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Autoreactive plasmablasts after B cell depletion with rituximab and relapses in ANCA-associated vasculitis

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OBJECTIVE: Autoreactive B cells are responsible for ANCA production in ANCA-associated vasculitis (AAV). Rituximab depletes circulating B cells including autoreactive ones. We aimed to evaluate changes and associations with relapse of the circulating autoreactive B cell pool following therapeutic B cell depletion in AAV.

METHODS: Sequential flow-cytometry was performed on 148 samples of peripheral blood mononuclear cells from 23 proteinase-3 (PR3)-ANCA+ AAV patients treated with rituximab for remission-induction and monitored off-therapy during long-term follow-up in a prospective clinical trial. PR3 was used as ligand to target autoreactive PR3-specific B cells (PR3+B cells). B cell recurrence was considered as the first blood sample with ≥10 B cells/μL after rituximab.

RESULTS: At B cell recurrence, PR3+B cell frequency among B cells was higher than baseline (p<0.01). Within both PR3+ and B cells, frequencies of transitional and naïve subsets were higher at B cell recurrence than at baseline, while memory subsets were lower (p<0.001 all comparisons). At B cell recurrence, frequencies of B cells and subsets did not differ between relapsers and patients remaining in remission. In contrast, the plasmablasts frequency within the PR3+B cell pool was higher in relapsers and associated with a shorter time to relapse. Frequencies of PR3+ plasmablasts higher than baseline were more likely to be found in patients who relapsed within the following 12 months compared to those in sustained remission (p<0.05).
CONCLUSION: The composition of autoreactive B cell pool varies significantly following rituximab treatment in AAV, and early plasmablast enrichment within the autoreactive pool is associated with future relapses.

KEYWORDS
Autoreactive B cells, ANCA-associated vasculitis; Proteinase 3, B cells; relapse.

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INTRODUCTION

The anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitides (AAV) are a group of systemic autoimmune diseases characterized by the presence of autoantibodies directed against proteinase 3 (PR3) or myeloperoxidase (MPO) (1–3) in the serum of most patients. In AAV, B cells are central in the development of the disease and the production of ANCA (4,5), which mediate the disease by a variety of pro-inflammatory mechanisms including the triggering of neutrophil activation and degranulation (6,7). ANCA levels are weakly associated with disease activity (8–10), and this association is affected by disease phenotype and remission-induction treatment, particularly in patients presenting with renal involvement, alveolar hemorrhage, and having severe relapses (11).

Rituximab (RTX), which induces B cell depletion by targeting CD20 on B cell surface, has become a standard treatment option for AAV based on the results of two randomized, controlled clinical trials (12–14), and the short- and long-term efficacy of RTX to control disease activity in patients with AAV has been confirmed in subsequent cohort studies and remission maintenance trials (15–20).

Autoreactive B cells are responsible for autoantibody production in autoimmune diseases, such as anti-nuclear antibodies in lupus and anti-citrullinated protein antibodies in rheumatoid arthritis (21–25). Similarly, autoreactive B cells are responsible for ANCA production in AAV (26). The existence of circulating B cells bearing a B cell receptor (BCR) specific for PR3 or MPO has been postulated for years, but their reliable detection has been elusive until recently (27,28). We previously developed a customized flow-cytometry method to evaluate circulating autoreactive PR3-specific (PR3+) B cells among
cryopreserved peripheral blood mononuclear cells (PBMCs)(27). We were able to characterize the phenotype and the function of PR3+ B cells in patients with PR3-AAV and healthy controls(27). However, after RTX-induced depletion of B cells, the reconstitution and subsequent persistence of circulating PR3+ B cells, their repartition between the different B cell subsets within the PR3+ pool, and their relationship with long-term treatment outcomes remain unknown.

Using this flow cytometry-based assay, we investigated the longitudinal changes of circulating PR3+ B cells in PBMCs from 23 patients with severe PR3-ANCA-positive AAV(12) who had achieved complete remission with RTX and glucocorticoids (GC) within 6 months after initiation of remission induction therapy in a clinical trial(12). For this proof-of-concept study, we hypothesized that RTX-induced B cell depletion would alter the proportions of circulating B cell subsets within the autoreactive pool and investigated whether any features of these changes during follow-up were associated with relapse.

