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ORIGINAL PAPER

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Development and characterization of the novel *MYD88* mutated, 6q deleted BCWM.2 cell line for Waldenström macroglobulinaemia

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Summary

Cell lines have enabled a comprehensive understanding of disease biology and the advancement of new therapeutics in Waldenström macroglobulinaemia (WM). Herein, we report the development of BCWM.2, a novel WM cell line derived from an untreated symptomatic WM patient. Immunophenotypic and genomic analyses confirmed its origin from primary bone marrow WM cells. Flow cytometry revealed expression of CD19, CD20, CD23, CD38, CD45RA, CD52, immunoglobulin M (IgM) heavy chain and λ light chain on BCWM.2 cells. Whole genome sequencing demonstrated that, unlike BCWM.1, MWCL-1 and RPCI-WM1, which harbour $MYD88^{L265P}$ mutations, BCWM.2 carries $MYD88^{S243N}$. BCWM.2 also exhibited hallmark WM genetic aberrations, including 6q deletion and 6p amplification, along with unique mutations in *LYN, AKAP9, HDAC5, RUNX3* and *SPI1*. BCWM.2 cells exhibited optimal growth when co-cultured with HS-5 stromal cells and developed subcutaneous flank masses in all five NOD-SCID mice, along with measurable serum human IgM levels. BCWM.2 cells also showed remarkable sensitivity to the B-cell lymphoma 2 inhibitor venetoclax and the Janus kinase 2/Interleukin-1 receptor-associated kinase 1 inhibitor pacritinib, underscoring their utility for identification of pharmacologically active agents. BCWM.2 represents a novel myeloid differentiation primary response 88-mutated, 6q-deleted cell line with distinct genomic features for in vivo and in vitro studies for WM.

KEYWORDS
6q deletion, BCWM.2, CXCR4, ibrutinib, *MYD88*, Waldenström macroglobulinaemia

INTRODUCTION

Waldenström macroglobulinaemia (WM) is an incurable B-cell malignancy distinguished by bone marrow (BM) infiltration with lymphoplasmacytic cells (LPCs) and an immunoglobulin M (IgM) monoclonal gammopathy.[1,2] Somatic activating mutations in myeloid differentiation primary response 88 (*MYD88*) (95%–97%) and *CXCR4* (30%–40%) mutations are highly prevalent in WM patients.[3–9] Heterozygous deletions of chromosome 6q (del6q) are observed in 30%–70% of WM patients and are associated with IgM monoclonal gammopathy of undetermined significance (MGUS) to symptomatic WM transition.[2,7–12] Moreover, del6q are associated with

adverse disease features including a higher International Prognostic Scoring System for WM (IPSSWM) score and C-reactive protein levels, as well as a shorter time to treatment initiation; progression-free and overall survival.[10–13] Many critical genes that regulate *MYD88*/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), B-cell lymphoma 2 (BCL-2) and apoptotic signalling are located within del6q.[14] Complete (homozygous) loss of del6q was also associated with disease progression in WM patients receiving ibrutinib.[15]

Cell lines occupy a pivotal role as indispensable disease models, enabling a comprehensive understanding of disease biology and the advancement of therapeutic strategies. Several cell lines derived from WM patients have been established and characterized, including WSU-WM,[16] BCWM.1,[17] MWCL-1[18] and RPCI-WM1.[19] The WSU-WM cell line exhibited a karyotype and morphology resembling Burkitt lymphoma rather than WM, and it also displayed discordant expression of κ and λ light chains compared to the primary patient cells. The authentication and neoplastic derivation of this cell line remained unresolved.[16] Subsequent attempts to establish WM cell lines have yielded more promising results, including BCWM.1 in 2007,[17] MWCL-1 in 2011[18] and RPCI-WM1 in 2013,[19] all of which express the *MYD88* p.Leu265Pro ($MYD88^{L265P}$) mutation. However, none of these cell lines express *CXCR4* or del6q. Importantly, BCWM.1 and MWCL-1 were instrumental in understanding the pro-survival signalling cascades associated with mutated *MYD88*.[20–25] This included the identification of Bruton's tyrosine kinase (*BTK*) as an important therapeutic target of mutated *MYD88* signalling that supported the investigation of BTK inhibitors and subsequent regulatory approval of ibrutinib and zanubrutinib for the treatment of symptomatic WM.[20,26,27] BCWM.1 and MWCL-1 cells have also been used to identify other druggable targets of mutated *MYD88* signalling and to gain valuable mechanistic insights into BTK inhibitor resistance.[28–32]

In this study, we report on the establishment and characterization of a novel *MYD88*-mutated, del6q WM cell line, BCWM.2, that was derived from the BM of a WM patient. Our study also offers a comparison of the key genomic features of BCWM.2 with three established WM cell lines (BCWM.1, MWCL-1 and RPCI-WM1) using whole genome sequencing (WGS). A novel WM xenograft mouse model using BCWM.2 cells is also described, as well as the use of BCWM.2 cells for evaluating novel therapeutic agents.

