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Original Article

Th17 cells promote tumor growth in an immunocompetent orthotopic mouse model of prostate cancer

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Abstract: Interleukin-17 (IL-17) has been demonstrated to promote development of a variety of cancers including prostate cancer in genetically modified mouse models. IL-17 is the main product secreted by T helper 17 (Th17) cells. A recent study has shown that Th17 cells and related genes are upregulated in human prostate cancers. However, there is no direct experimental evidence to demonstrate Th17's role in prostate cancer. In the present study, we co-implanted mouse prostate cancer MPC3-luc cells with Th17-polarized mouse splenocytes in the prostate of immunocompetent C57BL/6J male mice. We found that Th17-polarized splenocytes promoted orthotopic allograft prostate tumor growth compared to the control splenocytes. The numbers of IL-17-positive lymphocytes and macrophages were higher in the prostate tumors grown from co-implantation of MPC3-luc cells and Th17-polarized splenocytes, compared to the prostate tumors grown from co-implantation of MPC3-luc cells and control splenocytes. Our findings provide the first direct experimental evidence that Th17 cells may promote prostate cancer growth.

Keywords: Prostate cancer, IL-17, Th17, orthotopic, MPC3-luc

Introduction

Naïve CD4$^+$ T cells can differentiate into several effector subsets including T helper 1 (Th1), Th2, Th17, and regulatory T (Treg) cells [1]. Naïve CD4$^+$ T cells are induced to polarized into Th17 cells by a combination of transforming growth factor (TGF)-β and interleukin-6 (IL-6) [2-4], TGF-β and IL-1β [5, 6], or TGF-β and IL-21 [7]. IL-23 has been found responsible for the survival and expansion of Th17 cells [2-4]. Th17 differentiation can be enhanced through blocking Th1 and Th2 differentiation using anti-interferon γ (IFN-γ) and anti-IL-4 antibodies because more naïve CD4$^+$ T cells are polarized into Th17 lineage after blockade of Th1 and Th2 differentiation [8, 9]. The key transcription factor for Th17 differentiation is a thymus-specific isoform of the retinoid acid receptor-related orphan receptor C (*RORC*, or *RORγ*), also named *RORγT* [10], which orchestrates Th17 differentiation and transcription of IL-17A and IL-17F [11]. Another related orphan nuclear receptor RORα also promotes Th17 differentiation [12]. Other transcription factors also play important roles in Th17 differentiation such as signal transducer and activator of transcription 3 (STAT3) [13, 14], basic leucine zipper transcription factor and ATF-like (BATF), interferon regulatory factor 4 (IRF4), v-maf avian musculoapo-

neurotic fibrosarcoma oncogene homolog (c-Maf), E1A binding protein p300 (EP300) [15], and more [16]. Th17 cells secrete IL-17A, IL-17F, IL-17A/F, IL-22, IL-21, and other cytokines and chemokines that are important in host defense, autoimmunity, inflammation, and tumorigenesis [17, 18].

IL-17A, the first member of IL-17 cytokine family, acts through a heterodimer of IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC). Recently it has been reported that IL-17A, but not IL-17F or IL-17A/F, also acts through a heterodimer of IL-17RA and IL-17 receptor D (IL-17RD) [19]. Using *Il-17rc*-null and phosphatase and tensin homolog (*Pten*)-null mouse models, we have demonstrated that blockade of IL-17 signaling through *Il-17rc* knockout inhibits prostate cancer development [20]. Further studies have revealed that IL-17 induces expression of matrix metalloproteinase 7 (MMP7) to cleave E-cadherin, thus activating β-catenin-mediated epithelial-to-mesenchymal transition, which subsequently enhances development of prostate cancer in *Pten*-null mice [21]. IL-17 also promotes development of castration-resistant prostate cancer in *Pten*-null mice partially through increasing immunosuppressive M2 macrophages and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment [22]. These findings have been obtained through comparisons between *Il-17rc*-null and *Il-17rc*-wild-type mice with *Pten* conditional knockout background. Using an orthotopic allograft model in immunocompetent mice, we found that co-injection of recombinant IL-17A and mouse prostate cancer cells slightly increased primary tumor growth, however, metastasis (mainly to the pelvic lymph nodes) was significantly enhanced [23]. On the other hand, Th17 inhibitor SR1001 and anti-mouse IL-17A monoclonal antibody are able to partially inhibit formation of prostate cancer in *Pten*-null mice [24]. These studies suggest that Th17 cells and IL-17 cytokines secreted by Th17 cells may play important roles in development of primary prostate cancer and lymph node metastasis. However, no study has been performed to directly investigate the roles of Th17 cells in prostate cancer. The purpose of the present study was to determine the effects of Th17 cells on prostate tumor growth in an orthotopic allograft model in immunocompetent male mice.

Materials and methods

Animals

Animal protocol was approved by the Animal Care and Use Committee of Tulane University, which was in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. 35 to 38-week old male mice of C57BL/6J genetic background (Jackson Laboratory, Bar Harbor, ME) were used for harvesting splenocytes and orthotopic injection of mouse prostate cancer cells.

