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PRINCIPAL INVESTIGATOR: Wu, Lily

CONTRACTING ORGANIZATION:
University of California, Los Angeles

Los Angeles, CA 90095-1406

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Wu, Lily

E-Mail: lwu@mednet.ucla.edu

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UNIVERSITY OF CALIFORNIA, LOS ANGELES
11000 KINROSS AVENUE, STE 102
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14. ABSTRACT

Circulating tumor cells (CTCs) are tumor cells that are shed into the blood stream by a solid tumor such as prostate cancer. Current data supports CTCs likely denote the more aggressive tumor cells that have metastatic potential. It is extremely challenging to identify CTCs in context of 10^8 excess white blood cells in peripheral blood. The use of advanced microfluidic chip-based CTC detection method, such as the "Nano-Velcro" chip used in this project, has been shown to exhibit greatly enhanced CTC capture efficiency in prostate cancer patients, providing an earlier and more sensitive readout of treatment response than the FDA approved CellSearch™ CTC detection method, serum PSA or radiographic CT assessment. However, a limitation of the current detection technology is its inability to assess dynamic functional activity, such as the AR pathway, in the living CTCs as the immunohistochemistry approach of current methods can only provide static protein expression in the CTCs. This DOD funded project aims to incorporate the use of AR-driven reporter recombinant vectors to query dynamic AR functional status in viable CTCs captured by the Nano-Velcro chip.

15. SUBJECT TERMS
Prostate Cancer, Circulating Tumor Cells, Prostate-specific Adenoviral vectors, PSA, PSES

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Introduction

Analyses from prostate cancer patients so far indicated that circulating tumor cells (CTCs) represent an easily accessible liquid biopsy to assess the aggressive, metastatic tumor cells, as the number of CTCs is much higher in advanced, metastatic disease (1, 2). However, the detection of CTCs is hampered by their extreme low numbers amongst the great excess of red and white blood cells. To increase the certainty of detecting prostate cancer cells and its AR functional status, an important clinical drug response factor, we proposed to use an adenoviral mediated reporter gene transfer into cells in peripheral blood. The adenoviral vector will express green fluorescent protein driven by an amplified PSA or an amplified PSMA promoter, which are both prostate tissue-specific that is AR- or non AR-responsive, respectively. Thus, any nucleated cell in the circulation that expresses GFP should be a prostate cancer cell. Due to personnel and change of expertise issues, this project encountered significant delays. However, we have remedied some of the issues and are actively pursuing the revised 3 specific aims.

Body

Specific Aim 1: To generate two novel PSA- and a PSMA-driven fluorescent reporter Ads and assess the dual AR functional reporter capability in prostate tumor cell lines and tumor cell spiked blood samples.

Subtask 1- Construct the AdPSA-TSTA-GFP/CMV-RFP and AdPSMA-TSTA-GFP/CMV-RFP reporter Ad.

- We have initiated the construction of these 2 vectors. Final construct not completed yet.

Subtask 2- Assess the dual AR functional reporter capability of AdPSA-TSTA-GFP/CMV-RFP and AdPSMA-TSTA-GFP/CMV-RFP in prostate cancer cell lines spiked blood samples.

- Not initiated yet.

Specific Aim 2: To evaluate the functional capability of Ad-mediated CTC detection and the response to AR antagonists in the CTCs by the dual AR reporter in blood samples of CRPC patients.

- Amendment current IRB protocol to accommodate revised aims and adhere to HRPO guidance: Completed

Subtask 1: Evaluate the capability of AdPSE-TSTA-eGFP and AdPSES-TSTA-IFP to detection CTCs from blood samples of 10 CRPC patients..

- No initiated yet

Revised Specific Aim 3: Assess extracellular vesicles (EV) in blood samples of CRPC patients. We have begun to work on this revised SA by first assessing the functional activities of extracellular vesicles (EVs) from different tumor cells. We chose the murine kidney cancer RENCA line as the starting point because we have observed that the loss of VHL tumor suppressor gene in this model (RENCA VHL-KO) resulted in significant

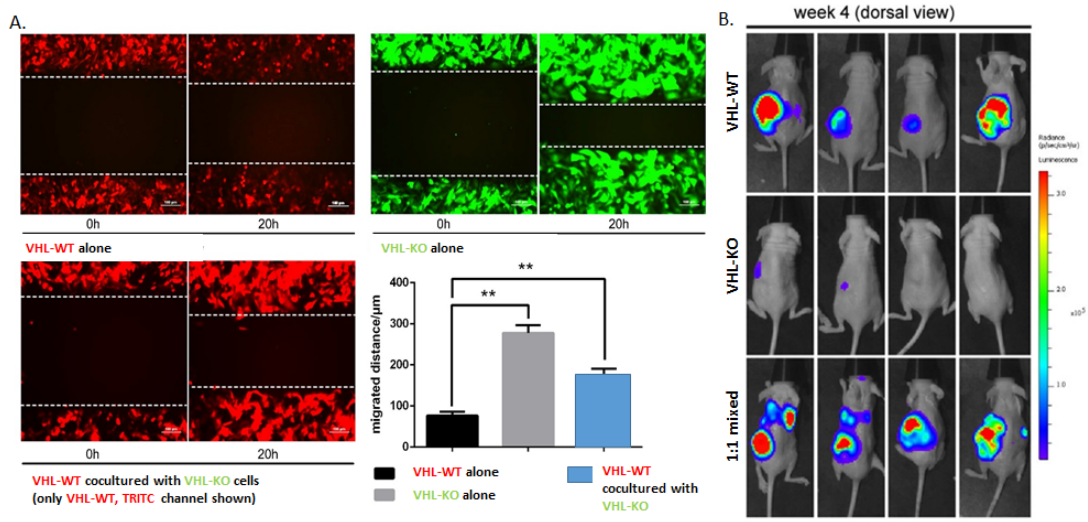

Figure 1. VHL-KO induce motility of VHL-WT cells, achieving cooperative metastasis *in vivo*. **A.** VHL-KO cells were marked with EGFP and VHL-WT cells with strawberry RFP. Cultured alone, VHL-KO cells migrated much faster VHL-WT cells. In coculture VHL-KO cells greatly enhanced the motility of VHL-WT cells. **B.** Tumors implanted into left kidney of mice with VHL-KO cells alone do not grow and those with VHL-WT cells alone grew primary tumor but no metastasis. Tumors with 1:1 mixed of both cells grew primary tumor and metastasize to lung rampantly.

increase in metastasis *in vivo*. This enhanced metastatic activity is due to a diffusible effect that VHL-KO cells conferred on VHL-WT, parental RENCA cells (**Figure 1**). Time lapse microscopy further suggest that VHL-KO cells are transferring EVs to VHL-WT cells in co-culture and inducing the VHL-WT cells to migrate (**Figure 2**). We have begun to isolate the EVs from VHL-KO cells to assess their functional activity in enhancing cellular motility. Briefly, the exosomes are

