Am J Cancer Res 2019;9(7):1382-1395
www.ajcr.us /ISSN:2156-6976/ajcr0097826

Original Article

Lenvatinib promotes antitumor immunity by enhancing the tumor infiltration and activation of NK cells

Qing Zhang[1,2*], Hongyan Liu[1,2,3*], Hanhan Wang[1,2*], Mengmeng Lu[1,2], Yangna Miao[1,2], Jiage Ding[1,2], Huizhong Li[1,2], Xiaoge Gao[1,2], Shishuo Sun[1,2], Junnian Zheng[1,2]

[1]Cancer Institute, Xuzhou Medical University, Xuzhou, Jiangsu, P. R. China; [2]Center of Clinical Oncology, Affiliated Hospital of Xuzhou Medical University, Xuzhou, Jiangsu, P. R. China; [3]The Second People's Hospital of Lianyungang, Lianyungang, Jiangsu, P. R. China. [*]Equal contributors.

Received January 22, 2019; Accepted June 7, 2019; Epub July 1, 2019; Published July 15, 2019

Abstract: Based on previous reports, the efficacy of lenvatinib against cancer is mainly attributed to its antiangiogenic activity and its ability to suppress tumor proliferation, which are mediated by targeting receptor tyrosine kinases (RTKs). However, the effects of lenvatinib on tumor immune modulation have rarely been explored. Here, we show that lenvatinib effectively inhibited murine melanoma and renal cancer, and this inhibition was associated with enhanced tumor infiltration by natural killer (NK) cells. Critically, lenvatinib-induced tumor growth inhibition was attenuated by antibody-mediated NK cell depletion or the blockade of NK cell chemotaxis with an anti-CXCR3 blocking antibody. In addition, the expression of natural cytotoxicity receptors (NCRs) by tumor-infiltrating NK cells and the expression of cytotoxic cytokines in the tumor tissue were also augmented by lenvatinib. These data thus suggest that lenvatinib may be used not only as a direct cytotoxic drug against tumor angiogenesis and proliferation but also as a potent adjunct for enhancing the efficacy of immune-based cancer therapies by enhancing the tumor infiltration and activation of NK cells.

Keywords: Lenvatinib, NK cell, tumor infiltration, activation, melanoma, renal cancer, chemokine

Introduction

Lenvatinib is an orally active inhibitor of receptor tyrosine kinases (RTKs), including KDR (VEGFR-2), FGFR1, Flt-1 (VEGFR-1), RET, PDGFR-β and c-kit [1]. Lenvatinib was granted an Orphan Drug Designation (ODD) for thyroid cancer in Japan, the United States and Europe between 2012 and 2013 [2]. The efficacy of lenvatinib was first confirmed in a phase II study that enrolled 58 patients with progressive radioiodine (RAI)-refractory differentiated thyroid cancer (DTC). The objective response rate (ORR) was 50%, 40% of patients achieved stable disease (SD), and 28% of patients experienced durable SD (≥23 weeks). The median progression-free survival (PFS) was 12.6 months. The beneficial effect of lenvatinib on PFS in RAI-refractory DTC was subsequently confirmed in a phase III clinical trial involving 392 patients. The ORR was 64.8%, and 15% of patients achieved SD. The median PFS rate was 18.3% [3].

In addition to thyroid cancer, combination therapy with lenvatinib and everolimus was also approved for advanced renal cell carcinoma (RCC) by the U.S. FDA based on the positive results of a clinical trial in 2016 [4, 5]. In the randomized, phase II, multi-center clinical trial (NCT01136733), 153 patients with advanced RCC were randomly allocated to receive single-agent lenvatinib (n = 52), single-agent everolimus (n = 50), or the combination of lenvatinib plus everolimus (n = 51). The median PFS was higher for the combination of lenvatinib plus everolimus (14.6 months) than for everolimus alone (5.5 months) or lenvatinib alone (7.4 months). The ORR was higher for the combination therapy (43%) than for the single-agent everolimus (6%) or single-agent lenvatinib therapy (27%).

Due to the broad-spectrum inhibitory potential of lenvatinib, lenvatinib was also tested in several other clinical trials, including trials of glioma (NCT01137604), hepatocellular carcinoma

(NCT00946153), non-small cell lung cancer (NCT00832819) and melanoma (NCT01136967, NCT01133977, NCT00121680). In a phase Ib study (NCT00121680), 32 patients with stage 4 or unresectable stage 3 melanoma were treated with a combination therapy of lenvatinib plus temozolomide. The overall ORR was 18.8%. SD ≥16 weeks was achieved in 28.1% of the patients [6]. In another phase I trial, lenvatinib showed an ORR of 17.2% in 29 enrolled melanoma patients [7].

Based on current reports, the efficacy of lenvatinib against these cancers is mainly attributed to the antiangiogenic activity and tumor proliferation suppression mediated through targeting RTKs [8-10]. In this study, we unveiled a new mechanism of promoting the tumor infiltration and activation of NK cells, through which lenvatinib exerts its therapeutic effect.

Materials and methods

Cell culture

Murine B16-F10 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco by Life Technologies, America) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO_2 air atmosphere. The murine RCC cell line Renca was maintained in RPMI1640 medium supplemented with 10% FBS, 0.1 mM MEM nonessential amino acids (NEAA) (11140-050, Gibco), 1 mM additional sodium pyruvate (111360-070, Gibco), and 2 mM additional L-glutamine (25030-081, Gibco) at 37°C in a 5% CO_2 atmosphere. All cell lines were authenticated and tested negative for mycoplasma.

In vivo therapeutic efficacy of lenvatinib

Female BALB/c or C57BL/6N mice, aged 6 weeks to 8 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Five to six mice were housed together in sterilized cages and maintained under specific pathogen-free conditions. The mice were acclimated for approximately 1 week in our animal facility before testing was initiated. The local committee for animal care approved all animal studies. A total of 2×10^5 B16-F10 cells or 5×10^6 Renca cells were suspended in 100 μl of DMEM or RPMI 1640 medium without FBS or penicillin/streptomycin and subcutaneously injected into the right flank of C57BL/6N or BALB/c mice, respectively. Three days later, the mice were randomly divided into 2 groups and treated with vehicle (5% methylcellulose in water) or lenvatinib (10 mg/kg) once per day. Two weeks later, the mice were anesthetized, and their tumors were harvested and weighed. The tumor tissue was cut and preserved in liquid nitrogen or formalin or processed into a single-cell suspension for further analysis.

In vivo NK cell depletion

A total of 2×10^5 B16-F10 cells were suspended in 100 μl of DMEM without FBS or penicillin/streptomycin and subcutaneously injected into the right flank of 6- to 8-week-old female C57BL/6N mice. Four days later, the mice were randomly assigned to 3 groups: untreated, lenvatinib+isotype control antibody (Len+Iso) and lenvatinib+NK cell depletion antibody (Len+PK136). The mice in the Len+Iso group and Len+PK136 group were treated with lenvatinib by gavage once per day on days 5-16. In addition, the mice in the Len+Iso and Len+PK136 groups received 200 μg isotype antibody or NK cell depletion antibody, respectively, via intraperitoneal injection (i.p.) once every three days. During the treatment, the mice were bled via the tail vein to detect the NK cell depletion effect. The mice were euthanized with anesthetics on day 17, and the tumors were removed and weighed. The tumor tissue was preserved in liquid nitrogen or formalin or processed into a single-cell suspension for further analysis. An anti-mouse NK1.1 monoclonal antibody (clone, PK136) and its isotype control monoclonal antibody mouse IgG2a, κ (clone, C1.18.4) were purchased from Bio X Cell.

Flow cytometry analysis

For sample preparation, the peripheral blood was collected via the tail vein and preserved in PBS containing 10 mM EDTA. Red blood cells were lysed by hypotonic lysis using RBC Lysis Buffer (64010-00-100, BioGems) after a 10-minute incubation in the dark at room temperature. The tumors were diced with ophthalmic scissors and then digested by incubating at 37°C for 1 hour in RPMI 1640 medium containing 2% FBS, 2 mg/mL Collagenase IV, and 2 mg/mL DNase I. After digestion, the cell suspensions were strained through a 70-μm mesh

filter. Then, the cells were washed twice with PBS containing 2% fetal bovine serum.

