The human bone marrow plasma cell compartment in rheumatoid arthritis - Clonal relationships and anti-citrulline autoantibody producing cells

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A majority of circulating IgG is produced by plasma cells residing in the bone marrow (BM). Long-lived BM plasma cells constitute our humoral immune memory and are essential for infection-specific immunity. They may also provide a reservoir of potentially pathogenic autoantibodies, including rheumatoid arthritis (RA)-associated anti-citrullinated protein autoantibodies (ACPA). Here we investigated paired human BM plasma cell and peripheral blood (PB) B-cell repertoires in seropositive RA, four ACPA+ RA patients and one ACPA− using two different single-cell approaches, flow cytometry sorting, and transcriptomics, followed by recombinant antibody generation. Immunoglobulin (Ig) analysis of >900 paired heavy-light chains from BM plasma cells identified by either surface CD138 expression or transcriptome profiles (including gene expression of MZB1, JCHAIN and XBP1) demonstrated differences in IgG/A repertoires and N-linked glycosylation between patients. For three patients, we identified clonotypes shared between BM plasma cells and PB memory B cells. Notably, four individuals displayed plasma cells with identical heavy chains but different light chains, which may indicate receptor revision or clonal convergence. ACPA-producing BM plasma cells were identified in two ACPA+ patients. Three of 44 recombinantly expressed monoclonal antibodies from ACPA+ RA BM plasma cells were CCP2+, specifically binding to citrullinated peptides. Out of these, two clones reacted with citrullinated histone-4 and activated neutrophils. In conclusion, single-cell investigation of B-cell repertoires in RA bone marrow provided new understanding of human plasma cells clonal relationships and demonstrated pathogenically relevant disease-associated autoantibody expression in long-lived plasma cells.

1. Introduction

During infection and autoimmune responses, activated B cells differentiate into antibody secreting cells as either short-lived plasma blasts or long-lived plasma cells (LLPC). LLPC in the bone marrow (BM) secrete large amounts of immunoglobulin and are the source of most of the serum IgG and IgA. They have a lifespan of years to a lifetime and are resistant to many B-cell targeted biopharmaceuticals, including anti-CD20 therapy. In rheumatoid arthritis (RA), seropositive patients develop rheumatoid factor (RF), anti-IgG Fc autoantibodies, and/or anti-citrullinated protein autoantibodies (ACPA) preceding diagnosis. ACPA are important diagnostic biomarkers, and evidence...
implicates that they also can have the capacity to directly affect RA pathogenesis in different ways [4]. In vitro and in vivo models have demonstrated that ACPA can activate osteoclasts and fibroblasts and mediate pain and inflammation [5–13]. Our previous single-cell studies have shown that ACPA can be produced locally by synovial fluid-derived antibody-secreting cells [14]. Others have reported that ACPA express have shown that ACPA can be produced locally by synovial fluid-derived pathogenesis in different ways [4]. In vitro and in vivo models have demonstrated that CD20 med [17], and ACPA have been reported to be also affected by other disease-modifying antirheumatic drugs [18–20], which would suggest that CD20 plasmablasts influenced by inflammation contribute to the serum ACPA pool. Yet, CD20-negative long-lived plasma cells may also produce ACPA, as implicated by the overall relatively stable levels over time and residual expression after CD20-targeted treatment. ACPA–memory B cells have a proliferative and activated phenotype [21], implicating constant antigen exposure, potentially promoting a continued replenishment of short-lived plasmablasts from the memory B-cell pool. However, ACPA+ B cells have also been reported to have an antigen-experienced transcriptional profile different from that of the more innate-like RF+ B cells, suggesting that ACPA+ B cells could be more primed to develop into LLPC compared to RF+ cells [22].

By generating human monoclonal antibodies derived from peripheral blood and synovial B cells, we and others have found that ACPA have several unusual features, such as a high degree of multireactivity to different citrulline peptides despite high level of somatic hypermutation (SHM) [10,14,15,23]. Almost all ACPA clones also carry N-glycosylation sites in their variable regions that SHM has introduced during affinity maturation [24–26]. However, for most clones, the glycosylation does not seem to affect citrulline-binding dramatically but has been speculated to influence antigen-independent B-cell selection or survival instead [25,27].

Even though the bone marrow plasma cell compartment is essential for our immunological memory, it has, due to limited accessibility, been much less studied than peripheral B cells in humans. The new single-cell sequencing technologies, which enable us to screen simultaneously the transcriptome and paired heavy and light chain Ig repertoire, can allow us to get additional insights into plasma cell repertoires. In the current study, we isolated human bone marrow plasma cells from RA patients using both conventional flow cytometry and droplet-based single-cell sequencing. We generated monoclonal antibodies from individual plasma cells followed by identification and characterization of ACPA+ bone marrow plasma cell clones. Our study also provides novel observations of the human plasma cell clonalities and B cell diversity in the bone marrow.