**METHODS**

**Study population and design**

Twenty-three subjects with PR3-ANCA-positive AAV from the RTX arm of the RAVE trial (NCT00104299)(12) who reached the primary endpoint of the study (BVAS=0 off GC at month 6) with available baseline and follow-up PBMC samples were selected for our analysis and provided 148 unique serial PBMC samples. PBMCs had been collected and cryopreserved upon enrollment, at month 6 and every 3 months until month 18, and annually after month 18, according to the trial protocol(13). See Supplementary Methods 1 for details.
According to the trial definition, B cell recurrence (or redetection) in RTX-treated trial participants was defined as at least 10 but less than 69 CD19+ cells per microliter, and reconstitution as 69 or more CD19+ cells per microliter or a return to baseline levels. All clinical data were obtained from the trial database.

To ensure comparability of the subsets of B cells and autoreactive cells between baseline and B cell return (i.e., assuring a minimum number of B cells at B cell recurrence, allowing an accurate assessment of B cell subsets), we considered the biological time-point “B cell recurrence” as the first blood sample at or after month 6 in which study participants had ≥10 B cells/μL.

Recombinant PR3 production and labeling

A recombinant PR3 (rPR3) was expressed in an epithelial cell line and labeled as previously described(27,28,30). See Supplementary Methods 2.

ANCA testing

PR3-ANCA IgG levels were determined by enzyme-linked immunosorbent assay (ELISA) (supplied by Euroimmun, Inc.) in all serum samples from all 23 patients as previously described(11).

PR3-reactive B cell detection and FACS analysis

PBMCs frozen in 20% DMSO/human AB serum were thawed, counted, and stained for flow cytometric analysis. Labeled PR3 was used as ligand to target autoreactive B cells. In brief, 1x10^6 cells were incubated on ice for 20 min with biotin-
labeled rPR3 (rPR3-biotin) and a cocktail of antibodies (anti-CD19-APC-Alexa Fluor 700, Anti-Human IgD-APC, anti-CD27-PC7, CD38-PC5.5 and anti-CD24-APC-Alexa Fluor 750, all from Beckman Coulter, Inc.), washed 3 times, incubated for 15 min with streptavidin-FITC, washed, and fixed. For each experiment, unstained cells as well as single color controls were included. Cell analysis was performed on a FACS Canto (BD Bioscience), as previously described(27). FACS data were analyzed and graphed using KALUZA (Beckman Coulter, Inc., Indianapolis IN) and FlowJo (Ashland, Oregon) software.

**Functional Validation of the PR3⁺ B-cell FACS method**

We studied whether PR3⁺ B cells identified by flow cytometry are able to secrete PR3-ANCA. PBMC of patients with active PR3-AAV were FACS-sorted based on streptavidin expression to isolate PR3⁺CD19⁺ and PR3⁻CD19⁺ cells. Sorted B cells were cultured and stimulated using modifications of previously published protocols to promote B cell activation (34,35) (Supplementary Methods 3). To validate PR3-ANCA secretion on a single B cell level, peripheral blood B cells from a patient with severe, untreated PR3-AAV were immortalized using Epstein-Barr virus (EBV) to obtain lymphoblastoid cell lines (LCLs) (32), and PR3⁺ and PR3⁻ cells were sorted for single cell culture. Cell culture supernatants of PR3⁺ and PR3⁻ LCL single cell clones were analyzed by ELISA to quantify anti-PR3 antibodies (IgM or IgG isotype) (Supplementary Methods 4).
Statistical analysis

Categorical data are presented as n (%), continuous data as median (range or interquartile range) or mean (SEM). Groups were compared using parametric or non-parametric tests when appropriate, Student T test or Mann-Whitney test for continuous data. Wilcoxon signed-rank test was used to analyze paired data before and after RTX, and Chi² or Fisher's test as appropriate for categorical data.

