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Polyclonal rabbit anti-human ovarian cancer globulins inhibit tumor growth through apoptosis involving the caspase signaling

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Most women with ovarian cancer are diagnosed at an advanced stage and there are few therapeutic options. Recently, monoclonal antibody therapies have had limited success, thus more effective antibodies are needed to improve long-term survival. In this report, we prepared polyclonal rabbit anti-ovarian cancer antibody (Poly Ab) by immunizing rabbits with the human ovarian cancer cell line SKOV3. The Poly Ab bound to SKOV3 and inhibited the cancer cells proliferation. Western blot analysis was conducted, which indicated that Poly Ab inhibited cancer cells through apoptosis involving the caspase signaling pathway including caspase-3 and caspase-9. Finally, compared with the control antibody, administration of Poly Ab reached 64% and 72% tumor inhibition in the subcutaneous and intraperitoneal xenograft mouse model, respectively. Our findings suggest that Poly Ab is an effective agent for apoptosis induction and may be useful as a safe anticancer agent for ovarian cancer therapy.

Although significant progression in the treatment of ovarian cancer have led to improved 5-year survival rates, ovarian cancers remain the leading cause of death from gynecologic cancers[1]. At diagnosis, most women present with advanced stage disease, which accounts for the high mortality rate. Despite initial treatment with cytoreductive surgery and platinum-based chemotherapy, relapse remains almost inevitable in patients with advanced disease.

The improved understanding of the genetics of ovarian tumor has led to the development of molecular targeted therapies[2]. Recently, a variety of tumor-associated antigens have been detected in ovarian cancer, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), α-folate receptor (αFr), and epidermal growth factor receptor family (Erbb family). Since these antigens play important role in tumor angiogenesis, survival, proliferation, and metastases, the targeted monoclonal antibodies are now entering clinical trials[3–6].

Unfortunately, ovarian cancers are etiologically heterogeneous and express numerous different cellular surface antigens. For example, only 11.4% ovarian cancers overexpress Erbb2[7]. Thus, targeting multiple antigens at the same time by efficient polyclonal antibodies is a potential alternative strategy[8,9].

In this study, we produced polyclonal rabbit anti-ovarian cancer antibody (Poly Ab) by immunizing rabbits with the human ovarian cancer cell line SKOV3. Poly Ab could inhibit proliferation and induce apoptosis of ovarian cancer *in vitro*. Furthermore, it significantly inhibited tumor growth in the subcutaneous and intraperitoneal mouse models. These results suggest that the therapeutic effects are related to multiple targets on ovarian cancer cells. Importantly, such strategies might allow selection of treatments based on the whole characteristics of tumors and bring us closer to an era of personalized medicine.

