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Stem-like tumor cells confer drug resistant properties to mantle cell lymphoma

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Abstract
We recently identified clonogenic malignant stem cell populations in human mantle cell lymphoma (MCL), a particularly deadly subtype of non-Hodgkin lymphoma (NHL). We discovered that CD45+CD19-MCL cells, which we termed MCL-initiating cells (MCL-ICs), are highly tumorigenic and display self-renewal capacity in vivo; in contrast, CD45+CD19+ MCL cells, which constitute the vast majority of cells within the tumors, show no self-renewal capacity and greatly reduced tumorigenicity. Given the newly appreciated role of cancer-initiating cells in the drug resistance of cancers, it is critical to investigate whether CD45+CD19-MCL-ICs play a role in the drug resistance of human MCL. We discovered that MCL-ICs were more resistant to clinically relevant chemotherapeutic agents, in combination or in a single regimen, compared to CD45+CD19+ cells, and that this drug resistance was largely due to quiescent properties with enriched ABC transporters. In conclusion, designing novel therapies to kill CD45+CD19-MCL-ICs may prevent relapse and increase patient survival.

Keywords: Mantle cell lymphoma, chemotherapy resistance, cancer stem cells, cancer-initiating cells, non-Hodgkin lymphoma

Introduction
Mantle cell lymphoma (MCL) is a particularly deadly subtype of non-Hodgkin lymphoma (NHL), which is the sixth most common type of human cancer in the United States [1–3]. Unlike small lymphocytic lymphoma/chronic lymphocytic lymphoma (SLL/CLL), which is relatively sensitive to chemotherapy, MCL is highly refractory to standard therapy and displays the worst survival rate among patients with NHL [3,4]. Furthermore, most patients with MCL respond poorly to new therapeutic interventions, such as monoclonal antibody-based therapy or stem cell transplantation, which have only minor effects on overall survival rates [3,5]. Therefore, MCL is essentially incurable using most conventional clinical regimens [6].

We recently identified CD45+CD3−CD34−CD19− MCL cells, which we termed MCL-initiating cells (MCL-ICs), in human patients [7] that represent the first identification of stem-like cells in any subtype of NHL. We discovered that CD45+CD19− MCL-ICs are highly tumorigenic and display self-renewal capacity in vivo. In contrast, CD45+CD19+ MCL cells, which constitute the vast majority of cells within the tumors, show no self-renewal capacity and have greatly reduced tumorigenicity. The frequencies of tumor-initiating cells in CD45+CD19− MCL populations are 48 000-fold higher than those in unfracti-
relapse of the disease [17–22]. Moreover, our CD45+CD19− MCL-ICs display quiescent properties [7], which may render them difficult to kill via conventional therapies that target cells with increased mitotic activity.

Therefore, in this study, we utilized clinically relevant chemotherapeutic regimens and tested the functional role of CD45+CD19− MCL-ICs in the drug resistance of MCL. Even though the drugs tested are widely used in the clinic, few studies have shown their efficacy in vitro using primary cells [23–25]. In addition, our study is the first to show that MCL stem-like cells are resistant to clinically relevant chemotherapeutic agents in vitro.

In the end, this study provides mechanistic approaches for targeting CD45+CD19− MCL-ICs and designing effective therapies to eliminate CD45+CD19− MCL-ICs, which could ultimately improve the outcome of patients with MCL.

Materials and methods

Patient samples

Patient blood specimens were obtained after informed consent, as approved by the M. D. Anderson Cancer Center and the University of Texas-Health Science Center institutional review boards. All primary patient peripheral blood mononuclear cells (PBMCs) were isolated from apheresis blood by standard Ficoll gradient methods. All patients were diagnosed with CD45+CD19− MCL-ICs and showed self-renewal capacities by limit dilution assays [7], which may render them difficult to kill via conventional therapies that target cells with increased mitotic activity.

