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Identification of the central tolerance checkpoint for autoreactive proteinase 3^{+} B cells in human bone marrow

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ABSTRACT

Major target antigens of ANCA-associated vasculitis (AAV) are myeloperoxidase (MPO) and proteinase 3 (PR3). High-affinity MPO- and PR3-ANCA immunoglobulins are produced by antigen-experienced, class-switched autoreactive B cells. To prevent autoreactivity, B cells are subjected to several self-tolerance checkpoints, from the early immature stages in the bone marrow (BM), collectively called "central tolerance", to late mature stages, collectively called "peripheral tolerance"; the latter was recently elucidated for autoreactive PR3^{+} B cells.

Here we investigated central tolerance controlling immature PR3^{+}B cells in the BM before their migration into the periphery as transitional B cells. We applied an established flow cytometry-based method using labeled recombinant PR3 (rPR3) to identify the PR3^{+}B cells to compare the phenotype of PR3^{+}B cells in paired samples of BM mononuclear cells (BMMC) and peripheral blood mononuclear cells (PBMC) of non-vasculitis controls (No-AAV), and PBMC of patients with PR3-ANCA-associated vasculitis (PR3-AAV).

We observed that the proportion of PR3^{+}B cells within BMMC was higher (median [IQR]; 1.98 % [1.77–2.75]) than within PBMC of No-AAV (0.9 % [0.63–1.44], $p < 0.01$ by paired comparison) and similar to their proportion within PBMC of patients with PR3-AAV (1.82 % [1.66–3.21]; $p > 0.05$). Within CD24^{++}CD38^{++} B cells, the subset of B cell migrating from BM to the periphery, BMMC contained a greater proportion of PR3^{+}B cells as compared to PBMC in No-AAV (3.35 % [1.99–4.92] versus 1.23 % [0.62–1.55], $p < 0.01$), showing different surface markers of maturation (i.e. higher proportion of CD27^{-}CD10^{+} and lower expression of CD21, IgD, IgM). Importantly, we observed a significant decline of the PR3^{+} fraction from the immature subset (IgD^{-}IgM^{+}; 2.80 % [1.23–4.02]) to the early transitional subset (IgD^{+}IgM^{+}; 1.76 % [0.96–2.68], $p < 0.01$) in BMMC, while no significant reduction was observed between the early transitional of BMMC and the transitional compartment of PBMC in No-AAV (1.26 % [0.62–1.56], $p > 0.05$).

In conclusion, to prevent PR3-related autoimmunity, autoreactive PR3^{+}B cells pass a stringent selection in the BM, and their removal by central tolerance checkpoint activity occurs mainly between T1-like/immature to T2-like/early transitional B cells of BMMC.

1. Introduction

In adult humans, B cells are generated in the bone marrow (BM), where they start their developmental process before the transition to the periphery [1,2]. In the BM, B cell development is accompanied by immunoglobulin gene rearrangements leading to the generation of a

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diverse immature B cell pool. The B cells strongly reactive with self-antigens are deleted, undergoes receptor editing functionally silenced by processes collectively known as central tolerance, whereas the remaining immature B cells can migrate into the peripheral compartment [3,4]. This process has been demonstrated in detail in mouse models, and preliminary evidence is emerging in humans [1,3].

In autoimmune conditions, B cells and their autoantibodies are central in the disease pathophysiology [2]. Patients with systemic lupus erythematosus produce autoantibodies directed against double-stranded DNA and other soluble cytoplasmic and nuclear antigens; patients with rheumatoid arthritis produce anti-citrullinated protein antibodies [5–7]. Similarly, antineutrophil cytoplasmic antibodies (ANCA) are found in the blood of patients with ANCA-associated vasculitis (AAV), and ANCA have a central role in the disease process [8,9]. Autoreactive B cells are responsible for the autoantibody secretion in AAV [10,11], ultimately triggering the immune response in this autoimmune condition.

We previously developed a customized flow cytometry-based method to identify circulating autoreactive B cells, using proteinase 3 (PR3), one of the main autoantigens in AAV, as the ligand to target autoreactive B cells ($PR3^+$ B cells) [12]. Using this system, we were able to characterize circulating autoreactive B cells in AAV, reporting their phenotypic perturbations as comparted to healthy and disease controls, proving their functional ability to produce autoantibodies, and the association of disease relapse with the re-detectability of autoreactive plasmablasts after B cell depletion with rituximab [10,11]. We were able to detect $PR3^+$ B cells in healthy controls (HC), and as previously suggested [32], we observed a higher percentage of autoreactive $PR3^+$ B cells in transitional and naïve compartments within AAV patients having ANCAs against PR3 or myeloperoxidase (MPO, the other major autoantigen in AAV) compared to HC, suggesting the presence of an early, antigen-independent "central" immune checkpoint at the BM level, in addition to the peripheral checkpoints [10]. Therefore, early developmental stages and checkpoints of autoreactive $PR3^+$ B cells in the BM in AAV remain largely unknown.

Since autoreactive B cells can be detected in the blood of HC, and since natural autoantibodies directed against PR3 and MPO after antigen-specific purification of isolated IgGs have also been detected in the serum of HC [12,15], it is conceivable that autoreactive B cells are present within the BM mononuclear cells (BMMC) of HC and likely undergo stringent selection before moving to the periphery. However, the presence of $PR3^+$ B cells has never been investigated within BMMC so far, and it is not clear if and at which level this postulated central tolerance checkpoint exists for these cells.

The aims of this study were 1) to investigate the presence and the specific phenotypic features of $PR3^+$ B cells within BMMC of non-AAV controls (No-AAV), and to compare them with paired PBMC of the same individuals as well as PBMC of patients with PR3-AAV, and 2) to evaluate at which level these immature $PR3^+$ B cells are removed in the postulated central tolerance checkpoint, before the remaining $PR3^+$ B cells migrate in the periphery as transitional cells.

