Paired single-B-cell transcriptomics and receptor sequencing reveal activation states and clonal signatures that characterize B cells in acute myeloid leukemia

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ABSTRACT
Background Acute myeloid leukemia (AML) is associated with a dismal prognosis. Immune checkpoint blockade (ICB) to induce antitumor activity in AML patients has yielded mixed results. Despite the pivotal role of B cells in antitumor immunity, a comprehensive assessment of B lymphocytes within AML’s immunological microenvironment along with their interaction with ICB remains rather constrained.

Methods We performed an extensive analysis that involved paired single-cell RNA and B-cell receptor (BCR) sequencing on 52 bone marrow aspirate samples. These samples included 6 from healthy bone marrow donors (normal), 24 from newly diagnosed AML patients (NewlyDx), and 22 from 8 relapsed or refractory AML patients (RelRef), who underwent assessment both before and after azacitidine/nivolumab treatment.

Results We delineated nine distinct subtypes of B cell lineage in the bone marrow. AML patients exhibited reduced nascent B cell subgroups but increased differentiated B cells compared with healthy controls. The limited diversity of SCR profiles and extensive somatic hypermutation indicated antigen-driven affinity maturation within the tumor microenvironment of RelRef patients. We established a strong connection between the activation or stress status of naïve and memory B cells, as indicated by AP-1 activity, and their differentiation state. Remarkably, atypical memory B cells functioned as specialized antigen-presenting cells closely interacting with AML malignant cells, correlating with AML stemness and worse clinical outcomes. In the AML microenvironment, plasma cells demonstrated advanced differentiation and heightened activity. Notably, the clinical response to ICB was associated with B cell clonal expansion and plasma cell function.

Conclusions Our findings establish a comprehensive framework for profiling the phenotypic diversity of the B cell lineage in AML patients, while also assessing the implications of immunotherapy. This will serve as a valuable guide for future inquiries into AML treatment strategies.

INTRODUCTION
Acute myeloid leukemia (AML) is a malignant hematological disorder characterized by the clonal expansion of myeloid precursor cells. It is the second most common adult leukemia, accounting for approximately 1% of adult cancer in the USA.1 Five-year overall survival for adult AML hovers around 30%, and for decades, the mainstay of treatment has been chemotherapy often followed by allogeneic stem cell transplant (alloSCT).

As the field of cancer immunotherapy advances, the interaction between AML and the host immune system has drawn particular interest. The success of alloSCT in inducing lasting remission is well-appreciated and appears to be largely driven by a graft-versus-leukemia effect.2,3 However, AML develops in the bone marrow where it is surrounded...
by immune effectors, yet manages to evade surveillance.\textsuperscript{1} More recently, investigations using immune checkpoint blockade (ICB) to induce antitumor activity in AML patients have yielded mixed results.\textsuperscript{5,6} Taken together, these observations suggest that AML can be susceptible to immune-mediated killing, yet AML cells are able to modulate host immunity to avoid detection and clearance. Whether this occurs by masking of neoantigens perceived as “other” by the immune system, or by co-optation of AML-reactive lymphocytes to adopt an immunosuppressive phenotype remains unknown, and dissecting the mechanisms by which AML evades immune clearance is of immunotherapeutic importance.

Much work has gone into understanding the anticaner role of T cells, largely because of their long-recognized role in mediating the graft-versus-leukemia effect, as well as the documented anticaner effects of tumor-infiltrating T cells.\textsuperscript{2,3,7,8} However, B cells and plasma cells also exist within the tumor microenvironment (TME), and their role has been less studied.\textsuperscript{9,10} A few recent studies have noted that B cell infiltration of solid tumors predicts response to ICB.\textsuperscript{11-13} Supporting the direct antitumor potential of B cells, several studies investigating solid tumors have demonstrated that tumor-infiltrating B cells and plasma cells are highly activated and differentiated, and demonstrate extensive somatic hypermutation (SHM), suggesting antigen-driven maturation within the TME.\textsuperscript{14,15} The role of B cells in AML specifically has only recently attracted attention. An earlier study used bulk RNA sequencing to characterize B-cell receptor (BCR) repertoires in pediatric and adult AML samples and found evidence of extensive activation, differentiation, and affinity maturation compared with healthy controls.\textsuperscript{16}

In the same study, patients with IgA-skewed polarization demonstrated worse survival, possibly related to an anti-inflammatory phenotype. A more recent study performed single-cell RNA sequencing on pediatric and adult AML samples and found evidence of extensive activation, differentiation, and affinity maturation compared with healthy controls.\textsuperscript{17} Further, atypical memory B cells were enriched in high-affinity AML and found to express suppressive markers, leading the authors to hypothesize that they contributed to AML immune escape.

Despite the advancements in AML research, there remains a significant gap in the comprehensive analysis of B cells in AML. Delving deeper into the dynamics of B cells during treatment, especially in the context of immunotherapies, represents uncharted territory in AML research. Moreover, associating the B cell phenotype with their BCR repertoire—which holds the potential to unveil intricate clonal dynamics and underlying biology—has yet to be undertaken in AML studies. This exploration is pivotal, emphasizing our commitment to shedding light on critical aspects that could redefine our understanding and treatment of AML. In this study, we pair single-cell transcriptomics with single BCR profiling in AML patients to evaluate how B cell phenotype and clonality vary at different stages of disease and treatment (figure 1A). We identify activation states, cellular interactions, and clonal signatures that appear to differentiate NewlyDx and RelRef patients, and which are likely directly related to immune control of AML. We further interrogate longitudinal clonotypic changes in B cell activation, differentiation, and expansion with ICB, and identify B cell populations and molecular features associated with ICB response. Taken together, our findings reveal the rich dynamic landscape of the B cell compartment in AML patients while also pointing to specific subpopulations that may be of prognostic or immunotherapeutic significance. To our knowledge, this study is the first to have interrogated the B cell compartment of AML patients with single-cell clonal resolution and fills an important knowledge gap by identifying specific B cell subpopulations and cellular interactions that may be targeted for therapeutic benefit.

**MATERIALS AND METHODS**

**Human participants and sample collection**

Six adult healthy donors and 32 patients with AML seen at The University of Texas MD Anderson Cancer Center were included in this study. Bone marrow biopsies were freshly frozen in freezing medium with 20% fetal bovine serum (FBS) and 10% dimethyl sulfoxide in Dulbecco’s modified Eagle medium. Then samples were stored in liquid nitrogen until thawing and library preparation.

