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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

There are an increasing number of more potent AR antagonists being developed and approved to treat advanced castrate resistant prostate cancer (CRPC). A diagnostic test capable of determining each patient's response to the new drug that could be applied before the initiation of treatments will be extremely valuable, especially toward implementing personalized medicine. Currently, the most common approach to assess treatment response rely on retrospective analysis of biomarkers obtained from the patient's bulk blood, serum or tumor samples (1). However, the known intratumoral cell heterogeneity in each patient may limit the accuracy and predictive power of these bulk tissue tests (2, 3). For this reason, a dynamic assay that can provide a read out of drug responsiveness at the single cell level after drug exposure should be more accurate to determine patient's response to AR blockade therapies (ABT). 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 (4, 5). Another important benefit of the proposed approach is that we will be query the drug responses in the living CTCs in the patients' blood without the need of long term laborious culturing. Mindful of all these objectives, we have begun to investigate and optimize the procedure of adenoviral mediated reporter gene transfer into cells in peripheral blood.

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.

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

- We completed the construction and large scale amplification of AdPSA-TSTA-GFP virus, which we have previously generated (6). This process was delayed 9 months due to contamination issues and inexperience of trainee in viral biology. We have since purified this virus and completed the amplification of this virus to pursue subtask 3 below. Please see difficulty encountered section below for more information.

<u>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.</u>

- We have confirmed the functional AR-reporting capability of AdPSA-TSTA-GFP virus in cell culture models as reported in last progress report.
- We have encountered technical difficulty in generating the PSMA-TSTA-GFP/CMV-RFP virus. Please see difficulties encountered section below for details.

<u>Subtask 3- Assess the dual AR functional reporter capability of AdPSA-TSTA-GFP/CMV-RFP and AdPSMA-TSTA-GFP/CMV-RFP in prostate tumor cells spiked blood samples</u>

- We substituted AdCMV-GFP and Ad PSA-TSTA-GFP to optimize blood samples processing and viral infection procedure with prostate tumor cells spiked blood samples.
- We have optimized the procedure for processing peripheral blood (from healthy volunteers) spiked with prostate cancer cells for viral infection (as reported in last progress report). Briefly, the peripheral blood processing procedure is:
  1) 12 ml of blood (with added prostate cancer cells (ranging 10-1000 per ml) was treated with Ficoll to remove the red blood cells (RBCs).
  2) The Ficoll treated samples (peripheral blood monoclear cells PMBCs + cancer cells) were resuspended in 4ml of RPMI media.
  3) Each ml of resuspended PBMC sample was infected with adenovirus (ranging from 10^7 to 10^10 infectious units) and incubate for 24hrs and analyzed the cells for reporter gene expression.

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.

- Not performed at this time. Will initiate this aim within 3-6 months. Please see difficulty encountered below for information.
- Co-PI Dr. Tseng's group has made major improvements in the development and fabrication of their third generation NanoVelcro chips (designated as CytoLumina's NanoVelcro PLGA Chips) to detect CTCs in patients. Progress in this technology will be described briefly below. We will apply this advanced CTC detection for SA2 and SA3.
-

Development and fabrication of of CytoLumina's NanoVelcro PLGA Chips. CytoLumina team had developed and produced the needed NanoVelcro Chip system components, i.e., 1) the imprinted NanoVelcro substrate, 2) an overlaid PDMS component, and 3) a slide-in & click-on chip holder (with a fluidic handler).
(i) Preparation of nano-imprinted NanoVelcro PLGA Substrates. Prior to fabricating NanoVelcro PLGA substrates, a new set of parental silicon nano-pillar (100-300 nm in diameter, 1.5 µm in length, 0.6-1.2 µm in spacing) arrays are prepared via e-beam lithography, followed by an inductively ICP-RIE process. With the increases in the nano-pillar aspect ratio and packing density, the cell-capture efficiency and specificity will be improved from earlier versions. Similar to our earlier approach, PDMS replicates are fabricated by molding the silicon nano-pillar arrays. A new workflow (**Figure 1**) are employed to fabricate the PLGA NanoVelcro substrates. First, a 5% PLGA polymer (MW. = 43-100 K) solution is spin-coated onto a LMD slide. Second, a "chlorobenzene (CB)-FREE" nano-imprinting approach (CB exhibits low to moderate toxicity as indicated by its LD50 of 2.9 g/kg.) had been developed and are employed to introduce nano-pillars onto a PLGA film (ca. 1.2-µm thick) on a LMD slide. Using this new approach PDMS is directly placed onto PLGA films. A metal sandwich holder is used to apply 150±20 g/cm2 onto the assembled layers. The molding process is carried out at 120oC, a temperature above the glass transition temperature of PLGA. Once the assembled layers are cooled down to 20°C, the PDMS

replicates are peeled off from the substrates to give the new PLGA NanoVelcro substrate. Immediately before CTC capture, the PLGA NanoVelcro Chips are conjugated with streptavidin using (N-hydroxysuccinimide) NHS chemistry (8, 9).

