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STUDYING THE ROLE OF MACROPHAGES IN CIRCULATING PROSTATE CANCER CELLS BY *IN VIVO* FLOW CYTOMETRY

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Metastasis is a very complicated multi-step process and accounts for the low survival rate of the cancerous patients. To metastasize, the malignant cells must detach from the primary tumor and migrate to secondary sites in the body through either blood or lymph circulation. Macrophages appear to be directly involved in tumor progression and metastasis. However, the role of macrophages in affecting cancer metastasis has not been fully elucidated. Here, we have utilized an emerging technique, namely *in vivo* flow cytometry (IVFC) to study the depletion kinetics of circulating prostate cancer cells in mice and determine how depletion of macrophages by the liposome-encapsulated clodronate affects the depletion kinetics. Our results show different depletion kinetics of PC-3 cells between the macrophage-deficient group and the control group. The number of circulating tumor cells (CTCs) in the macrophage-deficient group decreases in a slower manner compared to the control mice group. The differences in depletion kinetics indicate that the absence of macrophages facilitates the stay of prostate cancer cells in circulation. In addition, our imaging data suggest that macrophages might be able to arrest, phagocytose and digest PC-3 cells. Therefore, phagocytosis may mainly contribute to the depletion kinetic differences. The developed methods elaborated here would be useful to study the relationship between macrophages and tumor metastasis in small animal cancer models.

Keywords: Prostate cancer; macrophages; liposome-encapsulated clodronate; *in vivo* flow cytometer; circulating tumor cells.

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1. Introduction

Metastasis is a complex multi-step process, which consists of a series of sequential events involving sophisticated interactions between the cancer cells and their surroundings. The exact mechanism of metastasis has not yet been completely understood. In metastasis, the malignant cells must break off from the primary tumor and make their way into either blood or lymph circulation. Once entering the circulatory system, the cancer cells gain access to every portion of the body. The cancer cells usually get trapped in the first set of capillaries network when they encounter the downstream of their origin. The first vascular bed encountered by blood leaving most organs is in the lung since deoxygenated venous blood leaving many organs is returned to the lung for reoxygenation; only the intestines send their blood to the liver first. Accordingly, the lungs are the most common site of metastasis, followed by the liver.[1] After the cancer cells come to a rest at a secondary endothelial site, they may re-penetrate and pass through the endothelium layer, continue to proliferate until eventually another clinically detectable tumor is formed. Metastasis is a highly inefficient process,[2,3] most cancer cells die due to shear stress, lack of oxygen and nutrients, disruption of cell−cell contacts, immune surveillance, etc, yet a very few cancer cells may have the ability to survive and finally create a new tumor.

Prostate cancer is the second most frequently diagnosed cancer, and the third leading cause of cancer-related death among men in developed countries.[4] Like most other solid malignancies, prostate cancer can metastasize to distant organs such as the liver, lungs and brain. However, it also has a particular high favor of metastasizing to the bone as well as the lymph nodes. Most prostate tumors grow slowly and remain confined to the gland for many years. Early prostate cancer usually causes no symptoms. However, as the cancer advances, it can metastasize throughout other areas of the body and may cause bone pain, difficulty in urinating, problems during sexual intercourse, etc. The specific causes of prostate cancer remain unknown.[5]

Macrophages are versatile plastic cells which constitute a major component in virtually all malignancies.[6] Macrophages are conventionally considered to be cytotoxic effector cells.[7] The resident liver macrophages (Kupffer cells) have been shown to play a crucial role in arresting and killing disseminated tumor cells that enter the liver via the portal blood *in vivo*.[8,9] However, recent studies reveal that macrophages within the tumor microenvironment facilitate angiogenesis and extracellular matrix breakdown and remodeling and promote tumor cell motility, which enhances cancer metastasis.[10−12] Although some clinical studies have shown a correlation between the numbers of tumor-associated macrophages and poor prognosis of prostate cancer,[13] the role of macrophages in affecting circulating prostate cancer cells and metastasis has not been fully elucidated.