Isolation of naïve lymphocytes and Th17 polarization

Lymphocytes (called splenocytes) were prepared from fresh mouse spleens by gently grinding the spleens between two glass slides and filtering through 70-μm cell strainers (BD Biosciences) to make single cell suspension. Th17 polarization was performed according to our previous methods [25]. Briefly, cell culture dishes (60 × 15 mm in size) were first coated with anti-mouse CD3ε (5 μg/ml) at 37°C for overnight. After removing the antibody solution, the dishes were gently washed twice with phosphate-buffered saline (PBS). Naive splenocytes at 10×10^6/ml were plated in the coated dishes in Roswell Park Memorial Institute (RPMI) 1640 medium (Genesee Scientific) containing 2 mM glutamine, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 1% penicillin streptomycin cocktail (Mediatech, Inc., Manassas, VA), 1 nM IL-2, and 10% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA), in the presence of Th17 polarization medium consisting of anti-mouse CD28 (5 μg/ml), IL-6 (50 ng/ml), TGF-β1 (1 ng/ml), IL-23 (5 ng/ml), anti-mouse IL-4 (10 μg/ml), and anti-mouse IFN-γ (10 μg/ml). In parallel, separate dishes of splenocytes were cultured in the same medium without Th17 polarization medium as control splenocytes (control group). Cytokines (mouse IL-6, IL-23, and TGF-β1) and antibodies (anti-mouse CD3ε, CD28, IL-4) were purchased from BioLegend and anti-IFN-γ was purchased from R&D Systems. Three days after polarization, the cells were harvested for injection into the mice.

Cell culture

MPC3 cell line was established in the lab of Dr. Zhenbang Chen (Meharry Medical College, Nashville, TN) from *Pten*$^{-/-}$; *p53*$^{-/-}$ double knockout mouse prostate cancer and was transfected with a firefly luciferase construct to establish MPC3-luc cell line that stably expresses luciferase protein [23]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Genesee Scientific) supplemented with 5% FBS (Gemini Bio-Products, West Sacramento, CA), 1% penicillin streptomycin cocktail (Mediatech, Inc., Manassas, VA), and 200 μg/ml hygromycin (Sigma), in a 37°C humidified incubator with 5% CO_2.

Orthotopic cancer cell implantation

MPC3-luc cells were grown to near confluence on the day of surgery. 5 × 10^5 MPC3-luc cells in 10 μL PBS were mixed with 10 μL of Matrigel™ (Corning) for cancer cells only injection. For injection of a mixture of MPC3-luc cells and splenocytes, 5 × 10^5 MPC3-luc cells and 5 × 10^5 control splenocytes (control group) or 5 × 10^5 MPC3-luc cells and 5 × 10^5 Th17-polarized splenocytes (test group) were mixed in 10 μL PBS and 10 μL of Matrigel™ for injection. Mice were randomized into control or test groups by flipping a coin prior to surgery [23]. Mice in the control and test groups were operated on in an alternating fashion to avoid bias due to differences in cell viability over the course of the operation because the cells were kept on ice. Mice were initially anesthetized with 3% isoflurane at an induction chamber flow rate of 0.8 L/min. Mice were then positioned sternal on the surgical table with maintenance of 2-2.5% isoflurane through a nosecone. Mice received buprenorphine at a dose of 0.1 mg/kg body weight via intraperitoneal administration. The lower abdominal area was shaved with an electric trimmer and sterilized with 70% ethanol and then betadine solution. A 1-cm incision was cut in the midline of the lower abdomen to expose the abdominal cavity. The bladder was then identified through the surgical window and pulled out of the abdominal cavity to expose the ventral prostate lobes (VP). A 50-μL Hamilton syringe attached to a 26 s gauge needle was then used to take 20 μL of Matrigel™ containing MPC3-luc cells with or without splenocytes. The needlepoint was inserted just below where the bladder coalesced into the urethra, advancing only as far as the VP. The 20 μL of cell mixture was then administered slowly. After injection, the abdominal and skin wound was sutured separately with 5/0 chromic gut suture (CP Medical, Norcross, GA, USA). The animal was then positioned sternal in a cage for 10-15 minutes until becoming conscious [23].

In vivo bioluminescent imaging

Starting 3 or 7 days post-surgery, tumor growth was assessed using IVIS Lumina XRMS *In Vivo* Imaging System (PerkinElmer, Waltham, MA) as described previously [23]. Prior to imaging, the instrument was set to collect a minimum of 20,000 photon counts on the auto settings. Imaging parameters were prioritized as exposure time, binning, and *f* stop. D-luciferin potassium salt (catalogue #MB000102-R70170, Syd Labs Inc., Natick, MA) was resuspended in PBS to a final concentration of 20 mg/ml. Mice were dosed with 10 μL/g luciferin substrate intraperitoneally and waited for 10 minutes to allow for distribution of substrate prior to image acquisition. Animals were anaesthetized with 3% isoflurane with a 1 L/min induction chamber flow rate and maintained at 2% isoflurane at a 0.5 L/min imaging chamber flow rate. After the completion of imaging, mice were replaced in the cage in a sternal position and observed for 10-15 minutes until becoming conscious. Endpoint was determined in consulting with a veterinarian, including the following criteria: palpable tumor in the lower abdomen > 1.5 cm in diameter, inability to eat or drink, inability to ambulate (i.e. when animal failed to move when approached), inability to pass urine, loss of 15% body weight during the course of weekly weighing, or loss of bioluminescent signals on imaging. If an animal met any of the criteria, it would be euthanized for endpoint analysis. Prior to euthanasia, *in vivo* imaging was performed as described above. The animal was weighed intact. The entire genitourinary (GU) bloc was dissected out of the carcass, fixed in 4% paraformaldehyde overnight at 4°C, and subsequently embedded in paraffin.