2. Materials and methods

2.1. Donors and clinical samples

Five seropositive RA patients fulfilling the 2010 ACR RA classification criteria [3] and undergoing hip arthroplasty were included in the study (Supplemental Table 1). All patients gave informed consent and the regional ethics review board in Stockholm approved the study. Clinical data and peripheral blood samples were collected at inclusion. Four out of five patients had ongoing treatment with Methotrexate (doses 12.5–25 mg weekly) and two patients had concomitant Prednisolone (dose 5–15 mg). Bone marrow from the proximal femur surgical material was collected from the surgical room and processed immediately. A cell suspension was prepared, and bone marrow mononuclear cells (BMMC) were obtained by Ficoll-Paque gradient separation (Cytiva). All samples were immediately prepared for flow cytometry sorting. However, when possible, BMMC were also cryopreserved for subsequent single-cell transcriptomics analysis (10X Genomics). Heparinised blood was collected, and for four out of the patients, peripheral blood mononuclear cells were isolated and cryopreserved. Sera were tested for ACPA positivity using CCP2 IgG test (CCPlus, Svar Life Science) and RF positivity with RF IgM ELISA (Inova Diagnostics). Patients were genotyped for HLA-DRB1 [28] and determined to be shared epitope (SE) positive if carrying HLA-DRB1*01 (except *01:03, *04:01 or *10). 2.2. Flow cytometry sorting of bone marrow plasma cells

Fresh BMMC were stained according to Supplemental Table 2 with specific antibodies for cell surface markers, including CD19, CD138, and CD27. Individual CD19+ CD138+ B cells were isolated by flow cytometry on a BD Influx Cell Sorter into 96-well plates and kept at –80 until further processing. Single cell Ig sequences were generated as previously described [29].

2.3. 10X droplet capture and transcriptomics of bone marrow B cells

BMMC and PBMC were thawed, and single B cell suspensions were enriched by magnetic cell sorting using the B cell Isolation Kit II (Miltenyi Biotec) and processed using the 10X Genomics Chromium Controller following the manufacturer’s instructions. 5′ gene expression and VDJ libraries were prepared using Chromium Single Cell 5′ Library & Gel Bead Kit v3 and the Chromium Single Cell V(D)J Enrichment Kit (10X Genomics). Sequencing was performed using NovaSeq 6000 (Illumina) and data were processed using Cell Ranger pipeline (version 7.0.0, 10X Genomics) and downstream analysed using the R package Seurat (version 4.1.1, 2021-08-10) [30] together with R studio (version 4.1.1, RStudio Team, http://www.rstudio.com/). A detailed description of 10X capture, library preparation, sequencing and data processing is available in the Supplemental Material, and Supplemental Table 3.

2.4. Immunoglobulin repertoire analysis

For 10X data, immunoglobulin alignment was performed using the Cell Ranger multi function and the Ensembl GRCh38 V(D)J compatible reference (10X Genomics). Flow cytometry sorted and 10X immunoglobulin sequences were annotated using VQuest towards the international ImMunoGeneTics (IMGT) database [31]. A detailed description of data filtering is provided in the Supplemental Material. Clonotypes were defined by paired VH-VL CDR3 amino acid sequences. Variable region amino acid sequences were determined to have N-glycosylation sites if they carried the consensus motif N-X/S/T (whereby X is any amino acid except proline). Statistical analysis was performed using JMP 15 (SAS Institute Inc) where p-values < 0.05 were considered significant. Frequencies were compared using Fisher’s exact test. Data were visualised by JMP 15 (SAS Institute Inc), Prism 9 (Graphpad Software LLC), Circos Table Viewer v0.63–9 [32] or R Studio. Network analysis was performed using Python 3.9 and the visualization using the pyNetworkPlot package (https://github.com/Bioinformatics/pyNetworkPlot) (see Supplemental Material).

2.5. Monoclonal antibody expression

Recombinant human IgG monoclonal antibodies were expressed from plasma cell immunoglobulin variable region sequences as previously described [29] (see Supplemental Material).

2.6. Evaluation of mAb antigen binding

All expressed mAbs were screened at 5 μg/ml with CCPlus ImmunoScan ELISA (Svar Life Science) following the manufacturer’s instructions. The mAbs were also analysed for unspecific polyreactivity at 5 μg/ml using a soluble membrane protein (SMP) assay [23]. Expressed mAbs with adequate amounts were screened for binding to citrullinated peptides, native control peptides, and control antigens using a custom-designed antigen microarray, Supplemental Table 4 (Thermo Fisher Scientific, ImmunoDiagnostics) [33]. ACPA clones and germline
converted versions were further evaluated using in-house developed peptide ELISA for binding to citrullinated human histone 4 (Cit-His4_14-34 GAK-Cit-H-Cit-KVL-Cit-DNIQGITKPAI), hnRNP A1 (cyclic Cit-hnRNP A1_211-224 GDNDFG-Cit-GGNFGSR) and fibrinogen (Cit-Fib565-583 HHPGIAEFPS-Cit-GKSSSYSKQF) and the native arginine control peptide. The histone 4 and fibrinogen peptides have previously been reported as ACPA targets [34,35]. The hnRNP peptide was identified using an array screening (>50 000 cit-peptides; Nimblegen, Roche) [23]. Biotinylated peptides were captured on streptavidin-coated high-capacity plates (Thermo Fisher Scientific) at 1 μg/ml. In addition, we used modified citrullinated vimentin peptide plates (Ogentree Diagnostika; Cit-Vim_hn5-60 GRVYAT-Cit-SSAVR). Binding of the mAbs was evaluated as serial dilution from 5 μg/ml to 0.2 μg/ml IgG in RIA buffer (1% BSA, 325 mM NaCl, 10 mM Tris-HCl, 1% Tween-20, 0.1% SDS) and detection using HRP conjugated Fab’ goat anti-human IgG(γ) (Jackson Immunoresearch) and TMB substrate (Biolegend).