**Sample preparation, library construction, and sequencing**

Frozen tumor and normal cells were retrieved immediately before library preparation. To maximize cellular viability recovery, samples were processed in batches using a thawing protocol. In brief, cells were gently thawed at 37°C for 5

\[\text{min}\] in a water bath until partially thawed and immediately placed on ice. Next, cells were transferred to 10

\[\text{mL}\] room temperature media supplemented with 20% FBS and centrifuged (453×g for 5

\[\text{min}\]). Supernatants were disposed, and the cell pellets were carefully resuspended in 10

\[\text{mL}\] thawing media (RPMI1640 supplemented with 20% FBS containing 500 µL of heparin (stock 2

\[\text{mg/mL}\], catalog no. 9041-08-1; Sigma-Aldrich), 20 µL of DNase (stock 1 µg/mL, catalog no. 89835; Thermo Fisher Scientific), and 200 µL of MgSO4 (stock 200 mmol/L; Sigma-Aldrich)) and incubated at 37°C for 15

\[\text{min}\] to digest the DNA. Following incubation, cells were centrifuged and gently washed again in 2

\[\text{mL}\] of 0.04% trypsin (Gibco) in PBS. Then, cells were filtered with a 35 µm mesh strainer directly into BD Falcon 5

\[\text{mL}\] bottom tubes with cell strainer caps to remove cell clumps. Eventually, 10

\[\text{µL}\] of cell suspension was stained with 10

\[\text{µL}\] of 0.4% trypan blue, counted, and assessed for viability using a standard hemocytometer and light microscopy. Cells were centrifuged and resuspended in the desired amount of PBS to adjust the cell density. The 5

\[\text{’}\] gene expression libraries were generated by 10× Genomics Chromium Controller instrument and Chromium Single-Cell 5\’ V5.1 reagent kits (10× Genomics). Library quality was assessed using
High-Sensitivity D5000 DNA ScreenTape analysis and a Qubit dsDNA HS Assay Kit. Sequencing was conducted using an Illumina NovaSeq sequencer with 2×100bp paired reads to reach a depth of at least 50,000 read pairs per cell. The detailed methods of sequencing were described in our recent study.18

**Figure 1** Schematic design of the research and single-cell BCR repertoire features in the bone marrow of AML patients. (A) Schematic diagram of the research design, visualized by BioRender (https://biorender.com/). Bone marrow aspirates were obtained from 6 healthy donors, 24 NewlyDx, and 22 RelRef AML samples. Single-cell RNA sequencing (scRNA) and single-cell BCR sequencing (scBCR) data were generated for each sample. B cells and plasma cells were isolated for further analysis. (B) The relative clonal space occupied by the clonotypes divided by the bins of top 1:3, 4:50, 51:100, and 101:200 in each sample, was generated by the scRepertoire R package (V.1.7.0). (C) Clonotype diversity distribution of different sample types calculated as 1/Simpson’s clonal diversity index (mean±SD). (D) Boxplot depicted the somatic hypermutation frequency across three groups of samples (left). Per cent bar graph showing the somatic hypermutation categories composition among three sample types. Germline: mutation frequency=0; low: 0<mutation frequency≤3%; medium: 3%<mutation frequency≤6%; high: mutation frequency>6% (right). (E) Fraction of IGHG and IGHA isotypes in B cell lineage among three groups of samples. The significance test between RelRef versus normal and NewlyDx samples was performed by the Wilcoxon rank-sum test (left). Per cent bar graph showing the isotype categories composition among three sample types (right). RelRef samples in (B–E) figures referred to Relapsed/Refractory patients prior to azacytidine+nivolumab treatment (20, 560 cells).

Single-cell RNA transcriptome sequencing raw data processing, filtering, and B cell lineage identification

ScRNA raw sequencing reads were aligned using the Cell Ranger pipeline provided by 10× Genomics to generate the gene read count matrix, followed by quality control removing the genes that were detected in less than
three cells and cells expressing fewer than 200 or more than 7000 genes, as well as those with more than 10% of transcripts derived from the mitochondrial genome. Data processing, unsupervised clustering, and cell cluster differentially expressed genes (DEGs) identification were mainly implemented by the Seurat R package\textsuperscript{19} (V.4.1.1) without specific indication. The UMI count was normalized by library size, and highly variable genes were identified, excluding mitochondrial, ribosomal, and BCR genes, which accounted for approximately 75% of the cumulative variance. We used PCA linear dimensional reduction on the scaled data, with the number of top dimensions determined by the elbow plot. The batch effect caused by samples was corrected by Harmony\textsuperscript{20} (V.0.1.0). Cells were clustered using the Shared Nearest Neighbor graph-based clustering approach and visualized using the Uniform Manifold Approximation and Projection, a non-linear dimensional reduction technique. Differential expression analysis was performed using the FindAllMarkers function with the default parameters to identify DEGs in each cluster. These clustering procedures were repeated multiple times to remove doublets and to define subgroups within the B cell lineage.

We identified two primary clusters of B-lineage cells for further investigation based on their expression of specific marker genes. The B cell cluster specifically expressed CD79A, CD79B, CD19, and MS4A1, while the second cluster expressed JCHAIN, MZB1, and CD38, which are associated with plasma cells. In addition, we confirmed the presence of productive V(D)J sequences in these cells, except for pro/pre-B cells.

To eliminate doublets mixed in with the B-lineage cells, we used the DoubletFinder R package\textsuperscript{21} (V.2.0.3) to measure doublet scores and predict possible doublets. We then implemented the following cluster-based criteria: (1) doublets-dominated clusters likely exhibit an aberrantly high gene count, doublet scores, or are distributed dispersively across multiple clusters. (2) Cells in the B cell cluster expressing markers of other lineages, except for B cell canonical marker genes, were removed. This includes cells showing expression of canonical marker genes of T or myeloid cells, as described in our previous studies.\textsuperscript{15,22} Additionally, we detected cells that contained both productive T cell receptors and BCRs or had ≥2 productive T or BCRs, with the aid of TCR and BCR repertoire sequencing.

