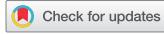


RESEARCH ARTICLE

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A novel anti-CD47 antibody with therapeutic potential for NK/T-cell lymphoma

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ABSTRACT

NK/T-cell lymphoma (NKTCL) is a rare type of non-Hodgkin lymphoma (NHL). Although L-asparaginase-based chemotherapy has significantly improved survival in early-stage patients, the prognosis is poor in advanced and relapsed or refractory patients. CD47 is a promising target for cancer immunotherapy. However, the expression of CD47 in NKTCL and the antitumor effect and mechanism of the anti-CD47 monoclonal antibody (mAb) AK117 in NKTCL remain unclear. Firstly, the expression level of CD47 protein in NKTCL cells was detected by immunoblot and flow cytometry. Secondly, in order to validate the role of CD47 downregulation in the proliferation, apoptosis, and cell cycle of NKTCL cells, we used shRNA transfection to knock down CD47 expression. We determined the effect of knocking down CD47 and the novel anti-CD47 antibody AK117 on the phagocytosis of NKYS and YTS cells by M2 macrophages *in vitro*. Finally, we assessed the ability of AK117 to inhibit tumor growth in an NKTCL xenograft model in which YTS cells were engrafted in SCID mice. The results showed that CD47 is relatively highly expressed in NKTCL cells. CD47 knockdown in NKTCL promoted phagocytosis by M2 macrophages in an *in vitro* coculture assay. The study also demonstrated that anti-CD47 mAb AK117 promoted phagocytosis of NKTCL cells by M2 macrophages. In addition, *in vivo* experiments showed that the anti-CD47 mAb AK117 significantly inhibited the growth of subcutaneous xenograft tumors in SCID mice compared to the control antibody IgG. Our results indicate that targeting CD47 monoclonal antibodies is a potential therapeutic strategy for NKTCL.

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Introduction

NK/T-cell lymphoma (NKTCL) is an aggressive non-Hodgkin lymphoma (NHL) derived from NK cells or cytotoxic T cells.¹ This condition is associated with Epstein–Barr virus (EBV) infection and is relatively common in Asia, especially in East Asia, but rare in Europe and North America. According to Chinese statistics, NKTCL constitutes 6.4% of non-Hodgkin lymphomas and more than 20% of mature T and NK cell lymphomas.^{2,3} Because of the rarity of this disease, no standard therapeutic regimen has been established.⁴ Previous studies have shown that cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy is ineffective mainly due to the intrinsic resistance of NKTCL cells to anthracycline caused by high expression of P-glycoprotein.⁵ Although L-asparaginase-based chemotherapy has improved response rates, nearly half of the patients with newly diagnosed NKTCL continue to experience disease progression, and the prognosis in patients with advanced and relapsed or refractory NKTCL remains unsatisfactory.⁶ In recent years, with the continuous exploration of and research on the pathogenesis and therapeutic targets of NKTCL, immunotherapy has brought new hope for patients; the most classic form of this

treatment is anti-PD1/PD-L1 monoclonal antibody (mAb). However, the effective rate of blocking the PD1/PD-L1 pathway in treating relapsed or refractory NKTCL is only 40–60%, the complete remission rate is very low, and the duration of efficacy is short.⁷ Therefore, it is important to explore new therapeutic targets for NKTCL.

The tumor microenvironment (TME) tends to be polarized to an immunosuppressive state to facilitate tumor immune evasion.^{8,9} Macrophages infiltrating the TME are termed tumor-associated macrophages (TAMs), which are the major component of immune cells in most tumors.¹⁰ Macrophages have been divided into two general subtypes: classically activated M1 macrophages and alternatively activated M2 macrophages.¹¹ Previous studies have shown that most of the macrophages in the tumor microenvironment are M2 macrophages.¹² Macrophages are an essential member of the innate immune system and play a critical role in the antitumor immune response.¹³ However, it remains unclear how NKTCL cells are able to escape the antitumor immune surveillance of M2 macrophages.

CD47, which was originally named integrin-associated protein (IAP), is a heavily glycosylated 50 kDa cell surface

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transmembrane protein of the immunoglobulin superfamily.¹⁴ This protein is ubiquitously expressed on all normal cells and is often overexpressed on various solid and hematologic tumor cells. CD47 plays an essential role in various cellular functions, including proliferation, apoptosis, adhesion, migration, and immune responses. For instance, CD47 stimulation induces cancer cell proliferation via a PI3K/Akt-dependent pathway in astrocytoma.¹⁵ Moreover, anti-CD47 mAb induces caspase-independent leukemia cell death in chronic lymphocytic leukemia.¹⁶ Generally, CD47 on normal cells acts as a “don’t eat me” receptor and plays an essential role in self-recognition by which normal cells protect themselves from phagocytosis.^{17,18} Several studies have shown that CD47 is upregulated in many malignancies to evade immune attack, and its overexpression is correlated with poor prognosis.^{19–22} Signal regulatory protein alpha (SIRPa), which is mainly expressed on the surface of macrophages, is the receptor for CD47. When CD47 interacts with its ligand SIRPa on macrophages, it transmits a “don’t eat me” signal to inhibit macrophage phagocytosis.^{23,24} However, interruption of the ligation of CD47 and SIRPa promotes tumor cell phagocytosis by macrophages in various malignancies.^{25,26} In several preclinical studies, anti-CD47 mAb treatment has shown significant antitumor effects in xenograft tumor models.^{27,28} However, the expression of CD47 in NKTCL cells and the anti-tumor effects of anti-CD47 mAb AK117 in NKTCL have not been investigated.

In this study, we investigated the expression of CD47 in NKTCL cells, performed a phagocytosis assay *in vitro* and established a subcutaneous xenograft NKTCL model to test the antitumor efficacy of a novel anti-CD47 antibody AK117. Our studies highlight the therapeutic potential of targeting CD47 for treating NKTCL.