(ii) <u>The PDMS-based chaotic mixer</u>. According to a recent study (10) on improving chaotic mixing for CTC capture, we proposed to alter the spacing of herringbone patterns and the width/length of the microchannel in our new PDMS chaotic mixer to further improve our CTC capture efficiency and specificity. In addition, the new chaotic mixer is prepared by thermally curing PDMS pre-polymer on a Si-based replicate mold where the herringbone patterns are fabricated first by photolithography then inductively coupled plasma dry etching. Compared to the SU-8 photolithographically deposited patterns used previously (11), the ICP-etched patterns on Si are much more durable for long-term, repeated usage. The fabrication of the Si replicate molds and PDMS chaotic mixers are supervised by CytoLumina's fabrication facility in UCLA Nanofabrication Lab.

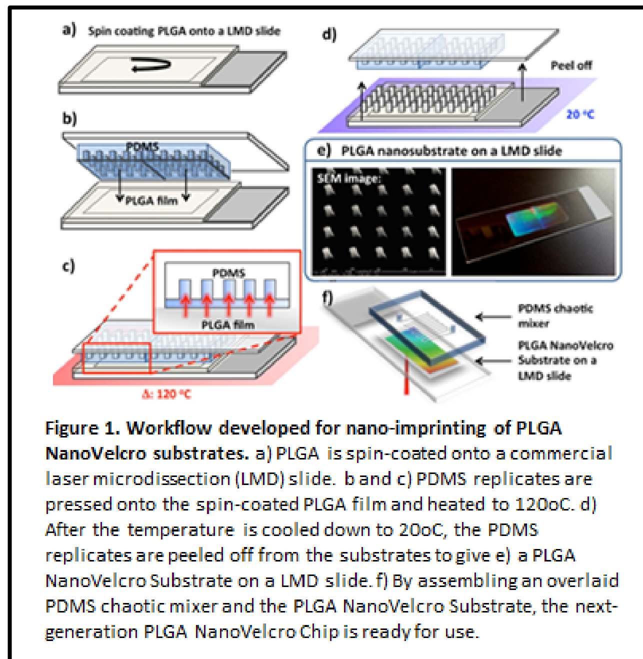

Figure 1. Workflow developed for nano-imprinting of PLGA NanoVelcro substrates. a) PLGA is spin-coated onto a commercial laser microdissection (LMD) slide. b and c) PDMS replicates are pressed onto the spin-coated PLGA film and heated to 120oC. d) After the temperature is cooled down to 20oC, the PDMS replicates are peeled off from the substrates to give e) a PLGA NanoVelcro Substrate on a LMD slide. f) By assembling an overlaid PDMS chaotic mixer and the PLGA NanoVelcro Substrate, the next-generation PLGA NanoVelcro Chip is ready for use.

(iii) <u>Design and fabrication of a digital fluidic handler for introducing blood and reagents into NanoVelcro Chip</u>. On the basis of manually operated syringe pumps used in earlier CTC enumeration, CytoLumina team had developed a fully automated fluidic handler (Figure 2) and a touch-screen user interface, capable of automated blood loading and CTC fixation. The fluidic handler is composed of four functional components: 1) a syringe and syringe pumps for injecting blood samples at variable flow rates (0.2-2 mL/h); 2) a rotary valve for controlled introduction of blood/reagents to NanoVelcro Chips, 3) reservoirs for storage of blood and reagents, and 4) a touch-screen panel that controls automation of fluidic handler. Here, LabView program are employed to control the automation of the digital fluidic handler. Since the system had just been developed, we will conduct 6 assays in parallel to achieve a desired throughput in the next 2 months.

(iv) <u>Slide-in & click-on chip holder are designed and fabricated by CytoLumina team with support from UCLA Machine Shop</u>. As shown in **Figure 2**, the chip holder is composed of upper and lower pieces. The lower piece is designed to house both of the NanoVelcro PLGA substrate and PDMS component. There are multiple "plug-in" alignment markers on the lower piece to help instant assembly of the individual components. The upper piece of the chip holder will be introduced onto the assembled lower one by sliding along the two tracks. The final step is to switch the "click-on" utility. The 4 built-in springs will compress to seal the PDMS module with NanoVelcro

substrate tightly. This slide-in & click-on design will reduce error during device assembly and CTC enumeration.

