed closest to (T2 in Figure 2B) or upon RA diagnosis (RA in Figure 2C; Supplemental Figure S3A). As a validation of the dynamic behavior of AMPA isotypes in this cohort, we observed a significant positive correlation between ACPA IgM and ACPA IgG levels (Supplemental Figure S3B) at the symptomatic phase (RA), consistent with ongoing maturation and class-switching of the ACPA response. This confirms that biologically meaningful relationships between isotypes can be detected in our data set. However, individuals who developed ACPA responses did not consistently show preceding AAPA IgM, suggesting that AAPA IgM is not a prerequisite for the generation of ACPA responses and AAPA IgM positivity does not result in higher ACPA levels (Figure 2B and 2C).

By stratifying individuals as AAPA IgM positive or negative at T1, we assessed whether the AMPA reactivity profile differed at the latest time point available (T2 or RA) when most AMPAs could be detected. As shown in Figures 2B through 2E, early AAPA IgM positivity is not required for the formation of other AMPAs. Although the group that was AAPA IgM negative at T1 did contain more AMPA-negative individuals at T2, the majority of individuals in this group developed a diverse AMPA response (Figure 2E). These results suggest that although AAPA IgM emerges earlier, it does not appear to be required for the development of other AMPA responses, indicating that it is unlikely to represent the sole initiating event of the AMPA response.

AAPA IgM is not transient in early RA. Given that AAPA IgM does not appear to be an essential initiating event in AMPA development and considering its presence in healthy individuals, we next asked whether this antibody response represents a spontaneously occurring, transient phenomenon or whether it persists into established disease, as has been previously described for ACPAs.[24–26] Due to the absence of a longitudinal collection of sera from healthy individuals, we could not evaluate the natural course of AAPA IgM in that setting. However, in patients with RA, we were able to examine whether AAPA IgM positivity remained stable over five years postdiagnosis in patients with early RA from the EAC cohort. Patients with RA were stratified by baseline AAPA IgM status, and follow-up sera were tested at two additional time points. Among those individuals who were AAPA IgM positive at baseline, some showed an increase in antibody levels, but the overall trend was a decrease over time (Figure 3A). Similarly, among individuals who were AAPA IgM negative at baseline, some displayed weak positive signals during follow-up according to the cut-off values used; however, ultimately only one patient seroconverted over time (Figure 3B). Patients with other forms of arthritis (non-RA) exhibited a similar trend, with AAPA IgM levels either decreasing or remaining stable compared to baseline values (Figure 3C and 3D). Overall, although AAPA IgM levels show some fluctuations such as titer decrease over time, they remain detectable in most AAPA IgM–positive individuals at baseline over time, indicating that the AAPA IgM response is not transient but rather a persistent response despite quantitative changes.

AAPA-expressing B cells in peripheral blood are rare and can express both germline and mutated BCRs. Based on our serological data, AAPA IgM responses can be detected in healthy individuals but, unlike ACPA IgM responses

