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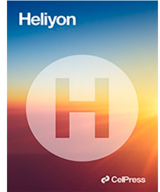

Synergistic efficacy of PI3Kδ inhibitor with anti-PD-1 mAbs in immune-humanized PDX model of endocrine resistance hormone receptor-positive advanced breast cancer

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ABSTRACT

Purpose: Endocrine resistance hormone receptor-positive (HR+) advanced breast cancer (ABC) is generally insensitive to immunecheckpoint inhibitors (ICIs). This study sought to determine whether PI3Kδ inhibitor could enhance the sensitivity of endocrine resistance HR + advanced BC to ICIs by reducing immune evasion.
Methods: Patient-derived HR + ABC xenografts were implanted into immune-humanized NSG mice and subsequently treated with YY20394 (PI3Kδ inhibitor) and camrelizumab. The mice were monitored for tumor progression, biochemical blood indicators, and peripheral blood T-cell subsets. The xenografted tumors were collected at the end of the treatment cycle and subjected to HE staining, immunohistochemistry and protein phosphorylation analysis. Besides, the xenografted tumors were also used to isolate primary breast cancer cells (BCCs) and regulatory T-cells (Tregs), which were subsequently used to evaluate drug sensitivity in vitro.
Results: The humanized PDX model showed a favorable initial treatment response to camrelizumab combined with YY20394 and manageable toxicity. YY20394 plus camrelizumab showed a strong inhibitory effect on HR + BC in vivo mediated by suppression of Treg activity and an increased proportion of $CD8^+$ T cells. Mice bearing tumors treated with YY20394 and camrelizumab had less invasion, mitotic figures, and ki67 expression, while having higher IL-12 expression compared with other groups. Mechanistically, YY20394 only effectively inhibited the PI3K pathway and proliferation activity in Tregs but not in BCCs.
Conclusion: Our study suggests PI3Kδ inhibitor could the enhance the efficacy of ICIs in HR + BC PDX models by combating immune suppression and provides a feasible approach that may overcome the resistance of ICIs in HR + BC patients.

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1. Introduction

Breast cancer is the leading cause of cancer-related deaths among females globally [1]. Hormone receptor-positive (HR+) breast cancer (BC) makes up 70% of breast cancer cases. Endocrine therapy is one of the main treatment options for HR + BC, but it often fails due to secondary or primary endocrine therapy resistance [2], while reversing resistance remains a challenge for clinicians. Accordingly, new therapeutic approaches are needed to improve the prognosis of patients with endocrine resistant advanced breast cancer.

The activation of intracellular signaling pathways is a major mechanism of endocrine therapy resistance and is consequently a major target of interventions aiming to reverse drug resistance [3–5]. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway plays essential roles in tumor initiation, cell survival, and angiogenesis [6]. Moreover, this pathway is also important for the "cross talk" involved in mediating resistance to antiestrogen therapy. More than half of HR + tumors harbor an alteration in the PI3K/Akt/mTOR pathway, leading to its dysregulated activation [7]. Class IA PI3Ks consist of a regulatory subunit and a catalytic subunit consisting of p110α, p110β, and p110δ (respectively encoded by the PIK3CA, PIK3CB, and PIK3CD genes). PI3Kα and PI3Kβ are widely distributed in tissues and play key roles in tumor growth, while p110δ is highly enriched in leukocyte subtypes and plays an important role in their recruitment and activation [8]. Approximately 40% of patients with HR + human epidermal growth factor receptor 2 (HER2)-negative advanced breast cancer harbor mutations in the PIK3CA gene. In general, PIK3CA mutations are often associated with treatment resistance and worse overall survival in advanced breast cancer [9,10]. Selective PI3K inhibitors targeting different PI3K isoforms have been extensively tested in clinical trials [8]. The efficacy of the PI3K inhibitor alpelisib, combined with fulvestrant, in PIK3CA-mutated advanced breast cancer has been recently confirmed in SOLAR-1 and X2101 trials [9]. PI3Kδ inhibitors are currently mainly used in the clinical treatment of hematological malignancies [8,11], but there is increasing evidence for the efficacy of PI3Kδ inhibitors in solid tumor immunotherapy [12,13]. However, the toxicity of PI3K inhibitors limits the applicable dose, leading to poor efficacy. As a consequence, PI3K inhibitors are currently not the main choice for the treatment of HR + breast cancer that progressed on previous endocrine therapy, except for clinical trials. The development of more effective combination therapies is currently needed to bring PI3K inhibitors back to the forefront of clinical treatment.

The immune system plays an essential role in tumor control. As an effective modality with fewer side effects, immune checkpoint blockade therapies have led to a dramatic shift in cancer treatment over the past decade [14]. Immunotherapy shows weak results in triple-negative breast cancer [15,16]. Nevertheless, HR + BC tumors are often considered "immunologically cold", harboring lower numbers of stromal tumor-infiltrating lymphocytes (TILs) compared to TNBC and HER2-overexpressing tumors, which makes them less sensitive to PD-1/PD-L1 axis blockade [17–19]. Anti-PD-1 mAbs monotherapy induced no significant change in patients with HR+/HER-2-negative Advanced breast cancer with PD-L1−positive tumors in a clinical trial [20]. Thus, it is still unclear how to extend this promising drug family to HR + breast cancer therapy. Recently, the combination of drugs targeting immune and signaling pathways has shown promising clinical results in a variety of solid tumors [21], but there is still no combination therapy for HR + BC. A greater appreciation of the complex roles of PI3K/mTOR signaling in the immune system has inspired unexpected ways to modulate the tumor microenvironment for therapeutic benefit. Insufficient lymphocyte infiltration is the main reason for the ineffectiveness of PD-1 in HR + BC [17]. PI3Kδ drives T cell differentiation and senescence, and supports immune homeostasis by affecting Treg subsets [22]. Notably, genetic or chemical inhibition of PI3Kδ induced antitumor immunity in several preclinical mouse models, including non-hematological solid tumors [13,23]. A phase II clinical study of head and neck cancer found that a PI3Kδ inhibitor (AMG319) activated intratumoral $CD4^+$ and $CD8^+$ T cells by reducing Treg subsets, and altered the cell distribution in the tumor microenvironment (TME) [12]. Another study found that inhibition of PI3Kδ can delay the terminal differentiation and exhaustion of $CD8^+$ T cells to prolong the antitumor response [24]. There is ample evidence that P110δ can restore immune surveillance, and many clinical trials targeting PI3Kδ combined with immunotherapy are also underway.