(v) Quality control (QC) protocols for ensuring the performance of NanoVelcro PLGA Chips. CytoLumina's experience in fabricating the devices has led to several standardized operation protocols that bring a higher yield of defect-free devices (>95%) and a fabrication capability >500 chips/day in UCLA Nano electronics Facility. Three QC steps, including 1) eye-inspection: a rainbow diffraction surface on the imprinted NanoVelcro PLGA substrate suggests the presence of regular nano-pillar features on the devices' surfaces, 2) SEM examination on the surface topographies of PLGA nanopillars arrays on glass slides, and 3) immunofluorescence chemistry for testing that homogeneity of streptavidin coating has been used to ensure the performance of PLGA NanoVelcro Chips. The continuous refinement of fabrication SOPs and QC protocols is documented in the CytoLumina database.

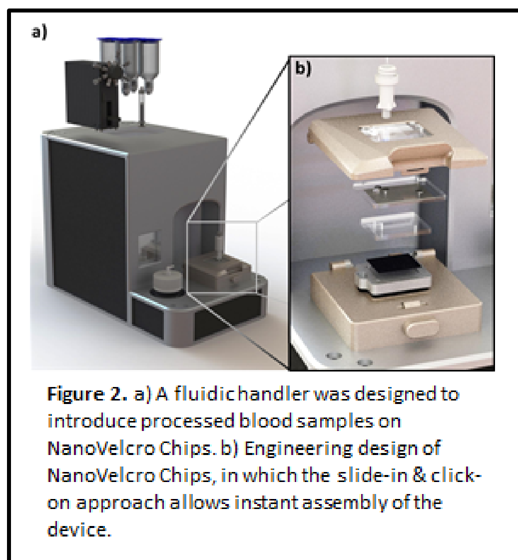

Figure 2. a) A fluidic handler was designed to introduce processed blood samples on NanoVelcro Chips. b) Engineering design of NanoVelcro Chips, in which the slide-in & click-on approach allows instant assembly of the device.

Specific Aim 3: To evaluate the therapeutic responses to AR antagonists in CTCs of CRPC patients before, during and after AR blockade treatment.

- The testing of adenoviral mediated transduction of patient's blood samples has not been initiated yet due to difficulties noted below. Will initiate this aim within 6-9 months.
- In a related study, we investigated novel treatment approaches for CRPC patients. Docetaxel chemotherapy is often given to patients who failed ADT. In a study under submission, 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. Please see enclosed manuscript entitled: Inhibition of TAMs improves the response to docetaxel in castration-resistant prostate cancer.

Difficulties Encountered:

- This project encountered major delays from May of 2017 to March of 2018 due to several family health crisis faced by Dr. Lily Wu, the PI of this project. She has been in contact with DOD's scientific program officers regarding these issues. These issues have taken a significant time and efforts from her as well as posing a major emotional toll on her. Thus, there is a 9-12 month delay on the progress of this project. The majority of these health issues has resolved and Dr. Wu has resumed her work in full capacity in the last 2 months. She is requesting for the resumption of this project with some modifications of research aims (see below) and a 1 year no cost extension of this project.
- A second issue that also hampered the progress of this project is in the shortage of investigators with appropriate expertise in adenoviral vectorology. Several trained urologists, specialized in prostate cancer, had worked on this project in the last 2

years. Unfortunately, their inexperience in molecular cloning and construction of adenoviral vectors has led to unsuccessful attempts to generate the new vectors proposed. To remedy this issue I have recruited a master degree student (Mr. Young-Hyeon) from Professor Chae-ok Yun's group at Hanyang University of South Korea to join my group. Professor Yun is a world leading expert oncolytic adenoviral therapy. https://www.researchgate.net/profile/Chae_Ok_Yun Mr. Choi will lead the efforts to complete the adenoviral vectors proposed. He will initiate the construction of the vectors in July 2018 and the completion of construction of the viral vectors is anticipated in December 2018.

- Our revised research aims for July 2018 to June 2019 will be:
  1) Construct and amplify AdPSA-TSTA-GFP/CMV-RFP and AdPSMA-TSTA-GFP/CMV-RFP (7/1/2018 to 12/31/2018)
  2) Test the ability of these 2 vectors to transduce healthy donor blood samples spiked with prostate cancer cells. Assess the specificity and sensitivity of these vectors to detect prostate cancer cells using the improved NanoVelcroPLGA Chips (noted above), in the presence or absence of MDV3100. (1/1/2019 to 3/1/2019)
  3) Test our Ad-mediated CTC detection methodology using patients' blood samples. Due to the time limitation, we will perform this task on the blood from 10 patients to assess the feasibility. (1/1/2019 to 6/30/2019) If the results are positive, we will extend our analysis to 40 patients as originally proposed, but the time to completion of this task will likely be beyond June 2019, due to time required for patient recruitment.
  4) We will use the improved NanoVelcroPLGA Chips to assess extracellular vesicles (EVs) such as exosome or oncosomes from patient's blood samples as an exploratory extension of this project. From our research on a different project on tumor heterogeneity and metastasis, we discovered that EVs are an instrumental method of cellular communication, especially during metastatic dissemination. Thus, we will take advantage of the sensitive NanoVelcroPLGA Chips to assess the presence of EVs. Current literature suggests EVs are much more prevalent than CTCs in patient's blood.

