Sustained molecular clearance of the *MYD88* p.L265P-mutant clone preceding resolution of cold agglutinin syndrome after autologous stem cell transplantation

Cold agglutinin disease (CAD) is a rare autoimmune hemolytic anemia mediated by immunoglobulin M (IgM) autoantibodies causing red blood cell agglutination at <37°C.[1] Cold agglutinin syndrome (CAS) occurs in B-cell lymphoproliferative disorders and is pathophysiologically distinct from CAD.[1] Autologous stem cell transplantation (ASCT) is a standard consolidation treatment for chemotherapy-sensitive relapsed diffuse large B-cell lymphoma (DLBCL). However, its application in CAS-complicated cases remains challenging because of the risk of temperature-dependent agglutination during apheresis and infusion.[2,3] Only five ASCT cases in patients with CAD/CAS have been reported,[2,4-7] with only one case achieving complete remission in the CA titer.[6] Whether ASCT can cure CAS and its underlying mechanisms remain unknown. Herein, we present a case of *MYD88*^{L265P}-mutated splenic marginal zone lymphoma (SMZL) with CAS that transformed into DLBCL and was successfully treated with ASCT.

This study followed the Declaration of Helsinki and was approved by the Institutional Review Board of the Institute of Medical Science, University of Tokyo (approval numbers: 26-112-270402; 2020-1-0422; 2024-103-0326). Cell-free DNA and genomic DNA were extracted using the Maxwell RSC ccfDNA Plasma Kit (Promega) and Gentra Puregene Blood kit (Qiagen). For targeted deep sequencing, libraries were prepared using the TruSight Myeloid Panel (Illumina). For whole-genome sequencing (WGS), libraries were prepared using TruSeq Nano DNA Library Prep kit and sequenced on NovaSeq 6000. The Genomon 2.6.2 pipeline identified somatic single nucleotide variants (SNV) and structural variants (SV) (https://genomon.readthedocs.io/ja/latest/); Control-FREEC estimated copy number alterations (CNA) and tumor purity (https://github.com/BoevaLab/FREEC). Clonal architecture was inferred using PyClone-VI, integrating variant allele frequencies (VAF) of SNV and SV with CNA profiles (https://github.com/Roth-Lab/pyclone-vi). Cancer cell fractions (CCF), defined as the fraction of cancer cells carrying each mutation, corrected for CNA and tumor purity, were estimated for each cluster. Phylogenetic trees were constructed using the pigeonhole principle.[8] Circulating tumor DNA (ctDNA) was quantified using digital polymerase chain reaction (PCR) targeting *MYD88*^{L265P} (QX200, Bio-Rad).

A 48-year-old woman presented with severe anemia, jaundice, and massive splenomegaly in October 2015. Laboratory findings revealed hemolytic anemia with a CA titer of 1:1,024, positive direct agglutination test, and negative direct antiglobulin test for IgG. Serum haptoglobin was decreased (2 mg/dL; normal range, 19-170) with elevated lactate dehydrogenase (LDH; 450 U/L; normal range, 124-222) showing an LDH1-predominant isozyme pattern (37.3%; normal range, 20-31) consistent with hemolysis. Serum immunofixation detected an IgM-κ monoclonal protein with elevated free κ light chain (314 mg/L; normal range, 3.3-19.4) and κ/λ ratio of 10.43 (normal range, 0.26-1.65). Serum IgM level was within the normal range (180 mg/dL; normal range, 35-220). Soluble interleukin 2 receptor (sIL-2R) level was elevated at 3,320 U/mL. Pathological examination of the extracted spleen confirmed SMZL. Targeted deep sequencing detected *MYD88*^{L265P} (VAF 14.6% in the spleen [SP] and 18.4% in the bone marrow [BM]), which was identified in serum ctDNA (VAF 3.2%), supporting lymphoma-associated CAS. Following splenectomy, hemolysis improved with normalization of haptoglobin (35 mg/dL) as ctDNA decreased to 1.0%, despite persistently high CA titers (1:512-1:2,048; Figure 1A). Eight cycles of rituximab achieved BM *MYD88*^{L265P} VAF reduction to 0.4% with sIL-2R normalization, though CA titers remained elevated (Figure 1A).