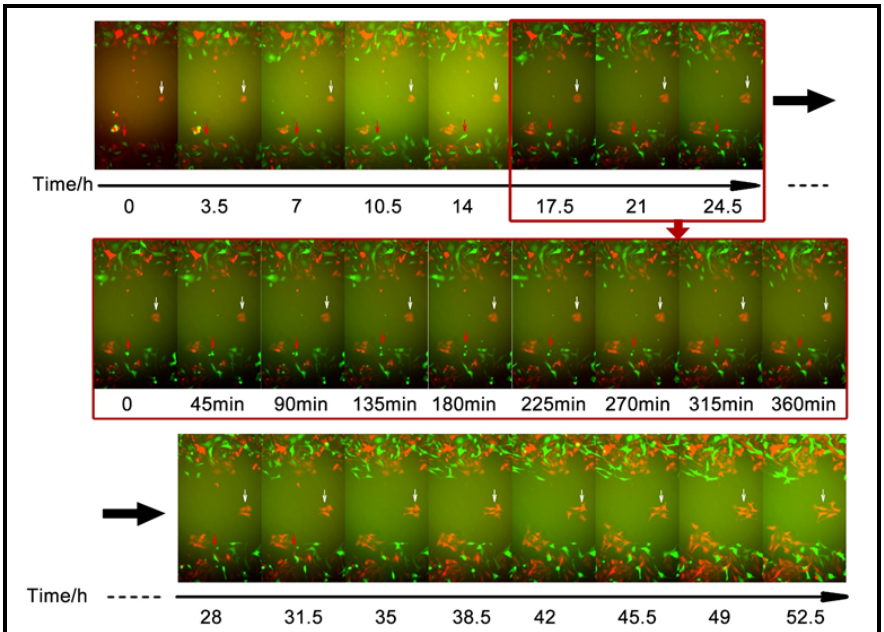

Figure 2. VHL-KO cells communicate with VHL-WT cells via EVs. Monitoring the coculture of VHL-KO (eGFP marked) and VHL-WT (sRFP marked) over 52 hrs revealed the transmission of green EVs from donor VHL-KO cells (red arrow) to a small cluster of recipient VHL-WT cells (white arrow). The period between 17.5 to 24.5 hr was further expanded in shorter time increments (second row). After receiving the EVs, the VHL-WT cells divided and migrated more rapidly, suggesting the EVs could be transmitting the pro-metastatic signals from VHL-KO cells.

purified from condition media of cells through sucrose gradient ultracentrifugation at 100,000g for 16 hrs. Two mls fractions were collected from the first centrifugation and re-suspended in PBS and spun at 100,000g for 1 hr to pellet the exosomes and re-suspended in PBS. To study the functional impact of exosome, exosome solution was added at 5% to culture media in the scratch assay on 24 well plates. Motility was examined by time lapse microscopy over 20 hrs. (**Figure 3**).

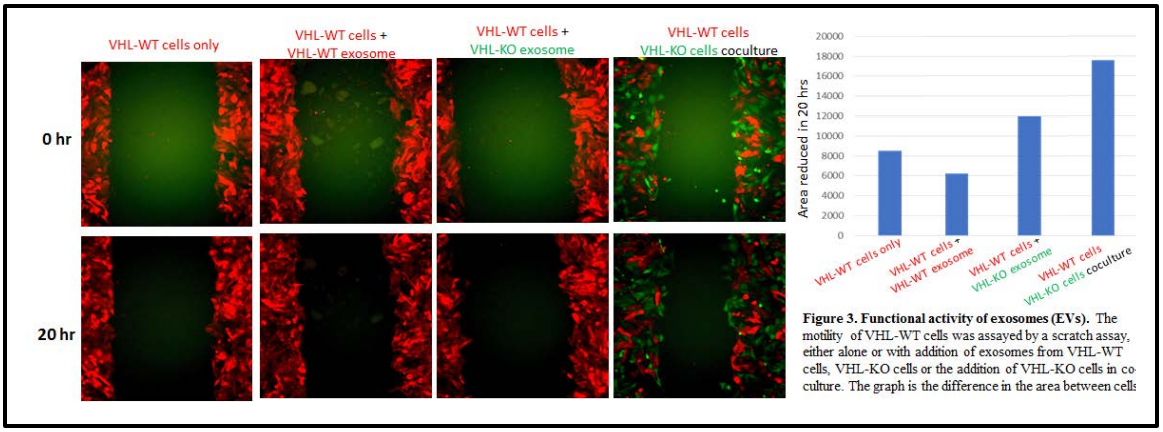

Figure 3. Functional activity of exosomes (EVs). The motility of VHL-WT cells was assayed by a scratch assay, either alone or with addition of exosomes from VHL-WT cells, VHL-KO cells or the addition of VHL-KO cells in co-culture. The graph is the difference in the area between cells

In a related study, we investigated novel treatment approaches for CRPC patients. Docetaxel chemotherapy is often given to patients who failed ADT. We showed that inhibition of tumor associated macrophages with CSF1R kinase inhibition in conjunction of ADT plus docetaxel significantly improved the durability of this treatment. This manuscript entitled: Inhibition of TAMs improves the response to docetaxel in castration-resistant prostate cancer, is accepted by Endocrine Related Cancer and is now in press. The galley proofs of this paper is enclosed.

Key Research Accomplishments

- We are in the process of generating AdPSA-TSTA-GFP/CMV-RFP and AdPSMA-TSTA-GFP/CMV-RFP.
- We have begun to develop exosome purification and functional characterization methods.
- A paper on improved therapeutic management of CRPC has been accepted for publication.

Reportable Outcomes

Guan W, Hu J, Yang L, Tan P, Tang Z, West B, Bollag G, Xu H, Wu L. Inhibition of TAMs improves the response to docetaxel in castration-resistant prostate cancer. *Endocrine Related Cancer*, 2018, in press.

Conclusion

Knowledge are becoming crystallized in that CTCs isolated from the blood stream of patients with advanced metastatic castrate resistant prostate cancer (CRPC) can reflect the tumor biology of the primary tumor or disseminated disease. The central focus of this technology-driven project is to further advance CTC diagnostic method to obtain clinical relevant functional activity in the CTCs. The novel strategy is to add a gene transfer step with a prostate-specific AR reporter Ad to the front end of current state-of-the-art microfluidic CTC capture platform. This approach will enable not only the identification of viable tumor cells of prostate origin, but more importantly, it will allow the assessment of the functionality of the AR pathway in CTCs in response to AR antagonists (e.g. MDV 3100), before the initiation of treatment. The later capability is not feasible with current technology. Our work so far shows that the concept is correct (3, 4). Furthermore, the approach proposed is feasible as PMBC and tumor cells within peripheral blood can maintain viability to be infected and express the viral mediated reporter genes. If successful, this advancement will provide real-time functional activity in the disease tissue to guide the use of latest generation of AR antagonists in patients with metastatic CRPC.