**Single-cell BCR repertoire data assembly, clonotypes identification, and analysis**

We conducted single-cell 5′ V(D)J sequence assembly using the Cell Ranger vdj pipeline with the GRCh38 genome as the reference provided by 10× Genomics. To assign clonal clusters to immunoglobulin (Ig) sequences, and reconstruct germline sequences, we employed the Change-O Repertoire clonal assignment toolkit\textsuperscript{23} and Shazam R package (V.1.1.1). Antibody isotypes for B cells and plasma cells were identified based on the presence of immunoglobulin constant region categories. SHM frequency was measured and categorized into four groups: germline (mutation frequency=0), low (0<mutation frequency≤3%), medium (3%<mutation frequency≤6%), and high (mutation frequency>6%). We evaluated BCR gene and gene family usage, clonal abundances, and diversity using the Alakazam R package (V.1.2.0). The clonal diversity was measured using the inverse of the Simpson index, and the clonal diversity index (D) for each group was calculated as the mean value of overall resampling realizations, with the confidence intervals of clonal diversity derived from the SD of the resampling realizations. We then integrated the paired scRNA data with scRNA-seq data based on their matched unique cell barcodes. B cell lineage subtypes were defined by combining scRNA and scBCR portraits, as explained in the relevant section of the results.

**Plasma cells and B cells differentiation trajectory reconstruction and cell type transformation inferring**

For naïve and memory B cells, we used Monocle\textsuperscript{24} (V.1.2.9) to present more complicated evolution trajectories constructed using DEGs derived from two comparable groups, CD27+ memory B cells versus naïve B cells and atypical memory B cells versus CD27+ memory B cells. Additionally, we used transcriptome similarity among B cell subsets and BCR transition index as auxiliary proofs of the rationalization of the differentiation route deciphered by Monocle3. We applied two different algorithms, TransferData function in the Seurat package and the SingleR package\textsuperscript{25} (V.1.10.0), to measure the transcriptome similarity of B cell subgroups and calculated the subtype-level BCR transition indexes using the STARTRAC R package\textsuperscript{26} (V.0.1.0).

We reconstructed the plasma cell differentiation trajectory using the monocle R package\textsuperscript{27} (V.2.24.1). The transcriptome dynamics along with pseudotime were inferred based on the top 500 DEGs between plasmablast and plasma cells with default parameters. We considered the side starting from plasmablast cells on the pseudo-temporal trajectory as the “root” state. The Monocle2 inferred direction of differentiation was confirmed by CytoTRACE\textsuperscript{28} (V.0.3.3), a computational method for predicting the relative differentiation state of cells without prior knowledge.

**Public scRNA-seq and bulk RNA-seq cohorts’ acquisition and processing**

We acquired an external processed and annotated scRNA-seq dataset generated by Lasry \textit{et al}\textsuperscript{41} from the Single Cell Portal at https://singlecell.broadinstitute.org/single_cell/study/SCP1987. A total of 4963 B-lineage cells from 25 bone marrow aspirates from adult AML (NewlyDx) and control samples were obtained and kept for further analysis. Furthermore, there were two bulk RNA sequencing datasets involved in our research, including TCGA AML cohort\textsuperscript{42} and Abbas \textit{et al} cohort.\textsuperscript{30} We restricted our analysis to adult bone marrow samples from newly diagnosed AML patients in non-M3, M6,
Additional statistical analysis

Enrichment analysis of common highly expressed AP-1 related naïve and memory B cell subtypes was performed using the Metascape resource, which identified enriched biological process (BP) terms and constructed the functional network. Other overexpression enrichment analysis and gene set enrichment analysis based on BP terms, KEGG pathways and Hallmark were using ClusterProfiler R package (V.4.4.4), where BP and Hallmark gene sets were download from MsigDB and KEGG pathways were acquired by KEGGREST R package (V.1.36.3). The genes used to measure AP-1 complex activity included FOS, FOSB, FOSL1, FOSL2, JUN, JUNB, and JUND. Gene set module scores in single cells were calculated using the AddModuleScore function in the Seurat package. Differential abundance of B cell lineage subtypes between NewlyDx and normal samples testing based on k-nearest neighbor graphs was implemented by milo R package (V.1.4.0). The kernel density enrichment of sample types and antibody isotypes distribution was assessed along the differentiation trajectory. Taking sample types as an example, we estimated the probability density distribution of AML and normal isotypes along pseudotime using Gaussian kernel functions. Subsequently, the ratio of each pseudotime point was calculated. For the analysis of ligand-receptor interactions between B cells and AML cells, we specifically examined genes that were expressed in more than 10% of cells within each cell subtype, based on experimentally confirmed interactions. Manually collected confirmed ligand-receptor interactions are presented in online supplemental table 5. To compare ligand-receptor gene expression levels in bulk datasets, we calculated the average expression value for each gene within each sample and then determined the overall average of all ligand-receptor genes. Smoothed Cox proportional hazard analysis were conducted using the phenoTest R package (V.1.44.0) and Kaplan-Meier Curves were visualized by survminer R package (V.0.4.9).

Statistical analyses and data visualization were implemented by R programming language (V.4.2.1). Two-sided Wilcoxon rank-sum and signed rank test and Kruskal-Wallis rank sum test were adopted to test the difference from two or more than two comparable groups, respectively. Correlation analyses were used the Spearman rank correlation test.

RESULTS

RelRef patients demonstrate restricted B-cell clonal diversity and extensive SHM

Paired single-cell RNA and BCR sequencing were conducted on a total of 52 bone marrow aspirate samples, consisting of 6 samples from healthy bone marrow donors (normal), 24 newly diagnosed (NewlyDx) AML bone marrow samples, and 22 samples from 8 relapsed or refractory (RelRef) AML patients who were assessed before and after receiving azacitidine/nivolumab treatment (NCT029397720) (figure 1A), which were described in detail in our previous research. In brief, the median age at AML diagnosis was 73 years (range: 52–88 years), with 75% of the individuals being male. Among them, 34% had secondary disease (eight cases of Newly Diagnosed and three cases of RelRef), where two and three patients, respectively, had undergone myeloid-directed therapy prior to AML diagnosis. Out of the total, 11 (34%) had diploid cytogenetics. The most prevalent mutations were in DNMT3A and IDH2, detected in 10 out of 32 patients (31%) and 9 out of 32 patients (28%), respectively. Of note, for the RelRef patients, longitudinal samples were collected whereby timepoint A designated the first collection at the time of first relapse or primary resistance, while timepoints B and C represent longitudinal samples corresponding to these patients following treatment with azacitidine/nivolumab on the clinical trial NCT029397720. The clinical and demographic characteristics of the samples included in this study were summarized in online supplemental figure 1A and online supplemental table 1 and were previously reported.