2. Material and methods

2.1. Study population and design

No-AAV adult subjects were selected among consecutive patients who underwent bone marrow aspirate to exclude hematologic conditions of myeloid origin and were found to be healthy or in long-term complete remission during follow-up of myeloid neoplasms. Patients with other types of hematologic diseases were excluded. To avoid any possible interference, those that were on conventional immunosuppressive treatment or patients with acute myeloid leukemia that were not found in molecular remission were excluded. Leukemic patients who had not been in remission for at least 12 months were also excluded. Both BMMC and PBMC were collected from these patients the same day, and these subjects were evaluated jointly by the hematologist (E.L., A. G.) and the rheumatologist (A.B., R.B.) the day of the sampling.

Adult patients with PR3-ANCA positive AAV (PR3-AAV) diagnosed clinically as granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA) and fulfilling EULAR/ACR criteria for GPA [43] or MPA [44] were selected among consecutive subjects with AAV seen in the Rheumatology Unit of the University of Trento. To be enrolled, they needed to be PR3-ANCA positive at disease onset and at the time of sampling, regardless of organs involved, disease activity and treatment received. PBMC were collected from the PR3-AAV patients.

BMMC and PBMC of No-AAV and PBMC of AAV patients were collected and cryopreserved upon enrollment. All clinical data were extracted from the medical records. In AAV, disease activity was measured using the Birmingham Vasculitis Activity Score version 3 (BVASv.3) [16]. Disease remission was defined as the stable absence of clinical and laboratory signs of disease activity (BVAS = 0 for vasculitis) for at least 12 months after finishing the remission induction treatment. For those patients treated with the B depleting agent rituximab (RTX) for remission induction, B cells needed to have reconstituted, defined as more than 69 $CD19^+$ cells per microliter, as previously reported [17,18].

This study was approved by the local Institutional Review Board (APSS Trento, session 31/01/2019, N. 2562) and was conducted in accordance with the amended Declaration of Helsinki. All patients gave written informed consent for their data to be stored electronically. The study was conducted according to STROBE guidelines (STrengthening the Reporting of Observational Studies in Epidemiology) for cohort, case-control, and cross-sectional studies. All the data can be obtained from the corresponding authors upon reasonable request.

2.2. Recombinant PR3 production and labeling

A recombinant PR3 (rPR3) was expressed in HEK-293 cells [19] as previously described [12,19–21]. This rPR3 variant corresponds to the mature form of the protein (deletion of the N-terminal propeptide, allowing a mature conformational state), enzymatically inactive (with the S195A point mutation to avoid the protease activity which could digest different proteins including immunoglobulins). This rPR3 is well recognized by PR3-ANCA from patients with GPA [22]. Culture supernatant of the stably transfected HEK-293 cells was harvested after 48 h starvation, and rPR3 was purified using a column loaded with the anti-human PR3 monoclonal antibody (Ab) MCPR3-2 [21] following recommendations from the supplier (CNBr-Activated Sepharose 4 Fast Flow, GE HealthCare), concentrated, and quantified by CoomassiePlus (Pierce, Rockford, IL). We biotinylated rPR3 using a commercial biotinylation kit (Lightning-Link Rapid Biotin Conjugation Kit, Innova Biosciences, Cambridge, UK), as previously described [12].

2.3. ANCA testing

PR3-ANCA IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) (supplied by Euroimmun, Inc.) in all serum samples from all the enrolled subjects, as previously described [23].

2.4. $PR3^+$ B cell detection and FACS analysis

BMMC and PBMC frozen in 10 % DMSO/human AB serum were thawed, counted, and stained for flow cytometric analysis. Briefly, 500.000 cells were incubated on ice for 20 min with rPR3-biotin and a cocktail of different antibodies (anti-CD19-PC7 cat# 560911 clone SJ25C1, anti-Human IgD-BV786 cat# 740997 clone IA6-2, anti-CD27-APC-Alexa fluor 750 cat# 561786 clone M-T271, CD38-PerCp 5.5 cat# 561106 clone HIT2, CD21-PE cat# 561768 clone B-ly4, CD24-BV480 cat# 746278 clone ML5, IgM BV421 cat# 562618 clone G20-127, CD20 APC-Alexa Fluor 700 cat# 560734 clone 2H7, CD10-BV650 cat# 563734 clone HI10a, all from Becton Dickinson, Inc.), washed 3 times, incubated for 15 min with streptavidin-FITC (cat# 554060, BD Biosciences), washed, and fixed. For each experiment, unstained cells as

well as single color controls were included. Cell analysis was performed on a FACSAria (BD Bioscience), as previously described [12]. FACS data were analyzed and graphed using FlowJo (Ashland, Oregon) software.

The high specificity of the staining of this customized FACS method has previously been shown using hybridoma cells expressing monoclonal IgG for either human or mouse PR3 on their cell surface [12]. The specificity of the method was subsequently further validated at the single cell level demonstrating that the great majority of the $PR3^+$ B cells identified in patients' blood are truly PR3-specific clones that produce anti-PR3 Ig [11]. Furthermore, a bulk correlation between the stained $PR3^+$B cells and functional production of PR3-ANCA in vitro and *in vivo* was previously shown using PBMCs and serum samples obtained at the same time from participants in a large clinical trial [10].

2.5. Statistical analysis

Categorical data were presented as n (%), continuous data as median (range or interquartile range [IQR]) or mean (SEM). Groups were compared using parametric or non-parametric tests when appropriate, Student T test or Mann-Whitney test for continuous data, and Chi^2 or Fisher's test for categorical data, where appropriate. Multiple comparisons between more than 2 groups were performed with one-way ANOVA or Kruskal-Wallis test, where appropriate. The multiple comparisons on B cell maturation were analyzed by using mixed-effects modeling. Findings in all the analyses were considered significant at $p < 0.05$ after adjustment for multiple comparisons by calculating the false discovery rate as described by Benjamini and Hochberg [24].

3. Results

3.1. Study cohorts

We compared BMMC and PBMC of a cohort of No-AAV subjects (n = 8), in whom BM blood and peripheral blood were collected the same day, and PBMC of a cohort of patients with PR3-AAV (n = 9). The included 8 consecutive No-AAV subjects that underwent BM aspiration were eventually identified as normal (2 healthy controls and 2 subjects with previous acute myeloid leukemia in stable prolonged remission) or with a chronic neoplasm of the myeloid lineage without signs of acute disease and not requiring any immunosuppressive treatment (2 subjects with a diagnosis of myelodysplastic syndrome, 1 with chronic myeloid leukemia, 1 with hypereosinophilic syndrome). None of the patients had a disorder of the B cell lineage, either in the past or at the time of sampling.