Immunohistochemical (IHC) staining

Paraffin blocks were cut into 4 μm-thick tissue sections, which were mounted on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA, USA). Sections were baked for 150 minutes (min) in a 60°C incubator. After being deparaf-

finized in xylene and rehydrated through a series of decreasing concentrations of ethanol, the sections were put in 0.01 M ethylenediaminetetraacetic acid in Tris buffer and boiled for 5 min for antigen retrieval. Then, the sections were cooled down at room temperature for 20 min, followed by treating with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Non-specific binding was blocked with 1.5% normal serum (Vector Laboratories, Burlingame, CA, USA) for 60 min as previously described [26]. The following primary antibodies were used: goat anti-IL-17 polyclonal antibodies (E-19, sc-6077; 1:100 dilution), rabbit anti-NOS2 [inducible nitric oxide synthase (iNOS), N-20, sc-651; 1:100 dilution], and goat anti-arginase I (V-20, sc-18345; 1:100 dilution) were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA. The sections were incubated with primary antibodies overnight at 4°C. Non-immune serum replacing primary antibodies was used as negative control. For IL-17 staining, after incubation with the primary antibodies, the sections were washed three times in PBS and then incubated with biotinylated secondary antibodies for 75 min, followed by avidin peroxidase using the Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA). The chromogenic reaction was carried out with 3'-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. Then, the sections were counterstained with a hematoxylin solution. Finally, the sections were dehydrated through a series of increasing concentrations of ethanol, cleared with xylene, and covered with cover glasses. For iNOS and arginase I staining, fluorescence-labelled secondary antibodies were incubated for 60 min at room temperature. Rhodamine Red™-X (RRX) affiniPure donkey anti-rabbit IgG (cat# 711-295-152; 1:200 dilution) and Alexa Fluor 488-conjugated affiniPure donkey anti-goat IgG (cat# 705-545-147; 1:200 dilution) were purchased from Jackson ImmunoReseach Laboratories, Inc. After three washes with 0.1% Tween-20 in PBS, the sections were stained with Hoechst 33342 solution (ImmunoChemistry Technologies; 1:200 dilution) for nuclear staining. Five representative high-power fields (400 × magnification) per tissue section were randomly selected in the tumor areas and evaluated by counting the number of positively stained cells in each high-power field. Then, the average number of positive cells was calculated from the five high-power fields to represent each specimen. Five tumor samples per group (n = 5) were used in IHC staining.

