Original Research Article

The Serum- and Glucocorticoid-Inducible Kinase 1 (SGK1) as a Novel Therapeutic Target in Mantle Cell Lymphoma

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Abstract

Introduction: Mantle cell lymphoma (MCL) is an aggressive and incurable B-cell-derived malignant disease. MCL is treated using general chemotherapy; however, disease progression and relapse are common; thus, the development of novel therapeutic targets for treatment of MCL is urgently required. Serum- and glucocorticoid-inducible kinase 1 (SGK1) is involved in various cellular activities, and its dysregulation contributes to the pathogenesis of multiple types of cancer. However, little is known regarding its functional roles and associated molecular mechanisms in MCL.

Methods: SGK1 inhibition mediated by either shRNA or treatment with SGK1 inhibitor (GSK650394) was conducted in MCL cell lines. Western blotting analysis was performed to figure out the expression of related proteins. MCL-cell-derived xenograft models were constructed to evaluate the anti-tumor effects of SGK1 inhibition or/and Bruton's tyrosine kinase (BTK) inhibition in vivo.

Results: In this study, it was shown that inhibition of SGK1 significantly reduced cell proliferation, invasion and migration, increased apoptosis and blocked cell cycle progression in MCL cells. Furthermore, SGK1 inhibition significantly reduced the activation of ERK, AKT/mTOR, JAK2/STAT3 and the NF-κB signaling pathways. Using MCL-cell-derived xenograft mice models, SGK1 inhibition decreased tumor cell proliferation and tumor growth. Importantly, SGK1 overexpression significantly promoted xenograft tumor growth. Moreover, simultaneous inhibition of SGK1 and Bruton tyrosine kinase (BTK) resulted in synergistic anti-tumor effects on MCL both in *vitro* and in vivo.

Conclusion: SGK1 may be a novel candidate therapeutic target and simultaneous inhibition of SGK1 and BTK may be a promising therapeutic strategy for MCL patients. Further pre-clinical and even clinical studies of SGK1 inhibitor or combination with BTK inhibitor are essential.

Keywords

mantle cell lymphoma, serum- and glucocorticoid-inducible kinase 1, bruton tyrosine kinase, targeted therapy, drug combination

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Introduction

Mantle cell lymphoma (MCL) is a rare subtype of B cell lymphoma that accounts for 6-8% of non-Hodgkin lymphoma cases.[1,2] Although traditional immunochemotherapy has significantly improved the survival rates of patients, MCL remains incurable with a median overall survival (OS) of 4-5 years.[1–3] Moreover, 60-90% of MCL patients that undergo immunochemotherapy either relapse or continue to exhibit progression.[4] In spite of the various novel drugs for treatment of MCL, such as bortezomib, ibrutinib and lenalidomide,[5–8] there are still no effective therapeutic regimens for MCL. Given the lack of successful treatment options for this disease, it is essential to explore novel therapeutic targets and rational combination treatment regimens to further improve the prognosis of MCL patients.

Serum- and glucocorticoid-inducible kinase 1 (SGK1) is a serine/threonine protein kinase and is a member of the AGC (PKA-, PKG- and PKC-related) family of protein kinases. Well-known members of the AGC family includes AKT, 3-phosphoinositide dependent kinase-1 (PDK1), ribosomal S6 kinase and ribosomal s6 kinase p90. In particular, SGK1 shares ~54% identity of its catalytic domain with AKT, indicating analogous functions.[9,10] SGK1 is closely related to AKT and is regulated upstream by the PI3K/mTOR signaling pathway.[11] Studies demonstrated that PDK1 is directly responsible for SGK1 phosphorylation on Thr256 and the phosphorylation on Ser422 performed by the mTOR complex (mTORC)2, which is the downstream target of PI3K.[12] Previous reports have demonstrated that SGK1 is also regulated by several other factors, including the tumor suppressor protein p53, growth factors and various cellular stressors, such as cell shrinkage, oxidative stress and DNA damage.[13–17] Additionally, SGK1 can phosphorylate various kinases that play essential roles in critical cellular processes, including cell survival, proliferation and apoptosis. Most notably, SGK1 is closely related to the development and progression of multiple types of tumors,[18] and upregulated expression of SGK1 has been observed in several tumors.[19–21] Glucocorticoid administration to patients leads to an increase in the expression of the anti-apoptotic gene SGK1 in ovarian tumor tissues, which may decrease the effectiveness of chemotherapy.[19] In lung cancer, the mRNA expression levels of SGK1 are significantly higher in squamous cell carcinomas, and its expression correlated with a poor prognosis.[20] In contrast, the relatively low expression of SGK1 is associated with a higher tumor grade and increased cancer relapse in prostate cancer.[21] Evidence has indicated that SGK1 induces cancer cell growth through multiple pathways, including the NF-κB, p27, c-fms and forkhead transcription factor (Foxo)3a pathways.[22–25] It has been demonstrated that SGK1 phosphorylates and activates IκB kinase β (IKKb) which is an important kinase of the NF-κB signaling pathway, then the activated IKKb phosphorylates Ser644 on FOXO3a. Serum-induced SGK or increased expression of SGK activates NF-κB transcriptional activity, whereas knocking down expression of SGK using small interfering RNAs targeting SGK reduced NF-κB activity.[22,23] Furthermore, previous work has established that stimulation of c-fms, with its ligand colony stimulating factor-1 (CSF-1), induces activation of PI3K, which then phosphorylates the downstream SGK1.[26] SGK1 may act as a downstream intracellular regulator of c-fms to promote the adhesiveness of breast cancer cells.[24] Moreover, in cancer cells, oncogenic activation of the PI3K/mTOR pathway may recruit SGK1 to impair the inhibition of cyclin-Cdk2 to block the cell cycle through driving cytoplasmic p27 mislocalization.[25] Therefore, SGK1 may serve as a potential therapeutic target for management of several types of cancer. However, the functional role and underlying mechanisms of SGK1 in MCL have not yet been elucidated, to the best of our knowledge.

SGK1 is considered a critical node of tumor cell resistance to various molecular inhibitors and chemotherapeutic drugs. It has been demonstrated that SGK1 is activated by PDK1 and contributes to the maintenance of residual mTORC1 activity through direct phosphorylation and inhibition of tuberous sclerosis complex-2 (TSC2), the expression of which is correlated with resistance to PI3Kα inhibitors. TSC-2 can restore the antitumor activities of PI3Kα inhibition in resistant cells through targeting either PDK1 or SGK1 to prevent mTORC1 activation.[27] Moreover, SGK1 is able to directly phosphorylate FOXO1 at residues T32 and S315,[28] and this has been shown to be associated with resistance to AKT inhibition.[29] Together, these studies suggest that SGK1 represents an essential target whose inhibition could synergize with small molecule inhibitors and may lead to a more favorable outcome when combined with standard therapeutic regimens. The aim of the present study was to assess the functional role and therapeutic potential of SGK1 in MCL, and assess the value of combined therapeutic regimens for the treatment of MCL patients.

Materials and Methods

Cell lines and culture conditions. The human MCL cell lines, Mino, Z138, Granta519, Jeko-1, JVM-2 and JVM-13, were kindly provided by Dr Fu from University of Nebraska Medical Center (Nebraska, USA). These cells were cultured in low-glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Biological Industries), penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and glutamine, in a humidified incubator supplied with 5% CO_2 at 37°C. Peripheral blood mononuclear cells (PBMCs) from whole blood of healthy donors were separated using BD Vacutainer CPT™ (BD Biosciences). Verbal informed consents were obtained from these healthy participants. PBMCs were collected in accordance with the ethical guidelines of Beijing Cancer Hospital & Institute. Then PBMCs were labeled with CD19-PE (Beckman Coulter, Inc.) for 20 min at 4°C, and B lymphocytes were sorted using a BD

FACSAria II cell sorter using wavelengths of 355, 488 and 633 nm.

Reagents and antibodies. GSK650394 (cat. no. HY-15192) and ibrutinib (cat. no. HY-10997) were purchased from MedChemExpress, Inc. and prepared using DMSO at room temperature, and then stored at −80°C until required. Information on the antibodies used is presented in Table S1.