To capture the diversity of the BCR repertoire, the sequencing data of 5’ end single-cell V(D)J were assembled and aligned, followed by the annotation of clonotype, identification of class-switched immunoglobulin isotype, and the evaluation of SHM frequency. In total, we identified 22,317 B cells containing productive V(D)J sequences. A higher clonal expansion was found in RelRef samples, compared with normal and NewlyDx samples, as shown by its high fraction of top clonotypes and lowest clonotype diversity index (figure 1B,C). These results suggested that a highly uneven distribution of clonotypes, dominated by a minority of expanded clones, exists at the time of relapse or primary resistance. Different usages of immunoglobulin variable genes in the CDR3 region across normal and AML samples also suggested that B cells have undergone distinct rearrangements (online supplemental figure 1B,C). Specifically, the CDR3 sequence in RelRef samples had the strongest antigen-driven selection pressure, reflected by their enhanced SHM frequency and the prevalence of class-switched isotypes, specifically IGHH and IGHA (figure 1D,E). Indeed, a higher SHM frequency and lower clonal diversity were observed for class-switched isotypes compared with IGHM and IGHD, whereas no significant difference was found among the class-switched isotypes (online supplemental figure 1D–F). The overall characteristics of BCR repertoires indicate clonal expansion and affinity maturation.
of RelRef samples, which may be associated with prior chemotherapy, highlighting the underlying connection of B cell repertoire dynamics and disease course in AML patients. Additionally, NewlyDx AML patients classified as adverse risk by ELN criteria, secondary AML, and non-diploid karyotype demonstrated lower clonal diversity and higher maturation in their BCR repertoire. These observations align with higher risk disease in these three classification types, reinforcing the inherent association between BCR features and the AML clinical presentation and prognosis (online supplemental figure 1G).

**Single-B-cell transcriptomics identifies differences in the AML-associated B-cell landscape at different stages of disease**

To further characterize the B cells within the AML TME, we identified 12,423 B-lineage cells with scRNA profile after stringent quality control and multiplets removal (see the Methods section). We first clustered samples by the average gene expression of B cells scRNA data and observed a pattern that roughly distinguished RelRef samples from others (online supplemental figure 2A). By integrating the scRNA and scBCR data, we defined 9 B-lineage cell subsets (pro/pre-B, immature B, naïve B, CD27+ memory B, Interferon (IFN)-induced memory B, atypical memory B, non-switched memory B, plasmablast, and plasma cells) using corresponding markers (figure 2A,B, online supplemental figure 2B,C, and online supplemental table 2). The pro/pre-B cell cluster did not exhibit productive V(D)J rearrangement sequences and highly expressed genes involved in the DNA cleavage phase during V(D)J recombination (RAG1 and RAG2 genes), as well as genes participating in the component of the surrogate light chain pre-BCR (VPREB1 and IGLL1 genes). CD10hi, CD44lo, CD24hi, and CD38hi expressions defined immature bone marrow B cells, that were predominantly composed of the IGHM isotype and germline CDR3 sequences. Naïve B cells are mature B cells characterized by the expression of IL4R, TCL1A, and FCER2, as well as the predominance of the IGHM/IGHD isotype. We identified four types of memory B cells with medium SHM frequencies and mixed class-switched isotypes, most prominently the classical CD27+CD24− memory B cells. IFN-induced memory B cells were identified by the expression of IFN-induced genes, indicating a unique transcriptional signature specific to this subset. CD27 CD24- atypical memory B cells were defined by the expression of FCRL3 and FCRL5, as well as features of B cell exhaustion such as CD11c (ITGAX). CD32b (FCGR2B), and CD72 (online supplemental figure 2C). The presence of atypical memory B cells was reported to play a dysfunctional role in patients with high-inflammation AML. The atypical memory B also exhibits the IGHD isotype (figure 2A and online supplemental figure 2B), indicating the existence of CD27 IgD+ memory B cells within the bone marrow of AML patients. Our analysis of the BCR repertoire in B lineage subsets revealed that atypical memory B cells exhibited higher clonal amplification and lower clonal diversity compared with CD27+ memory B cells, highlighting the selective pressures and distinct characteristics of these two types of memory cells (online supplemental figure 3A-C). The non-switched memory B cell cluster composed of B cells with IGHM isotype were predominantly from one patient (PT7) at time of relapse with secondary AML after myelodysplastic syndrome (online supplemental figure 2B,D,E). Given the peculiarity of this type of memory B cells, we analyzed the clonotypes in PT7, and found that the patient was dominated by the non-switched clone that further amplified after azacitidine/nivolumab therapy (online supplemental figure 3D). Moreover, non-switched memory B cells had a particular V(D)J rearrangement preference compared with other subgroups (online supplemental figure 3E). Plasma cells were characterized by high SHM frequency and the expression of MZB1 and CD138 (SDC1), and plasmablasts were characterized by additional expression of proliferative markers (figure 2B,C and online supplemental figure 2D).

To gain further insights into the association between B cell composition and AML phenotype, we assessed the variations in the abundance of different B-cell lineage subtypes (figure 2E,F and online supplemental figure 4A). The pro/pre-B and immature B cells exist most common in normal samples, occasionally in NewlyDx samples, and seldom or non-existent in RelRef samples, indicating that their presence diminishes as the disease progresses. Similarly, naïve B cells were fewer in RelRef samples than in other samples. On the other hand, the percentage of atypical memory B cells and plasma B cells showed a trend of increasing in AML patients than in normal donors. The proportion of CD27+ memory B cells showed no significant difference between NewlyDx and normal samples, but markedly increased in the RelRef group (online supplemental figure 4A). Taken together, the nascent B cell lineage was prominently manifested in the healthy specimens, whereas the AML subjects showed a higher proportion of differentiated B cells. To further confirm this, we analyzed a public AML scRNA dataset generated by Lasry et al. and verified the finding that AML subjects exhibit less nascent B cells and more differentiated B cells than normal samples (online supplemental figure 4B). In alignment with the observation that the BCR exhibited a heightened degree of affinity maturation in patient subgroups associated with more adverse clinical features, the categories of adverse and secondary AML patients also demonstrated an enrichment of differentiated B-cell subtypes (online supplemental figure 4C).