Due to the positive effect of PI3Kδ inhibitors on immune stimulation and reported primary immunotherapy resistance of HR + breast cancer, we evaluated the effect and therapeutic efficacy of PI3Kδ inhibitors combined with an anti-PD-1 antibody in a hu-PDX model of endocrine resistant HR + BC, and assessed the impact of combined treatment on the antitumor immune response in vivo. We also evaluated the effect of PI3Kδ inhibitor treatment on Tregs and primary BCCs in vitro. The results demonstrated that PI3Kδ inhibitor treatment enhanced the sensitivity to immune checkpoint inhibitor treatment in the HR + BC PDX model mainly by activating antitumor immunity.

2. Materials and methods

2.1. Tumor tissues

Eligible patients were women age≥18 years who had histologically confirmed advanced breast cancer that was estrogen and/or progesterone receptor positive and had progressed on at least one prior line of endocrine therapy. Unlimited prior endocrine therapies were allowed, the median patient age was 56 (range 39–65 years). All patients had secondary endocrine resistance defined by the ESO-ESMO guidelines as recurrence on but after the first 2 years of adjuvant endocrine therapy, or recurrence within 12 months of completing adjuvant endocrine therapy, or metastatic disease progression occurring on but ≥6 months after initiating first-line endocrine therapy. Prior endocrine treatments detailed in Table 1. This study was performed after approval by the Medical Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. All patients provided written informed consent.

3. Patient-derived human tumor xenograft models of breast cancer

Tumor tissues (2 cm *away from the tumor edge*) *were obtained at the time of chest* well biopsy *from 4 patients with breast cancer. After collection, the tissues were soaked in calcium-magnesium-free Dulbecco's phosphate-buffered saline* (*dPBS,* pH = 7.2) *for 5 min and then* were cut into 2 × 2 × 2-mm pieces. Before subsequent procedures, we kept the tissues pieces in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) supplemented with 1% penicillin and streptomycin (Invitrogen). For the mice models, the Patient-derived human tumor xenograft (PDX) tumors were generated and grown in female NSG mice (*6 weeks-old mice*) (*Shanghai Model Organisms Center*, Inc. China) prior to implantation in Hu-HSC Reconstruction mice. In a sterile operation, we buried the tissues pieces of *4 patients with breast cancer* in backs of *4* NSG *mice* subcutaneously while simultaneously supplementing with penicillin and streptomycin. When each xenografted tumor tissues grew to about 5 × 5 × 5-mm, we followed the aforementioned protocols to harvest the tissues from 4 mice and transplanted them into 4 weeks-old Hu-HSC Reconstruction mice (human CD34+haematopoietic stem cell-engrafted NSG mice) (*Shanghai Model Organisms Center*, Inc. China), which included multi-lineage human immune cells, one tissue was inoculated into 6 mice. After about two weeks, 4 *successful tumor-bearing mice were selected* to each patient were divided into four groups, and each treatment group had 4 mice (n = 4): model group (were given injection of equal volume of PBS), Camrelizumab (202011004F, Suzhou Shengdiya Biomedical Co., Ltd) group (were given tail vein injection of Camrelizumab, 100 mg/kg, on days 1 each week), Linperlisib (YY20394, sigma, USA) group (were given gavage injection of YY20394, 50 mg/kg, on day 3 and day 5 each week) and Camrelizumab combined YY20394 group. The administration lasted for 4 weeks, during which tumor size was measured once weekly with microcalliper and tumor volume was calculated as 0.5 × length × width × width, At the same time, the tumor inhibition rate was calculated. (Tumor inhibition rate (%) = [1 − (tumor volume of experimental group/average tumor volume of blank control group)] × 100%). At the end of the study, mouse peripheral blood was collected before incubation 0.2 mg/ml DNase (Thermo Scientific) in Hank's Balanced Salt Solution (Thermo Scientific) at 37 °C for 40 min. The samples were homogenised using repeated pipetting and were filtered through a 70-mm *cell strainer* (BD Biosciences) in supplemented dPBS (pH = 7.2, Thermo Scientific) to generate single-cell suspensions. After red blood cell lysis was accomplished using Pharm Lyse Buffer (BD Biosciences), all samples were washed and re-suspended in 200 μL dPBS. The sample was preprocessed by using a kit EasySep Human T Cell Isolation Kit/EasySep (novobiotec, Beijing, China) to sort T cells from peripheral blood. The flow cytometry was used for analysis of Tregs (CD4+Foxp3+) and $CD3^{+}CD8^{+}$ cytotoxic T cells on an FACSCalibur (BD Biosciences). The bearing tumors were stripped, and a portion of each tissue was quick-frozen in liquid nitrogen for proteins expression analyses and fixed using 4% paraformaldehyde for immunohistochemistry and hematoxylin-eosin staining.

3.1. Histological analysis and immunohistochemical staining

The tumor tissues were previously fixed in 4% formalin. After being paraffin-embedded, 4-μm paraffin sections were cut by microtome (Leica) and then stained with hematoxylin and eosin, respectively. For immunohistochemical staining, after deparaffinization and rehydration, the slices were immersed into 0.01 M sodium citrate buffer at 125 °C for 30 s, followed by 10 s at 90 °C for antigen recovery. Then, slices were covered with 3% hydrogen peroxide for 5 min to inactivate endogenous peroxidase. After the nonspecific binding sites were blocked by 10% goat serum in PBS, the slices were incubated with different primary antibody respectively at 4 °C for 24 h. Then, the slices were incubated with secondary HRP-conjugated antibody at 37 °C for 1 h. Sections were developed using freshly prepared DAB substrate, then counterstained with hematoxylin, dehydrated, and mounted. The primary antibodies included anti-Ki67 antibody and IL-12 antibody (Abcam, Cambridge, UK).

3.2. Western blotting

Protein phosphorylation was detected by western blotting. Briefly, total protein was extracted from cells and tissues using the M-PER and T-PER tissue protein extraction reagent (Pierce, IL, USA). Total equal proteins (20 μg) were boiled in sample buffer (Thermo Scientific) containing β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and subjected to SDS-PAGE (10–12% gel) and transferred onto nitrocellulose membranes. The blots were incubated with primary antibodies against human PI3K (1:400), P-PI3K (1:200), AKT1 (1:600), P-AKT1P53 (1:800), mTOR (1:800) and P-mTOR (1:1200) (Abcam), following incubated with the corresponding secondary HRP-conjugated anti-rabbit/mouse antibody (Abcam). After washing the membranes, the bands were detected by chemiluminescence and imaged with X-ray films. The relative phosphorylation of protein is expressed as the ratio of phosphorylated protein to total (Phosphorized + unphosphorylated).