Short hairpin RNA (shRNA)-mediated SGK-1 knockdown. Lentiviral vectors containing green fluorescent protein (GFP; shControl, target sequence: 5'-TTCTCCGAACGTGTCACGT-3') or SGK1-specific shRNAs (shSGK1 #68540, target sequence: 5'-GCTGAAATGTACGACAACATT-3'; or shSGK1 #68562 target sequence: 5'- AAGAAGTGTTCTATGCAGTCA -3') were constructed, packed and purified by Shanghai GeneChem, Co., Ltd. MCL cells (5×10^4/ml) were infected with shControl or shSGK1 at a multiplicity of infection (MOI) of 1:100, and cultured for >72 h before performing subsequent experiments. The depletion efficiency was evaluated by fluorescence microscopy and western blotting.

Cell viability assay. Cell viability was determined using the Cell Titer-Glo Luminescent Cell Viability Assay system (cat. no. G7572; Promega Corporation). Cells were plated in 96-well plates at a density of 3×10^4/ml, and incubated with different concentrations of GSK650394 for 72 h. Cells were transfected with shControl, shSGK1 (#68540 or #68562) vectors for >72 h, plated in triplicate at a density of 2×10^4/ml in 96-well plates, and cultured for the indicated time period. Next, 10 μL cell titer reagent was added to each well and incubated on a shaker at room temperature for 10 min. Luminescent signals were evaluated using an LMax II instrument (Molecular Devices, LLC).

Lentivirus-mediated overexpression of SGK1. Lentiviral overexpression vectors (GV492) were purchased from Genechem (Shanghai, China). Overexpression of SGK1 in MCL cells was performed using SGK1 lentiviral vector transfection according to the manufacturer's protocol. MCL cells (5×10^4/ml) were infected with overexpression lentivirus at a MOI of 1:100, and cultured for >72 h before being used in subsequent experiments. The overexpression efficiency was evaluated by western blotting.

Western blotting. Cells were harvested and lysed using RIPA lysis buffer (cat. no. 9806; Cell Signaling Technology, Inc.) supplemented with protease/phosphatase inhibitor cocktail (cat. no. 04693124001; Roche Diagnostics GmbH). Expression levels of signaling proteins were detected by western blotting as previously described.[30] Signals were visualized using a chemiluminescence detection system (Alpha Innotech, Inc.) according to the manufacturer's instructions.

Invasion and migration assays. The MCL cells were infected with shControl or shSGK1 (shSGK1 #68540 or shSGK1 #68562) at a MOI of 1:100, and cultured for >72 h for use in the invasion and migration assays. Similarly, MCL cells were pretreated with vehicle or different concentrations of GSK650394 for 12 h, and subsequently used for the invasion and migration assays. Invasion assays were performed using Matrigel coated Transwell chambers (cat. no. 354480; Corning Inc.) and cell migration was assessed using Transwell chambers that had not been coated with Matrigel (cat. no. 3422; Corning, Inc.), as described previously.[30]

Quantification of apoptosis and cell cycle. For apoptosis analysis, cells were harvested and washed once with PBS; then cells were resuspended in 1x Binding Buffer and labeled with Annexin-V-FITC and PI according to the protocol of the Annexin-V-FITC apoptosis detection kit (cat. no. AD10; Dojindo Molecular Technologies, Inc.). For cell cycle analysis, cells were collected and washed with PBS, then fixed using 75% ice-cold ethanol at 4°C overnight. The fixed cells were suspended in PBS containing 100 μg/mL RNAase A (cat. no. GE101; Transgen Biotech, Co., Ltd.) for 30 mins at 37°C, and then stained with PI staining buffer (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Finally, the labeled cells for apoptosis and cell cycle analysis were analyzed using a BD Accuri C6 flow cytometer (BD Biosciences, Inc.). Cell cycle distribution was calculated using ModFit LT software (Verity Software House).

Drug sensitivity assays. For SGK1 knockdown, cells transfected with shControl or shSGK1 (shSGK1 #68540 or shSGK1 #68562) were incubated with the indicated concentrations of ibrutinib for 72 h. For the SGK1 inhibitor, the cells were plated into 96-well plates and incubated with different concentration of GSK6650394 and ibrutinib for 72 h. The decrease in cell viability rates were assessed by measuring the viability of cells as above. CalcuSyn software was used to calculate the combination index (CI) value.

MCL-cell derived xenograft model. All animal experiments were blind trials, and performed in compliance with the Guide for the Care and Use of Laboratory Animals and approved from the Ethical Guidelines of Medical Ethics Committee from Beijing Cancer Hospital & Institute on March 1, 2019 (Beijing, China). The animal studies conformed to ARRIVE 2.0 guidelines[31] and followed the guide for the Care and Use of Laboratory Animals.[32] Female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice aged 7-8 weeks old were purchased from HFK Bioscience Co., Ltd. (Beijing, China) according to the animal model in previous study.[30] Animals were housed in a specific pathogen free (SPF) barrier at animal center of Beijing Cancer Hospital & Institute with 40~70% of humidity and 20~26°C of temperature. Animals had free access to autoclaved sterilized dry granule food and water. We have made efforts to minimize the number of animals utilized and to decrease their suffering. shControl or shSGK1 Z138 stable cells, or Z138 cells transfected with SGK1 overexpression vector in PBS with Matrigel (1:1 ratio), and each group consisted of 8 mice. For sensitivity analysis of SGK1 inhibitor (GSK650394), NOD/SCID mice were injected subcutaneously with 1×10^7 Z138 cells and each group consisted of 6 mice. For drug combination experiments, Z138 cells (1×10^7) in PBS with Matrigel (1:1 ratio) were subcutaneously injected into the area under the right flank of each mouse (n = 8 per group). When the