Clinical outcomes were correlated with B cells and PR3+B cells and their subsets. The estimated distributions of relapse and severe relapse were performed with the Kaplan–Meier method and the log-rank test.

To further assess the ability of the frequency of plasmablasts (PBs) within the PR3+B cell pool to distinguish between relapsers and patients remaining in long-term remission, a receiver operating characteristic (ROC) curve was constructed using logistic regression, with the % of the circulating biomarker as the predictor variable and relapse versus remission during follow-up as the dichotomous outcome. The area under the ROC curve (AUC or C-statistic) was calculated.

To assess a possible association between an increase of peripheral blood PB among the PR3+B cell pool (PR3+PB) and relapse, we performed a case-time-control analysis. It comprises a regular case-crossover of cases (relapse) and a case-crossover of controls (sustained remission). For this analysis, 148 samples, i.e., all available samples from the 23 patients studied, were used. For each individual study participant, the increase in frequency of PR3+PB after B cell depletion was defined as a PR3+PB level during follow-up equal or higher than the individual baseline level. For the patient to be considered at risk (“exposed”), the increase of PR3+PB level had to occur within the 12
months preceding the relapse. In order to calculate the effect of PR3+PB increase, we
used conditional logistic regression. The ratio between the odds ratio (OR) from the case
arm of the study and the OR obtained in the control arm generated the case-time-control
study OR risk for relapse if an increase in PR3+PB increase occurred.

A vector graphic editor program was used to build the Swimmer-plot (Affinity
Designer, Serif (Europe) Ltd, UK). Conditional logistic regression was calculated using
STATA (STATA-Corp LP, version 13.1). All other statistical analyses were performed
using the GraphPad Prism (San Diego, California) or JMP (Version 8, SAS Institute Inc)
software.
RESULTS

Remission, relapse and B cell recurrence in PR3-AAV

Clinical and demographic features of the study participants are reported in Supplementary Table 1. All patients were treated with RTX and GC for the induction of remission, achieved complete remission prior to month 6 after randomization and were off therapy (including GC) after month 6 unless a relapse occurred (Figure 1). Total median follow-up was 44 months (25-75% IQR, 31-54); 43 months (25-75% IQR, 35-54) for patients who suffered a relapse after achieving complete remission, and 48 months (25-75% IQR, 30-54) for patients who remained in complete remission for the duration of the trial (p=0.975). Ten subjects relapsed during follow-up (red timelines), eight of them had severe relapses, and five of them had multiple relapses during the observation period (Figure 1).

We compared cryopreserved PBMC collected at baseline with those collected at B cell recurrence. The median time point of B cell recurrence was month 12 (range, month 6-24). At B cell recurrence, absolute B cell counts and relative frequencies were significantly lower compared to baseline (Figure 2A). Among B cell subsets, the frequencies of transitional (Transi, CD19^{+}CD24^{high}CD38^{high}) and naïve B cells were significantly higher, while the frequencies of the memory B cell subsets were significantly lower at B cell recurrence than at baseline (subsets and gating strategy shown in Figure 2B-D and Supplementary Figure 1).
Identification and validation of the PR3-reactive B cells by FACS

Upon stimulation in culture, the staining of cytospinned B cells showed signs of activation and initial cytoplasmic IgG accumulation, suggestive of an initial differentiation of memory B cells towards antibody-secreting cells in both PR3+CD19+ and PR3-CD19+ sorted cells (Figure 2A-B). PR3-ANCA IgG was detected by ELISA only in the supernatant of PR3+CD19+ cell cultures and not of PR3-CD19+ cell cultures or in other negative controls (i.e. RPMI growth medium; supernatant of 4-day stimulated CD19+ B cell cultures from healthy controls; and purified MPO-ANCA IgG from patients) (Figure 2B), indicating that our detection methodology ensured a full recovery of circulating PR3-reactive B cells within the PR3+B cell pool as detected by FACS.