PATIENT, MATERIALS AND METHODS

Case description

The BCWM.2 cell line was derived from the BM aspirate of a 66-year-old male patient. He initially presented with fatigue and anaemia. Physical examination was unremarkable. Computed tomography scans showed mediastinal adenopathy and splenomegaly. A bone marrow examination revealed involvement by lymphoplasmacytic cells. His white blood cell count was 8.6 k/μL, haemoglobin 8.7 g/dL and platelet count 220 k/μL. Serum protein electrophoresis revealed an IgM λ monoclonal spike, and total serum IgM, immunoglobulin A and immunoglobulin G (IgG) levels were 2550, 34 and 548 mg/dL respectively. Both cryoglobulins and cold agglutinins were unremarkable. A repeat BM biopsy revealed 40% involvement of the intertrabecular space by lymphoplasmacytic cells. Flow cytometric analysis of the BM aspirate specimen indicated a population of $CD19^+$ $CD20^+$ B cells that displayed CD5, CD11c and CD52 expression; showed bright monotypic surface immunoglobulin λ; and were negative for CD10, CD23 and CD38. A BM aspirate from this biopsy was used for the establishment of the BCWM.2 cell line. The patient was subsequently treated with ibrutinib at a dose of 420 mg orally once daily and achieved a partial response. His haemoglobin reached 12.1 g/dL. As per protocol, serial response assessments including BM biopsies were performed at defined intervals. After 30 treatment cycles (each cycle being 4 weeks), the patient developed worsening anaemia and increased serum IgM levels consistent with disease progression.

Isolation of BCWM.2 WM cells

BM samples were collected following informed consent and with the approval of our Institutional Review Board. Mononuclear cells from the BM aspirate were isolated by density-gradient centrifugation using Ficoll-Paque Plus-1070 (Pharmacia Biotech, Piscataway, NJ). LPC immunoselection was performed using a $CD19^+$ cell isolation kit (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's instructions. Immortalized LPCs (BCWM.2) were cultivated in RPMI-1640 medium (Gibco, Baltimore MD) supplemented with 10% fetal bovine serum (FBS) for an extended period. Subsequently, LPCs were co-cultured with HS-5 stromal cells (ATCC, Manassas VA) in a tissue culture-treated Transwell system (Costar, Glendale AZ) using Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) supplemented with 20% heat-inactivated FBS, human interleukin-6 (IL-6) at 1 ng/mL (Invitrogen, Carlsbad CA), 100 U/mL of penicillin and 10 μg/mL of streptomycin (Mediatech, San Diego CA).

Phenotypic characterization of BCWM.2 cells

Flow cytometric analysis was performed using an LSRFortessa Analyzer (BD Biosciences). Staining patterns for all antibodies used were compared to their respective isotype controls. The following antibodies were employed: CD3 (Clone HIT3a, Biolegend, San Diego CA), CD19 (Clone SJ25C1, Biolegend), CD20 (Clone S18015E, Biolegend), CD45RA (Clone HI100, Biolegend),

CD11c (Clone S-HCL-3, Biolegend), CD138 (Clone MI15, Biolegend), CD117 (Clone 104D2, Biolegend), CD52 (Clone HI186, Biolegend), CD5 (Clone UCHT2, Biolegend), CD23 (Clone EBVCS-5, Biolegend), CD38 (HIT2, Biolegend), CD10 (Clone HI10a, Biolegend), IgM (Clone MHM-88, Biolegend), immunoglobulin D (IgD) (Clone IA6-2, Biolegend) and Ig light chain λ (Clone MHL-38, Biolegend). To characterize signalling pathways and survival processes activated in BCWM.2 cells and compare them with established WM cell lines (BCWM.1, MWCL-1 and RPCI-WM), cells were cultured in optimal media under resting conditions. Protein expression and phosphorylation levels were assessed by immunoblotting using the following antibodies: Phospho-Jak2 (Tyr1007/1008) (#3771, Cell Signaling), Janus kinase 2 (JAK2) (Clone D2E12, Cell Signaling), Phospho-signal transducer and activator of transcription 3 (STAT3) (Tyr705) (Clone D3A7, Cell Signaling), Phospho-STAT3 (Ser727) (#9134, Cell Signaling), STAT3 (Clone 124H6, Cell Signaling), Phospho-interleukin-1 receptor-associated kinase 4 (IRAK4) (Thr345/Ser346) (Clone D6D7, Cell Signaling), IRAK4 (#4363, Cell Signaling), Interleukin-1 receptor-associated kinase 1 (IRAK1) (Thr209) (#12756, Cell Signaling), IRAK1 (Clone D51G7, Cell Signaling), Phospho-Lyn (Tyr396) (Clone ARC1597, Invitrogen), Lyn (Clone C13F9, Cell Signaling), Phospho-hematopoetic cell kinase (HCK) (Tyr522) (PA5-37592, Invitrogen), HCK (Clone E1I7F, Cell Signaling), Anti-BTK (phospho Y223) (Clone EP420Y, Abcam), BTK (Clone D3H5, Cell Signaling), Phospho-NF-κB p65 (Ser536) (Clone 93H1, Cell Signaling), NF-κB p65 (Clone D14E12, Cell Signaling), Phospho-Bcl-2 (Ser70) (Clone 5H2, Cell Signaling), Bcl-2 (Clone D17C4, Cell Signaling), Phospho-extracellular signal-regulated kinase 1/2 (ERK 1/2) (Thr202/Tyr204) (Clone D13.14.4E, Cell Signaling), ERK 1/2 (Clone 137F5, Cell Signaling) and myeloid leukemia cell differentiation protein 1 (Clone D35A5, Cell Signaling). α-Tubulin (#2144, Cell Signaling) was used as a loading control.

Cytogenetic alteration and mutation identification by whole genome sequencing and whole exome sequencing

Genomic DNA was isolated using a genomic DNA purification kit (Qiagen, Ann Arbor MI). WGS of BCWM.2, the patient's primary BM CD19-selected WM cells and peripheral blood (PB) CD19-negative mononuclear cells for germline control, and the WM cell lines BCWM.1, MWCL-1 and RPCI-WM1 was performed as before using the Complete Genomics platform.[1,2] To verify the identity of BCWM.2 xenografted tumours, whole exome sequencing (WES) of tumour tissue and BCWM.2 cells was performed. Reads were aligned to the KnownGene HG19/GRCh37 reference using STAR. Read counts per gene were obtained using featureCounts from Rsubread and analysed using voom from the edgeR/limma Bioconductor packages in R.