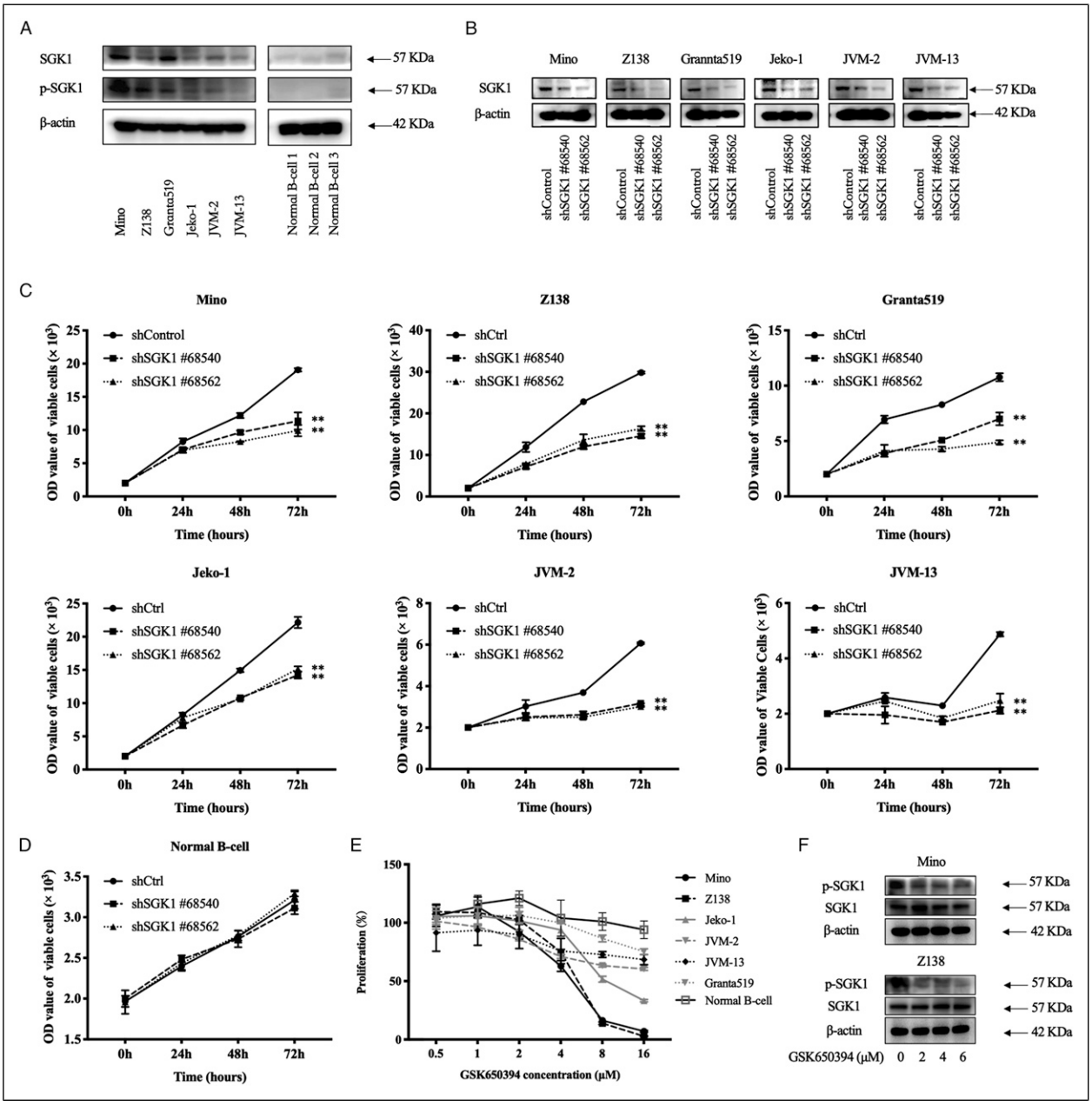


Figure 1. Downregulation of SGK1 using shRNA or GSK650394 significantly reduces the proliferation of MCL cells. **(**A) The expression levels of SGK1 and p-SGK1 in MCL cell lines and normal B-cells were analyzed by western blotting. β-actin was used as a loading control. (B) SGK1 knockdown using shRNA was confirmed using western blotting in the MCL cell lines. β-actin was used as the loading control. (C) MCL cells and (D) normal B-cells were transfected with shSGK1 vectors or shControl. The viability of the cells was analyzed using the Cell Titer-Glo Luminescent Cell Viability Assay. (E) MCL cell lines and normal B-cells from healthy donors were treated with the indicated concentrations of the SGK1 inhibitor GSK650394 for 72 h. The viability of the cells as a percentage of the control was calculated. (F) Mino and Z138 cells were treated with the indicated concentrations of GSK650394 for 24 h, and the levels of SGK-1 and p-SGK1 were determined using western blotting. Data are presented as the mean ± SD of three independent experiments. SGK1, serum- and glucocorticoid-inducible kinase 1; shRNA, short hairpin RNA; p-, phospho-; MCL, mantle cell lymphoma.

tumor volumes reached 100-150 mm^3, mice were randomly divided into different treatment groups and the indicated doses of compounds were administered daily. The mice were euthanized through the cervical dislocation. Touching the outside of the chest cavity to check the animal's heartbeat for 1-2 minutes without a heartbeat can be judged to be dead. The tumor tissue samples were collected after the final dose. Tumor volume was calculated as follows: $V = ab^2/2$, where a and b denote long and short diameters of the tumor, respectively.

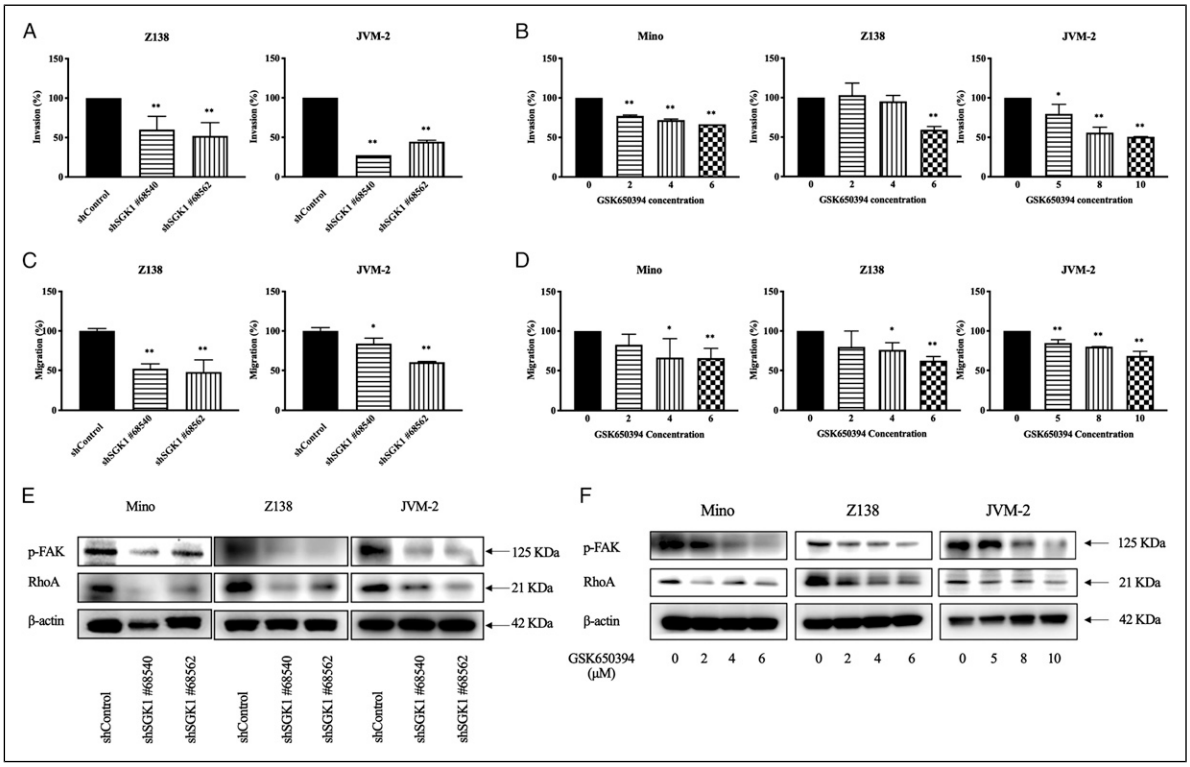


Figure 2. SGK1 inhibition reduces the invasion and migration of MCL cell lines. **(**A and C) MCL cells transfected with vectors carrying one of two shSGK1 constructs, shControl, or cells that had been pretreated with the indicated concentrations of SGK1 inhibitor GSK650394 were subjected to invasion assays using Transwell chambers precoated with Matrigel. (B and D) MCL cells transfected with vectors containing one of the two shSGK1 constructs, shControl, or cells that had been pretreated with the indicated concentrations of SGK1 inhibitor GSK650394 were subjected to migration assays in Transwell chambers. The number of cells in the lower chamber was determined using a Cell Titer-Glo Luminescent Cell Viability Assay. (E) MCL cells were transfected with shControl or one of two shSGK1 constructs for >72 h, or (F) treated with the indicated concentrations of GSK650394 for 12 h, and whole cell lysates were used to determine the levels of p-FAK and RhoA by western blotting. β-actin was used as the loading control. Data are presented as the mean ± SD of three independent experiments. Differences between groups were compared using a Student's t-test. *P < .05, **P < .001 vs the control group. SGK1, serum- and glucocorticoid-inducible kinase 1; shRNA, short hairpin RNA; p-, phospho-; MCL, mantle cell lymphoma; FAK, focal adhesion kinase; RhoA, Ras homolog family member A.

Statistical analysis. All experiments were repeated at least three times, and representative results are presented. SPSS version 22.0 (IBM Corp.) was used for all analyses. Data were analyzed using a paired or unpaired Student's t-test. $P < .05$ was considered to indicate a statistically significant difference.

Results

Knockdown and inhibition of SGK1 significantly reduces proliferation of MCL cells. To elucidate the essential role of SGK1 in the pathogenesis of MCL, the expression levels of SGK1 and phospho- (p-) SGK1 in MCL cell lines were first analyzed using western blotting. Immunoblotting analysis demonstrated that SGK1, the PI3K/AKT/mTOR signaling important regulator kinase, was activated in the six MCL cell lines to different degrees, and SGK1 protein expression was also increased compared with the normal B cells, which exhibited low expression levels of SGK1 and did not express p-SGK1 (Figure 1(a)). To further analyze the functional role of SGK1 in MCL cell viability and proliferation, the MCL cell lines were transfected with lentiviral particles carrying shSGK1 #68540 or shSGK1 #68562. A lentiviral vector containing a non-specific shRNA sequence was used as a negative control. The transfection efficiency and knockdown efficiency of the individual SGK1 shRNA in MCL cell lines was confirmed using fluorescence microscopy and western blotting analysis (Figure S1 and 1(b)). Cell viability assays have shown that the knockdown of SGK1 expression significantly reduced the proliferation of MCL cells (Figure 1(c)). In contrast, knockdown of SGK1 expression did not influence the proliferation of normal B-cells (Figure 1(d)).

