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Small molecule antagonist of CXCR2 and CXCR1 inhibits tumor growth, angiogenesis, and metastasis in pancreatic cancer

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Abstract

Pancreatic cancer (PC) has a poor prognosis, and current therapeutic strategies are ineffective in advanced diseases. We and others have shown the aberrant expression of CXCR2 and its ligands in PC development and progression. Our objective for this study was to evaluate the therapeutic utility of CXCR2/1 targeting using an small molecule antagonist, SCH-479833, in different PC preclinical murine models (syngeneic or xenogeneic). Our results demonstrate that CXCR2/1 antagonist had both antitumor and anti-metastatic effects in PC. CXCR2/1 antagonist treatment inhibited tumor cell proliferation, migration, angiogenesis, and recruitment of neutrophils, while it increased apoptosis. Treatment with the antagonist enhanced fibrosis, tumor necrosis, and extramedullary hematopoiesis. Together, these findings suggest that selectively targeting CXCR2/1 with small molecule inhibitors is a promising therapeutic approach for inhibiting PC growth, angiogenesis, and metastasis.

Keywords

Pancreatic cancer; CXCR2 antagonist; tumor growth; angiogenesis; neutrophils; metastasis

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Authors Contributions:

DRP, CL, and RKS conceived and developed the concept and helped in data interpretation, analysis. AP, SS, RS, BJD, and SK assisted with experiments, acquisition of data, analysis, and interpretation. DRP, CL, and RKS wrote the manuscript. RKS and SB were responsible for oversight and finds acquisition. All authors read and approved the final manuscript.

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Declaration of Interest

The authors declare no competing interests.

INTRODUCTION

According to Cancer Statistics 2023, pancreatic cancer (PC) is the fourth leading cause of cancer-related death in both males and females in the United States [1]. Unfortunately, by the time PC gets diagnosed, almost 80–85% of the patients have already reached an advanced stage or have metastases, leaving minimal treatment options [2]. This indicates the need for novel and effective therapeutic measures and a better understanding of the molecular mechanisms implicated in disease progression.

The G-protein-coupled receptors (GPCR) CXCR1 and CXCR2 and their ligands can regulate the growth of the tumor, angiogenesis, and metastasis in various cancers [3, 4]. CXCR2/1 are known to orchestrate immune responses in multiple diseases, including cancer [5, 6]. Several studies have confirmed the presence of CXCR2/1 and their ligands in human pancreatic ductal adenocarcinoma (PDAC) tissues and cell lines [7–15]. In addition to tumor cells, CXCR 1 and 2 are expressed in endothelial cells neutrophils, and immunosuppressive granulocytic myeloid suppressor cells (GR-MDSC) [16–21]. Neutrophils are one of the innate immune cell types which originate from the myeloid precursor [22] and can play a pro-tumor role in cancer [23, 24]. The expression of CXCR2 has also been reported on PDAC fibroblasts [25] and is a known mediator of tumor progression, angiogenesis, and metastasis in PDAC [10, 26, 27]. Furthermore, our lab has demonstrated the critical role of this signaling axis in regulating $KRAS^{(G12D)}$-induced autocrine growth of PDAC cells [28]. These findings suggest that CXCR2/1 can be a promising therapeutic target for PDAC.

Monoclonal antibodies and small molecule antagonists that block the CXCR2/1 axes have been undergoing clinical trials [4, 29, 30]. Earlier reports demonstrate that treatment with CXCR2/1 antagonists, SCH479833 and SCH527123, inhibited the *in vitro* cell proliferation and chemotaxis of malignant cells and inhibited tumor growth, angiogenesis, and metastasis in pre-clinical studies using human melanoma and colon cancer cells [31, 32]. Steele et al., [33] demonstrated that CXCR2 inhibition profoundly inhibits PDAC metastasis and augments immunotherapies. A recent report from Gulhati et al [34] demonstrated that targeting checkpoint in combination with CXCR2 antagonists have long-term impact in PDAC management. The utility of CXCR2 antagonists as anti-cancer agents in combination with immunotherapeutics is further supported by several published reports [12–15, 29, 33–42]. Our earlier report suggests a dynamic role of host CXCR2 axis in regulating PDAC immune suppression, tumor growth, and metastasis [43]. Host Cxcr2 loss led to increased extramedullary hematopoiesis and expansion of neutrophils and immature myeloid precursor cells in the spleen of tumor-bearing mice, leading to enhanced spontaneous and experimental metastasis [43]. However, the impact of pharmacological inhibition of CXCR2/1 in PDAC as monotherapy remains unclear.

The present study examined whether targeting the CXCR2/1 axis using an orally active small molecule antagonist SCH-479833 inhibits tumor growth, angiogenesis, neutrophil recruitment, fibrosis, necrosis, and metastasis in the preclinical murine models of PC. Our data demonstrate that selectively targeting CXCR2/1 with small molecule inhibitors is a promising therapeutic approach for inhibiting PC growth, angiogenesis, and metastasis.

MATERIALS AND METHODS

Cell lines and CXCR2/1 antagonists

Murine Cell line: UN-KC-6141 cell line [44] (referred to as KC cells in this study) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (HyClone®, Thermo Scientific, UT). We supplemented the medium with 5% Fetal Bovine Serum (FBS) (Atlanta Biologicals, GA), L-Glutamine (MediaTech, VA), Vitamin solution (MediaTech), and Gentamycin (Gibco, Life Technologies, NY).

Human Cell Lines: *CD18/HPAF* are human PDAC cell lines maintained in DMEM supplemented with 5% FBS, L-Glutamine, Vitamin solution, and Gentamycin. *HPNE & E6-E7-st* with or without KRAS mutation are a model of immortalized human pancreatic duct-derived cell lines with or without exogenous $KRAS^{G12D}$ expression. The model consisted of four cell lines hTERT-HPNE (referred to as HPNE), hTERT-HPNE-$KRAS^{(G12D)}$ (referred to as HPNE-KRAS), hTERT-HPNE-E6-E7-st (referred to as E6-E7-st), and hTERT-HPNE-E6-E7-st-$KRAS^{(G12D)}$ (referred to as E6-E7-st-KRAS). Generation and maintenance of hTERT-HPNE, E6-E7-st, and E6-E7-st-KRAS cells have been previously described by *Campbell et al.* [45]

All the cell lines were tested for mycoplasma using MycoAlert Plus Mycoplasma Detection kit (Lonza, Rockland, ME). We also had the cell lines authenticated by the Human DNA Identification Laboratory, UNMC, Omaha, NE, through short tandem repeat (STR) tests.

