Anti-CD20 and -PD-1 antibody effects on proliferation, apoptosis and cell cycle of endometrial cancer cells

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Received January 21, 2026; Accepted My 26, 2026

DOI: 10.3892/ol.2026.15712

Abstract. The present study aimed to systematically evaluate the functional effects of the anti-CD20 antibody Rituximab and the programmed cell death 1 (PD-1/PDCD1) antibody Pembrolizumab on endometrial cancer (EC) cells. The expression levels of membrane spanning 4-domains A1 (MS4A1) and PDCD1 in EC tissues and cells were analyzed using the The Cancer Genome Atlas database with R programming language. The impacts of Rituximab and Pembrolizumab treatment on proliferation, apoptosis and cell cycle progression were assessed in Ishikawa and HEC-1A EC cell lines by means of CCK-8 assays and flow cytometry. Both MS4A1 and PDCD1 were significantly upregulated in EC tissues and cells compared with normal controls. Rituximab and Pembrolizumab demonstrated concentration-dependent inhibitory effects on the proliferation of EC cells, induced cell apoptosis and arrested cell cycle progression. Furthermore, the combination of Rituximab and Pembrolizumab resulted in enhanced and more potent suppression of cancer cell growth than either monotherapy alone. In conclusion, the combination of anti-CD20 antibody and PD-1 antibody exerts synergistic antiproliferative effects on EC cells *in vitro*.

Introduction

Endometrial cancer (EC) is one of the most prevalent malignant tumors of the female reproductive system. Each year, EC develops in ~142,000 women worldwide, and an estimated 42,000 women die from this cancer. In recent years, its incidence has been on the rise globally, particularly in developed countries, and it is strongly associated with obesity, metabolic syndrome and elevated estrogen exposure (1). While patients with early-stage EC can achieve favorable prognoses through surgical intervention combined with adjuvant radiotherapy and chemotherapy, the management of advanced, recurrent and metastatic EC remains a significant challenge. Traditional therapies are prone to drug resistance and induce considerable toxicities and side effects, urgently calling for improvement in patient survival rates (2). Consequently, the development of novel targeted therapies and immunotherapeutic strategies has emerged as a critical focus of contemporary research.

Programmed cell death 1 (PD-1/PDCD1) signaling, as a fundamental immune checkpoint mechanism, downregulates inflammatory responses and maintains immune homeostasis (3). The PD-1/programmed cell death ligand 1 (PD-L1) signaling pathway not only serves as an important route for preventing autoimmune diseases but also exerts a significant impact on the delicate balance between tumor immune surveillance and immune tolerance (4). PD-1/PD-L1 inhibitors have emerged as a groundbreaking therapeutic approach, effectively reversing T-cell exhaustion by blocking negative regulatory signals (5). The U.S. Food and Drug Administration has approved anti-PD-1 antibodies as a second-line therapy for non-microsatellite instability-high and deficient mismatch repair advanced EC with a PD-L1 Combined Positive Score ≥1 (6). However, the response rates of other molecular subtypes to immune checkpoint inhibitors, particularly p53-mutated EC or special types of endometrioid carcinoma (e.g., serous papillary carcinoma, clear cell carcinoma, undifferentiated carcinoma, small cell carcinoma or mixed cell carcinoma), remain low. Consequently, exploring combination drug strategies to enhance therapeutic efficacy has become a key research focus.

Meanwhile, monoclonal antibodies targeting the CD20 antigen on the surface of B cells, (e.g., Rituximab) have been widely used in the treatment of B-cell lymphomas. Recent studies have demonstrated that CD20 is abnormally expressed in certain solid tumors, including breast cancer and ovarian cancer. High CD20 expression has also been detected in EC tissues, and anti-CD20 antibodies exert anti-tumor effects through multiple mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and the induction of tumor cell apoptosis (7,8). A previous Mendelian randomization analysis by our group revealed a significant association between CD20 and EC, suggesting that anti-CD20 antibodies may exert protective

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Key words: anti-CD20 antibody, PD-1 antibody, endometrial cancer, proliferation, apoptosis, cell cycle

effects against EC and reduce its risk (9). Furthermore, B-cell infiltration and abnormal activation are observed in the EC microenvironment, indicating that CD20 may play a role in regulating tumor progression. However, its expression patterns and biological functions in EC remain elusive, and its therapeutic potential has yet to be fully explored.

The occurrence and progression of EC are intricately associated with uncontrolled cell proliferation, impaired apoptosis and disrupted cell cycle regulation. Targeting these biological processes represents a central strategy in anti-tumor therapy (10). However, the synergistic mechanisms underlying the combination of anti-CD20 antibodies and PD-1 antibodies in EC have not been comprehensively investigated. Specifically, the effects of these agents on tumor cell proliferation, apoptosis and cell cycle regulation remain to be fully clarified. In light of this, the present study aims to investigate the biological impacts of anti-CD20 and PD-1 antibodies on EC cells. Through *in vitro* experiments, their effects on cell proliferation, apoptosis and cell cycle distribution were explored with the goal of providing a novel theoretical basis for combined immunotherapy in EC and promoting its translational research for clinical application.