Materials and methods

Cell lines and cell culture

Human NK/T-cell lymphoma cell lines (SNK1, SNK6, NKYS) were donated by Professor Tan Jing from the Cancer Center of Sun Yat-sen University. The human NK/T-cell lymphoma cell line YTS was donated by Professor Zhang Mingzhi from The First Affiliated Hospital of Zhengzhou University. The human monocytic cell line (THP-1), human peripheral T-cell lymphoma cell line (Hut78), and human diffuse large B-cell lymphoma cell lines (HBL1, OCI-LY8) were purchased from the American Type Culture Collection (ATCC). SNK1, SNK6, and NKYS cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS), interleukin-2 (100 U/mL), and 1% penicillin-streptomycin solution in a humidified incubator at 37°C and 5% CO₂. YTS cells were cultured in RPMI 1640 medium with 10% FBS, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin solution in a humidified incubator at 37°C and 5% CO₂. THP-1 and Hut78 cells were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin solution in a humidified incubator at 37°C and 5% CO₂. HBL1 cells were cultured in RPMI 1640 medium with 10% FBS, 1% MEM nonessential amino acids, 0.09% β-mercaptoethanol, and 1% penicillin-streptomycin solution

in a humidified incubator at 37°C and 5% CO₂. OCI-LY8 cells were cultured in IMDM with 10% FBS and 1% penicillin-streptomycin solution in a humidified incubator at 37°C and 5% CO₂.

Therapeutic antibodies

The anti-CD47 antibody B6H12 and isotype control mouse IgG1 antibody were purchased from Ebiosciences (San Diego, CA, USA). The novel fully human anti-CD47 antibody AK117 was obtained from Akeso Biopharma (Guangdong, China).

Immunoblot

Harvested cultured cells were lysed for 30 minutes in RIPA buffer (Cwbio, China) with protease/phosphatase inhibitor (Cwbio, China) on ice and then centrifuged at 12,000 × g at 4°C for 20 minutes. The BCA protein assay kit (Cwbio, China) was used to analyze the total protein concentrations. The proteins were separated by 12% SDS-PAGE (Epizyme, Shanghai, China) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The anti-CD47 antibody (1:1000, ab218810, Abcam) was incubated with PVDF membranes at 4°C overnight after blocking with 5% nonfat milk. The next day, the membranes were incubated with HRP-conjugated secondary antibody. A high-sensitivity chemiluminescence detection kit (Cwbio, China) was used to detect the protein bands. GAPDH was used as an endogenous control.

Flow cytometry analysis

The single-cell suspension was transferred to a centrifuge tube and mixed for cell counting. Then, 1 × 10⁶ cells were washed, resuspended in 100 μL of phosphate-buffered saline (PBS) and stained with PerCP/Cyanine 5.5-conjugated anti-human CD47 (clone CC2C6, BioLegend 323,109), PerCP/Cyanine 5.5-conjugated mouse IgG1κ isotype control (clone MOPC-21, BioLegend 400,149), PE-conjugated anti-human CD206 (clone 15–2, BioLegend 321,105), PE-conjugated mouse IgG1κ isotype control (clone MOPC-21, BioLegend 400,111), APC-conjugated anti-human CD206 (clone 15–2, BioLegend 321,109) and APC-conjugated mouse IgG1κ isotype control (clone MOPC-21, BioLegend 400,119) antibodies. All samples were incubated for 30 minutes at 4°C. After staining, the cells were washed twice with PBS, resuspended in 300 μL of PBS and immediately analyzed on an LSR II flow cytometer (BD Biosciences). At least 3 × 10⁴ events were collected for each analysis. All data were analyzed by FlowJo software (Tree Star, San Carlos, CA, USA). The relative mean fluorescence intensity (MFI) was calculated by dividing the experimental sample MFI by the isotype control MFI.

RNA interference (RNAi) and transfection

Lentivirus vectors that encoded either shCD47 or empty lentiviral vector (shControl) were constructed, packed, and purified by GeneChem (Shanghai, China). The RNAi sequences were as follows: shCD47 (sequence #1: 5'-GCCTTGGTTT ATTGTGACTT-3'; sequence #2: 5'-GTTACTAAT

ATGGAGGCACAA-3'; sequence #3: 5'-GCACAATTACT TGGACTAGTT-3'), shControl (5'-TTCTCCGAACGT GTCACGT-3'). Lentiviral transduction was performed according to the manufacturer's instructions. Briefly, 1×10^5 NKTCL cells (NKYS, YTS) were plated in a 12-well plate. Cells were infected with lentivirus vectors at a multiplicity of infection (MOI) = 100 and cultured for 12 hours. After 12 hours of transduction, the virus-containing medium was replaced with a fresh medium. Puromycin (1 μ g/mL) was used to select the cells that were successfully transfected. After two weeks, CD47 protein expression on NKTCL cells was assessed by immunoblot and flow cytometry.

Cell proliferation assay

The cells were seeded in a 96-well culture plate at a density of 5000 cells per 100 μ L. Then, the 96-well plates were cultured in a humidified incubator at 37°C and 5% CO₂ for 24 hours, 48 hours, and 72 hours. After incubation, 10 μ L of Cell Counting Kit-8 (CCK-8; Apexbio, USA) was added to each well at the indicated time points, and the plates were incubated at 37°C in the dark for 2 hours. We detected the absorbance of each well at a wavelength of 450 nm using a microplate reader (PerkinElmer, Waltham, MA, USA) and calculated the cell proliferation rate.

Cell apoptosis and cell cycle assays

The effect of the CD47 gene on apoptosis was assessed by detecting the percentage of apoptotic cells with an Annexin V-APC/PI apoptosis detection kit (Elabscience, China) according to the manufacturer's instructions. Briefly, shCD47 and shControl cells were seeded in 12-well plates at a density of 2×10^5 per 1 mL and cultured for 48 hours. After 48 hours, the cells were harvested, washed with PBS once, and then resuspended in 100 μ L of binding buffer (1 \times). Finally, 2.5 μ L of Annexin V-APC and PI per tube was added and incubated with the cell suspensions for 15 minutes in the dark at room temperature. Apoptotic cells were detected by flow cytometry within 1 hour, and the results were analyzed by FlowJo software (Tree Star, San Carlos, CA, USA). For cell cycle assays, cells were harvested and washed with PBS before being fixed overnight at -20°C with 70% ethanol. The next day, cells were stained in the dark for 30 minutes with 500 μ L of PI/RNase Staining Buffer (Abbkine, China). Finally, the cells were analyzed by flow cytometry within 24 hours.