Key Research Accomplishments

- We verified the ability of AdPSA-TSTA-GFP to query prostate tumor cells' responsiveness to AR antagonists such as MDV3100.
- We have refine the method to process peripheral blood for adenoviral infection.
- We demonstrated that PBMC and tumor cells in peripheral blood can be viable for infection by adenovirus, and capable to express the exogenous introduce reporter gene in the 24-48 hours after blood collection.
- Completion of a manuscript on improved therapeutic management of CRPC.

Reportable Outcomes

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

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 (6, 7). 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).

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

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Running title: Improve CRPC chemo-response by blocking TAMs

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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, that 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.

Keywords

CRPC, TAMs, docetaxel, CSF-1, CSF-1R

Introduction

Prostate cancer (PCa) is the second most common cancer in men after skin cancer, as 1 out of 7 men will be diagnosed with this disease in the United States[1]. It is estimated that 161, 000 newly diagnosed cases and 27,000 deaths will be attributed to this disease in 2017[1]. 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. In these cases, the 5-year survival rate drops precipitously to about 30% [1].

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[2]. 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) [3]. Abiraterone and enzalutamide are two newly approved potent ADT agents that inhibit CYP17A1 androgen synthetic enzyme and AR, respectively [4, 5]. Both agents are effective in prolonging the survival of castration-resistant prostate cancer (CRPC) patients who had progressed on first line ADT [6]. However, a significant proportion of CRPC patients either do not respond to either abiraterone or enzalutamide, or initially respond but subsequently progress on treatment [7]. Potential mechanisms of resistance include AR mutations, amplification and splice variant[8-10].

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 [11] [12, 13]. Belonging to the taxane family, docetaxel was initially postulated to suppress prostate cancer growth by interfering with microtubule function [14]. 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 [15].

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 [16-18] . 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), 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), 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.[19].

Statistical Analysis

Data are all presented as mean ± SEM. 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 (Figure 1A). We found that prostate cancer cells exhibit a wide range of sensitivity to docetaxel, with C4-2 (IC50 = 2nM) and CWR22Rv1 (IC50 = 5nM) being the most sensitive, PC-3 (IC50 = 30nM) as an intermediate responder and Myc-CaP (IC50 = 100nM) 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 (12, 13). Here we further inquired whether docetaxel in addition to 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 Figure 1B Myc-CaP or CWR22Rv1 cells treated with ADT plus docetaxel, dosed at each line's respective IC50, increased the expression of CSF-1 and IL-10. Likewise, the expression of these M2 cytokines were also induced when prostate cancer cells, including Myc-CaP, CWR22Rv1, PC3 and C4-2, were treated in the presence of macrophages (Figure 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 IC10 dose, the elevation of M2 cytokine expression was no longer observed (Figure 1D). These findings support that cell injury mediated by ADT plus docetaxel induces the heightened expression of M2 cytokines in PCa cells.

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

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 [17]. Next, we examine the impact of macrophage recruitment in the setting of docetaxel treatment. As shown in Figure 2A and 2B, 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 selective CSF-1Ri PLX3397 [16, 18, 20, 21] attenuated the enhancement in macrophage recruitment (Figure 2A and B).

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 4 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 40mg/kg/week. Comparing to diluent control treated tumors, PLX3397 only treatment significantly reduced the number of $CD11b^{+}$ $CSF1R^{+}$ TAMs, while docetaxel significantly increased TAMs (Figure 2C). 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 (Figure 2C). 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*.

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

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* [22]. 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 (Figure 2C-E). As expected, docetaxel treatment significantly retarded the growth of Myc-CaP tumor compared to control (Figure 2D, E). More importantly, docetaxel plus PLX3397 achieved the most significant tumor growth suppression in the 4 treatment groups, more effective than docetaxel alone (Figure 2D, 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, Figure 3A, B). 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 (Figure 3B). Treatment continued to day 42, at which point the animals were euthanized. Assessed either by BLI (Figure 3A, B) or by terminal tumor volume (Figure 3C), 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 (Figure 3D). This finding was further verified by F4/80