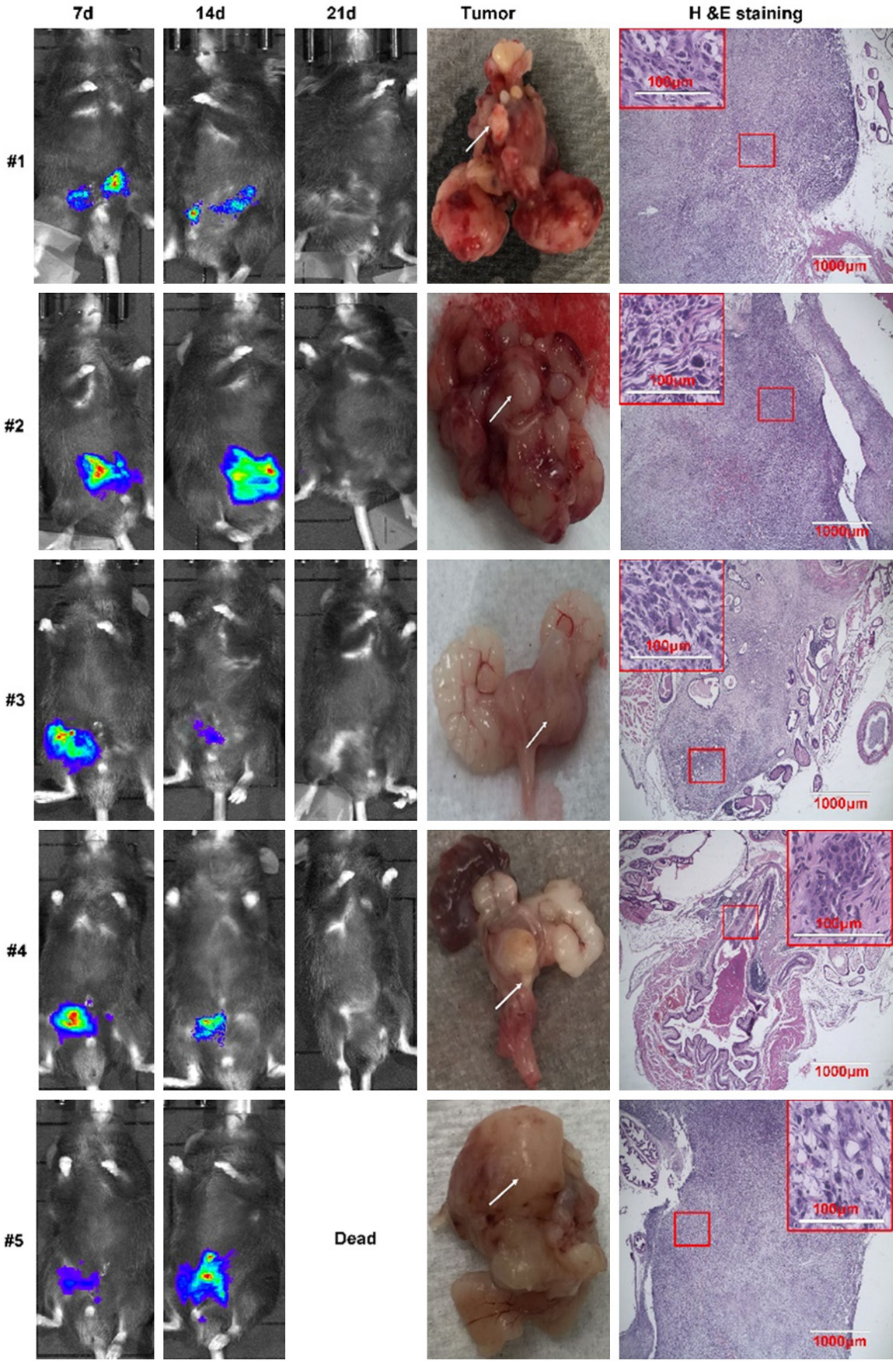
Statistical analysis

Quantitative data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism, version 6.0c (GraphPad Software, San Diego, CA). One-way analysis of variance (ANOVA) and unpaired Student's t test were used to analyze IL-17 staining data. Two-way ANOVA was used for analysis of iNOS and arginase I staining data. Two-way ANOVA and Student's t test were used for analysis of imaging data. $P < 0.05$ was considered statistically significant.

Results

Orthotopic allograft prostate tumor growth

In a pilot study, half-million MPC3-luc cells without any splenocytes were injected into the ventral prostate lobes of 6 male mice. Seven days (7 d) after implantation, bioluminescence imaging scan found signals in the lower abdomen of all animals (**Figure 1**). At 14 d, the signals increased in half of the animals, but at 21 d, the signals disappeared in all animals (**Figure 1**). However, necropsy revealed macroscopic tumor growth and tumor cells in the tumor tissue sections (**Figure 1**). Then, co-implantation of half-million MPC3-luc cells and half-million control splenocytes (control group) and co-implantation of half-million MPC3-luc cells and half-million Th17-polarized splenocytes (test group) were performed. Bioluminescence imaging scan found weak signals in the lower abdomen of all animals at 3 d (**Figure 2**). The signals were increased at 7d and peaked at 10-14 d in most animals, but faded at 21 d and 24 d (**Figure 2**). Quantification of the imaging signals showed that the signals of the test group were stronger than those of the control group at 7 d, 10 d, and 14 d (**Figure 3A**; $P < 0.05$ at 14 d with Student's t test and $P = 0.0529$ with two-way ANOVA comparing the two groups through the 6 time points). Of note, at 3 d, the imaging signals had no significant difference between the two groups ($P > 0.05$), suggesting that the numbers of cancer cells implanted were approximately equal between the two groups. Necropsy con-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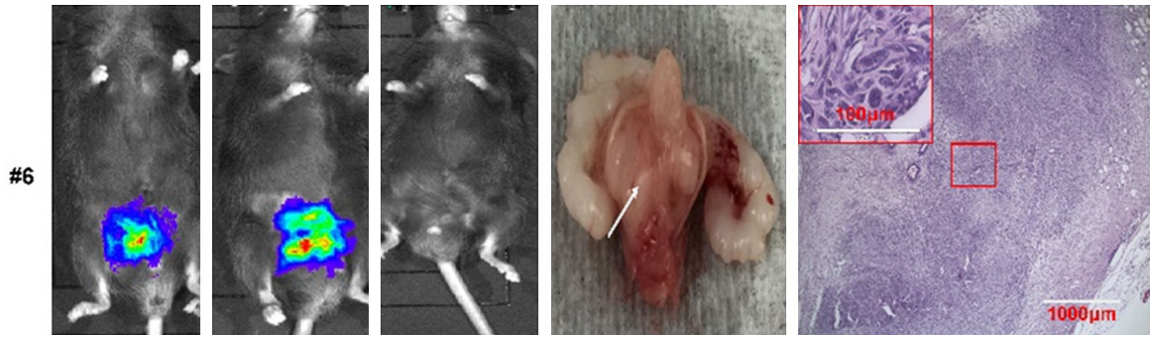


Figure 1. Orthotopic allograft prostate tumor growth in male mice implanted with MPC3-luc cells without any splenocytes. Six mice (#1 to #6) were longitudinally monitored with bioluminescence imaging at 7 days (7 d), 14 d, and 21 d post-implantation. Genitourinary blocs with tumors were harvested at the endpoints. Tumor tissue sections were stained with hematoxylin and eosin (H & E). Arrows in Tumor column indicate the tumors. Magnification in the H & E staining column: 40 × and scale bar = 1000 μm; of the insets, 400 × and scale bar = 100 μm.