M2 macrophages differentiation and identification

Differentiation of THP1 cells into M0 and M2 macrophages was performed as previously described.²⁹ Briefly, THP1 cells were differentiated into M0 macrophages by incubation with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 hours (Solarbio, Beijing, China). After adherence, the cells were transferred to a PMA-free medium to obtain M0 macrophages. These cells were then polarized to M2 macrophages by incubation with 20 ng/mL IL-4 (Solarbio, Beijing, China) for 48 hours. After induction, the cells were washed three times with PBS and digested with 0.25% trypsin-EDTA (Gibco).

Then, 1×10^6 cells were washed, resuspended in 100 μ L of PBS, and stained with PE-conjugated anti-human CD206 (clone 15-2, BioLegend 321,105) antibody. After staining, the cells were analyzed by flow cytometry.

In vitro phagocytosis assays

NKTCL cells (NKYS, YTS) were labeled with 1 μ M carboxy-fluorescein succinimidyl ester (CFSE) (AAT Bioquest, USA) according to the manufacturer's protocol. THP1-derived M2 macrophages were cultured in 6-well plates at a density of 5×10^4 cells per well. Then, 2×10^5 CFSE-labeled NKTCL cells were incubated with M2 macrophages in serum-free medium in the presence of RNAi, 10 μ g/mL IgG1 isotype or anti-CD47 antibody (B6H12, AK117) for 2 hours. After co-incubation, the plate was washed three times with warm PBS to remove unphagocytosed NKTCL cells. These cells were observed by fluorescence microscopy for the presence of fluorescently labeled cells in macrophages (indicated by arrows). For the flow cytometry assay, the cocultured cells were harvested from each well using 0.25% trypsin-EDTA (Gibco). Cell suspensions were stained with APC-conjugated anti-human CD206 (BioLegend) and analyzed on an LSR II flow cytometer (BD Biosciences). The data were analyzed with FlowJo software, and the phagocytic index was calculated as the percentage of CFSE⁺ CD206⁺ macrophages.

Apoptosis of NKTCL cells induced by anti-CD47 antibody

NKTCL cells were treated with an anti-CD47 antibody (AK117; 10 μ g/mL) or control antibody (IgG1; 10 μ g/mL) for 12 hours. Then, NKTCL cell apoptosis was evaluated by flow cytometry using an Annexin V-APC/PI apoptosis detection kit (Elabscience, China) according to the manufacturer's protocol.

In vivo antibody treatment of the NKTCL xenograft Model

All animal experiments were approved by the Animal Ethics Committee of Hunan Cancer Hospital and in compliance with the Guide for the Care and Use of Laboratory Animals. Female immune-deficient SCID mice, four weeks old, were purchased from SJA Laboratory Animal Co., Ltd. (Hunan, China). YTS tumor cells (5×10^6) in serum-free medium with Matrigel (1:1 ratio) were injected subcutaneously into the area under the right flank of each mouse. When the tumor reached 180 mm³, mice were randomly divided into two groups (IgG control, treated with AK117). There were six mice in each group. These mice were given daily intraperitoneal injections of 200 μ g mouse IgG isotype control or 200 μ g anti-CD47 antibody AK117 for two weeks. Tumor volume (mm³) and body weight (g) were monitored every day. The tumor volume was calculated using the formula (length \times width²/2). After two weeks, the mice were sacrificed by cervical dislocation and tumor tissue samples were collected from the two groups.

Statistical analysis

GraphPad Prism 8.0 software was used to perform statistical analyses. Statistically significant differences between

different groups were analyzed using the Student's *t* test. *p* values < .05 were considered statistically significant. All experiments were independently repeated more than three times.

Results

CD47 was highly expressed in NKTCL cells

Previous studies have reported that CD47 is widely expressed in many subsets of B-cell non-Hodgkin's lymphoma (B-NHL), including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), and marginal zone lymphoma (MZL).²⁵ We first detected the expression of CD47 protein in NKTCL cells (SNK1, SNK6, NKYS, YTS) by immunoblot. Compared to that in peripheral T-cell lymphoma cells (Hut78) and DLBCL cells (HBL1, OCI-LY8), CD47 was more highly expressed in NKTCL cells (Figure 1a,b). Considering that CD47 is a cell surface transmembrane protein that interacts with SIRPa, we investigated CD47 expression on NKTCL cells, peripheral T-cell lymphoma cells, and DLBCL cells by flow cytometry, and the relative mean fluorescence intensity (MFI) of CD47 was analyzed. Although CD47 protein was detectable in all cells, it was more highly expressed in NKTCL cells than in peripheral T-cell lymphoma and DLBCL cells (Figure 1c,d).

CD47 knockdown had no direct effect on the cell proliferation, apoptosis, and the cell cycle of NKTCL cells

To validate the role of CD47 downregulation in the proliferation, apoptosis, and cell cycle of NKTCL cells, we used shRNA transfection to knock down CD47 expression. Three lentivirus-mediated RNA interference (RNAi) vectors carrying GFP against CD47 demonstrated effective knockdown of CD47 in NKYS and YTS cells, and CD47#1 exhibited the highest efficacy. The effective knockdown was verified using immunoblot and flow cytometry (Figure 2a). Stable CD47-

transfected NKTCL cells exhibited no significant changes in tumor cell proliferation, apoptosis, or the cell cycle in contrast to cells transfected with empty vectors (Figure 2b-d).

CD47 knockdown increases phagocytosis of NKTCL cells by M2 macrophages in vitro

To investigate the role of CD47 in M2 macrophages phagocytosis of NKTCL, we determined the effect of knocking down CD47 on the phagocytosis of NKYS and YTS cells in vitro. First, THP1 cells were polarized into M2 macrophages by incubation with PMA and IL-4 in turn. After successful polarization, THP1 cells changed from suspended to adherent growth (Figure 3a). Flow cytometry was used to detect the expression of CD206, and the results showed that THP1-M2 cells had significantly higher levels than THP1 cells (Figure 3b). These results indicated that THP1 cells were successfully polarized into M2 macrophages. Then, we performed a CD47 knockdown experiment in NKYS cells and YTS cells using a lentivirus-mediated RNA interference method and confirmed the successful establishment of CD47 knockdown NKTCL cells. Finally, CFSE-labeled scramble control and CD47 knockdown NKTCL cells were incubated with M2 macrophages. The presence of CFSE-labeled cells (green) in M2 macrophages was observed by fluorescence microscopy (indicated by arrows) (Figure 4a). CD47 knockdown increased the phagocytosis of NKTCL cells by M2 macrophages compared with the scramble control (Figure 4b). These results show that the expression of CD47 in NKTCL was essential for the evasion of macrophage phagocytosis.