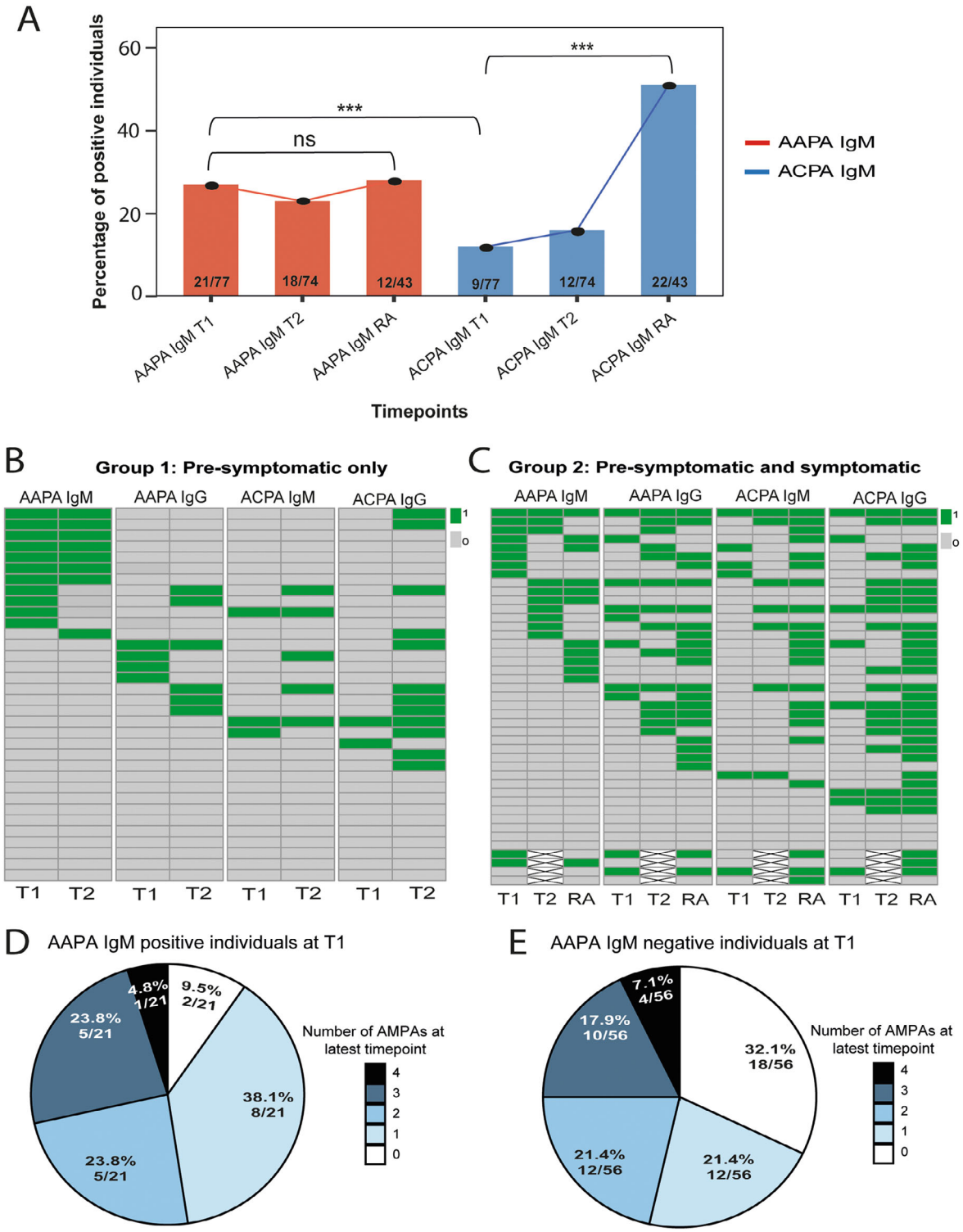

Figure 2. Longitudinal analysis of AAPA and ACPA IgM/G positivity in pre-RA individuals. (A) Percentage of individuals positive for AAPA and ACPA IgM at each time point. The proportions of AAPA IgM and ACPA IgM positivity at T1 were compared using a McNemar's test (***$P \leq 0.001$). The longitudinal change in autoantibody positivity over time was assessed using a linear mixed-effects model (***$P \leq 0.001$), yielding regression estimates of 0.1129 for AAPA IgM and 2.7637 for ACPA IgM. (B) Heatmap displaying AAPA and ACPA IgM/G positivity in presymptomatic-only (group 1) individuals and (C) symptomatic (group 2) individuals. White marked rectangles indicate missing samples, and positive time points are marked in green and negative in gray. (D) Pie chart illustrating the number of different AMPAs (AAPA and ACPA IgM/G) detected at the last available time point (T2 or RA) in individuals positive or (E) negative for AAPA IgM at the earliest (T1) time point. AAPA, anti-acetylated protein antibody; ACPA, anti-citrullinated protein antibody; AMPA, antimodified protein antibody; RA, rheumatoid arthritis; T1, time point 1 (asymptomatic); T2, time point 2 (asymptomatic).