Table 1
Detailed characteristics of xenografted HR-positive BC patients.

Patient	Age	Sex	cTNM	PIK3CA	ER (%)	PR (%)	Her-2	Ki67 (%)	Prior treatment
P1	54	F	T2N2bM1 IV	Mutation	90	30	1+	15	Letrozole
P2	39	F	T2N1bM1 IV	Mutation	90	70	2+ FISH (−)	40	Tamoxifen
P3	57	F	T2N3aM1 IV	wt	80	60	–	20	Fulvestrant
P4	65	F	T4aN1bM1 IV	wt	90	50	1+	30	Letrozole

3.3. Cell culture and treatments

Breast tumors from mice were respectively collected selectively. Then the tissues were cut and washed three times with dPBS (1% Penicillin-Streptomycin). About 100 mg of tissues were washed and cut into pieces using a sterile surgical scissor and tweezer and transferred to 50 ml sterile centrifuge tubes containing 10 ml 0.2% collagenase IV (sigma) for digestion at 37 °C for 3 h with shaking at 100 rpm. Single cells were obtained via a 100mesh cell strainer and centrifuged at 1000×*g* for 5 min. Primary culture breast tumor cells were then resuspended in DMEM *with* 10%FBS and subsequently plated in culture dishes and incubated at 37 °C in a 5% CO_2 atmosphere. When the adherent density reached 70% confluence, the primary breast cancer cells were passaged by trypsin digestion method. Isolation of $CD4^+CD25^+$ FoxP3+ Regulatory T Cells for mouse peripheral blood according to the previously published methods [25,26]. The Regulatory T cells were identified with FACS analysis using the anti-CD4 and anti-CD25 and anti-FoxP3 antibodies (Abcam). The *cells* were then resuspended in *RPMI 1640 medium* (*Invitrogen*) *with* 10% FBS and subsequently plated in culture dishes and incubated at 37 °C in a 5% CO_2 atmosphere.

Two kinds of cells were inoculated into 6-well culture plate (1×10^5 cells/well) respectively. After overnight under normal conditions, they were divided into four groups: control group, solvent group (adding the same volume of DMSO as YY20394), low-dose YY20394 treatment group (adding the final concentration of 1 nM YY20394), and high-dose YY20394 treatment group (adding the final concentration of 5 nM YY20394). After 48 h-treatment, some of the cells were collected for total protein extraction which was used to detect protein phosphorylation of PI3K and mTOR via western blotting. The remaining cells was re-inoculated to 96-well culture plate (3×10^4 cells/well) and continuously cultured at 37 °C under 5% CO2 for 72 h, the cells viability was examined at 24, 48, and 72-h using CCK-8 assay.

3.4. Statistical analysis

Statistical parameters (average values, SD, significant differences between groups) were calculated using Microsoft Excel and GraphPad Prism. Statistical significance between groups was determined by paired *t*-test or one-way ANOVA with post hoc Tukey's multiple comparison test ($p < 0.05$ was considered statistically significant).

4. Results

4.1. Synergistic inhibition of tumor growth by PI3Kδi combined with anti-PD-1 in a hu-PDX model of HR + BC

PI3Kδ inhibitors are generally used for treating B cell malignancies and they also show specific antitumor activity in the treatment of solid tumors [27]. YY20394 is a novel PI3Kδ inhibitor (linperlisib, Shanghai Yingli Pharmaceutical). It is characterized by a long half-life and is currently being investigated in several clinical trials for the therapy of solid tumors. Because the p110δ signal plays a

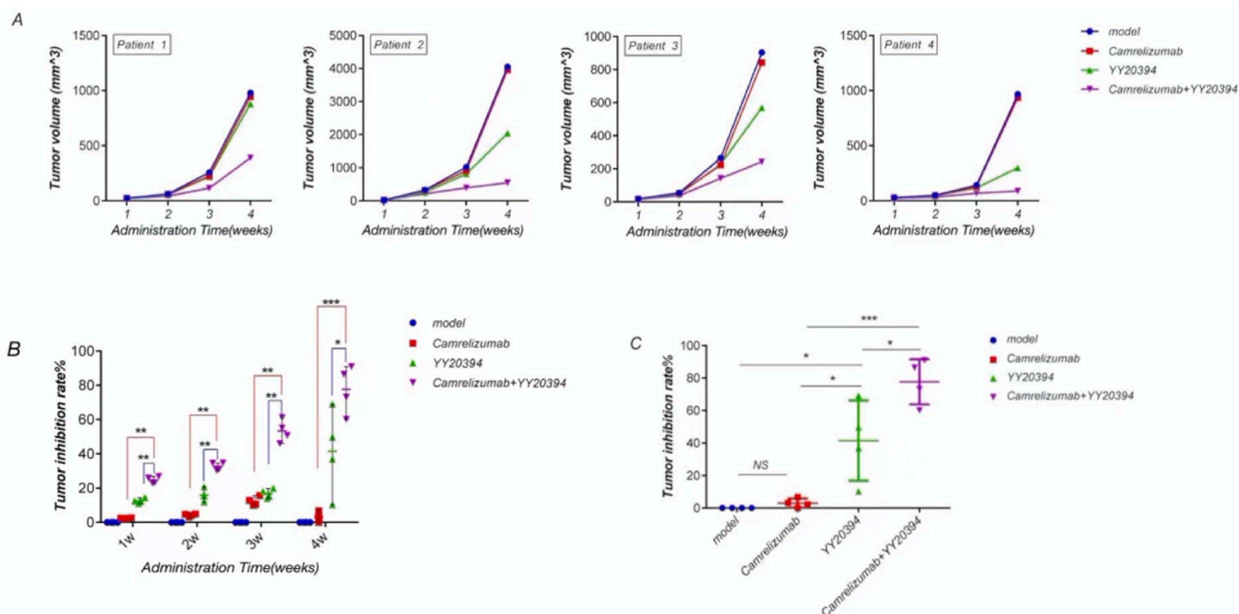

Fig. 1. The effects of YY20394, camrelizumab, camrelizumab + YY20394 on tumor growth. Xenografted tumor bearing mice were treated with YY20394, camrelizumab, camrelizumab + YY20394 for 4 week after palpable tumors were detected (each group, n = 4). Tumor growth was monitored. A: tumor volume of subcutaneous implants in mice over time. B: mean inhibition rate% at each monitoring time point. C: mean tumor inhibition rate% at the 4th week.