The novel anti-CD47 antibody AK117 enabled phagocytosis of NKTCL cells by M2 macrophages and had no direct effect on the apoptosis of NKTCL cells

Next, we investigated whether the novel anti-CD47 antibody AK117 could enable the phagocytosis of NKTCL cells by M2 macrophages in vitro. We incubated CFSE-labeled NKTCL

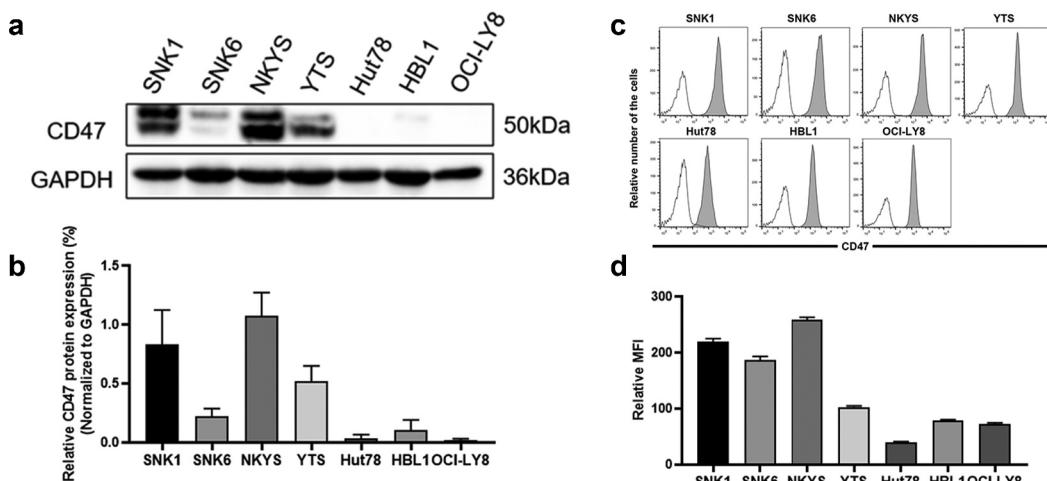


Figure 1. CD47 is more highly expressed on NKTCL cells than on peripheral T-cell lymphoma and DLBCL cells. (a, b) relative expression levels of CD47 were evaluated using immunoblot in SNK1, SNK6, NKYS, YTS, Hut78, HBL1, and OCI-LY8 cells. The data were normalized to GAPDH. (c, d) relative CD47 protein expression was determined in NKTCL cells, peripheral T-cell lymphoma cells (Hut78), and DLBCL cells (HBL1, OCI-LY8) by flow cytometry. Gray histograms show antibody-stained cells; white histograms are isotype controls. Numbers of CD47 expression indicate the relative mean fluorescence intensity (MFI) by flow cytometry.