2.7. ACPA binding to activated neutrophils

Whole blood from healthy donors was incubated 1:1 with 3% Dextran (Sigma-Aldrich) in 0.9% NaCl at RT until red blood cell segregation, followed by Ficoll Paque (Cytiva) separation. The granulocyte pellet was resuspended with 2 ml 0.2% NaCl for 45 s followed by washing the cells three times in ice-cold PBS. 1 × 10^6 cells/ml were seeded in 6-well plates with RPMI 1640 medium containing 1% l-Glutamine (Sigma-Aldrich) and 2% FBS (Sigma-Aldrich). To induce activation, neutrophils were stimulated with 25 μM A23187 (Sigma-Aldrich) for 45 min at 37°C. Cells were fixed and permeabilised with the BD Cytofix/Cytoperm kit (BD Biosciences) and stained with 50 μg/ml human ACPA/control mAb together with 20 μl FcR blocking reagent (Miltenyi Biotec). ACPA binding was detected with APC mouse anti-human IgG (BD Biosciences). Cells were fixed with 1% PFA before acquisition with FACSVerse (BD Biosciences) and analysis with FlowJo™ (BD Biosciences).

3. Results

3.1. Flow cytometry sorting and single-cell IgG amplification from bone marrow plasma cells

Bone marrow samples were obtained from five seropositive RA patients undergoing hip replacement surgery (Fig. 1a, Table 1 and Supplemental Table 1). One patient was ACPA+ RF+ (patient 1) by RF IgM and CCP2 IgG ELISA and four patients (patients 2–5) were ACPA+ RF+ with different profiles of ACPA fine-specificities (Fig. 1b). The CCP2+ patient did not have any cit-peptide reactivity above cutoff for the assay. HLA typing showed that all five carried HLA-DRB1 shared epitope alleles (Table 1). BM plasma cells were single-cell sorted by flow cytometry based on CD19 and CD138 positivity (Fig. 1c-e, Supplemental Fig. 1). The frequency of the plasma cells among lymphocytes (0.4–3.5%) or among CD19+ B cells (4.3–16%) varied between patients (Fig. 1c-d).

Single-cell multiplex PCR amplification of immunoglobulin transcripts generated 509 complete paired heavy-light chains from IgG BM plasma cells that were used for the downstream analysis (Table 1). We observed a high level of SHM (average 45–57 mismatches) in all patients (Fig. 1f). The frequency of IgG that carried N-glycosylation sites in their variable regions differed between patients (Fig. 1g), with the ACPA+ patient 1 displaying the lowest frequency (15%) and the ACPA+ patients 2 and 5 displaying the highest levels (32% and 34%, respectively).

3.2. 10X droplet-based single-cell RNA sequencing and Ig analysis of BM plasma cells

In addition, we performed an in-depth single-cell RNA sequencing analysis of BM B cells from two patients, patient 1 (ACPAS+CD19–) and patient 2 (ACPAS+), which were also included in the flow cytometry study. The BM mononuclear cells were carefully recovered from cryopreservation, and B cells were enriched using negative selection before analysis. The transcriptomics analysis confirmed that most of the analysed BM cells were B cells expressing CD19, CD20/MS4A, and immunoglobulins. Two small clusters were non-B cells displaying features of CD14+ monocytes and CD3+ T cells (Fig. 2a-b and Supplemental Fig. 2). Recovery and transcript quality differed between the samples with patient 2 having fewer transcriptomic events than patient 1 (n = 4625 and n = 896 events for patients 1 and 2, respectively). However, additional quality control filtering of cells and data integration using the Harmony package when merging datasets, ensured accurate clustering (Supplemental Fig. 3).

Plasma cell clusters were identified based on their characteristic expression profile including elevated MZB1, XBP1, and JCHAIN consistent with the literature (reviewed in Refs. [36–38]), as well as a high frequency of class-switched immunoglobulin transcripts (Fig. 2c-e, Supplemental Fig. 2). Differential expression analysis also revealed increased expression of DEBL3 and FKBPI1 in the plasma cells compared to the other B cells (Fig. 2c-d). Interestingly, we observed a separate cluster of cells primarily originating from patient 2, cluster 3, sharing some plasma cell transcripts features but at a lower frequency. Both the identified plasma cell clusters and cluster 3 demonstrated overall low CD19 and CD20/MS4A1 expression. For patient 1, a minor subset of the plasma cells (9%) had detectable CD19, and those cells were primarily IgA- cells (74%).