Nine patients with PR3-AAV were selected among consecutive subjects with AAV seen in our Unit, 7 with GPA and 2 with MPA. Of those, 3 patients had active disease a median BVAS.v3 of 20 [IQR:13–21] and 6 were in remission (BVAS.v3 of 0) when the blood was drawn; 2 of 3 patients with active disease were only on prednisone 1 mg/kg, and 4 out of 6 of those in remission were on azathioprine, 2 of which were also receiving low dose prednisone (5 mg daily or less). Further details on these two cohorts are summarized in Supplemental Tables 1 and 2, while baseline features of AAV subjects at the time of diagnosis are reported in Supplemental Table 2.

3.2. $PR3^+$ B cells are increased in BMMC of No-AAV and in PBMC of AAV patients

Using our customized flow cytometry-based assay, we studied the proportion of $PR3^+$ B cells of BMMC in No-AAV and compared them to their PBMC and to the PBMC of PR3-AAV patients. The proportion of total B cells ($CD19^+CD20^+$) were higher in BMMC as compared to PBMC of both AAV and No-AAV (Table 1). The gating strategy is represented in Supplementary Fig. 1A.

The proportion of $PR3^+$ B cells within BMMC (median [IQR]; 1.98 % [1.77–2.75]) was similar to their proportion within PBMC of PR3-AAV patients (1.82 % [1.66–3.21]; p = 0.863) (Fig. 1A), and higher than within PBMC of No-AAV (0.9 % [0.63–1.44], $p < 0.001$ by paired comparison) (Fig. 1B). The membrane expression of PR3-specific immunoglobulin on total B cells, measured as the MFI of labeled rPR3, was similar between BMMC and PBMC in No-AAV subjects, but lower in both BMMC and PBMC of this group compared to PBMC of patients with AAV (Fig. 1C), suggesting an efficient central and peripheral tolerance in No-AAV subjects compared to patients with AAV. PR3-ANCA IgG were detectable only in serum of patients with AAV (Supplementary Fig. 1B). $PR3^+$ B cell percentage did not change between AAV patients with active disease and patients in remission (Supplementary Fig. 2). Altogether, the percentage of $PR3^+$ B cells declined from BMMC to PBMC in No-AAV, whereas their proportion in PBMC of PR3-AAV patients was as high as that in BMMC of No-AAV subjects, leading to the hypothesis of the central tolerance checkpoint for $PR3^+$ B cells at the level of immature B cells, before migrating to the peripheral compartment, is altered in patients with PR3-AAV.

Table 1
Lymphocytes and B cell subsets of BMMC and PBMC of non-vasculitis controls (No AAV) (n = 8), and PBMC of PR3-AAV patients (n = 9).

Characteristics	PR3-AAV PBMC (n = 9)	No AAV PBMC (n = 8)	No AAV BMMC (n = 8)
Lymphocyte, %	62.9 [55.8,75.45]	81.50 [77.43,82.75]	54.25 [46.25 64.08]
Total B cells ($CD19^+CD20^+$), %	4.97 [2.36,5.90]	5.20 [3.00, 6.51]	7.78 [4.98, 8.12]
Transitional ($CD19^+CD20^+CD38^{++}CD24^{++}$), %	6.96 [1.14,13.95]	6.14 [1.59 8.91]	19.50 [12.38, 30.43]
T1 (IgD^- IgM^+)[a], %	24.50 [7.88, 29.70]	16.65 [10.53, 37.58]	36.05 [22.93, 44.08]
T2 (IgD^+ IgM^+)[a], %	43.30 [28.10, 70.95]	61.05 [23.28, 66.13]	15.10 [13.20, 17.33]
T3 (IgD^- IgM^-)[a], %	23.10 [9.54, 54,70]	19.15 [9.63, 50.65]	48.30 [38.98, 60.38]
Mature/Non transitional ($CD19^+CD20^+CD38^{low}CD24^{low}$), %	91.50 [81.20, 95.50]	91.85 [87.15, 96.45]	73.50 [62.53, 84.65]
Naïve ($CD19^+CD20^+CD27^-IgD^+$),%	57.00 [43.55, 72.00]	61.20 [51.45, 76.23]	–
Switched Memory ($CD19^+CD20^+CD27^+IgD^-$),%	14.40 [3.38, 17.85]	8.54 [4.08, 11.80]	–
Unswitched Memory ($CD19^+CD20^+CD27^+IgD^+$),%	5.75 [4.27,18.90]	5.99 [2.71, 13.93]	–
Double Negative ($CD19^+CD20^+CD27^-IgD^-$),%	17.30 [17.30, 31.00]	14.20 [10.69, 27.73]	–

Values are presented as median [25%–75 % Interquartile Range].
Abbreviations: PR3 = proteinase-3; ANCA = anti-neutrophil cytoplasmic antibodies; AAV = ANCA-associated vasculitis, No AAV = non-vasculitis controls.
[a] T1, T2, T3 refer to $CD24^{++}CD38^{++}$ B cell subsets within PBMC, while T1-like, T2-like, T3-like refer to $CD24^{++}CD38^{++}$ B cell subsets within BMMC.