**Loss of AP-1 activity in naïve and memory B cells points to an exhausted state in RelRef patients**

After stimulating primary B cells through the surface BCR and/or the CD40 receptor, AP-1 genes are upregulated. Further, AP-1 genes have been reported to play a multifaceted role in regulating the differentiation and proliferation of B cells. In line with this, we found significantly higher AP-1 activity of B cells in the NewlyDx samples...
Figure 2  B cell lineage definition and association with clinical manifestations. (A) Uniform Manifold Approximation and Projection (UMAP) embeddings of B cell lineage, colored by B cell and plasma cell subtypes (top) and highlighted by isotype categories (bottom). (B) Bubble plot depicted the canonical marker gene expression level in B-lineage subgroups. (C) UMAP embeddings and per cent bar graph showing the somatic hypermutation categories distribution among B cell and plasma subgroups. (D) UMAP embeddings exhibiting the distribution bias of B lineage cells from different sample types. (E) Violin plot displaying the B-lineage subtypes enrichment preference in NewlyDx compared with normal samples. The red and green colors represented the current cell type enriched in NewlyDx and normal samples, respectively. The enrichment value logFC was calculated by miloR. (F) Per cent bar graph showing the B cell and plasma cell categories composition among three sample types. RelRef in figure (F) referred to the samples prior to azacytidine+nivolumab treatment.
Figure 3  AP-1 activity defines subclusters of naïve and memory B cells in acute myeloid leukemia (AML) patients. (A) Violin plots exhibited AP-1 activity score between NewlyDx AML and normal samples in our data and Lasry cohort (top left). Uniform Manifold Approximation and Projection (UMAP) embeddings exhibiting the AP-1 activity score in naïve and memory B cells (10, 053 cells, top right) and the distribution of naïve and memory B cells across different sample types (bottom). (B) Scatter plot showing the consistency of altered gene expression in three comparable groups, in which X-axis and Y-axis denoted the log2-transformed fold change values of CD27+MemB AP1_hi versus CD27+MemB AP1_lo and NaiveB AP1_hi versus NaiveB AP1_lo groups, respectively. Dots were colored based on log2-transformed fold change values in NewlyDx versus normal samples, with red indicating upregulated and blue indicating downregulated genes (top). Heatmap depicting by the log2-transformed fold change values in different expressed genes (|log2FC|>0.3) of naïve and memory B cell in NewlyDx versus normal (middle) and atypical MemB AP1_hi versus atypical MemB AP1_lo (bottom). (C) Scatter plot exhibiting ranked hallmarks and KEGG pathways correlated with AP-1 in where red dots representing significant positive terms (Spearman correlation coefficient>0 and p<0.05) in our dataset. (D) Violin plots illustrating the differences in activity scores of functional terms between NewlyDx and normal in our data and the Lasry cohort. Significant p values between the two groups were calculated using the Wilcoxon rank-sum test. (E) Boxplot depicted the difference of AP-1 activity score between unique and expanded memory B cells. (F) B-cell receptor (BCR) clonotype diversity distribution between MemB AP1_lo and MemB AP1_hi cells, calculated as 1/Simpson’s clonal diversity index (mean±SD). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, ns, non-significant.
compared with the normal samples in our dataset and in Lasry et al dataset17 (figure 3A). On investigation of the transcriptome features of naïve and memory B cells, we discovered subclusters within naïve B cells, name CD27+ and atypical memory B cells that can be further divided by AP-1 activity. Notably, cells from the same patient could contain both high and low AP-1 signaling, suggesting that these findings were unlikely to be driven by different treatments or batch effect (online supplemental figure 5A). Though more B cells with strong AP-1 activity were found in NewlyDx samples, AP-1_hi B cells were rare in Rel/Ref samples (figure 3A and online supplemental figure 5B). Comparing the transcriptomes of naïve B cells and memory B cells with differing AP-1 activity revealed a strikingly similar pattern of expression changes that was also seen by comparing NewlyDx with normal samples (figure 3B and online supplemental table 3), indicating the same regulatory process associated with AP-1 activity.

AP-1 is known to regulate a wide array of cellular processes spanning from cell survival, activation, differentiation and apoptosis.45 Functional analysis revealed that upregulated genes in AP-1 groups were enriched in pathways related to kinase activity regulation, cellular stress response, programmed cell death regulation, and unfolded protein response (online supplemental figure 5C and online supplemental table 4). Pathways which correlated with B cell AP-1 activity in naïve and memory B cells, included MAPK pathway that has been shown to be important to activating AP-1 target genes,46 TNF that induces NFκB in chronic B lymphoid malignant cells,46 while also playing a crucial role in initiating a signaling cascade that activates c-Jun/AP-1, resulting in the proliferation of acute myelogenous leukemia blasts,46 and inflammatory pathway that requires AP-1 mediated gene expression as a response to TNF cytokine17 (figure 3C and online supplemental figure 5D). Additionally, these pathways were also significantly upregulated in B cells of NewlyDx patients compared with healthy donors, in both our dataset and an independent dataset (figure 3D). These analyses are in line with the well-documented role of AP-1 in responding to diverse cellular stresses and B-cell activation.50 In agreement with the activation of B cells, we found that memory B cells with high AP-1 activity were more likely to exhibit clonal expansion than other memory B cells (figure 3E,F). Although the causal effect of the disappeared AP-1+B cells observed in Rel/Ref AML patients is unclear, our results suggested that AP-1 inactivation probably undermine B cell responses that immunotherapy seeks to harness, specifically when combinational therapies where AP-1 activity might be strongly inhibited were considered, for example, immunotherapy in combination with MAPK inhibition.51 52

**Pseudotime analysis reveals potential transitional dynamics among B-cell lineage subgroups**

We next inferred the differentiation trajectory of naïve and memory B cells51 and observed two distinct developmental trajectories that both originated from naïve B cells with low AP-1 activity. One trajectory started with naïve B cells from normal samples and differentiated towards the memory B cells from RelRef samples, while the other trajectory began with naïve B cells and progressed to the CD27+ memory B cells from NewlyDx samples (figure 4A). Generally, B cells with high AP-1 activity were more differentiated than other B cells, and the atypical memory B subset represented the most differentiated B cells (figure 4B). Coincidently, B cells with AP-1 high activity were mostly overlap with the second trajectory. As expected, the classical CD27+ memory B cells that underwent class-switch recombination (CSR) harbored higher SHM and were more differentiated than other non-CSR cells (figure 4A).

To further confirm the differentiation trajectories, we used two independent methods to measure the similarity of single-cell gene expression between memory and naïve B cells (figure 4C). Both methods suggested that most AP1_hi CD27+ memory B cells originated from AP1_hi naïve B cells, whereas AP1_lo CD27+ memory B cells may originate from AP1_lo or AP1_hi naïve B cells. We also used the scBCR sequencing data to measure the pairwise transition index between B cell clones based on STAR-TRAC algorithm,26 and found that B cells with high AP1 activity exhibited higher transition index with each other (figure 4D). For example, Atypical MemB AP1_hi showed higher transition index with CD27+MemB AP1_hi and NaiveB AP1_hi than with MemB-AP1_lo and NaiveB-AP1_lo, respectively. The developmental trajectory of atypical memory B cells involved a transformation from CD27+MemB AP1_lo to Atypical MemB AP1_hi, followed by further development into the Atypical MemB AP1_hi cluster. This developmental pathway is distinguished by a significant clonal expansion in atypical memory B cells, which is notably more pronounced compared with CD27+ memory B cells (figure 4A,B,D and online supplemental figure 3A–C).