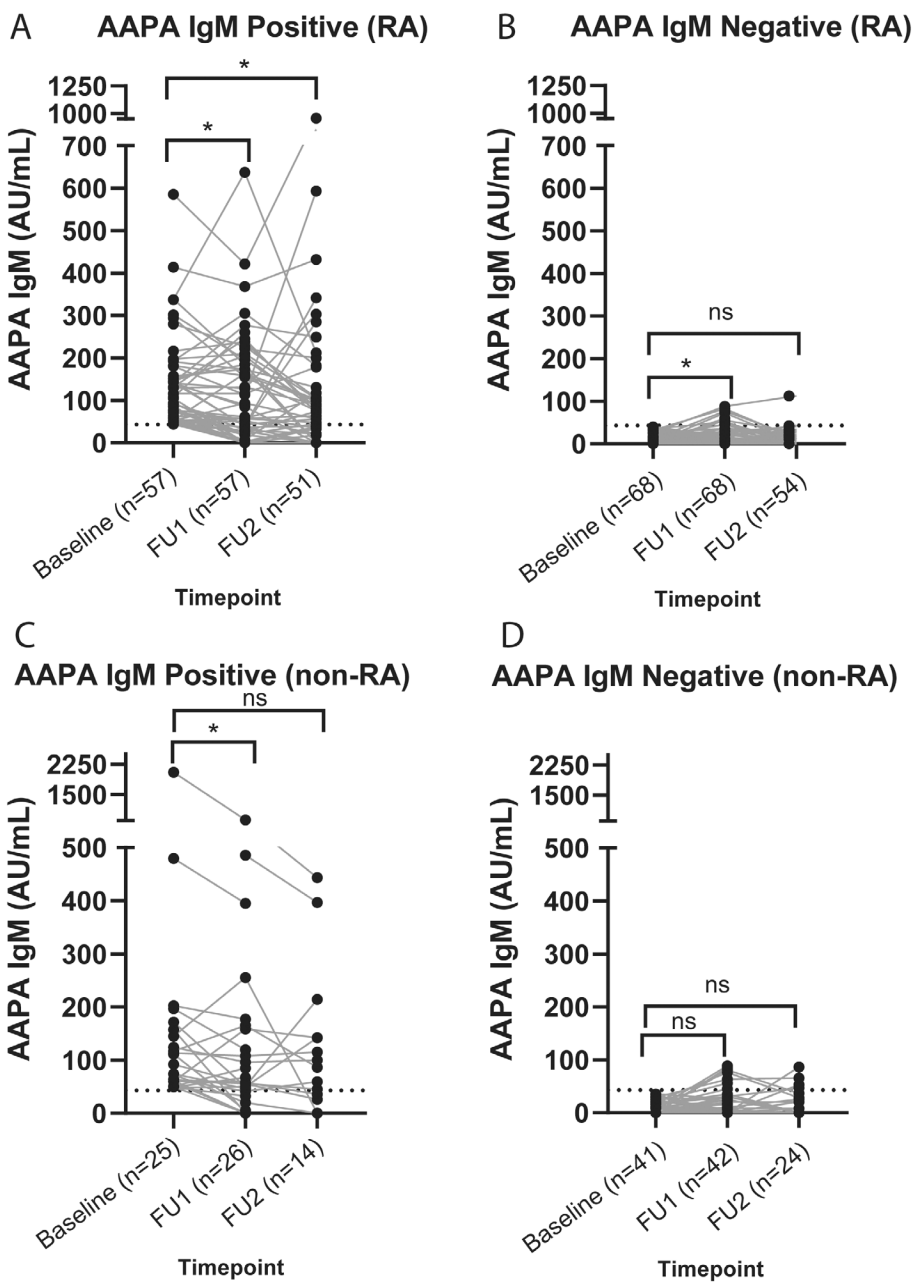

Figure 3. Longitudinal AAPA IgM levels up to five years after diagnosis. AAPA IgM levels in patients with early RA who were AAPA IgM (A) positive and (B) negative at baseline, across baseline, and two follow-up time points. AAPA IgM levels in patients with other forms of arthritis (non-RA) who were AAPA IgM (C) positive and (D) negative at baseline, across baseline, and two follow-up time points. The cut-off is shown with a dashed line. A Wilcoxon signed-rank test was used to compare AAPA IgM levels at follow-up time points to baseline. *$P \leq 0.05$. AAPA, anti-acetylated protein antibody; AU/mL, arbitrary units per mL; FU, follow-up; ns, not significant; RA, rheumatoid arthritis.

in the predisease phase, this does not correlate with disease progression. To determine if this difference extends to the cellular level, we aimed to identify and characterize AAPA-expressing B cells in patients with RA and healthy individuals. Specifically, we investigated whether these B cells display limited SHM, suggesting a T cell–independent origin, and are embedded in the normal B cell repertoire. We sorted single AAPA-reactive B cells from the blood of nine patients with RA and three HDs using acetylated (CAcP4)– and unmodified (CLysP4)–coupled tetramers (Supplemental Figure S4; Supplemental Table S1). After single-cell culture, AAPA production was confirmed by ELISA (Figure 4A). Although total Ig production indicated substantial single-cell outgrowth in the majority of cultures (Supplemental Figure S5; Supplemental Table S2), the frequency of AAPA-producing B cells was very low, with only four out of nine patients with RA (RA 1, 4, 7, and 9) and one of three HDs exhibiting substantial AAPA specificity ($\Delta OD > 0.5$) in at least one well (Figure 4B). In addition, despite total Ig levels being above