Cell preparation

Cell fractionation via bead-based selection. CD3+ T cells and CD34+ cells were removed from PBMC samples prior to all analyses using lineage-specific purified antibodies (CD3, 1:500 dilution; and CD34, 1:1000 dilution) and magnetic beads according to the manufacturer's protocol (Dynal® beads Methods). PBMCs (1 × 10^7 cells/mL) were first incubated with unlabeled CD3 and CD34 antibodies for 20 min at 4°C with gentle rotation. Cells were washed three times with Hank’s balanced salt solution (HBSS) buffer containing 2% heat inactivated fetal bovine serum (FBS). Dynal beads were added and incubated for 30 min at 4°C with gentle rotation. Beads were sorted with a magnet for 3–5 min and the supernatants were harvested for further analyses. These CD3+ and CD34-depleted cells were separated using CD19 lineage-specific antibodies (Biolegend, San Diego, CA) and Dyna-beads® (Invitrogen, Oslo, Norway). The purity of separated tumor cells (CD45+CD3−CD34−CD19+ and CD45+CD3−CD34−CD19− cells) was confirmed to be more than 95% by flow cytometric analysis.

Cell fractionation via fluorescence-activated cell sorting (FACS). CD3+ cells and CD34+ cells were deleted using the magnetic beads selection method as described above. The CD3- and CD34-depleted cells were stained by fluorescein isothiocyanate (FITC)-labeled anti-CD45 (BD Pharmingen, San Diego, CA) and phycoerythrin (PE)-labeled anti-CD19 (BD Pharmingen), and then fractionated using a BD FACSARia flow cytometer.

Cell culture. The cells were kept in complete RPMI 1640 (Cellgro, Manassas, VA) medium, which contained 10% heat-inactivated FBS, 2 mM glutamine, 100 μg/mL streptomycin, and 100 μg/mL penicillin.

Despite the similar marker expression between MCL-ICs and normal plasma cells, these MCL-ICs were able to form tumors in NOD/SCID (non-obese diabetic/severe combined immune deficiency) mice and showed self-renewal capacities by limit dilution assay in vivo, unlike normal plasma cells. We have calculated the frequencies of stem cells in MCL-ICs using limit dilution assays (http://bioinf.wehi.edu.au/software/elda) with a confidence level of 0.95 and single hit hypothesis. The frequencies of stem cells in the CD45+CD19− MCL-ICs are 1 in 1 (upper limit) to 1 in 1.08 × 10^2 (lower limit). The frequencies of stem cells in the CD45+CD19+ cells are 1 in 7 109 449 (upper limit) to 1 in 3.46 × 10^8 (lower limit). The frequencies of stem cells in unfractionated MCL cells are 1 in 48 702 (upper limit) to 1 in 2.27 × 10^5 cells. Therefore, CD45+CD19− cells are enriched with stem cells from 3.2 × 10^6 to 7 × 10^8 times compared to CD45+CD19+ cells, and MCL-ICs are enriched with stem cells from 2.1 × 10^3 to 4.8 × 10^4 times compared to unfractionated cells.

Antibodies

Commercially available antibodies were used: anti-CD45 (HI30, immunoglobulin G1 [IgG1], k), anti-CD19 (HB19, IgG1, k), anti-CD3 (FIT3a, IgG2a, k), anti-CD34 (581, IgG1, k), anti-CD5 (UCHT2, IgG1, k), anti-CD20 (2H7, IgG2b, k), etc. All antibodies were conjugated with appropriate fluorochromes based on the combinations of antibodies used in each experiment. Antibodies were purchased from BD or ebioscience.
Chemotherapeutic agents

The chemotherapeutic drugs used in our study were selected based on clinical usefulness in MCL treatment. All drugs were obtained from the pharmacy at the M. D. Anderson Cancer Center. We used drug concentrations determined based on our preliminary data employing MCL tumor samples, as well as concentrations reported in various studies on human hematological malignancies. We used the following chemotherapeutic regimens: R-CHOP at maximum doses (cyclophosphamide monohydrate, 320 μg/mL; doxorubicin, 2 μg/mL; vincristine, 2 μg/mL; prednisone, 20 μg/mL; rituximab, 20 μg/mL), R-CVAD (cyclophosphamide monohydrate, 320 μg/mL; vincristine, 2 μg/mL; doxorubicin, 2 μg/mL; dexamethasone, 10 μg/mL; rituximab, 20 μg/mL), R-DHAP (dexamethasone, 40 μg/mL; cytarabine, 12800 μg/mL; cisplatin, 160 μg/mL; rituximab, 20 μg/mL), and a fludarabine-based regimen (fludarabine, 320 μg/mL; cyclophosphamide monohydrate, 320 μg/mL; rituximab, 20 μg/mL). When the drugs were tested as a single agent, the following concentrations were used as maximum doses; rituximab at 80 μg/mL, cisplatin at 160 μg/mL, cyclophosphamide monohydrate at 1280 μg/mL, cytarabine at 6400 μg/mL, dexamethasone at 160 μg/mL, doxorubicin at 16 μg/mL, fludarabine at 320 μg/mL, prednisone at 100 μg/mL, and vincristine at 8 μg/mL.