3.3. The percentage of $PR3^+$ B cells within $CD24^{++}CD38^{++}$ B cells decreases from bone marrow to periphery

When in No-AAV subjects' frequencies of $PR3^+$ B cells were enumerated within $CD24^{++}CD38^{++}$ B cells, that subset of B cells that migrate from BM to periphery, it was found that BMMC contained the greatest proportion of $PR3^+$ B cells, as compared to PBMC (3.35 % [1.99–4.92] versus 1.23 % [0.62–1.55], p = 0.0078) (Fig. 2A, Supplementary Fig. 3A). In contrast, no significant difference was found between the $PR3^+$ fraction within $CD24^{++}CD38^{++}$ B cells within PBMC and the one within total mature B cells ($CD24^{dim}CD38^{dim}$) of No-AAV

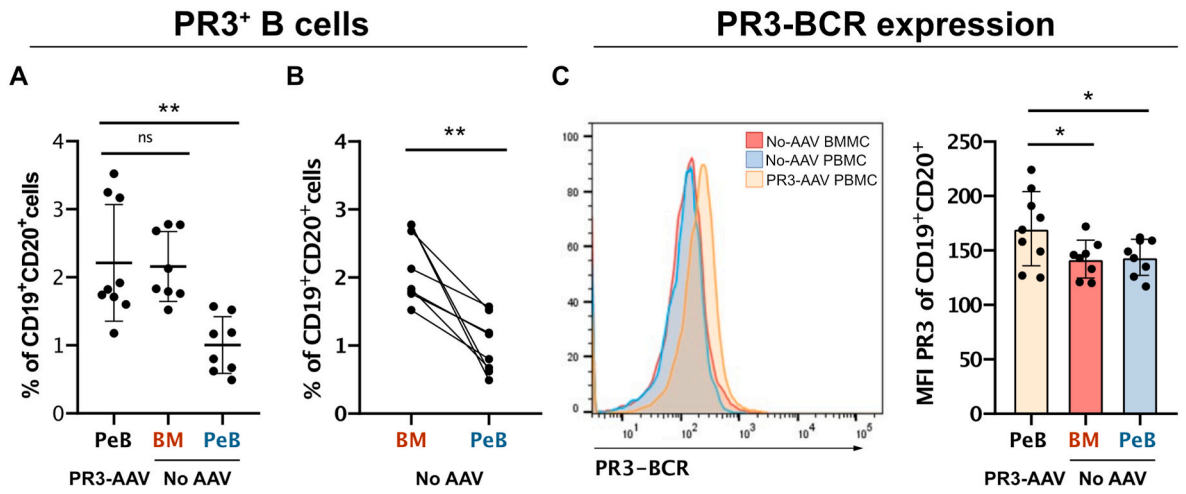

Fig. 1. PR3$^+$ B cells in BMMC and PBMC non-vasculitis (No-AAV) subjects and PR3-AAV patients. The proportion of circulating PR3$^+$ B cells in PBMC of PR3-AAV patients and in BMMC and PBMC of No-AAV subjects (A). Paired comparisons between BMMC and PBMC of No-AAV subjects (by means of Wilcoxon signed-rank test) (B); and PR3 membrane expression on total B cells of BMMC and PBMC of No-AAV subjects and of PBMC of PR3-AAV patients (C). Each point represents the frequency in an individual subject; horizontal lines show the median with 25–75 % IQR; each histogram represents mean (±SEM). P values were determined by 2-tailed Mann-Whitney test or Student T test, where appropriate. Multiple comparisons between more than 2 groups were performed with Kruskal-Wallis test. P values in the figures are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after correction for FDR with Benjamini and Hochberg test. *PeB: Peripheral Blood, BM: bone marrow (blood), AAV: ANCA associated vasculitis, PR3: proteinase 3.*

subjects (0.91 % [0.58–1.58], p = 0.742) (Fig. 2A, Supplementary Fig. 3A). Notably, PR3$^+$ proportions within mature B cells of PBMC were higher in AAV compared to No-AAV subjects (Supplementary Fig. 3A). The membrane expression of PR3-specific BCR on CD24^{++}CD38^{++} B cells was similar in BMMC and PBMC (Supplementary Fig. 3B). In aggregate, these descriptive data provide evidence that the main negative selection of PR3$^+$ B cells occurs in the BM of No-AAV subjects, before the transition of B cells to the peripheral compartment, suggesting that this mechanism might be defective or at least less efficient in AAV. We further characterized CD24^{++}CD38^{++} B cells in BMMC and PBMC, confirming a low-to-absent expression of CD27 (a marker of memory B cells) in CD24^{++}CD38^{++} B cells of both BMMC and PBMC, and a significantly higher proportion of CD27$^-$CD10$^+$ (highly expressed on B cell progenitors in the BM and then progressively disappearing with maturation [25]) among CD24^{++}CD38^{++} of BMMC as compared to those among PBMC (Fig. 2B) (CD27$^-$CD10$^+$: 72.50 % [21.18–84.28] versus 40.10 % [5.94–53.48], $p < 0.01$). In addition, the expression of CD21 (a marker of maturation), IgM and IgD was consistently lower on CD24^{++}CD38^{++} B cells in BMMC as compared to those in PBMC (Fig. 2C). Altogether, these findings confirm that CD24^{++}CD38^{++} B cells in BMMC and PBMC represent two separate developmental stages of B cell maturation rather than a single population in different blood compartments.

3.4. Isotype-defined subsets of CD24^{++}CD38^{++} B cells in bone marrow and peripheral blood

We further categorized CD24^{++}CD38^{++} B cells by IgM and IgD expression (Fig. 3A), as previously described [26,27]. As known, in PBMC T1 (IgM$^+$IgD$^-$) and T2 (IgM$^+$IgD$^+$) transitional B cells represent subsequent stages of maturation, while T3 (IgM$^-$IgD$^+$) transitional B cells have an intermediate profile of activation constituting a anergic state [26]. In BMMC, IgM$^+$IgD$^-$ represents a truly "immature" B cell phenotype, IgM$^+$IgD$^+$ shows a more advanced pattern of differentiation representing "early transitional" B cells, while IgM$^-$IgD$^+$ represents the initial stage of B cell maturation corresponding to "pre/pro B cells" [27].

Total CD24^{++}CD38^{++} B cells were significantly higher in BMMC than PBMC (of both No-AAV and AAV subjects) (Fig. 3B). The distribution of these isotype-defined subsets differed between BMMC and PBMC, with IgM$^+$IgD$^-$ being predominantly present in BMMC as compared to PBMC (of both No-AAV and AAV subjects) (Fig. 3, Table 1). These results reflected the immaturity of B cells in the BM before transitioning to the peripheral compartment, consistent with those previously published [28].