The analysis generated 176 BM plasma cell-derived paired Ig sequences for patient 1 and 245 for patient 2. We could detect both similarities and differences when comparing the Ig plasma cell repertoires between the two patients (Fig. 3). The plasma cells in both patients were dominated by IgA1+ cells (patient 1 = 42%, patient 2 = 41%) and IgG1+ (patient 1 = 21%, patient 2 = 36%) and (Fig. 3c-e). In the IgG compartment, patient 1 had a significantly higher frequency of IgG3 (26% vs 11% in patient 2, p = 0.02). Interestingly, the ACPA+ patient 2 had detectable IgG4+ plasma cells while they were not found in patient 1. In the IgA compartment, the ACPA– patient 1 had a higher frequency of IgA2 compared to the ACPA+ patient 2 (20% vs 11%, respectively). As expected, a majority of the class-switched clones displayed high level of somatic hypermutations, 91 and 92% of VH chains with more than 15 SHM and with an average of 49 ± 23 and 52 ± 23 IgG/IgA VH-VL SMH, in patient 1 and patient 2, respectively. Furthermore, the ACPA+ patient 2 had significantly higher frequency of bone marrow plasma cells expressing IgG with N-glycosylation sites, 35% compared to 17% in patient 2 (p = 0.0001) We could not observe any differences in VH gene usage based on VH families (Fig. 3a-b, f-g).

3.3. Overlapping clones in flow sorted and 10X BM repertoires

In the analysis we also compared the IgG BM plasma cell repertoires obtained either by flow cytometry sorting of CD19+ CD138+ cells or single-cell transcriptomics (Fig. 4). Collectively, we observed a similar degree of IgG VH-VL SHM in transcriptomics identified sequences compared to flow sorted (45 ± 19 vs 46 ± 19 for patient 1; 56 ± 24 vs 50 ± 24 for patient 2). Patient 1 displayed an expanded VH sequence in the flow cytometry data that were not expanded in the 10X data which was shown as a peak in the HCDR3 length histogram (Fig. 4a-b). There was a trend for a higher frequency of IgG sequences carrying Fab-glycosylation sites in the 10X captured cells compared to flow cytometry sorted (20% vs 16% for patient 1; 38% vs 32% for patient 2) but the difference was not statistically significant (Fig. 4e). We identified a few clonotypes that were shared between the plasma cells and other bone marrow B cell populations (Fig. 4c-d). Moreover, in patient 1 there was one clonotype that were found in both flow cytometry isolated plasma cells and in the 10X plasma cells, and two clonotypes that were overlapping between flow cytometry and other BM B cells sequences. Patient 2 had two clonotypes that were found in plasma cell identified either by flow cytometry or 10X sequencing, whereof one was also found among non-
Fig. 1. Study strategy and flow cytometry sorting of RA bone marrow plasma cells

a, Schematic overview of the bone marrow plasma cell study. b, Serum ACPA IgG fine-specificities by antigen microarray (Thermo Fisher Scientific), CCP2 IgG by CCPlas (Eurodiagnostics) and ELISA RF IgM reactivity (Inova Diagnostics) in the investigated RA patients. Values were scored 0–4 to generate the heatmap. c–e, Results from flow cytometry analysis of bone marrow B cells. c, Frequency of CD138+ cells among all bone marrow lymphocytes. d, Frequency of CD138+ cells among CD19+ cells. e, Gating of CD19+ CD138+ cells for sorting. f, g, Total number of IgG VH-VL somatic hypermutations (SHM) among plasma cell sequences obtained from flow cytometry sorting (N = 97; 92; 64; 194; and 62 paired heavy-light chain sequences in patients 1–5 respectively). Sequences were annotated by comparing to predicted germline VH/VL genes in IMGT. g, Frequency of variable region N-glycosylation sites (N-X-S/T where X = any amino acid except proline).
plasma cell BM B cells (Supplemental Tables 6–7).

3.4. Analysis of clonal relation with peripheral blood

For four of the RA patients (patients 1–4) cells were available for analysis of peripheral blood (PB) single B cells from the time of surgery by RNA sequencing (Figs. 5–6, Supplemental Fig. 4). The analysis showed that some of the samples were only partially enriched for B cells, and we therefore performed a pre-filtering step of the transcriptomics data to remove most non-B cells events before data integration with Harmony. Differential expression and analysis of expression of B cell lineage markers were used to identify naïve B cells (CD27 - IgD-; clusters 1, 3, 8, 10) memory B cells (CD27 + IgD-; clusters 2, 6, 7, 11), double negative (CD27 - IgD- ) and plasmablasts (CD27 + CD38hi, cluster 14) (Fig. 5 a-c). Within the naïve B cells, cluster 8 most likely represent CD27 - IgD- CD38 transitional B cells. The frequency of B cells subsets differed in the analysed patients, with for instance the plasmablast cluster being more prominent in patient 4 (Supplemental Fig. 4). Moreover, the recovery of paired BCR sequences varied (176 ± 58, Supplemental Table 5). The analysis confirmed an enrichment for class-switched immunoglobulins in the identified memory compartment and predominance for IgM expression in the naïve clusters (Fig. 5b). Repertoire analysis of paired VH-VL sequences illustrated gene usage and clonal diversity (Supplemental Fig. 5). Yet, despite the relatively low number of obtained of PB Ig sequences, we could identify paired VH-VL CDR3 clonotypes that were shared between blood and bone marrow in three out of four individuals (Fig. 5 d-e, Supplemental Tables 6–8). Notably, all peripheral blood clonotypes that were shared with bone marrow originated from memory B cell clusters. In patient 1, two of the PB-BM shared clonotypes had VH3-7/KV2-28 re-arrangement, although not clonally related. The other two clonotypes carried VH1-46/KV2-28 and VH3-74/KV3-3-15 pairing. In patient 2, the shared clonotypes displayed VH4-4/KV3-20 and VH6-9/KV1-39 re-arrangements (Supplemental Tables 6–8). Moreover, all shared clonotypes were of the same isotype and subclass, except one clonotype in patient 2 that were expressed as an IgA1 in the blood and an IgG1 in the bone marrow.