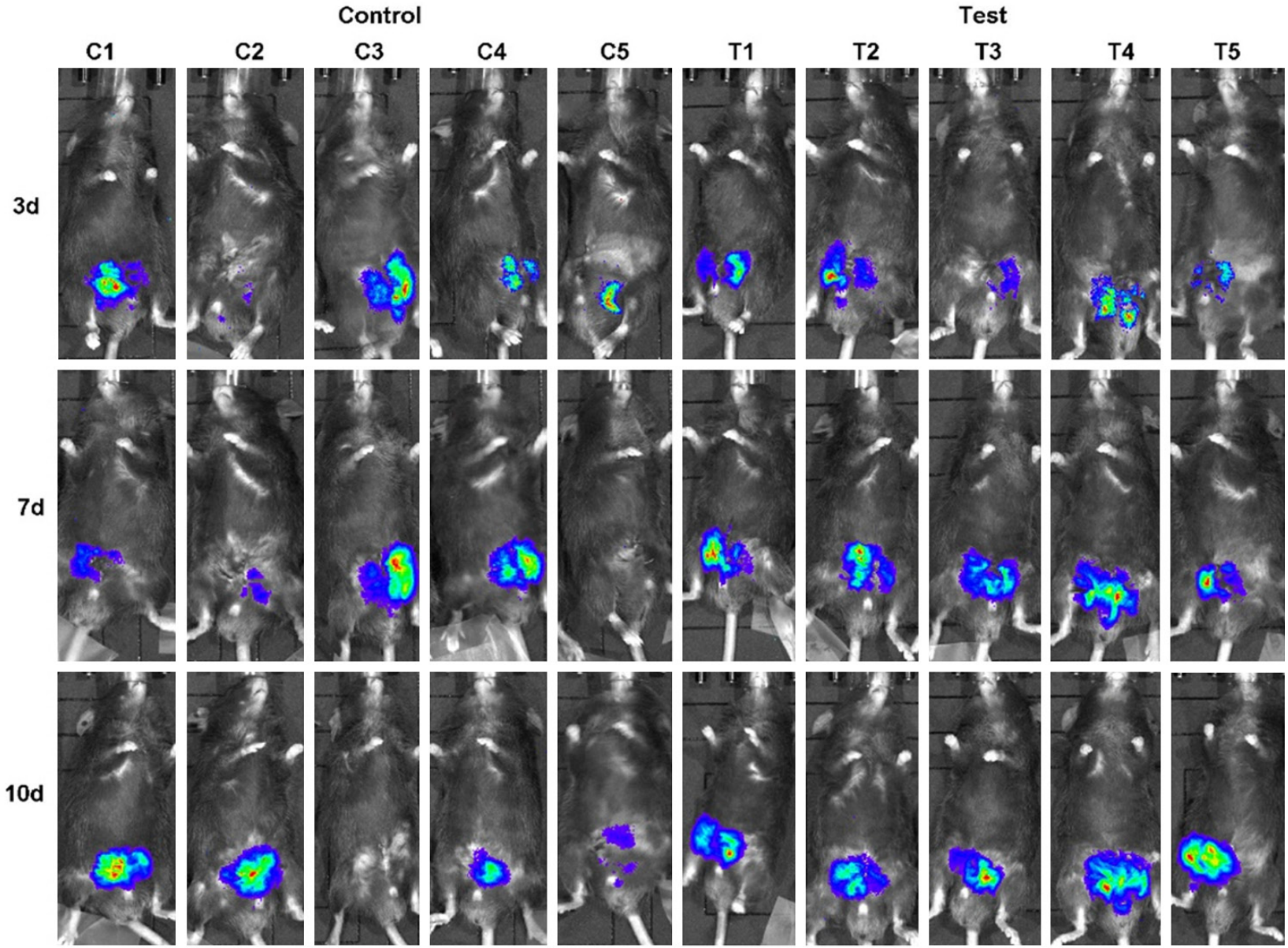
firmed orthotopic prostate tumor growth in all animals (**Figure 3B** and **3C**).

Th17 cells and macrophages in orthotopic allograft prostate tumors

We performed IL-17 staining to detect IL-17-positive lymphocytes (mainly Th17 cells) in orthotopic allograft prostate tumors. We found that the number of IL-17-positive lymphocytes was significantly more in the test group than the control group or MPC3-luc only group (**Figure 4**). Further, we checked M1 macrophages that express iNOS and M2 macrophages that express arginase I [22]. We found that the test group had more M1 and M2 macrophages than the control group (**Figure 5**).