Results

Preparation and evaluation of Poly Ab. Poly Ab was isolated using the affinity chromatography system and purification was confirmed by SDS-PAGE electrophoresis (Supplementary Fig. S1). ELISA analysis indicated that Poly Ab was binding to SKOV3 cells with 100000 times dilution (Fig. 1a). Meanwhile, flow cytometric analysis and immunofluorescence stain were carried out to identify the reaction abilities of Poly Ab against SKOV3 cells, which confirmed 98% and 95% SKOV3 cells could be detected by Poly Ab, respectively (Fig. 1b and 1c).
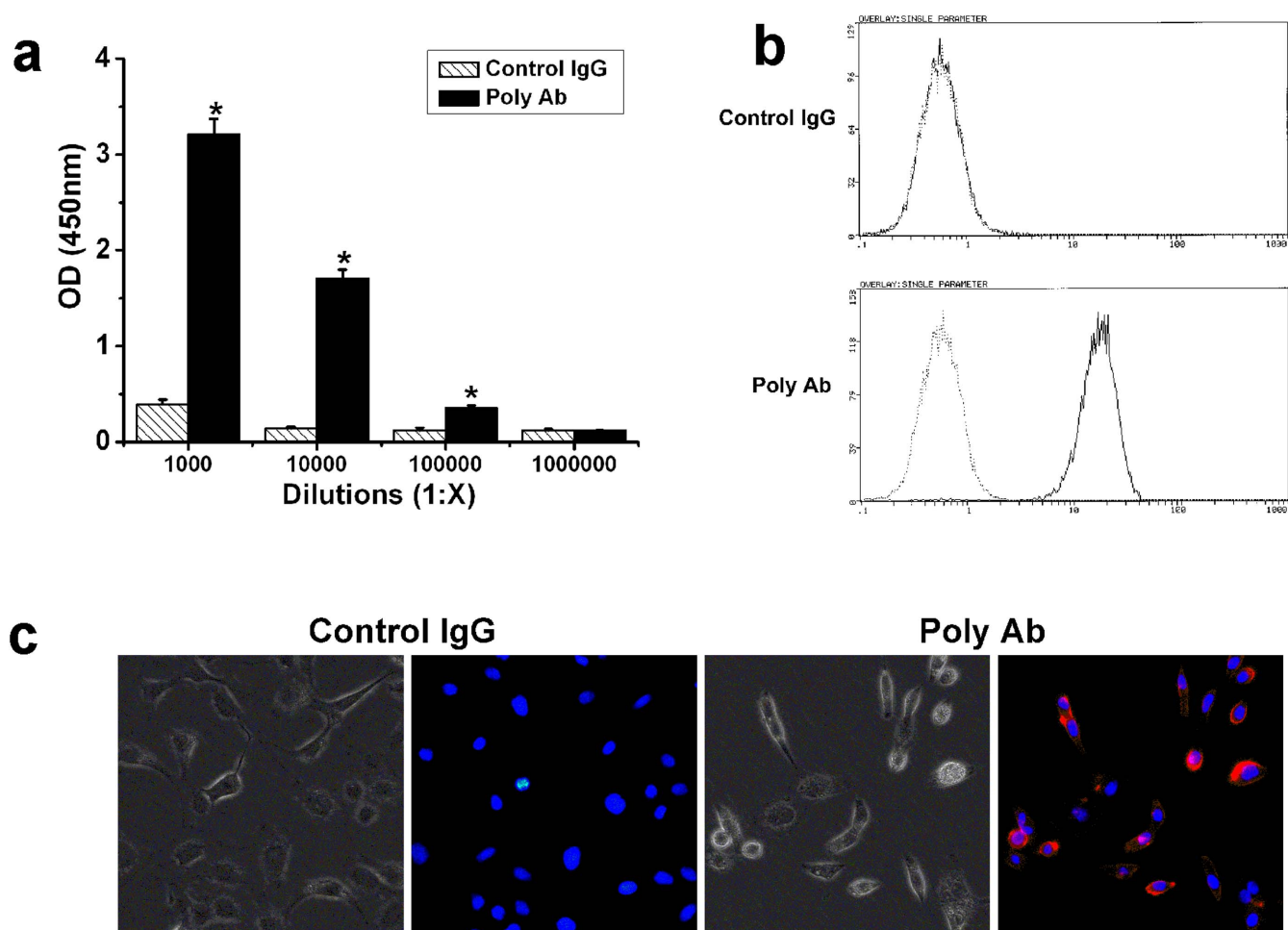
Figure 1 | Binding of Poly Ab to human ovarian cancer cells SKOV3. (a) ELISA was performed to determine the proper dilution of Poly Ab against SKOV3 cells. Poly Ab was binding to SKOV3 cells with 100000 times dilution. Columns, mean; bars, SD (n = 3; Student's t test; *, $P < 0.05$). (b) Flow cytometric analysis indicated 98% SKOV3 cells could be detected by Poly Ab. (c) Immunofluorescence stain indicated 95% combining capacity of Poly Ab against SKOV3 cells. Magnification, ×200.

Poly Ab inhibits cell proliferation and induces apoptosis. MTT analysis was performed to assess the potential effects of Poly Ab on cell proliferation. Poly Ab significantly inhibited tumor cell proliferation with an IC_{50} value of 0.82 mg/ml (Fig. 2a). We found the Poly Ab was specific to the SKOV3 and A2780 ovarian cancer cells, but did not show any inhibition in other kinds of tumor and the normal human cell line (Supplementary Fig. S2). We also speculated that Poly Ab might induce apoptosis in cancer cells. To examine this possibility, apoptosis was evaluated by Hoechst 33258 staining. Condensed nuclei were observed in 45.2% of treated cells, which was significantly more than that in control antibody group (Fig. 2b and 2c). Quantitative assessment was further done by flow cytometry to observe the exact number of apoptotic cells. 0.64 mg/ml Poly Ab treatment induced 41.6% cell apoptosis (Fig. 2d). Meanwhile, to demonstrate effect of the Poly Ab on cell proliferation, cell cycle was detected by flow cytometry. We found the Poly Ab caused concomitant increased cells in the G0/G1 phase and decreased cells in the S phase (Supplementary Fig. S3). These assays were repeated for three times.