3.5. Negative selection of PR3$^+$ B cells within bone marrow

To further investigate with more precision at which level the elimination of autoreactive B cells occurs in BMMC, we tracked the frequencies of PR3$^+$ B cells through the developmental stages of CD24^{++}CD38^{++} B cells in BMMC, followed by the main subsets in PBMC known to represent immune checkpoints in AAV patients, as previously described [12]. In all the BM samples, the proportion of PR3$^+$ B cells was close to 0 % in the IgD$^-$IgM$^-$ CD24^{++}CD38^{++} B cell subset (reflecting the absence of expression of a functional BCR at this developmental stage), highest in the IgD$^-$IgM$^+$ subset (2.80 % [1.23–4.02]), then significantly lower as B cells mature into the IgD$^+$IgM$^+$ subset (1.76 % [0.96–2.68], $p < 0.01$), while no significant reduction was observed between the latter subset and the transitional compartment of PBMC (1.26 % [0.62–1.56], $p = 0.2500$) (Fig. 4). In contrast, percentages of B cell subsets (including PR3$^+$ and PR3$^-$ B cells) decrease progressively from IgD$^-$IgM$^+$ to IgD$^+$IgM$^+$ in the BMMC ($p < 0.01$), and from IgD$^+$IgM$^+$ of BMMC to transitional B cells of PBMC ($p < 0.01$) (Supplementary Fig. 4, Table 1 for the median values). In other words, since only PR3$^+$ B cells declined from "immature" to "early transitional" compartments in BMMC, the early central tolerance checkpoint mostly contributing to the deletion of PR3$^+$ B cells in No-AAV subjects appears to be at this level.

Discussion

Autoreactive B cells need to pass a stringent selection in the BM. Virtually, all the autoreactive B cells are eliminated before B cells migrate to the peripheral compartment in healthy humans. However, a small percentage of these self-reactive B cells are still migrating to the periphery, where they release polyreactive, generally IgM idiotype, natural autoantibodies, a phenomenon described as natural autoimmunity [29–31]. In contrast to our knowledge about the fate of autoreactive B cell precursors in the BM of mice [3,32], our understanding of these mechanisms in humans remains scarce. In particular, nothing is known about the BM development of PR3$^+$ B cells, the precursors of

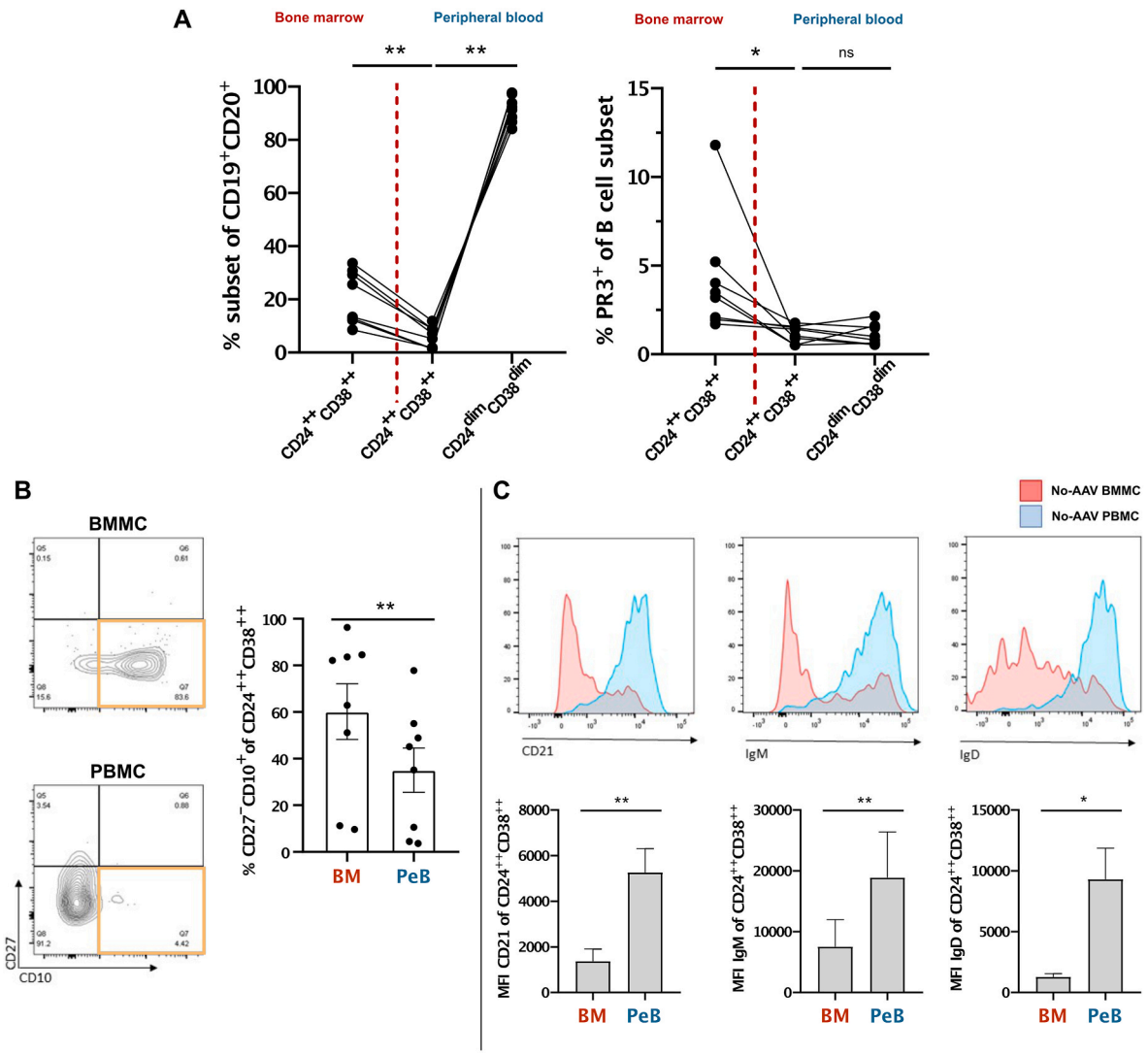

Fig. 2. Proportion of PR3$^+$ B cells within CD24^{++}CD38^{++} subset and its characterization in BMMC and PBMC. Proportion of B cells (A, left) and PR3$^+$ B cells (A, right) among CD24^{++}CD38^{++} of BMMC and PBMC and CD24^{dim}CD38^{dim} of PBMC in non-vasculitis (No-AAV) subjects (by means of Wilcoxon signed-rank test). Confirmatory gate of CD27$^-$CD10$^+$ on CD24^{++}CD38^{++} (B). Expression of markers of maturity on transitional B cells, CD21, IgM and IgD (C). Each point represents the frequency in an individual subject; horizontal lines show the median with 25–75 % IQR; each histogram represents mean (±SEM). P values were determined by 2-tailed Mann-Whitney test or Student T test, where appropriate. P values in the figures are indicated as * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *PeB: Peripheral Blood, BM: bone marrow (blood), BMMC: bone marrow mononuclear cells; PBMC: peripheral blood mononuclear cells.*

autoreactive B cells secreting PR3-ANCA IgG involved in the pathogenesis of AAV.