For cell staining, the single-cell suspensions prepared from the peripheral blood or tumor were stained with surface marker antibodies and analyzed on a FACS Canto II flow cytometer (Becton-Dickinson, USA) or Guava easyCyte (Merck Millipore, USA). FITC-conjugated anti-mouse CD45 (30-F11), FITC-conjugated rat IgG2b, k isotype (RTK4530), PE-conjugated anti-mouse NK-1.1 (PK136), PE-conjugated mouse IgG2a, k isotype (MOPC-173), FITC-conjugated anti-mouse CD49b (DX5), FITC-conjugated rat Lewis (LEW), PE-conjugated anti-mouse CD3e (145-2C11), PE-Cy7-conjugated Armenian hamster IgG (HTK888), PE-Cy7-conjugated anti-mouse VCAM-1 (429 (MVCAMA)), PE-Cy7-conjugated rat IgG2a, k isotype (RTK2758), PE-Cy7-conjugated anti-mouse CD16 (93), PE-Cy7-conjugated rat IgG2a, λ isotype, APC-conjugated anti-mouse CXCR3 (CXCR3-173) and APC-conjugated Armenian hamster IgG antibodies were purchased from Biolegend. FITC-conjugated anti-mouse NKp46 (29A1.4) and FITC-conjugated rat IgG2a, k isotype (Ebr2a) antibodies were purchased from eBioscience. 7-Aminoactinomycin D (CAT#A13-10) and Live/Dead Green dye (L23101) were purchased from Life Technologies. Data analysis was performed using FlowJo 10 software (Ashland, OR).

Hematoxylin and eosin staining and IHC

Tissue samples were dissected, paraffin-embedded and sectioned at 4 μm thick. To expose target proteins, heat-induced epitope retrieval (HIER) was performed in sodium citrate buffer (pH 6.0) for 20 minutes at 100°C. The sections were blocked in 10% BSA for 20 minutes at room temperature, and antibodies against CD161/NK1.1 (PK136, NB100-77528) were applied to the sections and incubated overnight at 4°C. Detection was performed using 100 μl of goat anti-mouse/rabbit secondary antibody from a two-step histostaining reagent kit (Beijing Zhong Shan Jinqiao Biotechnology Co., Ltd.) for 30 minutes at room temperature according to the manufacturer's protocol. The immunoreaction was visualized when a brown precipitate formed following incubation in diaminobenzidine. The sections were subsequently washed with water and counterstained with 0.5% hematoxylin for 3 minutes at room temperature. Finally, the tissue samples were visualized by an upright light microscope (Olympus Corporation, Tokyo, Japan).

Western blot

The B16-F10 cells were treated with vehicle, lenvatinib (1000 nM, 300 nM, 100 nM) or lenvatinib plus NK cell depletion antibody (PK136, 100 μg/mL). 4 hours later, cells were lysed in RIPA buffer with protease inhibitor for 30 minutes on ice. The supernatant was collected after centrifuging at 13,000 g for 15 minutes and quantified by BCA protein concentration assay kit. Equal protein was performed with SDS-PAGE and transferred to the PVDF membrane. The membrane was blocked with 5% non fat-dried milk for 1 hour at room temperature. Primary antibodies (phospho-Erk1/2 (4370S), Erk1/2 (4695S), Cell Signaling Technology, USA) were added and incubated overnight at 4°C. The next day, secondary antibodies (goat anti-rabbit IgG-HRP (AS001), Absin) were added and incubated for 1 hour at room temperature. Chemical signals were detected with Tanon imaging system (Tanon Science & Technology Co., Ltd, China).

Chemokine array analysis

Mouse melanoma B16-F10 tumors that received the indicated treatments were processed according to the manufacturer's protocol. Equal amounts of lysates were analyzed with a Proteome Profiler Mouse Chemokine Array kit (R&D Systems, ARY020). Quantification of the spot intensity in the arrays was conducted with background subtraction in ImageJ.

Real-time cell assay (RTCA)

The cytotoxic activity of NK cells sorted from tumors was determined using an xCELLigence RTCA TP instrument (ACEA Biosciences). First, 50 μL of B16-F10 cell culture media was added to each well of 2 × 8-well E-Plates (ACEA Biosciences), and the background impedance was measured and displayed as Cell Index. Then, B16-F10 cells were seeded at a density of 10000 cells/well of the E-Plate in a volume of 100 μL and allowed to passively adhere on the electrode surface. Post seeding, the E-Plate was kept at ambient temperature inside a lami-

nar flow hood for 30 minutes and then transferred to the RTCAMP instrument inside a cell culture incubator. Data recording was initiated immediately at 15-minute intervals for the entire duration of the experiment. After approximately 10 hours, the B16-F10 cells reached a logarithmic growth phase, and NK cells were added at an effector to target ratios (E:T) = 5:1 in a volume of 50 μL. After transferring the E-Plate back into the xCELLigence system, data acquisition was resumed to monitor the NK cell cytotoxicity based on the viability of attached target cells, as reflected by Cell Index values.

Statistical analysis

The data were analyzed using GraphPad Prism 5 software and presented as the mean ± SEM. The significance of differences between the results of two groups was evaluated using an unpaired two-tailed Student's t test. A *P* value less than 0.05 was considered statistically significant.

Results

Lenvatinib significantly inhibits tumor growth and promotes the infiltration of NK cells into tumors

To confirm the therapeutic efficacy of lenvatinib in vivo, murine melanoma models were established in C57BL/6N mice with the mouse melanoma cell line B16-F10. The protocols for model establishment and treatment are shown in **Figure 1A**. After 12 days of treatment, compared with vehicle, lenvatinib showed a 70.4% tumor weight reduction in the mice with B16-F10 xenografts (**Figure 1B**). These data demonstrated that lenvatinib can significantly inhibit the growth of melanoma in mouse models.

To investigate the effects of lenvatinib on NK cell infiltration, we first detected NK cells in formalin-fixed tumor tissue by immunohistochemical staining analysis with an anti-mouse NK1.1 antibody (Clone PK136). As shown in **Figure 1C** and **1D**, the positive rate of $NK1.1^+$ cells in the lenvatinib-treated tumor tissue was approximately 6-fold higher than that in the vehicle-treated tumor tissue. Second, we detected the frequency of NK cells in single-cell suspensions from the vehicle- or lenvatinib-treated tumors by flow cytometry analysis with a PE-conjugated anti-mouse NK1.1 antibody (Clone PK136). As shown in **Figure 1E** and **1F**, lenvatinib treatment led to an approximately 3-fold increase in the quantity of NK cells in the tumor tissue compared with the vehicle treatment. To further confirm these findings, we detected the mRNA expression of NK1.1 in vehicle- or lenvatinib-treated tumor tissue by qRT-PCR. The qRT-PCR results showed significantly increased NK1.1 mRNA expression in the lenvatinib-treated tumor tissue compared with that in the vehicle-treated tumor tissue (**Figure 1G**). Consistent results were also observed in the murine renal cancer models (**Figure 2**). NK cells represent a crucial component of the antitumor innate immune response. These data suggest that promoting NK cell infiltration into tumors may be an important mechanism through which lenvatinib exerts its antitumor effects.

Depletion of NK cells attenuates lenvatinib-induced tumor growth inhibition

If lenvatinib-induced NK cell tumor infiltration plays a dominant role in the antitumor immune response, then the removal of NK cells should attenuate the inhibitory effects of lenvatinib on tumor growth. To test this hypothesis, C57BL/6N mice with B16-F10 xenografts were pretreated with an NK cell depletion antibody (Clone PK136) [11], followed by lenvatinib treatment (**Figure 3A**). NK cells in the blood were continuously detected during the treatment. NK cells were almost completely removed in the mice treated with the depletion antibody during the treatment (**Figure 3B**). At the end of the treatment, tumor-infiltrating NK cells were detected. Almost no NK cells were detected in the tumor tissue of the mice that received the NK cell depletion antibody (**Figure 3C**, **3D**). More importantly, we found that NK cell depletion significantly attenuated the tumor growth inhibition induced by lenvatinib (**Figure 3E**). These data suggest that NK cells may play an important role in the lenvatinib-mediated antitumor effects.