Materials and methods

Bioinformatics. All raw and processed transcriptomic, genomic and clinical data for the Uterine Corpus Endometrial Carcinoma (UCEC) cohort were obtained from The Cancer Genome Atlas (TCGA) 1.0 release. The TCGA overall study accession number in the database of Genotypes and Phenotypes (https://www.ncbi.nlm.nih.gov/gap) is PHS000178, TCGA-UCEC dataset official identifier: TCGA-UCEC. Transcriptome data for patients with EC, initially including 537 tumor samples and 35 normal endometrial samples, were downloaded from the TCGA-UCEC database (https://portal.gdc.cancer.gov/projects/TCGA-UCEC). After exclusion of samples with zero expression values, the final analytic dataset comprised of 472 tumor samples and 35 normal endometrial samples.

Cell lines and culture conditions. EC cell lines [Ishikawa (99040201) and HEC-1A (HTB-112)] were obtained from the EC Cell Bank of the Chinese Academy of Sciences. Normal endometrial epithelial cells (hEEC) were purchased from Procell Life Science & Technology Co., Ltd. Ishikawa cells are estrogen receptor-positive, while HEC-1A cells show low expression of the estrogen receptor. Cells were cultured in corresponding media (McCoy's 5A for HEC-1A; Dulbecco's modified Eagle's medium for Ishikawa; both Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and incubated in a humidified incubator at 37˚C with 5% CO_2. Cells in the logarithmic growth phase were selected for use in subsequent experiments.

Reverse-transcription quantitative (RT-q)PCR analysis. Total RNA was isolated from tissue samples, cultured cells (hEEC, Ishikawa and HEC-1A cells) and exosomes using TRIzol reagent (Thermo Fisher Scientific, Inc.). The RNA was subsequently reverse-transcribed into complementary DNA (cDNA) using a reverse transcription kit (cat. no. RR036A; Takara Bio, Inc.) according to the manufacturer's instructions. Real-time quantitative PCR was carried out using SYBR Premix ExTaq™ II (cat. no. RR820A; Takara Bio, Inc.). According to the manufacturer's instructions, a 20-μl PCR mixture was used in a Q5 PCR instrument (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were applied: Pre-denaturation at 95˚C for 30 sec for 40 cycles; followed by 95˚C for 5 sec and 60˚C for 34 sec. The gene expression levels relative to β-actin were determined using the $2^{-\Delta\Delta Cq}$ method. All the steps were carried out in accordance with the manufacturer's instructions. The primer sequences used in the analysis are provided in Table I.

Antibody sources. Anti-CD20 antibodies (e.g., Rituximab; cat. no. HY-P9913) and immune checkpoint inhibitors (e.g., Pembrolizuma; cat. no. HY-P9902), with purity exceeding 95%, were procured from MedChemExpress.

Selection of drug working concentration and cell proliferation experiments. Ishikawa and HEC-1A cells in the logarithmic growth phase were seeded into 96-well plates at a density of $5x10^3$ cells per well. After 24 h of cultivation, various drug concentrations were subsequently added (Rituximab: 0,10, 20, 30, 40, 50 μg/ml; Pembrolizumab: 0,0.001, 0.01, 0.1, 1, 10 nM). At different time-points post-treatment (24, 48 and 72 h), CCK-8 reagent (Tongren Institute of Chemistry) was added to each well, followed by a 2-h incubation period. The absorbance values were measured as the optical density (OD) at a wavelength of 490 nm using a microplate reader to assess the inhibitory effects of Rituximab and Pembrolizumab. Based on these results, the optimal inhibitory concentration was determined. Subsequently, the optimal inhibitory concentration [half-maximal inhibitory concentration (IC_{50})] for the combination treatment group was screened. The cell proliferation inhibition rate was calculated using the following formula: Inhibition Rate (%)=(1-OD value of experimental group/OD value of control group) x100%. A drug concentration of 0 represents the negative control group.

Detection of apoptosis by phycoerythrin/7-amino-actinomycin D (PE/7-AAD) double staining. Ishikawa and HEC-1A cells in the logarithmic growth phase were seeded into 6-well plates. When the cells reached ~50% confluence, they were treated with drugs and divided into the following groups: Rituximab (10, 20, 30 μg/ml); Pembrolizumab (0.01, 0.1, 1 nM); Combination Groups (0.001, 0.01, 0.1 nM). After 48 h of incubation, the cells were harvested, digested with trypsin (Gibco; Thermo Fisher Scientific, Inc.), washed with PBS and resuspended in Binding Buffer. They were then stained with 5 μl PE and 10 μl 7-AAD (United Division) at 4˚C and incubated in the dark for 15 min. Apoptosis was analyzed using a flow cytometer.