Our study reveals that PR3$^+$ B cells within PBMC of patients with AAV are as high as those within BMMC of No-AAV subjects, in whom they are significantly lower in PBMC, suggesting a central tolerance checkpoint for autoreactive B cells at this level in patients with AAV. We explored the maturation of natural autoreactive PR3$^+$ B cells focusing on these CD24^{++}CD38^{++} B cell subsets in BM and the periphery. We observed that the most significant deletion of PR3$^+$ B cells in No-AAV subjects occurred between "T1-like/immature" and "T2-like/early transitional" B cell stages within BMMC (i.e. from IgD$^-$IgM$^+$ to IgD$^+$IgM$^+$ CD24^{++}CD38^{++} B cells), and not later, such as between BM T2-like and peripheral transitional B cell stages of maturation. Since transitional PR3$^+$ B cells within PBMC are more frequent in AAV than in No-AAV subjects, this data suggests that the checkpoint at this level can be defective or at least less efficient in AAV patients.

Even though only circulating autoreactive PR3$^+$ B cells of PR3-AAV are able to produce PR3-ANCA IgG detectable by conventional ELISA, the proportion of PR3$^+$ B cells were increased in BMMC compared to PBMC of No-AAV subjects. This is not surprising to us, since the overall frequency of autoreactive B cells in the BM starts at 50–80 %, and then decreases progressively to 10 % of mature naïve B cells in human adults [13,14]. Consistently, our findings on PR3-autoimmunity showed that PBMC of patients with PR3-AAV had significantly higher PR3$^+$ mature (CD24^{dim}CD38^{dim}) B cells compared to PBMC and BMMC of No-AAV controls. Several tolerance mechanisms operate in pre-immune B cells, before the acquisition of immunocompetence occurring in the naïve stage [3,33]. In the BM, BCR editing and clonal deletion occur at the stage of pre/pro B cells to immature B cells, while additional checkpoints at the level of early transitional stage can occur, leading to apoptosis or anergy of autoreactive B cells. In our experiments, pre/pro B cells (T3 CD24^{++}CD38^{++} B cells of BMMC) were not reactive for PR3, since no functional Ig are present on the B cell surface at this stage, and therefore, tolerance checkpoints cannot be studied with our flow cytometry method at this level. To address which tolerance checkpoints might be defective, we tracked the PR3$^+$ B cells in BMMC after pre/pro B cell stage. We found a significant reduction of PR3$^+$ B cells from immature to early transitional B cells, meaning that at this level there is

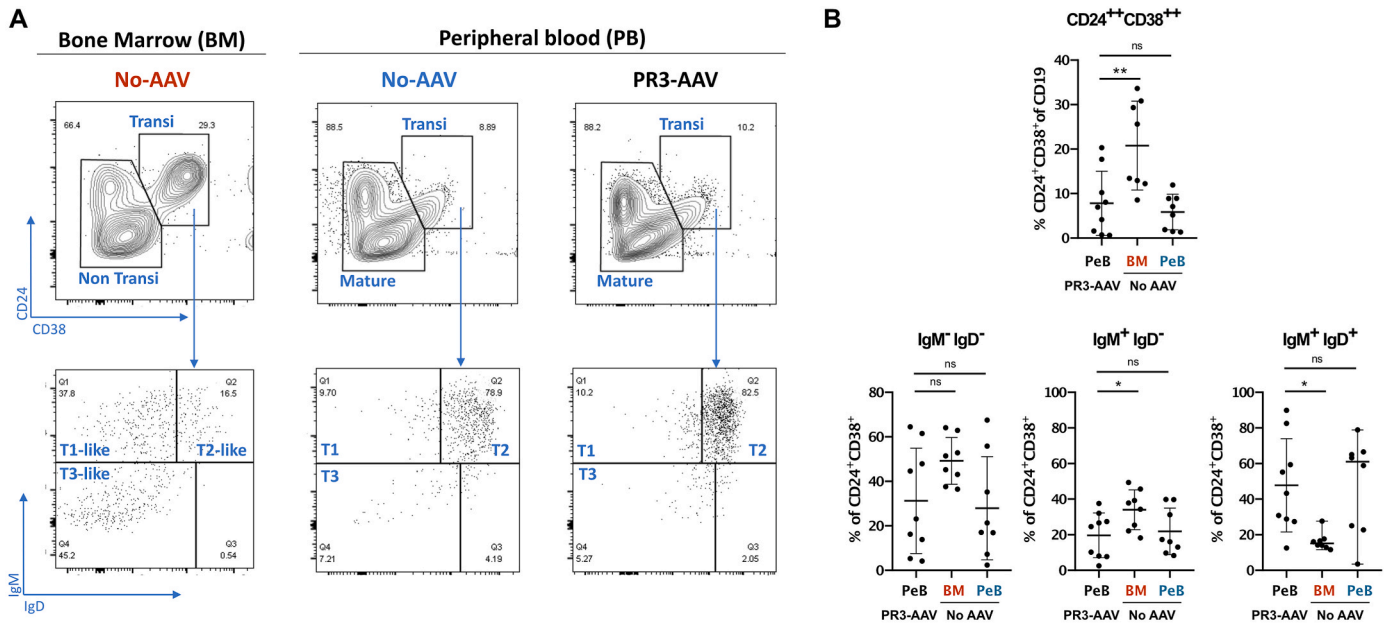

Fig. 3. Immature/Transitional B cells in bone marrow and peripheral blood. Gating strategy of $CD24^{++}CD38^{++}CD19^{+}CD20^{+}$ cells, further plotted by IgM and IgD (A); proportion of $CD24^{++}CD38^{++}$ within each subsets, i.e. T1 (or immature in BM), T2 (or early transitional in BM), and T3 (or pre/pro-B cells in BM) (B). Each point represents the frequency in an individual subject; horizontal lines show the median with 25–75 % IQR. Multiple comparisons between more than 2 groups were performed with Kruskal-Wallis test. P values in the figures are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after correction for FDR with Benjamini and Hochberg test. *PeB: Peripheral Blood, BM: bone marrow (blood).*