vital role in T cell differentiation and function [22], we investigated whether a PI3Kδ inhibitor can act as an immunomodulator to potentiate the antitumor effect of PD-1 blockade when combined in an immune-humanized PDX model of endocrine therapy-resistant advanced breast cancer. The Immune-humanized PDX model not only recapitulates patient-derived tumors on genomic, molecular, and cellular levels, but also offers an animal model that most closely resembles the human immune system, which makes it a powerful tool for analyzing tumor-immune system interactions and evaluating the efficacy of immunotherapy. Tumor samples from HR + BC patient (clinical evaluation confirming secondary endocrine therapy resistance, Table 1) were implanted into $CD34^{+}$hu-NGS mice. After the indicated incubation period, mice with palpable tumors were treated either with the vehicle, YY20394 (50 mg/kg twice weekly), camrelizumab (100 mg/kg on day 1 each week) or anti-PD-1 combined with YY20394. The treatment was continued for 4 weeks, after which the animals were euthanized. Although the PDX characteristics resulted in uneven growth rates for each model, our data indicate that YY20394 alone significantly inhibited tumor growth. However, combined treatment with YY20394 and anti–PD-1 had a more profound effect on the inhibition of tumor growth than YY20394 alone (Fig. 1A–C and Fig. 2B). By contrast, there was not significant inhibition of tumor growth in mice treated with anti-PD-1 alone compared with vehicle. Differences in the average tumor inhibition rates between anti-PD-1 plus YY20394 and anti-PD-1 alone were statistically significant at the 4th week, while differences between anti-PD-1 and YY20394, or YY20394 alone and YY20394 plus anti-PD-1 were also statistically significant. The ALT, AST, TBIL and LDH values of the mice were monitored regularly and were unremarkable (Fig. 3C). No significant weight loss was observed in any of the treatment groups.

At the end of treatment, tumor tissues were collected, and H&E staining showed that YY20394 plus anti–PD-1 induced widespread tumor cell damage and fragmentation (Fig. 2B). Immunohistochemistry (IHC) showed that Ki67 positivity, a marker of cell proliferation, significantly decreased in tumor tissues from the combined treatment group compared to the other groups (Fig. 2D). In addition, the results of IHC indicated significantly increased expression of IL-12in the tumor tissues from the combined treatment group compared with the single-drug group (Fig. 2C). IL-12 plays a critical role in modulating the tumor immune environment, including the activation and expansion of TILs. These data were consistent with the inhibition of tumor growth. Taken together, the results demonstrated that the efficacy of the combination therapy was superior to that of YY20394 alone, and especially to anti-PD-1 alone. Thus, YY20394 synergized with anti-PD-1 to induce considerable antitumor activity in the hu-PDX model of endocrine therapy-resistant HR + breast cancer.

4.2. PI3Kδ inhibitor monotherapy and combination therapy significantly downregulated downstream effectors of PI3K

In PDX models with four different genetic backgrounds, YY20394 combined anti-PD-1 displayed synergistic antitumor activity. In this study, the hu-PDX model mice were treated with YY20394 alone, anti-PD-1 alone, or YY20394 in combination with anti-PD-1, after which tumor tissues were collected for Western blot analysis. As shown in Fig. 3A and B, YY20394 alone and YY20394 plus anti-PD-1 significantly abrogated PI3K, Akt and mTOR phosphorylation, which was unrelated to the effect of anti-PD-1. Importantly,

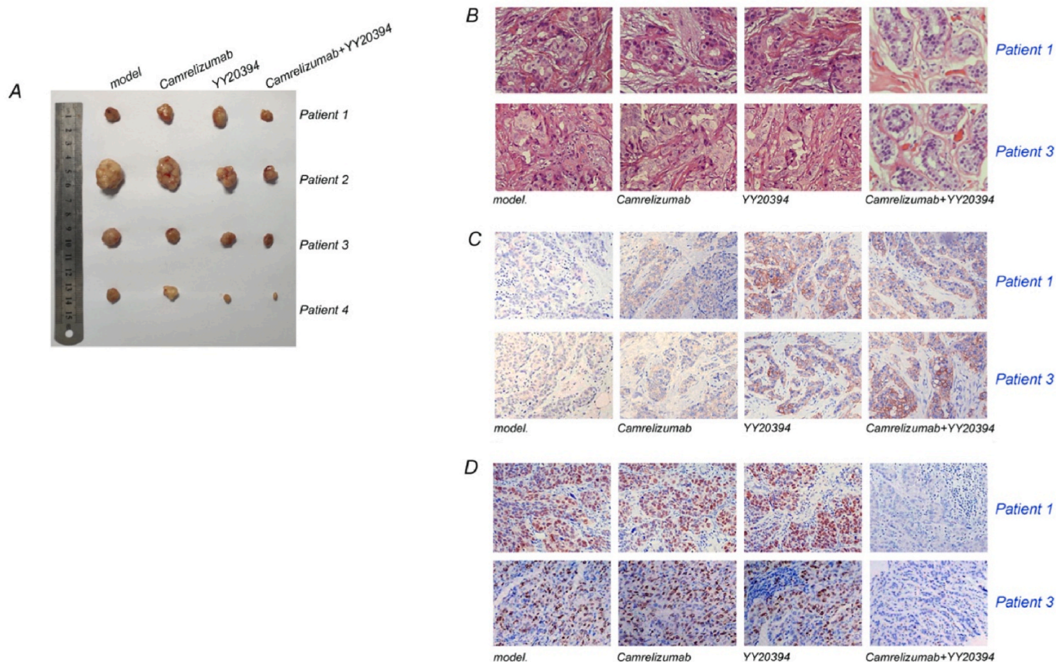

Fig. 2. A: Tumor photographs; camrelizumab + YY20394 significantly reduced the volume of PDX tumors compared with other groups. B: Tumor tissue were harvested and stained with hematoxylin and eosin (H&E). C: immunohistochemical staining showed expression level of IL12. D: Immunohistochemical staining showed expression level of Ki-67.