In August 2021, ctDNA levels increased to 7.6%, preceding the clinical progression of lymphoma by 3 months (Figure 1A). In November 2021, the patient developed cervical lymphadenopathy and was diagnosed with transformed DLBCL (activated B-cell type) by histological examination of the cervical lymph nodes. Serum free light chain analysis at relapse showed κ elevation (κ 58.8 mg/L, λ 8.5 mg/L, κ/λ ratio 5.92) with immunofixation-positive IgM-κ M-protein. Following one cycle of R-CHOP and three cycles of bendamustine and rituximab, the patient achieved a complete metabolic response on positron emission tomography–computed tomography and negative conversion of ctDNA. Considering her young age and chemotherapy-sensitive disease status, ASCT was planned as consolidation therapy for transformed DLBCL. Notably, the indication for ASCT was lymphoma control rather than CAS management, as active hemolysis had not recurred following splenectomy.

To define clonal relationships, we performed WGS of the SP and lymph node (LN) at relapse (average depth: SP 32.3×; LN 84.7×; and buccal swab 33.6×) (Figure 2). Across all specimens, 174,490 somatic variants were identified, of which 8,968 (5.1%) shared a common origin. Clonal decomposition identified ten distinct clonal clusters, with medi-

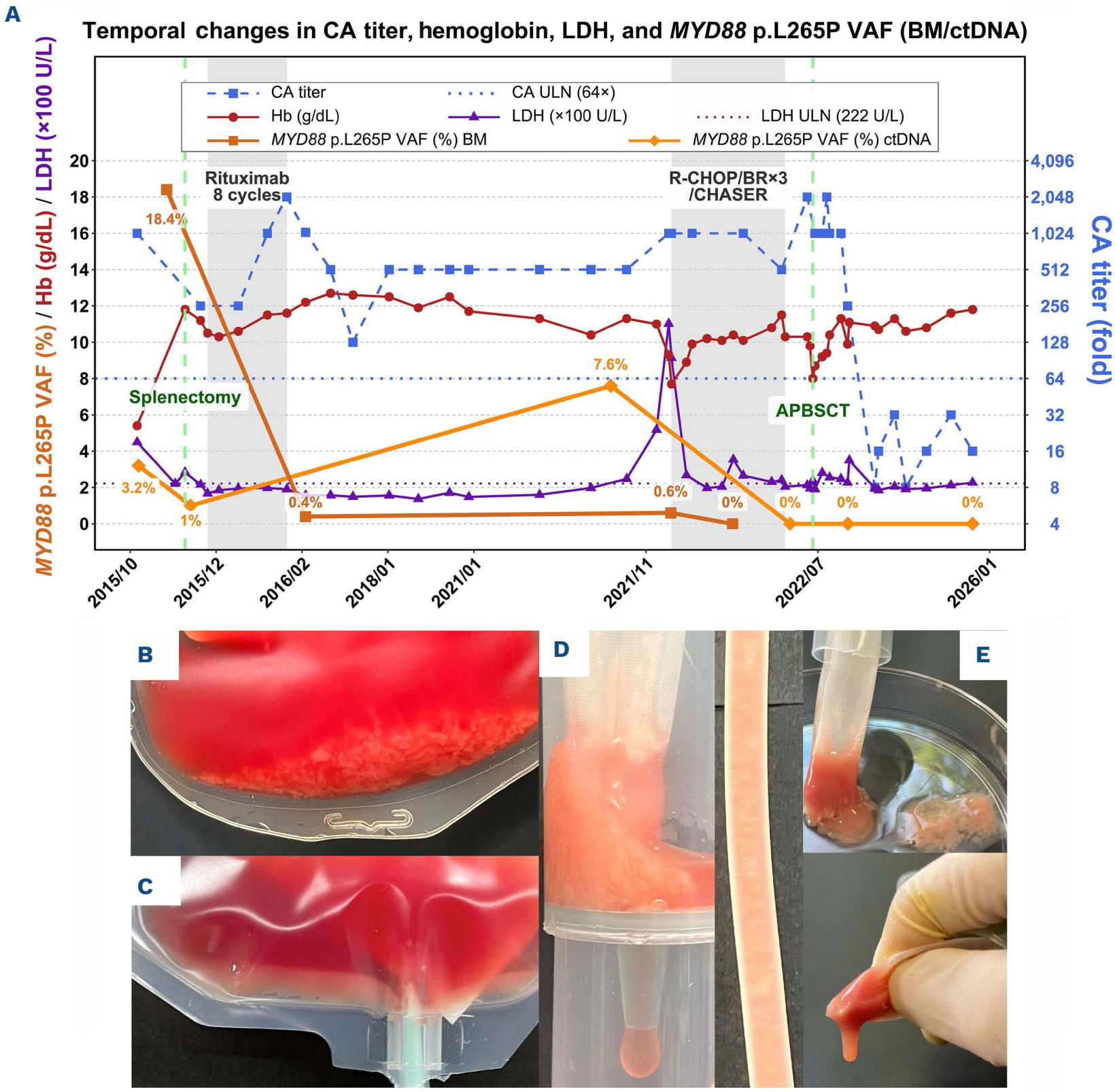


Figure 1. Clinical course, molecular monitoring, and macroscopic findings during autologous stem cell transplantation for *MYD88*-mutated splenic marginal zone lymphoma with cold agglutinin syndrome. (A) Longitudinal clinical course shows temporal changes in hemoglobin (Hb, red circles), lactate dehydrogenase (LDH, purple triangles, ×100 U/L scale), cold agglutinin (CA) titer (blue squares, logarithmic scale), and *MYD88*^{L265P} variant allele frequency (VAF) in bone marrow (BM, brown squares) and circulating tumor DNA (ctDNA, orange diamonds). Horizontal