In addition, a selective SGK1 inhibitor (GSK650394) was also used to assess the functional role of SGK1 in MCL cells. The data demonstrated that GSK650394 reduced the proliferation of MCL cells. However, the viability of normal B cells from healthy donors was not affected after incubation with the

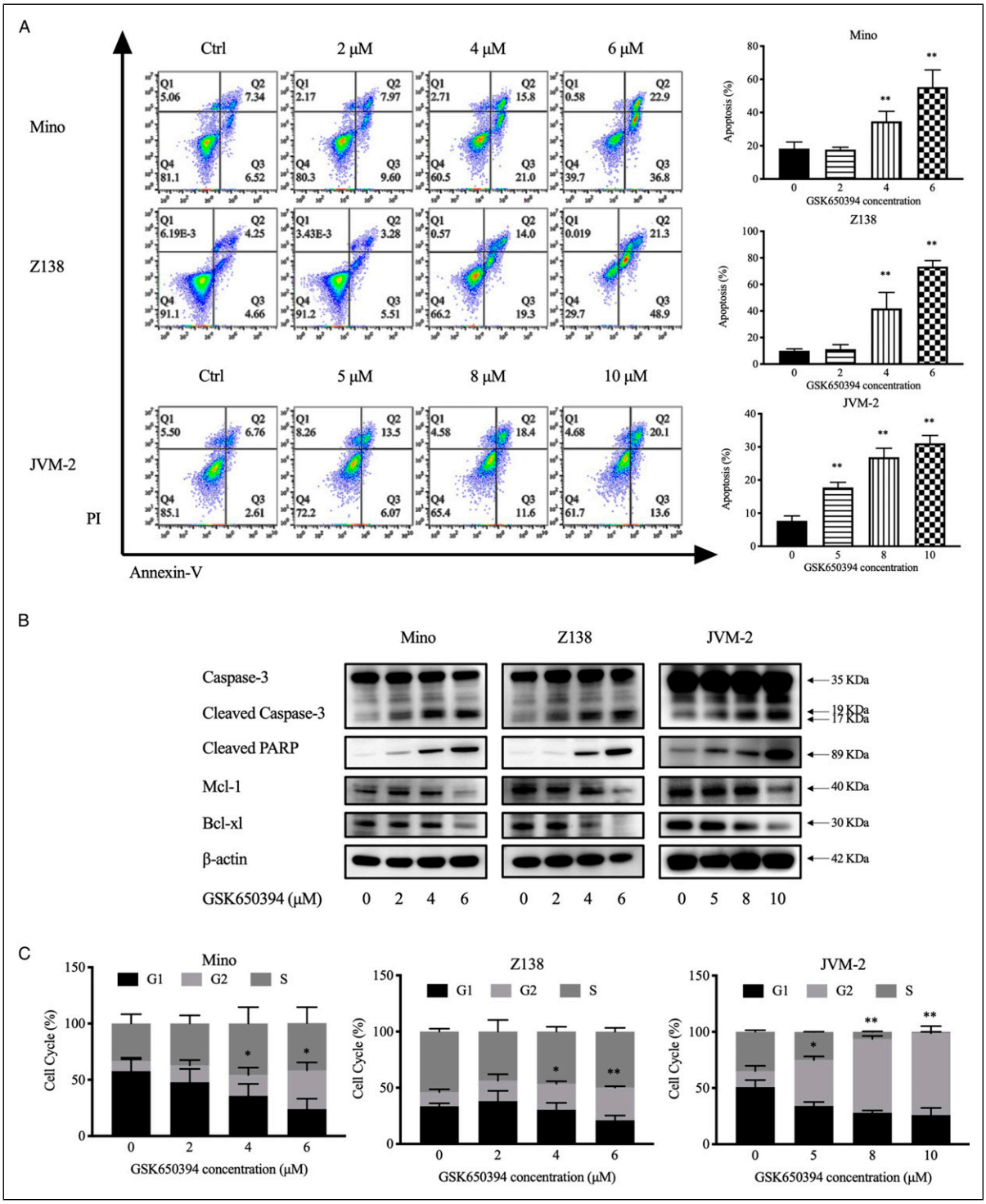


Figure 3. SGK1 inhibition significantly induces cell apoptosis and blocks cell cycle progression in MCL cell lines. (A) Mino, Z138 and JVM-2 cells were treated with the indicated concentrations of GSK650394 for 72 h, and the proportion of apoptotic cells was determined using flow cytometry. (B) MCL cells were treated with the indicated concentrations of GSK650394 for 72 h. Whole-cell extracts were used to determine the levels of Caspase-3, cleaved-PARP, Mcl-1 and Bcl-xl. β-actin was used as the loading control. (C) Mino, Z138 and JVM-2 cells were treated with the indicated concentrations of GSK650394 for 48 h, and the cell cycle distribution profiles were determined using flow cytometry. Data are presented as the mean ± SD of three independent experiments. Differences between groups were compared using a Student's t-test. *P < .05, **P < .001 vs the control group. SGK1, serum- and glucocorticoid-inducible kinase 1; PARP, poly-ADP ribose polymerase; MCL, mantle cell lymphoma; Mcl-1, myeloid cell leukemia-1.

indicated concentrations of GSK650394 (Figure 1(e)). Moreover, as the concentration of GSK650394 was increased in Mino and Z138 cells, p-SGK1 was significantly decreased in a dose-dependent manner (Figure 1(f)).

SGK1 inhibition reduces the invasion and migration of MCL cells. Given that invasion and migration are generally considered as important biological characteristics of cancer cells, invasion and migration assays were used to evaluate the effects of SGK1 inhibition on MCL cells. Similar to the cell viability experiments, compared with the cells transfected with shControl vector, the invasive and migratory activity of MCL cells were also significantly decreased following shSGK1 vector transfection (Figure 2(a) and (b)). Moreover, consistent with these results, treatment with the indicated concentrations of GSK650394 also efficiently reduced the invasion and migration of MCL cells in a dose-dependent manner (Figure 2(c) and (d)). Additionally, to determine the exact mechanism by which SGK1 inhibition reduced the invasive and migratory activity of MCL cells, signal proteins associated with invasion and migration were detected by western blotting. Consistent with the reduced invasion and migration of MCL cells, p-focal adhesion kinase (p-FAK) and ras homolog gene family, member A (RhoA) expression were notably decreased in MCL cells transfected with one of the shSGK1 vectors or in cells treated with GSK650394 for 12 h (Figure 2(e) and (f)).

SGK1 inhibition promotes apoptosis and induces G2/M phase arrest in MCL cells. To further investigate the mechanisms by which SGK1 inhibition resulted in cytotoxic effects on MCL cells, apoptosis was measured following treatment with GSK650394 were analyzed using flow cytometry. As shown in Figure 3(a), the percentage of apoptotic Mino cells was 17.7 ± 1.3%, 34.7 ± 6.0% and 55.3 ± 10.3% upon treatment with three increasing concentrations of the SGK1 inhibitor for 48 h, respectively, which suggested that SGK1 inhibition induced dose-dependent apoptotic cell death in MCL cell lines. Similar results were observed in Z138 and JVM-2 cells. Furthermore, immunoblotting analysis demonstrated that expression of the active forms of Caspase-3 and poly-ADP ribose polymerase (PARP) were significantly increased following treatment with the SGK1 inhibitor (Figure 3(b)). The results suggested that the cytotoxicity observed upon treatment with SGK1 inhibitor was Caspase-dependent. The Bcl-2 family of proteins play a critical role in regulating apoptosis, thus, the expression levels of two anti-apoptotic proteins, Bcl-xl and myeloid cell leukemia-1 (Mcl-1) were measured by immunoblotting analysis. As shown in Figure 3(b), the expression of these two proteins was noticeably decreased in response to treatment with the SGK1 inhibitor, suggesting that SGK1 inhibition promoted the induction of apoptosis via regulation of mitochondrion-related proteins.