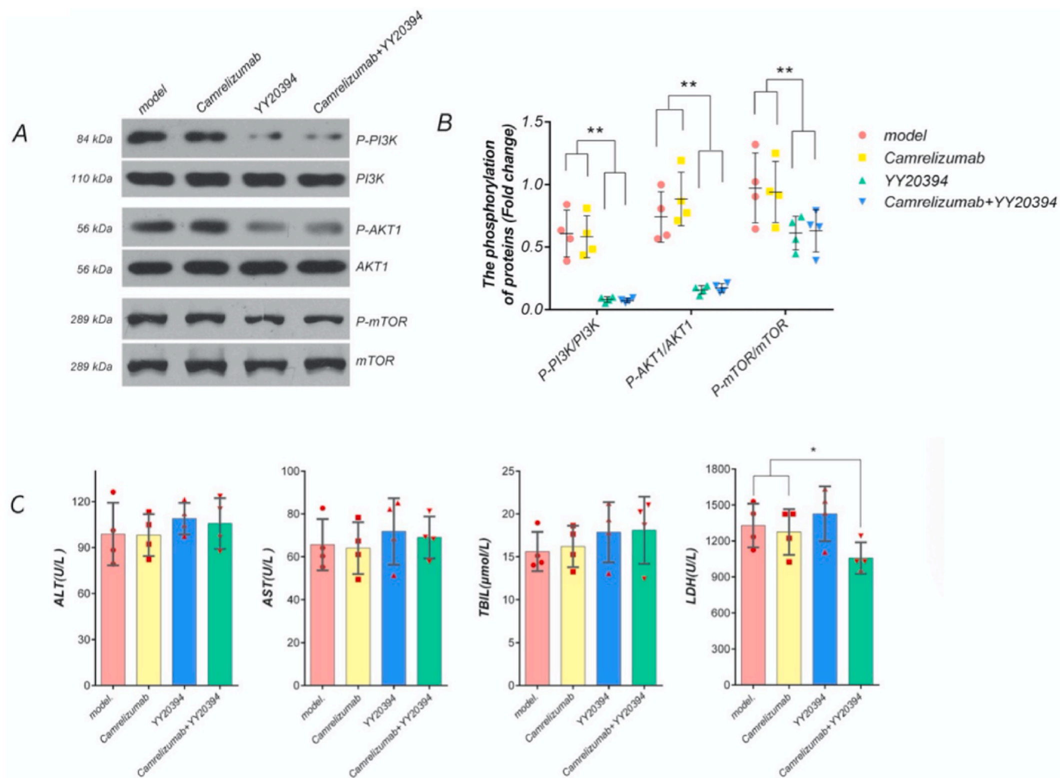

Fig. 3. A: PI3K pathways-related proteins in tumor tissue was determined by Western blot. B: To compare the phosphorylation levels of PI3K, AKT, and mTOR after treatment with YY20394, camrelizumab, camrelizumab + YY20394. C: Routine toxicity index monitoring of PDX mice in experimental endpoint.

the combined therapy group and YY20394 alone group showed the lowest mean levels of PI3K, Akt and mTOR expression. These results further confirmed that the synergistic effect of YY20394 combined with anti-PD-1 was due to the inhibition of PI3K pathway activation.

4.3. The PI3Kδ inhibitor promoted the effect of anti-PD-1 in combination therapy of HR + BC by immunostimulation

Our data clearly showed an enhanced antitumor effect of anti-PD-1 when combined with a PI3Kδ inhibitor. Notably, the combination therapy group exhibited increased IL-12 expression, which suggests that this drug schedule resulted in effective immune stimulation. To further evaluate the underlying mechanism, we analyzed the impact of YY20394 plus anti-PD-1mAb treatment on antitumor immunity. FACS analysis of peripheral blood was used to determine the proportions of $CD45^{+}$ immune cells, $CD8^{+}$ T cells, and Tregs in the humanized PDX mice. Consistent with the results of tumor growth inhibition, the proportion of $CD45^{+}$ immune cells and $CD8^{+}$ T cells in the peripheral blood was significantly higher in the groups that received the PI3Kδ inhibitor treatment (PI3Kδ inhibitor alone or in combination) compared with anti-PD-1 alone and the untreated model group (Fig. 4C and D). In particular, the proportion of $CD8^{+}$ T cells was highest in the combined treatment group, indicating that tumor killing mediated by $CD8^{+}$ T cells was activated. The group treated with YY20394 also showed a significant proportion of Tregs in comparison with the anti-PD-1 group and model group (Fig. 4A and B). These results indicate that the immunomodulatory effect of combination therapy resulted in a stronger antitumor milieu in the hu-PDX model of HR + breast cancer and demonstrated an augmentation of the antitumor immune response when the anti-PD-1 mAb was combined with a PI3Kδ inhibitor.

4.4. YY20394 leads to tumor growth inhibition mainly via an immunomodulatory effect

The above results clearly showed the effect of combining the anti-PD-1 mAb with YY20394 on tumor growth and immune-cell subsets in the hu-PDX model. To further evaluate whether the synergistic effect of YY 23094 combined with anti-PD-1 treatment is mediated mainly by affecting immunity, we isolated and cultured Tregs as well as breast cancer cells (BCCs) to test their sensitivity to treatment with YY20394 at different concentrations in vitro. The effects of the PI3Kδ inhibitor YY20394 on the growth and survival of Tregs and BCCs were assessed using the Cell Counting Kit-8 assay, as shown in Fig. 5B. YY20394 preferentially inhibits the proliferation of Tregs in a dose-dependent manner, but has no significant effect on the proliferation of BCCs·YY20394 significantly inhibited Treg proliferation at 5 nM, while the BCCs were less responsive to YY20394 at the same concentration. To investigate the mechanism