Liposome-encapsulated clodronate (lip-clo) is an efficient reagent for the selective depletion of macrophages and has been successfully applied in a number of immunological studies.[14,15] Free clodronate, which does not easily pass cell membrane, is not toxic for macrophages. However, lip-clo is readily taken by macrophages. The clodronate is accumulated intracellularly once delivered into macrophages using liposomes as vehicles. The macrophages are finally irreversibly damaged and undergo apoptosis after intracellular concentration of clodronate exceeds a toxic concentration threshold.[16] Macrophages can be depleted in the liver (Kupffer cells), lung (Alveolar macrophages), spleen (different macrophage subpopulations), peritoneal cavity, lymph nodes, etc, if liposomes are adequately administered.[17,18]

The *in vivo* flow cytometry (IVFC) has the capability to continuously and noninvasively quantify the number and flow characteristics of fluorescently labeled cells *in vivo*.[19−24] In IVFC measurement, when the fluorescent cells in fast flowing blood pass through the laser slit across the artery, the fluorescence signal could be excited and detected. IVFC allows researchers to acquire cytometric information from the circulation in live animals without extracting blood samples. Thus, IVFC is a particular useful technique for studying tumor on small animal models.[25,26] Fan *et al.* have recently used it for real-time monitoring of the rare circulating hepatocellular carcinoma cells in an orthotopic model of mice and found that the resection prominently restricts hematogenous metastasis and distant metastases.[27] Here, we have studied the role of macrophages in circulating prostate cancer cells by IVFC.

2. Materials and Methods

2.1. *Cells, animals and chemicals*

The human androgen-independent high metastatic potential prostate cancer cell line PC-3 was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Science, Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Balb/c nude mice and Kunming mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd and raised under specific pathogen-free conditions. The study was approved by the Ethical Committee of Animal Experiments of Institutes of Biomedical Sciences, Fudan University.

Lip-clo was a kind gift from Dr Nico van Rooijen at ClodronateLiposomes.org (Amsterdam, the Netherlands), and contained 5 mg of clodronate per 1 ml suspension.

2.2. *Toxic assay of lip-clo*

Peritoneal macrophages were harvested from eight-week-old male Kunming mice ($40\,g \pm 5\,g$) based on the well-developed protocol.[28] Peritoneal macrophages were seeded in 10-cm-diameter petri-dish and cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Jinuo Bio-pharmaceutical Tech. Co., Ltd., Hangzhou, China) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA). The cells were allowed to adhere to the dish surface by culturing for 2 h. Nonadherent cells were removed by gently washing three times with pre-warmed RPMI-1640 medium.

To verify the macrophages depletion effect of lip-clo, 0.2 ml lip-clo suspension (containing 1 mg clodronate) was added into the culture medium of the petri-dish where approximately 10^5 peritoneal macrophages were seeded. The macrophages were then cultured at 37°C and 5% CO_2 incubator overnight. The CCD camera mounted in our *in vivo* flow cytometer, which is coupled with a 40× microscopic objective (NA = 0.6), was used to monitor the viability of microphages and take images before/after lip-clo treatment.

2.3. *Macrophages depletion and depletion of circulating prostate cancer cells*

To obtain the macrophage-deficient mouse model, the lip-clo suspension was injected via the tail vein into Balb/c nude mice with a dose of 0.1 ml lip-clo (0.5 mg clodronate) suspension per 10 g mice weight. Lip-clo was warmed slowly to room temperature before intravenous injection each time. As lip-clo precipitated in storage, the suspension of lip-clo was shaken or stirred before injection in order to get a homogeneous suspension. Twenty-four hours after lip-clo injection, the macrophages were almost depleted when the mice were ready for experiments.[29]

To build tumor metastasis model, the PC-3 prostate cancer cells were cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Jinuo Bio-pharmaceutical Tech. Co., Ltd., Hangzhou, China) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA). For cell labeling, cell suspension was incubated at 37°C with 0.1 mM lipophilic carbocyanine DiD (Invitrogen, Eugene, OR, USA) for 30 min, and then washed by phosphate-buffered saline for three times. The cells were counted by hemocytometer and prepared to use. About 5×10^5 DiD-labeled cells were injected into six-week-old male Balb/c nude mice ($20\,g \pm 2\,g$) to establish metastatic prostate cancer model.

In the macrophage-deficient metastatic prostate cancer model, major types of macrophage populations, such as Kupffer cells in the liver and red pulp macrophages in the spleen, could remain depleted by lip-clo for as long as one to two weeks,[30,31] which makes it possible to study continuously how macrophages affect circulating prostate cancer cells by IVFC in the long term.

2.4. In vivo *flow cytometry*

In vivo flow cytometer (Fig. 1) is set up based on previous experience.[19] Briefly, we use transillumination with a $535\,nm \pm 15\,nm$ light-emitting diode (LED) to visualize the major veins and arteries of in the mouse ear. A typical artery of approximately 40 μm in diameter is selected for data acquisition. Light from a 633 nm He−Ne laser is focused into a slit by a cylindrical lens and imaged across the selected ear artery. The size of the slit at the focal plane of the sample is approximately $5\,\mu m \times 72\,\mu m$. The depth of focus (i.e., the full width at half-maximum of the light slit onto the sample in the axial direction) is approximately 50 μm, a value chosen to match the vessels of interest. The sample is positioned so that the long dimension of the slit traverses the width of the blood vessel.