We then analyzed the binding of 189 monoclonal antibodies from the supernatants of single-sorted immortalized B cells from a patient with active severe PR3-AAV as detected by PR3-specific ELISA, showing that 91% of the PR3+ve sorted B cells (n=96) had detectable PR3-specific antibodies in the supernatants (i.e. true positive), while 96% of the PR3-ve sorted B cells (n=93) did not have detectable antibodies against PR3 (i.e. true negative) (Figure 3C). This validated our customized FACS method, demonstrating that the great majority of the PR3+B cells identified in patient’s blood are truly PR3-specific clones.
Recurrence and subset redistribution of circulating autoreactive B cells following RTX-induced remission in PR3-AAV

The frequency of circulating PR3⁺ B cells was significantly higher at B cell recurrence compared to baseline (median (25-75% IQR), 5.82%, (4.11-7.87) vs. 4.25% (3.77-5.30), p=0.025; gating strategy Figure 4A, PR3⁺ as % of B cells in Figure 4B). Similar to what was observed for total B cells, the composition of the B cell subsets within the PR3⁺ reactive pool at B cell recurrence was substantially different from baseline, with an increase in frequency of Transi (in blue, Figure 4C) and a decrease in frequency of mature naïve subsets (in green Figure 4C), a decrease in frequency of mature switched (SW) and unswitched (UnSW) memory subsets (in orange and yellow, respectively; p<0.001 in all cases except UnSW, p=0.002), and no significant change in frequency of mature double negative (DN) or plasmablast (PB) subsets (in grey and red, respectively Figure 4C).

Pairwise comparisons showed that the frequency of circulating transitional and naïve subsets were higher, and the frequency of mature, UnSW and SW memory subsets were lower within the autoreactive pool at B cell recurrence compared to baseline (p<0.001 for all comparisons; Figure 4D).

Dynamics of total B cells and autoreactive B cells and relapse during long-term follow-up

Overall, the dynamics of circulating total B cell and PR3⁺ B cell counts in peripheral blood of AAV participants that achieved complete remission following RTX-induction were similar between relapsers and patients who remained in remission throughout the time
points evaluated ($p>0.05$ in comparisons) (Figure 5A-B, Supplementary Figure 2A-C). After RTX, total B cells and PR3+$B$ cells were depleted (<10 cells/$\mu$L) in all participants (Figure 5A-B). Total and PR3+$B$ cells repopulated during follow-up in all participants, but only two participants had detectable total B cells and autoreactive B cells at month 6; of those, one relapsed, and one maintained remission throughout the follow-up. Of note, all participants that relapsed repopulated total and PR3+$B$ cells before the clinical relapse occurred.

The composition of the peripheral blood subsets within PR3+$B$ cells and total B cells was similar at baseline between relapsers and patients who remained in remission (Supplementary Figure 2C-D). At B cell recurrence, no differences in the frequency of total B cells were observed between relapsers and those that stayed in remission (median (25-75% IQR); 5.44% (3.65-10.45) vs. 4.11% (2.30-7.17), $p=0.410$) or of total PR3+$B$ cells (median (25-75% IQR), 4.65% (3.18-7.02) vs. 6.5% (5.34-8.44), $p=0.121$). The frequency of all subsets evaluated within B cells at B cell recurrence were similar between these two disparate long-term outcome groups (Figure 5C); in particular, the frequency of Transi and PB were similar between relapsers and patients who remained in remission. However, a significantly higher frequency of PB was observed within the autoreactive pool at B cell recurrence in relapsers compared to patients who remained in remission (Figure 4D), and this subset was further enriched in severe relapsers compared to non-severe relapsers (Supplementary Figure 2E). Overall, enrichment in the frequency of PB within the PR3+$B$ pool at recurrence was the only B cell biomarker that differed between relapsers and patients with long-term remission.
Relationship of enrichment of plasmablasts within circulating autoreactive B cells with relapse

Since PB were enriched within the circulating autoreactive PR3⁺ B cell pool in relapsers compared to patients with long-term remission at B cell recurrence, we wanted to determine a metric of PB frequency within autoreactive B cells associated with relapse risk. The median follow-up from B cell recurrence to the last clinical evaluation was 31 months (25-75% IQR, 18-42) for the entire group; 31 months (25-75% IQR, 23-42) for future relapsers and 32 months (25-75% IQR, 15-40) for patients who remained in remission (p=0.574).