In vivo establishment of BCWM.2 xenograft model in NOD-SCID mice

All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (Dana-Farber Cancer Institute). Two million BCWM.2 cells were subcutaneously inoculated in a Matrigel suspension into 6- to 8-week-old female NOD-SCID mice (Charles River Laboratories, Cambridge MA). PB from the mice was serially collected from tail veins, and serum was evaluated using a human IgM ELISA Kit (Abcam, MA, USA). Flank masses developed ~4–5 weeks following BCWM.2 implantation, and the identity of the tumour was evaluated by immunohistochemical staining with anti-human CD20, CD138, MUM1, kappa, lambda, IgG, IgM and Ki67 antibodies; and by WES of the tumour mass, BCWM.2 cells and the original patient BM tumour sample.

Responses of BCWM.2 cells to pharmacological intervention

To evaluate the response of BCWM.2 cells to pharmacological intervention, 0.2 million BCWM.2 cells were treated in a 96-well plate with pharmacologically relevant agents that included the BTK inhibitors ibrutinib and zanubrutinib; the dual BTK/HCK inhibitor KIN-8194; the JAK2/SRC/IRAK1 inhibitor pacritinib; the BCL-2 inhibitor venetoclax; and the Lyn/Bcr-Abl inhibitor bafetinib (MedChemExpress Monmouth Junction NJ). BCWM.2 cells were incubated with these agents at concentrations of 0.5 μM and 1 μM over a duration of 20 hours. The induction of apoptosis and cell death in response to these treatments was subsequently evaluated through flow cytometric analysis, employing the Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego CA).

Evaluation of *LYN*^{I297N} mutation on the growth and cell death/apoptosis of BCWM.2 cells

To functionally characterize the *LYN*^{I297N} somatic mutation, Lyn-targeting shRNA constructs (shRNA#1: ACCG GCAGAGTGTCTTGGATGATTTCGTTAATATTCATAG CGAAATCATCCAGGACGCTCTGTTTT; shRNA#2: A CCGGGTGATGATGGAGTAGATTTGAGTTAATAT TCATAGCTCAAATCTACTCCATCGTCACTTTT; and non-targeting control shRNA: CCGGCAACAAGATGGA GAGCACTAAGTTAATATTCATAGCTTGGTGCTC TTCATCTTGTTGTTTT) were cloned into pRSI-U6-(shRNA)-CMV-GFP-Puro vector (Cellecta, Mountain View CA) and were transduced into BCWM.2 cells followed by puromycin selection. Knockdown of *LYN* was confirmed by western blot using an anti-LYN antibody (C13F9, Cell Signaling). Cell growth was measured using the alamarBlueTM HS Cell Viability Reagent (Invitrogen).

Cell death and apoptosis were evaluated by flow cytometry using Annexin V AF-647 and propidium iodide staining (Invitrogen).

RESULTS

Growth and morphological characteristics of BCWM.2 cells

Under optimized conditions with a concentration of 0.5–1 million cells/mL, BCWM.2 cells displayed a doubling time of 3 days when co-cultured with HS-5 stroma cells in IMDM medium supplemented with 20% FBS and IL-6.

Immunophenotypic characterization of BCWM.2 cells

By flow cytometry, BCWM.2 cells expressed CD19, CD20, CD23, CD38, CD45RA, CD52, surface IgM and surface immunoglobulin light chain λ. A small subset of cells expressed CD138 and surface IgD, while CD3, CD5, CD10, CD11c and CD117 were absent (Figure 1). The immunophenotype aligned with that of the patient's BM from which the BCWM.2 cells were derived, except for CD5, which was expressed in 81.4% of $CD19^+$ selected BM tumour cells.

Cytogenetic alterations and mutations identified by WGS and comparison to other established WM cell lines

WGS was performed on BCWM.2, primary ($CD19^+$) BM LPCs from the founder patient prior to treatment and at the time of progression; baseline PB patient germline ($CD19^-$) cells, and the WM cell lines BCWM.1, MWCL-1 and RPCI-WM1. BCWM.2, like the primary patient's BM tumour cells from which it was derived, carried a previously described activating $MYD88^{S243N}$ heterozygous mutation.[33–35] Conversely, BCWM.1, MWCL-1 and RPCI-WM1 cells were heterozygous for the $MYD88^{L265P}$ somatic mutation. The primary patient WM cells as well as all four cell lines (BCWM.1, BCWM.2, MWCL-1 and RPCI-WM1) were *CXCR4* wild-type. A novel, previously uncharacterized LYN^{I297N} somatic mutation was identified in BCWM.2. Additional mutations in BCWM.2 included $AKAP9^{R1276*}$, $HDAC5^{H236P}$, $RUNX3^{V141L}$ and $SPI1^{Q227E}$, the latter recently identified as a recurring mutation in WM[36] (Table 1). BCWM.2 closely matched its parental tumour conventional karyotyping profile with trisomy 3 and 12, as well as deletion of the entire arm of 6q and corresponding amplification of 6p. BCWM.1 exhibited no amplifications or deletions, whereas MWCL-1 displayed monoallelic deletions in 17p and 17q, as well as amplifications in 11q. RPCI-WM1 exhibited deletions in 3p, 6q, 9p, 13q, 18q, 19q and X, along with amplifications in 5p, 6p, 7q, 11q, 14q, 18q, 21q and acquired uniparental disomies on 17p, 18q and X.