Does NK cell depletion antibody treatment interfere with the receptor tyrosine kinase inhibitory activity of lenvatinib? To answer this question, we performed western blot analysis to detect downstream signals ERK and phosphorylated (Phospho-) Erk1/2 of receptor tyrosine kinases [8, 12, 13]. The B16-F10 cells

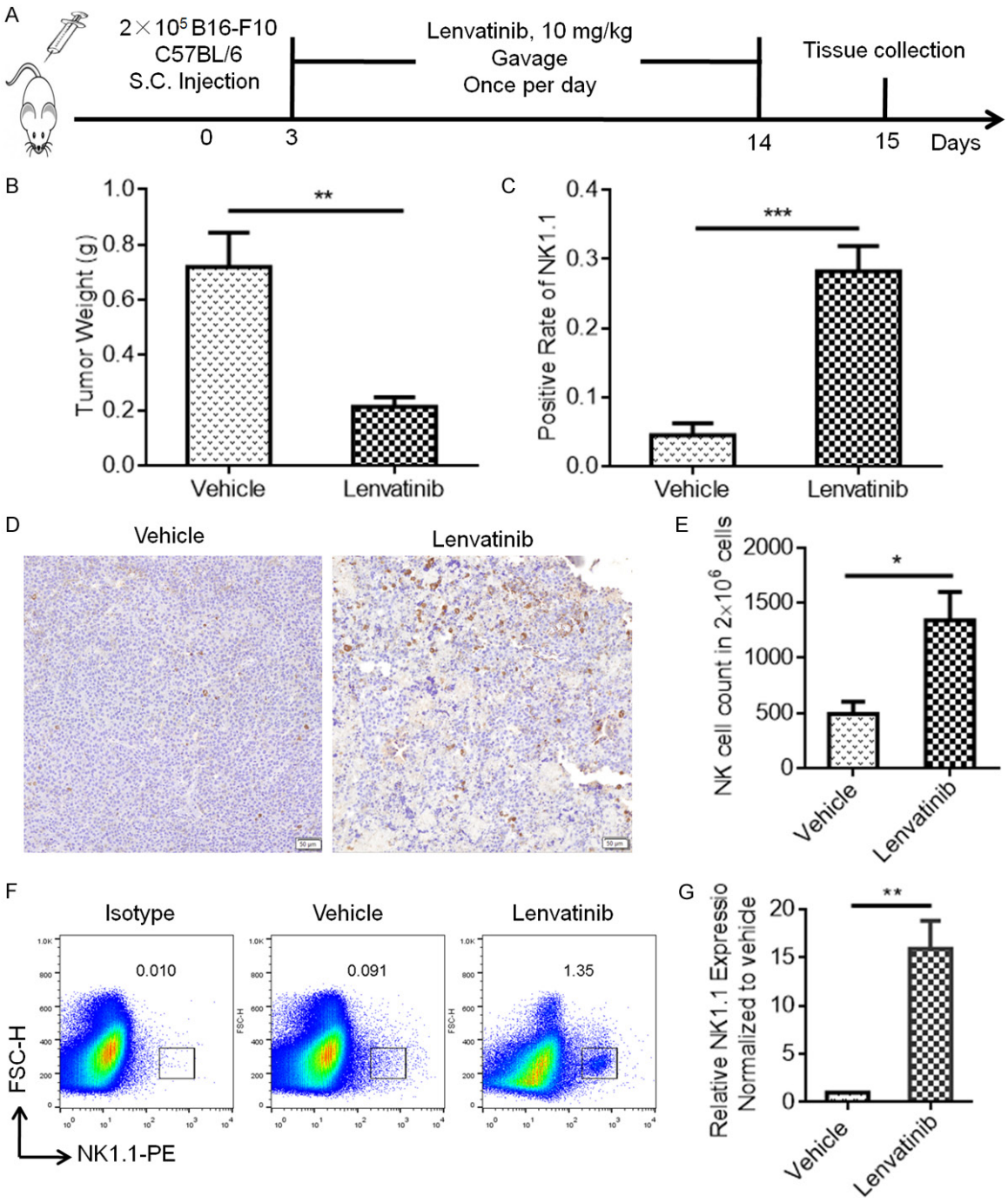

Figure 1. Lenvatinib suppresses tumor growth in murine melanoma models. A. Schematic diagram showing the treatment program of the mice. A total of 2 × 10^5 B16-F10 cells were inoculated into the right flank of C57BL/6N mice to establish a murine melanoma model. The tumor-bearing mice were divided into two groups and treated with lenvatinib (10 mg/kg) or vehicle (5% methylcellulose) for 12 days. B. The average tumor weight of each group of the C57BL/6N murine melanoma model at the end of the treatment. C and D. The summarized data and representative results of NK cell staining of vehicle- or lenvatinib-treated tumors by IHC. E and F. The summarized data and representative results show the numbers and frequencies of NK cells in the vehicle- or lenvatinib-treated tumors measured by flow cytometry analysis. G. The mRNA expression levels of NK1.1 were analyzed by qRT-PCR. n = 8 for each independent experiment. The experiment was repeated for three times. Representative results of one independent experiment are shown. *P<0.05, **P<0.01, ***P<0.001.

were treated with vehicle, lenvatinib (1000 nM, 300 nM, 100 nM) or lenvatinib plus NK cell depletion antibody (PK136, 100 μg/mL) for 4 hours. The dose of NK cell depletion antibody is