dotted lines indicate the upper limit of normal for CA titer (64×, blue) and LDH (222 U/L, purple). Gray shading denotes treatment periods; vertical dashed lines mark key interventions. Upon diagnosis, ctDNA VAF was 3.2% with CA titer 1,024× and elevated LDH (450 U/L). Following splenectomy, Hb improved and ctDNA declined to 1.0% despite persistent high CA titers. Rituximab reduced BM VAF to 0.4% with Hb stabilization and LDH normalization, but CA titers remained elevated (512-1,024×). In August 2021, ctDNA increased to 7.6%, preceding clinical relapse by 3 months. At diffuse large B-cell lymphoma transformation, LDH peaked at 1,102 U/L. After salvage chemotherapy and autologous peripheral blood stem cell transplantation (PBST), ctDNA (0%), CA titer (<64×), and LDH all normalized, paralleling sustained Hb improvement and demonstrating complete molecular and serological remission maintained for >3 years. (B-E) Macroscopic agglutination during autologous PBSC infusion. (B) Cryopreservation bag immediately post-thaw shows dense white cellular aggregates accumulating at the top of the product. (C) The same bag at 10 minutes post-thaw; agglutinated material has sedimented near the outlet port. (D) Filter obstruction during infusion. Left: sticky cellular aggregates adhering to the 40-µm filter mesh; progressive accumulation of such material led to repeated filter occlusion. Right: cellular aggregates visible in the downstream infusion line. Ten circuit changes were required during the 2-hour infusion. (E) Physical characteristics of the agglutinated product removed from the occluded filter: cohesive clumps in a petri dish (top) and gel-like material maintaining structural integrity when lifted (bottom), illustrating the severity of cold agglutinin-mediated cellular clumping. This progressive agglutination occurred despite maintaining the product at 36°C.