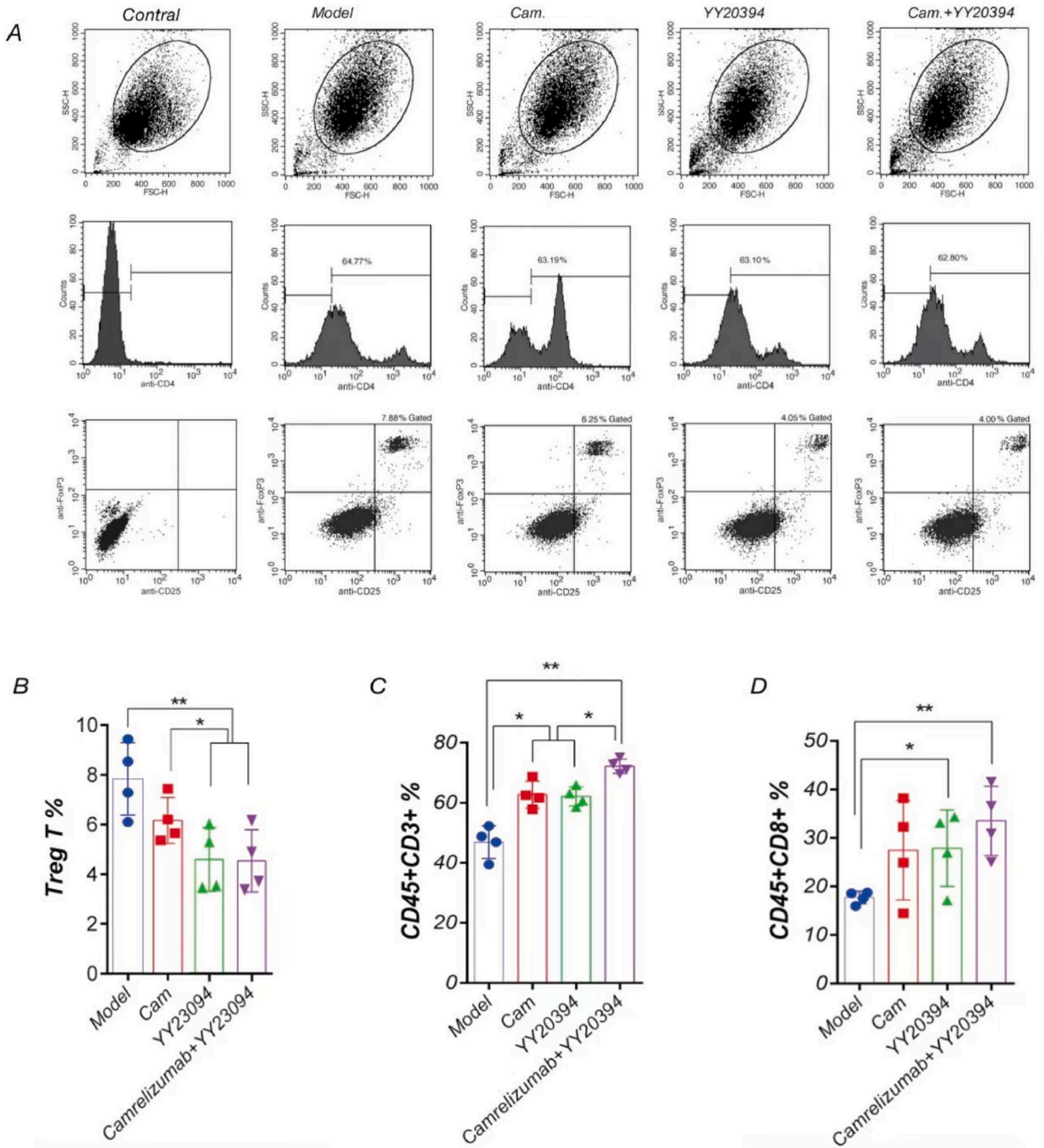

Fig. 4. A: Flow cytometric analysis results for Tregs ($CD4^+$CD25+Foxp3+). B: Results of quantification using flow cytometry of the proportion of Treg cells in CD4+T cells. C: proportion of $CD45^+$CD3+T cells. D: proportion of $CD45^+CD8^+$ T cells in CD3+T cells. Treg, regulatory; Cam, camrelizumab.

underlying the differential sensitivity to YY20394 in Tregs and BCCs, we next determined the levels of phosphorylated PI3K and AKT as indicators of PI3K pathway activity. As shown in Fig. 5C, YY20394 decreased the phosphorylation of both PI3K and AKT in a dose-dependent manner. YY20394 reduced p-PI3K and *p*-mTOR levels in Tregs at a concentration of 1 nM, while 5 nM YY20394 was required to achieve similar inhibition in BCCs. Thus, the phosphorylation of PI3K as well as downstream effectors AKT and mTOR in BCCs was blocked by 5 nM YY20394 in vitro. However, the proliferation of BCCs was not significantly inhibited at this concentration, indicating that PI3Kδ had a minimal direct effect on BCCs. It also suggests that low-dose PI3Kδ inhibitors may exert antitumor effects