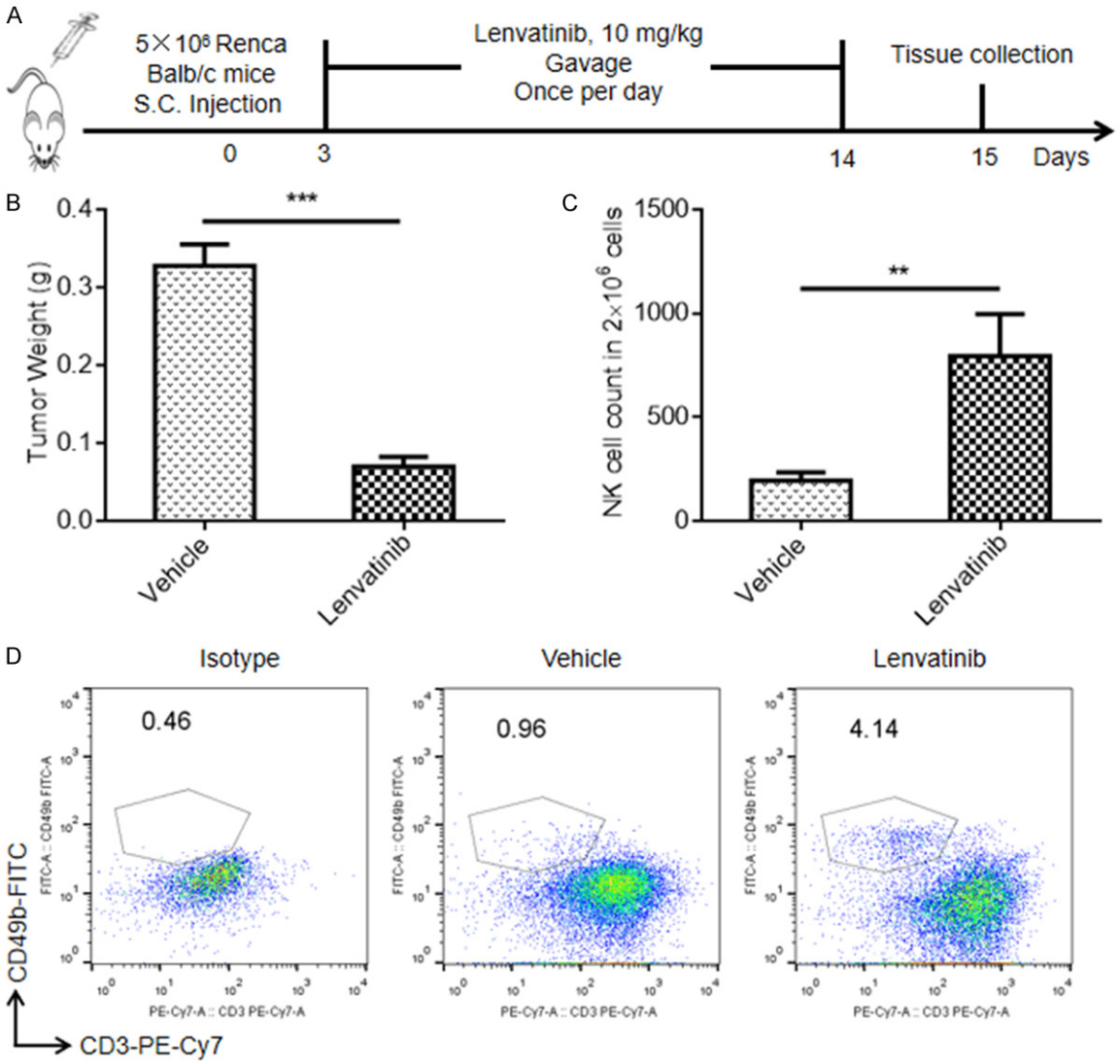

Figure 2. Lenvatinib suppresses tumor growth in murine renal cancer models. A. Schematic diagram showing the treatment program of the mice. A total of 5 × 10^6 Renca cells were inoculated into the right flank of BALB/c mice to establish a murine renal cancer model. The tumor-bearing mice were divided into two groups, which were treated with lenvatinib (10 mg/kg) or vehicle (5% methylcellulose) for 12 days. B. The average tumor weight of each group of BALB/c mice with murine renal cancer at the end of the treatment. C and D. The summarized data and representative dot plots show the numbers and frequencies of NK cells in tumors from BALB/c mice. NK cells are defined as $CD3^-CD49b^+$ cells in BALB/c mice. The experiment was repeated twice. n = 8 for each independent experiment. Representative results of one independent experiment are shown. $^{**}P<0.01$, $^{***}P<0.001$.

200 μg/mouse in in vivo experiments. The concentration of 100 μg/mL should be higher than the plasma concentration of the depletion antibody. As shown in **Figure 3F**, the phosphorylation of ERK was significantly inhibited by lenvatinib at the dose of 1000 nM, the NK cell depletion antibody did not attenuate the inhibitory efficacy of lenvatinib. Therefore, NK cell depletion antibody treatment does not interfere with the receptor tyrosine kinase inhibitory activity of lenvatinib. These data further demonstrated that NK cells play an important role in the lenvatinib-mediated anti-tumor effects.

Lenvatinib enhances the expression of NK cell infiltration-associated adhesion molecules and chemokines in tumors

Increased NK cell infiltration may occur because the lenvatinib treatment increases the recruitment of NK cells to the tumor site. These processes involve a complex sequence of

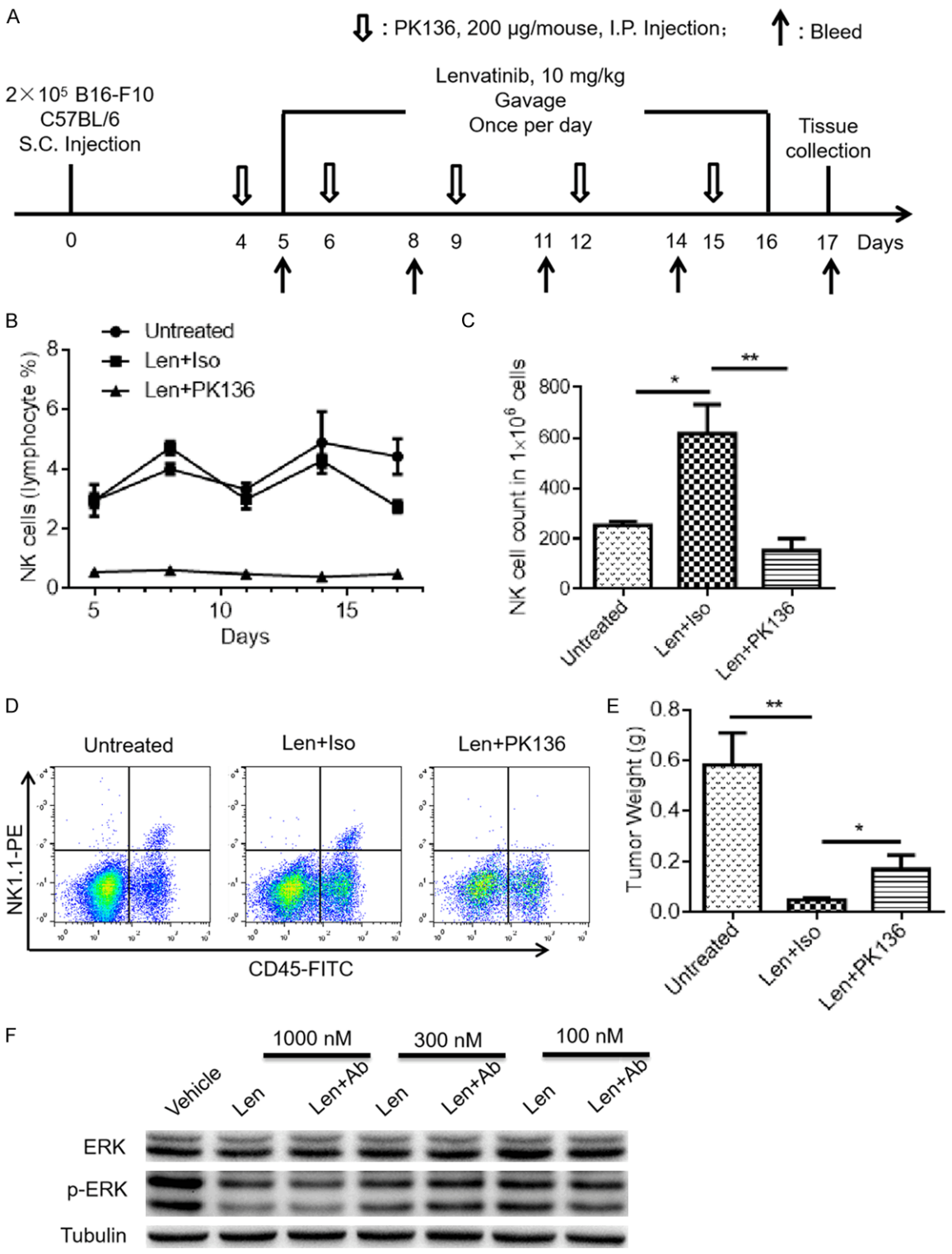

Figure 3. Depletion of NK cells attenuates lenvatinib-induced tumor growth suppression. A. Schematic diagram showing the treatment program of the mice. The mice were randomly assigned to 3 groups: Untreated, Lenvatinib+Isotype (Len+Iso) and Lenvatinib+PK136 (Len+PK136), n = 6. Intraperitoneal antibody injections were started four days after the subcutaneous inoculation of B16-F10 cells in the C57BL/6N mice. Lenvatinib treatment by gavage was started five days after the tumor cell inoculation. During these treatments, the mice were bled to detect NK cell depletion. B. Continuous NK cell depletion in the mice that received the NK cell depletion antibody. Fifty microliters of blood was collected from each mouse via the tail vein. After red blood cell lysis, the cells were stained with the

anti-NK1.1 antibody and detected by flow cytometry analysis. C and D. The summarized data and representativedot plots show the effect of NK cell depletion on tumors after PK136 treatment. The tumor tissue was processed into a single-cell suspension. A total of 1×10^6 cells were stained with the anti-NK1.1 antibody. The NK cell numbers were detected by flow cytometry analysis. E. The average tumor weight of each group at the end of the treatment. n = 6 for each independent experiment. The experiment was repeated twice. F. ERK and phosphorylated (Phospho-) Erk1/2 were detected by western blot. The B16-F10 cells were treated with vehicle, lenvatinib (1000 nM, 300 nM, 100 nM) or lenvatinib plus NK cell depletion antibody (PK136, 100 μg/mL) for 4 hours. Total protein was extracted. ERK and phosphorylated (Phospho-) Erk1/2 were detected by western blot. The experiment was repeated for three times. The results are consistent. Representative results of one independent experiment are shown. *P<0.05, **P<0.01.

events, beginning with the adhesion of NK cells to endothelial cells and followed by chemokine-chemokine receptor interactions that modulate the extravasation of NK cells into tumor tissue [14]. Integrin α4β7 is found on the majority of peripheral lymphocytes, including NK cells. Integrin α4β7 binds its ligands, including VCAM-1 (CD106), on tumor vascular endothelial cells and plays an important role in lymphocyte adhesion and the directional migration of blood lymphocytes into extravascular tumor tissue [15]. To investigate the mechanism driving the increased infiltration of NK cells into the tumor site, we detected the expression of these adhesion molecules in tumor vascular endothelial cells and tumor-infiltrating NK cells. Our data showed significantly increased cell surface expression of integrin α4β7 in tumor-infiltrating NK cells (**Figure 4A**, **4B**) and of VCAM-1 in tumor vascular endothelial cells (**Figure 4C**, **4D**). To further address the effects of lenvatinib treatment on the expression of chemokines in the tumor, we performed a chemokine array analysis with the tumor tissue. This analysis revealed a statistically significant upregulation of the expression of the chemokines CXCL9 and CXCL10 in the lenvatinib-treated tumors compared with that in the vehicle-treated tumors (**Figure 4E**, **4F**). These data suggest that the increased NK cell infiltration at the tumor site may be due to the increased expression of adhesion molecules and chemokines caused by the lenvatinib treatment.