Increased activity of caspases and executioners. In the study, we indicated increased levels of Bax, Bad, and released cytochrome c after Poly Ab treatment. Bcl-2, a major antiapoptotic molecule that inhibited mitochondrial cytochrome c release, was decreased significantly after treatment. We also observed significant increase

of the cleaved PARP, caspase-9 and caspase-3. The increased levels of Bax, released cytochrome c, activated caspases, cleaved PARP along with other results clearly demonstrated enhanced apoptosis after Poly Ab administration in SKOV3 ovarian cancer cells (Fig. 3). 3 independent experiments were performed and had the similar results.

Poly Ab inhibits tumor growth *in vivo*. We used 2 xenograft tumor models to investigate the effect of Poly Ab on tumor growth. In the first experiment, SKOV3 subcutaneous xenografts were treated for 3 weeks through tail vein. Meanwhile, SKOV3 intraperitoneal xenografts were treated intraperitoneally for 3 weeks. Compared with the control group, administration of Poly Ab reached 64% and 72% inhibition against subcutaneous xenografts and intraperitoneal xenografts, respectively (Fig. 4a and 4b). No adverse effects such as skin ulcerations or weight loss occurred in Poly Ab group. Furthermore, toxic pathologic changes in main organs were not detected by microscopic examination. These data demonstrated that the inhibition of tumor growth was not attributable to systemic toxicity (Fig. 4c).

After mice were sacrificed, the tumor samples were taken out and applied for H&E staining to investigate the microscopic changes. The significant necrosis was observed in the center of tumor tissues in mice treated with Poly Ab. Meanwhile, tumor cells grew well and no obvious necrosis was observed in other groups (Fig. 5a). We also
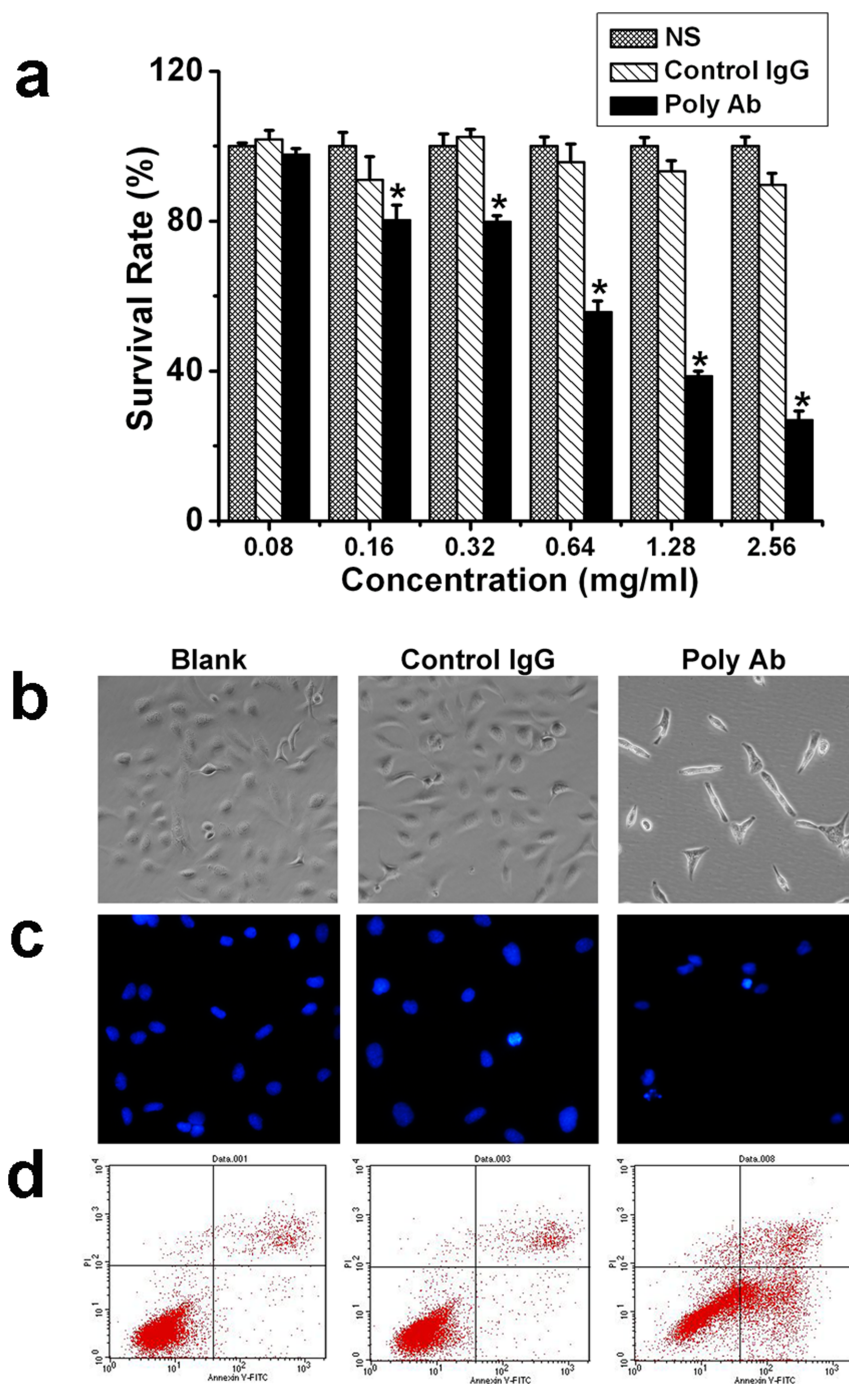
Figure 2 | Poly Ab inhibited cell proliferation and induced apoptosis. (a) MTT assay was used to assess the effect of Poly Ab on the cell viability. NS indicated normal saline treated group. Time course experiments were performed which revealed that Poly Ab significantly reduced cell viability in comparison with control antibody. Columns, mean; bars, SD (n = 3; Student's t test; *, $P < 0.05$). (b) SKOV3 cells were inhibited by Poly Ab and imaged under the optical microscope. Magnification, ×200. (c) Apoptosis was evaluated by Hoechst 33258 staining. Condensed nuclei were observed in 45.2% of treated cells, which was significantly more than that in control antibody group (n = 3; Student's t test; $P < 0.05$). 3 independent experiments were performed. Magnification, ×200. (d) Annexin V-FITC and PI staining was used to quantitatively assess apoptosis at an early stage by flow cytometric analysis. Living cell populations were clustered in the Q3 quadrant; cells in early apoptosis were in the Q4 quadrant; late apoptotic/necrotic cells were in the Q2 quadrant. 0.64 mg/ml Poly Ab treatment induced 41.6% cell apoptosis.