Discussion

We have previously demonstrated that IL-17 promotes prostate cancer development in genetically modified mouse models [20-22, 27]. We have shown that recombinant IL-17 cytokines enhanced prostate cancer cell migration and invasion *in-vitro* [28] and promoted lymph node metastasis *in-vivo* [23]. In this exploratory study, we showed that Th17-polarized splenocytes promoted prostate tumor growth in an immunocompetent orthotopic mouse model. A recent study found that Th17 cells and related genes were upregulated in human prostate cancers from the World Trade Center responders [29]. Our findings provide the first experimental evidence to support a tumor-promoting role of Th17 cells, which are consistent with the findings in human prostate cancers [29]. The number of IL-17-positive lymphocytes was more in the test group than the control group, indicating that the implanted Th17-polarized splenocytes indeed contained Th17 cells. The fact that more M1 and M2 macrophages were found in the test group compared to the control group further supports that IL-17 cytokines were likely secreted by Th17 cells in the prostate tumors, because it has been shown previously that IL-17 could attract macrophages [30]. It is not clear why M1 macrophages are more than M2 macrophages in the test group, yet the tumor growth appears to be promoted in the test group compared to the control group, since it has been known that M1 macrophages inhibit tumor growth while M2 macrophages promote tumor growth [30-32]. To better understand the tumor immune microenvironment, we also tried to detect myeloid-derived suppressor cells (MDSCs), but we failed to identify any MDSCs, though we used the same methods as previously described [22]. This could be due to a technical problem or it could be that few MDSCs exist in the tumors. It is worth mentioning that in the pilot experiment with implantation of MPC3-luc cells only, the tumor growth appears to be faster than co-implantation of MPC3-luc cells and splenocytes in the later experiment, though we could not directly compare the two experiments because they were not performed simultaneously using the same batch of MPC3-luc cells. We speculate that some splenocytes could become inhibitory cells such as cytotoxic T cells when injected *in-vivo*. Future studies using purified Th17 cells may clarify the findings.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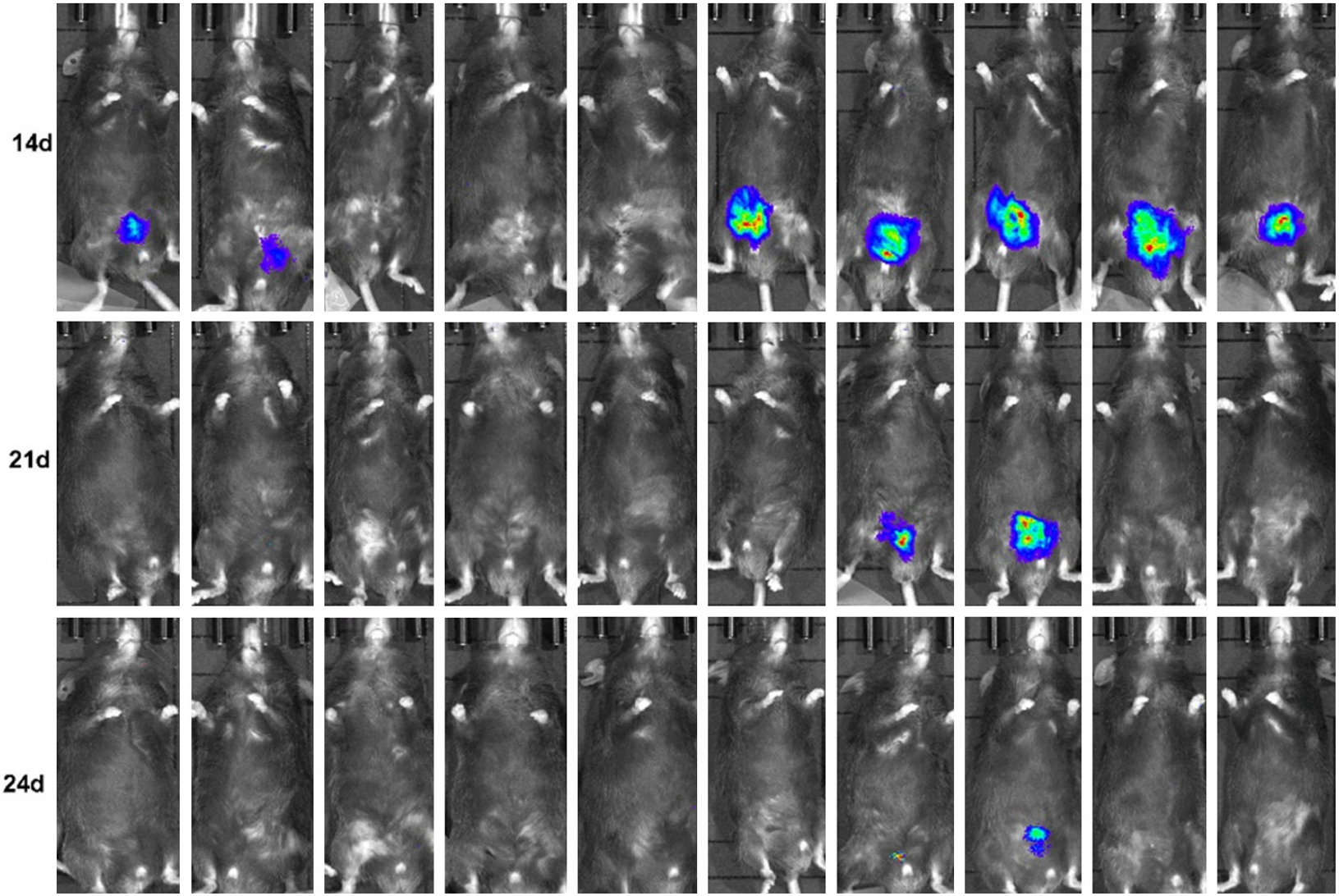

Figure 2. Orthotopic allograft prostate tumor growth in male mice implanted with MPC3-luc cells plus control splenocytes (control group) or Th17-polarized splenocytes (test group). Five mice per group (C1-5 in the control group and T1-5 in the test group) were longitudinally monitored with bioluminescence imaging at 3 days (3 d), 7 d, 10 d, 14 d, 21 d, and 24 d post-implantation.

