Simultaneous blockade of PD-1 and VEGFR2 induces synergistic antitumour effect in vivo

Short title: Synergistic antitumour effect by dual blockade of PD-1 and VEGFR2

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Summary

Recent basic and clinical studies have shown that PD-L/PD-1 pathway has a significant role in tumour immunity and its blockade has a therapeutic potential against several human cancers. We hypothesized that antiangiogeneic treatment might augment the efficacy of PD-1 blockade. To this end, we evaluated combining blockade of PD-1 and VEGFR2 in a murine cancer model using Colon-26 adenocarcinoma. Interestingly, simultaneous treatment with anti-PD-1 and anti-VEGFR2 mAbs synergistically inhibited tumour growth in vivo without overt toxicity. Blocking VEGFR2 significantly inhibited tumour neovascularization as demonstrated by the reduced number of microvessels, while PD-1 blockade had no impact on tumour angiogenesis. PD-1 blockade might promote T cell infiltration into tumours and significantly enhanced local immune activation as shown by the upregulation of several proinflammatory cytokine expressions. Importantly, VEGFR2 blockade did not interfere with T cell infiltration and immunological activation induced by PD-1 blockade. In conclusion, simultaneous blockade of PD-1 and VEGFR2 induced a synergistic in vivo antitumour effect possibly through different mechanisms that might not be mutually exclusive. This unique therapeutic strategy may hold significant promise for future clinical application.

Introduction

Blocking immune checkpoints can potentially activate and sustain T cell response against tumours [1]. CTLA-4 (CD152) is well known to serve as a dominant inhibitory receptor on T cells, and it plays a key role in immune tolerance and homeostasis [2, 3]. CTLA-4 blockade has been long expected as a new cancer immunotherapy. A recent large-scale randomized clinical trial nicely demonstrated that immunotherapy using anti-human CTLA-4 monoclonal antibody had a significant antitumour response and improved overall survival in metastatic melanoma [4]. That was the first therapy to extend the overall survival in humans and one of the most successful cancer immunotherapies. However, immune-related adverse events had been also reported, and some patients died due to severe toxicities related to the study drugs. Therefore, another treatment targeting T cell negative regulatory pathway with less toxicity as well as the substantial efficacy for anticancer treatment would be desirable.

Programmed death 1 (PD-1, CD279) is another potent immune-checkpoint receptor [5-7]. PD-1/programmed death lingand-1 (PD-L1) also functions as a negative regulator of T cell activation and contributes to the prevention of autoimmune diseases. A number of previous studies have shown that PD-1/PD-L1 pathway has clinical importance in several human malignancies and its blockade has a significant antitumour effect in rodent models

[8-10]. Furthermore, recent Phase I clinical trials have shown that anti-human PD-1 or PD-L1 antibodies were tolerable for clinical use and might hold great promise as new anticancer treatment for several advanced human malignancies [11, 12]. However, the effect of targeting PD-1/PD-L1 alone may be insufficient especially for advanced or intractable malignant tumours that are resistant to conventional anticancer treatments including chemotherapy and radiotherapy. Therefore, it should be important to investigate the combination treatments for augmenting the potency of PD-1 blockade.

It is known that angiogenesis is a key feature in cancer development and metastasis [13, 14]. Among various regulators of angiogenesis, VEGF and its receptor VEGFR are thought to be essential [15]. Basic findings have shown that blocking of VEGF/VEGFR pathway disrupts tumour microvessels and inhibits tumour growth. Furthermore, it has been also reported that VEGF/VEGFR blockade could also normalize abnormal tumour vessels and increase tumour oxygenation, drug supply and immune cells [16-19]. Indeed, anti-VEGF treatment is currently standard therapy for several human malignancies. However, it is also insufficient as a single treatment and usually administered with other cytotoxic anticancer drugs.

In this study, we hypothesized that antiangiogenesis treatment may enhance the

antitumour effect of targeting PD-1 pathway without enhancing toxicity by efficiently inducing T-cell infiltration into tumours. To this end, we employed an anti-VEGF receptor-2 (VEGFR2, CD309) monoclonal antibody (mAb), designated DC101. VEGFR2 is a major receptor for VEGF and plays a central role in tumour angiogenesis [20]. Furthermore, this mAb has been proven to have certain antitumour effect in murine models [21-24].

Materials and methods

Animal and cell line

Female BALB/c mice (5–6-weeks old) were obtained from CLEA JAPAN (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. All experiments were conducted under a protocol approved by our institutional review board. A murine Colon-26 adenocarcinoma was obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum.