performed Ki67 staining to assess the effect of Poly Ab on tumor cell proliferation. The inhibition of Ki67 expression was observed in tumor tissues treated with Poly Ab (Fig. 5b). In addition, the TUNEL assay revealed that more apoptotic cells were observed in tumor tissues of Poly Ab group (Fig. 5c). Finally, by CD31 staining, we indicated the increased apoptotic tumor cells induced less tumor angiogenesis (Fig. 5d).

Discussion

Cytoreductive surgery followed by systemic chemotherapy with paclitaxel and cisplatin is the current standard therapy for advanced ovarian cancer, but the majority of patients experience disease recurrence. For recurrent ovarian cancer, the second-line treatment regimens have limited efficacy, especially in patients with platinum-resistant disease[10,11]. In recent years, monoclonal antibodies
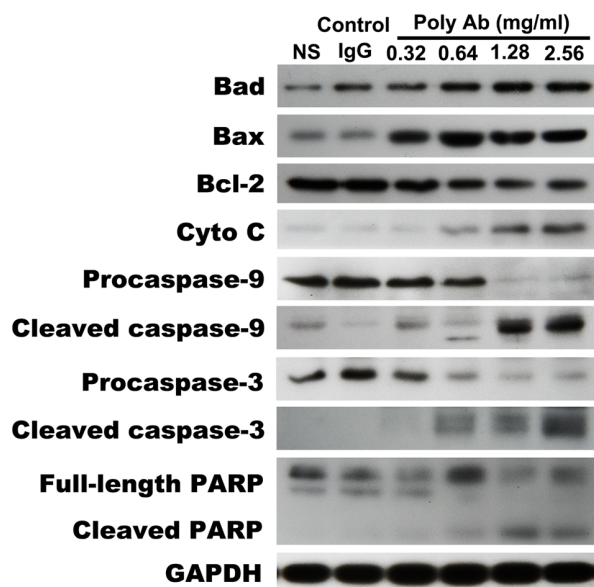
Figure 3 | Western blot analysis indicated increased activity of caspases and executioners after Poly Ab administration in SKOV3 ovarian cancer cells. The PVDF membranes were reprobed for GAPDH expression to show that similar amounts of protein were loaded in each lane. NS indicated normal saline treated group. The cropped blots images are shown in the figure and the full-length blots are presented in Supplementary Figure S4.