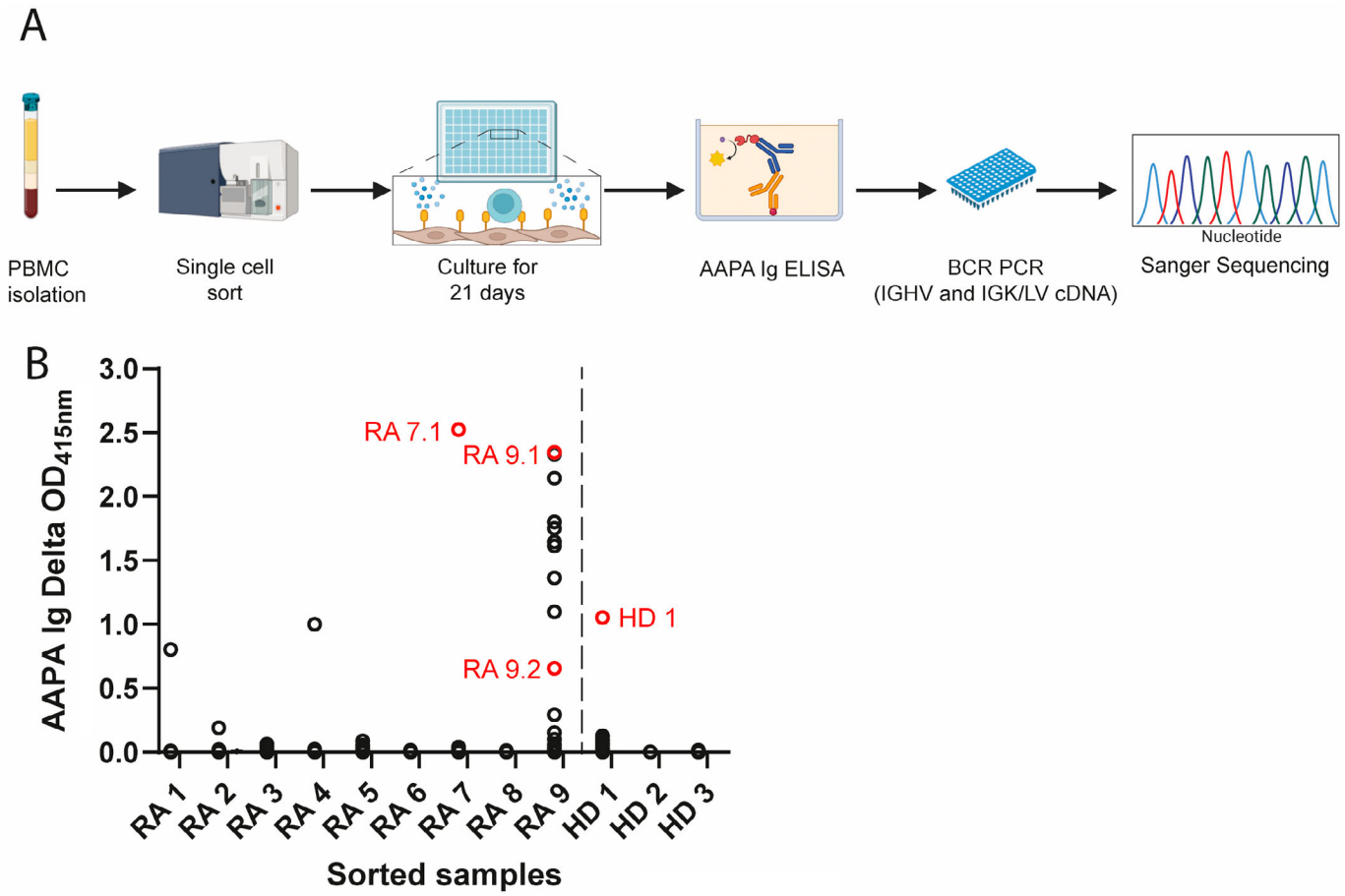

Figure 4. AAPA-expressing B cell single-cell sorts. (A) Schematic overview of the experimental workflow for analyzing AAPA-expressing BCRs, created using Biorender. PBMCs were isolated from blood samples, after which AAPA-expressing B cells were single-cell sorted and cultured for 21 days. Culture supernatants were then tested for AAPA Ig reactivity using an ELISA, and finally AAPA-expressing BCRs were sequenced. (B) Dot plots showing the ΔOD values of AAPA Ig detected by cross-linking AAPA ELISA in the supernatants from individual single-cell cultures. All sorted cells are shown, and events with identical signals overlap in the plot. Samples on which BCR sequencing was performed are shown in red. Sample IDs are labeled as RA/HDx.y, where "x" represents the individual patient and "y" denotes distinct B cell clones derived from that patient. AAPA, anti-acetylated protein antibody; BCR, B cell receptor; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; HD, healthy donor; OD, optical density; IGHV, Ig heavy chain variable region; IGK/LV, Ig κ/λ light chain variable region; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RA, rheumatoid arthritis.

background, the quantity of AAPA produced may still have been too low to be detected by ELISA, suggesting that not only the frequency of AAPA-producing B cells but also the amount of antibody produced per cell may be limited in these cultures.

In total, 16 AAPA-expressing B cells were identified after stimulation of sorted single cells across all patients with RA as well as a single cell in a HD. To determine whether this relatively low detection rate reflects a true low frequency or is the consequence of methodologic limitations, we shifted to bulk cultures to improve detection of circulating AAPA-expressing B cells. We analyzed AAPA and ACPA IgM and IgG production in total PBMC population, as well as enriched B cells, assessing both stimulation-induced and spontaneous antibody production in patients with RA and HDs. Spontaneous production in this context would reflect antibody secretion by pre-existing circulating plasmablasts, whereas stimulation-induced production would reflect antibody secretion following activation-driven differentiation of B cells in culture. Despite robust B cell outgrowth, as indicated by total Ig production (Supplemental Figure S6), AAPA IgM levels remained very low, confirming that AAPA IgM B cells are present at low frequencies (Figure 5A). Detectable AAPA IgM production (ΔOD > 0.1) from B cell populations was observed in one patient with RA (RA 4) and two HDs (HD 4 and 6) only after stimulation of B cells in vitro. Given that AAPA IgM was also undetectable in donor plasma, these findings suggest that AAPA IgM B cells in these donors are not part of the activated B cell pool (Figure 5A). Similarly, ACPA IgM production was minimal (Figure 5B). In contrast, AAPA IgG and ACPA IgG production were substantially higher, particularly in patients with RA, in both stimulated PBMC and B cell cultures (Figure 5C and 5D). Collectively, these findings confirm that AMPA IgM–expressing B cells can be present in low frequencies in the peripheral blood of both