Next, cell cycle arrest was evaluated using flow cytometry to further characterize the mechanism underlying the anti-tumor activity of SGK1 inhibitor. Flow cytometry analysis of Z138 cells showed that the number of cells in the G2 phase significantly increased from 12.7 ± 2.5% in the control group to 18.3 ± 5.8%, 23.3 ± 2.2%, 29.3 ± 1.1% in the three increasing dose treatment groups, respectively. Similar effects were also observed in Mino and JVM-2 cells (Figure 3(c)). These results demonstrated that SGK1 inhibition exhibited an anti-proliferative effect on MCL cells by inducing cell cycle arrest at the G2/M checkpoint.

SGK1 inhibition downregulates activation of ERK, AKT/mTOR, JAK2/STAT3 and NF-κB signaling in MCL cells. To clarify the potential effect of SGK1 inhibition on the downstream signaling pathways, the related cascades were further investigated by immunoblotting analysis. In Z138 cells, GSK650394 substantially decreased the phosphorylation of downstream signaling cascades in a dose-dependent manner after 12 h of treatment, including that of the ERK, AKT, mTOR, JAK2, STAT3 and NF-κB signaling pathways (Figure 4(a) and (b)). Similar results were observed in Mino and JVM-2 cells (Figure 4(a) and (s)2). Furthermore, compared with cells transfected with the shControl vector, Z138 cells transfected with shSGK1 (shSGK1 #68540 or shSGK1 #68562) exhibited reduced phosphorylation of ERK, AKT, mTOR, JAK2, STAT3 and NF-κB (Figure 4(c) and (d)). Similar results were observed in Mino and JVM-2 cells (Figure 4(c) and (s)3). These data demonstrated that SGK1 inhibition mediated by either shRNA or SGK1 inhibitor exhibited an anti-proliferative effect on MCL cells through downregulating the transduction and activation of the ERK, AKT/mTOR, JAK2/STAT3 and NF-κB signaling pathways.

Effects of SGK1 inhibition or overexpression in the MCL-cell-derived xenograft model. The aforementioned results provided in vitro evidence that SGK1 inhibition reduced MCL development. Next, the functional role of SGK1 was investigated in MCL tumor development using an in vivo subcutaneous xenograft mouse model. First, a lentiviral-based shSGK1 stable knockdown Z138 cell line was established. The knockdown efficiency of the Z138 cells was confirmed by immunoblotting. Next, 8-week-old NOD/SCID mice were subcutaneously implanted with shControl or shSGK1 Z138 stable cells on day 0, and the tumor volume was measured every 2-3 days until the mice were sacrificed on day 26. As shown in Figure 5(a), SGK1 knockdown markedly reduced the growth of tumors in the MCL-cell implanted mice based on the decrease in tumor volume ($P = .011$ and $P = .012$ for shSGK1 #68540 and shSGK1 #68562, respectively). In addition, to determine the impact of SGK1 on MCL growth in vivo, a stable SGK1-overexpressing Z138 cell line was also established and subcutaneously implanted into the NOD/SCID mice. First, the overexpression vector transfection efficiency was examined by western blot (Figure 5(b)). Compared with the effect of shSGK1, SGK1 overexpression significantly increased tumor volume (Figure 5(c)).

To explore the effect of SGK1 inhibition on MCL growth in vivo, a xenograft model was established through

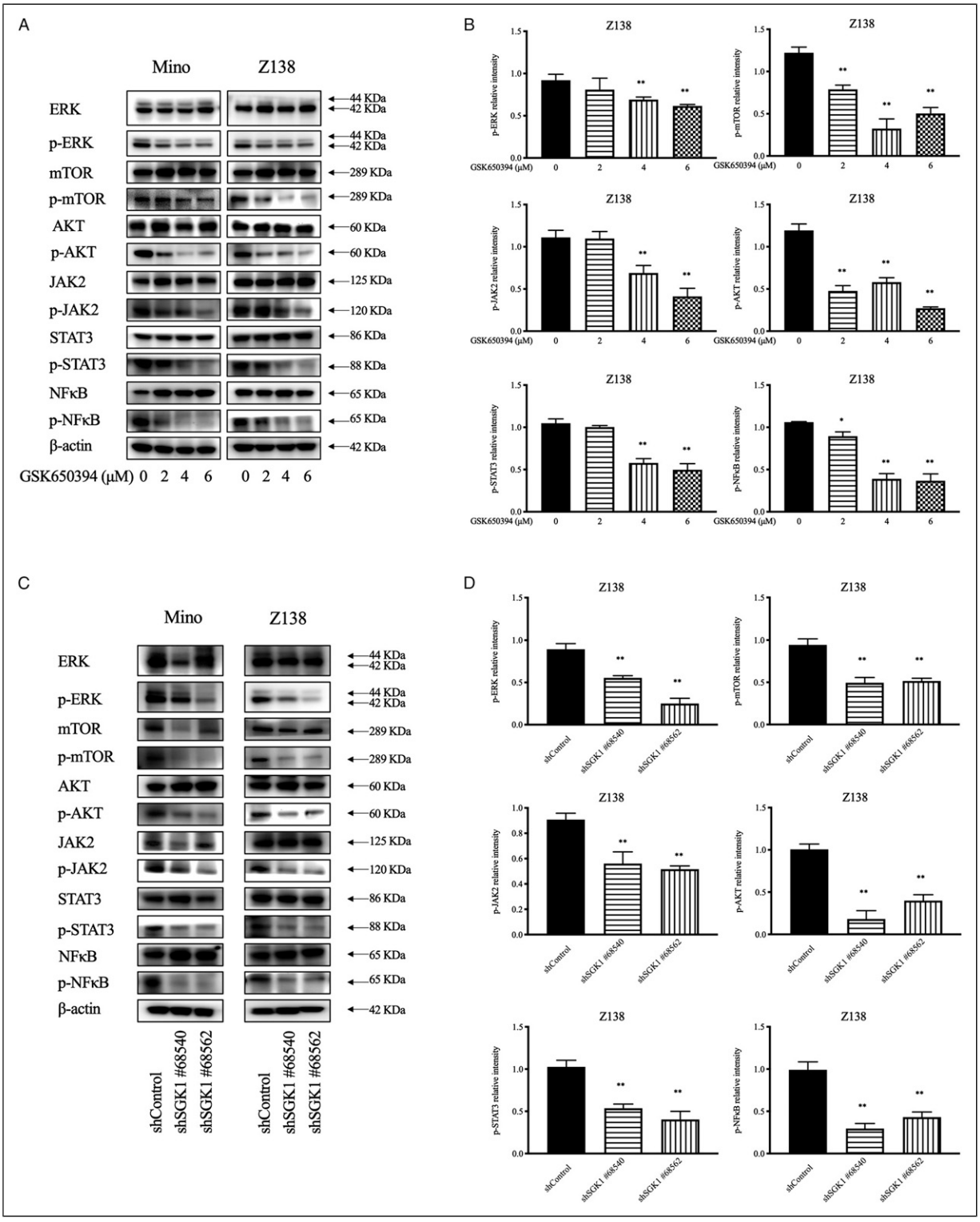


Figure 4. SGK1 inhibition reduces the activation of the ERK, AKT/mTOR, JAK2/STAT3 and NF-κB signaling pathways. **(**A) Mino and Z138 cells were treated with the indicated concentrations of GSK650394 for 12 h. Then whole cell lysates were used to detect ERK, p-ERK, AKT, p-AKT, mTOR, p-mTOR, JAK2, p-JAK2, STAT3, p-STAT3, NF-κB and p-NF-κB protein expression levels using western blotting. β-actin was used as the loading control. (B and D) The phosphorylation levels of signaling mediators were quantified by measuring the relative intensity of phosphorylated bands with the respective total bands. Results are presented as the mean ± SD of three independent repeats. *P < .05, **P < .001 vs control group. (C) Mino and Z138 cells were transfected with vectors carrying one of two shSGK1 constructs or shControl, and western blotting analysis was used to determine the expression levels of ERK, p-ERK, AKT, p-AKT, mTOR, p-mTOR, JAK2, p-JAK2, STAT3, p-STAT3, NF-κB, p-NF-κB. β-actin is shown as a loading control. SGK1, serum- and glucocorticoid-inducible kinase 1; p-, phospho; shRNA, short hairpin RNA.