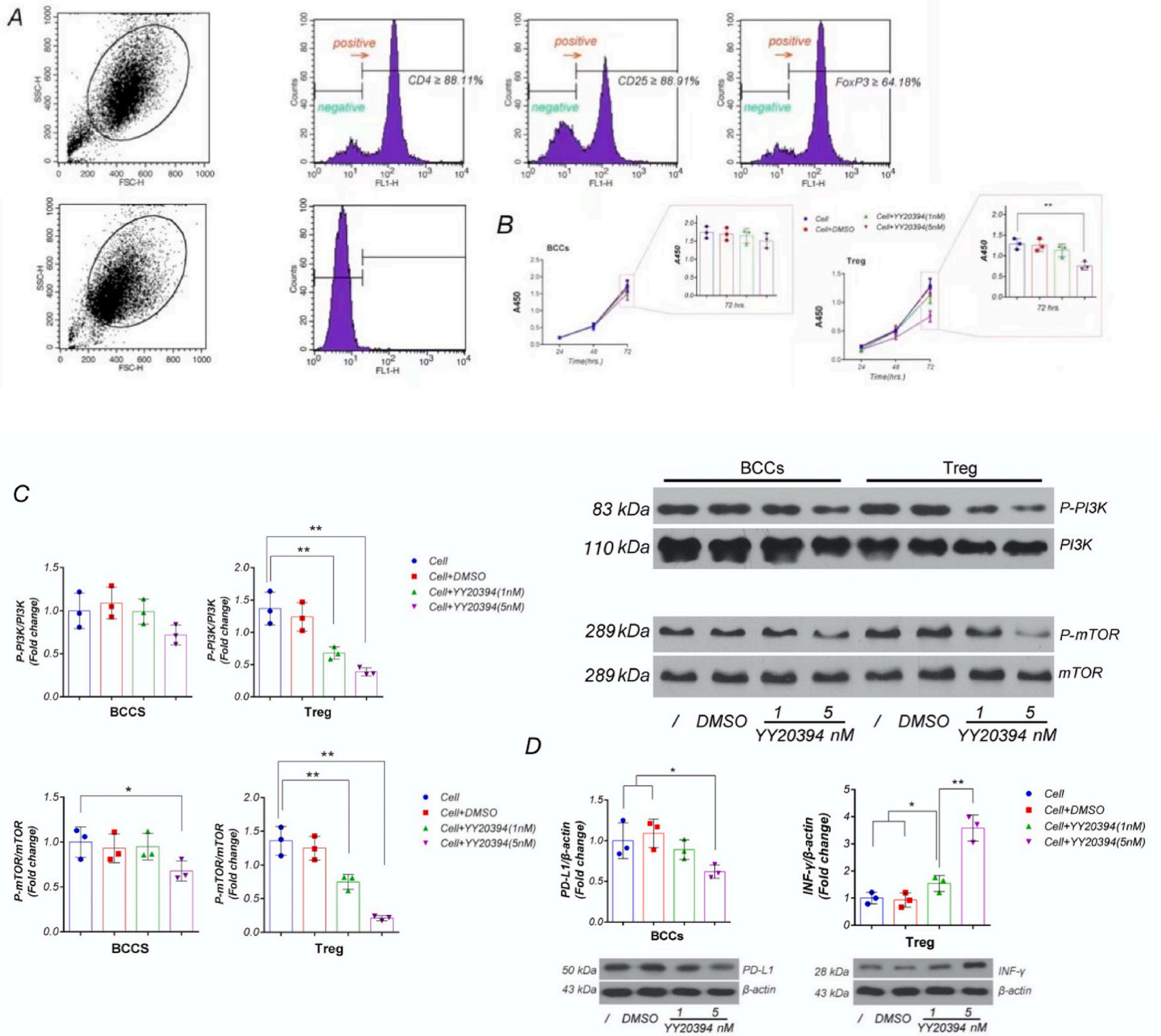

Fig. 5. Primary BC cells obtained from tumor tissue of PDX mice and Treg cells obtained from mouse peripheral blood were stimulated with YY20394 (1 nm and 5 nm) and DMSO for 48 h. A: Treg were identified with Face analysis. B: After 48 h-treatment, the cells viability was examined at 24, 48, and 72-h using CCK-8 assay. C: YY20394 affect expression levels of p-PI3K and *p*-mTOR in two kinds of cells. D: YY20394 affect expression levels of PD-L1 in four group of BCCs and YY20394 affect expression levels of IFN-r in four group of Tregs. DMSO, Dimethyl Sulfoxide.

by affecting immune cells, further reducing the toxicity and side effects of PI3Kδ inhibitors. Next, we determined PD-L1 expression in the YY20394-treated BCCs. PD-L1 can be expressed by some breast cancer cells and surrounding immune cells to mediate the inhibition of TILs. The PI3Kδ inhibitor downregulated PD-L1 expression, and the high-dose (5 nm) treatment group had the lowest mean level of PD-L1 expression (Fig. 5D). The expression level of IFN-γ in Tregs was examined by immunohistochemistry, which correlated well with the functional dysregulation of Tregs. As shown in Fig. 5D, YY20394 upregulated IFN-γ production in a dose-dependent manner. This finding is consistent with the immunostimulatory effect of YY20394.

5. Discussion

The effect of PI3Kδ inhibition on antitumor immunity has been partly elucidated [3]. Considering the heterogeneity of breast cancer and the multiple roles of the immune system in tumor growth and response to immunotherapy [28], we used the $CD34^+$ humanized PDX model to test drug combinations, which not only preserves the patient's tumor characteristics and gene expression, but also approximates the response to the PI3Kδ inhibitor plus anti-PD-1 in the human immune system as closely as possible, which will facilitate a better understanding of the impact of PI3Kδ inhibition on anti-tumor immunity in HR + BC. The PI3Kδ inhibitor combined

with an anti-PD-1 mAb exerted a potent antitumor effect in the hu-PDX model of HR + BC, confirming that the PI3Kδ inhibitor restore immune checkpoint blockade sensitivity to activate antitumor immunity. Our data demonstrate that combination of an PI3Kδ inhibitor and anti-PD-1 mAb is a promising strategy to overcome drug resistance while showing manageable toxicity in a mouse model.

We further explored the mechanism of this effective combination therapy, our study demonstrates that the mechanism underlying the synergistic effect of the anti-PD-1 mAb and PI3Kδ is based on the reconstitution of immune surveillance in the HR + breast cancer PDX model. On the face of it. The significant antitumor effect of the combination therapy in the mouse model may be partly due to the inhibition of the PI3K/AKT/mTOR pathway. As shown in Fig. 1, YY20394 monotherapy also resulted in more substantial tumor growth inhibition than anti-PD-1 monotherapy. Although the PI3K pathway plays a complex and still somewhat unclear roles in the occurrence and development of breast cancer as well as the development of endocrine therapy resistance [2]. However, it should be noted that the combination therapy induced significant immune stimulation, which might be the basic mechanism of resistance reversal of immunotherapy in HR + BC.

Immune checkpoint inhibitors (ICIs) were demonstrated to induce an enduring clinical response in various solid tumors [29]. HR + BC is the most common subtype of malignant tumors of the breast [2]. However, it is a canonical "immunologically cold " tumor, and clinical trials of ICIs have not shown satisfactory results [30] [31]. Nevertheless, this "consensus" generalizes breast cancer heterogeneity, the patient disease status, and the complexity of the immune system. The effectiveness of immunotherapy depends on the accumulation and activity of immune effector cells. Previous research consistent with our data showed that PI3Kδ inhibition can promote antitumor immunity by affecting T cell subsets [12,13,23,24], which provides a basis for the addition of immunotherapy. PI3Kδ inhibition is the optimal combinatorial strategy to enhance the efficacy of anti–PD-1/PD-L1–based immunotherapy. This combination was able to reconstitute immune surveillance in the HR + BC mouse model by modulating the proliferation and function of immune cells. Our study found that YY20394 exerts its antitumor effect through an immunological mechanism related to a decreased proportion of Tregs and increased proportion of antitumor lymphocytes. There was a remarkably increased the abundance of $CD8^+$ T cells in the PDX group treated with YY20394 plus anti-PD-1, resulting in superior tumor regression compared with monotherapy. This was consistent with previous clinical studies that found a higher proportion of $CD8^+$ T cells in tumor tissues to be associated with a better prognosis and better response to immunotherapy [32].