have entered the field of ovarian cancer therapy. Some antibodies served as antagonists of the oncogenic receptors (EGFR or HER2) and directed against tumor growth. On the other hand, bevacizumab was used to block vascular endothelial growth factor-A (VEGF-A), slowing formation of the ovarian cancer vasculature and ascites[12–14].

However, human cancers frequently demonstrate a great variation in DNA content, and cancer cells are known to be heterogeneous with regard to their antigenic properties[15]. The genetic instability of tumor cells enables them to mutate and develop mechanisms of resisting anticancer therapies, which is more likely to occur when a single tumorigenic pathway is targeted than when multiple pathways are targeted simultaneously. The polyclonal antibodies could target multiple epitopes simultaneously, precluding the signal compensation and immune escape of tumor cells[16]. Furthermore, polyclonal antibodies were obtained from hyper-immunized animals and identified the most immunogenic epitopes in ovarian cancer, which was effective at engaging Fcγ receptors on NK cells, macrophages, and neutrophils. Antibody engagement of these receptors led to the killing of target cells by antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent phagocytosis (ADP)[17,18].

Apoptosis is the process of programmed cell death that occurs under various physiological and pathological conditions. Resistance to apoptosis is the hallmark of cancer, which is due to mutations of specific genes and provides cancer cells with a distinct advantage for survival and proliferation. Activation of apoptosis in cancer cells offers the potentially useful approach to improve therapeutic response of the tumor patients[19].

In the present study, we prepared Poly Ab by immunizing rabbits with the human ovarian cancer cell line SKOV3. Poly Ab bound to SKOV3 and inhibited the cancer cells proliferation, migration, invasion, and colony formation. Meanwhile, Poly Ab inhibited tumor growth in the subcutaneous and intraperitoneal xenograft mouse models. Various experiments, including the Annexin V-FITC/PI staining and TUNEL assay, showed increased apoptosis after Poly Ab treatment *in vitro* and *in vivo*, which was the possible cause of

increased tumor necrosis, cell proliferation and decreased angiogenesis observed in xenografts.

The induction and execution of apoptosis need the cooperation of a series of molecules, including signaling molecules, receptors, and enzymes. Caspases are interleukin-1β converting enzyme family proteases that initiate apoptosis in mammalian cells. To detect the underlying mechanism of apoptosis, western blot was applied to monitor the increased expression of death factor in SKOV3 cells. We observed increased protein levels of cleaved caspase-9 and caspase-3, which were maximally expressed 48 h after Poly Ab treatment. The increased levels of executioner caspases, which were directly responsible for the cleavage of apoptotic executioner molecules, clearly showed enhanced apoptosis in SKOV3 cells after treatment. PARP is a DNA-binding protein that preferentially binds to damaged DNA containing excised nucleotides. PARP cleavage has been shown to occur early in the apoptotic response as a result of caspase-3 activation. Here, we observed a significant increase of cleaved PARP accompanied by chromatin condensation and fragmentation, which was one of the hallmarks of apoptosis[20,21].

There are still several limitations in our study. For example, the nature of the antigens recognized by Poly Ab is at present unclear, and their characterization is now in progress. Meanwhile, our approach may be limited by the immunosuppressive tumor microenvironments. Overcoming immunosuppression and enhancing the quality of the immune response may require two antibodies against PD-L1 and CTLA4, respectively[14,22]. Thus, combination of antibody therapeutics will be required for developing maximum effects in cancer immunotherapies.