Colorimetric cell viability assay

Cytotoxicity was assessed with a colorimetric cell viability assay using CellTiter-Blue® (Promega, Madison, WI). Briefly, cells were seeded into 24-well plates at a concentration of 1.5–2 × 10⁵ cells per well and incubated for 6 h at 37°C in triplicate, with various determined doses of chemotherapeutic drugs and the combinations. CellTiter-Blue reagents (20 μL) were added to cells in suspension (80 μL) and these were incubated in 96-well plates for 4 h at 37°C. The fluorescent signal was measured at 560nm/590nm using a fluorescence plate reader equipped with SoftMax Pro software (Molecular Devices, Sunnyvale, CA), and the level of fluorescent resorufin was calculated. As preliminary tests, we serially cultured separated primary CD45+CD19+ and CD45+CD19– MCL cells at different time periods between 3 and 24 h to optimize the culture time. Primary MCL cells began to die rapidly after incubation for 6 h, even with no treatment [Supplementary Figure 1(A)]. The survival rates of CD45+CD19+ MCL cells and CD45+CD19– MCL-ICs were similar after the 6 h incubation [Supplementary Figures 1(B) and 1(C)].

Dose–response curves were calculated based on the cell viability assay of cells treated with each chemotherapeutic drug. Cell viability was assessed based on the value of the fluorescent signal of live cells with no drug treatments. The viabilities of drug-treated cells were calculated based on a ratio of the fluorescent signals using the following equation:

\[
\text{Cell viability(\%)} = \frac{V_{\text{max}} \times S_x}{S_0}
\]

where \( V_{\text{max}} \) is the full range of cell viability, i.e. 100%; \( S_x \) is the value of the fluorescent signal of live cells at \( x \) drug concentration; and \( S_0 \) represents the value of the fluorescent signal of live cells without drug treatments.

\( IC_{50} \)

The IC₅₀ value (the concentration of a drug required for 50% inhibition in vitro) was used to indicate the...
Chemotherapy resistance of MCL stem cells

(A) Relative Ratio of IC50

(B) Survival Fraction (%)

(C) Cell Survival Rate

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quantitative measure of the different cell killing effect of drugs. The Hill–Slope logistic model was used to calculate IC$_{50}$ using Microsoft Office Excel software.

Drug combination assay

The cytotoxic effects of combined anti-MCL regimens were determined by the combination index (CI) method based on the Chou and Talalay equation [26], and analyzed using CompuSyn software (ComboSyn, NJ, USA). Briefly, the CI equation is a quantitative measure of the degree of drug interaction in terms of synergism and antagonism of a given endpoint of the effect measurement [27], and the following median-effect equation: 

\[
f_{x} / f_{u} = (D / D_{m})^{m}
\]

where \(n\) is the number of combined drugs, \((D_{i})\) is the dose of drug \(i\) alone that inhibits \(x\)%, and \((D)\) is the portion of drug \(i\) in the drug combination that also inhibits \(x\%). Synergy is present when the CI is less than 1.0, there is an additive effect when CI equals 1.0, and antagonism when CI is greater than 1.0.

Flow cytometry

For the apoptosis assay, drug-treated or -untreated cells were washed with cold phosphate buffered saline (PBS) and stained with PE-labeled annexin V (BD Pharmingen) or FITC-conjugated annexin V and 7-amino-actinomycin (7-AAD) according to the manufacturer’s instructions. Stained cells were analyzed immediately by flow cytometry. For cell cycle analyses, treated or untreated cells were washed with cold PBS, and fixed with cold 70% ethanol. Fixed cells were stained with Hoechst 33342 and Pyronin Y, and analyzed by flow cytometry. For cell cycle analysis, treated or untreated cells were washed with cold phosphate buffered saline (PBS) and stained with PE-labeled annexin V (BD Pharmingen) or FITC-conjugated annexin V and 7-amino-actinomycin (7-AAD) according to the manufacturer’s instructions. Stained cells were analyzed immediately by flow cytometry. For cell cycle analyses, treated or untreated cells were washed with cold PBS, and fixed with cold 70% ethanol. Fixed cells were stained with Hoechst 33342 and Pyronin Y, and analyzed by flow cytometry. All assays were performed in duplicate.