3.5. Clonality and public clones in BM

Expanded BM plasma cell clonotypes in 2–3 copies were found in four out of five RA patients, defined by identical paired VH-VL CDR3 amino acid sequences (Fig. 6 a–c). Analysis of variable gene usage in the expanded clonotypes showed different preferential gene usage in the different patients (Fig. 6 b–c). Clones with identical complete VH-VL amino acid sequences as well as clonally related sequences with minor amino acid differences were observed. Patient 1 generally had a higher frequency of expansions than the other patients in both flow and 10X derived clones. There were no VH-VL public or stereotypic clones that were shared between patients. However, when analysing the VH separately, we found three VH bone marrow public clonotypes. One expanded VH sequence were shared between flow cytometry sorted cells from patient 1 and patient 5 with identical HCDR3 amino acid sequence. There were also an additional VH-clonotype shared between patient 1 and 5 and one that were shared between patient 1 and 2. Nevertheless, these clones differed significantly based on light chain expression (Fig. 6 d-e, Supplemental Table 9).

3.6. Evidence of receptor revision and clonal convergence in bone marrow plasma cells

The availability of paired heavy-light chain Ig sequences made it possible to perform a unique analysis of clonal lineages in the BM. We identified several cells with identical clonotypes by HCDR3 but different LCDR3 sequences. After excluding lineages with the same V-J recombination and LCDR3 length, where different LCDR3 could be ascribed to SHM, five SHM class-switched lineages in the ACPA patient 1, one lineage in the ACPA patient 4 and three in patient 5 contained clonal plasma cells with the same heavy chain but different light chains (Fig. 7 a–c; Supplemental Table 10). There was also an example of an IgA lineage outside of the plasma cells compartment in patient 1 with identical heavy chains but different light chains (Supplemental Table 11). Notably, all complete VH sequences had the same length, with no insertions or gaps. Interestingly, in some lineages, most light chains were identical or similar, but with one or two examples of different light chains. However, there were also examples of lineages with completely identical heavy chains, but a wide diversity of light chain combinations as visualised by network analysis (Fig. 7d).

3.7. Expression of recombinant IgG and identification of ACPA+ clones

Our previous data [10,14,25] show that most ACPA+ clones carry high SHM and N-glycosylation sites. Consequently, we here used these features as criteria when selecting BM plasma cell clones for recombinant IgG expression. We expressed clones from the flow cytometry single-cell data set for all five RA patients and additional clones from the 10X BM data set for patient 2, resulting in 55 expressed IgG1 mAbs, of which 50 clones were from plasma cells (44 from ACPA+ patients) and five clones were from cluster 3 in patient 3 that shared some plasma cell features. Most clones originated from IgG B cells, but five clones from patient 2 were originally IgA whereof four originated from plasma cells. All purified mAbs were screened for ACPA positivity by CCP2 assay at 5 μg/ml. We identified three ACPA+ clones with CCP2 reactivity, two
from patient 2 (254:17D08, CCP2 = 371 AU; 254:C7X1604, CCP2 = 610 AU) and one from patient 4 (268:07G01, CCP2 = 35 AU) (Table 2).

Interestingly, none of the ACPA clones were expanded in the patients and we could not find any ACPA-related clonotypes, neither based on VH-VL gene usage, or by aligning to either VH or VL separately (Supplemental Fig. 6). Moreover, the identified ACPA clones did not show any CDR3 sequence similarities (Table 2). The three BM-derived plasma cell ACPA+ clones were subsequently evaluated for citrulline fine-specificity and neutrophil interactions.

3.8. Citrulline binding properties of BM-derived ACPA

All expressed mAbs were investigated for additional citrulline reactivity with a peptide array and ELISA. Only the three CCP2 reactive clones showed specific reactivity to citrullinated peptides (Figs. 8b and 9a-b). Importantly, the ACPA clones did not have any reactivity to the tested native peptides and control antigens. The two clones with the highest CCP2 reactivity (254:17D08 and 254:C7X1604) showed binding to ‘classical’ RA autoantigen, i.e., citrullinated vimentin, fibrinogen, and histone 4. The third clone (268:07G01), with low CCP reactivity, only showed reactivity to citrullinated hnRNP A1 (Fig. 9b). When all the SHM of the ACPA clones (96 SHM 254:17D08, 135 SHM 254:C7X1604, 60 SHM 268:07G01) were reverted to the predicted germline (Supplemental Fig. 7), the citrulline reactivity was lost for 254:17D08 and 254:C7X1604. Interestingly, while the germline 268:07G01 also lost all CCP2 reactivity (Fig. 9a and b), the reactivity to citrulline hnRNP A1 was attained. Neutrophil extracellular traps (NETs) generated by infiltrating synovial neutrophils have been suggested to be a source of ACPA-targeted autoantigens in RA, and citrullinated histones have been especially pinpointed. Therefore, we investigated binding by flow
cytometry of the ACPA clones and germline versions to ionophore activated primary human neutrophils. Indeed, the two BM-derived Cit-histone 4-reactive ACPA bound A23187 activated human neutrophils, as detected by flow cytometry in three different donors (Fig. 9 c-d). None of the germline versions showed any binding. Hence, our data confirm that ACPA produced in the bone marrow could have an active role in RA pathogenesis through neutrophil and NET targeting.