Taken together, our studies indicate that Poly Ab is an effective agent for apoptosis induction involving the caspase signaling pathway. Poly Ab and its derivates are promising candidates as ovarian cancer drugs.
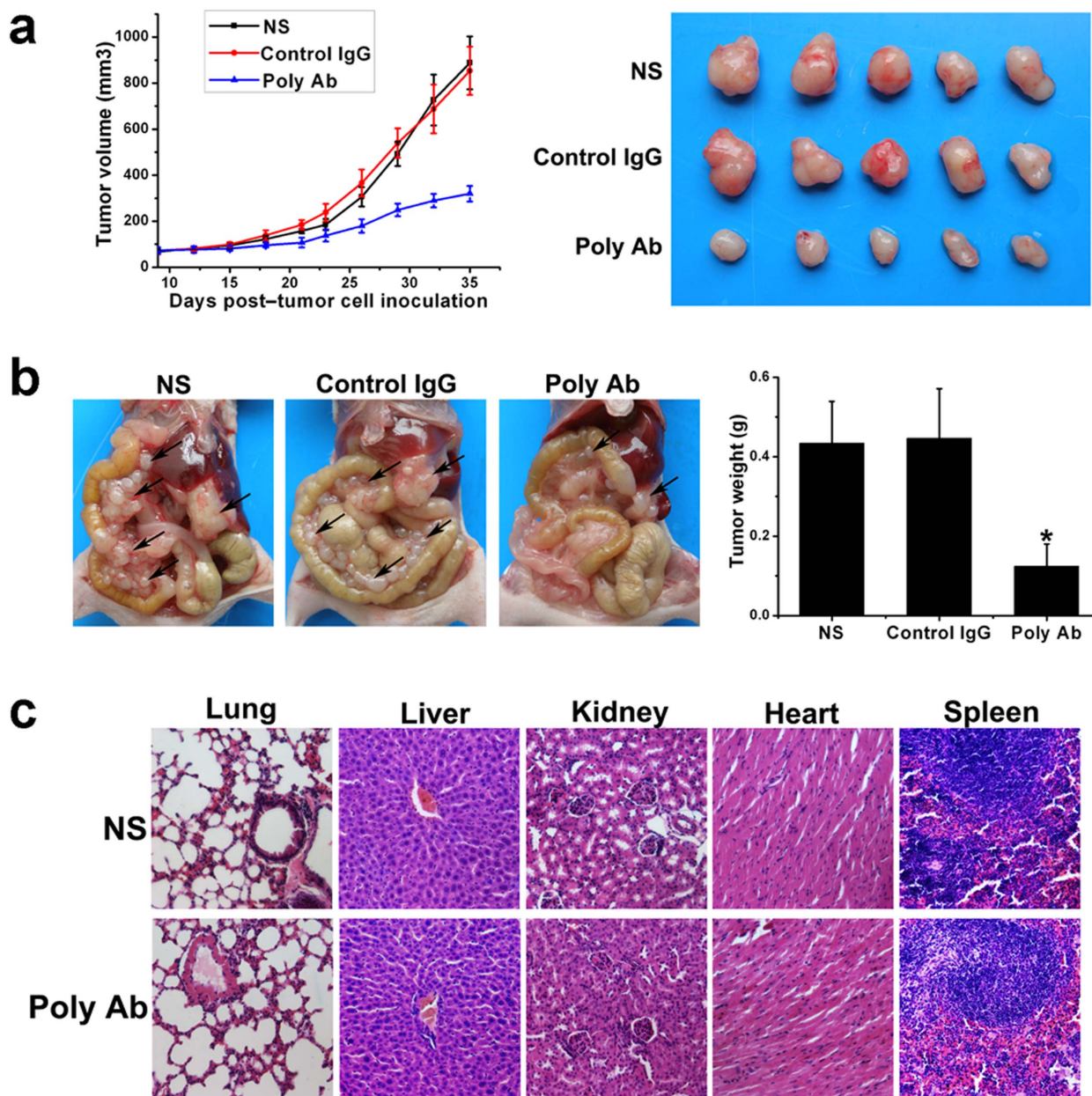
Methods

Figure 4 | Antitumor effects of Poly Ab *in vivo* and toxicity evaluation. We used 2 xenograft tumor models to investigate the effect of Poly Ab on tumor growth. Compared with the control group, administration of Poly Ab reached significant inhibition against subcutaneous xenografts (n = 6; Student's t test; $P < 0.05$, Poly Ab *vs.* control antibody). Meanwhile, administration of Poly Ab reached 72% inhibition against intraperitoneal xenografts. Columns, mean; bars, SD (n =6; Student's t test; *, $P < 0.05$, Poly Ab *vs.* control antibody). (c) H&E staining of paraffin-embedded sections of the lung, spleen, kidney, heart, and liver. Poly Ab did not cause obvious pathologic abnormalities in main organs. NS indicated normal saline treated group. Magnification, ×400.

with indicated concentrations of poly Ab (0.08–2.56 mg/ml) for 48 h. Each assay was replicated 3 times.