Rhodamine 123 staining

Cells were suspended at 10$^6$ cells/50 μL of HBSS containing 5% FBS. Rhodamine 123 (Invitrogen) was added to a final concentration of 0.1 μg/mL. Cells were incubated for 20 min at 37°C in the dark followed by washing twice with HBSS. Cells were resuspended to allow efflux at 37°C for 2 h. Cells were washed once with HBSS and stained with antibodies before fluorescence activated cell sorting (FACS) analysis.

ABC transporter expression and activity

CD45+CD19$^-$ MCL-ICs and CD45+CD19$^+$ MCL cells were stained in culture medium at 37°C with Rhodamine 123 and chased for 1 h. Following adenosine triphosphate (ATP)-binding cassette (ABC) transporter inhibitors, verapamil (100 μM; Brandi), MK571 (for ABCC1 inhibition, 25 nM; ALEXIS), and PGP-4008 (for ABCB1 inhibition, 10 nM; ALEXIS) were added during the chase.

Quantitative reverse transcription-polymerase chain reaction

mRNA samples were subjected to RNase-free DNase treatment performed according to the RNasy® Mini Kit (Qiagen, Germantown, MD) and reserve transcribed to cDNA using the SuperScript® III First Strand Synthesis System (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. We used the following primers: ABCB1 transporter sense 5’ GCCCTGACTATGCCAAAGC 3’; ABCB1 transporter antisense 5’ TCTTCACCTCCAGGCTT 3’. Quantitative reverse transcription-polymerase chain reactions (qRT-PCRs) were performed using RT$^2$ SYBR Green/ROX$^TM$ qPCR Master Mix (SABiosciences, Foster City, CA) according to the manufacturer’s instructions. Human β-actin genes were used as internal controls. All samples were run in duplicate using the ABI 7900HT Fast Real-Time PCR System equipped with SDS Software v2.3 (Applied Biosystems), and data were analyzed using the comparative Ct method (ΔΔCt).

Statistical analysis

All assays were performed in triplicate, and data are expressed as mean value ± standard deviation. Statistical analyses were performed using the software SPSS for Windows version 12.0. The statistical significance of differences between the CD45+CD19$^+$ and CD45+CD19$^-$ cells was evaluated by Student’s t-test or Mann–Whitney–Wilcoxon test. p-Values <0.05 were considered statistically significant.

Results

CD45+CD19$^-$ MCL-ICs are more resistant to clinically relevant combined chemotherapeutic regimens in vitro as compared to CD45+CD19$^+$ MCL cells

This study was based on the cell survival rate analysis of in vitro culture cells. To optimize the culture conditions, we first serially cultured separated primary CD45+CD19$^+$ MCL cells and CD45+CD19$^-$ MCL-ICs at different time periods between 3 and
24 h. Primary MCL cells began to die rapidly after incubation for 6 h, even with no treatment [Supplementary Figure 1(A)]. The survival rates after the 6 h incubation between CD45+CD19− MCL cells from three different patients were comparable with those of CD45+CD19− MCL-ICs, both by the microscopic counting of live cells and by the CellTiter-Blue cell viability assay [Supplementary Figures 1(B) and 1(C)]. Although bead selection had a tendency to induce more cytotoxicity compared to FACS fractionation, the cell survival rates between CD45+CD19− MCL cells and CD45+CD19− MCL-ICs were comparable regardless of the method of separation [Supplementary Figures 1(D) and 1(E)].

Table I. IC50 values* (drug concentrations that were inhibitory for 50% of cells) of each drug in regimens calculated using the Hill–Slope logistic model by construction of dose–response curves.

<table>
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*The data are the results of experiments repeated in triplicate. CD45+CD19− MCL-ICs require much higher doses of chemotherapeutic drugs to achieve the same 50% cell growth inhibition as CD45+CD19− MCL cells.