Cell cycle analysis [propidium (PI) staining]. Cells treated for 48 h were harvested, washed with prechilled PBS three times and fixed in 70% ethanol at 4˚C overnight. Centrifugation was performed at 300 x g for 5 min at 4˚C to remove the ethanol. The cells were resuspended in a staining solution containing 50 μg/ml PI and 100 μg/ml RNase A (Thermo Fisher

Table I. Primer sequences (5'-3').

Gene name	Forward	Reverse
MS4A1	TGATGCTGATCTTTGCCTTCTTCC	TCGTCTCTGTTTCTTCTTCTTCCTC
PDCD1	GCTGCACTAATTGTCTATTGGG	CACAGTAATTCGCTTGTAGTCG
β-actin	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT

PDCD1, programmed cell death 1; MS4A1, membrane spanning 4-domains A1.

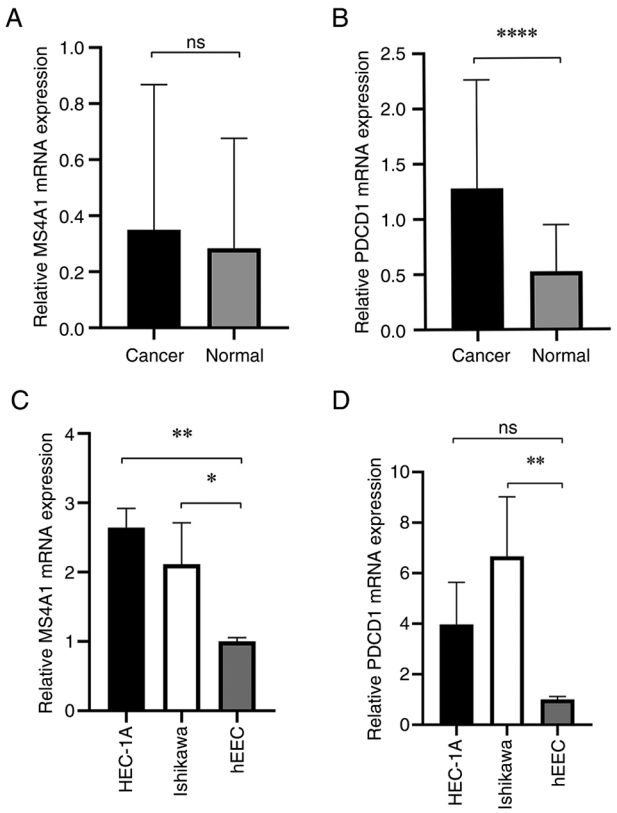

Figure 1. Differential expression of MS4A1 and PDCD1. (A) Statistical graph of MS4A1 mRNA expression in TCGA data, including 472 tumor samples and 35 normal endometrial samples. (B) Statistical graph of PDCD1 mRNA expression in TCGA data. (C) Statistical graph of MS4A1 mRNA expression in EC cells and normal cells. (D) Statistical graph of PDCD1 mRNA expression in EC cells. *P<0.05; **P<0.01; ****P<0.0001; ns, no significance. TCGA, The Cancer Genome Atlas; EC, endometrial cancer; PDCD1, programmed cell death 1; MS4A1, membrane spanning 4-domains A1.

Scientific, Inc.), and incubated at 37˚C in the dark for 30 min. The cell cycle distribution (G0/G1 phase, S phase, G2/M phase) was analyzed by flow cytometry and the proportions of each phase were quantified using FlowJo software (v10.10.1; BD Biosciences).

Statistical analysis. The experimental data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 8.0 software (Dotmatics). Intergroup differences were assessed using one-way ANOVA (Tamhane's T2 test) or unpaired t-tests, and P<0.05 was considered to indicate statistical significance. All experiments were independently repeated three times. In the CCK-8 assay, for the same conditions, 5 parallel wells were used. After eliminating the two wells with the greatest difference, a total of 3 parallel wells remained.

Results

Differential expression of MS4A1 and PDCD1. The differential expression analysis of MS4A1 and PDCD1 was conducted using the EC dataset from the TCGA database. MS4A1 encodes the CD20 protein, while PDCD1 encodes PD-1. R programming language was utilized to evaluate the expression levels of these two genes in EC tissues compared to normal endometrial tissues. The results showed that MS4A1 exhibited higher expression in EC tissues; however, this difference was not statistically significant (Fig. 1A). By contrast, PDCD1 demonstrated significantly higher expression in EC tissues compared to normal endometrial tissues (Fig. 1B). Meanwhile, RT-qPCR was used to assess the differential expression of MS4A1 and PDCD1 in EC cells. Compared with normal endometrial cells, the expression of MS4A1 and PDCD1 in EC cells was markedly upregulated (Fig. 1C and D).