In vivo establishment of BCWM.2 tumour mass in NOD-SCID mice

To assess the tumour-forming capability of BCWM.2 cells in immunodeficient NOD-SCID mice, 2 million BCWM.2 cells were subcutaneously injected per mouse. A subcutaneous flank tumour mass emerged at the injection site approximating 500 mm^3 4 weeks post-injection in in all five mice. Importantly, serum human IgM levels increased 2 weeks post-injection in BCWM.2 xenografted mice (Figure 2A,B). Pathological hematoxylin and eosin (H&E) staining of the BCWM.2 xenografted tumours revealed that the tumour cells were predominantly intermediate in size with plasmacytoid differentiation, and immunohistochemical staining confirmed expression of human $CD20^+$ and $MUM1^+$, along with clonality for human λ light chain and IgM heavy chain. The proliferation index, as determined by Ki67 staining, was 60% (Figure 2C). Further confirmation of the xenografted tumour origin was achieved through WES that showed the $MYD88^{S243N}$ and LYN^{I297N} variants.

Responses of BCWM.2 cells to pharmacological intervention and comparative analysis of signalling activation and survival processes with other WM cell lines

To evaluate therapeutic vulnerabilities, BCWM.2 cells were subjected to treatment with several active agents including those in development. BCWM.2 cells showed a modest response to the BTK-inhibitors ibrutinib and zanubrutinib, both of which are Food and Drug Administration (FDA)-approved therapies for WM (Figure 3). Similarly, treatment with the dual BTK/HCK inhibitor KIN-8194 yielded a modest apoptotic response (Figure 3). In contrast, treatment with the BCL-2 inhibitor venetoclax elicited a significant apoptotic response (Figure 3). Pacritinib, an FDA-approved kinase inhibitor primarily used in myelofibrosis that targets key pro-signalling pathways implicated in mutated *MYD88* signalling, such as IRAK1, JAK2/STAT3 and SRC,[30] also showed robust apoptosis in BCWM.2 cells (Figure 3). To further explore the signalling features of BCWM.2 cells in comparison to other WM cell lines, we assessed key pro-survival pathways (Figure 4). BCWM.2 cells exhibited lower BTK activation but showed clear activation of BCL-2 and STAT3. These signalling characteristics may explain the limited response to BTK inhibitors while supporting sensitivity to the BCL-2 inhibitor venetoclax and the STAT3-targeting inhibitor pacritinib.

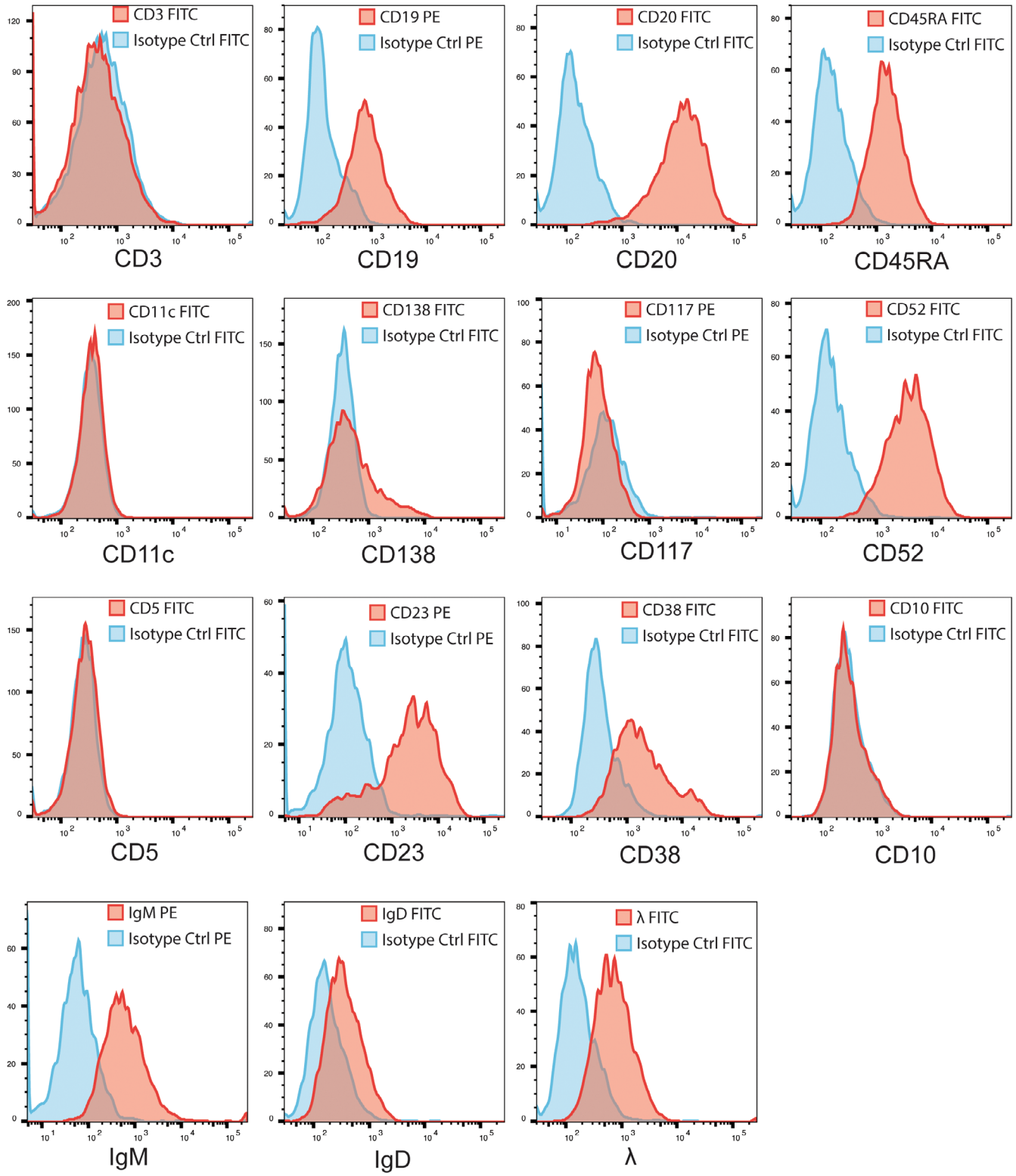

FIGURE 1 Immunophenotypic characterization of BCWM.2 cells. FITC- and Phycoerythrin (PE)-conjugated antibodies were used for phenotyping of the BCWM.2 cell line by flow cytometry. Blue peaks represent isotype staining, and red peaks represent antigen-specific staining.