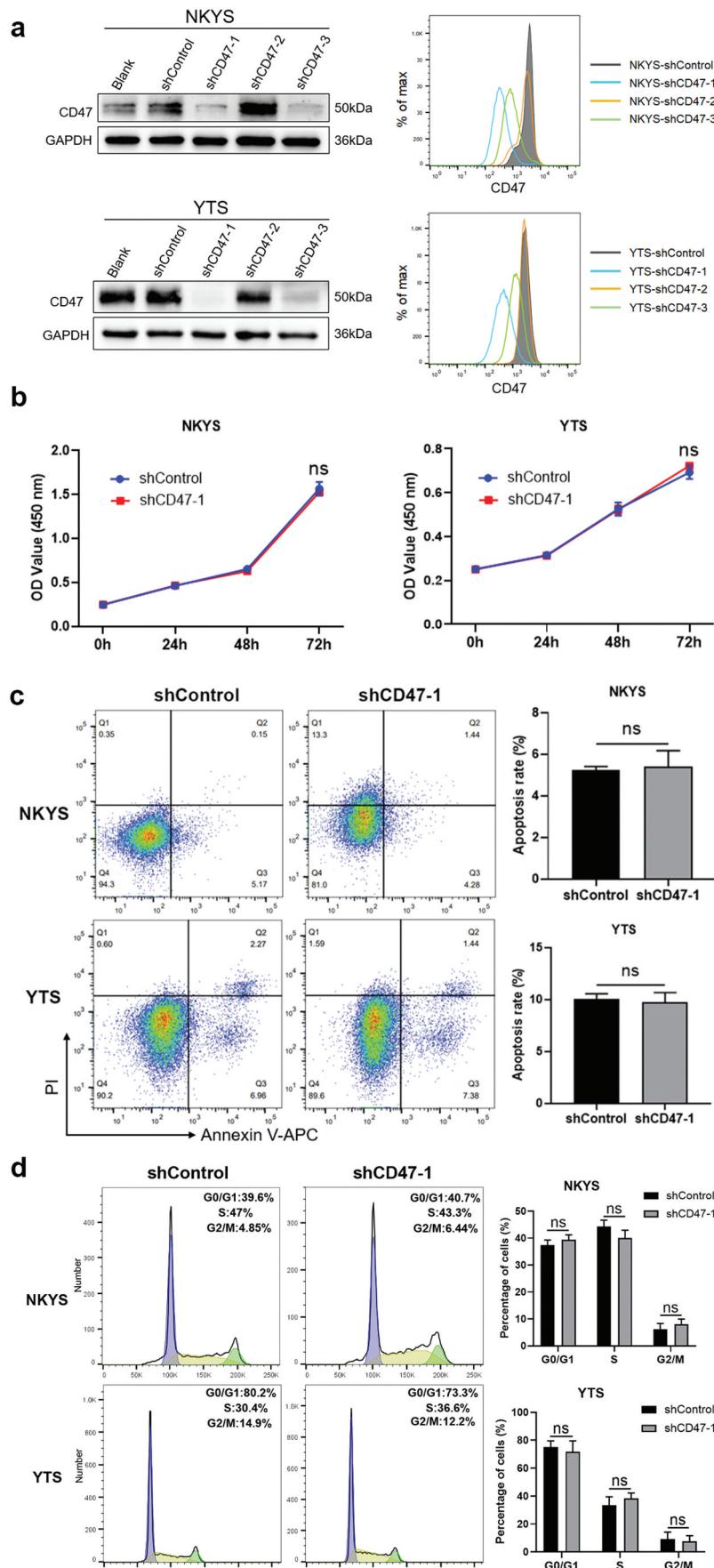


Figure 2. CD47 knockdown had no direct effect on the cell proliferation, apoptosis, and cell cycle of NKTCL cells. (a) Relative expression levels of CD47 were evaluated using immunoblot and flow cytometry in stably transfected NKYS and YTS cells in contrast to those with the empty vectors. (b) Effect of CD47 downregulation on the proliferation of NKTCL cells. The cell viability of tumor cells was determined using the cell counting kit-8 assay. (c) Effect of CD47 downregulation on the apoptosis of NKTCL cells as assessed by flow cytometric analysis with Annexin V-APC/PI staining. (d) The effect of CD47 downregulation on the cell cycle of NKTCL cells was detected using flow cytometry. The results are shown as the mean \pm SD, and the data are representative of three independent experiments (ns, not significant).

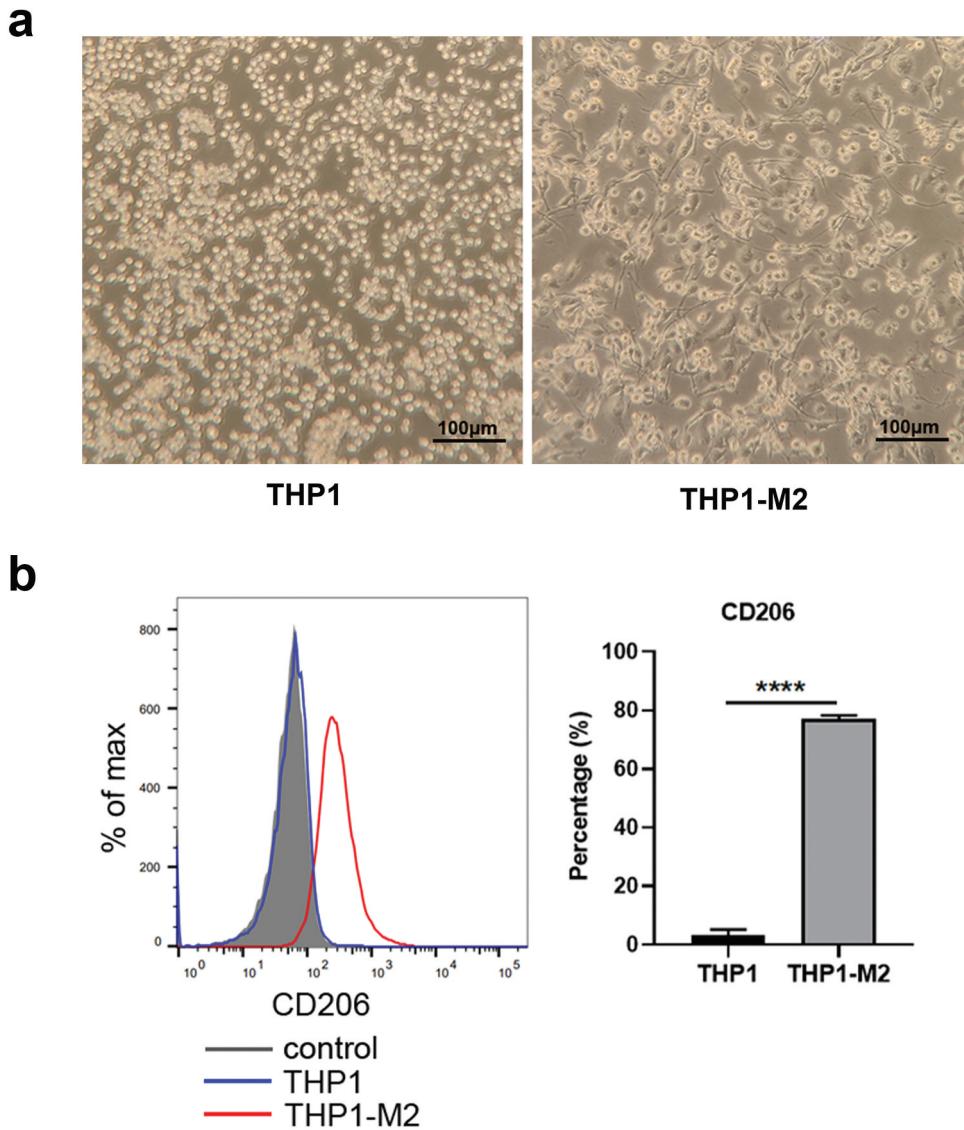


Figure 3. M2 macrophages differentiation and identification. (a) THP1 cells were induced by PMA and IL-4 to differentiate into M2 macrophages and then changed from suspended to adherent growth. (b) The expression of CD206 was significantly increased after THP1 cells were polarized into M2 macrophages (**** $p < .0001$).

cells with THP1-derived M2 macrophages in the presence of an IgG1 isotype control and anti-CD47 antibody (B6H12 and AK117) and measured phagocytosis by fluorescence microscopy (indicated by arrows) or flow cytometry. The anti-CD47 antibodies B6H12 and AK117 significantly enabled phagocytosis of NKYS or YTS cells compared with the IgG1 isotype control antibody (Figure 5a,b). However, there was no significant difference in phagocytosis between B6H12 and AK117 (Figure 5b). In addition, our results showed that the anti-CD47 antibody AK117 could not promote the apoptosis of NKTCL cells (Figure 5c). These results demonstrate that the novel anti-CD47 antibody AK117 promoted M2 macrophages phagocytosis of NKTCL cells.

The novel anti-CD47 antibody AK117 inhibits the growth of NKTCL xenografts in vivo

Finally, we assessed the ability of AK117 to inhibit tumor growth in an NKTCL xenograft model in which YTS cells were engrafted in SCID mice. When the tumor reached

180 mm³, mice were randomly divided into two groups and treated with daily 200 μg intraperitoneal injections of IgG or AK117 for 14 days. Tumor volume (mm³) and body weight (g) were monitored every day. Compared with the IgG control-treated group, the tumor volume was significantly smaller in the mice treated with AK117 (Figure 6a,b,c). Additionally, the tumor weight of the AK117 treatment group was significantly lower than that of the IgG control group (Figure 6d). Treatment was well tolerated, and there was no difference in mouse body weight between the two groups (Figure 6e). These results suggest that the novel anti-CD47 antibody AK117 significantly inhibited the growth of the NKTCL xenografts in vivo.