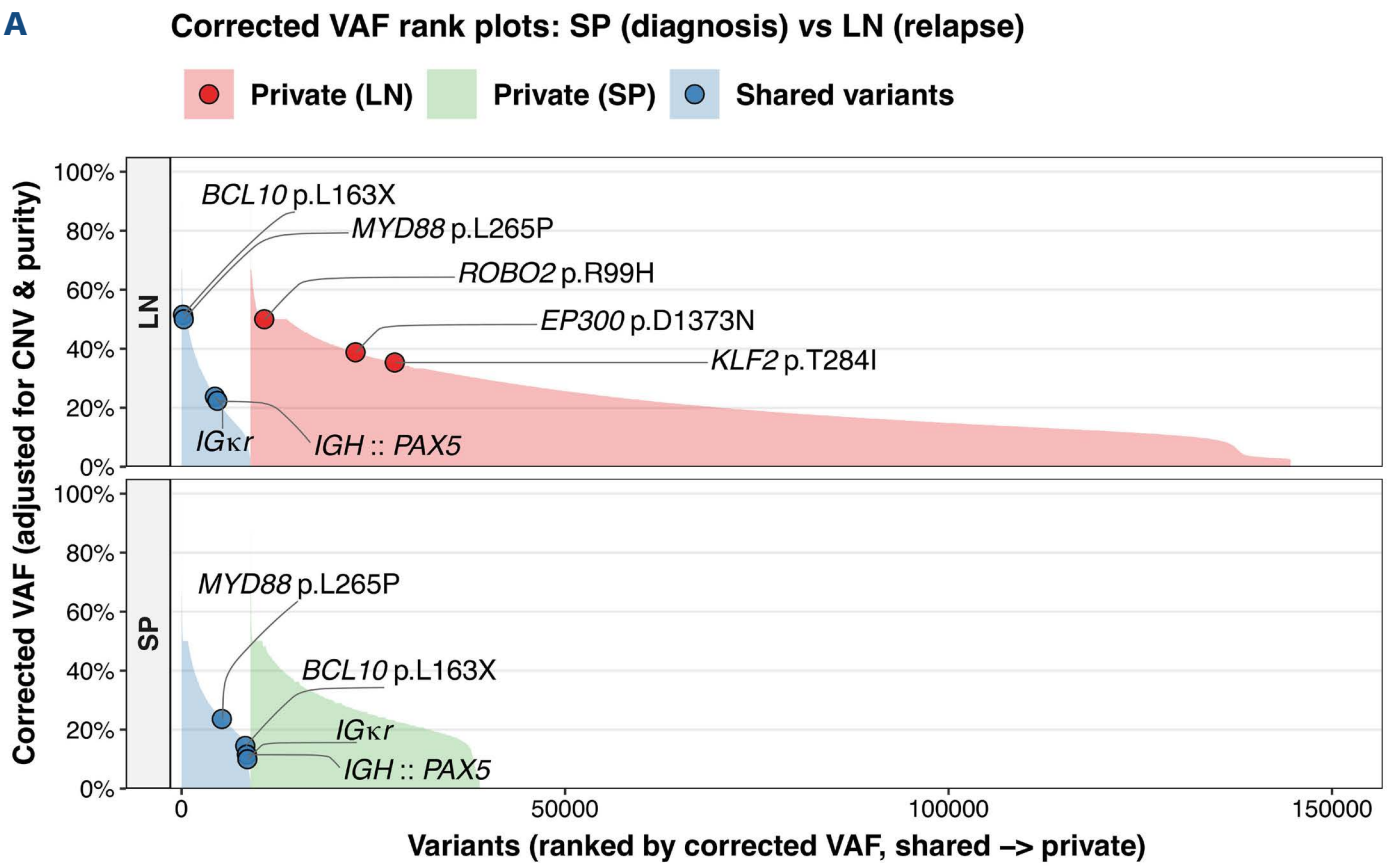
an CCF ranging from 10.1-100.0%. Phylogenetic inference revealed a founder clone (C6; CCF: SP; 80.4%, LN; 100.0%, N=1,398) harboring the pathogenic *MYD88*^{L265P} (Figure 2). Two evolutionary branches emerged: (i) an LN-specific transformation-associated branch acquiring *ROBO2*, *EP300*, and *KLF2* mutations and (ii) a branch (C5; *BCL10*^{L163X}) diverging into SP-specific (C4/C0) and shared (C2/C3; *IGH::PAX5*) sub-branches. All malignant cells remained anchored to the founding *MYD88*^{L265P}-mutant trunk.

Mobilization was performed using cyclophosphamide, cytarabine, dexamethasone, etoposide, rituximab (CHASER) chemotherapy, followed by granulocyte colony-stimulating factor and plerixafor. Compared with most reported cases,[2,4-6] prophylactic plasma exchange (PE) was not performed (Table 1) because the patient experienced no clinical hemolysis after splenectomy despite persistently high CA titers. Temperature management during apheresis was achieved using only a blood warmer (36°C) on the

Table 1. Clinical characteristics, peri-transplant management, and outcomes of reported cases of autologous stem cell transplantation in cold agglutinin syndrome.