CXCR2/1 antagonists—SCH-479833 [46] was synthesized at Schering-Plough (Kenilworth, NJ) and formulated in hydroxypropyl-beta-cyclodextrin (HPβCD, Acros Organics, Morris Plains, NJ). The inhibition constant (K_i) of CXCR1 (17 nM) and CXCR2 (0.17 nM) for SCH-479833 were calculated from the IC_{50} value using the Cheng-Prusoff equation [47].

In vitro Cell proliferation assay

Tumor cells were seeded in 96-well plates at a low density (3000 cells/well). Following overnight adherence, cells were incubated with media alone or medium containing serum (5%) with SCH-479833 or media control for 72 h. Four different concentrations (5, 10, 25, and 50 ug/ml) of the antagonist were used. Cell proliferation was determined by MTT assay [48]. Growth inhibition was calculated as a percent $(\%) = [((A-B)/A) \times 100]$, where A and B are the absorbances of untreated and treated cells, respectively.

Cell invasion assay

To investigate the effect of SCH-479833 on PC cell migration, cells (1×10^5 cells/well) in serum-free media were plated in the top chamber of Matrigel-coated polyethylene terephthalate membranes (six-well insert, 8 μm pore size; Becton Dickinson, Franklin Lakes, NJ). The bottom chamber contained 1.0 ml serum-free media with or without SCH-479833 (at different concentrations). The cells were incubated overnight at 37°C, and unmigrated cells were removed. Per the manufacturer's instructions, cells that passed through the membrane pores were stained using the Hema 3 kit (Fisher Scientific Company L.L.C.,

Kalamazoo, MI). Cells were counted in ten random fields (200x) and expressed as the average number of cells per field of view. The data is represented as the average of three independent experiments.

Tumor growth and metastasis assay

All procedures performed in mice followed institutional guidelines approved by the University of Nebraska Medical Center (UNMC) Institution Animal Care and Use Committee.

Syngeneic Mouse Model: C57BL/6 wild type (WT), 6–8 week old, *immunocompetent* mice were obtained from Charles River Laboratories (Wilmington, MA). *Murine PC cells* KC were inoculated (1×10^5/mouse in 50 μl HBSS) orthotopically into the pancreas of these mice. The mice were randomly divided into two groups a week following the inoculation. The control group (n = 5) was given intraperitoneal (IP) injections of 100 μl HPβCD ((2-Hydroxypropyl)-Beta-Cyclodextrin), while the treatment group (n = 5) was given CXCR2 antagonist (SCH-479833; 100 mg/kg body weight diluted in 100 μl HPβCD) for seven days a week. The mice were euthanized after 8 weeks; gross metastatic spread was evaluated, and a portion from each tumor was processed for histopathological and immunohistochemistry analysis.

Xenogeneic Mouse Model: Athymic nude mice (6–8 weeks) were obtained from Charles River Laboratories (Wilmington, MA). *Human PC cells* CD18/HPAF and E6-E7-st-KRAS cells (1×10^6/mouse in 50 μl HBSS) were orthotopically inoculated into the pancreas of these mice. Seven days post-inoculation, the mice were randomly sorted into the HPβCD and SCH-479833 antagonist treatment groups, as mentioned in the syngeneic model. The mice were euthanized after four weeks and examined for tumor growth and metastasis. The primary tumors were resected and processed for histopathological and immunohistochemistry analysis.

Immunohistochemical analysis

To explore the expression of various tissue markers, we performed IHC staining. First, 4–5 μm thick, formalin-fixed, paraffin-embedded tissue sections were pre-warmed at 65 °C for 2 hours on the slide warmer and then deparaffinized with graded xylene. The slides were rehydrated through diluted concentrations of ethanol in water. Afterward, antigen retrieval was performed using sodium citrate buffer (pH = 6.0) and heating in the laboratory microwave for 10 minutes. Endogenous peroxidase was blocked by incubating with 3% hydrogen peroxide for 5 minutes. After blocking non-specific binding by incubating with serum, slides were probed with respective primary antibodies (Table 1) and incubated overnight at 4°C. The following day, slides were washed and incubated with appropriate secondary antibodies. The ABC Elite Kit and DAB substrate kit (3, 3 Diaminobenzidine) (Vector Laboratories, Burlingame, CA) were used to detect immunoreactivity per the manufacturer's protocols. A positive IHC staining gave a brown color (cytoplasmic, nuclear, membrane, or all, depending on the proteins' location). At the same time, the nuclei were counterstained blue with Meyer's Hematoxylin (Thermo Scientific, Fremont, CA). Then the slides were dehydrated with ascending concentrations of ethanol and xylene. Finally, the

slides were mounted with permanent media Permount (Fisher Scientific, Fair Lawn, NJ). Two independent observers evaluated IHC staining.

For quantitative evaluation, in each tissue section(n=3–5), positive cells were counted in five areas with significant staining at 200X magnification. The average was used for plotting the graph. Similarly, the number of positive endothelial cells was counted for blood vessels to evaluate microvessel density as described by *Weidner et al. and* Saad et al. [49, 50]. Single or clusters of positive endothelial cells were considered countable vessels, but the presence of blood cells or fibrin without any detectable endothelial cells was disregarded. All the above analyses were done, and the representative photomicrographs were captured with a Nikon Eclipse E800 microscope and its NIS-Elements BR 5.11.00 software (Nikon, Melville, NY).

Hematoxylin and Eosin Staining (H & E)

To demonstrate the general histological architecture of tissue, we did regressive hematoxylin and eosin (H&E) staining. Hematoxylin demonstrates nuclear detail, and eosin provides cytoplasmic and connective tissue details. The slides were stained in our Tissue Science Facility with an automated stainer Tissue-Tek® Prisma™ (Sakura, Torrance, CA). Briefly, 4–5 μm thick, formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin (CV select HemaMax, BBC Biochemical, McKinney, TX) for 5 minutes and with eosin (CV select Eosin Y, BBC Biochemical, McKinney, TX) for 90 seconds intermingled with pre-and post-staining xylene, alcohol, and water wash steps. The slides (n=3–5) were examined under Nikon Eclipse E800 light microscope after mounting with a permount mounting medium.