Discussion

In the present study, we demonstrated that CD47 was highly expressed in NKTCL cells. In addition, CD47 knockdown using shRNA transfection in NKTCL cells promoted M2

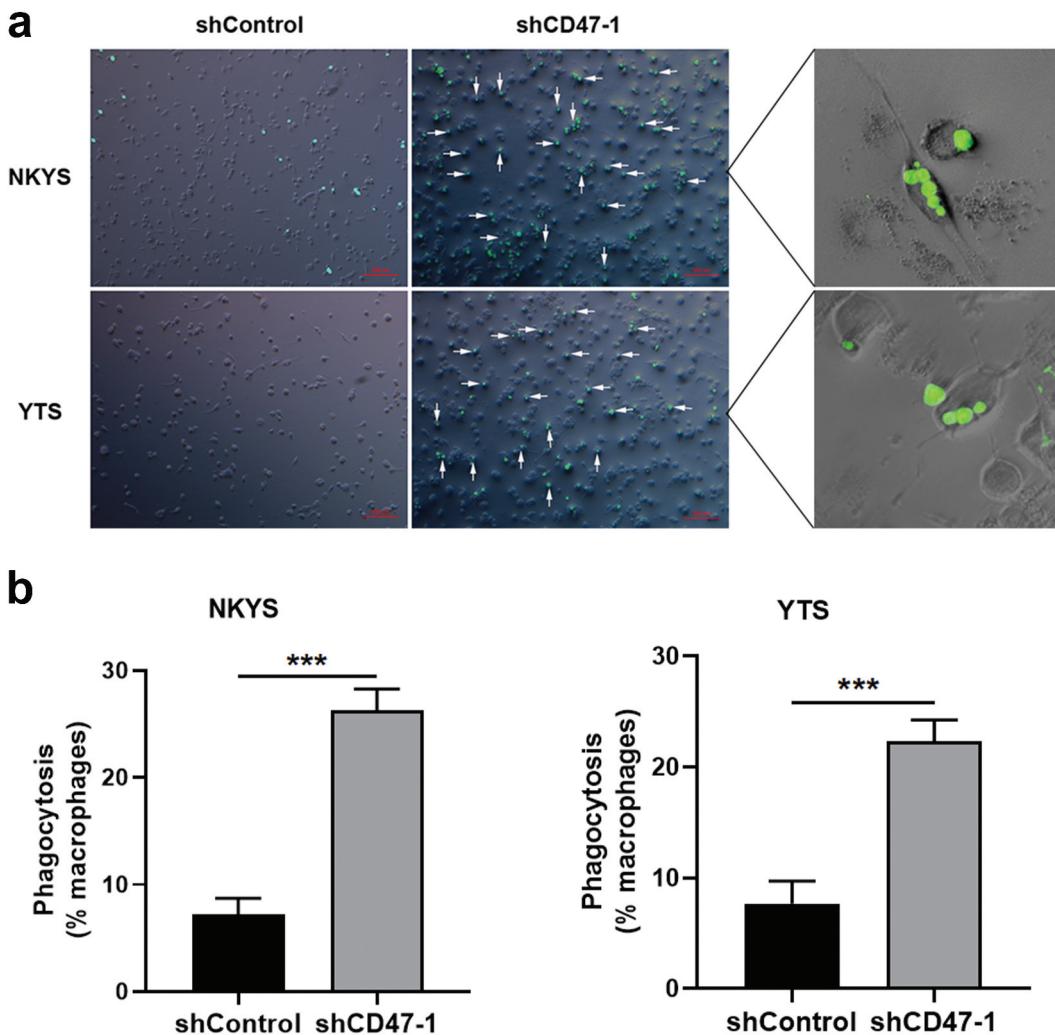


Figure 4. CD47 knockdown increases phagocytosis of NKTCL cells by M2 macrophages in vitro. (a) Representative images of the phagocytosis assay in which cfse-labeled scramble control and CD47 knockdown NKTCL cells (NKYS or YTS) were incubated with M2 macrophages. These cells were assessed by fluorescence microscopy for the presence of fluorescently labeled NKYS or YTS cells in M2 macrophages. The white arrows point to the M2 macrophages that phagocytosed NKYS or YTS cells (green). (b) The phagocytic index was calculated as the percentage of CFSE⁺ CD206⁺ macrophages by flow cytometry. Data are shown as the mean \pm SD (**p < .001).

macrophages phagocytosis in vitro and had no direct effect on cell proliferation, apoptosis, or the cell cycle, suggesting that high expression of CD47 on the surface of NKTCL contributes to immune evasion and resistance. We also showed that the novel anti-CD47 antibody AK117 enabled the phagocytosis of NKTCL cells by M2 macrophages and significantly inhibited the growth of NKTCL xenografts in vivo. Together, these results establish a rationale for the use of an anti-CD47 antibody as a therapy for NKTCL.

CD47 is a cell surface transmembrane protein expressed in hematopoietic cells and other normal tissues. It acts as a membrane receptor involved in many normal and pathophysiological processes.³⁰ CD47 expressed on tumor cells interacts with SIRPa expressed on macrophages and then transmits a “don’t eat me” signal, inhibiting the phagocytosis of tumor cells.^{31,32} Previous studies have shown that CD47 is highly expressed on many subsets of B-cell NHL, including diffuse large B-cell lymphoma (DLBCL), B-cell chronic lymphocytic leukemia (B-CLL), follicular lymphoma (FL), mantle cell lymphoma (MCL), and marginal zone lymphoma (MZL). Higher CD47 expression was associated with adverse prognosis in

DLBCL, B-CLL, and MCL.²⁵ However, the expression of CD47 in T-cell non-Hodgkin’s lymphoma, especially in NKTCL, is still largely unknown. Our study showed that CD47 was highly expressed in NKTCL cells. Knockdown of CD47 by shRNA in NKTCL cells enhanced M2 macrophages phagocytosis. Since inhibitory antibody administration is more clinically applicable, we further blocked the CD47 signaling pathway using the novel anti-CD47 antibody AK117 in NKTCL cells. Similar to CD47 knockdown, the novel anti-CD47 antibody AK117 increased M2 macrophages phagocytosis in vitro. Therefore, targeting CD47 should be a promising and fruitful therapeutic approach for treating NKTCL. In addition to blocking the “don’t eat me” signal, other mechanisms also contribute to the antitumor effects of anti-CD47 antibody therapy. For example, previous studies have shown that CD47-blocking antibodies could directly induce the apoptosis of chronic lymphocytic leukemia (CLL) cells.³³ However, our results suggested that the antitumor effects of the novel anti-CD47 antibody AK117 were mediated by the interruption of the CD47-SIRPa pathway instead of promoting the apoptosis of NKTCL cells.