Antibodies

The anti-mouse PD-1 blocking monoclonal antibody (mAb) (RMP1-14, rat IgG1) was generated as previously described [25]. The anti-mouse VEGFR2 blocking mAb (DC101, rat IgG1) was kindly provided by ImClone Systems (New York, NY). The anti-mouse CD34 mAb (MEC 14.7, rat IgG2a), the anti-CD4 Ab (sc-7219, rabbit polyclonal), and the anti-CD8 mAb (EP1150Y, rabbit IgG) was purchased from Abcam (Tokyo, Japan), Santa Cruz Biotechnology, Inc (Santa Cruz, CA), and Novus Biologicals (Littleton, CO), respectively.

Animal experimental protocol

One million Colon-26 cells were subcutaneously inoculated in the flank of syngeneic BALB/c mice with 100 μl of cell suspension with an equal volume of Matrigel (BD Bioscience). When tumour reached 4-5 mm in diameter around 3 days after tumour inoculation, treatment was started. In the antibody treatment arm, mice were intraperitoneally injected with 0.25 mg of RMP1-14, and/or 0.8mg of DC101 every other day for 5 times. Control mice received control rat IgG. The doses were determined on the basis of our preliminary experiments and previous studies [8, 23, 24]. Tumour size was determined by electric caliper measurements. In some

mice, mice were sacrificed and tumours were removed for further analysis at 11 days after tumour establishment.

Cell viability analysis

Cell viability was determined using the Cell-titer 96 aqueous one solution cell proliferation assay kit, according to the instruction manual (Promega Corporation, Madison, WI, USA). Briefly, aliquots of 3×10^3 cells per well were cultured in 96-well plates with control IgG, RMP1-14, DC101 or both of RMP1-14 and DC101 for 72 h. Antibody was used at a concentration of 1 or 10 μg/ml. Cell-titer 96 aqueous one solution was added to each well and incubated for an additional 1 h. The absorbance at 490 nm was recorded with a 96-well plate reader. Each experiment was performed in triplicate and repeated at least thrice.

Immunohistochemistry and tumour vessel density measurement

Formalin-fixed or zinc-fixed, paraffin-embedded tissues of primary tumour were cut into 5-μm sections, deparaffinised, and rehydrated in a graded series of ethanol. To block endogenous peroxidase, sections were immersed in 0.3 % solution of hydrogen peroxide in absolute methanol for 5 min at room temperature and washed thrice in fresh PBS, each of 5

min duration. Purified rat anti-mouse CD34 mAb, rabbit anti-CD4 Ab, or rabbit anti-CD8 mAb diluted with Antibody Diluent (DAKO, Tokyo, Japan) was added and incubated for 1 h at room temperature or overnight at 4℃. Sections were washed thrice in PBS, each of 5 min duration, and then Histofine Simple Stain MAX PO (NICHIREI, Tokyo, Japan) was added and incubated at room temperature for 30 min. After washing thrice, the Histofine DAB substrate kit (NICHIREI) was added and incubated at room temperature for 5 min. Sections were rinsed thrice in distilled water, counterstained with haematoxylin, dehydrated in ethanol, cleared in Hemo-De, and coverslipped. For tumour vessel density measurement, slides were scanned at low power fields (×40) to identify areas of highest vascularity. 20 high-powered (×200) fields were then selected randomly within these areas, and tumour vessel densities were calculated based on the number of CD34-positive luminal structures. To rule out the possibilities that staining kit reacted with antibodies which had been used for the treatment in mice, we confirmed no positive signals in samples stained without primary anti-CD34 mAb.

Extraction of total RNAs and real-time reverse- transcriptase PCR analysis

Total RNA was isolated using RNAspin Mini (GE Healthcare, Tokyo, Japan) and the first-strand cDNA was synthesized from 1 μg RNA using a High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Real-time quantitative PCR analysis was carried out using an ABI Prism 7700 sequence detector system (Applied Biosystems). All primer/probe sets were purchased from Applied Biosystems. PCR was carried out using the TaqMan Universal PCR Master Mix (Applied Biosystems) using 1 μl of cDNA in a 20 μl final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression level of the housekeeping gene β_2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all specimens. The ratio of the mRNA level of each gene was calculated as follows: (absolute copy number of each gene)/(absolute copy number of β_2-microglobulin).

Statistical analysis

Results were expressed as mean values ± standard error, and Student's t test or Welch's t test was used for evaluating statistical significance. A value less than 0.05 was considered for statistical significance.