4. Discussion

In the current study, we had the opportunity to investigate the seropositive rheumatoid arthritis bone marrow compartment. For the first time, we can show that ACPA can be produced by plasma cells in the bone marrow and that BM-derived ACPA monoclonal antibodies have disease-relevant properties by targeting activated neutrophils. Additionally, our results provide important insights into human bone marrow
plasma cell clonality and diversity. We demonstrate differences between individuals in subclass, variable gene usage, and repertoire diversity. We show plasma cell clonal expansions and evidence of receptor revision in class-switched BM plasma cells in RA patients.

We used two strategies to isolate, identify, and investigate bone marrow plasma cells; flow cytometry sorting of CD19^+ CD138^+ cells and single-cell sequencing using the 10X Genomics Chromium platform. We found ACPA-expressing cells using both approaches. These cells did not belong to any expanded clonotypes, and we could not find any related clones in our analysis. Intriguingly, one of the identified ACPA^+ clones (254:17D08) was of the IgG4 subclass. Serology has previously shown that while serum ACPA is predominantly IgG1, it can also be IgG4 [39]. It can be speculated that this results from a chronic immune response. We could here observe an overall higher frequency of IgG4^+ BM plasma cells in the ACPA^+ patient compared to the ACPA^- RF^+ patient (both carriers of a genetic RA risk allele). While no conclusion about BM can be made from only two individuals, we previously reported elevated frequency of circulating IgG4^+ B cells in ACPA^+ RA compared to healthy individuals [40]. Importantly, the patients included in the current study were representative for patients in need to arthroplasty large joint surgery, i.e. seropositive, and with a history of destructive disease. Hence, BM in early RA may not necessarily share the same features.

Both single-cell methods identified a majority of cells as class-switched and with a high level of SHM, consistent with plasma cells. Current mounting evidence suggests that bona fide long-lived plasma cells both in the gut and bone marrow have a low CD19 expression [41, 42]. In contrast, the CD19^+ plasma cells in the bone marrow have been reported to represent antibody secreting cells that are more short-lived,
although still with a lifespan of years. Based on the protocols when the study was initiated, we only flow cytometry sorted CD19+ plasma cells. Re-analysis of the flow cytometry data showed that CD138+CD19+ plasma cells were indeed present in the BMMC samples (16–42% of CD138+ cells, Supplemental Fig. 1), but these cells were unfortunately not flow cytometry captured for further studies. From 10X sequencing, on the other hand, all identified plasma cell clusters had low CD19 levels compared to the other bone marrow B cell clusters, and we could not discriminate any distinct subpopulations based on CD19 transcripts. Notably, flow cytometry was run on fresh samples while cryopreserved samples were used for the subsequent 10X pipeline, which may influence the results. Hence, we assume that we captured partly different subsets of antibody secreting cells with these two approaches. Even so, we identified shared clonotypes between the flow cytometry sorted and single-cell transcriptomics plasma cell subsets.

The identified plasma cell clusters had transcription profiles consistent with other reports, with increased expression of genes associated with protein folding and transportation compared to other B cells and decreased expression of HLA/MHC class II and other genes involved in T-cell interactions [43,44]. We could detect plasma cell markers in four
different clusters (clusters 6, 9, 12, and 13) with robust expression of features (e.g. XBP1, MZB1). One cluster, primarily originating from patient 2, cluster 3, shared some plasma cell features possibly implicating an intermediate state. Interestingly, the plasma cells had higher coverage both in the transcriptomics library and the VDJ library than the other clusters, presumably due to their size and high transcription activity.

Interestingly, although the LLPC repertoire reflects our immunological memory of life-long encounters and should cover a large diversity of antigens, we still found several expanded clonotypes. Human B-cell phenotypes have previously been observed to be stable between different BM sites [45] and the plasma cell antibody repertoire relatively persistent over time [46]. Hence, we found it remarkable that we could find paired VH-VL clonotypes that were shared between the peripheral memory B cell and bone marrow repertoires.