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Appendices

Guan et al manuscript enclosed below.

Supporting Data

None (relevant data inserted into the body section and appended manuscript).

RESEARCH

Inhibition of TAMs improves the response to docetaxel in castration-resistant prostate cancer

Wei Guan[1,*], Junhui Hu[1,2,3,*], Lu Yang[4], Ping Tan[4], Zhuang Tang[4], Brian L West[5], Gideon Bollag[5], Hua Xu[1] and Lily Wu[3,6,7,8,9]

[1]Department of Urology and Institute of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[2]Department of Paediatric Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[3]Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, USA
[4]Department of Urology, Institute of Urology, West China Hospital of Sichuan University, Chengdu, China
[5]Plexxikon Inc., Berkeley, California, USA
[6]Department of Urology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, USA
[7]Department of Pediatrics, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, USA
[8]Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California, USA
[9]Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California, USA

Correspondence should be addressed to L Wu: lwu@mednet.ucla.edu

*(W Guan and J Hu contributed equally to this work)

Abstract

For men with castration-resistant prostate cancer (CRPC), androgen-deprivation therapy (ADT) often becomes ineffective requiring the addition of docetaxel, a proven effective chemotherapy option. Tumor-associated macrophages (TAMs) are known to provide protumorigenic influences that contribute to treatment failure. In this study, we examined the contribution of TAMs to docetaxel treatment. An increased infiltration of macrophages in CRPC tumors was observed after treatment with docetaxel. Prostate cancer cells treated with docetaxel released more macrophage colony-stimulating factor (M-CSF-1 or CSF-1), IL-10 and other factors, which can recruit and modulate circulating monocytes to promote their protumorigenic functions. Inhibition of CSF-1 receptor kinase signaling with a small molecule antagonist (PLX3397) in CRPC models significantly reduces the infiltration of TAMs and their influences. As such, the addition of PLX3397 to docetaxel treatment resulted in a more durable tumor growth suppression than docetaxel alone. This study reveals a rational strategy to abrogate the influences of TAMs and extend the treatment response to docetaxel in CRPC.

Key Words
- CRPC
- TAMs
- docetaxel
- CSF-1
- CSF-1R

Endocrine-Related Cancer (2019) **26**, 1–10

Introduction

Prostate cancer (PCa) is the second most common cancer in men after skin cancer, as one out of seven men will be diagnosed with this disease in the United States (Siegel *et al*. 2017). It is estimated that 161,000 newly diagnosed cases and 27,000 deaths will be attributed to this disease in 2017 (Siegel *et al*. 2017). A great majority of PCa patients, 70–80%, present with localized, organ-confined disease and their outcome is very favorable, having 10-year survival rate above 95%. However, 20–30% of patients will present with characteristics of high risk, advanced disease such as high Gleason grade or distant metastases.

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In these cases, the 5-year survival rate drops precipitously to about 30% (Siegel *et al.* 2017).

For PCa patients with advanced disease, androgen-deprivation therapy (ADT) is the first line of treatment, developed by Dr Huggins more than 75 years ago to deplete androgen, a key growth factor for prostate cancer cells (Esch *et al.* 2014). Over the years, effective strategies of ADT include the depletion of the body's source of androgen by inhibiting androgen biosynthesis pathways and by blocking the activation of androgen receptor (AR) (Merseburger *et al.* 2015). Abiraterone and enzalutamide are two newly approved potent ADT agents that inhibit CYP17A1 androgen synthetic enzyme and AR, respectively (de Bono *et al.* 2011, Scher *et al.* 2012). Both agents are effective in prolonging the survival of castration-resistant prostate cancer (CRPC) patients who had progressed on first-line ADT (Ryan *et al.* 2015). However, a significant proportion of CRPC patients either do not respond to either abiraterone or enzalutamide, or initially respond but subsequently progress on treatment (Silberstein *et al.* 2016). Potential mechanisms of resistance include AR mutations, amplification and splice variant (Antonarakis *et al.* 2014, Azad *et al.* 2015, Romanel *et al.* 2015).

Docetaxel has been established as the standard first-line chemotherapy agent to treat CRPC since 2004. It was approved by FDA for this purpose as several large clinical trials showed docetaxel containing regimens provided survival benefits over other chemotherapies for CRPC patients (Petrylak *et al.* 2004, Tannock *et al.* 2004, Sweeney *et al.* 2015). Belonging to the taxane family, docetaxel was initially postulated to suppress prostate cancer growth by interfering with microtubule function (Petrylak 2003). However, subsequent research supported that the therapeutic activity of taxanes in prostate cancer could arise from its interference with androgen signaling via the nuclear translocation process (Gan *et al.* 2009).

Given taxane-based chemotherapy is one of a few effective treatments for CRPC, we investigate a rational combination regimen to improve its therapeutic efficacy. Recent findings from our group and others showed that tumor-associated macrophages (TAMs) contribute significantly to treatment failure in PCa and other solid cancers via their wound-healing and protumorigenic functions (Xu *et al.* 2013, Escamilla *et al.* 2015, Brown *et al.* 2017). In this study, we employed a small-molecule CSF1R kinase inhibitor (CSF-1Ri), PLX3397, to block TAMs in CRPC models. In combination with ADT and docetaxel, PLX3397 was able to significantly reduce the number of infiltrating TAMs and lower their protumorigenic influences. We showed that the addition of PLX3397 extended the therapeutic response to ADT and docetaxel in CRPC models.

Materials and methods

Cell culture and drugs

The murine macrophage RAW264.7 (RAW) cells (ATCC) and MyC-CaP cells (a kind gift from Dr. Charles Sawyers, Memorial Sloan Kettering New York) were cultured with DMEM (high glucose) while PC3 (ATCC), CWR22Rv2 (a kind gift from Dr. David Agus, Cedars-Sinai Medical Center) and LNCap-C4-2 (C4-2) cells (ATCC) were cultured in RPMI-1640. Both media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. PLX3397, 5-[(5-chloro-1H-pyrrolo[2,3-b] pyridin-3-yl)methyl]-N-[[6-(trifluoromethyl)-3-pyridyl] methyl]pyridin-2-amine was synthesized at Plexxikon Inc. The detailed synthetic procedure is shown by Tap *et al.* (2015).

Transwell coculture and migration assay

In coculture assay, 1.0×10^6 RAW macrophages were seeded in transwell inserts with membrane pore size at 4 µm (BD Falcon) in media supplemented with 2 µM PLX3397, 1 µM GW2580 or DMSO vehicle. The chamber was inserted in a 6-well plate with conditioned media from Myc-Cap, PC3, CWR and C4-2 cells treated with docetaxel (100 nM for MyC-CaP, 5 nM for CWR22Rv1, 30 nM for PC3 and 2 nM for C4-2 cells) or DMSO. Total RNA was extracted from tumor cells after 48 h and analyzed by RT-PCR. The methods for RT-PCR is described in Supplementary data (see section on supplementary data given at the end of this article) and primers are listed in Supplementary Table 1.