At B cell recurrence, the frequency of PB within the circulating autoreactive B cell pool ranged from 0.0% to 5.0%, with a median of 1.6% (Figure 6A). We hypothesized that those with higher levels of PB within autoreactive PR3⁺ B cells where at higher risk of relapse. The optimal level of PB within autoreactive PR3⁺ B cells at B cell recurrence to discriminate patients who relapsed from those who remained in remission using a receiver operating characteristic curve was 1.6% (AUC=0.79, p=0.013, Supplementary Figure 3). We observed that study participants with ≥1.6% of PB within PR3⁺B cells at B cell recurrence had a significantly shorter time to first relapse and to first severe relapse (Figure 6B; log-rank test p-value=0.026; Figure 6C, log-rank test 0.007, respectively).

To explore whether an increased frequency of PB among circulating PR3⁺ B cells occurs before each relapse, we performed a case-crossover analysis to evaluate if each relapse was preceded by a rise of PB in autoreactive B cells within the previous 12 months. After B cell depletion, PR3⁺PB cells were redetected in all participants at some point during the follow up. Sixteen relapses occurred in 10 of the 23 participants studied
(Figure 1). Among relapsers, the increase of PBs within the autoreactive pool in the 12 months preceding each relapse was approximately 2-fold and significantly more likely (OR 2.10; 95%CI 1.07-4.11, p=0.031). In contrast, the increase of PR3+PB was smaller and non-significant among patients who remained in remission (OR 1.40; 95%CI 0.76-2.59, p=0.278), resulting in a case time-control OR for the autoreactive PB increase of 1.50.

Since the increase of PR3-ANCA has previously been shown to anticipate severe relapses in patients treated with RTX (11), we repeated the previous analysis with PR3-ANCA. At baseline, PR3-ANCA was detectable in all the subjects regardless the future relapsing status (210 RU/mL, 95%IQR: 70.9-327; versus 266 RU/mL, 95%IQR: 86.35-366, p=0.403). Although PR3-ANCA levels were increased in relappers compared to patients who remained in remission at B cell recurrence (p<0.05, Supplementary Figure 2F), PR3-ANCA levels higher than the median at B cell recurrence (36.1 RU/mL; range, 3.4 to 331) was not associated with shorter time to relapse or severe relapse at time-to-event analyses (log-rank test p=0.157 and p=0.414, respectively).

Consistently, after B cell depletion, increases of PR3-ANCA levels (if the assay had previously become positive) or reappearance (detectable from undetectable levels, if the assay was previously negative) did not anticipate the PR3+PB increase or reappearance (Supplementary Table 2).
DISCUSSION

This study shows a major change in the composition of B cell subsets within the circulating autoreactive pool after RTX-induced B cell depletion in patients with AAV that achieved complete remission, and it supports the hypothesis that an early enrichment of the PB fraction within autoreactive B cells during the reconstitution process is indicative of future relapse off therapy. This finding significantly contributes to clarifying the biological mechanisms underpinning disease relapse and prolonged remission in AAV, further providing mechanistic insights of the dynamics of B cells and autoreactive B cells in response to targeted therapy in AAV.

Specifically, we performed a detailed longitudinal analysis of autoreactive, antigen-specific PR3+ B cells before and after B cell depletion in patients with PR3-ANCA positive AAV who were successfully treated with RTX and did not receive any other potentially confounding treatment after remission induction. We compared baseline with the timepoint of B cell recurrence, which is characterized by an increase in the frequency of transitional and a reduction in the frequency of mature B cells. The depletion of B cells after RTX lasted for approximately 1 year in the majority of patients, and B cell counts remained lower than baseline at B cell recurrence. Clinically, the prolonged depletion of B cells after RTX treatment in AAV is well recognized (15,36), and it may impact on the B cell ontogeny at reconstitution, as shown by the increased proportion of the peripheral blood transitional B cells and correspondingly reduced memory B-cell compartments after RTX in patients with rheumatoid arthritis, lupus and pemphigus, and during reconstitution after hematopoietic stem cell transplantation (37–42) (reviewed in (40)). Notably, the timing of B cell repopulation following RTX therapy seems to be different in different
diseases, with AAV patients experiencing reconstitution of B cells later compared to patients with rheumatoid arthritis, connective tissue diseases and pemphigus (20, 36, 41).