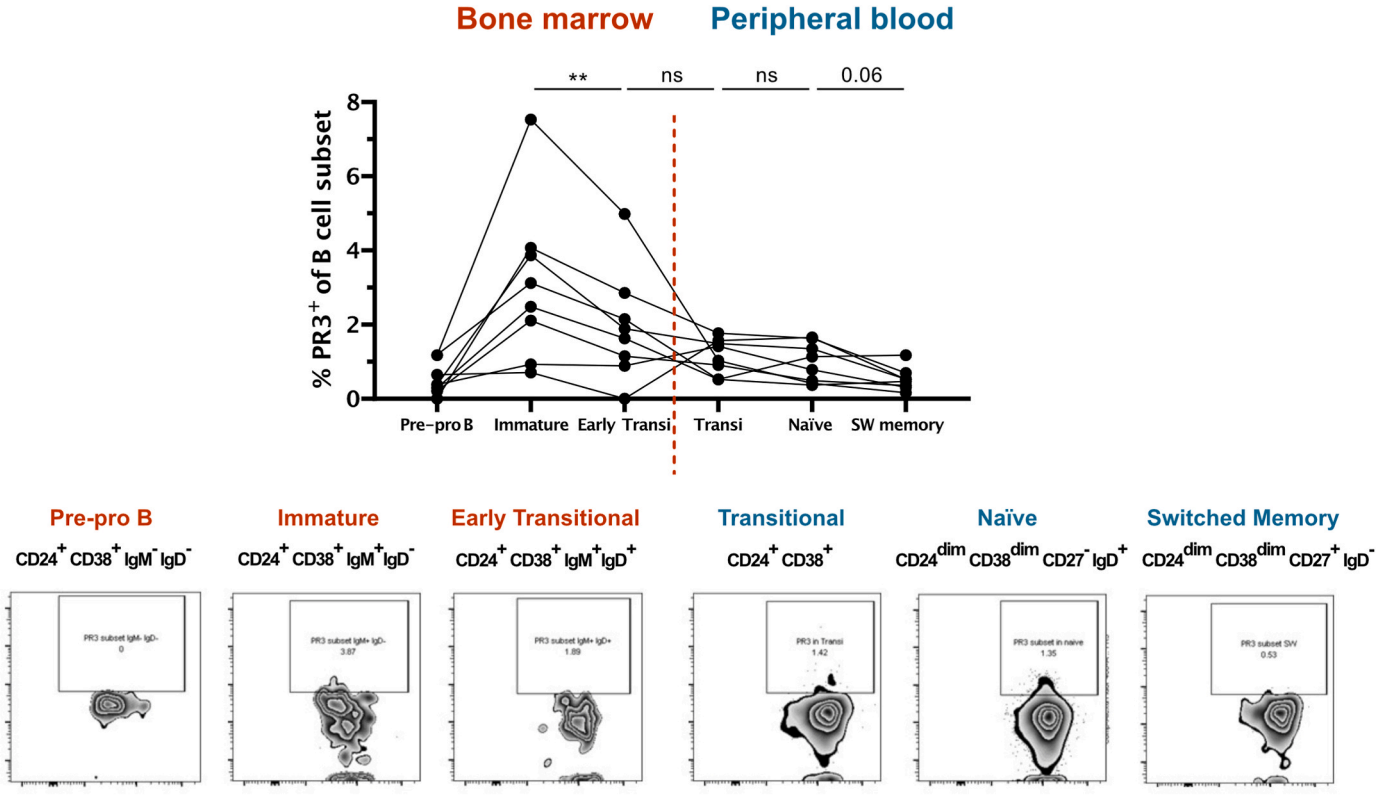

Fig. 4. Comparison of the proportions of $PR3^{+}$B cells among the different bone marrow B-cell subsets to peripheral B cell subsets in non-vasculitis (No-AAV) subjects. $PR3^{+}$ B cells are significantly vetted from the T1-like to T2-like B cells compartments in non-vasculitis controls, and not after, indicating that the central immune checkpoint for PR3+ B cells mostly contributing to deletion of these autoreactive B cells occurs at that level (upper figure). Proportions of $PR3^{+}$ B cells in these different B-cell subsets in a No-AAV subject (bottom). Each point represents the frequency in an individual subject. The multiple comparisons on B cell maturation were analyzed by using mixed-effects modelling. P values in the figures are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after correction for FDR with Benjamini and Hochberg test. *SW memory: Switched memory (B cells), PR3: proteinase 3.*

the checkpoint contributing the most to the removal of $PR3^+$ B cells in No-AAV subjects, checkpoint likely less efficient in AAV patients.

Several central mechanisms balancing positive and negative selection of autoreactive B cells have been identified (summarized in Ref. [3]). In BALB/c and C57BL/6 mice, a positive selection for ANA^+ autoreactive B cell at early transitional stage in bone marrow and has been reported by some authors [34]; similarly, a positive selection at the transitional T1 and T2 cell stages in the spleen (recent immigrants from the BM to the spleen) is well documented. Overall, evidence is limited for BM [34,35]. In the BM, the BCR tonic signaling in immature B cells promotes the positive selection of cells with unligated receptors through a phosphoinositide 3-kinase-dependent pathway, whereas BCR ligation promotes negative selection by hindering this pathway and likely by downregulating BCR levels [3]. Our findings showed a downregulation of PR3-BCR expression on the surface of B cells in the No-AAV B cells (both among PBMC and BMMC) compared to B cells from PBMC of AAV patients. However, the proportion of $PR3^+$ B cells was similar between No AAV BMMC and AAV PBMC, while being significantly lower in the No AAV PBMC. This data is consistent with the current thinking that both deficiency of central tolerance (i.e. less efficient negative selection in the BM) probably allowing the escape of (poly)autoreactive B cells through the early tolerance checkpoints, and deficiency of peripheral tolerance (i.e. due to maturation in germinal centers or outside of germinal centers in the periphery) lead to the increased frequency of autoantigen-specific reactivity in autoimmune diseases [1,3].