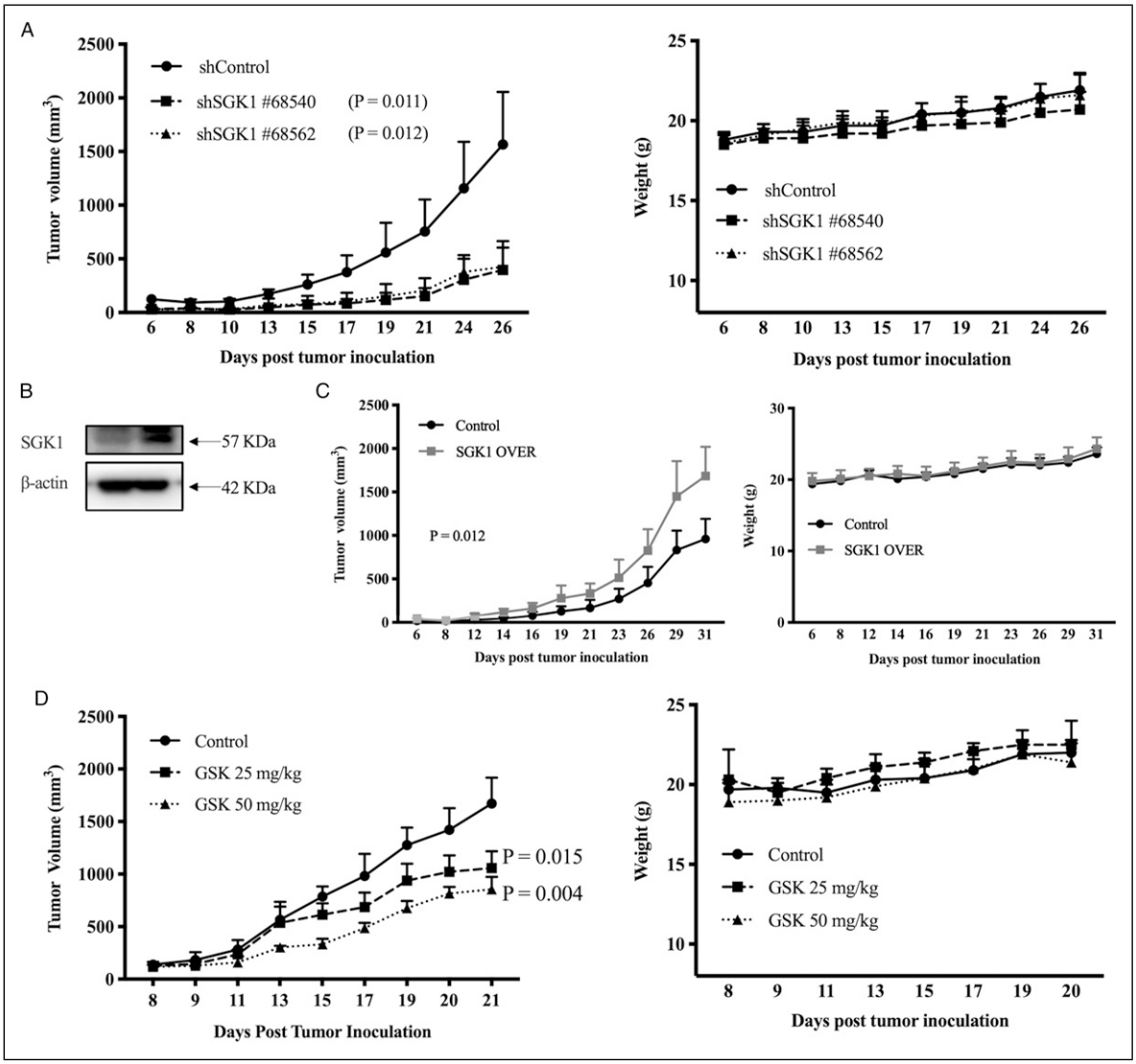
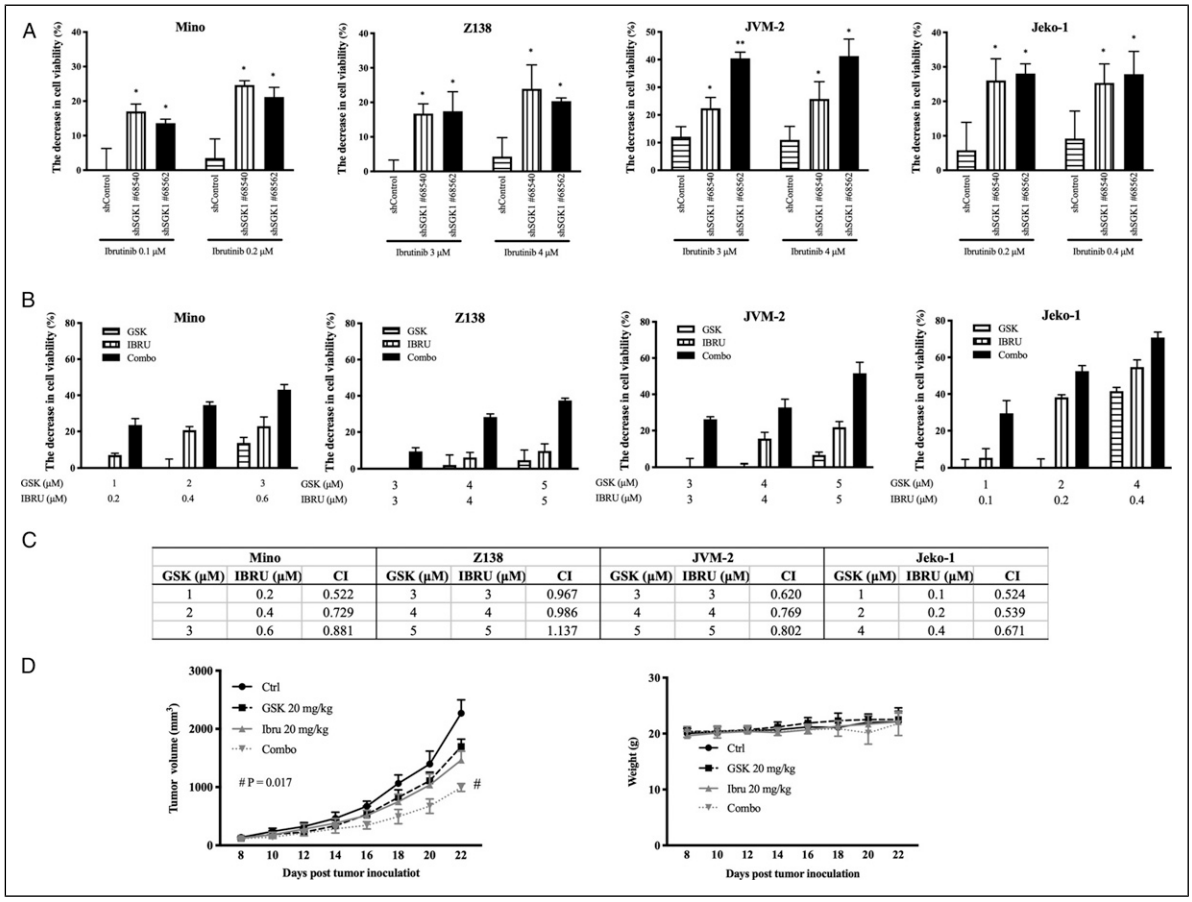


Figure 5. Effects of SGK1 inhibition or overexpression in the MCL-cell-derived xenograft model. NOD/SCID mice were subcutaneously injected with 1×10^7 (A) shControl or shSGK1 Z138 stable cells, or (C) Z138 cells transfected with SGK1 overexpression vector in .1 mL PBS with Matrigel (1:1 ratio), and each group consisted of 8 mice. Body weights and tumor volumes of mice were measured every 2-3 days during the experimental procedure. (B) The transfection efficiency of SGK1 overexpression vector was detected by western blot in Z138 cells. β-actin was used as the loading control. (D) NOD/SCID mice were injected subcutaneously with 1×10^7 Z138 cells and each group consisted of 6 mice. GSK650394 (25 or 50 mg/kg) was administered intraperitoneally into mice daily for 14 days. The tumor volume and body weight were measured every 2 days during treatment. GSK, GSK650394. Data are presented as the mean ± SD. Differences between groups were compared using a Student's t-test. *P < .05, **P < .001 vs control group. SGK1, serum- and glucocorticoid-inducible kinase 1; shRNA, short hairpin RNA; MCL, mantle cell lymphoma.

subcutaneously injecting Z138 cells into NOD/SCID mice, followed by treatment with GSK650394 daily for 14 days by intraperitoneal injection according to the dose of GSK650394 used in vivo experiment.[33] As shown in Figure 5(d), the SGK1 inhibitor reduced tumor growth by 36.8% (P = .015) and 48.9% (P = .004) in response to 25 and 50 mg/kg GSK650394 treatment, respectively. The body weight of tumor-bearing mice was not significantly affected in mice treated with the SGK1 inhibitor. Taken together, these results clearly demonstrated that inhibition of SGK1 effectively reduced tumor growth and overexpression of SGK1 increased tumor proliferation of MCL cells in vivo.