Parameter	Reference, first author (year)					
	Crowther *et al.* (2006)[7]	Badami *et al.* (2017)[4]	Morigi *et al.* (2021)[6]	Thompson *et al.* (2023)[2]	Castonguay *et al.* (2025)[5]	Present case
Underlying disease	Relapsed DLBCL with CAS	MZL with CAS	Relapsed DLBCL with CAS	Plasmacytoma and AL chain/ rimary amyloidosis with CAS	CAD → DLBCL with CAS → DLBCL with CAS (relapse)	SMZL with CAS → DLBCL transformation with CAS (relapse)
Age, years, sex	62 M	61 F	56 F	62 M	61 M	48 F
CA titer (°C)	1:512 (RT)	1:64 (4)	1:256 (4)	>1:512 (4)	1:4,096 (4) 1:128 (37)	1:1,024 (4)
Clinical hemolysis present	No	Yes	Yes (resolved)	No	Yes (transfusion-dependent)	Yes (resolved post-splenectomy)
MYD88^{L265P} mutation	NA	NA	NA	NA	NA	Positive
Splenectomy	Post-ASCT	No	No	No	No	Yes (pre-ASCT)
Pre-harvest plasma exchange performed (N of times)	Yes (1)	Yes (2)	Yes (1)	Yes (1)	Yes (5)	No
Thermal management/ product handling	Room 30°C; warming blanket; return line warmer; 37°C-controlled product warming; warm saline washing	Room 28°C; warming blanket; inlet/return line warmer; 37°C-controlled product warming; no washing	Room >30°C; no line warmer; no product warming; no washing	Room 24°C; warming blanket; inlet/return line warmer; 37°C-controlled product warming; no washing	Room 30°C; warming blanket; return line warmer; 37°C-controlled product warming; no washing	Standard room temperature; return line warmer; no product warming; no washing
Infusion-related complications	None	None	None	None	None	Massive agglutination, 10 circuit changes
Engraftment (neutrophils/ platelets)	Day +14/+28	Day +16/+38	Day +20/+25	Day +21/+18	Engrafted	Day +10/+17
Lymphoma treatment response (months)	CR (15)	Relapsed	CR maintained	NA	CR (12)	CR (43)
CA titer normalization	NA	No	Yes	NA	No	Yes (13 months after mCR; sustained >30 months)

Features that distinguish the present case, include the longest documented follow-up with sustained lymphoma remission and cold agglutinin normalization. AL: amyloid light chain; ASCT: autologous stem cell transplantation; CA: cold agglutinin; CAD: cold agglutinin disease; CAS: cold agglutinin syndrome; CR: complete remission; DLBCL: diffuse large B-cell lymphoma; F: female; M: male; mCR: molecular complete remission; MZL: marginal zone lymphoma; NA: not available or applicable; RT: room temperature; SMZL: splenic marginal zone lymphoma.