Effects of different concentrations of Rituximab and Pembrolizumab on the proliferation of Ishikawa and HEC-1A cells. Cell proliferation was assessed via the CCK-8 assay. The results showed that various concentrations of Rituximab and Pembrolizumab markedly inhibited the viability of Ishikawa and HEC-1A cells, with the inhibitory effect increasing as the drug concentration increased. Notably, Rituximab at a concentration of 30 μg/ml and Pembrolizumab at a concentration of 1 nM exhibited the most pronounced inhibitory effects after 48 h (P<0.05). Thus, 48 h was selected for subsequent experiments (Fig. 2 and Table II). The relatively weaker inhibitory effect of rituximab on HEC-1A cells at 48 h may be due to a plateau effect or mild compensatory cell proliferation after prolonged treatment. Additionally, the optimal concentration of Rituximab (30 μg/ml) and the concentration gradient of Pembrolizumab in the combination group were investigated (Table III). The concentration of Rituximab (30 μg/ml) was determined based on preliminary dose-response experiments in EC cells. This concentration produced a moderate but significant inhibitory effect on cell proliferation without excessive cytotoxicity, which was suitable for evaluating the synergistic effect in combination with pembrolizumab. The findings revealed that the optimal concentration of Pembrolizumab in the combination group was 0.1 nM, which was subsequently utilized for further experiments (Table IV).

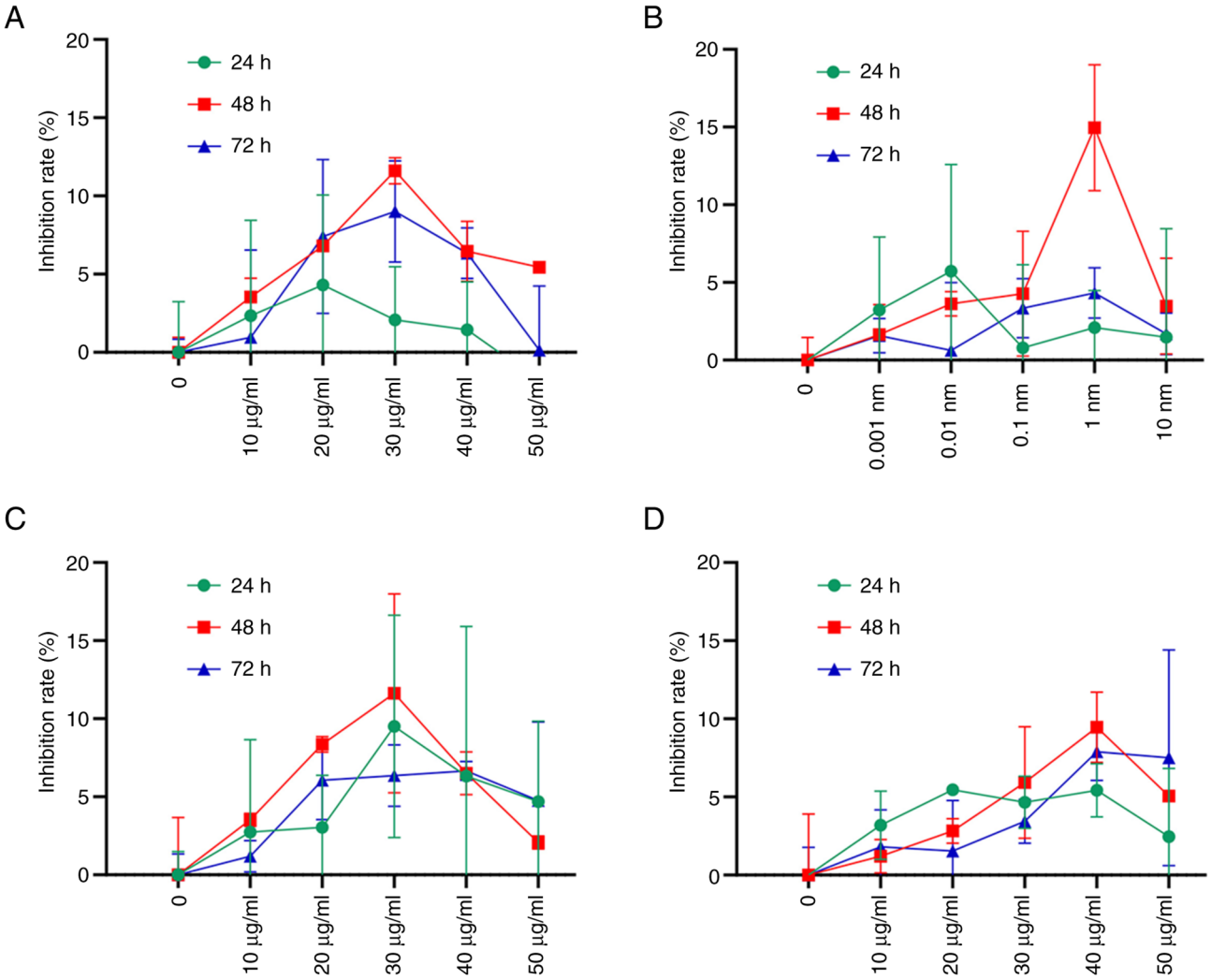

Figure 2. Effects of Rituximab and Pembrolizumab on EC-cell proliferation. (A) Inhibitory effect of Rituximab on Ishikawa cell proliferation; (B) inhibitory effect of Pembrolizumab on Ishikawa cell proliferation; (C) inhibitory effect of Rituximab on HEC-1A cell proliferation; (D) inhibitory effect of Pembrolizumab on HEC-1A cell proliferation. Data are presented as the mean ± standard deviation.