SGK1 inhibition sensitizes MCL cells to a Bruton tyrosine kinase (BTK) inhibitor in vitro *and* in vivo. Inhibition of the B-cell receptor (BCR) signaling pathway using a BTK selective inhibitor, has generated favorable responses in patients with MCL.[5] Thus, whether SGK1 inhibition could improve the sensitivity to ibrutinib in MCL was assessed. ShControl or shSGK1-transfected Mino cells were incubated with ibrutinib (.1 μM) for 72 h. As shown in Figure 6(a), shSGK1



Mino			Z138			JVM-2			Jeko-1		
GSK (μM)	IBRU (μM)	CI	GSK (μM)	IBRU (μM)	CI	GSK (μM)	IBRU (μM)	CI	GSK (μM)	IBRU (μM)	CI
1	0.2	0.522	3	3	0.967	3	3	0.620	1	0.1	0.524
2	0.4	0.729	4	4	0.986	4	4	0.769	2	0.2	0.539
3	0.6	0.881	5	5	1.137	5	5	0.802	4	0.4	0.671

Figure 6. SGK1 inhibition sensitizes MCL cells to a BTK inhibitor in vitro and in vivo. (A) MCL cells were transfected with vectors carrying one of two shSGK1 constructs or shControl were incubated with the indicated concentrations of ibrutinib for 72 h. The viability of the cells was determined using the Cell Titer-Glo Luminescent Cell Viability Assay. The decrease in cell viability rates were calculated as follows: (1-Dosing/Vehicle)x100%. (B) MCL cells were seeded in opaque-walled 96-well plates at a density of 3×10^4 cells/ml, and incubated with increasing concentrations of GSK650394 in the presence or absence of different concentrations of ibrutinib at a constant ratio for 72 h. The viability of the cells was determined using the Cell Titer-Glo Luminescent Cell Viability Assay. The decrease in cell viability rates were calculated as described above. (C) The CI values at different concentrations of GSK650394 and ibrutinib in MCL cell lines were measured using CalcuSyn software. A CI value <1.0 indicates a synergistic interaction between the two agents. (D) NOD/SCID mice were subcutaneously injected with 1×10^7 Z138 cells (n = 8 per group). GSK650394 (20 mg/kg) and ibrutinib (20 mg/kg) was administered to the tumor-bearing mice daily for 14 days. Body weights and tumor volumes of mice were measured every other day during treatment. Data are presented as the mean ± SD of three independent experiments. Differences between groups were compared using a Student's t-test. *P < .05, **P < .001 vs control group; #P < .05 vs ibrutinib treatment group. CI, combination index; MCL, mantle cell lymphoma; SGK1, serum- and glucocorticoid-inducible kinase 1; shRNA, short hairpin RNA; IBRU, ibrutinib; Combo, GSK650394 combined with ibrutinib.

transfection effectively enhanced the antitumor effects of the ibrutinib with the decrease in cell viability of 18.8% (shSGK1 #68540) and 18.3% (shSGK1 #68562) in Mino cells compared with cells transfected with shControl (3.5%). Similar results were observed in Z138, JVM-2 and Jeko-1 cells. These data demonstrated that SGK1 knockdown could significantly enhance the antitumor effects of ibrutinib in MCL.

To further confirm the synergistic activity of the combination of SGK1 inhibition and BTK inhibition, MCL cells were incubated with a fixed-ratio concentration of the SGK1 inhibitor, GSK650394, and the BTK inhibitor, ibrutinib, for 72 h. As shown in Figure 6(b), the combination treatment with both reagents resulted in significantly greater anti-tumor activity than that afforded by either agent alone. Of note, the combination treatment did not increase the cytotoxicity to normal B cells. Next, the CI values were used to verify the synergistic anti-tumor effects of these two agents (Figure 6(c)). Analysis of CI values revealed the synergistic combination of the SGK1 inhibitor and BTK inhibitor as it exhibited CI values <1, which suggested that SGK1 inhibition greatly improved the anti-tumor activities of common therapeutic regimens in MCL clinical treatment.

In addition, to determine whether the SGK1 inhibitor could enhance the sensitivity to the BTK inhibitor in vivo, GSK650394 and ibrutinib were co-administered in the Z138-xenograft model. According to the dose of GSK650394 used

in Figure 5(d) and that of ibrutinib administrated in vivo experiment,[34] the mice were randomized into four treatment groups according to tumor volume and body weight when the tumor reached 100−150 mm^3: Vehicle control, GSK650394 (20 mg/kg), ibrutinib (20 mg/kg), and the combination of these two agents. Mice were treated with GSK650394 intraperitoneally and ibrutinib orally once daily for 14 days. As shown in Figure 6(d), exposure to GSK650394 and ibrutinib alone resulted in 25.3 and 35.5% reduce of tumor growth in the Z138 xenograft model, respectively. However, tumor growth was reduced by 56.2% in mice treated with the combination of SGK1 inhibitor and BTK inhibitor, highlighting the synergistic activity of these two agents. All treatment groups were tolerated with no increased loss of body weight. These data suggested that SGK1 inhibition sensitized MCL cells to BTK inhibition in vivo.

Additionally, the combination of SGK1 inhibitor and chemotherapeutic drugs (doxorubicin/vincristine) exhibited synergistic anti-tumor effects on MCL cells in vitro (Figure S5).

Discussion

MCL is an aggressive and incurable type of B-cell non-Hodgkin's lymphoma. Traditional immunochemotherapy for MCL has limited efficacy, with the majority of patients experiencing relapse or continuing to progress.[4] Although a wide range of novel treatment agents, such as bortezomib, ibrutinib and lenalidomide, have been applied in clinical therapy for salvage treatment,[5–8] the prognostic outcomes of MCL patients are still far from acceptable. Therefore, there is an urgent need to explore novel therapeutic targets and develop effective combination-based therapeutic regimens for treating this disease. In the present study, it was demonstrated that genetic and pharmacological inhibition of SGK1 markedly reduced the proliferation, invasion and migration of MCL cells, and the activation of several important signaling pathways (ERK, AKT/mTOR, JAK2/STAT3 and NF-κB signaling) in MCL. In addition, the SGK1 inhibitor promoted apoptosis and induced G2/M phase arrest in MCL cells, and delayed tumor growth in MCL-cell-derived xenograft models. Importantly, SGK1 inhibition enhanced the sensitivity of MCL cells to a BTK inhibitor, both in vitro and in vivo, which suggested a novel combination treatment regimen for management of MCL.

SGK1, a serine/threonine protein kinase with 54% homology in its catalytic domain to AKT, has a critical role in several oncogenic signaling cascades involved in cell transformation, tumor progression and chemo/radioresistance.[18,27,35,36] SGK1 has been shown to be upregulated in several types of cancer,[37–41] and is closely associated with tumor invasion and metastasis.[23,42] Somatic mutations of SGK1 have also been detected in diffuse large B cell lymphoma of germinal center (GC) B-type as well as in follicular lymphoma,[43,44] indicating a pathogenic role in GC B-cell-derived lymphomas. In addition, high expression of SGK1 has been observed in nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) combined with a high rate of apoptosis after SGK1 inhibition, suggesting SGK1 exerts an oncogenic function in NLPHL.[45] Likewise, downregulation of SGK1 expression resulted in a decreased number of viable cells in multiple myeloma.[40] Taken together, these findings highlight SGK1 as a key element in the development and/or progression of cancer. However, to the best of our knowledge, there have been no studies investigating the functional role and underlying mechanisms of SGK1 in MCL. In the present study, the data showed that intermediate/high expression of SGK1 was seen in all six MCL cell lines assessed, and relatively low expression of SGK1 was observed in normal B-cells from healthy donors. Moreover, MCL cell lines expressed different levels of p-SGK1, whereas normal B-cells exhibited very low levels of p-SGK1. These results indicate that SGK1 may play a critical role in the pathogenesis of MCL and may serve as a potential therapeutic target in the management of MCL. Additionally, the present is the first to assess the functional role of SGK1 in MCL. It was shown that SGK1 inhibition by either shRNA or using an SGK1 inhibitor significantly reduced the survival, proliferation, invasion and migration of MCL cells, consistent with previous studies in other types of cancer. In the preliminary experiments, we used immunohistochemistry to detect the expression of SGK1 in tumor tissues of 42 MCL patients. With 10% positive staining per a tissue as the cut-off value for what was considered positive expression, the expression of SGK1 was positive in 25 MCL patients. Unfortunately, survival analysis could not be performed due to missing follow-up data. In our next work would explore the correlation between SGK1 expression and clinical characteristics and survival in MCL patients.