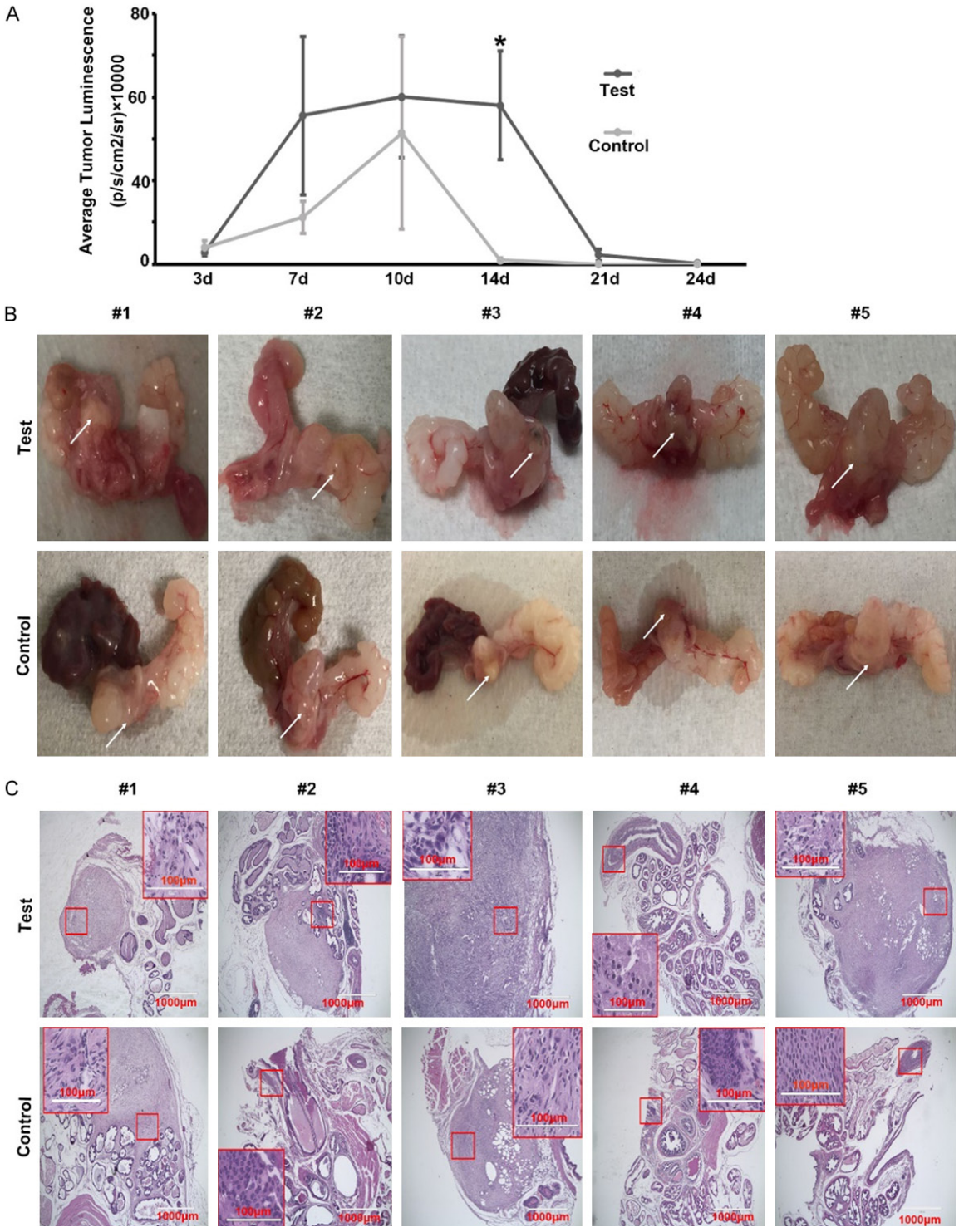

Figure 3. Quantification and necropsy of orthotopic allograft prostate tumor growth in male mice implanted with MPC3-luc cells plus control splenocytes (control group) or Th17-polarized splenocytes (test group). A. Quantification of bioluminescence imaging signals at different time points; error bars represent SEM; *P < 0.05 at 14 d with Student's t test; P = 0.0529 with two-way ANOVA comparing the two groups through the 6 time points. B. Genitourinary blocs harvested at the endpoints; arrows indicate the tumors. C. Tumor tissue sections were stained with hematoxylin and eosin (H & E); magnification: 40 × and scale bar = 1000 μm; of the insets, 400 × and scale bar = 100 μm.

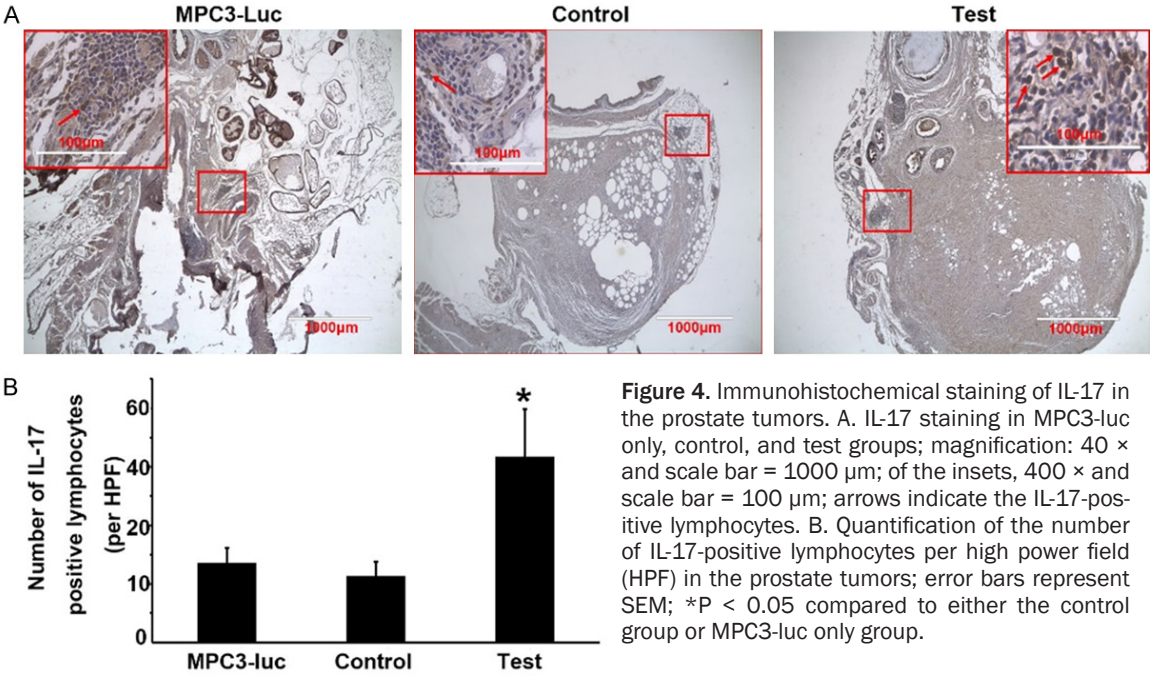

Figure 4. Immunohistochemical staining of IL-17 in the prostate tumors. A. IL-17 staining in MPC3-luc only, control, and test groups; magnification: 40 × and scale bar = 1000 μm; of the insets, 400 × and scale bar = 100 μm; arrows indicate the IL-17-positive lymphocytes. B. Quantification of the number of IL-17-positive lymphocytes per high power field (HPF) in the prostate tumors; error bars represent SEM; *P < 0.05 compared to either the control group or MPC3-luc only group.