In migration assay, 1.0×10^5 RAW cells were seeded in transwell inserts with membrane pore size at 8 µm assembled in 24-well plates. The number of migrated cells was evaluated after 6 h of incubation at 37°C, and then treated with 3% paraformaldehyde (PFA) and stained with 0.1% (w/v) crystal violet solution. Random 10 fields/well at 4× magnification were sampled and quantified with ImageJ2.

ELISA assay

1.0×10^6 MyC-CaP, PC3, CWR and C4-2 cells were cocultured with or without RAW cells as mentioned earlier, with or without Docetaxel or PLX3397 at tumor cells' IC_{50} or IC_{10} concentrations.

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Supernatant of all cell culture media were harvested after 48 h. 96-well Nunc MaxiSorp Plates (Cat#44-2404-21, Thermo Scientific) were coated with the anti-M-CSF antibody (1:300, Cat#sc-365779, Santa Cruz Biotech) in coating buffer diluted from Coating Solution Concentrate Kit (KPL) at 4°C overnight. Then, the plate was washed with 1× wash buffer (KPL) and blocked with 1% BSA Blocking Solution (KPL) for 1 h at room temperature. Cell supernatant was added to the wells and incubated for 1 h at room temperature in the shaker at 220 rpm. After washing with 1× wash buffer (KPL), each well was incubated with the second anti-M-CSF antibody (1:300, Cat#sc-13103, Santa Cruz Biotech) overnight at 4°C. The wells were washed four times, 5 min for each and incubated with 100 µL of HRP-conjugated goat-anti-rabbit IgG (1:5000, Cat# 111-035-045, Jackson Laboratory) for 1 h at room temperature. The wells were washed four times, 5 min for each and incubated with 100 µL of ABTS ELISA HRP Substrate (KPL). Absorbance at 410 nm was measured by Synergy HT microplate reader (BioTek).

Flow cytometry

MyC-CaP cells were cocultured with or without RAW cells, docetaxel (IC_{10} or IC_{50}) for 48 h before cells were trypsinized. Single cell suspension was rinsed with PBS twice and incubated with APC conjugated anti-IL-10 antibody (Cat#17-7101-82, eBiosicence) for 30 min at 4°C at darkness. Cell acquisition was done on a BD LSR-II flow cytometer (Beckman Coulter) and data were analyzed by FlowJo software (TreeStar).

For tumor tissue analysis, single cell suspension was prepared by digestion of collagenase II at 0.1% for 1 h. Then, cells were counted and incubated with APC-conjugated anti-CD11b antibody (Cat#17-0112-81, eBioscience) and PE-conjugated anti-CSF1R antibody (Cat#12-1152-82, eBioscience) for 30 min at 4°C in darkness.

MyC-Cap subcutaneous xenograft model

All animal experiments were approved by the Animal Research Committee of the University of California, Los Angeles. For MyC-CaP s.c. xenograft model, 16 FVB male mice that are 6–8 weeks old from Taconic Biosciences were adopted and kept at BSL2 animal facility. After trypsinization and rinsing with cooled PBS, 1.0×10^6 MyC-CaP cells were resuspended in 200 µL PBS/Matrigel (1:2) (356230, Corning) and injected with insulin syringe into the subcutaneous space on the right back of FVB male mice ($n=16$). One week after the cell injection, mice were castrated and divided randomly into four groups, receiving DMSO vehicle + control chow, DMSO vehicle + chow containing PLX3397, docetaxel + control chow or docetaxel + chow containing PLX3397. The PLX3397 dosage is 40 mg/kg/day on average and docetaxel dosage is 40 mg/kg/week. Tumor size was measured by digital calipers and calculated by the formula $V=0.5*a*b^2$, in which a is the larger and b is the smaller index of the two perpendicular indexes of the tumor.

CWR22Rv1 orthotopic xenograft model

After trypsinization and rinsing with precooled PBS, 1×10^5 CWR22Rv1 cells, stably expressing firefly luciferase, were resuspended in 10 µL of PBS/Matrigel (1:2) (356230, Corning) and injected by insulin syringe into the left anterior lobe of prostate gland of 6–8 weeks old SCID-beige male mice (Jackson Laboratory). All mice were castrated on day 14 post injection and randomly divided into two groups, receive docetaxel + control chow or docetaxel + PLX3397 chow (40 mg/kg/day). The docetaxel treatment started on day 19 at 10 mg/kg/week. The *in vivo* BLI were performed every week and the luminescence count was recorded as previously described (Palmeri *et al.* 2008). All mice were killed on day 42.

Statistical analysis

Data are all presented as mean ± S.E.M. Student *t*-test was used for comparison between two groups while two-way ANOVA was used for comparisons between multiple groups.

Results

Docetaxel-mediated tumor cell injury induces the expression of M2 cytokines

To study the impact of docetaxel in prostate cancer, we first examined the dose response of this chemotherapeutic agent on several prostate cancer cell lines (Fig. 1A). We found that PCa cells exhibit a wide range of sensitivity to docetaxel, with C4-2 ($IC_{50}=2$ nM) and CWR22Rv1 ($IC_{50}=5$ nM) being the most sensitive, PC-3 ($IC_{50}=30$ nM) as an intermediate responder and MyC-CaP ($IC_{50}=100$ nM) being the most resistant. As we have shown in previous studies, conventional cytotoxic therapies such as radiation therapy and ADT all can induce PCa cells to express M2 cytokines (Xu *et al.* 2013, Escamilla *et al.* 2015). Here, we further inquired whether docetaxel in addition to