Masson Trichrome Staining (MT)

We used Masson trichrome to stain the tissue's collagen, differentiating it from muscle fibers and cytoplasm. The 4–5 μm thick, formalin-fixed, paraffin-embedded tissue sections were stained with Masson Trichrome Stain Kit-Aniline Blue (BBC Biochemical, McKinney, TX) as per manufacture protocol. In brief, staining with Aniline Blue for 2 minutes and other reagents in the kit gives a blue color to the collagen, while the muscle fibers stain red and the cytoplasm stains pink. The slides (n=3–5) were mounted with a permount mounting medium and examined under the Nikon Eclipse E800 microscope. The amount of fibrosis (blue-stained collagen) was analyzed qualitatively & semi-quantitatively by the pathologist.

Pro- and anti-apoptotic gene expression analyais

Total RNA was isolated from homogenized tumor tissues using the standard Trizol (Invitrogen, Carlsbad, CA) protocol. Reverse Transcription was performed with 5 μg RNA using oligo (dT) (Fermentas, Hanover, MD, USA) and Superscript® II RT (Invitrogen) or iScript™ Reverse Transcription Supermix for RT-qPCR (BIO-RAD, Hercules, CA, USA). Quantitative real-time PCR reactions were performed using FastStart SYBR Green Master Mix (Roche; Indianapolis). Primer sets used for the study are; *Bcl2*, 5'-AATGTCCAGGTGGGTCAGAG-3', and 5'-TCCTGCTGGATCTGCCTAGT-3'; *Bax*, 5'-TGCAGAGGATGATTGCTGAC-3', and 5'-GGAGGAAGTCCAGTGTCCAG-3'; and *Rpl13a*, 5'-ACTCTGGAGGAGAAACGGAAGG-3' and 5'-

CAGGCATGAGGCAAACAGTC-3'. The C_t values of the target genes were normalized to mean C_t values of the endogenous control, ribosomal protein large 13 A (*Rpl13a*); [$-\Delta C_t = C_t(Rpl13a) - C_t$(target gene)]. The ratio of mRNA expression of target genes versus *Rpl13a* was defined as $2^{(-\Delta Ct)}$. Melting curve analysis was performed to check the specificity of the amplified product.

Statistical analysis

We performed the statistical analysis using GraphPad Prism 9.0.0 software (San Diego, CA). All the values are expressed as mean ± standard error of the mean (SEM). Differences between the groups were compared using an unpaired two-tailed *t-test*. *In vivo* analysis was performed using the Mann-Whitney U-test of significance. A value of $p<0.05$ was deemed significant.

RESULTS

SCH-479833 treatment decreased cell proliferation and invasion of PC cells in vitro

The overall growth of the tumor is determined by the interaction of tumor cells with the host microenvironment. In previous studies, it has been reported that chemokine signaling not only helps in the migration of endothelial cells and, thus, angiogenesis but may also help in the motility and invasion of malignant cells. Therefore, we investigated whether treating CXCR2/1 antagonists would affect tumor cell proliferation and motility. Our data demonstrated inhibition of cell proliferation with SCH-479833 treatment compared to the control (Fig. 1A and B). Cells treated with SCH-479833 also showed a significant inhibition ($p<0.05$) in the number of invading cells in the invasion assay (Fig. 1C and D).

CXCR2/1 antagonist inhibited tumor growth and metastasis.

Xenogenic models: We examined the effect of the CXCR2/1 antagonists on mice bearing human PC cells CD18/HPAF. Animals were randomized into control and treatment groups (Fig. 2A). The control group received HPβCD, while the treatment group received the CXCR2/1 antagonist intraperitoneally. Mice were euthanized on day 28. We observed that the mice receiving the CXCR2/1 antagonist had low tumor weight (Fig. 2B) and reduced size (Fig. 2C) compared to control-treated mice. Also, the CXCR2/1 treated animals had a lower regional and distant metastasis incidence than controls (Fig. 1C & D).

There was no difference in the histopathology of the tumors derived from the two different groups, as both have moderately-to-poorly differentiated morphology on H&E staining (Fig. 1E). There was decreased tumor necrosis in the CXCR2/1 antagonist group (Fig. 1E). We also investigated the ability of the CXCR2/1 antagonist to impact fibrosis in murine PC by staining with Masson's Trichrome (MT). We observed decreased fibrosis in the tumors derived from mice treated with the CXCR2/1 antagonist (Fig. 1E).

In the xenogeneic mouse model bearing the human PC cell hTERT-HPNE-E6-E7-st-KRAS, animals were randomized into control and treatment groups (Fig. 3A). Mice were euthanized and examined for primary tumor growth and gross metastasis (day 28). The CXCR2/1 antagonist-treated mice had low tumor weights (Fig. 3B) compared to those receiving

HPβCD. We observed no regional or distant metastasis in control or treated groups. We did not observe any noticeable difference in the histopathology of the tumors derived from the two different groups, as both have moderately-to-poorly differentiated morphology on H&E staining (Fig. 3C). We observed increased necrosis (Fig. 3C) and fibrosis (Fig. 3C) in the CXCR2/1 antagonist treatment group.

Syngeneic model: We utilized the murine PC cell line, KC, to generate the syngeneic mouse model and examine the efficacy of the CXCR2/1 antagonist in immunocompetent animals. As per the schematic shown in Fig. 4A, the animals were randomized into control and treatment groups. The control group was given HPβCD, while the treatment group was given a CXCR2/1 antagonist intraperitoneally. The mice were euthanized at 8 weeks, and tumors were resected for analysis. We observed reduced primary tumor weight in CXCR2/1 treated group compared to controls (Fig. 4B). We observed enlarged spleen in all treated and control animals. There was no significant difference in the weight of the spleens in both groups (Fig. 4C). However, immunohistochemical analysis of the spleen of treated and untreated mice showed higher levels of Ly6G neutrophils in antagonist-treated animals as compared to untreated animals (Supplemental Fig. 1A).

HPβCD and CXCR2/1 antagonist-treated mice had lymph node and distant metastasis, and the incidence of metastasis was lower in CXCR2/1 treated group as compared to the controls (Fig. 4D and E). Like the xenogeneic models, both groups had similar morphology. However, both groups have less aggressive well-differentiated tumors, as seen on H&E staining (Fig. 4E). Similarly, the necrosis and fibrosis were higher in the CXCR2/1 antagonist group than in the HPβCD group (Fig. 4E).