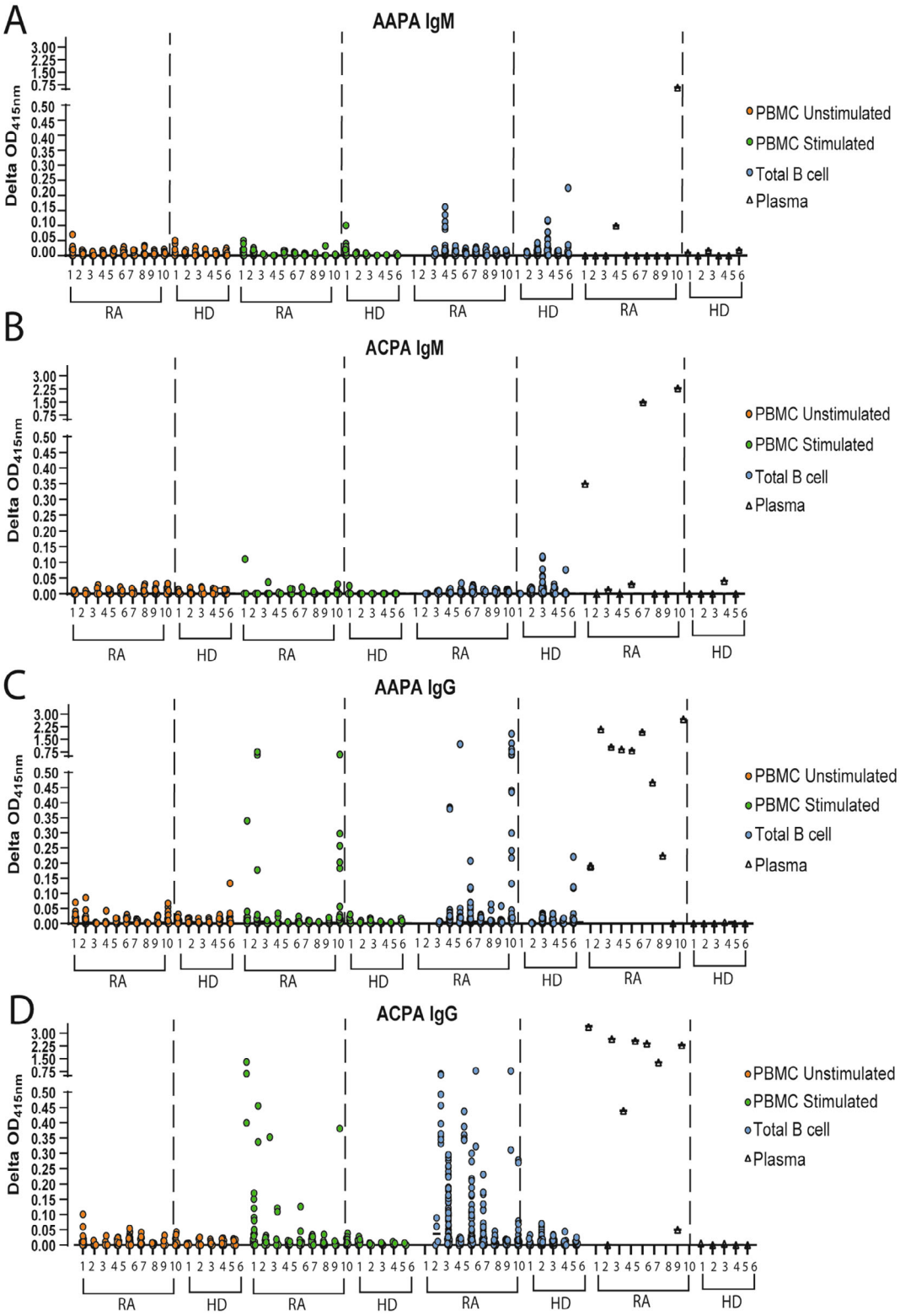

Figure 5. AMPA Ig production in patients with RA and HD cell cultures. Dot plots representing (A) AAPA IgM, (B) ACPA IgM, (C) AAPA IgG, and (D) ACPA IgG production in PBMCs or total B cell cultures. Cells were isolated from patients with RA and HDs, separated by dashed lines in the dot plots. PBMCs were cultured either in medium alone (orange) or with cytokines (green), whereas total B cells were cultured with cytokines (blue). Supernatant was collected at day 10 and tested on AAPA/ACPA IgM/G enzyme-linked immunosorbent assays. Matched plasma samples collected from peripheral blood during PBMC isolation were also analyzed (triangles). Results are presented as ΔOD values where each dot represents one well. AAPA, anti-acetylated protein antibody; ACPA, anti-citrullinated protein antibody; HD, healthy donor; OD, optical density; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis.

patients with RA and HDs, as indicated by both single-cell and bulk culture approaches.