Circulating autoreactive B cells were detectable in all the AAV subjects following B cell recurrence. The frequency of B cell subsets within the PR3+B cell pool differed significantly between B cell recurrence and baseline, and as for total B cells, there was a relative increase of the transitional and naïve subsets and a reduction of the memory subsets at B cell recurrence. Relapsers exhibited an enrichment of the PR3+PBs fraction (i.e., the precursor of the cells producing PR3-ANCA) compared to patients who remained in remission. Previous data have suggested that increased frequency of total PBs during remission is related to higher disease relapse in granulomatosis with polyangiitis patients (44). In contrast, our study shows that total PBs were not different between relapsers and patients who remained in remission at B cell recurrence, and only a small minority of total PBs were PR3+ at this stage (between 0 and 5%). Among these, it is likely that only a fraction of them will be able to terminally differentiate into plasma cells and produce high affinity PR3-ANCA IgG. An alternative explanation for the diverging findings by von Borstel et al. is that those patients received a variety of different additional conventional immunosuppressive treatments which may have affected the final results (44). For all those reasons, we believe that changes in PB-PR3+ could be more biologically meaningful than changes in total PBs in PR3-ANCA+ AAV.

Consistently, a higher frequency of PB within circulating autoreactive PR3+ B cells at B cell recurrence was associated with shorter time to relapse. Additionally, increased levels of PR3+PB were more likely to be found in subjects that relapsed in the following 12 months, and this increase was 50% more likely to occur when compared to patients
who remained in remission, strongly linking autoreactive PB to relapse. In addition, the
return of PR3-ANCA or increases in PR3-ANCA levels did not anticipate the return or
increase of the PR3⁺PB subset. In other words, autoreactive PBs are significantly higher
in relapsers compared to patients who remained in remission, further increasing in the 12
months preceding each relapse, being detected concurrently or before a PR3-ANCA titer
increase. Because of these associations in relation to the relapse timeline, one could
speculate that expansion of PB within the circulating autoreactive B cell pool may provide
a source of high affinity antibody secreting cells that promote relapses in AAV. In patients
with lupus undergoing B cell targeted therapy with belimumab, the percentage of
autoreactive naïve B cells decreased from baseline during follow-up while anergic B cells
seems to increase(22,40). In patients with rheumatoid arthritis treated with methotrexate
and TNF inhibitors, the posttreatment frequencies of autoreactive mature naïve B cells
were elevated and similar to pretreatment(45). Thus, there are differences and similarities
between different autoimmune diseases in their autoreactive B cell responses to different
immune-modulating therapies: our findings show that RTX in AAV does significantly affect
the autoreactive B cell pool composition in contrast with methotrexate and TNF inhibitor-
treated rheumatoid arthritis, by reducing the accumulation of autoreactive naïve B cells,
as occurs in belimumab-treated lupus patients. Taken altogether, our methodology may
prove useful in the future to assess restoration of tolerance in response to different
therapeutic agents in AAV.

From a methodological perspective, our customized flow cytometry-method using
rPR3 as a ligand has been proven to be specific for human PR3-BCR expressed by
hybridoma cell lines (27). We have also shown that only the B cells from patients with
PR3-AAV were able to secrete PR3-ANCA IgG as compared to MPO-AAV patients and HC in PBMC cultures and ELISPOT analysis (26).