Results

Simultaneous blockade of PD-1 and VEGFR2 induced synergistic antitumour effect

First, we investigated the efficacy of simultaneous blockade with both PD-1 and VEGFR2 in vivo using murine colon cancer model. Tumour cells were inoculated subcutaneously with 1×10^6 in the right flank of BALB/c mice and treated with anti-PD-1 mAb (RMP1-14) and/or anti-murine VEGFR2 mAb (DC101). Control rat IgG was used as a control. In vivo treatment either with anti-PD-1 mAb or anti-VEGFR2 mAb induced a substantial antitumour effect and significantly inhibited tumour growth compared to control (Fig 1). There was no significant difference in tumour growth between PD-1 and VEGFR2 blockade. Furthermore, dual blockade of both PD-1 and VEGFR2 significantly inhibited tumour growth compared to each mAb treatment (Fig 1). Thus, the combination therapy of anti-PD-1 and anti-VEGFR2 mAb showed a synergistic antitumour effect in tumour growth. There were no overt toxicities in treated mice.

Effect of PD-1 and VEGFR2 blockade on cancer cell in vitro

To analyze the underlying mechanisms in tumour growth inhibition induced by PD-1 and VEGFR2 blockade, we evaluated in vitro effect of anti-PD-1 and anti-VEGFR2 mAb on

Colon-26. A total of 3000 Colon-26 were co-cultured with anti-PD-1 mAb, anti-VEGFR2 mAb, or both mAbs. Control rat IgG was used as a control. The survival rate of Colon-26 was determined by MTS assay. As a result, anti-PD-1 mAb and anti-VEGFR2 mAb did not affect cell survival. Thus, blockade of PD-1 and VEGFR2 does not have any direct effect on cancer cell growth (Fig 2).

VEGFR2 blockade inhibited tumour neovascularization

Then, we analyzed tumour neovascularization by immunohistochemistry with antibody against CD34 (Fig 3A). Treatment with anti-VEGFR2 mAb or combination therapy significantly inhibited the development of tumour microvessels compared with control (Fig 3B). Furthermore, anti-PD-1 mAb had no effect on tumour neovascularization (Fig 3B). Thus, PD-1 blockade did not interfere with anticancer treatment targeting tumour angiogenesis.

PD-1 blockade enhanced T cell recruitment into tumours

We next evaluated tumour T cell-infiltrations by immunohistochemistry and quantitative real-time PCR analysis. Although there were no significant statistical differences, there was a constant tendency of increase in CD4+ and CD8+ T-cell infiltration in tumour tissues

treated with anti-PD-1 mAb alone or combination of anti-PD-1 mAb and anti-VEGFR2 mAb compared to control or anti-VEGFR2 mAb alone (Fig 4). Even though anti-VEGFR2 mAb disrupted tumour vessels as shown above, T cell infiltration in tumours treated with anti-VEGFR2 mAb or combination did not decrease. Thus, VEGFR2 blockade did not abrogate recruitment of T lymphocytes into tumours induced by PD-1 blockade. In addition, we examined FOXP3 expression in tumours as a marker for regulatory T cells. We found that FOXP3 expression was not reduced by anti-VEGFR2 treatment and elevated by anti-PD-1 treatment (data not shown).

PD-1 blockade activated local immunity

To analyze local immune status in tumours, we evaluated the several potent pro-inflammatory cytokines and mediators. Treatment with anti-PD-1 mAb or combination therapy induced significant increase of expressions of IFN-γ, TNF-α, and granzyme B in comparison with control (Fig 5). Thus, PD-1 blockade enhanced T cell recruitment and activated local immune status, thereby resulting in tumour reduction. However, VEGFR2 blockade alone did not induce local immune activation in this model.

Discussion

This is the first study to investigate the synergistic antitumour effect induced by dual blockade of PD-1 and VEGFR2. Recent multicenter Phase I clinical trials have shown that PD-1 or PD-L1 mAbs were safe in patients with various types of cancer and hold promise as new anticancer agents. However, in order to enhance antitumour efficacy of strategies targeting PD-1/PD-L pathway, combination therapy may be desirable especially for refractory tumours such as pancreatic cancer. Since practical reagents targeting PD-1 and VEGF pathways are currently available, our proposed strategy may have actual clinical relevance. There are several advantages in dual blockade of completely different pathways. First, the combination therapy may reduce the harmful effect if they have different profiles of toxicity, since they can be used at reduced doses while preserving efficacy. This is important because both PD-1 and VEGFR blockades are thought to cause unique adverse events. For instance, it is known that PD-1 blockade have a risk to induce autoimmune reactions and diseases [10, 11, 26]. The therapeutic dose of anti-PD-1 Ab may cause significant harmful effect. Its reduced, but optimal dose can be achieved by the combination therapy. Although we have observed no overt toxicity in mice during and after treatment, careful observations will be required in