immunohistochemistry stain to detect macrophages (Figure 3E). 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 (Figure 3F, G). Taken all 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[23], head and neck cancer[24], and non-small cell lung cancer[25]. In the Chemohormonal Therapy versus 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, achieving a very significant prolongation of their median survival by 17 months compared to ADT alone (8). 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 [17]. Congruent with this concept, we observed a significant increase in the expression of M2 cytokines, such as CSF-1 and IL-10 in all 4 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. 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 (Figure 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 [33]. Cabazitaxel exhibits low affinity for P-glycoprotein and has been shown to be effective in docetaxel-refractory PCa patients [26, 34]. Although the cancer vaccine Sipuleucel-T was approved for CRPC, current clinical

experience suggests this therapy has limited efficacy for aggressive large volume disease [31, 35]. 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 CD3 ζ chain [36, 37]. Interestingly, a recent study by Gordon et al [38] 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.

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 [16], to the implementation of ADT for more advanced disease [18], 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 [22]. A large volume of literature shows that macrophages are educated and polarized by the tumor microenvironment towards the protumorigenic M2 subtype [17]. 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[31], 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[39]. 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.

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Figure Legends

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 IC50 and IC10 values. (**B**) Myc-CaP and CWR22Rv1cells, cultured alone were treated with docetaxel at the IC50 dose for each cell (Myc-Cap at 100 nM and CWR22Rv1 at 5nM). Expression of CSF-1 and IL-10 and Ki-67 in response to docetaxel treatment was shown. (**C**) The impacts of docetaxel treatment at IC50 on PCa cells in the presence of macrophages (RAW cells) were shown for Myc-CaP, CWR22Rv1, PC3 (30nM) and C4-2 (2nM) cells were shown (**D**) When treated at their respective IC10 doses of 20 and 1 nM, Myc-CaP and CWR22Rv1 cells showed no significant change in CSF-1, IL-10 or Ki67 expression. All cells were cultured in media supplemented with charcoal-stripped serum. (*: $p<0.05$)

Figure 2. CSF-1R inhibitor PLX3397 abrogated the increased recruitment of macrophages induced by docetaxel treatment *in vitro* and *in vivo*.

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 2μM CSF-1Ri PLX3397 to the docetaxel treated conditioned media abrogated the increased macrophage recruitment induced by both cell lines. The effects of combination therapy 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 (**C**). Longitudinal tumor volume and final tumor size were analyzed in the 4 treatment groups (**D, E**). (*: $p<0.05$, **: $P<0.01$)

Figure 3. The addition of PLX3397 to docetaxel improves therapeutic efficacy in CRPC by reducing the protumorigenic influences of TAMs. Intraprostatic CWR22Rv1 tumors were established with firefly luciferase marked cells, and longitudinal tumor growth were monitored by *in vivo* BLI (**A, B**). Tumor growth suppression was more effective in the docetaxel + PLX3397 group compared to the docetaxel only group (**C**). Likewise the

PLX3397 containing treatment group was significantly reduced in the proportion of CD11b+ CSF1R+ TAM as analyzed by flow cytometry (**D**), and immunohistochemistry with F4/80 macrophages (**E**) and the tissue remodeling marker MMP-9 (**F**). Gene expression profiling by qRT-PCR revealed a reduction in VEGF-A, MMP-9 and Arg-1 with CSF-1Ri treatment (**G**). (*: $P<0.05$; **: $p<0.01$)

Figure 4. Schematic illustration of TAMs involvement and tumor progression in prostate cancer. 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.