Next, we performed BCR sequencing on four AAPA IgM B cells that produced AAPA IgM in the single-cell cultures (three from patients with RA and one from a HD) (Figure 4B). Specifically, from the AAPA-reactive wells identified by ELISA, we first excluded AAPA IgG–expressing cells (n = 3) identified by retrospective indexing. Of the remaining AAPA IgM–positive wells, four contained sufficient BCR material of adequate quality for sequencing. Clones RA 9.1, RA 9.2, and HD 1 harbored limited Ig heavy variable region mutations (Supplemental Table S3), consistent both with the typical mutation range reported for IgM BCRs and with the few previously described ACPA and AAPA IgM sequences.[15,27,28] Notably, a fully germline-configured IgD+ AAPA BCR (RA 7.1), lacking variable heavy or light chain mutations was identified in peripheral blood (Supplemental Table S3). This directly demonstrates that AAPA IgM BCRs can exist in both unmutated and minimally somatically mutated forms, highlighting the presence of autoreactive germline precursors in the peripheral repertoire.

Using a similar approach, ACPA-expressing B cells were sorted with CCP2- and CArgP2-coupled tetramers to investigate whether unmutated ACPA BCRs also exist in peripheral blood (Supplemental Figure S7A). Identifying such BCRs, as shown above for AAPA, would indicate that AMPA responses may originate through distinct developmental pathways, including those that do not rely on SHM. Because ACPA B cells often carry somatic mutations, we used retrospective index sort analysis to specifically focus on IgD+ cells because these are the most likely to express unmutated or germline-like BCRs (Supplemental Figure S7B). Indeed, three CCP2-reactive ACPA B cells were identified (RA 10, RA 11.1, and RA 11.2; Supplemental Table S3). Their reactivity was validated by expressing the BCRs as mAbs, further confirming CCP2 binding by (cross-linking) ELISA (Figure 6A and 6B).

We next investigated whether these antibodies were directed against only one PTM or were cross-reactive to multiple PTMs. Culture supernatant from the unmutated AAPA IgM clone RA 7.1 was tested against different PTMs, using previously characterized mAbs as positive and negative controls (Supplemental Figure S8).[13] RA 7.1 exhibited cross-reactivity to homocitrulline (HCit) but showed no reactivity toward citrulline (Figure 6B). Among the three germline-encoded ACPA B cells identified, two clones (RA 10 and RA 11.1) also displayed cross-reactivity with HCit but did not recognize acetyl modifications, whereas clone RA 11.2 was specific for citrulline only (Figure 6A and 6B).

Together, our data indicate that both AAPA and ACPA BCRs can exist in a germline configuration, indicating that both anti-acetylated and anti-citrullinated protein reactivity could potentially serve as the starting point for AMPA responses in RA. In addition, we show that such germline-configured B cells can exhibit cross-reactivity to other PTMs, such as HCit, offering valuable insights into the origin of promiscuity of AMPAs in RA.

DISCUSSION

The data presented in this manuscript provide new insight into the origins of AMPA responses, showing that AAPA IgM does not emerge during early life as has been described for natural antibodies, yet they can be detected years before RA symptom onset. Notably, although AAPA IgM often appears before ACPA IgM, the two responses differ markedly: ACPA IgM prevalence closely mirrors disease maturation, as it increases toward symptom onset, whereas AAPA IgM remains stable over time and does not reflect disease progression. In presymptomatic individuals, AAPA IgM and IgG appear to be largely mutually exclusive, whereas this pattern was less evident upon disease onset (Figure 2B and 2C). This may reflect temporal maturation of the AAPA response, with increased class-switching and co-occurrence of isotypes closer to clinical onset. Such dynamics were not observed for ACPA IgM and IgG, further underscoring fundamental differences in the developmental trajectories of AAPA and ACPA responses. Furthermore, we provide evidence that AAPA IgM does not appear to be an obligatory precursor for the development of other AMPAs because these readily emerge in individuals pre-RA, independent of AAPA IgM status.

Natural antibodies, typically low-affinity, polyreactive IgM, can be present from birth and can be detected in cord blood and early childhood, recognizing self- and microbial structures without being associated with overt autoimmunity.[21] In contrast, we did not detect AAPA IgM in cord blood samples or children up to three years old (Figure 1), suggesting that AAPA IgM does not belong to this constitutive natural antibody pool but instead reflects an acquired, antigen-driven response. Supporting this interpretation, anti-dsDNA IgM (a typical natural antibody) was readily detectable in young children and even displayed higher median levels than in healthy adults. This pattern has been previously observed for some natural IgM specificities, likely reflecting repertoire evolution and skewing.[21]

Although the exact timing and antigenic trigger of AAPA IgM formation remain unknown, we hypothesize that this response arises later in life, potentially during adulthood and years before symptom onset, following repeated exposure to acetylated environmental antigens. A recent study demonstrated that acetylated bacterial proteins can be recognized by human AMPAs and by AMPA-expressing B cells, suggesting that bacterial antigens may serve as potential triggers for the AAPA response.[29] These findings support the hypothesis that AAPA IgM may arise in response to environmental antigens, particularly at mucosal sites. Although the previous study also demonstrated that immunization of mice with bacterial acetylated proteins can induce cross-reactive AMPA responses, our findings suggest that acetylated antigens are unlikely to be the sole source responsible for the