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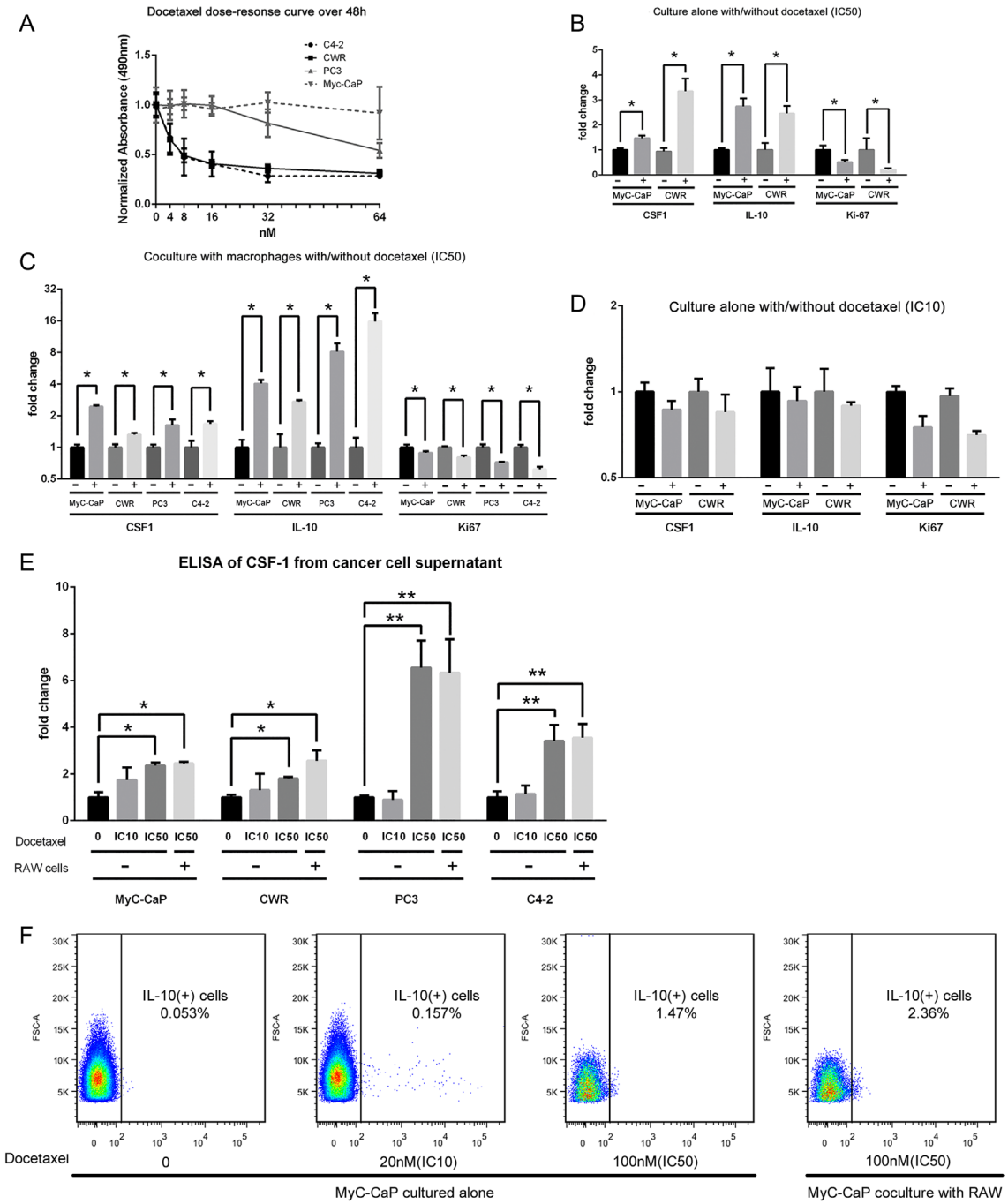

Figure 1

Cytokine expression upon docetaxel treatment in prostate cancer cells. (A) The dose–response curve was plotted in different working concentrations of docetaxel for C4-2, CWR22Rv1, PC3, MyC-CaP cells to determine their respective IC_{50} and IC_{10} values. (B) MyC-CaP and CWR22Rv1cells, cultured alone were treated with docetaxel at the IC_{50} dose for each cell (Myc-Cap at 100 nM and CWR22Rv1 at 5 nM). Expression of CSF-1 and IL-10 and Ki-67 in response to docetaxel treatment was shown. (C) The impacts of docetaxel treatment at IC_{50} on PCa cells in the presence of macrophages (RAW cells) were shown for MyC-CaP, CWR22Rv1, PC3 (30 nM) and C4-2 (2 nM) cells were shown (D). When treated at their respective IC_{10} doses of 20 and 1 nM, MyC-CaP and CWR22Rv1 cells showed no significant change in CSF-1, IL-10 or Ki67 expression. (E) The level of secreted CSF-1 in culture supernatant from MyC-CaP, CWR22Rv1, PC3 and C4-2 cells treated with docetaxel at IC_{10} or IC_{50}, and with and without co-cultured with RAW macrophages, were analyzed by ELISA. (F) Intracellular IL-10 expression in MyC-CaP cells treated with docetaxel at IC_{10} or IC_{50}, with and without co-cultured with RAW macrophages, were analyzed by flow cytometry. All cells were cultured in media supplemented with charcoal-stripped serum (*$P<0.05$, **$P<0.01$).

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ADT would also induce the expression of M2 cytokines such as CSF-1 and IL-10. To mimic ADT, all prostate cancer cells were cultured in media supplemented with charcoal-treated fetal bovine serum (FBS) to remove the androgens. As shown in Fig. 1B MyC-CaP or CWR22Rv1 cells treated with ADT plus docetaxel, dosed at each line's respective IC_{50}, increased the expression of CSF-1 and IL-10. Likewise, the expression of these M2 cytokines was also induced when PCa cells, including MyC-CaP, CWR22Rv1, PC3 and C4-2, were treated in the presence of macrophages (Fig. 1C). This ADT plus docetaxel treatment resulted in a significant reduction in cell proliferation, as indicated by the decrease in the proliferative marker Ki67. Interestingly, when the PCa cells were treated with a lower dose of docetaxel at the IC_{10} dose, the elevation of M2 cytokine expression was no longer observed (Fig. 1D). Docetaxel treatment induced increase in CSF-1 and IL-10 in the tumor cells were further analyzed and verified at the protein level by CSF-1 ELISA (Fig. 1E) and IL-10 flow cytometry (Fig. 1F). This induction of M2 cytokines is likely not restricted to docetaxel alone. We observed very similar effects with paclitaxel treatment of all four PCa cell lines (Supplementary Fig. 1). Collectively, these findings support that cell injury mediated by ADT plus docetaxel induces the heightened expression of M2 cytokines in PCa cells.

Docetaxel induces CSF-1 expression and increases the recruitment of macrophages *in vitro*

CSF-1 or M-CSF is a cytokine critical not only in the differentiation and proliferation of myeloid cells but also in the recruitment and polarization of protumorigenic M2 macrophages (Brown *et al.* 2017). Next, we examine the impact of macrophage recruitment in the setting of docetaxel treatment. As shown in Fig. 2A and B, CWR22Rv1 and C4-2 PCa cells treated with docetaxel were able to recruit more macrophages in an *in vitro* transwell assay compared to chemo-naïve cells. The elevated CSF-1 produced by the docetaxel-treated PCa cells likely contributed to the increased macrophage recruitment, as the addition of the CSF-1Ri PLX3397 attenuated the