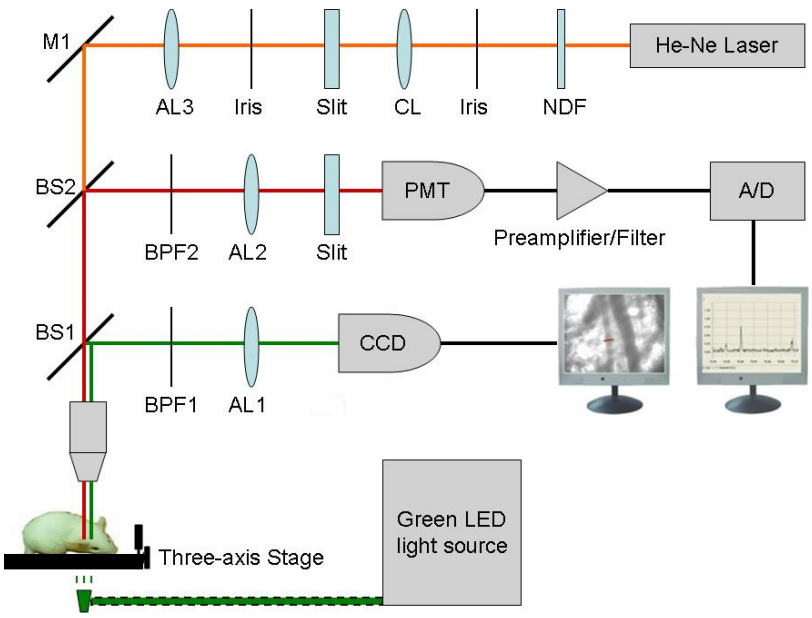


Fig. 1. Schematic of the *in vivo* flow cytometer setup: He−Ne laser light 633 nm is focused into a slit by a cylindrical lens (CL) and imaged across the selected blood vessel with a microscope objective lens (40x, NA = 0.6). The fluorescence from excited labeled cells in blood stream is collected by the same microscope objective, directed through the dichroic beam splitter BS1, which reflects green LED light to image sample onto CCD camera, reflected by a second beam splitter BS2, and imaged onto a 200 μm × 3000 μm mechanical slit, which is confocal with the excitation slit. BPF1−2: bandpass filter. AL1−3: achromatic lenses. NDF: neutral density filter. A/D: analog-to-digital converter. M1: mirror.

Fluorescently labeled cells are excited one by one as they flow through the chosen artery and traverse the slit, producing a burst of fluorescence for each cell. Fluorescence is detected with a photomultiplier tube (PMT) placed directly behind the mechanical slit, sampled at a rate of 5 kHz with a data acquisition card, and displayed/stored on a computer.

In animal experiments, the mice are anesthetized with sodium pentobarbital and then placed on a heated stage. An artery in the ear is chosen for obtaining measurements. Fluorescence signal is excited as the labeled circulating cells pass through the laser slit focused across the blood vessel [Fig. 2(a)]. Detecting the excited fluorescence confocally enables researchers to monitor labeled cells in the very same vessel continuously. Signal is recorded at a rapid rate (5 kHz) to ensure the measurement of fast-flowing cells [Fig. 2(b)]. The blood flow velocity in the artery is 2.3−7.0 mm/s.[19] The slit width is approximately 5 μm. The number of fluorescent peaks, along with the height and full width at half maximum of each peak, is determined by a fully automated algorithm using wavelet-based denoising and dynamic peak picking method,[24] which is more objective in the analysis than previous line-separating method.[19]