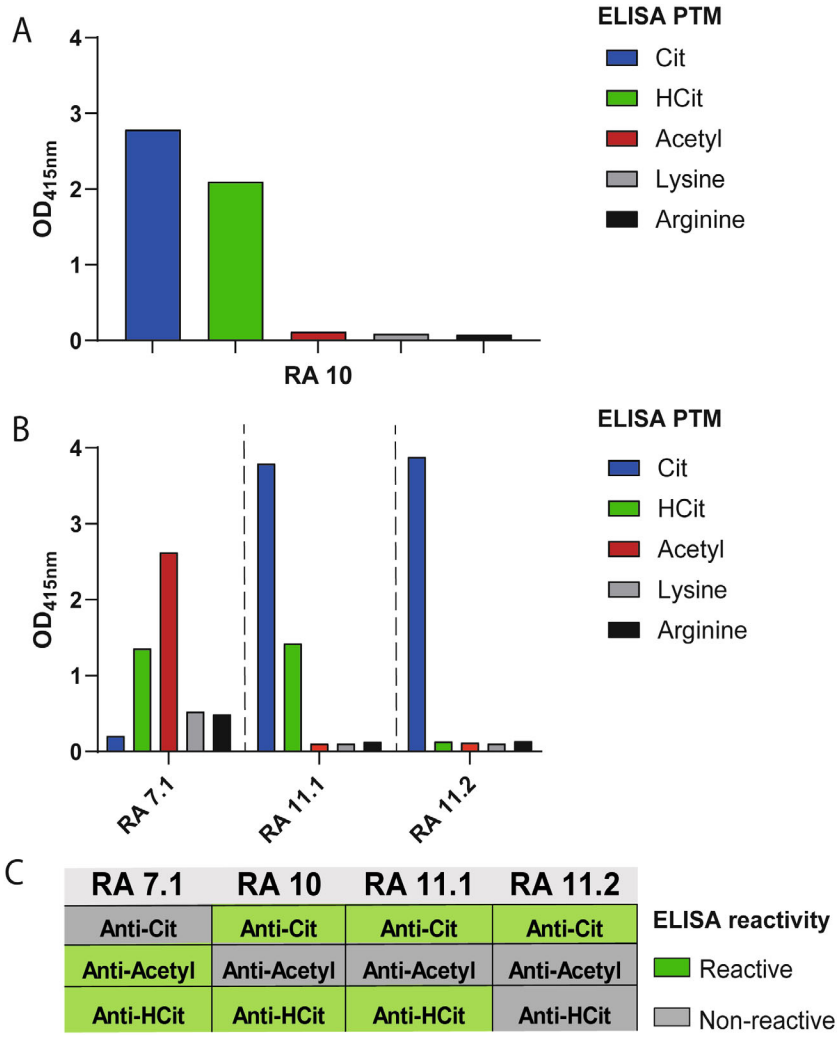

Figure 6. Single PTM reactivity and cross-reactivity of germline-configured antimodified protein antibody B cells. (A) ELISA of supernatant of HEK293-F cells transfected with the sequence of clone RA 10, demonstrating anti-PTM reactivity using second-generation cyclic modified peptides (CXP2). (B) Cross-linking ELISA of culture supernatant from clone RA 7.1 and supernatants from HEK293-F cells transfected with clones RA 11.1 and RA 11.2, tested for reactivity against PTM peptides. Each clone was assessed using peptides corresponding to the peptide generation applied during sorting (CXP4 for anti-acetylated protein antibody clone RA 7.1 and CXP2 for anti-citrullinated protein antibody clones RA 11.1 and RA 11.2). Each clone is separated by a dashed line. (C) Summary table of all germline-encoded B cell receptor clones, showing reactivity against individual PTM peptides. Reactivity was determined by comparing OD values for modified versus unmodified peptides, with clones considered reactive when $\Delta OD > 0.5$. Positive signals are indicated in green, and negative signals are shown in gray. Acetyl, acetylated; Cit, citrulline; ELISA, enzyme-linked immunosorbent assay; HCit, homocitrulline; PTM, post-translational modification; OD, optical density; RA, rheumatoid arthritis.

initial breach of tolerance leading to AMPA development.[29] In particular, the distinct serological profile of AAPA IgM compared to ACPA IgM further supports that these are separate responses, with AAPA IgM potentially reflecting a more physiologic, nonpathogenic immune response, whereas ACPA IgM is more closely associated with the development of RA. Despite key differences in the serological profiles of these two AMPA IgM responses, the longitudinal behavior of AAPA IgM in patients with early arthritis is similar to ACPAs. Antibody levels generally decrease after diagnosis, a phenomenon previously reported for ACPAs and likely attributable to immunomodulatory treatment rather than shared underlying biology.[24,30–33] Overall, we provide evidence that the AAPA IgM response arises after the period of early childhood and, when present, is not transient because AAPA IgM seropositivity tends to persist over time.