Blockade of NK chemotaxis attenuates NK cell infiltration and tumor growth inhibition caused by lenvatinib treatment

A previous report showed that CXCL9 and CXCL10 are implicated in NK cell chemotaxis from the periphery through interacting with their receptor (CXCR3) expressed on the surface of NK cells [16]. To further confirm the role of the two chemokines in the current study, we first detected the expression of CXCR3 on the surface of tumor-infiltrating NK cells by flow cytometry analysis. Our data showed that more than 50% of the tumor-infiltrating NK cells were CXCR3-positive, although there was no significant difference between the vehicle and lenvatinib treatment groups (data not shown). This result is consistent with that of a previous report [17]. To determine whether CXCL9 and CXCL10 are essential for lenvatinib-mediated tumor regression, the B16-F10 tumor-bearing C57BL/6N mice were treated with an anti-mouse CXCR3 blocking antibody (CXCR3-173) in conjunction with the lenvatinib treatment (**Figure 5A**) [17]. CXCR3 blockade significantly attenuated lenvatinib-mediated tumor growth inhibition (**Figure 5B**) and increased NK cell infiltration at the tumor site (**Figure 5C**, **5D**). Altogether, these results clearly indicated that the CXCL9- and CXCL10-dependent recruitment of NK cells to the tumor site was essential for the lenvatinib-mediated antitumor immune responses.

Lenvatinib promotes the activation of tumor-infiltrating NK cells

We have demonstrated that NK cells play an important role in the antitumor effects exerted by lenvatinib and mechanistically linked lenvatinib with NK cell infiltration into tumors. Can lenvatinib treatment enhance the activation and cytotoxicity of tumor-infiltrating NK cells? Natural cytotoxicity receptor (NCR)-mediated cytotoxicity through NKp46, NKp44 and NKG2-D and CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) are two of the main mechanisms, through which NK cells release cytotoxic cytokines upon interacting with target cells [18].

To confirm these mechanisms, we first examined the effect of lenvatinib treatment on the expression of CD16, NKp46 and NKG2-D on tumor-infiltrating NK cells by flow cytometry analysis. The results showed that lenvatinib

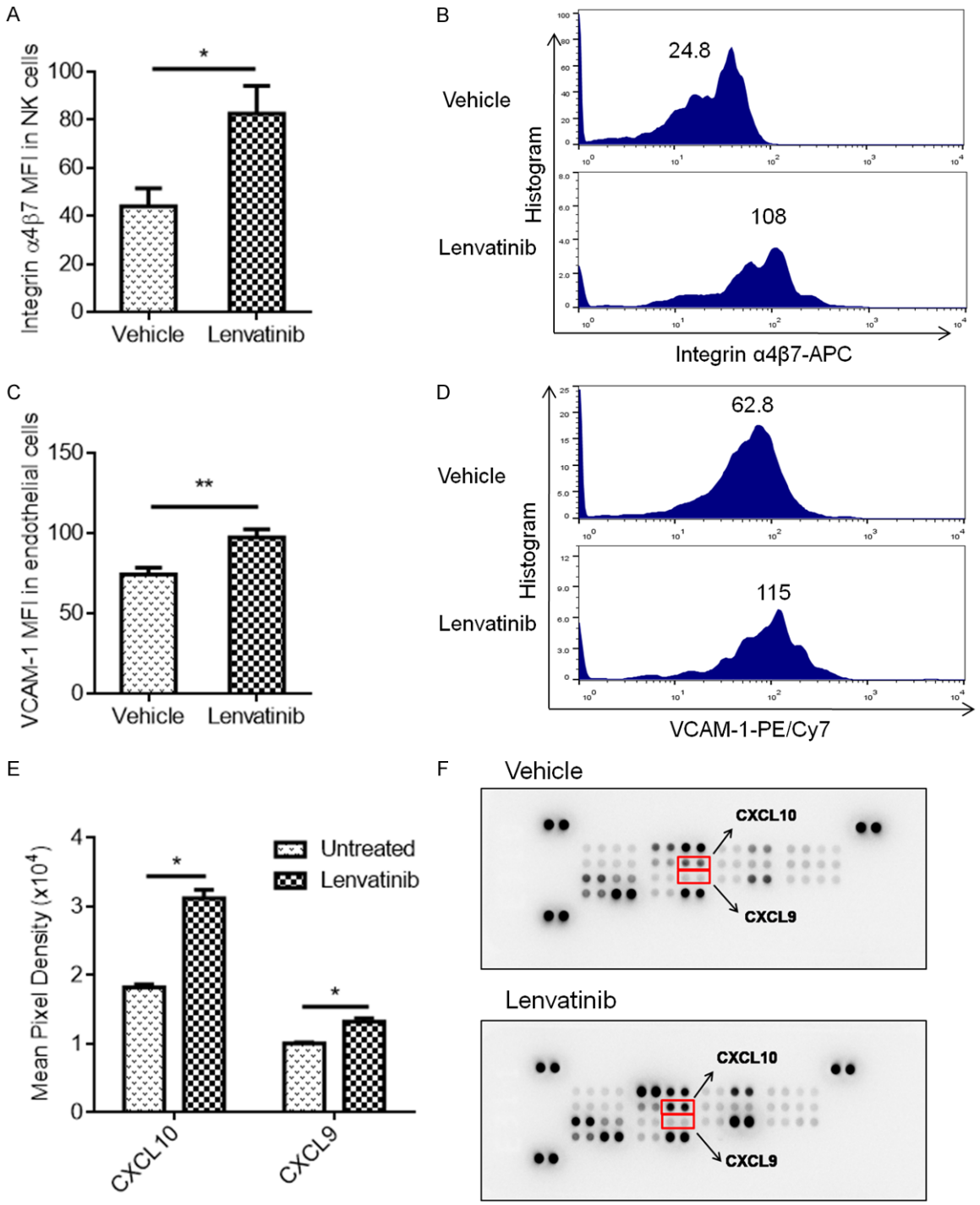

Figure 4. Lenvatinib enhances the expression of NK cell infiltration-associated adhesion molecules and chemokines. Samples from the C57BL/6N mice that received the vehicle or lenvatinib treatment are shown in **Figure 1**. A and B. The summarized data and representative results show the integrin α4β7 expression of the tumor-infiltrating NK cells. C and D. The summarized data and representative results show the VCAM-1 expression of the tumor vascular endothelial cells. E and F. The summarized data and representative films show the expression of the chemokines CXCL9 and CXCL10 in the B16-F10 tumor tissues measured by a mouse chemokine array (n = 2).

treatment significantly increased the cell surface expression of CD16 (**Figure 6A**, **6B**) and NKp46 (**Figure 6C**, **6D**) on the tumor-infiltrating NK cells. Although the lenvatinib treatment increased the expression of NKG2-D on the tumor-infiltrating NK cells compared with the vehicle treatment, the difference was not statistically significant (data not shown). To further