Evaluation of *LYN*^{I297N} mutation to BCWM.2 growth and survival

Since *LYN*^{I297N} is located in the regulatory hinge region of LYN, we investigated its role in BCWM.2 cell growth and survival by performing *LYN* knockdown. Western blot analysis confirmed successful knockdown (Figure S1A). However, neither shRNA construct affected BCWM.2 cell proliferation or apoptosis (Figure S1B–C). Moreover, BCWM.2 cells treated with the Lyn/Bcr-Abl

TABLE 1 Mutated genes identified within WM cell lines through filtering algorithms in whole genome sequencing (WGS) using ExAC allele frequencies and variant damaging scores with SIFT, PolyPhen and CADD, and transcriptome validation.

WM cell line	Symbol	Location	Allele	Protein position	Amino acid	SIFT	PolyPhen	ExAC AF	CADD	BCWM2 patient Mut/ Tota	BCWM2 cell line Mut/Total
BCWM.2	*MYD88*	3:38182292–38182292	G/A	251	S/N	Deleterious(0)	Probably_damaging (0.983)	-	29.7	88/134	71/106
BCWM.2	*LYN*	8:56879436–56879436	T/A	297	I/N	Deleterious(0)	probably_damaging (0.984)	-	33	0	19/54
BCWM.2	*AKAP9*	7:91646405–91646405	C/T	1276	R/*	-	-	-	36	15/28	16/33
BCWM.2	*HDAC5*	17:42165957–42165957	T/G	236	H/P	Deleterious(0)	possibly_damaging (0.747)	-	23.6	25/57	18/42
BCWM.2	*RUNX3*	1:25254083–25254083	C/A	141	V/L	Deleterious(0.01)	probably_damaging (0.986)	-	35	17/35	19/37
BCWM.2	*SPI1*	11:47376915–47376915	G/C	227	Q/E	Deleterious(0)	possibly_damaging (0.738)	-	27.2	8/20	5/23
BCWM.1, RPCI-WM1, MWCL-1	*MYD88*	3:38182641–38182641	T/C	265	L/P	Deleterious(0)	probably_damaging (1)	0.0002	32		
BCWM.1	*RAPGEF6*	5:130846087–130846087	C/G	242	R/P	Tolerated(0.15)	possibly_damaging (0.897)	-	26.3		
RPCI-WM1	*CDK18*	1:205495302–205495302	G/A	189	R/Q	Deleterious(0.01)	probably_damaging (0.995)	-	35		
RPCI-WM1	*BATF3*	1:212860284–212860284	C/T	78	R/Q	Deleterious(0)	probably_damaging (0.957)	-	26.6		
RPCI-WM1	*TP53*	17:7577120–7577120	C/T	273	R/H	Tolerated(0.09)	benign(0.303)	0.0002	27.3		
MWCL-1	*TP53*	17:7578502–7578502	A/G	143	V/A	Deleterious(0)	probably_damaging (0.997)	-	23.1		