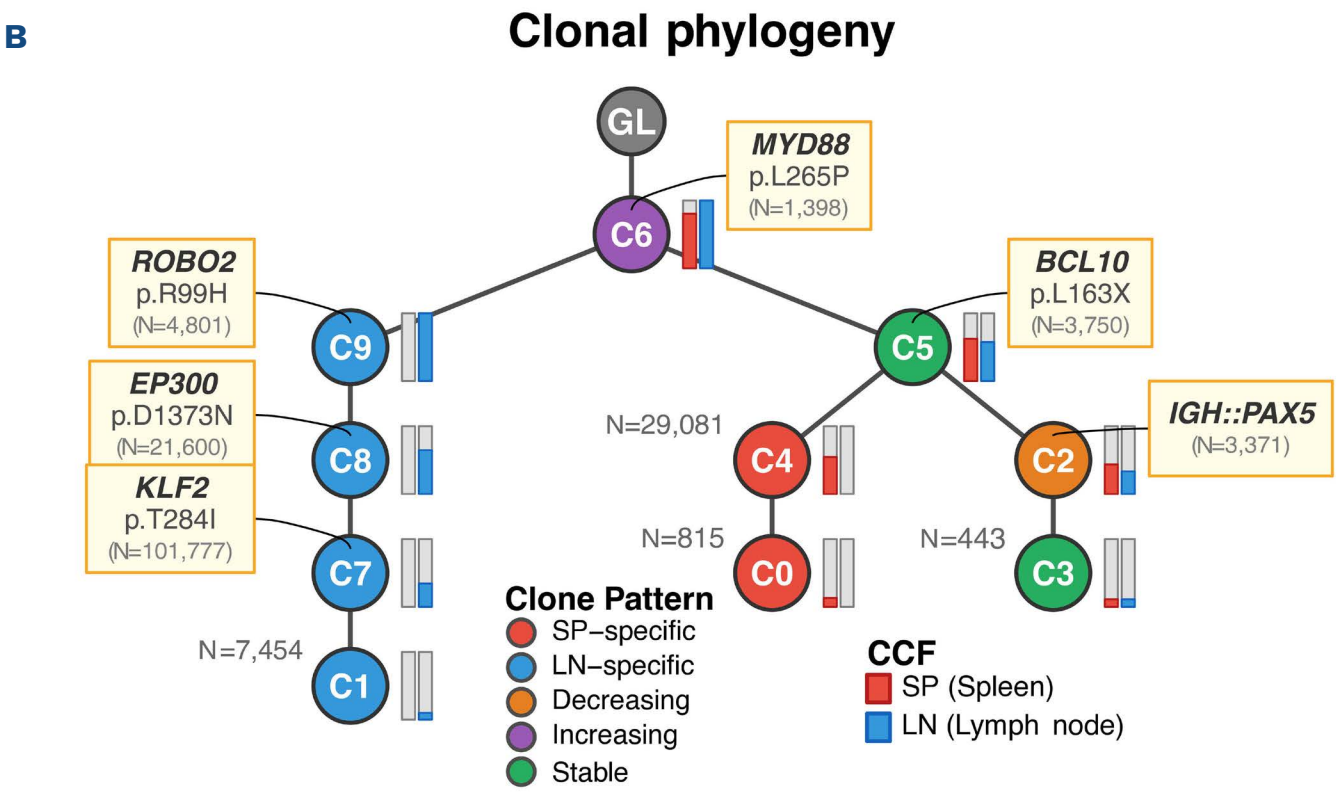


Figure 2. Clonal architecture and phylogenetic analysis of splenic marginal zone lymphoma-to-diffuse large B-cell lymphoma transformation. (A) Rank plots of corrected variant allele frequency (VAF) demonstrate clonal relationships between splenic marginal zone lymphoma (SMZL) (spleen [SP], upper panel) and transformed diffuse large B-cell lymphoma (DLBCL) (lymph node [LN], lower panel). Somatic single nucleotide variants (SNV) and structural variants (SV) are ranked in descending order of corrected VAF (local copy number [CNV]- and tumor purity-adjusted VAF). Shared variants (blue, detected in both samples) are prioritized over private variants (green for SP; red for LN). Key driver mutations are annotated: *MYD88*^{L265P}, *BCL10*^{L163X}, *IGH::PAX5* are shared between compartments, confirming a common clonal origin. Transformation-associated mutations, including *ROBO2*^{R99H}, *EP300*^{D1373N}, and *KLF2*^{T284I}, are exclusively LN-private. The dramatically extended tail in the LN plot (>150,000 variants) reflects the exceptionally high mutation burden within subclone C7, consistent with ongoing genomic instability during transformation. (B) Clonal phylogeny reconstructed from PyClone-VI cancer cell fraction (CCF) estimates using the pigeonhole principle. Nodes represent clonal clusters; edges indicate ancestral relationships. The tree originates from germline (GL). Node colors denote clone patterns; red (SP-specific), blue (LN-specific), orange (decreasing CCF from SP to LN), purple (increasing CCF), and green (stable). Adjacent bar plots show CCF in spleen (red) and lymph node (blue). The founding clone C6 harbors *MYD88*^{L265P} (N=1,398 mutations) and anchors all subsequent evolution. Two major branches emerge; (i) an LN-specific transformation-associated branch (C9→C8→C7→C1) sequentially acquiring *ROBO2*, *EP300*, and *KLF2* mutations, with C7 showing exceptionally high mutation burden (N=101,777) and (ii) a *BCL10*-mutant branch (C5) diverging into SP-specific (C4→C0) and shared (C2→C3) sub-branches, the latter harboring *IGH::PAX5*. Numbers along branches indicate private mutations acquired at each node.

return line. Collection over 2 days yielded 2.78×10^6 CD34$^+$ cells/kg without complications. The product was cryopreserved without previous washing using ABO-compatible fresh-frozen plasma with 10% dimethyl sulfoxide. Post-thaw viability was excellent (88.4-93.7%), with no visible agglutination before cryopreservation.