The present study revealed that not all MCL cells were sensitive to the SGK1 inhibitor, such as JVM-2, Granta519 and JVM-13 cell lines, whose cell proliferation curves did not exhibit a distinct dose-dependent response. It was shown that cells treated with an SGK1 inhibitor exhibited an enlarged volume 24 h after treatment compared with the untreated cells, and this increase became even more prominent after 48 h of treatment with the SGK1 inhibitor. Previous studies have demonstrated that SGK1 inhibition, mediated by either GSK650394 or SGK1 shRNA, induced G2/M phase arrest in human prostate cancer.[46] Therefore, it is hypothesized that the SGK1 inhibitor induced cell cycle arrest and inhibited cell division in JVM-2, Granta519 and JVM-13 cells, which was validated in subsequent cell cycle analysis.

Previous studies have reported that FAK and RhoA played important roles in modulating the motility of malignant lymphoma cells. FAK is a non-receptor tyrosine kinase that localizes to sites of cell adhesion with the extracellular matrix, which controls cell movement, invasion, survival and gene expression through its kinase or scaffolding function.[47] RhoA is a member of the Rho GTPase family, which regulates the cytoskeleton and cell migration.[48] Consistent with previous reports, suppressing the expression of SGK1 reduced the

migration and invasion of MCL cells by decreasing FAK and RhoA expression.

Previous studies have demonstrated that increased levels of cleaved-Caspase-3 can result in cleavage of PARP to an inactive form, thereby disrupting DNA repair, leading to apoptosis. The role of the Bcl-2 family of proteins in the regulation of Caspase activation has been well characterized in the mitochondrial apoptotic pathway. The Bcl-2 protein family plays an important role in apoptosis, which include pro-apoptotic proteins, such as Bcl-2-associated X protein, Bcl-2 homologous antagonist/killer, and antiapoptotic proteins, such as Bcl-2, Bcl-xl and Mcl-1. A low level of Bcl-2 results in mitochondrial dysfunction and induces the release of intermembrane proteins, thereby activating Caspase signaling.[49] In the present study, it was shown that inhibition of SGK1 increased the expression levels of cleaved-Caspase-3 and PARP, and decreased Bcl-xl and Mcl-1 expression.

SGK1 promotes cancer cell proliferation through multiple pathways, including NF-κB, c-fms, p27 and Foxo3a/FKRHL1.[22–25] Previous studies have revealed that a high level of SGK1 can increase NF-κB transcriptional activity though phosphorylating IKKβ to enhance IKK activity.[23] Consistent with the aforementioned findings, the results of the present study revealed that SGK1 inhibition, mediated either by shRNAs or a SGK1 inhibitor, significantly decreased the phosphorylation of NF-κB at Ser536 based on western blot analysis. In addition, accumulating evidence suggests that mTOR activation facilitates an increase in SGK1 phosphorylation, thereby activating the SGK1 signaling pathway.[12,25] Moreover, SGK1 silencing significantly decreased the phosphorylation of mTOR and SGK1 overexpression, and stimulated the activity of mTOR.[46] The results further showed that SGK1 inhibition by shRNA or a SGK1 inhibitor also markedly reduced the phosphorylation of mTOR. Recently, research data demonstrated that PDK1-mediated SGK1 activation contributes to the maintenance of residual mTORC1 activity through direct phosphorylation and inhibition of TSC2, which further confirms our findings. Moreover, STAT3 signaling is positively regulated by the PI3K/mTOR pathway,[50] indicating a mechanism by which the administration of SGK1 inhibitor could significantly reduce activation of JAK2/STAT3 signaling.

SGK1 has been considered as a critical node in tumor cell resistance to various molecular inhibitors, chemotherapeutic drugs (such as PI3Kα inhibitors, AKT inhibitors, paclitaxel and temozolomide) and radiation therapy, suggesting that suppression of SGK1 may lead to a more favorable outcome when combined with standard therapies.[11,27,29] Inhibition of the BCR signaling pathway using a BTK inhibitor, ibrutinib, resulted in a favorable response in MCL.[5] However, ~1/3 of patients do not respond well to ibrutinib, and disease relapse or progression on ibrutinib is almost universal.[51] Therefore, novel combined treatment strategies designed to target the molecular mechanisms underlying the development of MCL are urgently required. NF-κB activation is an important pathogenic mechanism in MCL, and it is a critical downstream signaling pathway of BCR signaling that contributes to cell survival and proliferation.[52] Given that SGK1 inhibition could significantly reduce the activation of NF-κB, it is hypothesized that the combination of a BTK inhibitor and SGK1 inhibitor may synergistically enhance the anti-tumor activity in the treatment of MCL. By establishing stable shSGK1-MCL cells, SGK1 inhibition using SGK1 shRNA or an SGK1-selective inhibitor combined with the BTK inhibitor ibrutinib exhibited synergistic anti-tumor activity in MCL both in vitro and in vivo, indicating that SGK1 inhibition could increase the sensitivity of the BTK inhibitor ibrutinib in MCL, and the combination of the SGK1 inhibitor and BTK inhibitor may be a novel promising therapeutic strategy for the treatment of MCL. In addition, Amato *et al* observed that ectopic IL-2 receptor expression in renal carcinoma cells was able to activate SGK1 through PI3K.[53] Activated SGK1 mediated hyper-proliferation and survival, and induced resistance to doxorubicin through a FAS/FASL (CD95^{-}CD95 L) dependent mechanism.[53] Consistent with this previous study, in the present study it was also shown that MCL cells overexpressing SGK1 exhibited low sensitivity to conventional chemotherapy, including doxorubicin and vincristine, and combined treatment with the SGK1 inhibitor and doxorubicin/vincristine acts synergistically to enhance the anti-tumor activity on MCL cells.

Conclusion

In summary, the present study provides evidence of the functional role of SGK1 in the survival, growth and motility of MCL. Knockdown and inhibition of SGK1 markedly reduced the oncogenic potential of MCL cells, and delayed disease progression in MCL-cell-derived xenograft models. In addition, SGK1 acts as an important factor affecting the response of MCL cells to the BTK inhibitor ibrutinib, and SGK1 inhibition increased the sensitivity to ibrutinib in MCL both in vitro and in vivo. Therefore, SGK1 may be a novel candidate therapeutic target for management of MCL, and the combined treatment of an SGK1 inhibitor and BTK inhibitor may be a novel promising therapeutic strategy for the treatment of MCL. It is essential to perform pre-clinical or even clinical research on SGK1 inhibitor or combination with BTK inhibitor.

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Authors' Contributions

Jun Zhu and Yuqin Song were involved in the conception and design of the study. Jiao Li performed in vitro experiments and analyzed data. Jiao Li and Hui Yu mainly performed the animal experiments

and analyzed data, and Xing Wang, YingYing Ye and Wei Fang helped to complete the animal experiments. Jiao Li, Hui Yu, and Xing Wang provided the peripheral whole blood samples of healthy donors. Ning Ding guided the performance of experiment and developed the methodologies. Lan Mi, Lingyan Ping and Xing Wang acquired the data. Jiao Li wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Ethical Approval

All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals and in accordance with the ethical guidelines of Beijing Cancer Hospital & Institute. Peripheral blood mononuclear cells (PBMCs) from peripheral whole blood samples of healthy donors were collected in accordance with the ethical guidelines of Beijing Cancer Hospital & Institute.

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Supplemental Material

Supplemental material for this article is available online.

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