Decreased in-situ cell proliferation and increased apoptosis in SCH-479833 antagonist-treated animals

Next, we examined whether inhibition in tumor growth was due to reduced PC cell proliferation and/or survival by Ki-67 and CC3 immunostaining, respectively. Ki-67 is a nuclear protein and a biomarker used frequently to measure the proliferative activity of tumor tissues. At the same time, cleaved caspase 3 (CC3) is a standard method for detecting cellular apoptosis that results from caspase cascades. We observed that treating animals with the antagonist decreased tumor cell proliferation (Fig. 5 A and B) in both xenogeneic and syngeneic animal models. In contrast, the number of apoptotic cells was higher in the antagonist-treated group (Fig. 5C and D). In addition, we observed significantly higher expression levels of *Bax* and lower expression of *Bcl2* in antagonist-treated tumors (Figure 5F).

Tumor angiogenesis and neutrophil recruitment were decreased in SCH-479833 antagonist-treated animals

The role of a CXCR2/1 is well-established in inflammation and angiogenesis [29]. Therefore, we examined whether inhibition of PC growth by SCH-479833 antagonist affected angiogenesis and inflammatory responses. We evaluated tumor angiogenesis by performing IHC using the blood vessel (angiogenesis) marker CD31. Microvessel density was lower in the tumors that received CXCR2/1 antagonist treatment than in HPβCD

treatment (Fig. 6) in both syngenic and xenogenic animal models. Similarly, the number of MPO-positive neutrophils was significantly lower in the CXCR2/1 antagonist treatment group compared to controls (Fig. 6). We did not observe infiltration of tumor-associated macrophages in antagonist-treated and untreated murine KC tumors (Supplemental Fig. 2). Overall, treatment with the CXCR2/1 antagonist in xenogeneic and syngenic mouse models decreased angiogenesis and neutrophil infiltration in the pancreatic tumors.

DISCUSSION

In the present study, we demonstrated that intraperitoneal administration of the CXCR2/1 antagonist SCH-479833 in syngenic and xenogenic murine PC models inhibited the growth and incidence of metastasis. Our studies showed that the antitumor efficacy of SCH-479833 resulted from a decrease in *in-situ* cell proliferation, angiogenesis, neutrophil recruitment, and enhanced apoptosis. In addition, we observed reduced invasion and cell proliferation of human and murine PC cells upon treatment with the antagonists.

The use of small molecule inhibitors represents an attractive targeted therapeutic approach. Previously, we demonstrated that CXCR2 and its ligands play an important role in PC growth and metastasis [19, 29]. Therefore, targeting these receptors was a logical next step. CXCR2/1 antagonists have shown encouraging results for various cancer, including pancreatic cancer, melanoma, colon cancer, and esophageal and prostate cancer treatment [13, 14, 30, 31, 33, 34, 37, 46, 51–54]. Our *in vivo* studies revealed that mice treated with the CXCR2/1 antagonist reduced tumor volume and incidence of lymph node and distant metastasis in the treatment groups compared to the control group. These findings indicate the potential of small molecule CXCR2/1 antagonists as novel therapeutics for PC. Our previous observations support it, associating the CXCR2/1 axes with PC aggressiveness [13, 14, 29, 30, 33, 34, 37].

The overall growth of the tumor *in vivo* depends on several factors, including the tumor cell proliferation rate, survival, and vascularization of the tumor tissue, along with the invasive capacity of the tumor cells [55, 56]. While evaluating the effect of SCH-479833 antagonists on proliferation and apoptosis, we observed that tumors derived from antagonist-treated mice were associated with decreased cell proliferation and increased apoptosis, as detected by Ki-67 and CC3 immunostaining. The *in vitro* studies also supported the above finding, thus, demonstrating the antiproliferative effect of the CXCR2/1 antagonist. These results support our previous work that showed the critical role of CXCR2 and its ligands in modulating PC phenotypes associated with growth and metastasis [29, 43]. An earlier report by Steele et al. also supports that CXCR2 antagonists suppress metastasis in PDAC [33].

The CXCR2/1 axes are well-known in inflammation and angiogenesis. Our lab has already demonstrated that CXCR2 knockout reduced angiogenesis in pancreatic tumors [57]. In the present study, we observed that the treatment with CXCR2/1 antagonist also decreased tumor vascularity. This was similar to earlier observations from our laboratory and others in melanoma, breast, and colon cancer models [31, 32, 58, 59].

Neutrophils are known crusaders of inflammation and tumor progression. Our study demonstrated that treatment with the CXCR2/1 antagonist decreases neutrophil recruitment in the pancreatic tumor. These results affirmed our recently published CXCR2 knockout study [57]. White et al. showed that the CXCR2 antagonist inhibited the migration of neutrophils [60]. Moss et al. also concluded that CXCR2 antagonism might be a practical approach for modulating airway inflammation in patients with cystic fibrosis by reducing the recruitment of neutrophils at the site of inflammation [61]. As inflammation is critical in the progression of cancer, reducing it with the antagonist can also benefit pancreatic cancer. Tazzyman et al. had favorable results in decreasing neutrophils using CXCR2/1 antagonist in lung adenocarcinoma [62].

Many studies have revealed that CXCR2 expression in cancer cells can drive their proliferation, invasion, and migration [63, 64]. By inhibiting CXCR2, malignant cell proliferation, and motility can be controlled. Moreover, we discovered in our study that the CXCR2/1 antagonist inhibits PC cell proliferation and invasiveness. *Steele et al.* also demonstrated that tumor cell proliferation was reduced after treatment with CXCR2 antagonist in PDAC [33].

Fibrosis plays a vital role in pancreatic cancer. Studies have reported a cross-talk between tumor cells and cancer-associated fibroblasts (CAFs), aiding tumor progression, metastasis, and therapy resistance [48, 65, 66]. Our study shows that treatment with CXCR2/1 antagonist decreases fibrosis in CD18/HPAF xenogenic mouse model while increasing it in KC syngeneic models. Interestingly, fibrosis in these models was positively associated with necrosis. The precise cause for these differences remains unclear.