We illustrated how $PR3^+$ B cells are removed by central tolerance mechanisms in BMMC under homeostatic conditions in individuals without vasculitis. Despite this well-structured system of central tolerance checkpoints to avoid autoimmunity, functionally active $PR3^+$ B cells clones still emerge in PR3-AAV patients, indicating overall that pathogenic autoimmunity configures as a dysregulation of natural homeostatic autoimmunity rather than the new onset of a previously absent self-recognition [36]. More in general, defects in the early removal of autoreactive B cells do not only occur only in autoimmune diseases, but also in primary immunodeficiency [14]. In this regard, alterations of BCR and Toll-like receptor signaling pathways result in a defective central checkpoint leading to the failure to counter-select immature autoreactive B cells in the BM [4,14,37,38]. These phenomena have been shown in humans, resulting in a high frequency of autoreactive transitional B cells in patients with either autoimmune disease or several types of immunodeficiency [37,38].

This work has strengths and limitations. One strength is that the ontogeny of human autoreactive B cells has not been thoroughly studied to date, probably due to the difficulty of obtaining BMMC samples of healthy/non-vasculitis patients for research purposes. Our findings, therefore, provide new insights into the mechanisms that control PR3-autoimmunity occurring in pre-immune cells. Another strength is that we used a wide panel of antibodies for the flow cytometry analysis, for instance considering only double positive $CD19^+CD20^+$ as B cells. In fact, considering all the $CD19^+$ as B cells, as is usually done for PBMC, would have been imprecise for BMMC, since this would have led to the inclusion of mature plasma cells within the immature B cell pool which is usually $CD20^-$ and residing in the BM [39], and some early precursors of B cells without surface BCR, such as pro-B cells [3].

This work also has potential limitations. First, the numbers of BMMC samples and patients in the AAV cohort are relatively small. Yet, the data were consistent throughout all cohort samples. For the AAV patients, one could also speculate that different treatments could have affected the numbers of $PR3^+$ B cells. However, we have previously not detected an effect of conventional treatments (with or without glucocorticoids) on the proportions of $PR3^+$ B cell proportions [12]. This is in contrast to rituximab, which by depleting B cells also depletes $PR3^+$ B cells, and we have documented previously in patients from the RAVE trial cohort that during B cell reconstitution after rituximab treatment, $PR3^+$ B cells are even higher as compared to untreated patients with active disease [11]. Consequently, it is unlikely that treatments affected the number of $PR3^+$ B cells in this AAV cohort, because patients were either still untreated or on glucocorticoids alone when sampled at the time of active disease, or on azathioprine, low dose of glucocorticoids or no therapy when sampled during remission. The only exception was one patient who received on demand redosing with rituximab whenever B cells were reconstituted, and this patient was sampled at the time of reconstituted B cells.

Moreover, we did not have access to BMMC of patients with AAV, which would have definitely proven a defect of the B-cell tolerance machinery at this level. Obtaining BM blood is an invasive and quite painful procedure, which would have unethical to perform without clinical indication solely for research purposes in patients with AAV. Third, all the samples studied were frozen, possibly affecting the quality of the material studied. However, previously published studies suggested that cryopreserved cells can be stored for at least 12 years with no general tendency toward cell loss over time [40]. Furthermore, no statistically significant changes in the percent cell viability according to the length of time frozen have been reported, and without changes in fluorescence intensity in subsequent flow cytometry for most of the subsets (including CD19 cells) [41,42].

In addition, HLA characterization of the studied subjects could not be performed. Therefore, we cannot exclude the possibility that the frequency of $PR3^+$ B cells is indirectly affected by PR3-reactive CD 4^+ T cells from AAV patients, which have recently been shown to be associated with the HLA-DPB1*04:01 PR3-AAV risk allele [45]. Further studies in larger patient populations focusing specifically on autoreactive $PR3^+$ B-cells during active and inactive disease are needed, particularly to further investigate potential relationships between the frequency of $PR3^+$ B cells and their PR3-ANCA production and PR3-reactive $CD4^+$ T cells and their interferon-g production [45]. Finally, in vitro functional evaluations of PR^+ B cells will also be needed to better understand their role in the disease.

In conclusion, the proportion of autoreactive $PR3^+$ B cells within BMMC is higher than within PBMC of No-AAV subjects, and similar to their proportion within PBMC of PR3-AAV patients, suggesting the presence of a central tolerance checkpoint for $PR3^+$ B cells at the level of immature/transitional B cells. The negative selection of $PR3^+$ B cells occurs at the level of T1-like/immature to T2-like/early transitional B cells in BMMC of non-vasculitis controls under normal circumstances, thus preventing PR3-related autoimmunity, as opposed to no significant reduction between the T2-like and the transitional compartment of PBMC.

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CRediT authorship contribution statement

Alvise Berti: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Michele Tomasi:** Writing – review & editing, Formal analysis, Data curation. **Isabella Pesce:** Writing – review & editing, Formal analysis, Data curation. **Enrico Lista:** Writing – review & editing, Data curation. **Anna Guella:** Writing – review & editing, Data curation. **Roberto Bortolotti:** Writing – review & editing, Data curation. **Giuseppe Paolazzi:** Writing – review & editing, Data curation. **Sophie Hillion:** Writing – review & editing, Supervision, Formal analysis, Data curation. **Ulrich Specks:** Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Guido Grandi:** Writing – review & editing, Methodology, Investigation, Data curation. **Divi Cornec:** Writing – review &

editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Dr. U. Specks, has received research grants from Amgen, AstraZeneca, BristolMyerSquibb, GSK, Novartis, NS Pharma and Roche/Genentech, unrelated to the present publication. Dr. A. Berti has served on advisory boards and has received lecturing fees from GSK, all unrelated to the present publication. Dr. D. Cornec has no personal conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2024.103330.

Data availability

Data will be made available on request.

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