To confirm whether the in vivo antitumor effect of the PI3Kδ inhibitor is independent of direct effects on tumor cells, we isolated and cultured four groups of primary BCCs and Tregs. Our data demonstrate the preferential impact of YY20394 on Tregs compared with primary BCCs, whereby YY20394 abrogated downstream biological effects on Treg proliferation and function. In Tregs, YY20394 upregulated IFN-γ production in a dose-dependent manner, which was correlated with functional destabilization of Tregs. In addition, the Tregs were significantly more sensitive to the antiproliferative effect of YY20394 than primary BCCs in vitro. These results proved that the antitumor activity of YY20394 is not mainly based on a direct effect on tumor cells, but that it indirectly enhances antitumor immunity by affecting Tregs. This again confirms our hypothesis that although the capacity of the PI3Kδ inhibitor to induce cell death in primary BCCs is limited, it may act as an immune modulator, converting the intrinsically immunosuppressive microenvironment of HR + BC to an immunosupportive one. As 40% of PIK3CA mutations are expressed in luminal breast cancer [33], PI3Kα inhibitors have been more extensively evaluated in breast cancer-related clinical trials than other isoform-selective PI3K inhibitors [34,35]. The PI3Kα inhibitor alpelisib was the first PI3K inhibitor approved in combination with endocrine therapy for the treatment of HR + HER-2--negative advanced breast cancer [33]. However, it only had a curative effect in patients with PIK3CA mutations in various clinical trials [33]. Importantly, our data indicate that both PIK3CA-mutated and PIK3CA wild-type PDX models respond to treatment with the combination of YY20394 with anti-PD1 or YY20394 alone, demonstrating that the efficacy of PI3Kδ inhibition may be not dependent on the PIK3CA mutation status of cancer cells, but is rather explained by the fact that the PI3Kδ inhibitor reestablishes immune surveillance by inhibiting the proliferation of Tregs. Based on our in vivo and in vitro observations, we hypothesize that the efficacy of PI3Kδ inhibitors as immunomodulators is different from that of PI3Kα inhibitors. Thus, PI3Kδ inhibitors combined with anti-PD-1 therapy can exert an active anti-tumor effect by regulating the immune system, independent of PIK3CA mutation status. Our findings undoubtedly offer hope for increasing the patient population where combination therapy can be applied, and more patients may benefit from it. Nevertheless, additional studies with larger sample sizes are needed for further verification.

Genetic or chemical inhibition of the PI3Kδ isoform in animal models results in reduced Treg numbers and delayed tumor growth through an immune-mediated mechanism [23,36]. Consistent with these earlier reports, we found that the PI3Kδ inhibitor can act as an immunomodulator to alter lymphocyte subsets in the immune microenvironment of HR + BC. However, Lim et al. found that PI3Kδ inactivation results in impaired $CD8^+$ T cell-mediated antitumor effects that partially offset the impact of Treg dysfunction, highlighting the antagonistic relationship between PI3Kδ inhibition and immune checkpoint blockade [37]. The proliferation and survival of Tregs depend on PI3Kδ signaling, but another study revealed that in conventional T cells, PI3Kα and PI3Kβ have functional redundancy with PI3Kδ [13]. Because the functional dichotomy in class IA PI3K isoform, YY20394 can specifically target Tregs in vivo without inhibiting the function of cytotoxic T-lymphocytes (CTLs), providing an opportunity to regulate the tumor immune microenvironment through the PI3K signal difference between Tregs and CTLs. Moreover, we found that combination therapy elicited a durable response in mice treated for four weeks with an intermittent dose schedule of YY20394. This data is reminiscent of previous studies supporting the idea that rather than simply suppressing Tregs, PI3Kδ inhibitors induce long-term immune memory by delaying the terminal differentiation of $CD8^+$ T cells and maintaining the memory phenotype [24]. This offers novel insights into how PI3Kδ inhibitors exert their activity by reprogramming/modulating the TME and provides a rationale for concurrent targeting of PI3Kδ and immune checkpoints in HR + BC. Preclinical studies have shown that PI3Kδ knockdown does partially impair $CD8^+$ T cell function, but it still results in a net antitumor effect. The degree of PI3K signal output determines the balance of antitumor versus immunosuppressive lymphocyte subsets, which largely determines tumor progression and the efficacy of immunotherapies [3]. Differences of dose and schedule can significantly impact the efficacy of targeted antitumor therapy. The data indicate that our intermittent dosing

strategy is successful, demonstrating that considerable antitumor effects can be achieved without needing continuous inhibition of PI3Kδ when combined with PD-1. Our in vitro experiment also verified this point. Although low-dose PI3Kδ inhibitors cannot significantly inhibit tumor cell proliferation, they can substantially affect Treg at the same dose, which may make up for the shortcoming of excessive toxicity and many side effects of PI3Kδ in clinical application. However, whether our dosing sequence and regimen can maximize antitumor immunity while minimizing toxicity in the clinical setting remains unknown.

6. Conclusions

The PI3Kδ inhibitor YY20394 combined with an anti-PD-1 mAb exhibited a synergistic tumor-suppressive effect against HR + BC PDX model by dysregulating Tregs to restore sensitivity to immune blockade therapy. Furthermore, our data provide new evidence that PI3Kδ inhibition may induces an antitumor effect mainly by regulating and reconstituting the immune surveillance of HR + BC, rather than via a direct effect on tumor cells. Overall, this study may provide a theoretical basis for the combined application of PD-1 and PI3Kδ inhibitors in immunologically ‘cold’ tumor like HR + BC, although further confirmation required a larger sample size.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Medical Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (No. SYDW2022-109).

Author contribution statement

Yingjue Li: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shuhui Li: Performed the experiments.

Dan Lu: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Yiwen Li: Conceived and designed the experiments.

Yu Yang; Yuwei Deng: Conceived and designed the experiments; Analyzed and interpreted the data.

Zhaoqi Yan; Bochen Zhao: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Yixin Li; Linbo Liu: Analyzed and interpreted the data.

Xiangdong Ni; Wen He: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18498.

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