CD19+, CD45+CD19+ mantle cell lymphoma (MCL) cells; CD19−, CD45+CD19− MCL-initiating cells (MCL-ICs); R-CHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; R-CVAD, rituximab, cyclophosphamide, doxorubicin, vincristine, dexamethasone; R-DHAP, rituximab, dexamethasone, cytarabine, cisplatin; CTX, cyclophosphamide monohydrate; DOX, doxorubicin; VCR, vincristine; Pd, prednisone; RTX, rituximab; Dexe, dexamethasone; Ara-C, cytarabine; CDDP, cisplatin; Flu, fludarabine; FluBR, fludarabine-based regimen.
were compared. CD45+CD19− MCL-ICs from all five tested patients demonstrated resistance to R-CHOP, R-CVAD, R-DHAP, and fludarabine-based regimens, which are the most commonly used chemotherapeutic agents for MCL (p < 0.05) [Figure 1(B) and Supplementary Figures 2(A) and 2(B)].

However, due to patient-to-patient variability, the difference of resistance between treatments showed some variation. Figure 1(C) shows a heat map illustrating clusters of the survival rates of CD45+CD19− MCL-ICs and CD45+CD19+ MCL cells in response to each concentration of

![Graph showing survival rates](image)

Figure 2. Annexin V staining profiles confirm increased cell survival in MCL-ICs after drug treatment. Representative histograms show annexin V staining of cells (patient 1) that were treated with different chemotherapeutic agents. Percentages show the proportions of annexin V negative cells, which indicate live cells after chemotherapy treatments. We treated the cells with the following doses of chemotherapeutic regimens: R-CHOP (CTX, 160 μg/mL; DOX, 1 μg/mL; VCR, 1 μg/mL; Pd, 10 μg/mL; RTX, 10 μg/mL), R-CVAD (CTX, 160 μg/mL; VCR, 1 μg/mL; DOX, 1 μg/mL; Dexa, 5 μg/mL; RTX, 10 μg/mL), R-DHAP (Dexa, 20 μg/mL; Ara-C, 6400 μg/mL; CDDP, 80 μg/mL; RTX, 10 μg/mL), FluBR (Flu, 160 μg/mL; CTX, 160 μg/mL; RTX, 10 μg/mL). FluBR, fludarabine-based regimen; CTX, cyclophosphamide monohydrate; DOX, doxorubicin; VCR, vincristine; Pd, prednisone; RTX, rituximab; Dexa, dexamethasone; Ara-C, cytarabine; CDDP, cisplatin; Flu, fludarabine.
Chemotherapy resistance of MCL stem cells

To understand the molecular mechanism of CD45+CD19+ MCL-IC drug resistance, we examined the cell cycle status of CD45+CD19+ MCL-ICs using Rhodamine 123 (Rho123), the efflux of which can be a measurement of the presence of functional ABC transporters [33]. ABC transporter family members, especially ABCB1 (P-glycoprotein), mediate multidrug resistance in various cancers [34,35]. Decreased Rho123 accumulation (high ABCB1) also indicates inactive mitochondria in these cells, suggesting a possible quiescent state. Previous studies reported that human peripheral B cells express functional ABCB1 [36]. Flow cytometric analysis of MCL cells showed that CD45+CD19− fractions contained decreased Rhodamine 123 accumulation (increased Rho123 low, 17%) [Figure 4(A)]. The expression of ABCB1 transporters was much higher in CD45+CD19− MCL-ICs than in CD45+CD19+ MCL cells, as measured by real-time PCR [Figure 4(B)]. We also treated CD45+CD19− cells with various inhibitors that block ABC transporter activity, to determine which drug transporters mediate Rho123 extrusion in the tumors. Extrusion of Rho123 was inhibited by verapamil, which blocks all ABC transporters, or PGP-4008, which is specific for ABCB1 transporters [Figure 4(C)]. To determine whether inhibition of the ABCB1 drug transporter affects MCL-ICs drug resistance, primary CD45+CD19− MCL-ICs were treated with PGP-4008 (specific ABCB1 inhibitor) and were then treated with vincristine, which is a substrate of ABCB1 [37] and a common drug in anti-MCL regimens. We used vincristine instead of combined regimens to analyze the more specific effects of ABCB1 inhibitors on CD45+CD19− MCL-IC drug resistance. The cytotoxicity of PGP-4008-treated CD45+CD19− MCL-ICs in the presence of vincristine was higher than that of untreated CD45+CD19− MCL-ICs [Figure 4(D)].