clinical applications. Second, when combining two reagents that have different properties, enhanced efficacy may be anticipated because of synergistic interactions. Our data clearly indicates that synergistic in vivo antitumour effect can be successfully induced by combining PD-1 and VEGFR2 blockade.

Although the underlying mechanisms are not fully elucidated, several interpretations may be drawn from our data. First, as expected, anti-VEGFR2 mAb treatment resulted in significant decrease of tumour microvessels. Reducing tumour vasculature deprives the tumours of blood supply, thereby leading to the necrosis or apoptosis of tumour cells [19, 21]. This was not observed in tumours treated with anti-PD-1 mAb. Second, anti-PD-1 mAb treatment enhanced the infiltrations of T cells into tumours. Furthermore, significant increases of several pro-inflammatory cytokines were also confirmed in tumours treated with anti-PD-1 mAb. Thus, PD-1 blockade induced T cell infiltrations, thereby resulting in local immune activation against tumours. Interestingly, although tumour vessels were significantly reduced by VEGFR2 blockade, tumour T cell infiltration was not interfered with the treatment. This paradoxical phenomenon may be explained by the normalization of tumour vessels induced by anti-angiogenesis treatment [16]. The normalized tumour vessels restore blood flow and improve the ability to transport oxygen, anticancer drugs as well as immune cells to the

tumour [27-30]. Consistent with our data, previous studies investigating the combination of tumour immunotherapy with antiangiogenic therapy also have shown that anti-angiogenesis treatment does not impede the infiltrations of immune competent cells into tumours [19, 21, 31]. In addition, since regulatory T cells also selectively express PD-1, it is possible that PD-1 blockade suppressed regulatory T cells and inhibited tumour growth [32]. However, our data analyzing FOXP3 expression suggested that regulatory T cells did not play a significant role in this model. Interestingly, there were no direct effects of PD-1 or VEGFR2 blockade on cancer cell growth as demonstrated by in vitro studies. Therefore, combining PD-1 and VEGFR2 blockades may exert their antitumour efficacy through controlling tumour microenvironments by activating tumour–infiltrating lymphocytes and inhibiting tumour neovascularization. Taken together, anti-angiogenesis strategy may be a good candidate for combination with immune checkpoint blockade in cancer therapy.

Immunotherapy has long been expected to become a powerful anti-cancer treatment that can be tumour-specific and less toxic [33]. It includes cancer vaccine and adoptive cell therapy. However, to date, there are few definitive evidences for their efficacy in clinical cancers. Besides these conventional immunotherapies, monoclonal antibody-based treatments of targeting T cell negative regulatory pathways, CTLA-4 and PD-1, have been recently

introduced and evaluated. A recent large-scale randomized clinical trial demonstrated that immunotherapy using anti-human CTLA-4 monoclonal antibody improved overall survival in metastatic melanoma [4]. To our knowledge, that is the first strong evidence that immunotherapy has worked in actual human cancer. In general, there are many pathways and mechanisms involved in tumour development and progression. Thus, it may be difficult to induce complete cure by monotherapy or single anti-cancer method especially for intractable tumours. Toward future clinical application, other combinational therapy with blockade of immune checkpoints should be evaluated in order to achieve synergistic antitumour effect and less systematic toxicity. In fact, several previous preclinical in vivo studies have shown that the combination of blockade of PD-L1/PD-1 pathway with the simultaneous use of gemcitabine [8], anti-LAG-3 [34], or anti-TIM3 mAb [35] exerted a significant antitumour efficacy without overt toxicity. Furthermore, the other immune checkpoints including B7-H3 [36], LAG3 [34], or TIM3 [35] should be also evaluated in the combination of anti-angiogenesis treatment. In addition, VEGFR1 has become recognized to have unique and diverse activities including cancer cell survival and migration [37]. Therefore, combination of PD-1 and VEGFR1 blockades warrants further investigation.