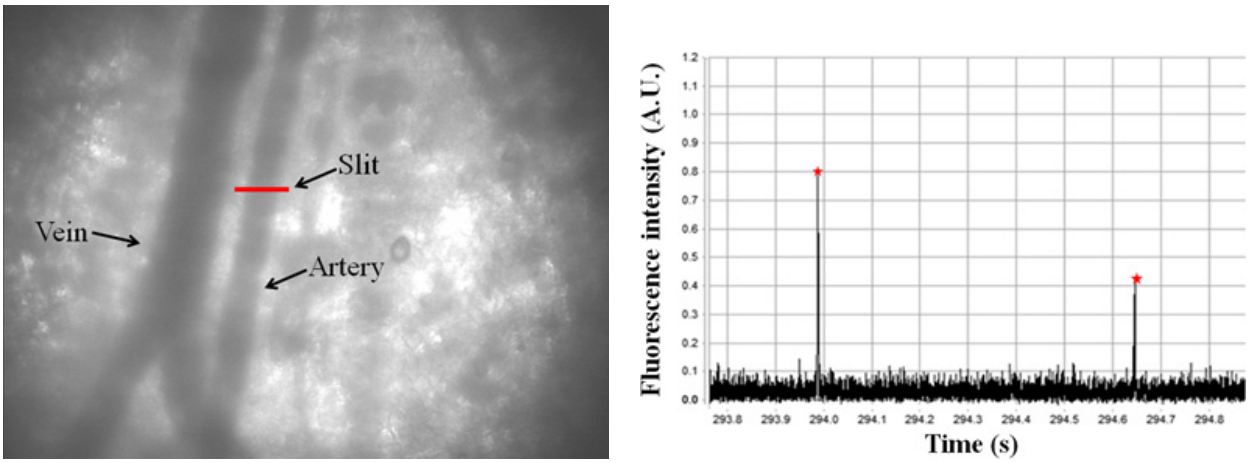
2.5. *Confocal laser scanning microscopy*

To study the phagocytosis of prostate cancer cells by macrophages, DiD-labeled PC-3 cells were co-cultured with the peritoneal macrophages in a 10-cm-diameter petri-dish and cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Jinuo Bio-pharmaceutical Tech. Co., Ltd., Hangzhou, China) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA) overnight. A confocal laser scanning microscope (CLSM, Leica TCS SPE, Leica Microsystems) with an excitation laser wavelength of 635 nm and an emission bandwidth PMT of 650−700 nm were used for acquiring images.

3. Results

3.1. *Macrophages were depleted by lip-clo* in vitro

Before lip-clo was introduced into mice by intravenous injection, a validation test was performed to verify the depletion of macrophages by lip-clo *in vitro*. About 0.2 ml lip-clo suspension, which contains 1 mg clodronate was added to 10^5 peritoneal macrophages



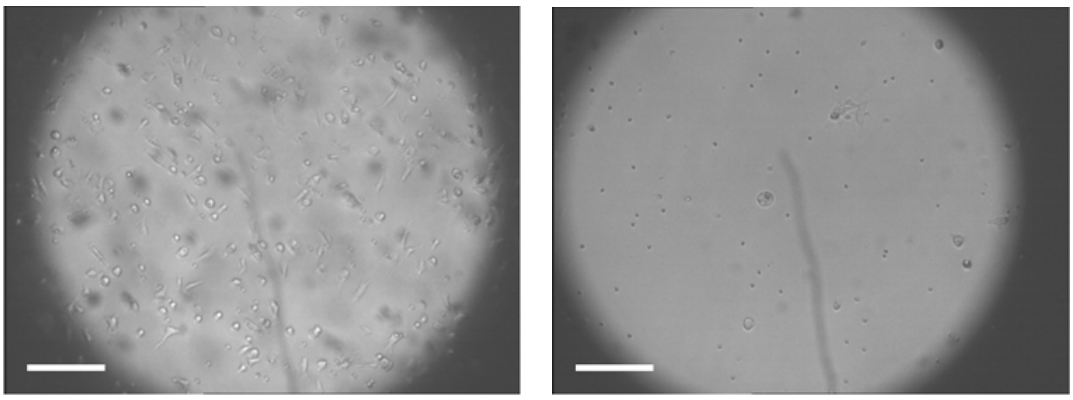
(a) (b)

Fig. 2. (a) The mouse is anesthetized and placed on a heated stage. An artery in the ear is chosen for the measurement; (b) A trace of DiD-labeled circulating PC-3 prostate cancer cell measured by the *in vivo* flow cytometer. Each peak (red star) within the trace indicates that a fluorescently-labeled circulating PC-3 cell passes through the slit of light and thus gives a burst of fluorescence. A.U. denotes arbitrary unit.

(final concentration: 100 μg clodronate per 1 ml culture medium) for overnight incubation. Images of macrophages were taken before and after lip-clo treatment by a CCD camera (Fig. 3). The images showed that the majority of macrophages were successfully depleted overnight by lip-clo *in vitro*.