Effects of Rituximab and Pembrolizumab on EC cell apoptosis. To explore the dose-dependent effect of the drugs and revalidate their optimal concentrations, apoptosis was assessed using PE/7-AAD double staining. The results demonstrated that varying concentrations of Rituximab and Pembrolizumab significantly suppressed the viability of Ishikawa and HEC-1A cells, with the inhibitory effect increasing as the drug concentration rose. Further analysis revealed that the proportion of apoptotic cells in the combination treatment group was significantly higher than in the single-drug groups, suggesting a synergistic effect of the two antibodies in inducing apoptosis (Fig. 3).

Effects of Rituximab and Pembrolizumab on EC cell cycle. The cell cycle distribution was analyzed using PI staining. The results demonstrated that gradients of Rituximab at different concentrations elevated the proportion of Ishikawa and HEC-1A cells in the G0/G1 phase, indicating that Rituximab inhibits cell proliferation by inducing G0/G1 phase arrest. Conversely, different concentrations of Pembrolizumab raised the proportion of Ishikawa and HEC-1A cells in the S phase, suggesting that Pembrolizumab suppresses cell proliferation by inducing S-phase arrest. The combination of the two antibodies further elevated the cell ratio in the S and G2/M phases, indicating that the two antibodies inhibit cell proliferation by inducing S- and G2/M phase arrest, with Pembrolizumab playing a predominant role (Fig. 4).

Discussion

In recent years, immunotherapy has achieved remarkable breakthroughs in the treatment of malignant tumors, particularly with the widespread clinical application of immune checkpoint inhibitors and monoclonal antibodies. To date, the roles of anti-CD20 antibodies and immune checkpoint inhibitors (e.g., PD-1 antibodies) in EC have been confirmed. However, systematic explorations of the synergistic effects of these two therapeutic modalities remain relatively scarce. This study was the first, to the best of our knowledge, to comprehensively evaluate the individual and combined effects of the anti-CD20 antibody Rituximab and the PD-1 antibody Pembrolizumab on EC cells, thus offering novel insights into the clinical management of this prevalent gynecological malignancy.

Table II. Effects of Rituximab on endometrial cancer cell proliferation.

	Ishikawa		HEC-1A	
Concentration, μg/ml	Mean±SD	P-value	Mean±SD	P-value
0	0±1.58		0±0.56	
10	2.44±0.79	0.14	1.93±1.98	0.37
20	2.93±0.15	0.08	4.88±3.91	0.04
30	9.20±1.67	<0.001	9.09±1.90	<0.001
40	5.99±1.51	0.00	4.88±0.32	0.04
50	3.82±2.47	0.03	4.35±1.55	0.06

Table III. Effects of Pembrolizumab on endometrial cancer cell proliferation.

	Ishikawa		HEC-1A	
Concentration, nm	Mean±SD	P-value	Mean±SD	P-value
0	0±3.47		0±0.75	
0.001	4.15±0.68	0.18	2.35±1.30	0.05
0.01	6.20±0.40	0.09	5.17±0.83	<0.001
0.1	6.50±0.71	0.06	7.05±1.26	<0.001
1	13.75±1.09	<0.001	9.72±1.50	<0.001
10	7.98±0.29	0.01	4.63±0.21	<0.001

Table IV. Effects of combination treatment on endometrial cancer cell proliferation.

	Ishikawa		HEC-1A	
Pembrolizumab concentration, nm	Mean±SD	P-value	Mean±SD	P-value
0	0±3.76		0±2.46	
0.001	7.01±3.03	0.01	7.61±4.50	0.01
0.01	9.89±1.37	<0.001	6.31±1.77	0.04
0.1	15.45±1.04	<0.001	14.60±1.38	<0.001
1	11.44±1.81	<0.001	3.70±2.50	0.19
10	7.61±0.71	0.01	5.02±2.31	0.09

Anti-CD20 antibodies can effectively inhibit tumor progression through diverse antitumor mechanisms. CD20 is a transmembrane cellular protein that has been validated as a therapeutic target for B-cell malignancies (11). Rituximab, the first CD20 monoclonal antibody approved for use in cancer patients, is a human/mouse chimeric anti-CD20 monoclonal antibody. Additionally, it exhibits an excellent safety profile in patients with various CD20+ lymphoid malignancies (12). Studies have shown that anti-CD20 antibodies exhibit effective therapeutic outcomes in patients with multiple sclerosis (13). Schlaak *et al* (14) demonstrated that although melanoma has low CD20 expression, anti-CD20 antibody treatment remains efficient. The anti-tumor mechanisms of anti-CD20 antibodies include: i) High-density CD20+ B cells may possess anti-tumor immunity potential; ii) certain tumor cell lines express CD20, and anti-CD20 antibodies can directly kill tumor cells through ADCC and CDC; iii) anti-CD20 antibodies may indirectly enhance T cell-mediated anti-tumor immune responses by depleting immunosuppressive B-cell subsets, such as regulatory B cells (15). Tertiary lymphoid structures (TLS) are ectopic lymphoid aggregates that occur in inflamed, infected or neoplastic tissues. These structures share features analogous to lymph node architecture, including the presence of large clusters of CD20-positive B lymphocytes, which are critical for mediating adaptive immune responses (16). Previous studies have demonstrated an association between TLS and favorable prognosis as well as enhanced immunotherapy responsiveness in patients with endometrial cancer (EC) (17,18). A high level of CD20+ B-cell infiltration has been observed in EC, with significant CD20 expression detected in EC tissues (19,20),