Figure 1

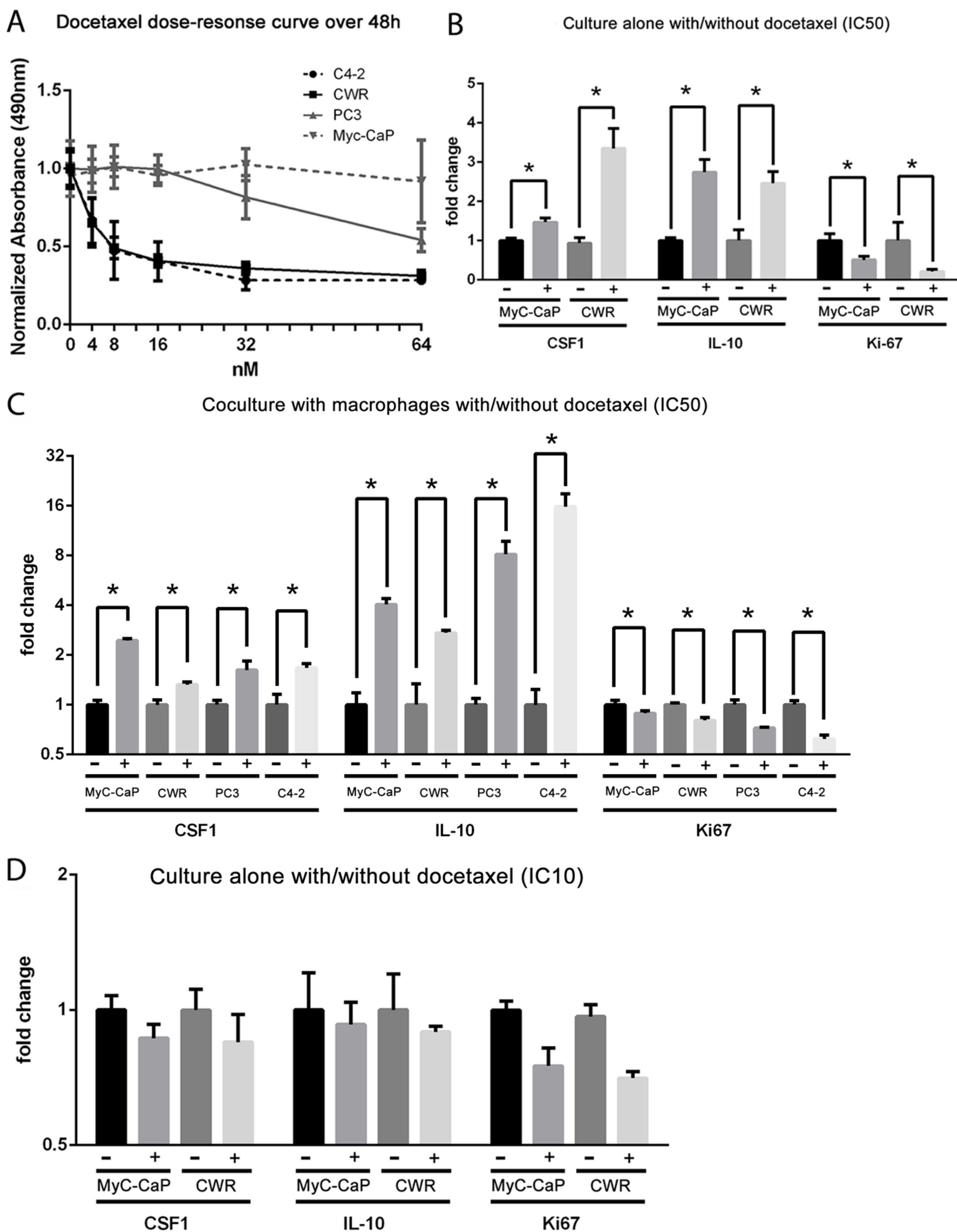


Figure 2

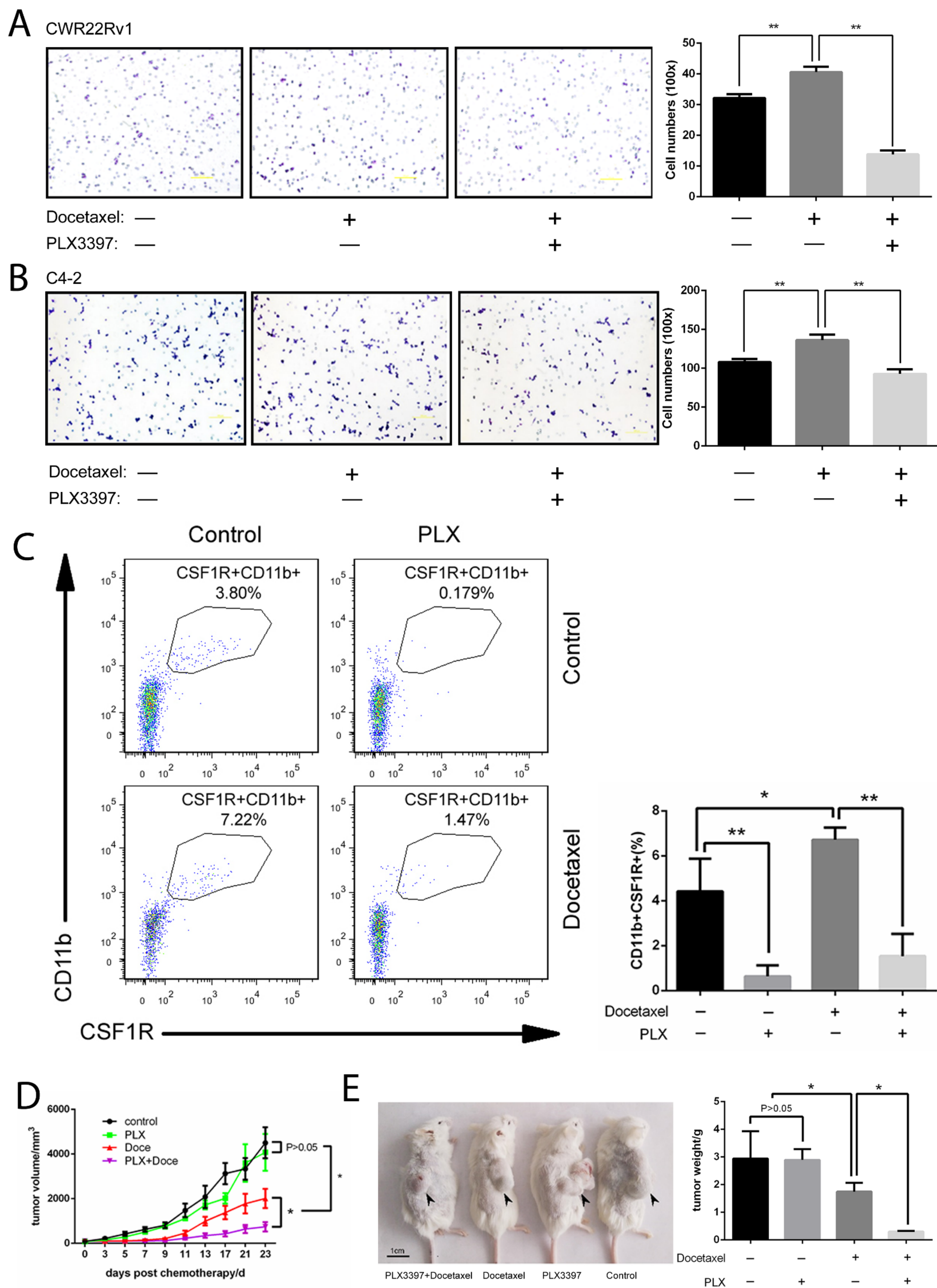
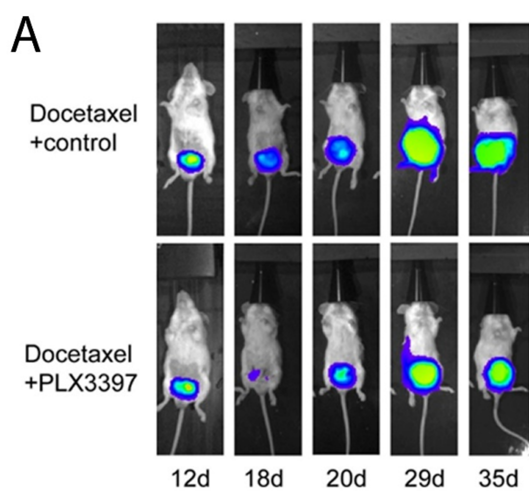