Clearly, further studies will be required to achieve definitive conclusions. First, long-term treatment of combination of PD-1 and VEGFR2 blockade needs to be assessed. In this study, tumour growth became noticeable after withdrawal of antibody treatment. It may be desirable that immunotherapy can induce tumour-specific memory cells that prevent tumour recurrence. Therefore, the sustained beneficial and adverse effects by long-term administration of both mAbs need to be evaluated. Second, more fundamental mechanistic studies should be also performed, since some of our data failed to demonstrate statistical significance.

In conclusion, we have shown for the first time that the combination of PD-1 and VEGFR2 had induced a synergistic in vivo antitumour effect without overt toxicity. This unique strategy may have clinical relevance and should have a potential to be evaluated in future clinical trial.

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Disclosure

None of the authors has any financial conflicts to disclose.

Figure legends

Fig. 1. Simultaneous blockade of PD-1 and VEGFR2 induced synergistic antitumour effect in vivo. BALB/c mice were subcutaneously inoculated with Colon-26 cells and were given with control rat IgG, anti-PD-1 mAb, anti-VEGFR2 mAb or both mAbs for 5 times (*arrow*). Data are presented as mean ± standard error of 7-10 mice of each group. *$P < 0.05$; **$P < 0.01$.

Fig. 2. PD-1 and VEGFR2 blockade did not have any direct effect on cancer cell growth in vitro. A total of 3000 Colon-26 cells were co-cultured with anti-PD-1 mAb, anti-VEGFR2 mAb or control rat IgG for 72 hours, and cell proliferation was determined by MTS assay.

Fig. 3. Treatment with anti-VEGFR2 mAb inhibited tumour neovascularization. (a) Immunohistochemistry analysis by staining with CD34. Representative tumours from mice treated with control rat IgG, anti-PD-1 mAb, anti-VEGFR2 mAb, or both mAbs. (b) Tumour microvessels were counted at 200x magnification. Data are collected from 4-7 mice of each group. *$P < 0.01$; **$P < 0.001$.

Fig. 4. (a) Immunohistochemical staining of CD4+ and (b) CD8+ T cells in tumour tissue at day 11. Representative pictures of mice for each treatment are shown. PD-1 blockade and combination treatment seemed to induce more CD4+ and CD8+ T-cell infiltration compared to control and VEGFR2 blockade. (c) Quantification of tumour-infiltrating CD4+ and (d) CD8+ T-cells by real time PCR. There was a tendency toward increased T-cell infiltration by

the treatment of anti-PD-1 mAb and combination treatment. Anti-VEGFR2 mAb treatment did not interfere with T-cell infiltration. Data are collected from 4-7 mice of each group.

Fig. 5. Expression of IFN-γ, TNF-α, and granzyme B was significantly upregulated by anti-PD-1 mAb or combination mAb treatment compared with control. Treatment of anti-VEGFR2 mAb alone did not increase each cytokine expression. Data are collected from 4-7 mice of each group. *$P < 0.05$.