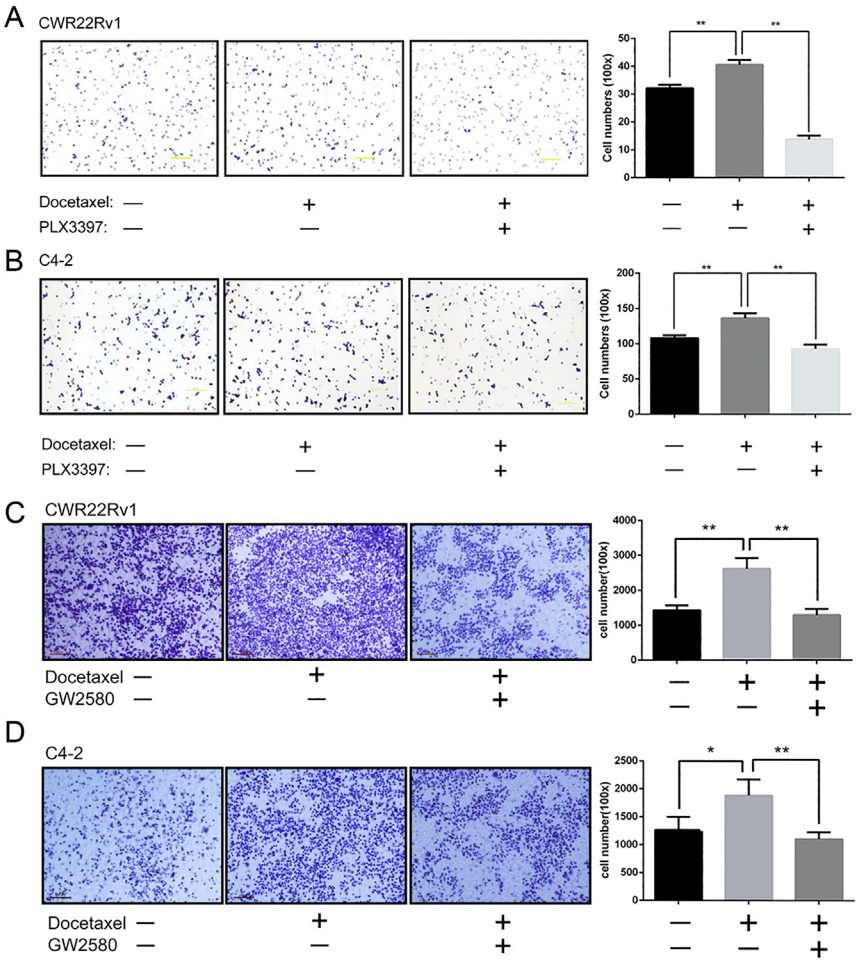

Figure 2
CSF-1R inhibitor PLX3397 abrogated the increased recruitment of macrophages induced by docetaxel treatment *in vitro*. Conditioned media of CWR22Rv1 (A) and C4-2 (B) prostate cancer cells treated with docetaxel was able to recruit more RAW macrophages migrating across a transwell porous membrane than media from untreated cells. The addition of 2 μM CSF-1Ri PLX3397 to the docetaxel treated conditioned media abrogated the increased macrophage recruitment induced by both cell lines. In a second set of similar study, the increase in RAW macrophages migration by CWR22Rv1 (C) and C4-2 (D) conditioned media was inhibited by 1 μM GW2580, a selective CSF-1R kinase inhibitor (*$P<0.05$, **$P<0.01$).

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enhancement in macrophage recruitment *in vitro* (Fig. 2A and B), as we and others have previously reported (Xu *et al.* 2013, Escamilla *et al.* 2015, Moughon *et al.* 2015, Butowski *et al.* 2016).

PLX3397 is known to also inhibit c-Kit (Tap *et al.* 2015). We employed a second highly selective CSF-1R kinase inhibitor GW2580 to substantiate that CSF1/CSF1R as the key signal axis for macrophage recruitment (Priceman *et al.* 2010). As shown in Fig. 2C and D, the enhancement of macrophage recruitment across a transwell mediated by docetaxel-treated PCa cells was dampened significantly by the addition of GW2580.

Adding CSF-1R kinase inhibitor, PLX3397, to docetaxel regimen enhances therapeutic efficacy in CRPC

Next, we investigated the impact of docetaxel treatment on macrophage recruitment *in vivo* in CRPC tumors. We first evaluated TAMs in the MyC-CaP tumors engrafted subcutaneously in syngeneic FVB male mice. One week after tumor cell implantation, tumor-bearing mice were treated with surgical castration as ADT, and divided into four treatment groups receiving (i) diluent control, (ii) oral PLX3397, (iii) docetaxel or (iv) docetaxel plus PLX3397. The PLX3397 treatment was administered orally via rodent chow and docetaxel was administered IP at 40 mg/kg/week. Comparing to diluent control=treated tumors, PLX3397 only treatment significantly reduced the number of $CD11b^+$ $CSF1R^+$ TAMs, while docetaxel significantly increased TAMs (Fig. 3A and B). Importantly, the addition of PLX3397 to docetaxel-treated group was able to not only reverse the chemotherapy-induced TAM influx but suppressed the TAM level in the tumor below that of the control treated group (Fig. 3A and B). These results demonstrate the importance of CSF-1/CSF-1R axis in the recruitment of macrophages and the effectiveness of PLX3397 in blocking this CSF-1R-mediated TAM recruitment *in vitro* and *in vivo*.

In our previous therapeutic studies, we consistently observed that CSF-1R blockade treatment alone can reduce the infiltration of TAMs but exert negligible impact on tumor growth *in vivo* (Priceman *et al.* 2010, Xu *et al.* 2013, Escamilla *et al.* 2015, Butowski *et al.* 2016). The same result was observed here in the MyC-CaP tumors: no significant reduction in tumor growth was observed after oral PLX3397 treatment alone despite clear reduction in the level of TAMs in the tumor (Fig. 3C, D and E). As expected, docetaxel treatment significantly retarded the growth of MyC-CaP tumor compared to control (Fig. 3C, D and E). More importantly, docetaxel plus PLX3397 achieved the most significant tumor growth suppression in the four treatment groups, more effective than docetaxel alone (Fig. 3C, D and E).

Next, we asked whether the benefit of PLX3397 in combination with docetaxel in the subcutaneous MyC-CaP model can also be observed in the orthotopic prostatic environment of the CWR22Rv1 model. SCID/Beige male mice received intraprostatic injection of firefly luciferase-labeled CWR22Rv1 cells, such that tumor growth can be monitored in real time by bioluminescence imaging (BLI, Fig. 3F and G). On day 14 after tumor cell implantation, mice received ADT via surgical castration. On day 19, tumor-bearing mice received either docetaxel with control or docetaxel plus oral PLX3397 (Fig. 3B). Treatment continued to day 42, at which point the animals were killed. Assessed either by BLI (Fig. 3F and G) or by terminal tumor volume (Fig. 3H and I), the docetaxel plus PLX3397 group consistently showed significantly greater efficiency in suppressing tumor growth over docetaxel treatment alone. Again, corroborating our prior findings, the added oral PLX3397 drastically reduced the level of CD11b+ CSF1R+ TAMs from 10.6% in the docetaxel only group to 0.1% in the docetaxel plus PLX3397 group, as analyzed by flow cytometry (Fig. 3J). This finding was further verified by F4/80 immunohistochemistry stain to detect macrophages (Fig. 3K). The functional consequences of TAM inhibition by PLX3397 included lowering angiogenic drive, tissue remodeling and immunosuppression as assessed by VEGF-A, MMP-9 and Arg-1 expression respectively (Fig. 3F and G). Taken together, we have shown that the use of a selective CSF-1Ri PLX3397 can block the infiltration of TAMs into prostate tumor and thus reduce the protumorigenic influences of M2 macrophages by lowering tumoral angiogenesis, tissue remodeling and immunosuppression leading to more effective treatment response to docetaxel.