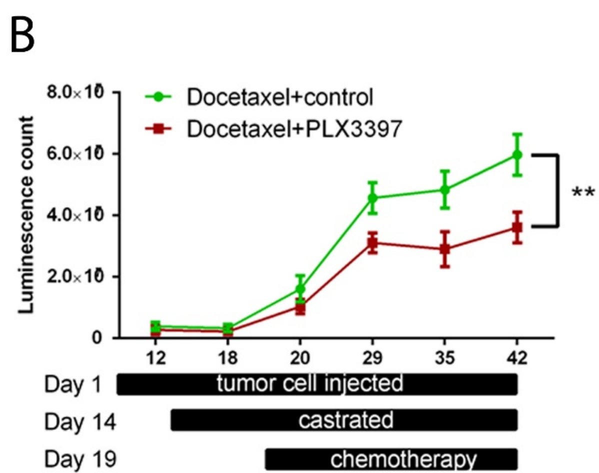
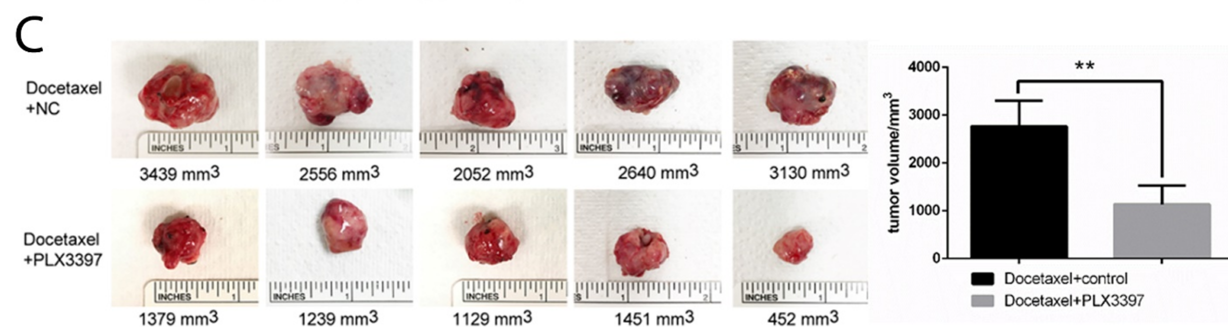
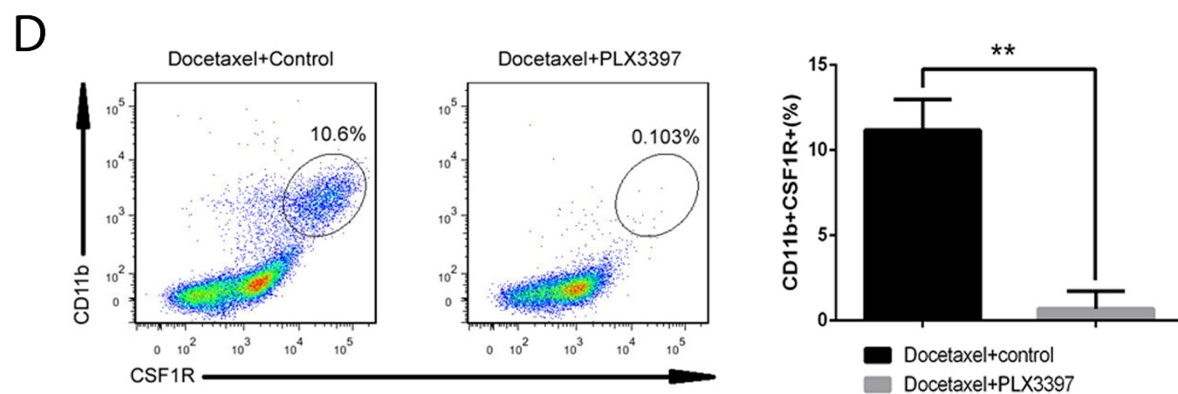