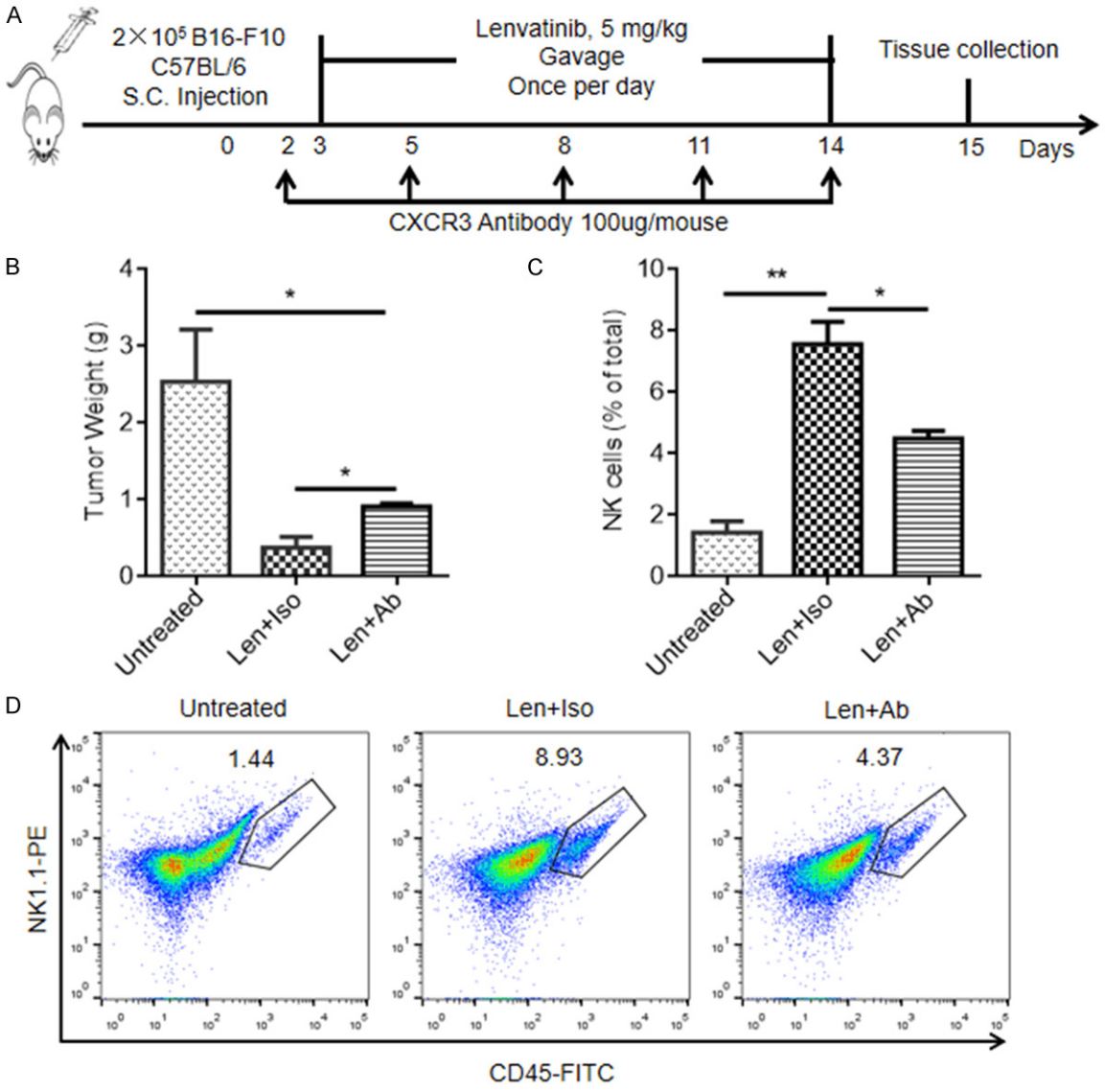

Figure 5. Blockade of NK chemotaxis with the CXCR3 blocking antibody attenuates lenvatinib-induced NK cell infiltration and tumor growth inhibition. A. Schematic diagram showing the treatment program of the mice. The mouse models were set up as described above, and then the mice were randomly assigned to 3 groups: Untreated, Lenvatinib+Isotype (Len+Iso) and Lenvatinib+CXCR3 Antibody (Len+Ab), n = 6. Intraperitoneal antibody injections were started two days after the subcutaneous inoculation of the B16-F10 cells. Lenvatinib treatment by gavage was started three days after the tumor cell inoculation. The mice were euthanized with anesthetics on day 15, and the tumors were removed and weighed. The tumor tissue was processed into a single-cell suspension for further analysis. B. The average tumor weight of each group. C and D. The summarized data and representative dot plots show the frequency of NK cells in the tumor. The tumor tissue was processed into a single-cell suspension. A total of 1×10^6 cells were stained with the anti-mouse CD45 antibody and anti-mouse NK1.1 antibody and were analyzed by a Guava easy Cyte flow cytometer. n = 6 for each independent experiment. The experiment was repeated twice. Representative results of one independent experiment are shown. *P<0.05, **P<0.01.

confirm whether lenvatinib treatment improves cytotoxicity of NK cells, NK cells were sorted from tumors of C57BL/6N mice that received vehicle or lenvatinib treatment (10 mg/kg) and cocultured with B16-F10 cells at an E:T ratio = 5:1 respectively. The direct cytotoxicity of NK cells to B16-F10 cells was analyzed by real-time cell assay (RTCA) (**Figure 6E**). The corresponding quantitative analysis results at 70 h showed that NK cells from tumors of lenvatinib-treated mice show stronger cytotoxicity than their concomitants from vehicle-treated mice