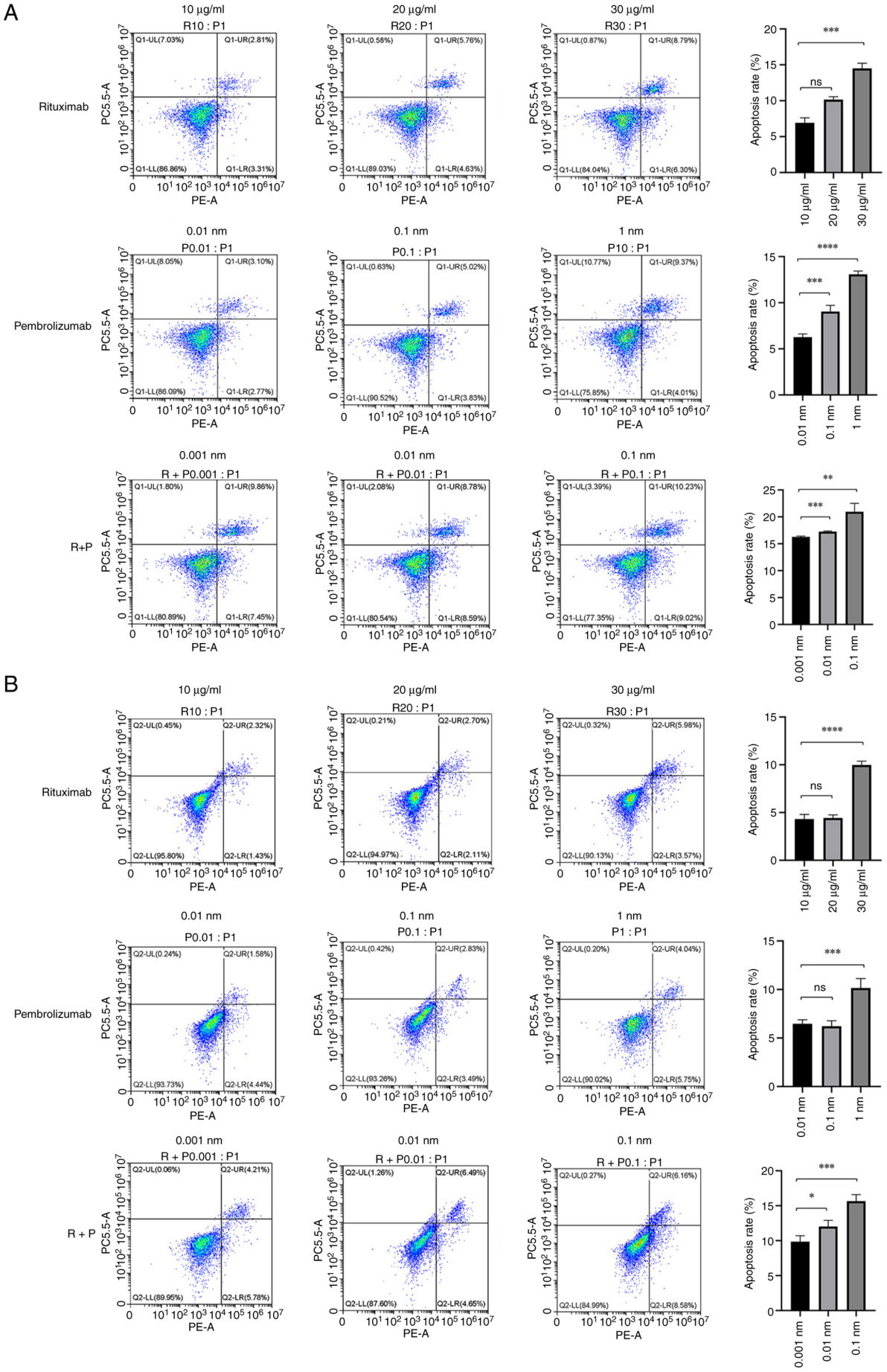

Figure 3. Effects of Rituximab and Pembrolizumab on endometrial cancer cell apoptosis. (A) Apoptosis rate of Ishikawa cells; (B) Apoptosis rate of HEC-1A cells. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001; ns, no significance. Q, quadrant; UL, upper left; LR, lower right; R+P, Rituximab + Pembrolizumab.