**Atypical memory B cells interact closely with AML malignant cells and are associated with worse outcomes in AML**

B cells are professional antigen-presenting cells that play a critical role in adaptive immune response.53 Promoted by the finding of abundant atypical B cells in our AML cohort and in Lasry et al dataset,17 and their previously reported association with other immune-dysregulated states,54 55 we next investigated atypical B cells by comparing with other B cell clusters. Interestingly, we found that atypical memory B cells showed higher BCR signaling activity than classical memory B cells and exhibited the highest activity of antigen processing and presentation, and endocytosis pathways (figure 5A,B and online supplemental figure 6A,B). The same observations were also found in Lasry et al dataset (online supplemental figure 6C–E). In line with this, it was recently reported that atypical memory B cells had the capacity to capture, internalize, and transport antigens at even higher levels than classical naïve and classical memory B cells.56

With the increased antigen presentation activity of atypical memory B cells, we explored their correlation with AML cells by inferring ligand-receptor interactions in NewlyDx patients. This demonstrated that atypical memory B cells exhibited a distinct pattern of interaction with AML cells compared with naïve and classical memory B cells (Figure 5C and online supplemental figure 6F). AML has the potential to activate the NOTCH signaling pathway in atypical memory B cells through the NOTCH2 receptor, which may be associated with increased B-cell activation and the determination of B-cell differentiation fate.57 58

Figure 4  Pseudotime analysis reveals potential transitional dynamics among B-cell lineage subgroups. (A) Naïve and memory B cells developmental trajectories were inferred using Monocle3, and colored by cell type subsets, inferred pseudotime, sample types, somatic hypermutation (SHM) categories and AP-1 activity score, separately. AP1_lo and AP1_hi represent B cells characterized by low AP-1 activity and high AP-1 activity, respectively. (B) Boxplot depicted the difference of CytoTRACE score in memory B cell subgroups. (C) The transcriptome similarity between CD27+ memory B cells and naïve B cells was measured by SingleR and presented as Uniform Manifold Approximation and Projection (UMAP) plot and pie charts (top), as well as percent bar graph calculated by the TransferData function in Seurat package (bottom). (D) Heatmap depicting the transition index between major B cell subclusters, was generated using STARTRAC. The color red indicated a high probability of transition, with 0.03 being the maximum truncation value. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, ns, non-significant.
Figure 5  Atypical memory B cells were specialized for antigen presentation and interacted tightly with acute myeloid leukemia (AML) malignant cells. Violin plot showing the difference of B-cell receptor (BCR) signaling pathway (A) and antigen processing and presentation pathway scores (B) across B cell subclusters. (C) Ligand-receptor interaction between naïve/memory B cell subgroups and myeloid cells in newlyDx samples. Heatmaps were used to display the normalized expression levels of the ligand-receptor genes, with highly expressed genes shown in red. (D) The lollipop chart visualized the enriched biological process term of receptor genes in AML cells. The circles on the chart were colored based on their Benjamini-Hochberg (BH)-adjusted p values. (E) Cox proportional hazard analysis smoothed by ligand-receptor gene expression level, illustrating their relationship with overall survival (OS) in the TCGA LAML cohort. Samples were divided into low (L), medium (M), and high (H) groups based on their gene expression levels. The red curve represents the results obtained using the Cox proportional hazards model, while the dotted curves indicate the 95% CI of the log HR. (F) Kaplan-Meier curves displaying differences in OS probability among TCGA LAML patients whose tumors had high, medium, or low levels of the ligand-receptor gene signature. The log-rank p value was calculated between the high and low patient groups. (G) Boxplot showing the difference in LSC17 signature scores between patients whose tumors had low and high levels of the ligand-receptor gene signature in the TCGA LAML cohort. Significant p values between the two groups were calculated using the Wilcoxon rank-sum test. (H) Cox proportional hazard analysis smoothed by ligand-receptor gene expression level, illustrating their relationship with OS in the Abbas et al cohort. Samples were divided into low (L), medium (M), and high (H) groups based on their gene expression levels. The red curve represents the results obtained using the Cox proportional hazards model, while the dotted curves indicate the 95% CI of the log HR. (I) Kaplan-Meier curves displaying differences in OS probability among patients in Abbas et al cohort whose tumors had high, medium, or low levels of the ligand-receptor gene signature. The log-rank p value was calculated between the high and low patient groups. (J) Boxplot showing the difference in LSC17 signature scores between patients whose tumors had low and high levels of the ligand-receptor gene signature in Abbas et al cohort. Significant p values between the two groups were calculated using the Wilcoxon rank-sum test.
Furthermore, atypical memory B cells possessed the intriguing ability to interact with specific cell adhesion molecules such as ITGA4 and SELL, which are known to be associated with the malignant phenotype of AML cells.\textsuperscript{59,60} Remarkably, the interaction between FLT3 ligand (FLT3LG) and the FLT3 receptor in atypical memory B cells and AML cells has been observed. Notably, FLT3 was commonly found to be constitutively activated in AML, and the interplay of FLT3 ligand and FLT3 stimulated AML cell proliferation, consequently promoting leukemia progression.\textsuperscript{61,62} Furthermore, the receptor genes of AML cells regulated by ligands from atypical memory B cells were enriched by multiple pathways that have been reported to be associated with the tumorigenesis of hematological malignancies\textsuperscript{63–65} (figure 5D). Notably, higher overall expression levels of these ligand-receptor genes are linked to worse overall survival, as shown by Cox proportional hazards model and Kaplan-Meier analysis in both the TCGA LAML cohort\textsuperscript{29} and the cohort from Abbas et al\textsuperscript{20} (figure 5E,F). Additionally, elevated overall expression of these genes is associated with increased AML stemness, measured by the 17-gene signature\textsuperscript{31} (figure 5G,J). Our study revealed a previously unknown association between these atypical memory B cells and the stemness of AML malignant cells, highlighting the potential importance of these atypical memory B cells in the course of AML development.