Nuclear chromatin staining for apoptosis. As described previously[24], 48 h after incubation with Poly Ab, SKOV3 cells were fixed for 15 min in 4% paraformaldehyde and stained with 1 μg/ml Hoechst 33258 (Sigma, USA). Finally, apoptosis was visualized with a fluorescence microscope (Carl Zeiss, Germany). 3 independent experiments were performed.

Apoptosis assay by Annexin V-FITC and PI staining. AnnexinV-FITC and PI staining were used to detect apoptosis at an early stage according to the method of Vermes *et al.*[25]. Briefly, SKOV3 cells were harvested 48 h after treatment. Cells were resuspended at 1×10^6/ml in binding buffer containing AnnexinV-FITC and PI. The samples were evaluated with the FACSAria flow cytometer (BD Biosciences, USA) at an excitation wavelength of 488 nm and observation wavelengths of 530 nm.

Western blot analysis. The SKOV3 cells were incubated with Poly Ab for 48 h and lysed with buffer containing 1% Triton X-100, 1% deoxycholate, and proteinase inhibitor cocktail. Cellular protein from each sample was probed with specific antibodies (1 : 1000, Cell Signaling Technology, USA) including Bad, Bax, Bcl-2, cytochrome c, procaspase-9, cleaved caspase-9, procaspase-3, cleaved caspase-3, full-length PARP, and cleaved PARP. Blots were developed with HRP-conjugated secondary antibodies and chemiluminescent substrate (Thermo Scientific, USA) on Kodak X-ray films. Blot data were quantified using densitometry with Quantity One (Supplementary Figure S5).