High-dose conditioning with cyclophosphamide, etoposide, melphalan, and dexamethasone was administered before ASCT. During stem cell infusion, massive agglutination occurred within minutes of thawing, causing repeated filter occlusion requiring ten circuit changes over 2 hours (Figure 1B-E). Besides transient oxygen desaturation, no severe adverse events occurred and the entire cell dose was successfully infused. Neutrophil engraftment occurred on day +10 and platelet engraftment on day +17. At 3 months post-ASCT, serum immunofixation showed no detectable M-protein with normalization of free light chain levels (κ <0.5 mg/L, λ <0.8 mg/L). Importantly, CA titers progressively declined from 1:1024 at transplantation to complete normalization (<1:64) at 14 months post-ASCT, which was maintained for >30 months (Figure 1A), followed by sustained ctDNA negativity for *MYD88* p.L265P. The patient remained in sustained complete remission of DLBCL and CAS at the last follow-up.

Here, we report a case of *MYD88*^{L265P}-mutated SMZL transformed into DLBCL with a high-titer CAS (1:1,024), in which ASCT led to sustained remission in both conditions. This case illustrates three key observations relevant to lymphoma-associated CAS management. First, complete normalization of CA titers was achieved post-ASCT, representing a rare and second most reported outcome in the literature. Second, the CA titers did not parallel the molecular kinetics of *MYD88*^{L265P} in real time. Finally, high CA titers pose a significant technical risk of agglutination during stem cell infusion, despite the absence of active clinical hemolysis.

Previous reports have suggested that CA titers track lymphoma activity, with CAS often preceding lymphoma diagnosis or accompanying aggressive disease courses.[9,10] In our case, the CA titers did not parallel the ctDNA kinetics of *MYD88*^{L265P}. Despite a marked decline in ctDNA levels following splenectomy and rituximab-based therapy, CA titers have remained elevated for years. Considering the short half-life of circulating IgM (5-10 days),[11] this persistence reflects continued CA production by long-term, chemo-insensitive plasma cells[12] that may be outside the *MYD88*-mutant clonal hierarchy yet depend on the lymphoma-associated microenvironment for survival. Complete CA normalization occurred only after sustained molecular remission post-ASCT, suggesting that the CA-producing population ultimately depended on the lymphoma clone or its associated microenvironment.[13] Clinically, persistent CA titers after apparent molecular remission should not be interpreted as evidence of refractory lymphoma.[14,15]

Although splenectomy is considered ineffective in CAD in which hemolysis occurs predominantly through hepatic C3b-mediated clearance,[1,14] our patient's improvement likely resulted from tumor debulking (confirmed by ctDNA reduction), removal of a major site of extravascular hemolysis, and cold avoidance limiting *in vivo* antibody binding.[1,14] The negative direct antiglobulin test for IgG argues against a significant warm autoimmune component to the hemolysis. Regarding transplantation management, although prophylactic PE was omitted because of the absence of clinical hemolysis after splenectomy, massive agglutination occurred during stem cell infusion, possibly owing to residual high-titer CA within the cryopreserved product.[3] Among five previously reported CAS/CAD-associated ASCT cases (Table 1),[2,4-7] most employed extensive preventive measures, including PE, and only one achieved complete CA normalization.[7] Our case demonstrates delayed yet durable CA normalization occurring 13 months after confirmed molecular clearance, with sustained serologic remission for more than 30 months at last follow-up. Retrospectively, preventive measures, such as pre-apheresis PE[2,4-7] or warm saline washing of the apheresis product,[7] may warrant consideration in high-titer CAS to mitigate infusion-related complications.

Overall, this case provides molecular evidence that durable remission of CAS is achievable post-ASCT. *MYD88*^{L265P} serves as a valuable traceable biomarker for disease activity, with sustained ctDNA negativity preceding eventual CA normalization. Although ASCT should not be precluded by high CA titers in eligible patients with CAS, meticulous preparation for infusion-related agglutination remains essential.

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Contributions

Conception and design by KY and YN. Provision of study materials or patients by JK, AT, KO, and TN-I. Collection and assembly of data by KY, NY, ES, and SI. Data analysis and interpretation by KY and YN. Manuscript writing by KY and YN. Final approval of manuscript by all authors, who are also accountable for all aspects of the work.

Data-sharing statement

All available data are presented in the text and figures of this case report. Additional requests for information can be made to the corresponding author.

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