3.2. *Macrophages influence depletion kinetics of circulating prostate cancer cells significantly measured by IVFC*

To explore if there is any relationship between macrophages and the depletion kinetics of circulating prostate cancer cells. We took advantage of the IVFC to monitor circulation kinetics in both macrophage-deficient mice group and the control group. The human androgen-independent prostate cancer cell line PC-3 was used in this study to show circulation kinetics of high metastatic prostate cancer cells. Compared with the human androgen-dependent low metastatic prostate cancer cell line LNCaP, PC-3 cells demonstrate significant difference in depletion kinetics in the first 2 h after tail-vein injection (Fig. S1). Figure 4 shows the normalized circulating tumor cell (CTC) quantitation of macrophage-deficient mice group and the control group. The first IVFC measurements were acquired



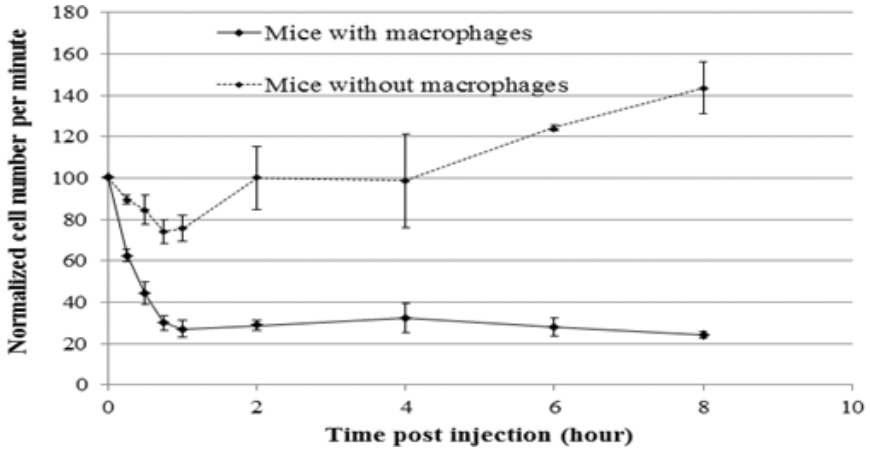
(a) (b)

Fig. 3. Lip-clo depletes macrophages *in vitro*. (a) Macrophages cultured in a petri-dish before the addition of lip-clo; (b) Macrophages cultured in a petri-dish after the addition of lip-clo and incubated overnight. Scale bar: 100 μm.

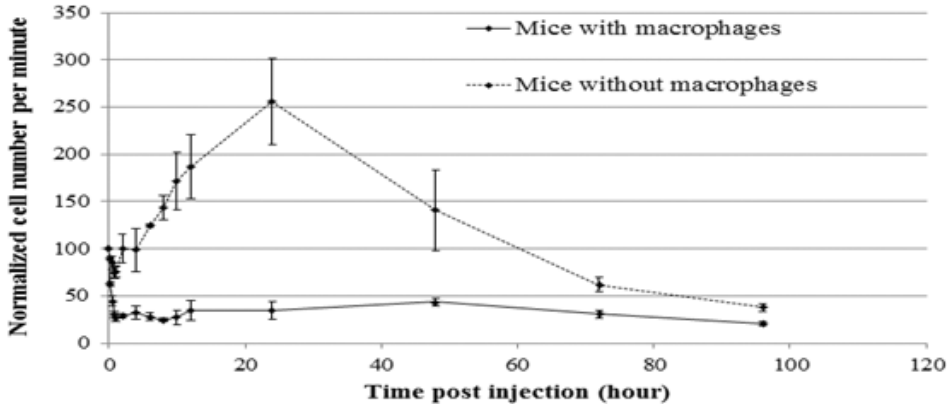
within 5 min after injection. Additional measurements were acquired at the very same vessel location at 15, 30, 45 min; 1, 2, 4, 6, 8, 10, 12 h; as well as after 1, 2, 3, 4 days. In the control group, more than 70% PC-3 cells are depleted within the first hour. After the initial depletion, there is a slight increase in CTC number, quickly followed by a second depletion. By 8 h, $76\% \pm 3.6\%$ PC-3 cells are depleted from the circulation. Although the CTC number slightly fluctuates, it remains consistently low during a period of four days. In comparison, in the macrophage-deficient mice group induced by lip-clo, only 30% PC-3 cells are depleted within the first hour, followed by an apparent increase of CTC number with the peak number appearing at 24 h. The number of circulating PC-3 cells at 24 h is approximately 2.5-fold of the cell count measured right after injection. For the long-term monitoring, the cell number keeps dropping during day 2−4. $62\% \pm 4.0\%$ PC-3 cells are depleted from the circulation at 96 h. The differences in depletion kinetics indicate that removing macrophages facilitates the stay of prostate cancer cells in the circulation.