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Fig 1

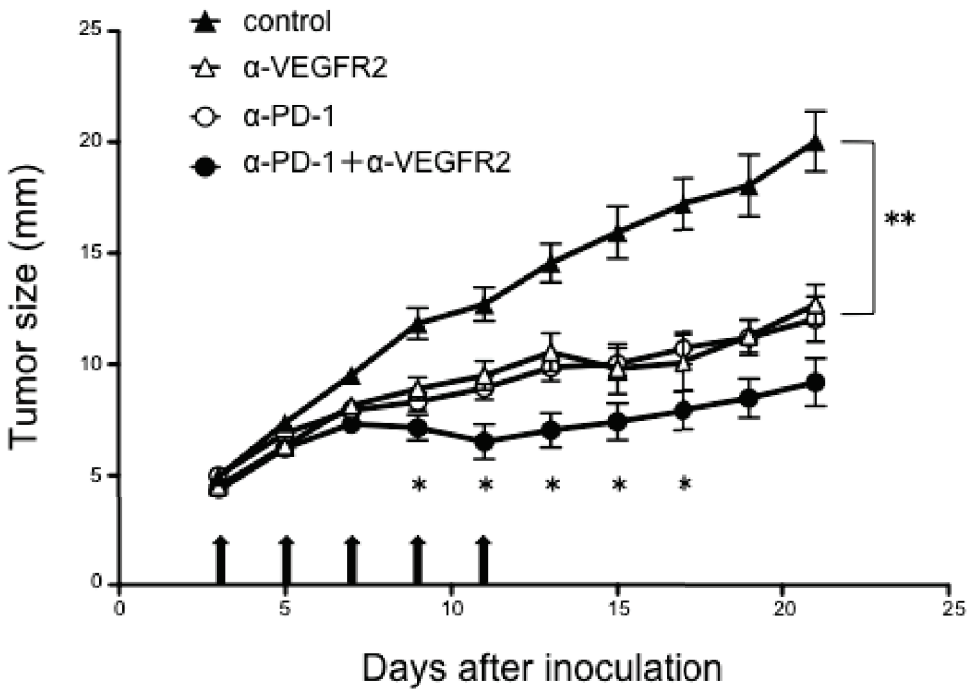