Furthermore, our findings provide evidence that AMPA responses can originate from multiple, independent B cell clones with distinct PTM reactivities, even with BCRs in germline configuration. Specifically, we identified both AAPA- and ACPA-expressing IgM B cells with unmutated BCRs, supporting the idea that autoreactive B cells in RA may arise from the germline-encoded B cell repertoire rather than through extensive SHM

and affinity maturation.[34,35] This aligns with prior observations that immature and naive B cells can exhibit autoreactivity, in addition to their inherent ability to recognize foreign antigens.[36,37] Such promiscuity suggests that the presence of AMPA B cells with unmutated, germline BCRs may reflect a physiologic, low-level autoreactive state that becomes pathogenic upon failure of peripheral tolerance mechanisms during B cell development. Similar to previous reports using germline-reverted AMPA IgM monoclonals, we demonstrate that the germline-encoded AMPA B cells identified in this study exhibit cross-reactivity.[15] A recent publication, however, suggested that ACPA specificity arises through affinity maturation because a germline-reverted ACPA mAb lost citrulline reactivity while retaining anti-CarP reactivity.[38] In contrast, our findings demonstrate that AMPA B cells can be identified in a germline configuration, encompassing both cross-reactive clones and clones restricted to citrulline reactivity only. Although our study does not directly address clonal lineage relationships between AAPA IgM and AMPA IgG responses, together, our findings support the notion that B cells expressing a BCR in germline configuration may play a role in the development of AMPA responses and that cross-reactivity can be an intrinsic property of these early B cell clones.

Subsequent exposure to microbial antigens bearing PTMs could promote the activation of these naive clones that, upon affinity maturation, ultimately drive the expansion of autoreactive and hypermutated AMPA clones, potentially giving rise to both restricted or cross-reactive AMPA IgG responses.[8] Our data demonstrate that circulating AAPA IgM antibodies and AAPA-expressing B cells in germline or mutated configurations can be found in both HDs and patients with RA, whereas ACPA presence, as established in previous reports, is mainly restricted to patients with RA.[16,39] This key difference could lie in the presence of additional triggers in patients with RA, such as antigen-specific T cell help, an inflammatory environment, or genetic predispositions like the HLA-shared epitope alleles, which may promote the activation and persistence of these autoreactive ACPA B cells.[40–42] Specifically HLA-shared epitope alleles have been shown to be associated with ACPA IgG in RA but not with other AMPAs or ACPA positivity in asymptomatic individuals, underscoring their potential role in the expansion of ACPA responses.[43,44] In the absence of such factors in healthy individuals, autoreactive B cell activation may be limited, explaining why AAPA IgM may be present without further progression to class-switched AMPA responses.

Our study has several limitations, including the low number of AAPA-expressing B cells analyzed and the low levels of AMPA IgM produced in culture, which impaired accurate estimation of their frequency in both healthy individuals and patients with RA. Additionally, the low yield from single-cell sorts constrained full characterization of AAPA clonality and diversity. Another limitation is the absence of serological measurements for anti-CarP antibodies, which could not be performed due to limited sample availability in the pre-RA cohorts. However, we address this gap functionally by demonstrating that some germline-encoded AMPA B cells exhibit cross-reactivity to HCit peptides. A strength of our study lies in the dual-level analysis of the AAPA IgM response, both through secreted antibodies and direct BCR characterization, which offers a more comprehensive understanding of their origin. Unlike previous studies that relied on BCRs reverted to germline configuration to infer germline AMPA reactivity, to our knowledge, our study provides the first direct evidence of naturally occurring germline-encoded AMPA B cells in the peripheral blood of patients with RA.[15,38] This novel finding sheds light on the early stages of autoreactive B cell development in RA and may inform future strategies to target pathogenic B cells before full maturation.

Moving forward, it will be essential to investigate whether and how these germline-encoded AMPA B cells become activated and mature into effector memory B cells and antibody-producing plasmablasts, as well as to map their prevalence and clonal evolution in both patients with RA and healthy individuals. Such studies will help identify critical checkpoints in which tolerance breaks down and offer valuable insights into timing and strategies for early intervention. All in all, our study provides compelling evidence that AAPA IgM is part of the normal B cell repertoire yet unlikely to be the sole driver of AMPA responses in RA. Importantly, we demonstrate for the first time that germline-encoded B cells reactive to PTM antigens naturally exist in the peripheral blood of patients with RA, some of them possessing cross-reactive properties. This challenges the notion that autoreactive AMPA responses and cross-reactivity arise only after extensive affinity maturation and instead suggests that early breakdown of tolerance permits these immature autoreactive B cells to persist and potentially contribute to disease development. These findings offer new insights into the origins of autoreactivity in RA and underscore the importance of targeting early B cell checkpoints to restore immune tolerance as a strategy for novel therapies.

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AUTHOR CONTRIBUTIONS

All authors contributed to at least one of the following manuscript preparation roles: conceptualization AND/OR methodology, software, investigation, formal analysis, data curation, visualization, and validation AND drafting or reviewing/editing the final draft. As corresponding author, Dr van der Woude confirms that all authors have provided the

final approval of the version to be published and takes responsibility for the affirmations regarding article submission (eg, not under consideration by another journal), the integrity of the data presented, and the statements regarding compliance with institutional review board/Declaration of Helsinki requirements.

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