ELISA of culture supernatants from bulk-sorted B cells and almost 200 single cell-sorted PR3+ and PR3- immortalized B cell clones showed that the PR3+B cell pool from PR3-AAV patients, as detected by cell surface staining with autoantigen in flow cytometry, contains antibody-secreting cells that are specific sources of PR3-ANCA. These data demonstrate that autoantigen-specific staining by FACS can accurately identify PR3-ANCA+ B cells in AAV, similar to what has been documented for other rheumatic diseases(22,46). However, in primary B cells, our FACS method may also identify a subset of B cells expressing BCRs with low affinity to PR3. To which extent low-affinity anti-PR3 BCR+ B cell populations significantly contribute to the observed PR3+B cell pool and produce PR3-ANCA with pathogenic potential, requires future studies at the single cell level.

One strength of our work is that it is based on the analysis of data and samples obtained during the conduct of a clinical trial; and subjects included in this study underwent standardized prolonged clinical monitoring and systematic blood sampling, and were treated with what is now considered standard-of-care (13). By means of the novel flow cytometry method that we used to detect autoreactive B cells in this longitudinal analysis, we were able to identify the repopulating autoreactive PR3+B cells throughout the course of the disease, providing new insights into the mechanisms of relapse, as shown by enrichment of PBs within the autoreactive pool.

This is a discovery study that has limitations. First, the number of patients studied is small and limited to PR3-AAV, and the % of PR3+PB is derived from a post-hoc analysis
that requires confirmation in future longitudinal studies. A precise, longitudinal characterization of antigen-specific B cells in different samples requires a lot of effort from both individual subjects and the study team. The subjects included were all treated with RTX to effectively induce remission and were off treatment during the follow-up according to the trial protocol, including GCs, which is known to be lymphocytolytic and to interfere with B cells (45,46), representing an exceptional model to study B cell dynamics. Second, not all the subjects had the same length of follow-up, and patients who experienced a severe relapse were retreated with additional cycle(s) of RTX, whereas non-severely relapsing subjects only received GC, according to the trial protocol. Third, all the samples studied were cryopreserved, which could theoretically have affected our findings on autoreactive B cell subsets. However, previously published studies have indicated that cryopreserved cells can be stored for decades without general tendency toward cell loss over time (47), without significant decrease in viability, and without changes in fluorescence intensity in subsequent flow cytometry for most of the subsets (including CD19 cells) (48). Furthermore, others have reported the percentage and absolute numbers of cryopreserved PB to be similar to those freshly analyzed (44). Finally, thanks to the use of cryopreserved samples, we were able to process all the samples under the same conditions (same flow cytometer calibration, etc.) potentially protecting the experiment from intercurrent non-biological variables usually referred to as batch effect.

In conclusion, our findings show the restructuring of B cell and autoreactive B cell compartments in peripheral blood after B cell depletion in AAV successfully treated with RTX and demonstrate that early changes within the autoreactive B cell pool are
associated with the future outcome. Our results indicate that enrichment of PB within the autoreactive pool after successful treatment with RTX is linked to subsequent relapse, identifying a potential mechanistic target for more durable treatment strategies in AAV.
Disclosures

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Contributions

AB, US and DC designed the study. AB, AH, and DC acquired the data. AB, SH, MCM, US and DC analyzed the data, AB, DC and US drafted the manuscript. MFK designed and conducted LCL single cell experiments and analyzed the data. All coauthors interpreted the results and analyzed critically the manuscript for important intellectual content and approved the final version.
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REFERENCES


cells for flow cytometry analysis effects on cell subset proportions and fluorescence intensity. *J Clin Lab Anal* 1991;5.
FIGURE LEGENDS

Figure 1. Swimmer plot showing response to RTX, relapse and B cell dynamics in PR3-AAV trial participants during follow-up (A). After the induction of remission with RTX and GC, complete remission off therapy including GC was achieved by all subjects (blue dots), before month 6th after randomization (grey shadow). Ten subjects relapsed during follow-up (red timelines), eight of them had severe relapse (red dots; non-severe-relapse, yellow dots), and five of them had multiple relapses during the observation period. All the patients repopulated B cell during follow up (green bars), in all relapsing patients and no patient relapse before B cells repopulated (B cells > 69 cells/μL).