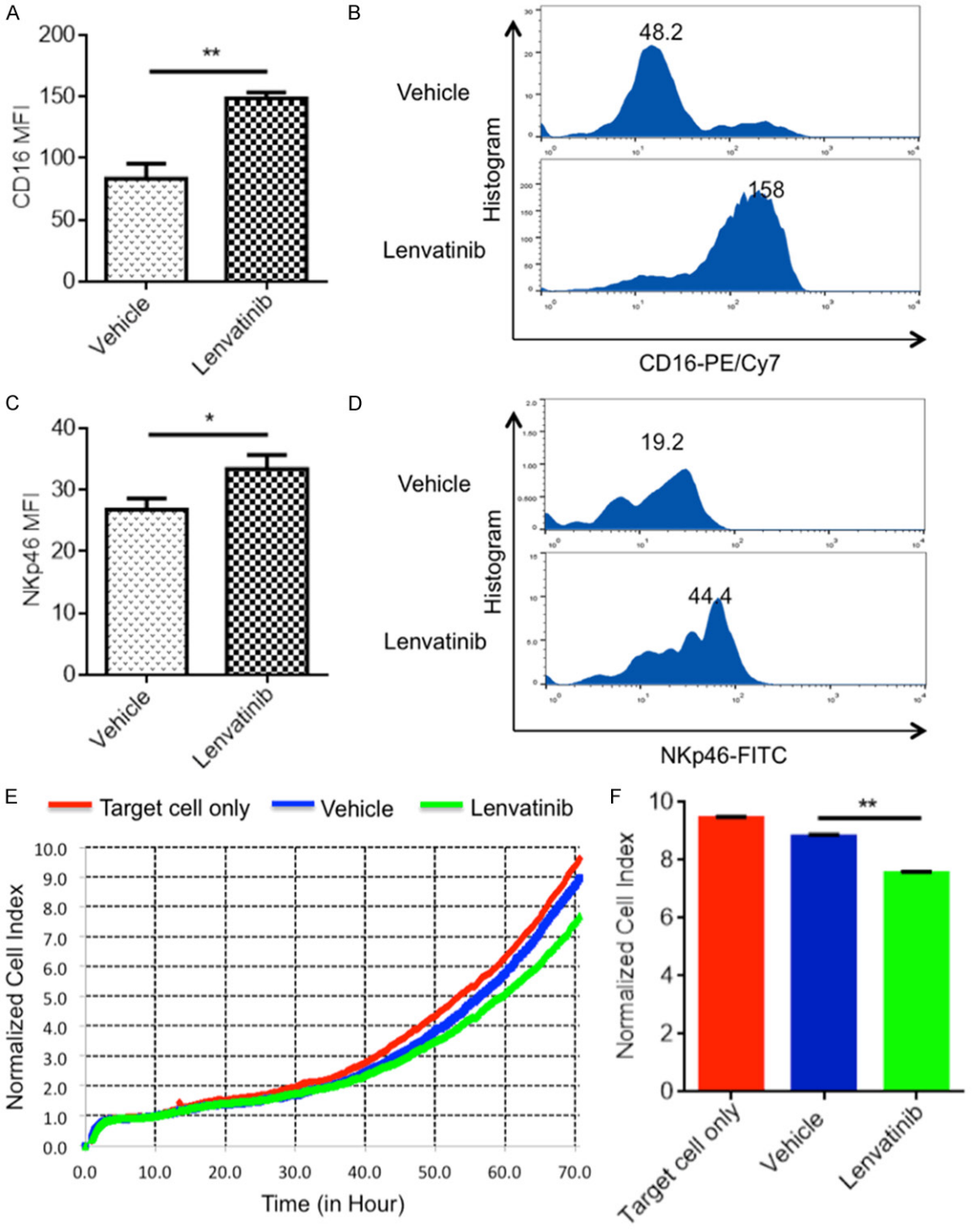

Figure 6. Lenvatinib promotes the activation of tumor-infiltrating NK cells. Samples from C57BL/6N mice that received vehicle or lenvatinib treatment are described in **Figure 1**. For (A-F), the tumor tissues were processed into single-cell suspensions. The expression levels of NCRs in the tumor-infiltrating NK cells were detected by flow cytometry analysis. (A, B) The summarized data and representative results show the expression level of CD16 in the tumor-infiltrating NK cells. (C, D) The summarized data and representative results show the expression level of NKp46 in the tumor-infiltrating NK cells. (E, F) The summarized data and representative results show the cytotoxicity of tumor infiltrating NK cells. NK cells were sorted from tumors received vehicle or lenvatinib treatment and cocultured with B16-F10 cells at an E:T ratio = 5:1. The direct cytotoxicity of NK cells to B16-F10 cells was analyzed by real-time cell assay (RTCA) **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

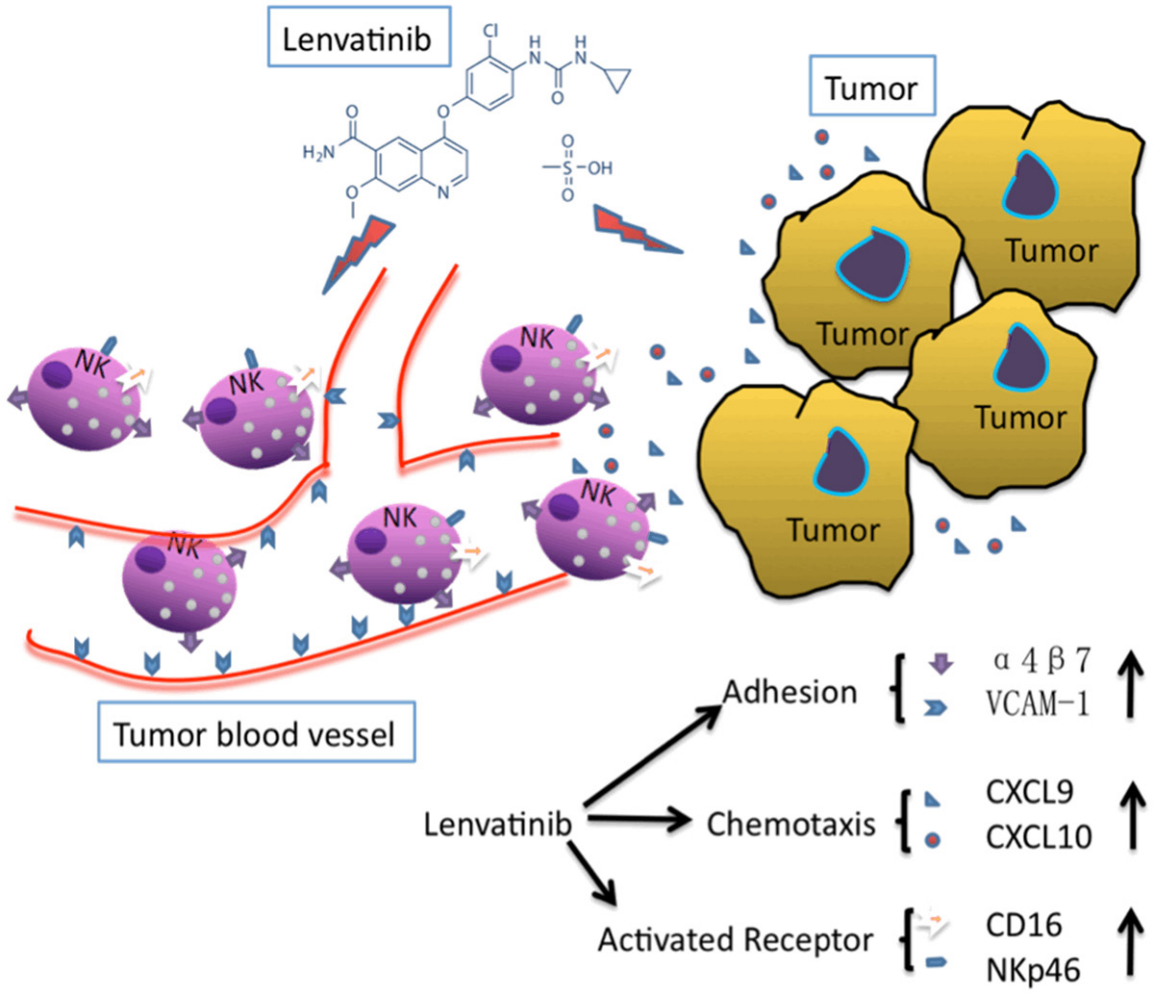

Figure 7. The mechanism through which lenvatinib exerts anti-tumor effects through NK cells. On the one hand, Lenvatinib treatment enhances the tumor infiltration capacity of NK cells by increasing the expression of chemokines CXCL9 and CXCL10 in tumor tissues, as well as adhesion molecules on NK cells (α4β7) and tumor vascular endothelial cells (VCAM-1). On the other hand, lenvatinib treatment enhances cytotoxicity of NK cells by up-regulating the expression of natural killer receptors CD16 and NKp46 on the surface of NK cells.

(**Figure 6F**). These data suggest that lenvatinib treatment can promote the activation and cytotoxicity of tumor-infiltrating NK cells.

Discussion

In recent years, increasing evidence has shown that chemotherapeutic drugs can not only kill tumor cells but also enhance antitumor immunity through different mechanisms [19]. Chemotherapeutic agents can negatively target immunosuppressive cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [20], and may also promote the function of antigen presenting cells, T lymphocytes and NK cells [21]. In recent years, lenvatinib has shown broad antitumor activity in clinical patients and preclinical human tumor xenograft models [22-24]. Several studies aimed at understanding the mechanism(s) of lenvatinib therapy have shown that this therapy exerts its therapeutic effects mainly through targeting the VEGFR/FGFR signaling pathways and inhibiting tumor cell proliferation and tumor angiogenesis [8-10, 12, 13, 25]. However, the immunomodulatory effects of lenvatinib on cancer remain unclear.

Here, we demonstrated that NK cell-mediated innate immunity is necessary for the tumor growth inhibition effects of lenvatinib through following mechanism: on the one hand, Len-

vatinib treatment enhances the tumor infiltration capacity of NK cells by increasing the expression of chemokines CXCL9 and CXCL10 in tumor tissues, as well as adhesion molecules on NK cells (α4β7) and tumor vascular endothelial cells (VCAM-1); on the other hand, lenvatinib treatment enhances cytotoxicity of NK cells by up-regulating the expression of NCR CD16 and NKp46 on the surface of NK cells (**Figure 7**).

Growing evidence in the field of immunobiology has now proven that multiple components of the human immune system, especially cytotoxic T lymphocytes and NK cells, have important roles in fighting cancer. This insight has led to many studies aimed at using chemotherapeutic drugs and other methods to bolster antitumor immunity in patients with cancer [18]. In this study, we showed that lenvatinib promotes NK cell infiltration and induces the upregulation of NCR expression by tumor-infiltrating NK cells and the expression of cytotoxic cytokines in tumor tissue. These findings highlight that lenvatinib may have an advantageous effect on antitumor immune activation and suggest the value of using this drug as a potent immunomodulatory agent in a future combination therapy with NK cell therapy or other immunotherapeutics for cancer.