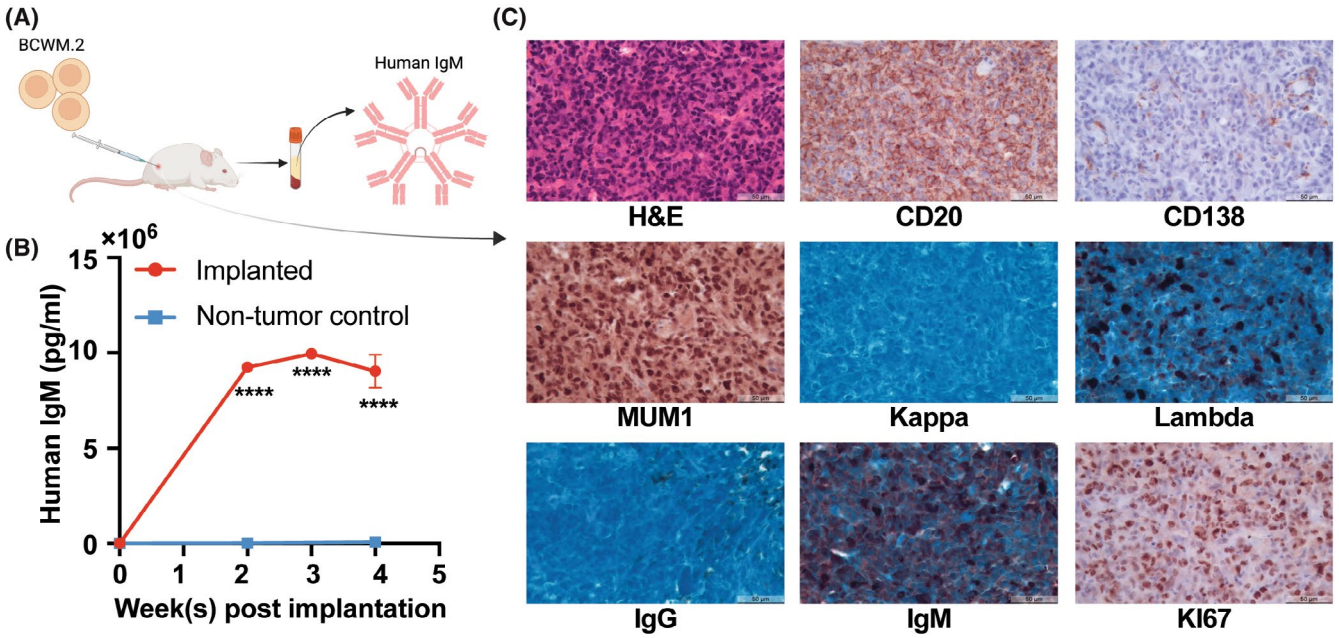

FIGURE 2 In vivo establishment of BCWM.2 tumour in NOD-SCID mice. (A) Schematic representation of establishing BCWM.2 xenograft mouse model and collecting specimens for human IgM evaluation in serum. (B) NOD-SCID mice were subcutaneously injected with BCWM.2 cells. Serum was collected, and human IgM levels were monitored once a week by ELISA (non-tumour control $n = 1$, BCWM.2 implanted mice $n = 5$). Data represent mean ± SEM, $^{****}p < 0.0001$; One-way ANOVA Dunnett's multiple comparisons test, each timepoint compares to the timepoint of implantation (Time 0). (C) Representative slides of pathological evaluation of the tumour mass from the implanted mice by hematoxylin and eosin (H&E) and immunohistochemical staining for human CD20, CD138, MUM1, kappa, lambda, IgG, IgM and Ki67.

inhibitor bafetinib showed only a modest level of apoptosis (Figure 3).

DISCUSSION

Cell lines have been invaluable to the understanding of the biology and development of therapeutics for WM. However, only three established cell lines are currently available for use in WM.[17–19] In this study, we report the establishment of a new WM cell line, BCWM.2, which bears the activating $MYD88^{S243N}$ mutation that differs from the more common activating $MYD88^{L265P}$ mutation found in 95%–97% of WM patients. Although *MYD88* mutations other than $MYD88^{L265P}$ are rare in WM patients, they constitute a significant portion of all *MYD88* mutations in patients with the ABC subtype of diffuse large B-cell lymphoma (ABC DLBCL) and CLL.[33,35–37] Like $MYD88^{L265P}$, $MYD88^{S243N}$ mutation triggers NF-κB activation.[33] Our signalling pathway data indicate that $MYD88^{S243N}$-mutated BCWM.2 cells exhibit distinct IRAK1 activation and STAT3 phosphorylation patterns compared to $MYD88^{L265P}$-mutated WM cell lines, consistent with the findings of Ngo et al.[33] on $MYD88^{L265P}$ and $MYD88^{S243N}$ mutants. Differences in disease presentation have been reported based on *MYD88* variant type in patients with ABC DLBCL and CLL. Therefore, BCWM.2 provides a valuable tool for investigating differences between mutated *MYD88* L265P and *MYD88* non-L265P biology and signalling.

Noteworthy, the BCWM.2 cell line also carries heterozygous del6q, which is the most prevalent cytogenetic abnormality in WM affecting up to 70% of WM patients.[2,7–12] Del6q impacts multiple genes with critical regulatory functions related to BTK (*IBTK*), apoptosis (*FOXO3*), *BCL-2* (*BCLAF1*) and NF-κB (*TNFAIP3*, *HIVEP2*) signalling.[14] Therefore, BCWM.2 offers a model for systematically studying copy number losses associated with del6q. Moreover, homozygous del6q loss has also been reported in patients progressing on ibrutinib, affording a model for the use of BCWM.2 to study the consequences of complete del6q loss on the development of resistance to BTK-inhibitors.[15] Other mutations identified in BCWM.2 included LYN^{I297N}, $AKAP9^{R1276*}$, $HDAC5^{H236P}$, $RUNX3^{V141L}$ and $SPI1^{Q227E}$. The mutation in *LYN* was of particular interest since *LYN* is part of the BCR signalling apparatus and this mutation was located within the regulatory hinge region of *LYN*. Moreover, CD79 activating mutations that trigger BCR complex signalling are frequently found in *MYD88*-mutated ABC DLBCL, primary CNS lymphoma and transformed WM.[33,38,39] However, knockdown of *LYN* did not impact the growth or survival of BCWM.2. Moreover, treatment of BCWM.2 cells with the *LYN* inhibitor bafetinib[40] resulted in only a modest apoptotic response. Therefore, the functional significance of this mutation, if any, remains to be clarified.

While *AKAP*, *HDAC5* and *RUNX3* mutations have not been reported in WM, the finding of the $SPI1^{Q227E}$ was of great interest since this mutation was found in 6% of a