In addition, we analyzed the change in the Rho123 low fraction, which mostly contained CD45+CD19− MCL-ICs, before and after chemotherapy.
to confirm whether the Rho123 low fraction was indeed resistant to chemotherapy. Flow cytometric analysis showed that Rho123 low cell proportions were increased approximately three-fold after chemotherapy [Figure 5(A)]. To confirm that CD45+CD19− MCL-ICs were resistant to chemotherapy, we also analyzed the change in the CD45+CD19− fraction in a single unsorted population that was CD3- and CD34-depleted and contained CD19+ and CD19− cells both before and after chemotherapy, using FACS analysis. The CD19 flow cytometric profile showed that the CD19− cell fractions were increased from 2–3% to 13–19%, which demonstrates that CD45+CD19− MCL-ICs show greater survival after chemotherapy compared to CD45+CD19+ bulk MCL cells [Figure 5(B)]. These results indicate that CD45+CD19− MCL-ICs are enriched for Rho123 efflux activity, which is mediated by functional drug transporters, and that these cells are maintained in a quiescent state. From Hoechst/Pyronin Y-stained flow cytometric analysis, we determined that CD45+CD19− MCL-ICs are largely maintained in the G0 phase of the cell cycle compared to non-stem MCL cells (Supplementary Figure 5).
Non-Hodgkin lymphomas (NHLs) are a heterogeneous group of hematological malignancies [1,2]. NHL is the sixth most common cancer in the United States, and the incidence of NHL has nearly doubled in the past two decades, with 60,000 new cases every year. The reason behind the rising incidence and the cause of NHL remain poorly understood [1,2]. Although the use of combined chemotherapy regimens have dramatically improved the survival outcome of patients with NHL [38], significant numbers of patients still fail to achieve complete remission [39–41], suggesting that the current therapy may be limited by the presence of subpopulations of drug-resistant tumor cells. Therefore, it is necessary to design effective therapies that selectively target these cells.

Various reports have shown that some C-ICs are resistant to conventional chemotherapy and radiotherapy, indicating that these resistant cells may be responsible for relapse of the disease [17,19,20,42]. Based on our recent discovery of stem-like cells in MCL, in this study we investigated whether CD45+CD19− MCL-ICs contribute to the drug resistance properties of MCL. MCL is a particularly deadly subtype of B-cell NHL, is highly refractory to standard therapy, and displays the worst survival rate among patients with NHL [3,4]. Typical immunophenotypes for clinical diagnosis of MCL are CD20+, CD79a+, and CD45+ [43,44]; however, MCL displays widespread cellular heterogeneity, which leads to only a partial response to conventional therapies [39,40,45]. This report is the first to show the chemotherapy resistance properties of MCL using stem-like cells isolated from primary patient cells. In addition, we tested several chemotherapeutic regimens that are widely used in clinics, such as anthracycline-containing regimens (CHOP), alkylating agent-based regimens (CVP;
cyclophosphamide, vincristine, prednisone), anti-metabolite-containing regimens (DHAP), dose-intensive combination chemotherapy regimens (Hyper CVAD-MA; hyperfractionated CVAD-methotrexate, cytarabine), and purine analog-based regimens [6,39,44,46]. We included rituximab in all regimens because rituximab has remarkably improved the survival and prognosis of patients with B-cell lineage lymphomas, including MCL [47,48]. Because there are only a few published studies on testing drug treatments in MCL primary cells, we determined the drug concentrations based on our own preliminary experiments using MCL primary cells and chemotherapy sensitivity studies of other hematologic malignancies [23,49–51].

Our data clearly show that CD45+CD19− MCL-ICs are more resistant to conventional chemotherapeutic regimens compared to CD45+CD19+ bulk MCL cells [Figures 1(B) and 1(C) and Supplementary Figures 2(A) and 2(B)]. Apoptosis assays using annexin V FACS analyses further support this conclusion (Figure 2). However, we noted some differences in the live cell fractions, as measured by colorimetric assay and annexin V staining, because annexin V can detect cells that have already undergone apoptosis and cells in early stages of apoptosis [52–54].