**AML-associated plasma cells are highly differentiated and activated**

Recent studies have suggested that plasma cells could be further differentiated within TME.\textsuperscript{15} To explore this in the context of AML, we reconstructed the developmental trajectory of plasma cells.\textsuperscript{27} Along with the trajectory that started from plasmablasts, both the number of expressed genes and the CytoTRACE score of plasma cells decreased, indicating a further differentiation process (figure 6A). Plasma cells of IgG isotypes were more differentiated than those of IgA isotypes, as indicated by the significantly lower CytoTRACE score and their enrichment at the end of the differentiation trajectory (figure 6B,C and online supplemental figure 7A). Coincidentally, plasma cells from AML samples also show higher expression IGHG genes (figure 6D), enrichment at the end of trajectory (figure 6E), and lower CytoTRACE score than normal samples (figure 6F). These results suggested that IgG plasma cells from AML exhibit a more differentiated state, a conclusion that was also supported by the Lasry et al dataset\textsuperscript{17} (online supplemental figure 7B). Notably, a recent study also reported the enrichment of IgG+ plasma cells in hepatoma that promotes disease progression and impedes effector T cell immunity.\textsuperscript{66}

We then examined the changes in gene expression over pseudotime by ordering the top 3000 highly variable genes and observed an enrichment of genes involved in B cell activation, B cell immunity, and immunoglobulin production at the terminal of differentiation trajectory (figure 6G and online supplemental table 6), reminiscent of the long-lived plasma cells that were reported recently.\textsuperscript{15} In line with this, we found the enrichment of CD19\textsuperscript{hi}CD38\textsuperscript{hi}CD138\textsuperscript{hi} cells at the terminal of trajectory (figure 6H), suggesting the existence of long-lived plasma cells.\textsuperscript{67} Comparing plasma cells from NewlyDx AML samples versus normal samples reproduced the same collection of enriched pathways (online supplemental figure 7C and online supplemental table 7). These findings suggest that plasma cells in AML patients are under dynamic regulation and exhibit significant heterogeneity, with a tendency towards further differentiation and long-lived IgG producing plasma cells.

To investigate the potential developmental origin of plasma cells, we examined the resemblance between plasma cells and memory B cells. While previous studies proposed that atypical memory B cells could be precursors to plasma cells,\textsuperscript{68,69} our finding indicated a lower likelihood of transformation from atypical memory B cells to antibody-secreting cells compared with classical memory B cells in AML bone marrow environment (figure 6I). Consistent results were also observed in the Lasry cohort\textsuperscript{17} (online supplemental figure 7D), and the analysis of BCR transition index further supported this conclusion (figure 4D).

**Expansion of B cell clones and plasma cell abundance is associated with clinical response to ICB**

The combination treatment of azacitidine and nivolumab has been successful in a subset of patients with relapsed/refractory AML.\textsuperscript{5} While several studies highlighted the role of B cells in ICB blockade therapy in solid cancers,\textsuperscript{11–13} little is known about the impact of this combination on the B cell lineage of AML patients. We leveraged this rare cohort of longitudinally collected patient samples prior to (timepoint A) and following treatment (timepoints B and C) with azacitidine/nivolumab to examine the effects of ICB therapy on B cell and plasma subtypes in AML patients. Our analysis revealed that the overall proportion of B cell and plasma subtypes remained unchanged after treatment (online supplemental figure 8A,B). However, the top clonotypes were amplified to a greater extent, and the BCR repertoire showed decreased clonal diversity post-treatment (figure 7A,B). Notably, the expanded B cell clonotypes were associated with complete responses to ICB-based therapy (figure 7C). These findings were reminiscent to the T-cell repertoire dynamics that we previously reported in these same patients.\textsuperscript{22} This suggests that T and B cells follow similar dynamics.
post-ICB-based therapies in AML. In addition, we observed that plasma cells differentiated further in patients undergoing this ICB-based therapy, with a smaller CytoTRACE score (figure 7D). Regarding naïve and memory B cells, cells from pretreatment and post-treatment samples were predominantly distributed in non-AP-1 groups (online supplemental figure 5B, online supplemental figure 8A,B).

Mechanistically, ICB-based therapy upregulated AP-1 complex and tumor necrosis factor alpha signaling via the NFKB pathway in non-AP-1 naïve and memory subtypes (online supplemental figure 8C,D), while reducing the antigen processing and presentation capabilities (online supplemental figure 8E), as well as their B cell-mediated adaptive immune response in CD27+ memory B cells (online supplemental figure...
These findings suggest that ICB-based therapy may have a significant impact on B cell function in AML patients. We noted a significant attenuation in AP-1 activity and tumor necrosis factor alpha signaling through the NFKB pathways in RelRef patients who attained complete responses to ICB-based therapy (figure 7E,F). Conversely, individuals manifesting complete responses exhibited heightened levels of protein export and enhanced protein processing within the endoplasmic reticulum (online supplemental figure 8G,H). Remarkably, a concurrent increase in plasma cell proportions was discerned in patients with complete responses, underscoring the pivotal role of plasma cells in augmenting the efficacy of immunotherapy (figure 7G).

However, our study was limited by the low number of B cells in the relapsed/refractory samples, which hinders a comprehensive analysis of the B cell lineage affected by ICB-based therapy. Future studies with larger sample sizes will be required to fully elucidate the effects of ICB-based therapy on B cell function in AML.

**DISCUSSION**

A growing body of work points to the important immunological role B cells play in cancer pathogenesis. In this study, we paired single-B cell transcriptomics with clonotypic BCR analysis to evaluate the dynamic landscape of AML-associated

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**Figure 7** Characterization of B cells and plasma cells undergoing azacytidine+nivolumab treatment in relapsed/refractory patients. (A) The relative clonal space occupied by the clonotypes was divided by the bins of top 1:5, 6:100, 101:200, and 201:500 in RelRef samples before and after azacytidine+nivolumab treatment. (B) Clonotype diversity distribution of RelRef samples before and after azacytidine+nivolumab treatment, calculated as 1/Simpson's clonal diversity index (mean±SD). (C) The bar graph displays the proportion of immunotherapy response among the novel emerging, expanded, and contracted clonotypes in the post-treatment group, compared with the samples collected before treatment. (D) Boxplot depicted the difference of CytoTRACE score of plasma cells between RelRef samples collected before and after azacytidine+nivolumab treatment. Boxplot showing the difference of AP-1 (E) and tumor necrosis factor alpha signaling via NFKB pathway scores (F) among RelRef patients with different immunotherapy response. (G) Plasma cell fraction in relation to immunotherapy response among RelRef patients. Significant p values between the two groups were calculated using the Wilcoxon rank-sum test. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, ns, non-significant. CR, complete response; NR, no response; PR, partial response; SD, stable disease.
B cells. Our ability to exploit and modulate B cells for immunotherapeutic benefit in hematological malignancies like AML depends on better understanding how exactly B cells may be acting as agents of cancer versus agents of host immunity. Evidence from solid tumor studies indicates that tumor-infiltrating B cells represent a heterogenous mix of actors with both pro and antitumor activity.\textsuperscript{10, 15, 70} Understanding the roles of these B cell subpopulations hinges on observing how individual B cell clones expand, contract, and change phenotype over the course of disease and treatment. By evaluating the B cell compartment in AML patients with single-cell resolution, we fill an important knowledge gap and identify specific B cell populations and cellular interactions for further investigation.