3.3. *Macrophages might arrest and digest prostate cancer cells* in vitro

As a type of specialized phagocytic cells, macrophages may help to arrest and digest cancer cells, thus contribute to the depletion kinetics differences of circulating cancer cells. An *in vitro* test of



(a)



(b)

Fig. 4. Depletion kinetics of circulating prostate cancer cells PC-3 in macrophage-deficient and control BALB/c nude mice. (a) The normalized numbers of circulating cells per minute are shown during 8 h following injection of the DiD-fluorescently labeled cells to illustrate short-term depletion process; (b) The normalized numbers of circulating cells per minute are shown during 96 h following injection of the DiD-fluorescently labeled cells to illustrate long-term depletion process. The mean values and standard deviations (SD) are shown ($n \geq 3$).

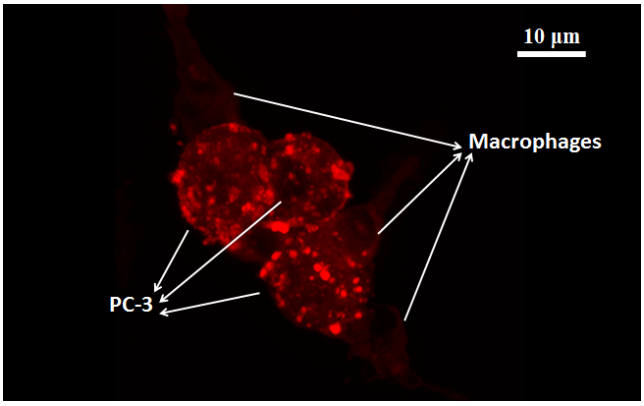


Fig. 5. Prostate cancer cells PC-3 were arrested, phagocytosed and digested by peritoneal macrophages *in vitro*. PC-3 was labeled by DiD and co-cultured with peritoneal macrophages. The image was taken by a Leica confocal microscope mounted with a water immersion objective lens (40×, NA = 0.8).

macrophage's phagocytic ability to prostate cancer cells was performed. The DiD fluorescently-labeled PC-3 cells and the peritoneal macrophages were co-cultured overnight. Laser confocal microscopy was used to monitor the behavior of PC-3 cells and macrophages. We frequently observed that the PC-3 cells were captured by macrophages. In addition, the DiD signals were not only present in PC-3 cells, but also detected in macrophages, which implies that macrophages could digest the prostate cancer cells after arresting them (Fig. 5).

4. Discussion

The relationship between macrophages and circulating cancer cells is complex and is not yet fully understood. In our study here, we have used the *in vivo* flow cytometer, combined with a macrophage-depletion drug, lip-clo, to continuously monitor the depletion kinetics of malignant PC-3 prostate cancer cells in mice and study how removal of macrophages by lip-clo affects PC-3 cell depletion.

The depletion kinetics differences of PC-3 cells in BALB/c nude mice between macrophage-deficient mice and the control mice could be explained by two major reasons: (i) after tail-vein injection, most cancer cells usually immediately get trapped in the first set of capillaries network that they encounter downstream of their origin, such as the lung and liver. As an integral arm of the immune system and one of the body's main lines of defense, macrophages involve the arrest and killing of CTCs. For example, Bayon *et al.* reported that Kupffer cells, the resident macrophages in liver, played a role in arresting circulating colon carcinoma cells at the liver sinusoid and were mainly responsible for clearing neoplastic cells from the liver parenchyma.[9] The observation that PC-3 cells were arrested and digested by co-cultured macrophages *in vitro* in our study is consistent with those findings view. Thus, a fraction of intravenously injected PC-3 cells could be trapped in capillaries and physically arrested/killed by macrophages. Depletion of macrophages by lip-clo would let more trapped PC-3 cells leave capillaries and cruise in the circulatory system; (ii) besides direct physical arrest and digest, the macrophages could also cooperate with other cells by cytokines to affect the metastasis behavior of CTCs. For example, Huh *et al.* reported that neutrophils helped melanoma cells transiently trapped in lung capillaries to anchor to vascular endothelium and facilitated further metastasis by a cytokine called interleukin-8 (IL-8), which could be paracrine produced and secreted by melanoma cells.[32] Meanwhile, IL-8, as an angiogenic factor, could also be produced by monocytes/macrophages.[33] Chen *et al.* also found out tumor associate macrophages could up-regulate IL-8 expression and have a close correlation with tumor angiogenesis and patient survival in non−small-cell lung cancer.[34] Thus, tumor-associated macrophages may enhance the anchoring and metastasis abilities of circulating cancer cells by secretion of certain cytokines such as IL-8. Depletion of macrophages by lip-clo could disrupt this cytokine link between tumor associate macrophages and cancer cells, which makes CTCs harder to tether/anchor to the endothelium and therefore have more chances to get access to the blood flow again. To investigate the particular mechanism(s), further research works are currently underway.