Fig.2

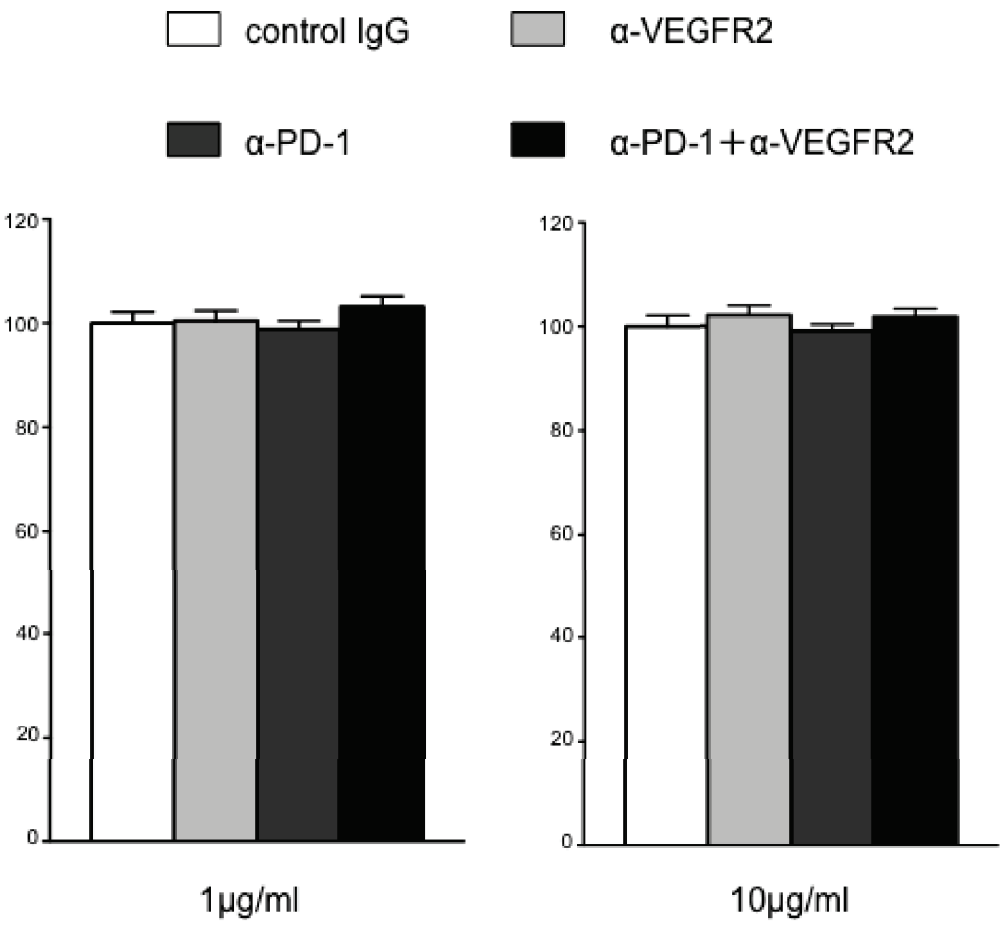
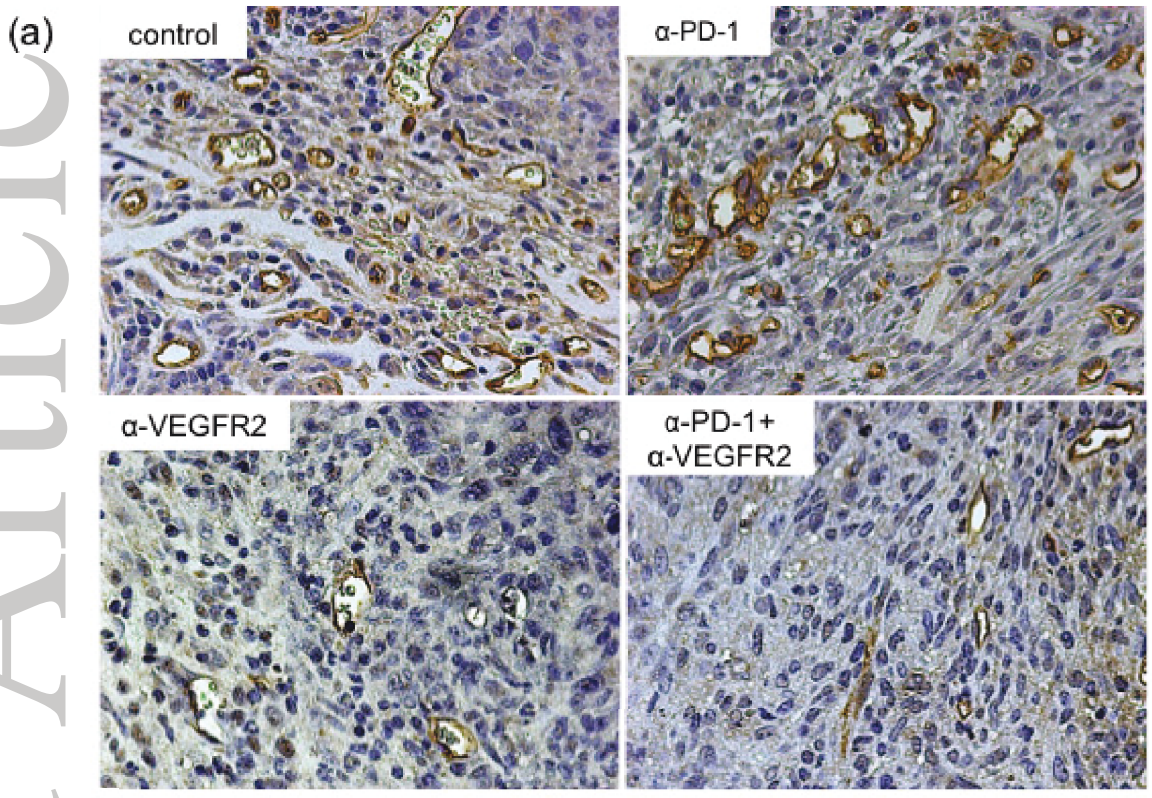