Moreover, our analysis of the plasma cell compartment revealed class-switched clonotypes carrying high SHM with identical heavy chain VDJ rearrangements but different light chains. The identical heavy chains were identified based on CDR3, but in many cases, they were identical on nucleotide level in the complete VDJ sequence. We

Fig. 6. Clonal diversity in RA bone marrow plasma cells

a, Clonotype frequencies in the different patients for the 20 most abundant clonotypes identified by VH-VL CDR3 amino acid sequences. b, Bubble graph visualization of VH-VL gene pairing of unique clonotypes based on VH-VL CDR3. Red colour and size of the bubbles are proportional to frequency (%) of unique VH-VL CDR3 clonotypes. c, VH-VL pairing in expanded BM plasma cell clonotypes in patient 1–4. No expanded clonotypes were detected in the sequences from patient 5. Both clonotypes identified in 10X and flow cytometry were included for patient 1–2. d–e, Network representation of bone marrow plasma cells by complete VH VDJ variable region amino acid sequences (d) and by the complete VH-VL V(D)J amino acid sequences (e). Each node represents a single sequence; red links indicate identical sequences, and black links similar sequences with up to eight amino acid differences. Nodes are coloured based on patients. Squares represent immunoglobulin sequence with any N-glycosylation sites in either VH or VL.
speculate that these could either represent receptor revision, i.e., receptor editing and re-arrangement of the light chain second allele in mature antigen-experienced cells [47]; alternatively clonal convergence, i.e., the heavy chain rearrangement is critical for antigen recognitions, while the light chain is not, and the observed clonotypes have evolved as parallel events. Notably, the heavy chain members were of the same subclass which would point towards a peripheral receptor revision event. On the other hand, we found three lineages that shared HCDR3 but not light chains between two patients. It has recently been reported that there is indeed a previously unappreciated high frequency of shared clonotypes, so-called public clones or stereotypic receptors, in human B-cell repertoires [48]. Preferential pairing, gene usage, and stereotyped BCRs have been observed in both infection and autoimmune responses [49]. Interestingly, the public VH clonotypes found here used VH3-7, VH1-69 and VH4-39 that have previously been reported in stereotypic receptors, yet the clones all carried SHM.

IgA expressing plasma cells in the RA bone marrow were frequent (46–53%), consistent with previous studies reporting around 40% IgA+ plasma cells in healthy human BM [50,51]. Serum IgA primary originating from expression in the bone marrow and has a higher proportion of IgA1 vs IgA2 than mucosal IgA. Yet, proteomics and repertoire analysis have shown that there is a clonal relationship between gut and serum IgA [52] and that IgA expressing cells originating from mucosal immune responses can be found in the bone marrow [50]. Although we cannot draw any conclusions from the two individuals, it is interesting that the proportion of IgA2+ BM plasma cells was lower in the ACPA+...
than in the ACPA− individual. We have previously detected higher serum levels of total IgA in ACPA+ RA but a lower frequency of circulating IgA+ B cells compared to healthy controls [40]. In the current study, the flow cytometry approach only included IgG transcript amplification, hence not permitting a direct comparison between IgA+ plasma cells in the different methodologies. Our previous studies have identified IgA+ ACPA+ circulating memory B cells in RA [10]. Naturally, further investigation of ACPA IgA expression in bone marrow would be relevant.

We expressed 44 mAbs from bone marrow plasma cells from ACPA+ RA patients.

### Fig. 8. Expression of human recombinant monoclonal antibodies from bone marrow plasma cells

Monoclonal recombinant IgG1 were expressed from selected bone marrow plasma cell clones. All expressed clones carried high SHM and variable region N-glycosylation sites (N-X-S/T). a, Distribution of IgG+ (left) and IgA+ (right) plasma cells SHM, with expressed clones highlighted in red. b, Citrulline reactivity was assessed with citrullinated peptide array, CCP2 ELISA, citrulline peptide ELISA, and polyreactivity by the soluble membrane protein SMP assay. The heatmap shows reactivities scored 0–5 with red being high binding. 55 expressed IgG1 mAbs of which 50 clones originated from identified plasma cells (denoted with *). We identified three clones as ACPA+ with specific citrulline reactivity (two from flow cytometry and one from 10X transcriptomics) from two different ACPA+ RA patients.
RA patients and found three plasma cells expressing CCP2 specific immunoglobulins (7%). However, we used a pre-selection strategy where we expressed clones with high SHM (>$15$ SHM in either chain), and variable region N-glycosylation sites to increase the success rate. The rationale was based on previous data showing that these are significant features of synovial and blood ACPA+ cells $[10,14]$. Hence, the real frequency of ACPA-producing clones could be closer to three out of $657$ (0.5%) due to the total number of analysed cells from the ACPA+ patients. Notably, a majority of BM IgG+ and IgA+ plasma cells in the current study had high mutation levels. However, the frequency of N-glycosylation sites varied. Yet, for the ACPA positive patients, the frequency (22–35% of IgG) was in the higher range compared to the 15–25% expected from serum IgG in healthy individuals (reviewed in Ref. $[53]$). Interestingly the ACPA negative patient had a lower frequency (17%). We think that the selection strategies increased our probability of finding ACPA+ clones, yet we may have missed ACPA+ clones that did not fulfil our criteria for expression. It is also important to acknowledge that N-glycosylation, although a feature of ACPA, is also present in other antibody responses $[54]$.