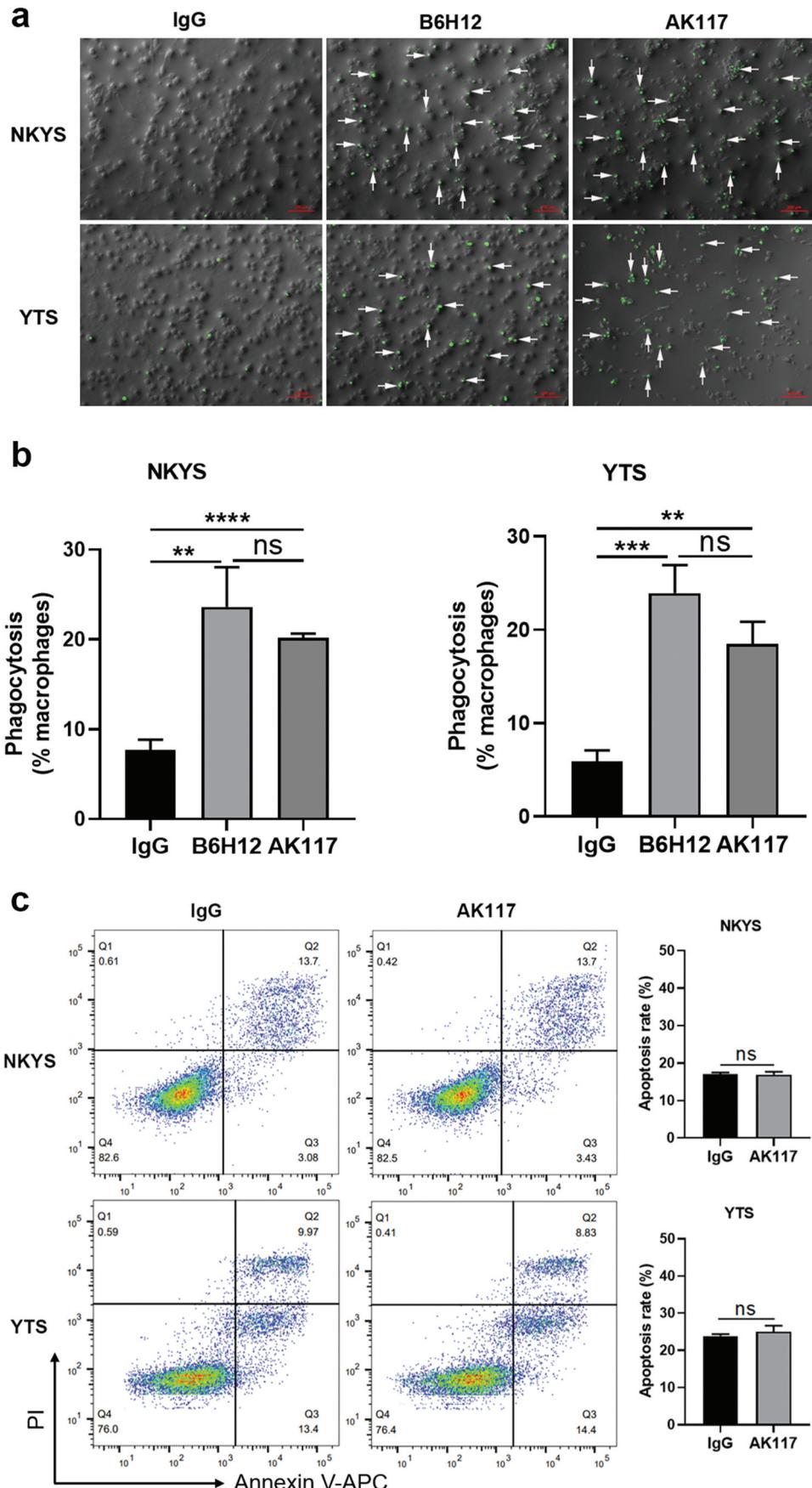


Figure 5. The novel anti-CD47 antibody AK117 enables phagocytosis of NK/TCL cells by M2 macrophages and has no direct effect on the apoptosis of NK/TCL cells. (a) CFSE-labeled NKYS or YTS cells were incubated with THP1-derived M2 macrophages in the presence of IgG1 isotype control or anti-CD47 antibodies (B6H12 and AK117). These cells were assessed by fluorescence microscopy for the presence of fluorescently labeled NKYS or YTS cells in M2 macrophages. Representative photomicrographs are shown with NKYS or YTS cells (green), and arrows indicate M2 macrophages containing phagocytosed NKYS or YTS cells. (b) Flow cytometry