It has long been hypothesized that B and T cell exhaustion and polarization to regulatory phenotypes may be important mechanisms by which patients lose immunological control of cancer, resulting in relapse or treatment refractoriness.\textsuperscript{71–73} We found that B cells in NewlyDx patients demonstrated both high AP-1 expression (a marker of activation) as well as high clonotypic diversity compared with healthy controls, presumably reflecting a rich repertoire of activated B cells that are responding to AML antigens in the acute phase of disease. On the other hand, we observed a stark loss of AP-1 expression in B cells from RelRef patients, as well as a dramatically restricted repertoire of B cell clonotypes. Interestingly, B cells in RelRef patients were also highly differentiated with extensive class-switching and SHM, suggesting antigen-driven maturation of specific B cell clones within the AML microenvironment. It is notable that similar restriction of B cell diversity paired with extensive differentiation and SHM has been observed in other advanced cancers.\textsuperscript{14, 16, 74} Importantly, we observed that AP-1 activity is restored and that specific B cell clones expand after ICB, which is associated with respond to treatment. Notably, recent T cell work by our group demonstrated a similar restriction of TCR repertoire diversity in RelRef AML patients, as well as selective post-ICB expansion of specific T-cell clones among ICB responders.\textsuperscript{22} Taken together, our findings suggest that impaired B cell activation and loss of plasticity may be important features of AML relapse, and that AML-specific B cells in RelRef patients may be terminally differentiated into a maladaptive immunophenotype. Our observation that this pathological phenotype can be broken by ICB in certain RelRef patients is promising, and mechanistically supports further investigations into ICB in AML, which have thus far been frustrated by mixed results.\textsuperscript{3, 22}

Cancer-associated B cells are notably heterogeneous, and efforts to better understand their role have been obscured by inconsistent findings across cancer types. It has been frequently observed that tumor-infiltrating B cell and plasma cell populations that appear protective in some cancers are correlated with worse outcomes in others.\textsuperscript{19} A population of particular interest in our study was atypical memory B cells which appear to associate closely with AML cells based on our receptor-ligand transcriptomic analysis, and which were associated with poorer outcomes in RelRef AML patients. Interestingly, atypical memory B cells have been previously documented to correlate with positive prognosis in a range of solid cancers.\textsuperscript{75–77} The mechanisms by which atypical memory B cells might exert their effects remain elusive, but atypical memory B cells in other cancers seem to have high antigen presenting activity, as well as a close spatial association with CD8 T-cells.\textsuperscript{17, 56, 77} While atypical memory B-cells expressed several markers of exhaustion, we found that they did not express canonical regulatory or suppressive markers. Nevertheless, their close physical association with AML cells points to a more direct role in AML survival through alternative mechanisms that warrant further investigation. It seems plausible that atypical memory B cells represent a functionally heterogeneous population, and further dissecting the mechanisms by which they may be protective in some cancers and deleterious in others will be key to understanding their immunological relevance.

Another population of interest in our analysis were plasma cells, which represent a high proportion of the extensively somatic-hypermutated clones in our RelRef patients. Plasma cells in RelRef patients were notably highly stimulated and expressed primarily IgA and IgG. Tumor-infiltrating plasma cells have been generally associated with positive prognosis in a range of solid cancers,\textsuperscript{10, 78, 79} presumably owing to their ability to secrete antitumor antibodies that can clear cancer cells via complement fixation, opsonization, and antibody-dependent cell-mediated cytotoxicity.\textsuperscript{80} Alternatively, it has been hypothesized that tumor-infiltrating plasma cells may aid some cancers by secreting large quantities of immunoglobulins that promote local immunomodulation by saturating Fc receptors on other tumor infiltrating immune cells, similar to the immunoregulatory action of IVIG.\textsuperscript{81, 82} It remains unclear what role plasma cells play in AML, but our observation that certain plasma cell clones expanded in ICB-responding patients suggests an anti-tumor role. It is also worth acknowledging that the prevalence of plasma cells in our analysis relative to other B cell populations does not reflect the “natural history” of B-cell-AML interactions, as plasma cells are much more likely to have survived the intensive chemotherapy these patients have previously experienced. Nevertheless, our data point to AML-associated plasma cells as another promising target for future immunotherapeutic efforts.

Perhaps the biggest limitation of our study is the lack of serial samples from AML patients who achieve sustained remission. Future studies longitudinally comparing RelRef patients with AML patients in remission will help identify patterns of B cell activation, differentiation, and clonotypic evolution that predict immunological control of AML. Additionally, AML is a remarkably heterogeneous entity encompassing a range of genetic lesions and clinical features that likely shape immune-AML interactions in ways that may be obscured by our relatively small patient panel.\textsuperscript{83, 84} Follow-up efforts using expanded patient
panels that recapitulate the mutational and cellular diversity of AML will allow us to better characterize the specific immunological differences among AML subtypes and will allow us to more rationally target specific immune cell populations and cellular interactions for immunotherapy.

A technical limitation of our study is the relatively low absolute numbers of B-cells incorporated into our analysis, especially among RelRef patients. This was a function of both the intrinsically lymphodepleted state of RelRef patients, as well as the throughput limitations of currently available scRNA sequencing techniques. Follow-up studies incorporating larger samples and bulk sequencing will allow us to interrogate the BCR repertoire with greater depth and breadth, complementing the high-resolution phenotypic and clonotypic data generated by single-cell techniques. Finally, the immunotherapeutic potential of AML-associated B cells is predicated on the assumption that they recognize AML antigens, yet the exact antigen-specificity of these cells remains unknown. Recently developed techniques may soon allow us to reliably identify AML-specific B cells and their cognate antigens, filling an important gap in our understanding of the immunological role of AML-associated B cells, and informing novel anti-AML therapies including vaccines, biologics, and cellular therapies.

In summary, the work presented here represents an important early step in dissecting the complex role B cells play in AML pathogenesis and immune control. Our findings identify particularly interesting cell populations and B-cell-AML interactions for further study while highlighting important outstanding questions. We believe continued work by our group and others promises to yield immunological insights that will inform the next generation of AML-directed immunotherapies.

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