We have previously shown that monoclonal ACPA have extensive multireactivity to citrullinated antigens, explained by recognition of small peptide epitopes consisting of the citrulline residue and a few critical flanking amino acids, which can be present in different antigens $[10,14,23]$. Indeed, one out of three BM ACPA clones showed a high degree of multireactivity, while the other two were more restricted in their citrulline binding patterns. ACPA reactivity extends beyond the regular RA citrullinated candidate antigens $[23]$. Hence, it is difficult to predict the pathogenic target of ACPA in RA. Nevertheless, we were intrigued to find a weak CCP2+ ACPA clone with strong mono-reactivity to the cit-hnRNP A1 peptide. This peptide is displaying a gly-cit-gly motif and both the gly-cit and cit-gly motifs were previously found to be prevalent targets of ACPA. While native hnRNP-A1/B1 (RA33) $[55]$ and citrullinated hnRNP DL $[56]$ have been demonstrated to be auto-antibody targets, anti-cit-hnRNP A1 has not been previously reported. Notably, hnRNP A1 is one of the most highly abundant proteins in the nucleus with multiple functions mediated by RNA binding $[57]$. Intriguingly, nucleic acid binding proteins are commonly targeted by autoantibodies, suggesting the formation of stimulatory immune complexes. Moreover, the germline converted version of the cit-hnRNP A1 positive clone maintained the same peptide reactivity, implicating that the naïve B cell may recognise this antigen. However, it should also be acknowledged that predicting the ancestral germline V(D)J rearrangement is difficult in antibodies with high SHM without extensive lineage information, not the least due to incomplete coverage of allelic variation in the reference IMGT database. In the CDR3 junction, we converted mismatches in the V-region and J-chain alleles. Yet, since original residues in the non-template nucleotide insertions (N-insertions) region cannot be predicted, the CDR3s mostly remained intact.

In addition, we investigated neutrophil and citrullinated histone 4 interactions of the ACPA. While we know from our previous studies that
different RA anti-modified protein autoantibody clones can bind activated neutrophils and NETs by recognizing different antigens [58]. However, citrullinated histone 4 has been particularly implicated as an ACPA NET target in the literature [34]. Indeed, the two ACPA with cit-histone 4 binding also bind to ionophore activated neutrophils. Neutrophils and NETs have been hypothesised to contribute to RA pathogenesis as a source of autoantigens and drivers of inflammation (reviewed in Ref. [59]). Hence, ACPA binding to citrullinated histones and active NETs may be important. Yet, the role of Ca-induced PAD-dependent NETosis compared to other pathways can be debated, and neutrophils contribute to pathogenesis through cytotoxic pathways leading to induction of hypercitrullination [60]. ACPA can target many different autoantigens in the complex RA citrullinome, which may be potential candidates for driving disease [61].

5. Conclusions

Our findings contribute to new understanding of the bone marrow compartment with BM plasma cell repertoire data showing high frequency of expanded clonotypes, shared blood and BM clonotypes, presence of VH public clones, and evidence of receptor revision and clonal convergence. It also demonstrates the power of single-cell technologies and analysis of paired heavy-light chain sequences and full transcriptome. The complete paired Ig data enable both new understandings of clonalities and, importantly, permit generation of recombinant antibodies for investigation of specificity and functionality. We also show that ACPA can be produced by long-lived plasma cells in the bone marrow of RA patients, which is essential for current and future B-cell targeted therapeutic strategies.

Author contributions

VM, AC, AH and CG designed and planned the study and interpreted the data. LK initiated monoclonal work and provided additional scientific input. AH recruited patients and coordinated the study. RW performed orthopedic surgeries and facilitated collection of surgical samples. LI, JH and RT performed flow cytometry sorting, single-cell processing, and sequencing. BH conducted single-cell data processing and transcriptomics analysis and interpretation. PS expressed and characterised monoclonal antibodies. MH and LMA performed autoantigen array analysis of serum and mAbs. ChG contributed with cell processing and flow cytometry analysis and BR with neutrophil experimental design. JSDB performed network analysis. LMDG guided and designed computational analysis and interpretation. CG conducted V(D)J analyses and visualizations and wrote the first manuscript draft. All authors were involved in drafting the article and revising it critically. All authors participated in discussions finalizing the manuscript and approved the final version.

Funding sources

This work was supported by the Swedish Research Council, Sweden (2019-01664; 2020-02260), Sweden; Swedish Rheumatism Association, Sweden (R-940981; R-969403); Innovative Medicines Initiative (IMI), EFPIA, Belgium (Joint Undertaking project RTCure 777357).

Author statement

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Declaration of competing interest

Linda Mathsson-Alm is employed by Thermo Fisher Scientific. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Drs Per Wretenberg and Rüdiger Weiss for performing orthopedic surgeries and facilitate collection of tissues. We are also thankful to Dr Leonid Padyukov for HLA typing of the cohorts and informative discussions. We thank Gloria Rostvall, Susana Hernandez Machado and Julia Boström, for managing the cohort biobanking and handling of blood samples. We thank Dr Heidi Wåhämää for help with processing BM tissue. We would also like to thank Dr Khaled Amara for his contributions in 10X sample processing and Dr Peri Noori for technical support and advice regarding 10X experiments. We thank Ragnarh Stålesen for monoclonal antibody production and validation, and Annika van Vollenhoven for flow cytometry sorting. We also thank Sarks Tafnakaji for contributing to antibody cloning and expression. We thank Dr Holger Bang, Orgentec Diagnostika, for the generous gift of modified vimentin peptide assays. We are especially grateful to the RA patients in the study for their contribution to research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2023.103022.

References