Xenograft mouse model. Briefly, 5×10^6 SKOV3 cells were injected s.c. into six-week-old female athymic mice. 7 days later, the mice bearing tumors of approximately 50 mm³ were randomly assigned to three independent treatment groups (6 mice per group): (a) normal saline; (b) 500 μg control antibody; (c) 500 μg Poly Ab. The drugs were injected through the tail vein every 3 days for 3 weeks. The tumor volume was measured with a caliper every 3 days, using the formula: volume = length × width²/2.
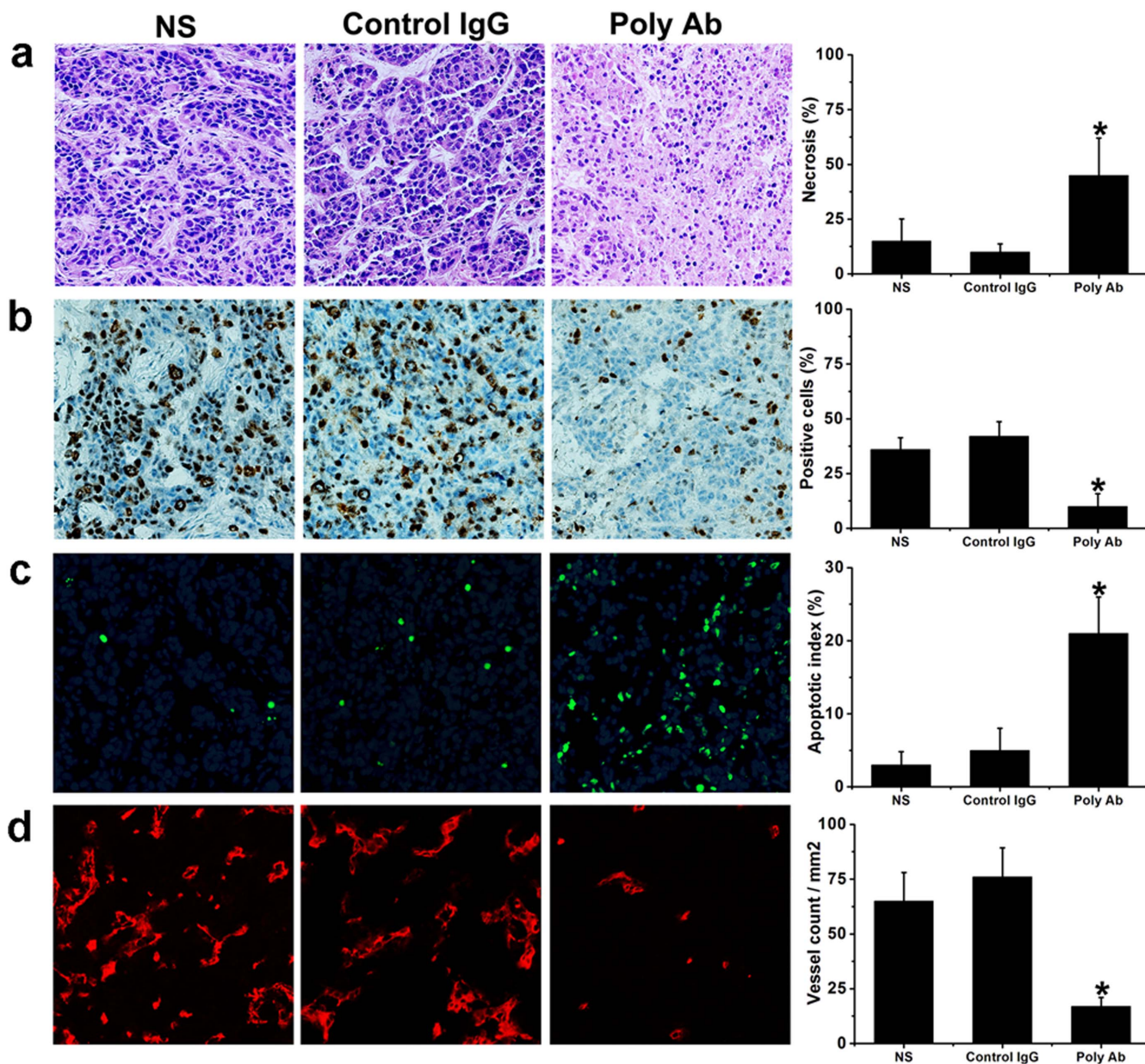
Figure 5 | Poly Ab inhibited cell proliferation and induced tumor apoptosis *in vivo*, which correlated with inhibition of SKOV3 xenograft growth. (a) H&E staining showed more necrosis in Poly Ab group than that in control groups. (b) Ki67 staining was performed to assess the effect of Poly Ab on tumor cell proliferation. The inhibition of Ki67 expression was observed in tumor tissues treated with Poly Ab. (c) The TUNEL assay revealed that more apoptotic cells in tumor tissues were observed in Poly Ab group than that in other groups. (d) CD31 staining indicated the increased apoptotic tumor cells induced less tumor angiogenesis. Columns, mean; bars, SD (n = 3; Student's t test; *, $P < 0.05$, Poly Ab *vs.* control antibody). NS indicated normal saline treated group. Magnification, ×200.

Furthermore, 5×10^6 SKOV3 cells were injected i.p. into athymic mice in another experiment. Drug therapies were administered intraperitoneally with the same dosing schedules mentioned above. 30 days after tumor cell inoculation, mice were sacrificed and the solid i.p. lesions were recorded and expanded for further study.

Toxicity evaluation. To investigate potential toxicity during the treatment, the mice were observed continuously for relevant indexes such as weight loss, diarrhea, anorexia, and toxic deaths. The tissues of liver, heart, kidney, lung, and spleen in intraperitoneal model were stained with hematoxylin and eosin (H&E).

Immunohistochemistry. To explore whether Poly Ab could effectively inhibit cell proliferation *in vivo*, anti-Ki67 antibody (Thermo Scientific, CA, USA) was used to determine cellular proliferation in tumor tissues. Meanwhile, to explore whether the antitumor effects could influence tumor angiogenesis, vessel density in tumor tissue was detected as described previously[26].

Detection of apoptosis. Cell apoptosis in SKOV3 xenograft tumors was determined using the TUNEL assay following the manufacturer's instructions (Promega, USA). The number of TUNEL-positive cells was quantified by fluorescence microscopy, and the apoptotic index in 6 random fields per group was counted.

Statistical analysis. SPSS 18.0 was used for statistical analysis. Data were analyzed statistically using two-tailed Student's t-test. Differences were considered significant if $P < 0.05$.

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Acknowledgments

Author contributions

Y.W. and H.D. carried out study design and manuscript preparation. S.Z. and M.Y. participated in animal study, literature research, data analysis, and manuscript editing. G.S. performed immunohistochemistry and other pathological experiments. All authors reviewed the manuscript.

Additional information

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