In conclusion, our data show that CXCR2/1 antagonists are effective against PC in preclinical models. The inhibitory effects of the antagonist resulted from decreased proliferation, survival, and invasion of PC cells. Furthermore, angiogenesis and recruitment of neutrophils were also inhibited, which is extremely important in tumor growth and progression. Based on these preclinical findings and published reports, CXCR2/1 represents a potential novel therapeutic agent for PC management.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. This study evaluated the therapeutic utility of orally active small molecule antagonists targeting CXCR2/1, SCH-479833, using different PC preclinical murine models (syngeneic or xenogeneic).
2. We demonstrate that systemic administration of CXCR2/1 antagonist had both antitumor and anti-metastatic effects in PC. CXCR2/1 antagonist treatment inhibited tumor cell proliferation, angiogenesis, and recruitment of neutrophils, while it increased apoptosis.
3. Our data suggest that selectively targeting CXCR2/1 with small molecule inhibitors is a promising therapeutic approach for inhibiting PC growth, angiogenesis, and metastasis

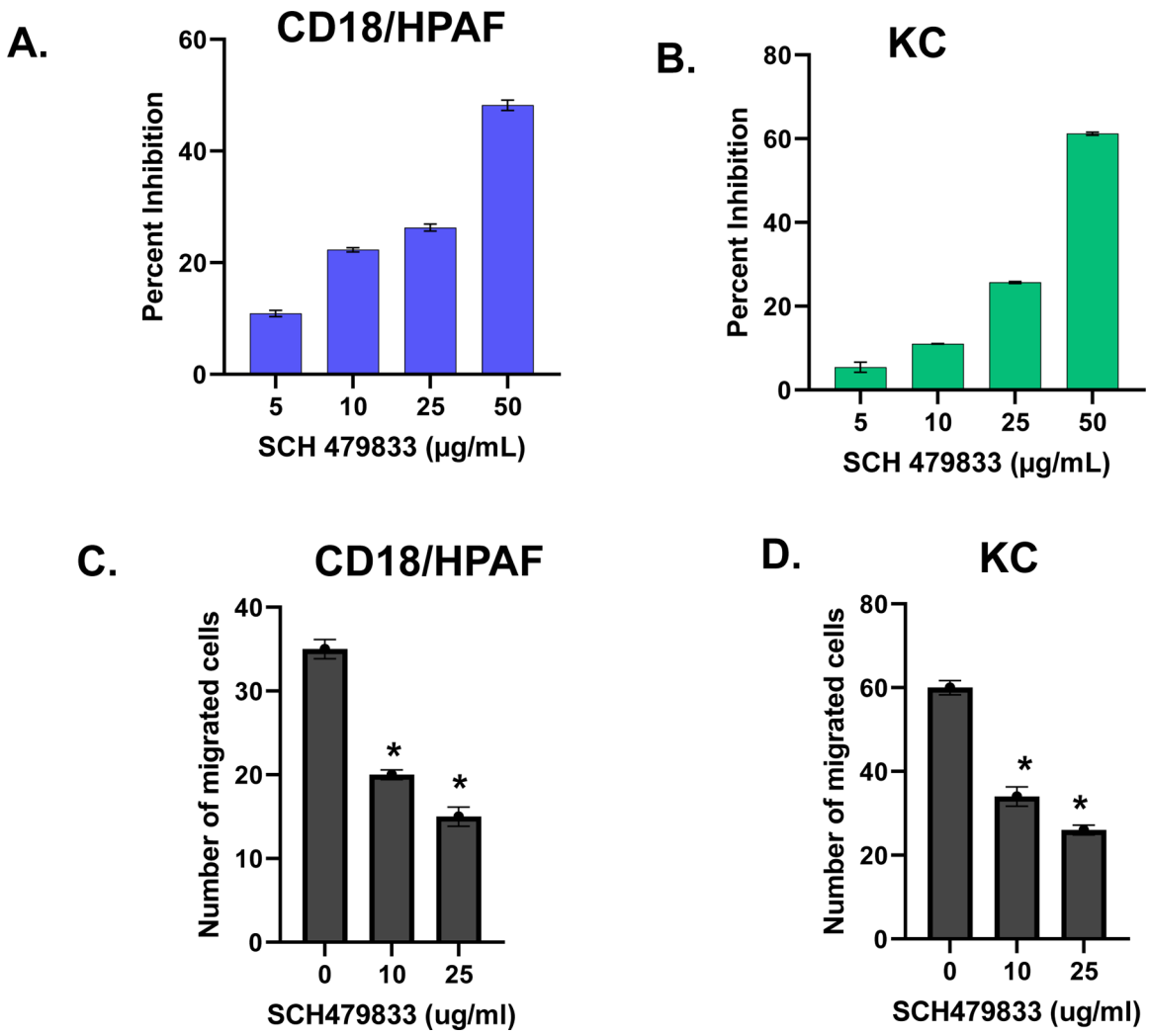
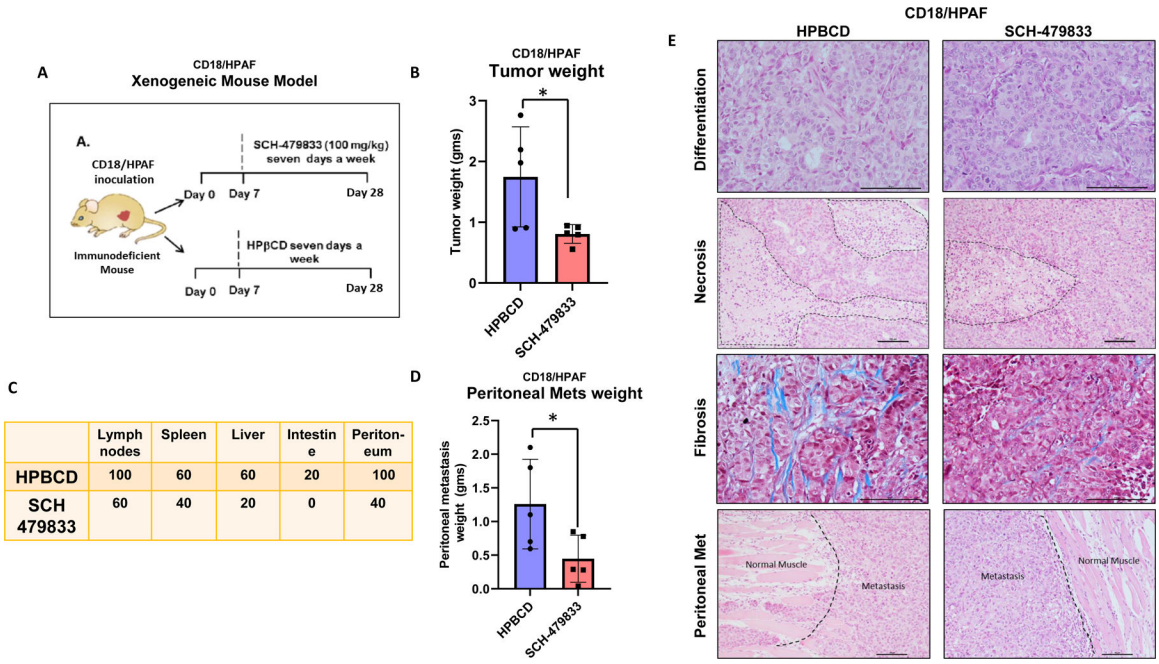