In conclusion, the developed methods here would be useful to study the relationship between macrophages and tumor metastasis in small animal cancer model. Our study of macrophages on circulating prostate cancer cells might provide new ways for anti-cancer therapies.

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References

1. E. Ruoslahti, "How cancer spreads," *Sci. Amer.* **275**, 72−77 (1996).
2. L. Weiss, "Metastatic inefficiency," *Adv. Cancer Res.* **54**, 159−211 (1990).
3. C. W. Wong, A. Lee, L. Shientag, J. Yu, Y. Dong, G. Kao, A. B. Al-Mehdi, E. J. Bernhard, R. J. Muschel, "Apoptosis: An early event in metastatic inefficiency," *Cancer Res.* **61**, 333−338 (2001).
4. J. E. Damber, G. Aus, "Prostate cancer," *Lancet* **371**, 1710−1721 (2008).
5. A. W. Hsing, A. P. Chokkalingam, "Prostate cancer epidemiology," *Front. Biosci.* **11**, 1388−1413 (2006).
6. A. Sica, V. Bronte, "Altered macrophage differentiation and immune dysfunction in tumor development," *J. Clin. Invest.* **117**, 1155−1166 (2007).
7. S. J. Oosterling, G. J. V. D. Bij, G. A. Meijer, C. W. Tuk, E. V. Rooijen, N. V. Rooijen, S. Meijer, J. R. V. D. Sijp, R. H. Beelen, M. V. Egmond, "Macrophages direct tumour histology and clinical outcome in a colon cancer model," *J. Pathol.* **207**, 147−155 (2005).
8. G. Heuff, H. S. Oldenburg, H. Boutkan, J. J. Visser, R. H. Beelen, N. V. Rooijen, C. D. Dijkstra, S. Meyer, "Enhanced tumour growth in the rat liver after selective elimination of Kupffer cells," *Cancer Immunol. Immunother.* **37**, 125−130 (1993).
9. L. G. Bayon, M. A. Izquierdo, I. Sirovich, N. V. Rooijen, R. H. Beelen, S. Meijer, "Role of Kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver," *Hepatology* **23**, 1224−1231 (1996).
10. J. Condeelis, J. W. Pollard, "Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis," *Cell* **124**, 263−266 (2006).
11. J. Y. Shih, A. Yuan, J. J. W. Chen, P. C. Yang, "Tumor-associated macrophage: Its role in cancer invasion and metastasis," *J. Cancer Molec.* **2**, 101−106 (2006).
12. E. Y. Lin, A. V. Nguyen, R. G. Russell, J. W. Pollard, "Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy," *J. Exp. Med.* **193**, 727−740 (2001).
13. I. F. Lissbrant, P. Stattin, P. Wikstrom, J. E. Damber, L. Egevad, A. Bergh, "Tumor associated macrophages in human prostate cancer: Relation to clinicopathological variables and survival," *Int. J. Oncol.* **17**, 445−451 (2000).
14. P. Seiler, P. Aichele, B. Odermatt, H. Hengartner, R. M. Zinkernagel, R. A. Schwendener, "Crucial role of marginal zone macrophages and marginal zone metallophils in the clearance of lymphocytic choriomeningitis virus infection," *Eur. J. Immunol.* **27**, 2626−2633 (1997).
15. J. W. Tyner, O. Uchida, N. Kajiwara, E. Y. Kim, A. C. Patel, M. P. O'Sullivan, M. J. Walter, R. A. Schwendener, D. N. Cook, T. M. Danoff, M. J. Holtzman, "CCL5/CCR5 interaction provides anti-apoptotic signals for macrophage survival during viral infection," *Nat. Med.* **11**, 1180−1187 (2005).
16. N. V. Rooijen, A. M. Sanders, T. K. V. D. Berg, "Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine," *J. Immunol. Methods* **193**, 93−99 (1996).
17. N. V. Rooijen, A. Sanders, "Liposomes mediated depletion of macrophages: Mechanism of action, preparation of liposomes and applications," *J. Immunol. Methods* **174**, 83−93 (1994).
18. N. V. Rooijen, J. Bakker, A. Sanders, "Transient suppression of macrophage functions by liposome-encapsulated drugs," *Trends Biotechnol.* **15**, 178−185 (1997).
19. J. Novak, I. Georgakoudi, X. Wei, A. Prossin, C. P. Lin, "*In vivo* flow cytometer for realtime detection and quantification of circulating cells," *Opt. Lett.* **29**, 77−79 (2004).
20. I. Georgakoudi, N. Solban, J. Novak, W. L. Rice, X. Wei, T. Hasan, C. P. Lin, "*In vivo* flow cytometry: A new method for enumerating circulating cancer cells," *Cancer Res.* **64**, 5044−5047 (2004).
21. S. Boutrus, C. Greiner, D. Hwu, M. Chan, C. Kuperwasser, C. P. Lin, I. Georgakoudi, "Portable two-color *in vivo* flow cytometer for real-time detection of fluorescently-labelled circulating cells," *J. Biomed. Opt.* **12**, 020507-1−020507-3 (2007).
22. D. A. Sipkins, X. Wei, J. W. Wu, J. M. Runnels, D. Cote, T. K. Means, A. D. Luster, D. T. Scadden, C. P. Lin, "*In vivo* imaging of specialized bone marrow endothelial microdomains for tumor engraftment," *Nature* **435**, 969−973 (2005).
23. W. He, H. F. Wang, L. C. Hartmann, J. X. Cheng, P. S. Low, "*In vivo* quantitation of rare circulating tumor cells by multiphoton intravital flow