Discussion

Docetaxel is a widely used chemotherapeutic agent in treating breast cancer (Palmeri *et al.* 2008), head and neck cancer (Rapidis *et al.* 2008) and non-small-cell lung cancer (Fossella 2002). In the Chemohormonal Therapy vs Androgen Ablation Randomized Trial for Extensive Disease (CHAARTED) randomized phase III trial, men with hormone-naive metastatic PCa were randomly assigned to receive docetaxel plus ADT or ADT alone, with nearly 400 men in each arm. In particular, patients who had high-volume disease benefited the most with docetaxel,

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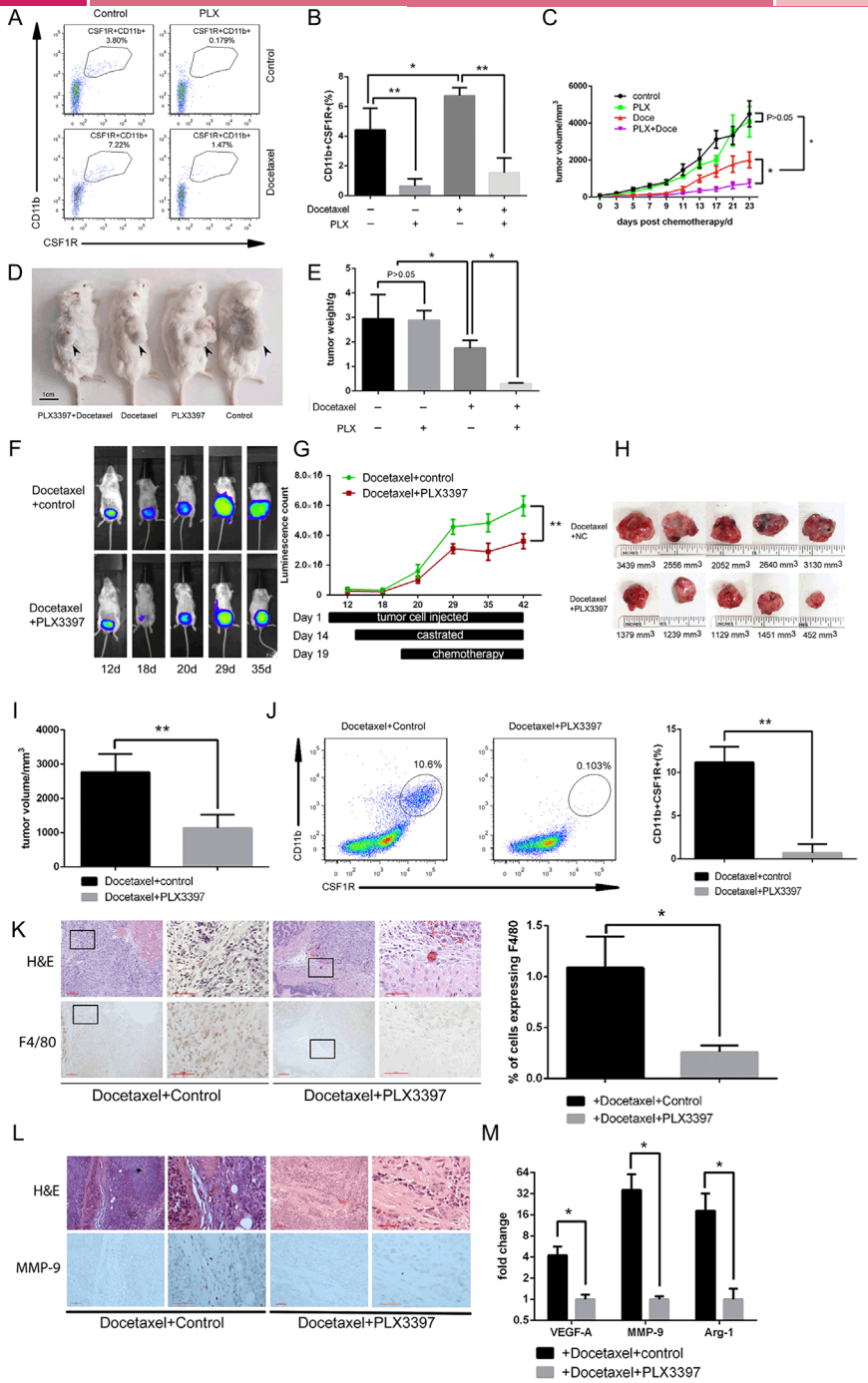

Figure 3

The addition of PLX3397 to docetaxel improves therapeutic efficacy in CRPC by reducing the protumorigenic influences of TAMs. The therapeutic effects of combining CSF-1Ri PLX3397 with docetaxel were evaluated in subcutaneous MyC-CaP tumors established in FVB male mice. Seven days after tumor cell implantation, all mice received surgical castration and randomly assigned to 4 treatment groups: (i) control, (ii) PLX3397, (iii) docetaxel or (iv) docetaxel + PLX3397. Flow cytometric analyses of CD11b+CSF1R+ TAM population in the tumor were shown as individual representative flow plots (A) and for each treatment cohort (B). Longitudinal tumor volume (C) and final tumor size (D and E) were shown for the four treatment groups. Intraprostatic CWR22Rv1 tumors were established with firefly luciferase marked cells, and longitudinal tumor growth were monitored by *in vivo* BLI (F and G). Tumor growth suppression was more effective in the docetaxel + PLX3397 group compared to the docetaxel only group (H and I) as assessed by terminal tumor volume. Likewise the PLX3397 containing treatment group was significantly reduced in the proportion of CD11b+ CSF1R+ TAM as analyzed by flow cytometry (J), and immunohistochemistry with F4/80 macrophages (K) and the tissue remodeling marker MMP-9 (L). Gene expression profiling by qRT-PCR revealed a reduction in VEGF-A, MMP-9 and Arg-1 with CSF-1Ri treatment (M) (*$P<0.05$, **$P<0.01$).