Figure 1. SCH-479833 inhibits PC cell proliferation and invasiveness:

A and **B.** PC cells (3000 cells/well) in a 96-well plate were cultured in a medium with or without serum (5%), and SCH-479833 in different concentrations. Cellular proliferation was determined at 72h by MTT assay. The values are mean percent inhibition of proliferation ± SEM. **C** and **D**. Cells were plated on Matrigel-coated membranes for invasion assays and incubated overnight. Serum-free medium with SCH-479833 (different concentrations) or HPβCD was added to the lower chamber. The cells that did not migrate through the Matrigel and/or pores in the membrane were removed, and cells on the other side of the membrane were stained and photographed at 200x magnification. Cells were counted in ten random fields (200x) and expressed as the average number of cells per field of view. The values are the number of migrated cells ± SEM. This is representative of three experiments done in triplicate. *Significantly different from control (p<0.05).



	Lymph nodes	Spleen	Liver	Intestine	Periton-eum
HPBCD	100	60	60	20	100
SCH 479833	60	40	20	0	40

Figure 2. CXCR2/1 antagonist to nude mice bearing CD18/HPAF-xenograft tumors inhibits tumor growth and metastasis.

A. Experimental design for treating pancreatic cancer in CD18/HPAF-xenogeneic mouse model with HPβCD (control) and SCH-479833 (CXCR2 antagonist). **B.** The bar graph shows a difference in the weight of CD18/HPAF tumors derived from mice treated with HPβCD and SCH-479833. **C.** The incidence of various metastasis in two treatment groups. **D.** The graph showing difference in weight of peritoneal metastasis in the two treatment groups. **E.** Representative photomicrographs of H&E staining of tumors, showing similar morphology (moderately-to-poorly differentiated carcinoma) in both the groups; necrosis, fibrosis (Masson Trichrome staining), and peritoneal metastasis. The error bars in the graphs represent the SEM. Scale bar=100 μm. *Significantly different from control (p<0.05).

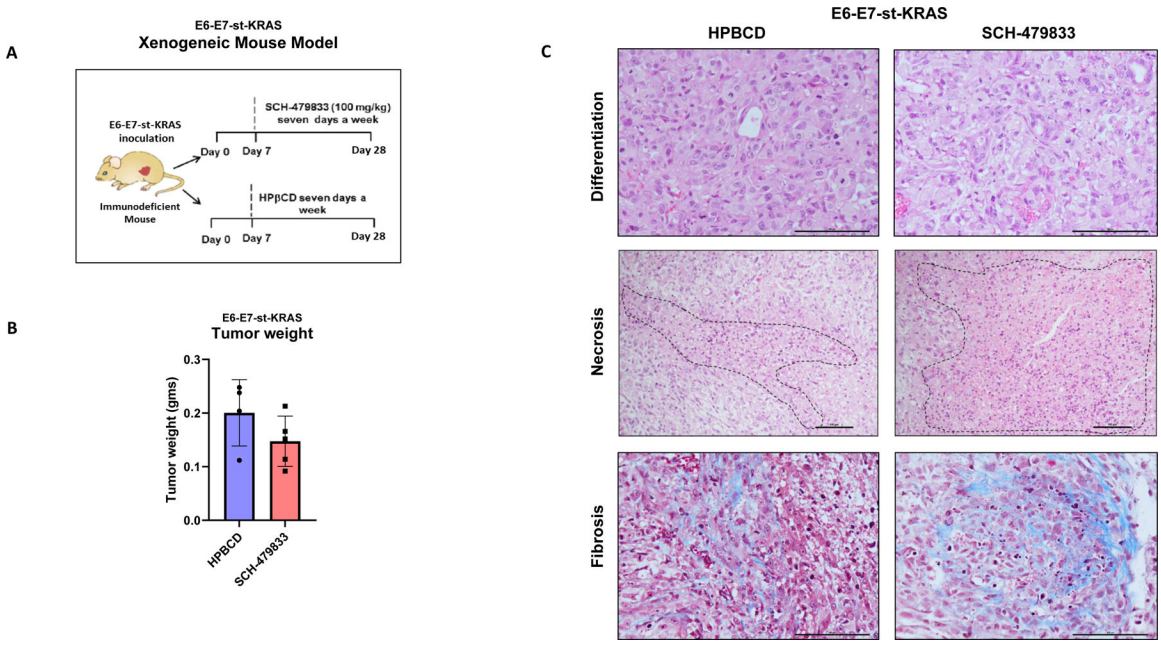


Figure 3. CXCR2/1 antagonist treatment to mice bearing E6-E7-st-KRAS xenograft tumors decreases tumor weight while increasing necrosis and fibrosis.

A. Experimental design for treating pancreatic cancer in E6-E7-st-KRAS-xenogeneic mouse model with HPβCD (control) and SCH-479833 (CXCR2 antagonist). **B.** The bar graph shows a difference in PC tumor weight of E6-E7-st-KRAS tumors derived from mice treated with HPβCD and SCH-479833. **C.** Representative photomicrographs of H&E staining of tumors showing similar morphology (Moderately to poorly differentiated carcinoma) in both the groups; the difference in necrosis (dotted area) between two groups (more necrosis with CXCR2 antagonist treatment); and fibrosis (Masson Trichrome staining) in different treatment groups. The error bars in the graphs represent the SEM. Scale bar=100 μm.