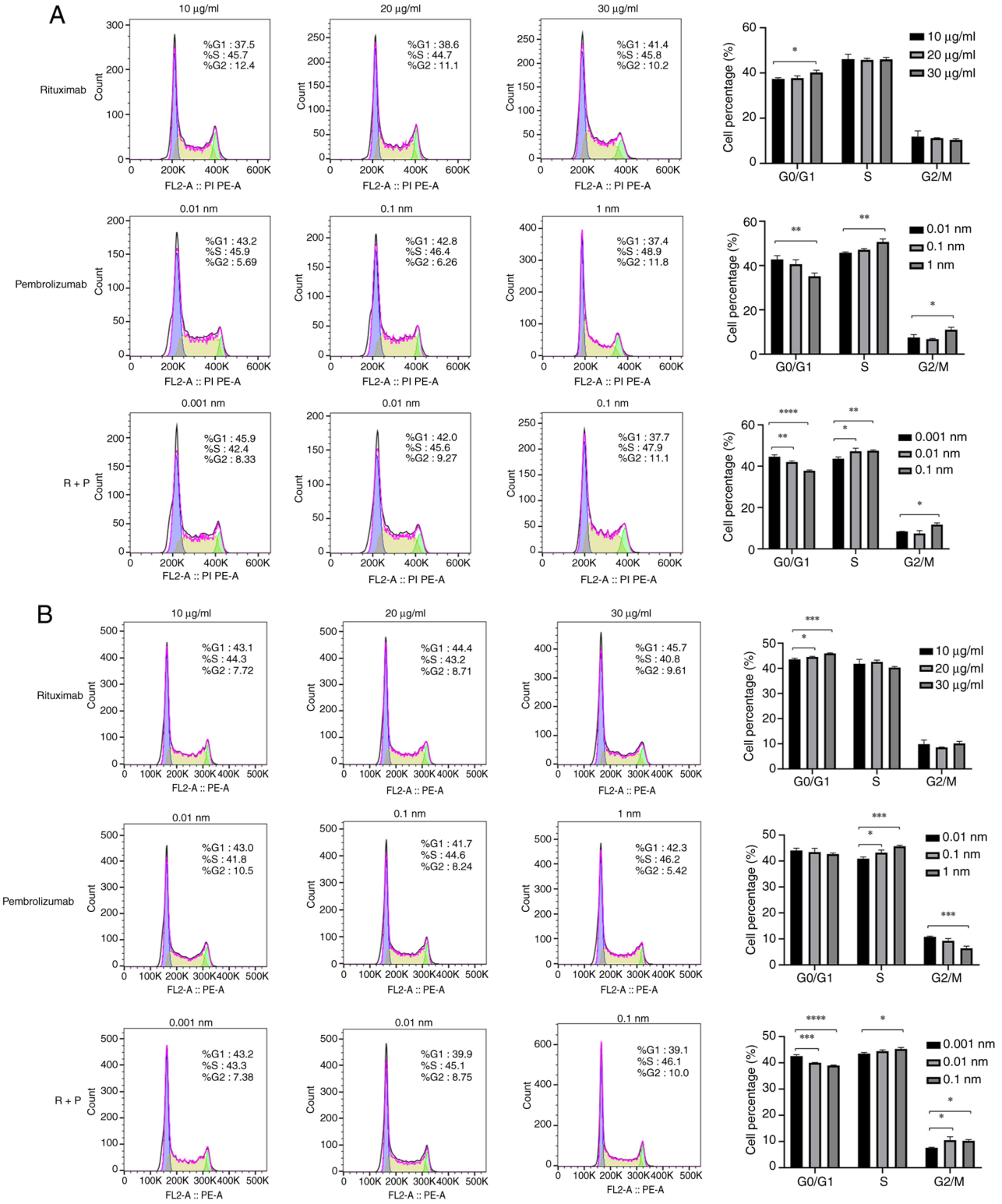

Figure 4. Effects of Rituximab and Pembrolizumab on endometrial cancer cell cycle. (A) Cell cycle distribution of Ishikawa cells; (B) cell cycle distribution of HEC-1A cells. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. R+P, Rituximab + Pembrolizumab.

corroborating the findings from a bioinformatics analysis performed as part of the present study regarding the elevated expression of MS4A1. However, the difference was not statistically significant, potentially attributable to the limited sample size. CD20 is highly expressed in EC tissues but it remains unknown whether CD20 is highly expressed on the tumor cells themselves or on the infiltrating B cells within the tumor microenvironment. Mendelian randomization analyses have revealed a strong correlation between CD20 and EC, suggesting that anti-CD20 antibodies may exert protective effects and reduce EC risk (9). Consistent with this hypothesis, the current study found that Rituximab inhibits EC-cell proliferation and promotes apoptosis by inducing G0/G1-phase arrest, with the inhibitory effect increasing in a concentration-dependent

manner, thereby validating the protective role of anti-CD20 antibodies in EC cells.