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achieving a very significant prolongation of their median survival by 17 months compared to ADT alone (Azad *et al.* 2015). Hence, docetaxel is an important therapeutic agent in the armamentarium against CRPC.

In this study, we investigated whether TAMs, an important component of the tumor microenvironment, could influence CRPC's response to docetaxel. We postulate that cellular damage sustained during docetaxel treatment induces PCa cells to produce cytokines and chemokines that recruit and polarize macrophages to the protumorigenic, alternatively activated M2 subtype (Brown *et al.* 2017). Congruent with this concept, we observed a significant increase in the expression of M2 cytokines, such as CSF-1 and IL-10 in all four prostate cancer cell lines, MyC-CaP, PC-3, CWR22Rv1 and C4-2, after docetaxel treatment. The elevated CSF-1 led to increased infiltration of macrophages *in vitro* and TAMs in MyC-CaP and CWR22Rv1 tumors after ADT and docetaxel treatment. We observed that treatment with another chemotherapeutic agent, paclitaxel, also elicited an increase in M2 cytokine expression in PCa, parallel the findings of a comprehensive chemotherapeutic study in preclinical breast cancer (DeNardo *et al.* 2011). Importantly, these findings support the rational combination of CSF-1Ri with docetaxel to lower the recruitment and M2 polarization of TAMs, which in turn reduce the protumorigenic influences of TAMs and significantly increase the efficacy of tumor growth suppression of ADT and docetaxel treatment (Fig. 4).

As the emergence of resistance to the current therapies is expected, what new and effective therapies will be incorporated to treat CRPC? A second-line taxane, cabazitaxel, was developed to overcome this resistance problem. The effectiveness of docetaxel is limited by its affinity for P-glycoprotein, an ATP-dependent drug efflux pump that decreases the intracellular concentrations of drugs (Bradshaw & Arceci 1998). Cabazitaxel exhibits low affinity for P-glycoprotein and has been shown to be effective in docetaxel-refractory PCa patients (de Bono *et al.* 2010, Paller & Antonarakis 2011). Although the cancer vaccine Sipuleucel-T was approved for CRPC, current clinical experience suggests this therapy has limited efficacy for aggressive large volume disease (Schellhammer *et al.* 2013, Mok *et al.* 2014). New immunotherapeutic strategies for CRPC need further exploration. In this regard, TAMs could have multiple negative influences. For instance, M2 macrophages are well known to impair T-cell responses by depleting essential nutrients through arginase I or by inhibiting T-cell receptor CD3ζchain (Rodriguez *et al.* 2004, Munder *et al.* 2006). Interestingly, a recent study by Gordon *et al.* (2017) further implicated that PD-1 expressing TAMs are inhibiting tumor immunity, which might further empower the efficacy of the PD-1 or PD-L1 checkpoint blockade.

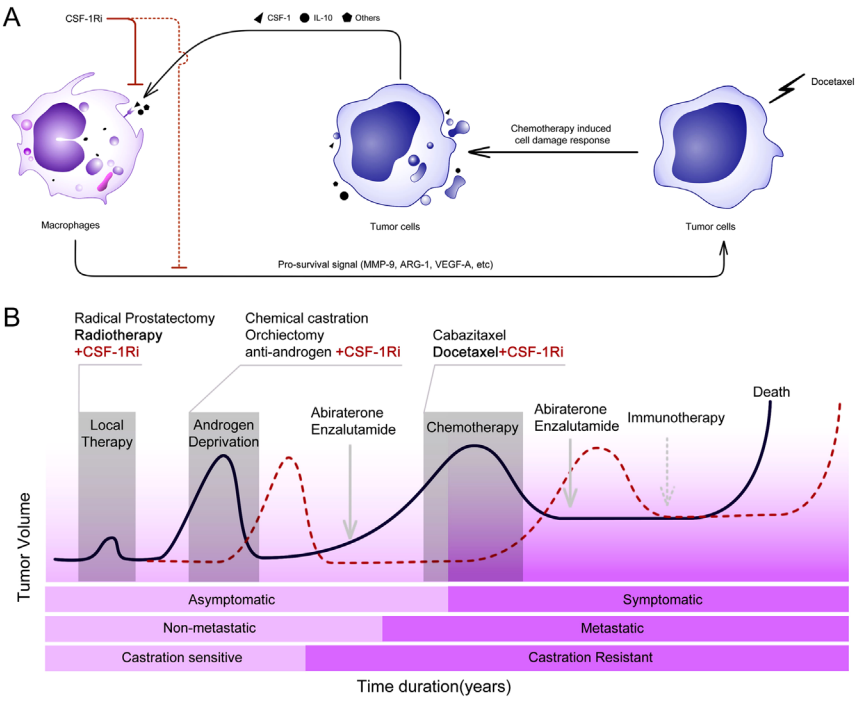

Figure 4
TAMs' contribution to docetaxel treatment failure in prostate cancer. A schematic illustration of impact of docetaxel treatment in PCa. The cellular damage caused by docetaxel heightens expression of M2 cytokines such as CSF-1 and IL-10, which recruit and polarize more M2 TAMs to foster their protumorigenic influences in the tumor microenvironment. The use of CSF1-R inhibitors could disrupt this TAM mediated vicious cycle.

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In our collective experience of studying TAM's influences in cancer therapy, we observed that TAMs contribute to every stage of PCa progression and therapy. From the control of local disease by radiation therapy (Xu *et al*. 2013), to the implementation of ADT for more advanced disease (Escamilla *et al*. 2015), to the use of docetaxel in recurrent CRPC studied here, blocking TAMs with CSF-1Ri in conjunction with these conventional therapies consistently improved therapeutic outcome by prolonging the duration of tumor growth suppression. Of note, the use of CSF-1Ri alone has no therapeutic impact in numerous preclinical models we have studied, including PCa, melanoma and lung cancer (Priceman *et al*. 2010). A large volume of literature shows that macrophages are educated and polarized by the tumor microenvironment towards the protumorigenic M2 subtype (Brown *et al*. 2017). We deduced that in the face of cellular injuries induces by conventional therapies, tumor cells secrete a higher level of M2 cytokines and chemokines such as CSF-1, CCL2 and IL10 that accentuate the protumorigenic functions of TAMs. Thus, combining CSF-1Ri with conventional cytotoxic therapies is a rational approach to improve their effectiveness. As we have shown that CSR-1Ri can improve the efficacy of adoptive T-cell therapy (Mok *et al*. 2014), it will be prudent to consider the incorporation of TAM blockade in combination for future immunotherapy strategies developed for CRPC, be it checkpoint inhibition or CAR T-cell therapy or others (Bilusic *et al*. 2017). Given the critical role of TAMs in therapeutic setting for PCa, we envision that the incorporation of TAM blockade could extend the efficacy of all phases of treatment. In doing so, we could extend the survival of PCa patients and achieve the goal of transforming PCa into a chronic and survivable malignancy.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/ERC-18-0284.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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