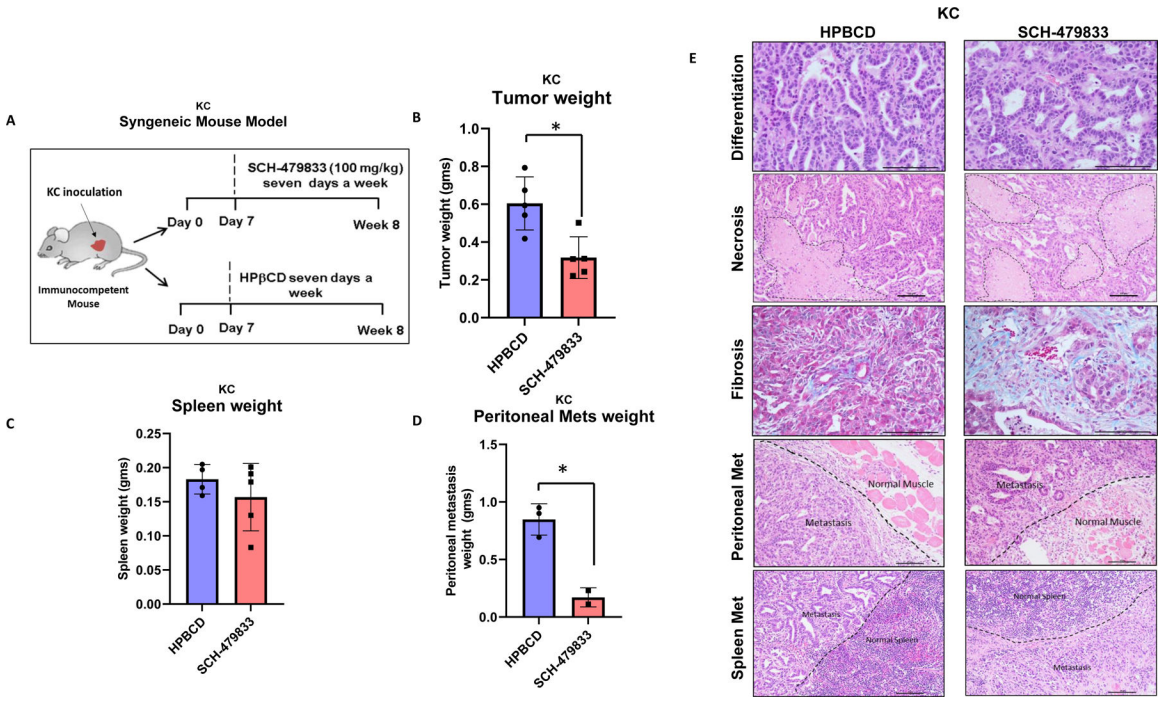


Figure 4. CXCR2/1 antagonist inhibited syngenic KC tumor growth and metastasis:

A. Experimental design for treating pancreatic cancer in KC-syngeneic mouse model with HPβCD (control) and SCH-479833 (CXCR2/1 antagonist). **B.** The bar graph shows a difference in the weight of KC tumors derived from mice treated with HPβCD and SCH-479833. **C.** The bar graph shows a difference in the weight of spleens from the mice in the two treatment groups. **D.** The bar graph shows a difference in the weight of peritoneal metastasis in the two treatment groups. **E.** Representative photomicrographs of H&E staining of tumors showing similar morphology (well-differentiated carcinoma) in both the groups; the difference in necrosis (dotted area) between two groups (more necrosis with CXCR2 antagonist treatment); increased fibrosis (Masson Trichrome staining) in CXCR2/1 antagonist treatment; H&E staining showing infiltration of muscles by malignant cells (metastasis) in the peritoneal region in both groups; and H&E staining shows infiltration of the spleen by malignant cells (metastasis) in both groups. The Error bars in the graphs represent the SEM. Scale bar=100 μm.

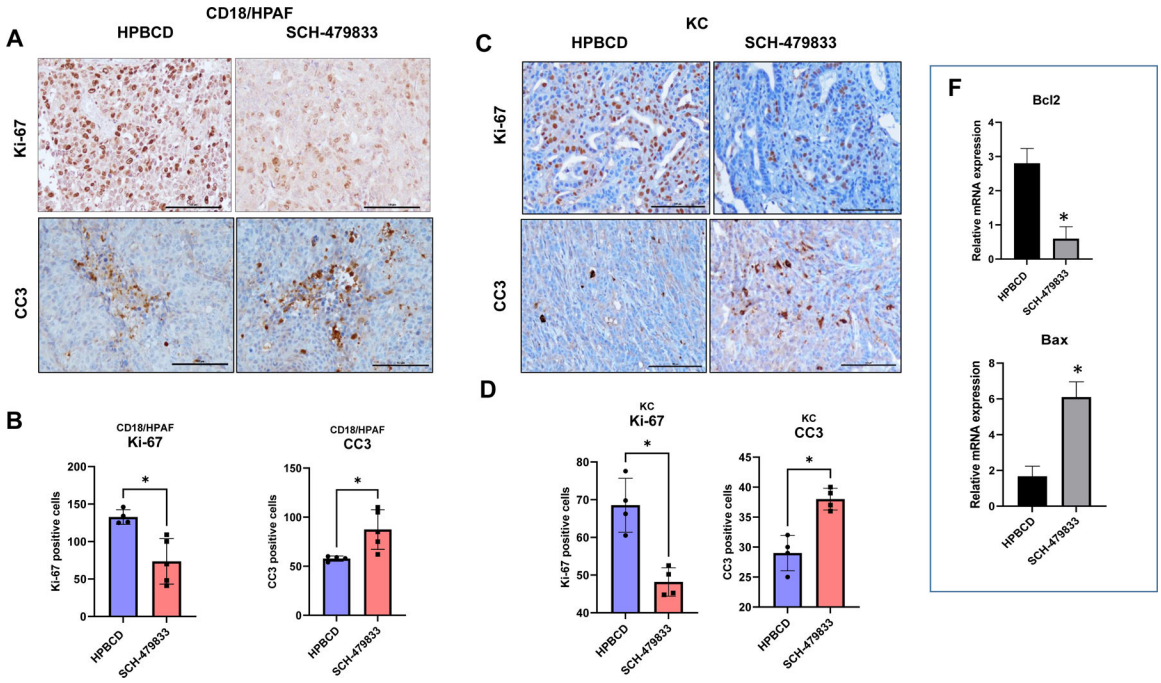


Figure 5: SCH-479833 inhibits *in-situ* PC cell proliferation and survival.