PD-1 antibodies can effectively inhibit tumor progression through diverse anti-tumor mechanisms.PD-1 receptor and its ligand, PD-L1, constitute a critical immune checkpoint pathway responsible for regulating T-cell activation (21). PD-1 is primarily expressed on T cells and PD-L1 on tumor, immune and stromal cells. PD-L1 is upregulated on various cancer cells, facilitating tumor immune escape. Over the past decade, therapeutic antibodies targeting the PD-1/PD-L1 axis have been developed to alleviate the immunosuppression caused by these two proteins (22,23). Despite extensive research, the prognostic significance of PD-1 remains controversial and its functional role within the tumor microenvironment awaits further elucidation (24). Regarding the anti-tumor mechanism, the PD-1 antibody specifically binds to PD-1 on the surface of tumor cells or immune cells, thereby blocking its interaction with PD-L1. This action releases T cells from their inhibitory state, restoring their cytotoxic capabilities against tumors. Upon blockade of the PD-L1/PD-1 signaling pathway, T cells regain their capacity for proliferation, cytokine secretion (e.g., IFN-γ, TNF-α) and direct tumor cell killing. Additionally, the PD-1 antibody may indirectly activate dendritic cells and natural killer cells, enhancing antigen presentation and the overall anti-tumor immune response (25). In gastric cancer treatment, the use of PD-1 antibodies effectively prolongs progression-free survival (PFS) and overall survival (OS), while minimizing adverse reactions and improving clinical outcomes (26). PD-1 antibody therapy, either as monotherapy or in combination with chemotherapy, has shown significant improvements in OS and PFS in patients with lung cancer and high PD-1 expression (27). As adjuvant therapy for high-risk stage III melanoma, PD-1 antibodies have significantly prolonged recurrence-free survival when compared to placebo (28). In advanced or recurrent EC, the addition of Pembrolizumab to standard chemotherapy resulted in significantly longer PFS compared to chemotherapy alone (29). The bioinformatics analysis of the present study revealed that PDCD1 expression was significantly upregulated in EC tissues relative to normal endometrial tissues. Furthermore, cellular experiments indicated that Pembrolizumab could inhibit EC-cell proliferation by inducing S-phase arrest and promoting apoptosis, thereby clarifying the underlying mechanism of PD-1 antibody action on EC cells.

Combined anti-CD20 and PD-1 antibodies can effectively inhibit tumor progression through diverse anti-tumor mechanisms.Two Phase II studies demonstrated that the combination of Pembrolizumab and Rituximab effectively blocked PD-1 signaling in patients with relapsed or refractory follicular lymphoma, achieving an objective response rate of 67%. This finding suggests that the combination of anti-CD20 antibodies and PD-1 antibodies exhibits synergistic anticancer effects (30). Additionally, certain studies have indicated that incorporating PD-1 antibodies into anti-CD20 antibody regimens may counteract the poor prognosis associated with aberrant activation of the PD-1/PD-L1 pathway (31). Furthermore, the present study revealed that the proportion of apoptotic cells in the combination group was significantly higher than that in the single-agent group, accompanied by an increased proportion of cells in the S and G2/M phases, indicating a synergistic apoptosis-inducing effect of the two antibodies. However, the underlying mechanism of interaction remains elusive. It may be hypothesized that the anti-CD20 antibody improves the tumor immune microenvironment by regulating B cells, thereby enhancing the T-cell activating effect of the PD-1 antibody; further investigation is therefore warranted.

Regarding advantages and limitations, the present study reports the mRNA expression levels of MS4A1 and PDCD1; however, the protein expression levels have not yet been verified. The effects of anti-CD20 and PD-1 antibodies on the proliferation, apoptosis and cell cycle of EC cells were only demonstrated *in vitro*, but no *in vivo* experiments and no clinical experiments were performed. Meanwhile, the absence of immune cells in cell culture limits the interpretation of ADCC/CDC mechanisms; such mechanisms cannot be inferred without immune cells.

In conclusion, the present *in vitro* results demonstrate that combined treatment with anti-CD20 and PD-1 antibodies effectively suppresses EC-cell proliferation and markedly promotes apoptosis, providing preliminary experimental evidence supporting further mechanistic and *in vivo* validation.

Acknowledgements

Not applicable.

Funding

This project was supported by the National Natural Science Foundation of China (grant no. 82160443), the Guangxi Natural Science Foundation (grant no. 2020GXNSFAA159023), the independent research project of the Regional Key Laboratory of Early Prevention and Treatment of High-incidence Tumors in 2021 (grant no. GKE-ZZ 202147), the 18th batch of Guangxi 'New Century Ten Hundred Thousand Talents Project' second-level candidate special fund (grant no. 2015226) and the Guangxi Medical High-level Backbone Talent Training '139' Plan special fund (grant no. G201903032).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JS and HW designed the research. YY and JD collected and analyzed the data. MF, QH and BL collected and analyzed the data and supervised the study. JS drafted the article and HW edited it. JS and HW confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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