cytometry," *Proc. Natl. Acad. Sci. USA* **104**, 11,760−11,765 (2007).

24. Y. Li, J. Guo, C. Wang, Z. Fan, G. Liu, C. Wang, Z. Gu, D. Damm, A. Mosig, X. Wei, "Circulation times of prostate cancer and hepatocellular carcinoma cells by *in vivo* flow cytometry," *Cytometry A* **79**, 848−854 (2011).
25. V. V. Tuchin, A. Tárnok, V. P. Zharov, "*In vivo* flow cytometry: A horizon of opportunities," *Cytometry A* **79**, 737−745 (2011).
26. C. M. Pitsillides, J. M. Runnels, J. A. Spencer, L. Zhi, M. X. Wu, C. P. Lin, "Cell labeling approaches for fluorescence-based *in vivo* flow cytometry," *Cytometry A* **79**, 758−765 (2011).
27. Z. C. Fan, Y. Jun, G. D. Liu, X. Y. Tan, X. F. Weng, W. Z. Wu, J. Zhou, X. B. Wei, "Real-time monitoring of rare circulating hepatocellular carcinoma cells in an orthotopic model by *in vivo* flow cytometry assesses resection on metastasis," *Cancer Res.* **72**, 2683−2691 (2012).
28. M. Yin, S. Li, L. Yuan, H. Dai, "Separation, cultivation and identification of mouse peritoneal macrophages," *Med. J. Wuhan University* **27**, 203−205 (2006).
29. K. H. V. D. Hoek, S. Maddocks, C. M. Woodhouse, N. V. Rooijen, S. A. Robertson, R. J. Norman, "Intrabursal injection of clodronate liposomes causes macrophage depletion and inhibits ovulation in the mouse ovary," *Biol. Reprod.* **62**, 1059−1066 (2000).
30. N. V. Rooijen, N. Kors, G. Kraal, "Macrophage subset repopulation in the spleen: Differential kinetics after liposome-mediated elimination," *J. Leukoc. Biol.* **45**, 97−104 (1989).
31. M. B. Jordan, N. V. Rooijen, S. Izui, J. Kappler, P. Marrack, "Liposomal clodronate as a novel agent for treating autoimmune hemolytic anemia in a mouse model," *Blood* **101**, 594−601 (2003).
32. S. J. Huh, S. Liang, A. Sharma, C. Dong, G. P. Robertson, "Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development," *Cancer Res.* **70**, 6071−6082 (2010).
33. A. E. Koch, P. J. Polverini, S. L. Kunkel, L. A. Harlow, L. A. DiPietro, V. M. Elner, S. G. Elner, R. M. Strieter, "Interleukin-8 as a macrophage-derived mediator of angiogenesis," *Science* **258**, 1798−1801 (1992).
34. J. J. Chen, P. L. Yao, A. Yuan, T. M. Hong, C. T. Shun, M. L. Kuo, Y. C. Lee, P. C. Yang, "Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: Its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer," *Clin. Cancer Res.* **9**, 729−737 (2003).