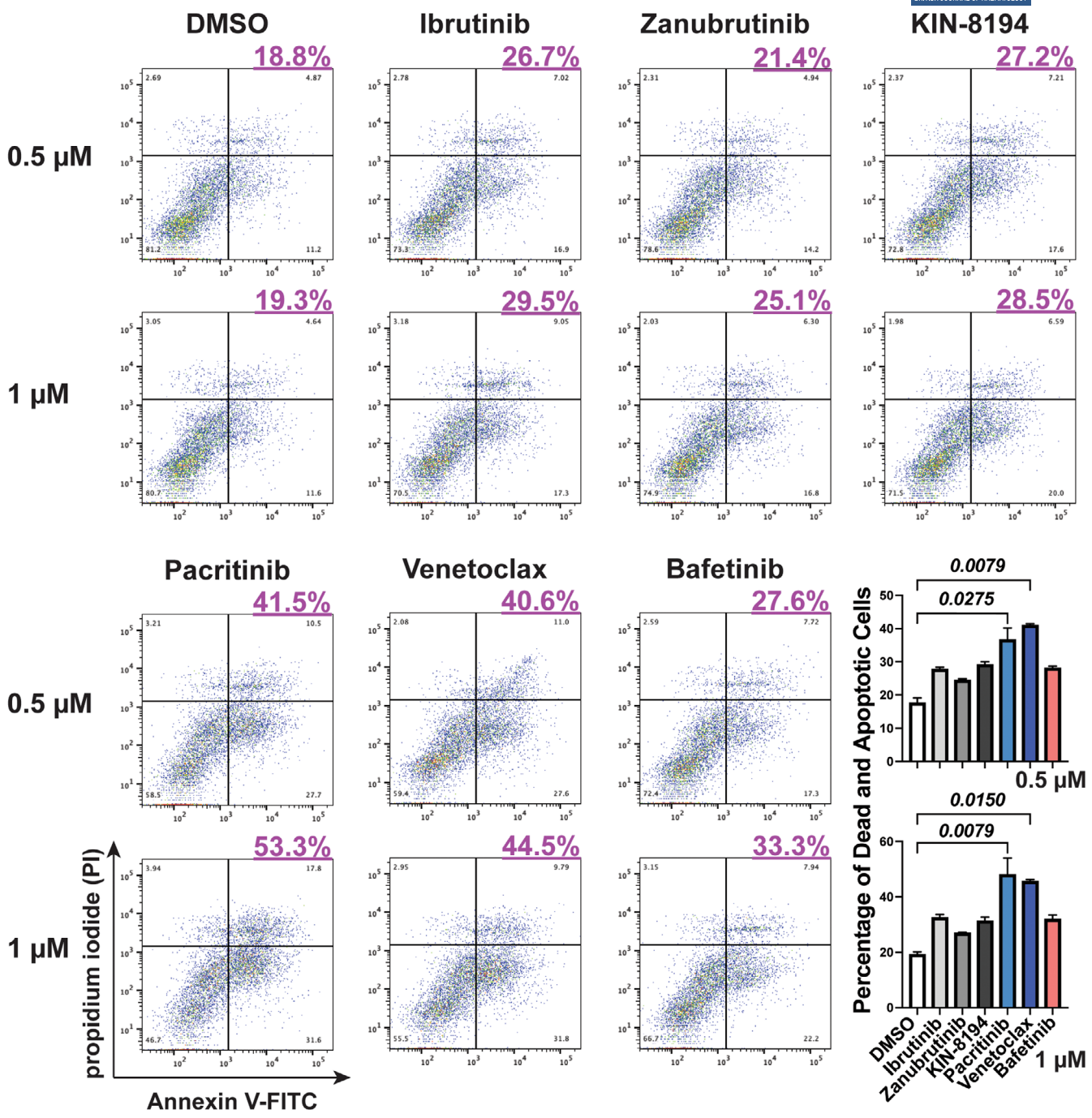

FIGURE 3 Impact of pharmacological agents on BCWM.2 cells. BCWM.2 cells were treated with the BTK inhibitors ibrutinib and zanubrutinib; KIN-8194, a dual inhibitor of BTK and HCK; pacritinib that targets JAK2, SRC and IRAK1; venetoclax, targeting BCL-2; and bafetinib, a Lyn/Bcr-Abl inhibitor. Treatments were administered at the indicated concentrations overnight. Evaluation of apoptosis was conducted via flow cytometry, utilizing FITC-Annexin V and propidium iodide staining. Representative flow cytometry plots of three independent experiments are presented with summarized data. Error bars represent mean ± SEM, and statistical analysis was performed using the Friedman test multiple comparisons test. Percent of BCWM.2 cells that underwent apoptosis is shown above each histogram for that drug treatment.

discovery and validation cohort in studies by Roos-Weil et al.[36] The expression of $SPI1^{Q227E}$ (identified as $SPI1^{Q226E}$ in their studies due to different isoform sequencing) was associated with inferior overall survival. Moreover, their functional studies showed that the $SPI1^{Q227E}$ mutation modified the DNA-binding specificity of the wild-type protein to promote binding to DNA recognition sequences of other classes of ETS proteins, while its expression showed enrichment in transcription signatures that promoted cell proliferation and blocked B-cell differentiation. BCWM.2 therefore offers a cell model for the study of this recently recognized recurrent somatic mutation in WM.

Our pharmacological studies with BCWM.2 also provided new insights for drug development in WM. While