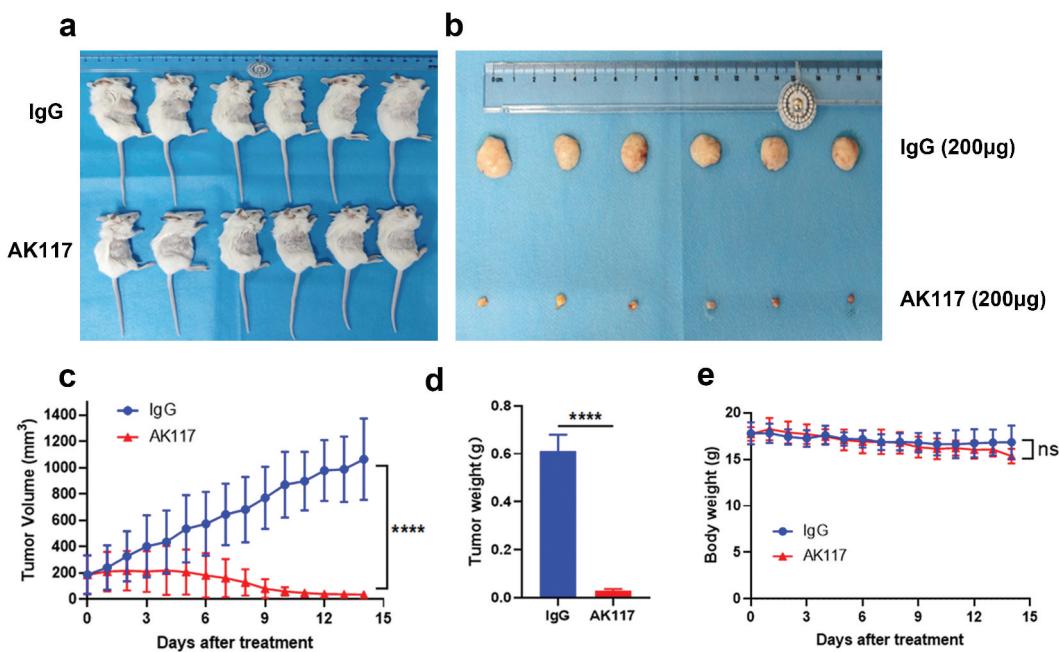


Figure 6. The novel anti-CD47 antibody AK117 inhibits the growth of NKTCL xenografts in mouse models *in vivo*. (a) Photograph of the YTS subcutaneous xenograft SCID mouse model treated with IgG and AK117. (b) Photograph of resected tumors derived from the YTS xenograft mouse model. (c) Tumor volume curves derived from the YTS xenograft mouse model. (d) The data represent the tumor weight for the control IgG-treated and AK117-treated groups. (e) Body weight curves derived from the YTS xenograft mouse model. Data are shown as the mean \pm SD (ns, not significant; **** p < .0001).

This study employed an immunodeficient SCID mouse model for xenograft experiments *in vivo*. The SCID mouse lacks B cells and T cells, but it has intact phagocytic functions in macrophages.³⁴ The scope of our study is focused on the antitumor function of macrophages. Therefore, we used SCID mice to evaluate the antitumor effect of the novel anti-CD47 antibody AK117 in NKTCL. The results suggested that AK117 could significantly inhibit the growth of NKTCL. Together, these results establish the rationale for the use of the novel anti-CD47 antibody AK117 as a therapy for human NKTCL.

In the tumor microenvironment, M2 macrophages inhibit adaptive immunity and promote tumor progression, whereas M1 macrophages can inhibit tumor growth. Previous research has demonstrated that most macrophages in the tumor microenvironment are M2 macrophages.¹² It has been reported that targeting CD47 may alter the behavior of resident M2 macrophages to inhibit tumor growth.³⁵ In this study, we assessed the efficacy of THP1-derived M2 macrophages on NKTCL. Our results have shown that knockdown of CD47 or anti-CD47 antibody treatment could increase the phagocytosis of NKTCL cells by M2 macrophages.

To our knowledge, CD47 is widely expressed in hematopoietic cells and other normal tissues, which could potentially lead to toxic effects from anti-CD47 antibody therapy. Recent studies employing CD47 monoclonal antibodies have shown that anemia and thrombocytopenia are the most

common adverse events (AEs). This effect was due to the high expression of CD47 on the surface of red blood cells (RBCs) and platelets. Blocking CD47 on RBCs with anti-CD47 antibodies can lead to phagocytosis by macrophages, eventually resulting in anemia and hemagglutination.³⁶ Hu5F9-G4 (5F9), also known as magrolimab, is the most commonly used anti-CD47 antibody and is currently undergoing clinical trials.³⁷ Although it showed good tolerance in clinical phase I experiments, a large proportion of patients still had anemia and hemagglutination.³⁷ Therefore, a novel anti-CD47 antibody with low affinity for red blood cells and fewer adverse effects needs to be developed. AK117, also known as ligufalimab, is a novel human IgG4 monoclonal antibody. Because of the unique conformation of the AK117/CD47 complex, AK117 has no hemagglutination effect. A phase I clinical study of AK117 showed that AK117 was well tolerated, and no hematologic treatment-related adverse events were observed in patients.³⁸ Several studies have shown that anti-CD47 antibodies might directly induce the apoptosis of tumor cells.³⁹⁻⁴¹ However, our results showed that AK117 had no effect on the apoptosis of NKTCL cells. In the present study, AK117 significantly inhibited the growth of the NKTCL xenograft model *in vivo*, and there was no significant difference in mouse body weight compared with that of the IgG control group. Therefore, targeting CD47 should be a promising and fruitful therapeutic approach for treating NKTCL.

results of phagocytosis assays in which NKYS or YTS cells were incubated with M2 macrophages. The phagocytic index was calculated as the percentage of CFSE⁺ CD206⁺ macrophages in total macrophages. The IgG1 isotype control, B6H12, and AK117 were incubated at a concentration of 10 µg/mL. (c) The effect of the novel anti-CD47 antibody AK117 on the apoptosis of NKYS and YTS cells was assessed by flow cytometry. Data are shown as the mean \pm SD (ns, not significant; ** p < .01; *** p < .001 and **** p < .0001).

Conclusions

In this study, we have shown that CD47 was highly expressed in NKTCL cells. Blocking CD47 signaling promoted the phagocytosis of tumor cells by M2 macrophages. The *in vivo* study found that treatment with the novel anti-CD47 antibody AK117 could significantly inhibit the growth of the NKTCL xenograft model. In conclusion, our results suggest that CD47 is a potential therapeutic target in NKTCL.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Hui Zhou conceived this project. Liping Qin and Yajun Li designed the experimental contents and plans. Liping Qin performed the experiments. Ruolan Zeng, Yizi He, Xiaoyan Chen and Ling Xiao analyzed the data. Liping Qin wrote the original manuscript, and subsequently meticulously scrutinized and revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Ethics statement

The animal experiments were approved by the Animal Ethics Committee of Hunan Cancer Hospital and in compliance with the Guide for the Care and Use of Laboratory Animals.

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