Fig.3



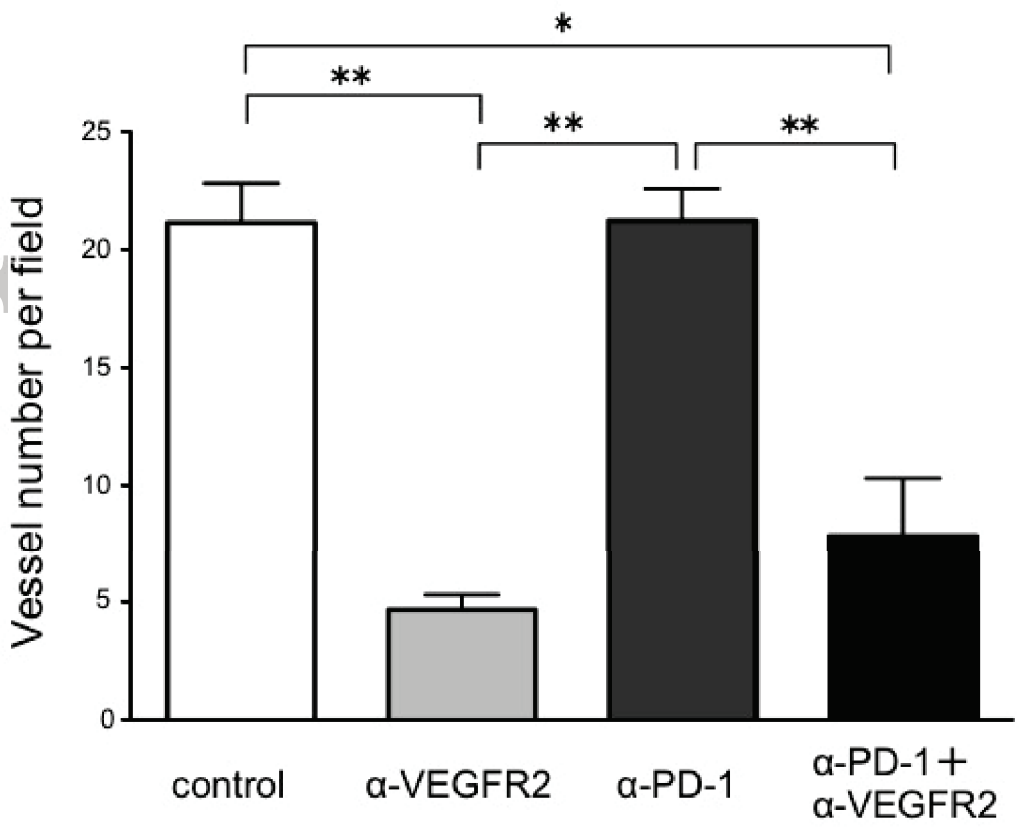


Fig.4

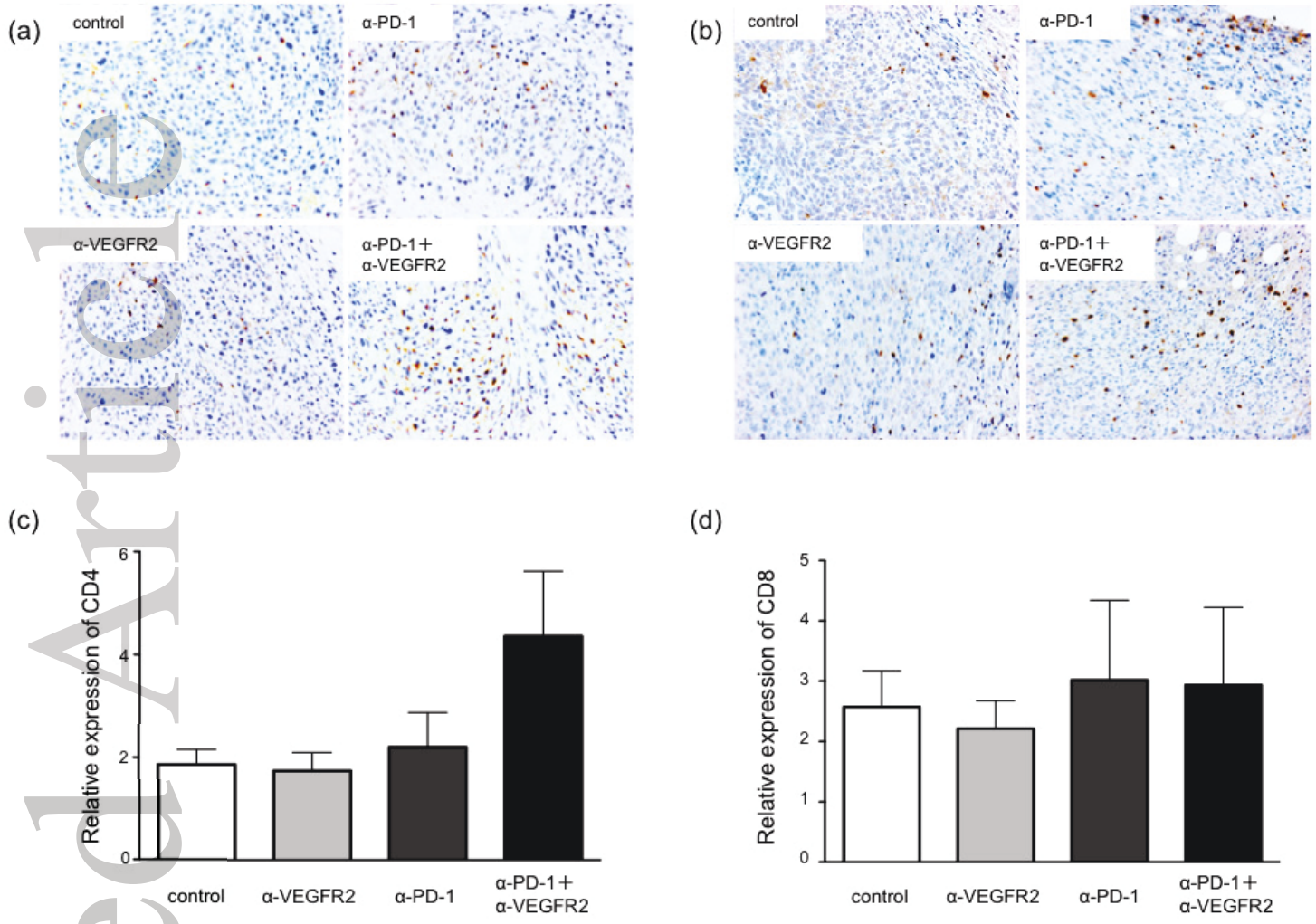


Fig.5