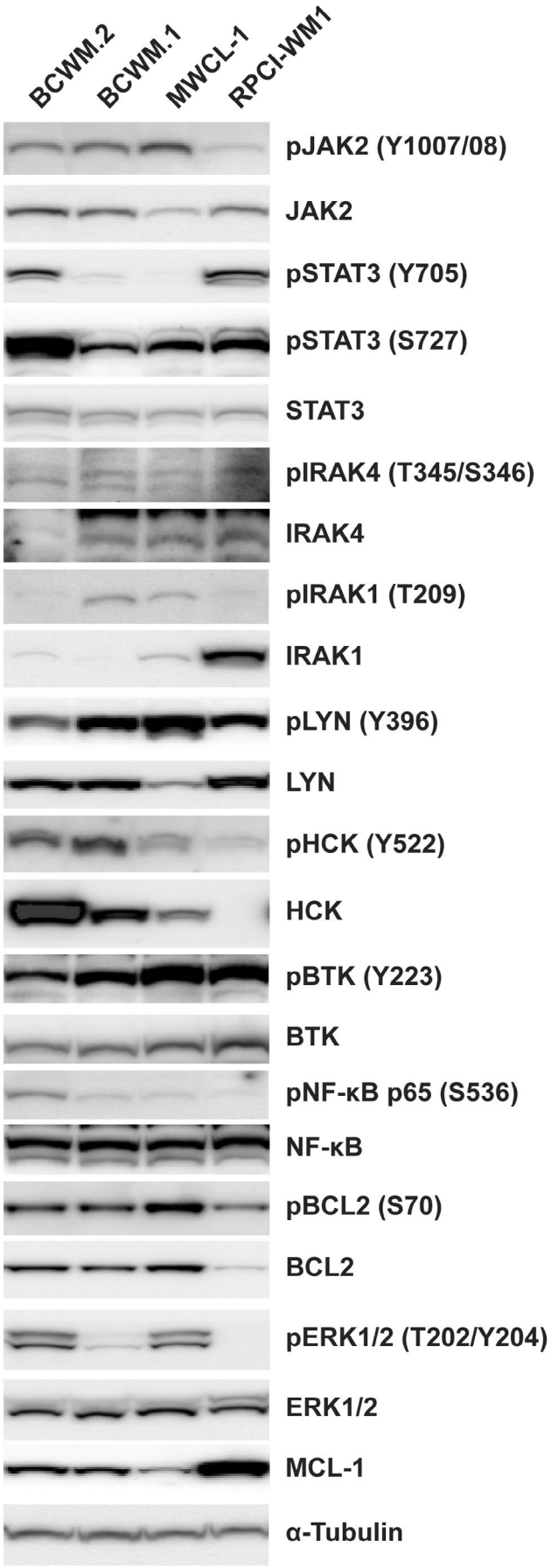

FIGURE 4 Activation profiles of signalling pathways and survival processes in BCWM.2 cells compared to established WM cell lines. Indicated protein expression and phosphorylation levels were assessed by western blot in resting BCWM.2 cells and the established WM cell lines BCWM.1, MWCL-1 and RPCI-WM1. α-Tubulin was used as a loading control. Experiments were performed at least twice, and representative blots are shown.

BCWM.1 and MWCL-1 are sensitive to BTK inhibitors and have played a key role in identifying BTK-targeted therapies for symptomatic WM, BCWM.2 appears to be resistant to BTK inhibition. In contrast, treatment of BCWM.2 cells with pacritinib, a compound that targets multiple pro-survival kinases within the mutated *MYD88* signalling pathway such as *JAK2/STAT3, SRC* and *IRAK1* triggered extensive apoptosis.[30] Particularly impressive was the robust apoptotic effect triggered by venetoclax. Our pro-survival signalling analysis revealed distinct activation patterns in BCWM.2 cells compared to other WM cell lines, including strong BCL-2 and STAT3 activation—both previously linked to $MYD88^{S243N}$ mutantion.[33] These findings highlight the potential of BCWM.2 as a unique model for developing targeted therapies against BCL-2, JAK2/STAT3 and non-L265P *MYD88* mutations.

Animal models have been crucial for the investigation of disease biology and therapeutic development in WM. In this study, we established a novel animal model using BCWM.2 cells that were xenografted into NOD-SCID mice. All five subcutaneously inoculated animals developed robust flank tumour growth within 2 weeks post-inoculation. The origin of the tumour from BCWM.2 cells was confirmed by immunohistochemistry and by WES that identified both $MYD88^{S243N}$ and LYN^{I297N} mutations. Importantly, BCWM.2 xenografted mice showed an increase in human serum IgM following tumour development, whose levels can be monitored as a useful biomarker of response assessment to novel drug development.

In summary, BCWM.2 represents a novel WM cell line that harbours $MYD88^{S243N}$, del6q and $SPI1^{Q227E}$ variants, thereby providing a novel cell model for in vitro and in vivo studies of WM pathogenesis and targeted drug development.

AUTHOR CONTRIBUTIONS

SPT, GY, YC, XL and SL conceived the experimental design. SPT and SL wrote the first draft. CFBY, AK, KMS, JP, AGC, YC, GY, LX, NT, HS, MLG and SL performed experiments. JJC, SRS, CJP and AG provided patient care and collected samples. ZRH performed genomic analysis, and RDC provided pathology review. PCG oversaw animal studies. All authors reviewed the first draft and were provided the opportunity to submit revisions. The final draft was approved by all authors.

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CONFLICT OF INTEREST STATEMENT

SPT received research funding and/or consulting fees from Abbvie/Pharmacyclics Inc., Janssen Oncology Inc., Beigene Inc., Eli Lilly Pharmaceuticals, Bristol Myers Squibb, and Ono Pharmaceuticals. JJC received research funds from Abbvie, AstraZeneca, Beigene, Cellectar, LOXO, and Pharmacyclics, and honoraria from Abbvie, AstraZeneca, Beigene, Cellectar, Kite, LOXO, Janssen, and Pharmacyclics. SRS received research funding and/or consulting fees from Beigene, Cellectar Biosciences, and ADC Therapeutics. JMH receives consulting fees from Arbella Therapeutics. GY is currently employed at Blueprint Medicines, Inc. All other authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data in support of the findings for this study are available from the corresponding author (SPT) and are available upon reasonable request. Sequencing data will be deposited in the Sequence Read Archive (SRA).

ETHICS STATEMENT

Sample use was approved by the Dana-Farber/Harvard Cancer Centre Institutional Review Board.

PATIENT CONSENT STATEMENT

Written patient consent was obtained for human samples used in these studies using an institutional review board-approved consent form.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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