Acknowledgements

This work was supported in part by grants from the National Science Foundation of China (81773253), Jiangsu Province Social Development Projects (SBE2018740637, BE20166-43), the Natural Science Key Project of Jiangsu Provincial Education Department (17KJA32-0011), the 333 Project of Jiangsu Province (BRA2016398), and the Qing Lan Project of Jiangsu Province. Jiangsu Provincial Key Medical Discipline, the Project of Invigorating Health Care through Science, Technology and Education (NO.ZDXKA2016014).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Junnian Zheng, Cancer Institute, Xuzhou Medical University, Xuzhou 221002, Jiangsu, P. R. China. Tel: +86-516-85582530; E-mail: jnzheng@xzhmu.edu.cn

References

[1] Okamoto K, Kodama K, Takase K, Sugi NH, Yamamoto Y, Iwata M and Tsuruoka A. Antitumor activities of the targeted multi-tyrosine kinase inhibitor lenvatinib (E7080) against RET gene fusion-driven tumor models. Cancer Lett 2013; 340: 97-103.

[2] Krajewska J, Kukulska A and Jarzab B. Efficacy of lenvatinib in treating thyroid cancer. Expert Opin Pharmacother 2016; 17: 1683-1691.

[3] Krajewska J, Gawlik T and Jarzab B. Advances in small molecule therapy for treating metastatic thyroid cancer. Expert Opin Pharmacother 2017; 18: 1049-1060.

[4] Roviello G, Corona SP, Bozza G, Aieta M, Generali D, Rodriquenz MG, Mileo AM, Imperatori M, Ianza A, Conca R and Sobhani N. Lenvatinib for the treatment of renal cell carcinoma. Expert Opin Investig Drugs 2018; 27: 507-512.

[5] Studentova H, Vitaskova D and Melichar B. Lenvatinib for the treatment of kidney cancer. Expert Rev Anticancer Ther 2018; 18: 511-518.

[6] Hong DS, Kurzrock R, Falchook GS, Andresen C, Kwak J, Ren M, Xu L, George GC, Kim KB, Nguyen LM, O'Brien JP and Nemunaitis J. Phase 1b study of lenvatinib (E7080) in combination with temozolomide for treatment of advanced melanoma. Oncotarget 2015; 6: 43127-43134.

[7] Hong DS, Kurzrock R, Wheler JJ, Naing A, Falchook GS, Fu S, Kim KB, Davies MA, Nguyen LM, George GC, Xu L, Shumaker R, Ren M, Mink J, Bedell C, Andresen C, Sachdev P, O'Brien JP and Nemunaitis J. Phase I dose-escalation study of the multikinase inhibitor lenvatinib in patients with advanced solid tumors and in an expanded cohort of patients with melanoma. Clin Cancer Res 2015; 21: 4801-4810.

[8] Matsuki M, Adachi Y, Ozawa Y, Kimura T, Hoshi T, Okamoto K, Tohyama O, Mitsuhashi K, Yamaguchi A, Matsui J and Funahashi Y. Targeting of tumor growth and angiogenesis underlies the enhanced antitumor activity of lenvatinib in combination with everolimus. Cancer Sci 2017; 108: 763-771.

[9] Yamamoto Y, Matsui J, Matsushima T, Obaishi H, Miyazaki K, Nakamura K, Tohyama O, Semba T, Yamaguchi A, Hoshi SS, Mimura F, Haneda T, Fukuda Y, Kamata JI, Takahashi K, Matsukura M, Wakabayashi T, Asada M, Nomoto KI, Watanabe T, Dezso Z, Yoshimatsu K, Funahashi Y and Tsuruoka A. Lenvatinib, an angiogenesis inhibitor targeting VEGFR/FGFR, shows broad antitumor activity in human tumor xenograft models associated with mi-

crovessel density and pericyte coverage. Vasc Cell 2014; 6: 18.
[10] Tohyama O, Matsui J, Kodama K, Hata-Sugi N, Kimura T, Okamoto K, Minoshima Y, Iwata M and Funahashi Y. Antitumor activity of lenvatinib (e7080): an angiogenesis inhibitor that targets multiple receptor tyrosine kinases in preclinical human thyroid cancer models. J Thyroid Res 2014; 2014: 638747.
[11] Cheng L, Du X, Wang Z, Ju J, Jia M, Huang Q, Xing Q, Xu M, Tan Y, Liu M, Du P, Su L and Wang S. Hyper-IL-15 suppresses metastatic and autochthonous liver cancer by promoting tumour-specific CD8+ T cell responses. J Hepatol 2014; 61: 1297-1303.
[12] Matsuki M, Hoshi T, Yamamoto Y, Ikemori-Kawada M, Minoshima Y, Funahashi Y and Matsui J. Lenvatinib inhibits angiogenesis and tumor fibroblast growth factor signaling pathways in human hepatocellular carcinoma models. Cancer Med 2018; 7: 2641-2653.
[13] Nakagawa T, Matsushima T, Kawano S, Nakazawa Y, Kato Y, Adachi Y, Abe T, Semba T, Yokoi A, Matsui J, Tsuruoka A and Funahashi Y. Lenvatinib in combination with golvatinib overcomes hepatocyte growth factor pathway-induced resistance to vascular endothelial growth factor receptor inhibitor. Cancer Sci 2014; 105: 723-730.
[14] Muller WA. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 2003; 24: 327-334.
[15] Perez-Villar JJ, Zapata JM, Melero I, Postigo A, Sanchez-Madrid E and Lopez-Botet M. Expression and function of alpha 4/beta 7 integrin on human natural killer cells. Immunology 1996; 89: 96-104.
[16] Griffith JW, Sokol CL and Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014; 32: 659-702.
[17] Seo H, Kim BS, Bae EA, Min BS, Han YD, Shin SJ and Kang CY. IL21 therapy combined with PD-1 and Tim-3 blockade provides enhanced NK cell antitumor activity against MHC class I-deficient tumors. Cancer Immunol Res 2018; 6: 685-695.
[18] Childs RW and Carlsten M. Therapeutic approaches to enhance natural killer cell cytotoxicity against cancer: the force awakens. Nat Rev Drug Discov 2015; 14: 487-498.
[19] Galluzzi L, Senovilla L, Zitvogel L and Kroemer G. The secret ally: immunostimulation by anticancer drugs. Nat Rev Drug Discov 2012; 11: 215-233.
[20] Alizadeh D, Trad M, Hanke NT, Larmonier CB, Janikashvili N, Bonnotte B, Katsanis E and Larmonier N. Doxorubicin eliminates myeloid-derived suppressor cells and enhances the efficacy of adoptive T-cell transfer in breast cancer. Cancer Res 2014; 74: 104-118.
[21] Casares N, Pequignot MO, Tesniere A, Ghiringhelli F, Roux S, Chaput N, Schmitt E, Hamai A, Hervas-Stubbs S, Obeid M, Coutant F, Metivier D, Pichard E, Aucouturier P, Pierron G, Garrido C, Zitvogel L and Kroemer G. Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death. J Exp Med 2005; 202: 1691-1701.
[22] Matsubara N, Naito Y, Nakano K, Fujiwara Y, Ikezawa H, Yusa W, Namiki M, Okude T and Takahashi S. Lenvatinib in combination with everolimus in patients with advanced or metastatic renal cell carcinoma: a phase 1 study. Int J Urol 2018; 25: 922-928.
[23] Mahajan P, Dawrant J, Kheradpour A, Quintanilla NM, Lopez ME, Orth RC, Athanassaki I and Venkatramani R. Response to lenvatinib in children with papillary thyroid carcinoma. Thyroid 2018; 28: 1450-1454.
[24] Kudo M. Lenvatinib may drastically change the treatment landscape of hepatocellular carcinoma. Liver Cancer 2018; 7: 1-19.
[25] Lee YS, Kim SM, Kim BW, Chang HJ, Kim SY, Park CS, Park KC and Chang HS. Anti-cancer effects of HNHA and lenvatinib by the suppression of EMT-mediated drug resistance in cancer stem cells. Neoplasia 2018; 20: 197-206.