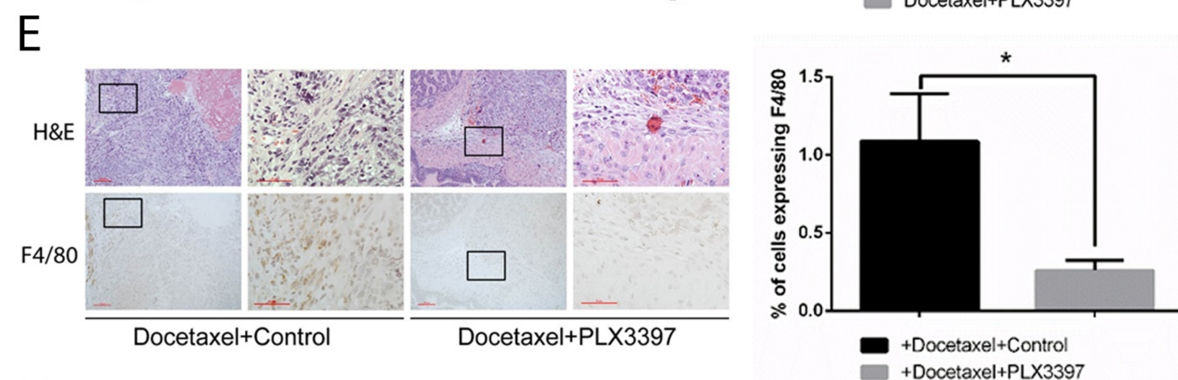
Figure 3

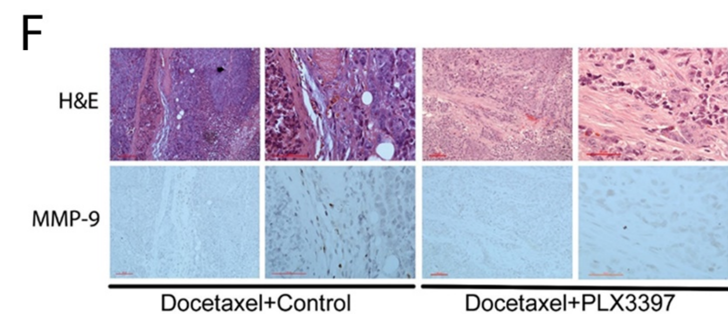












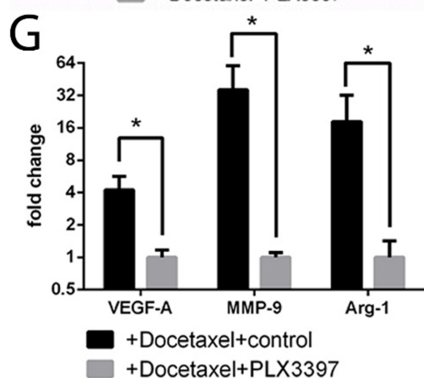


Figure 4