Figure 2. Pairwise comparisons of absolute count (top panel) and frequency (lower panel) of B cells among cryopreserved PBMC between baseline and B cell recurrence (A). B cell subset gating strategy (B). Pairwise comparisons of the frequency of subsets within circulating B cells between baseline and B cell recurrence (C-D): at B cell recurrence, a median of 37.56% (25-75%IQR, 28.35%-51.56%) of circulating B cells expressed a transitional phenotype. PB (CD19+CD24-CD38high) did not significantly change from baseline (2C). Within the mature B cell pool (CD19+CD24low/highCD38low), the frequency of naïve (CD27-IgD+) cells was significantly higher; the frequency of unswitched (UnSW, CD27+IgD+), switched (SW, CD27+IgD-), and double negative (DN, CD27-IgD-) memory subsets was significantly lower at B cell recurrence compared to baseline (2D). Significant p value was reported as * when <0.05, ** when <0.01, *** when <0.001, **** when <0.0001.
Figure 3. Functional validation of autoreactive PR3+ B cells by sorting PR3+ and PR3− B cells. PR3+ and PR3− B cells were sorted and cultured (A). PR3-ANCA IgG as measured by ELISA after 4-day culture from bulk sorted PR3+ and PR3− B cells (n=3 PR3-AAV patients) (B, left histograms), B cells from HCs (n=4) (B, central histograms), and purified PR3-ANCA and MPO-ANCA from patients used as positive and negative controls (n=4), respectively (B, right histograms). Representative staining of cytospin smears at day 4 with May-Grünwald Giemsa to assess B cell activation and with anti-human IgG AMCA in immunofluorescence to assess initial cytoplasmic IgG accumulation (B, right images).

PR3-specific immunoglobulin secretion as measured by PR3-specific ELISA in cell culture supernatants (week 4) of single-cell sorted PR3+ and PR3− EBV-immortalized B cells from a patient with severe, untreated PR3-AAV (C). Native PR3 ELISA was used to measure PMSF-treated anti-human IgG and IgM. Red bars show mean background-corrected ODs and standard deviation. Cut-off for positivity was define as the 95th percentile of PR3- B cells. Recombinant patient-derived IgG anti-PR3 is shown as a positive control. Significant p value was reported as * when <0.05, ** when <0.01, *** when <0.001, **** when <0.0001.
Figure 4. Gating strategy for autoreactive B cell subset (A). Pairwise comparisons of frequency of PR3⁺B cells between baseline and B cell recurrence (B). Frequencies of B cells subsets within PR3⁺B cell pool at baseline and B cell recurrence (C). Pairwise comparisons of circulating PR3⁺B cell subsets between baseline and B cell recurrence (D). Significant p value was reported as * when <0.05, ** when <0.01, *** when <0.001, **** when <0.0001.

Figure 5. Counts of total B cells (cells/10⁶L) and autoreactive PR3⁺ B cells (cells/10⁶L) at baseline and during follow up (relapsers in red; patients who remained in remission in black) (A-B). All the patients repopulated total B cells and autoreactive B cells during follow up; two patients had detectable total B cells and autoreactive B cells at month 6. Frequency at B cell recurrence of subsets within total B cells (C) and autoreactive B cells (D) in subjects that relapsed (open bars) or maintained long-term remission (black bars). Significant p value (<0.05) was reported as * in the figure.

Figure 6. Distribution of PB frequency within autoreactive PR3⁺B cell pool at B cell recurrence by the median value of the cohort (blue <1.6%; red≥1.6%) (A). Time-to-relapse and time-to-severe relapse in AAV by PB within autoreactive PR3⁺B cell levels (blue <1.6%; red≥1.6%) (B-C).
Day 0: sorting and B cell cultures

Day 4: B cell activation upon stimulation and PR3-ANCA IgG detection in the supernatant

PR3 ELISA: Binding of monoclonal Ig from single cell-sorted PR3+ (n=96) and PR3- (n=93) immortalized B cells

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