All figures show representative photomicrographs of IHC staining with different markers in CD18/HPAF (**A** and **B**) and KC (**C** and **D**) tumors derived from mice treated with HPβCD (control) and SCH-479833 (CXCR2/1 antagonist). **A.** Ki-67 and CC3 IHC shows a decrease in cell proliferation increased apoptosis after CXCR2 antagonist treatment in CD18/HPAF tumor-bearing mice. **B.** The bar graph shows the difference in the number of Ki-67 and CC3 positive cells in HPβCD and SCH-479833 treatment groups in CD18/HPAF tumor-bearing animals. **C.** Ki-67 and CC3 IHC show decreased cell proliferation and increased apoptosis after CXCR2 antagonist treatment in KC-tumor-bearing mice. **D.** The bar graph shows the difference in the number of Ki-67 and CC3 positive cells in HPβCD and SCH-479833 treatment groups in KC-tumor-bearing animals. The error bars in the graphs represent the SEM. Scale bar=100 μm. F. Relative mRNA expression of Bcl2 and Bax genes as determined by qRT-PCR.

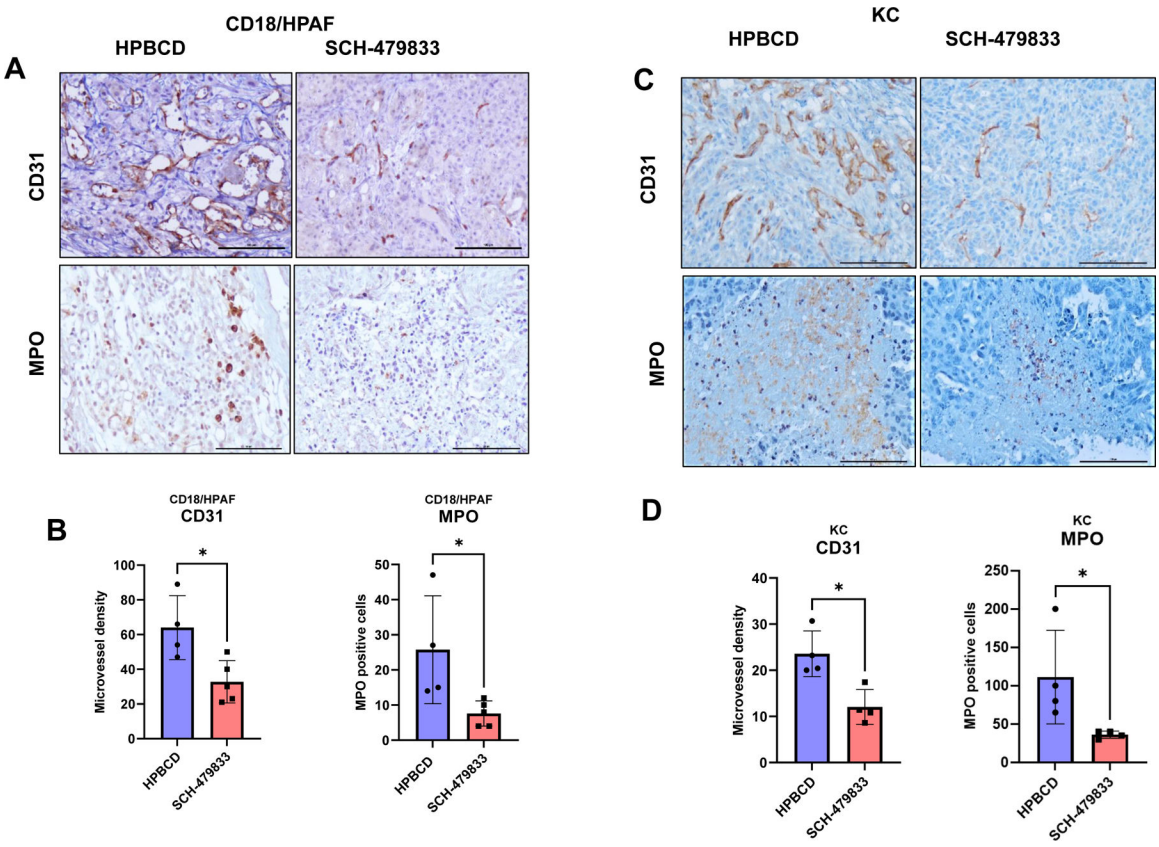


Figure 6. SCH-479833 inhibits microvessel density and neutrophil recruitment in human and murine PC tumors.

A. CD31 and MPO IHC show decreased cell proliferation and increased apoptosis after CXCR2 antagonist treatment in CD18/HPAF tumor-bearing mice. **B.** The bar graph shows the difference in the number of CD31 and MPO-positive cells in HPβCD and SCH-479833 treatment groups in CD18/HPAF-tumor-bearing animals. **C.** CD31 and MPO IHC show decreased cell proliferation and increased apoptosis after CXCR2 antagonist treatment in KC-tumor-bearing mice. **D.** The bar graph shows the difference in the number of CD31 and MPO-positive cells in HPβCD and SCH-479833 treatment groups. The error bars in the graphs represent the SEM. Scale bar=100 μm. The error bars in the graphs represent the SEM. Scale bar=100 μm.

Table 1:

Antibodies used in this study

No.	Primary Antibody	Marker For	Source	Catalog No.	Dilution
1	Anti-Ki-67	Cell Proliferation	Santa Cruz, TX, USA	sc-23900	1:100
2	Anti-MPO (Myeloperoxidase)	Neutrophils	Abcam, MA, USA	ab9535	1:100
3	Anti- CC3 (Cleaved Caspase 3)	Apoptosis	Cell Signaling, MA, USA	asp175–5A1E-9664	1:200
4	Anti- CD31 (Cluster of Differentiation 31)	Blood Vessels	Abcam, MA, USA	ab28364	1:200
5.	F4/80	Macrophages	Abcam	Ab6640	1:100
6	Ly6	Neutrophils	Thermo Scientific	MA1–40038	1:50