The comparison of the cytotoxic effects of rituximab and combination regimens containing rituximab between non-heat-inactivated human serum and heat-inactivated FBS revealed that rituximab and other tested regimens induced greater CD45+CD19+ MCL bulk cell cytotoxicity in unheated human serum than in heat-inactivated FBS. However, CD45+CD19−
MCL-ICs showed similar resistance to chemotherapeutic regimens between the addition of human serum and of fetal calf serum (Figure 3). These findings suggest that CD45+CD19– MCL-ICs might have an immunological mechanism to escape complement-dependent cytotoxicity.

The IC50 values of chemotherapeutic drugs using CD45+CD19– MCL-IC samples were much higher than those using bulk tumor cells, suggesting that CD45+CD19– MCL-ICs comprise the major populations that contribute to MCL drug resistance. Importantly, the IC50 values that inhibited CD45+CD19– MCL-IC growth exceeded clinically achievable drug concentrations, indicating that CD45+CD19– MCL-IC survival pathways should be targeted to improve the outcome of patients with MCL.

Combined chemotherapeutic regimens have been essential in cancer therapies [55,56], because they facilitate lower doses of drugs with higher anti-tumor effects. These synergistic effects also reduce treatment-related toxicity, which is often associated with single-drug regimens [57–59]. We treated CD45+CD19– MCL-ICs using single-drug or combination treatments and analyzed the synergistic anti-tumor effects of these treatments by CI plots based on the Chou and Talalay method. The CIs of anti-MCL regimens for CD45+CD19+ MCL cell cytotoxicity were mainly less than 1.0 at all the effect levels analyzed, which indicated synergistic cytotoxicity, in contrast with the CIs of CD45+CD19– MCL-ICs, which were usually greater than 1.0 (Supplementary Figure 4). These data suggest that CD45+CD19– MCL-ICs have a tendency to be resistant to the synergic tumor suppression effect of combined drug regimens. It should be noted, however, the variations between regimens.

The drug resistance properties of CD45+CD19– MCL-ICs are partially due to the elevated expression of multidrug-resistant (MDR) gene products, including ABC transporters [Figure 4(B)], which are large transmembrane proteins that mediate the ATP-dependent transport of various molecules across cell membranes [60,61]. The presence of functional ABC transporters can be detected by measuring the efflux of dyes such as Rho123. In this study, we showed that CD45+CD19– MCL-ICs are maintained in a quiescent state with markedly increased Rhodamine 123 efflux activity (Rho123 low) compared to CD45+CD19+ MCL cells. Rho123 is a fluorescent dye that preferentially accumulates in mitochondrial membranes proportional to the activation state of the cell [62,63]. Rho123 is a substrate for P-glycoprotein, an efflux pump responsible for multidrug resistance in tumor cells that contributes to drug resistance by acting as a unidirectional drug efflux pump that decreases the accumulation of drugs within cells [62,63]. In the murine hematopoietic system, Rho123 low (resting) cells show a 20-fold greater ability to form colonies in vitro and display higher in vivo reconstitution activity compared to Rho123 high (active) cells [63]. Therefore, an increased intensity of Rho123 staining could directly correlate with the degree of hematopoietic progenitor cell differentiation. Decreased Rho123 accumulation further reflects inactive mitochondria in these cells, suggesting quiescence. Moreover, the Rho123 low fraction within CD34+CD38– cord blood has been shown to be four times as enriched with cells that can repopulate in NOD/SCID mice compared with the Rho123 high fraction [63]. Therefore, the quiescent properties of CD45+CD19– MCL-ICs are largely maintained in the quiescent G0 phase of the cell cycle compared to non-stem MCL cells (Supplementary Figure 5)[7].

In conclusion, CD45+CD19– MCL-ICs are resistant to clinically relevant anti-MCL chemotherapeutic regimens, and importantly, synergic drug combinations that are not effective in inhibiting CD45+CD19– MCL-IC growth. These findings clearly support that CD45+CD19– MCL-ICs play an important role in MCL chemotherapy resistance. Understanding the molecular mechanisms of CD45+CD19– MCL-IC survival and their contribution to drug resistance during treatment will provide the groundwork for the design of novel therapeutic strategies to treat or prevent MCL in humans.

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Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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Supplementary material available online

Figures showing additional results