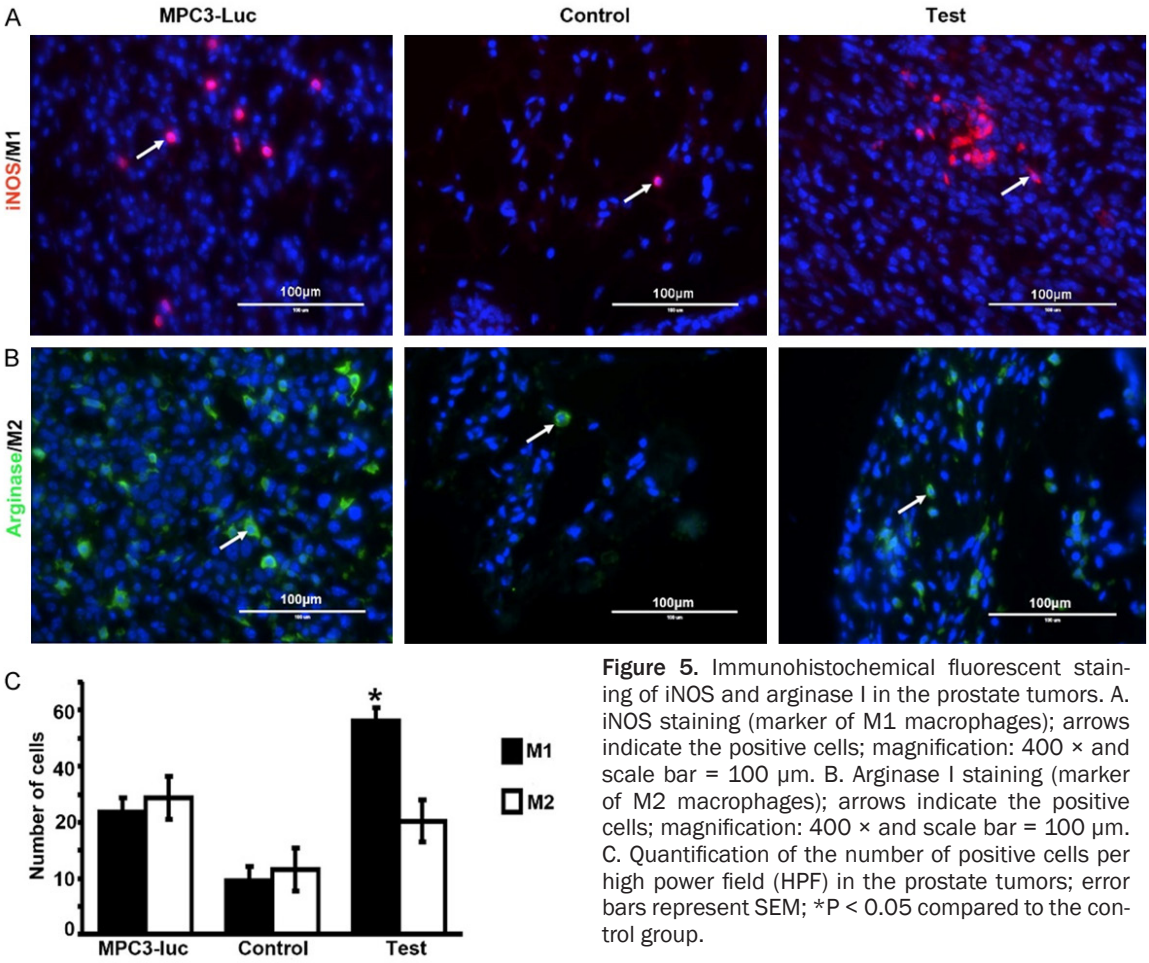

Figure 5. Immunohistochemical fluorescent staining of iNOS and arginase I in the prostate tumors. A. iNOS staining (marker of M1 macrophages); arrows indicate the positive cells; magnification: 400 × and scale bar = 100 μm. B. Arginase I staining (marker of M2 macrophages); arrows indicate the positive cells; magnification: 400 × and scale bar = 100 μm. C. Quantification of the number of positive cells per high power field (HPF) in the prostate tumors; error bars represent SEM; *P < 0.05 compared to the control group.

The limitations of the present study are obvious. First, the number of animals is small, which limits the power of statistical analysis. Second, the MPC3-luc cells may be a heterogeneous population with some cells lacking luciferase expression. We found that some tumors do not show bioluminescence signals, but live tumor cells are found in the tumor tissue sections. We are working to purify the cells to homogenously express luciferase protein. And finally, MPC3 cells were derived from a mixed genetic background, thus host rejection is possible when injected into pure C57BL/6J mice. What could be done is to serially transplant the cells in C57BL/6J mice to select cell clones that can survive in the host. Alternatively, Myc-CaP and Myc-CaP/CR cell lines may be used in mice with FVB genetic background [33].

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Disclosure of conflict of interest

None.

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