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CCL2 is induced by chemotherapy and protects prostate cancer cells from docetaxel - induced cytotoxicity

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Abstract

Background—Metastatic prostate cancer is either inherently resistant to chemotherapy or rapidly acquires this phenotype after chemotherapy exposure. In this study, we identified a docetaxel-induced resistance mechanism centered on CCL2.

Methods—we compared the gene expression profiles in individual human prostate cancer specimens before and after exposure to chemotherapy collected from previously untreated patients who participated in a clinical trial of preoperative chemotherapy. Subsequently, we used the gain- and loss- of function approach *in vitro* to identify a potential mechanism underlying chemotherapy resistance.

Results—Among the molecular signatures associated with treatment, several genes that regulate the inflammatory response and chemokine activity were upregulated including a significant increase in transcripts encoding the CC chemokine CCL2. Docetaxel increased CCL2 expression in prostate cancer cell lines *in vitro*. CCL2 specific siRNA inhibited LNCaP and LAPC4 cell proliferation and enhanced the growth inhibitory effect of low-dose docetaxel. In contrast, overexpression of CCL2 or recombinant CCL2 protein stimulated prostate cancer cell proliferation and rescued cells from docetaxel-induced cytotoxicity. This protective effect of CCL2 was associated with activation of the ERK/MAP kinase and PI3K/AKT, inhibition of docetaxel-induced Bcl2 phosphorylation at serine 70, phosphorylation of Bad, and activation of caspase-3. The addition of a PI3K/AKT inhibitor Ly294002 reversed the CCL2 protection, and was additive to docetaxel induced toxicity.

Conclusion—These results support a mechanism of chemotherapy resistance mediated by cellular stress responses involving the induction of CCL2 expression, and suggest that inhibiting CCL2 activity could enhance therapeutic responses to taxane-based therapy.

Keywords

prostate cancer; chemotherapy resistance; CCL-2; inflammation

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Introduction

Chemotherapy using docetaxel or mitoxantrone is a standard treatment option for patients with metastatic castration-resistant prostate cancer [1,2]. Unfortunately, resistance to treatment severely limits the potential of these agents to improve the lives of patients with advanced prostate cancer [3,4]. Understanding of the mechanisms underlying the tumor response and resistance are urgently needed to develop strategies that can enhance the chemotherapy efficacy and circumvent treatment resistance. We hypothesized that such mechanisms can be identified by comparing the *in vivo* gene expression profiles acquired before and after the administration of cytotoxic drugs. In an ongoing clinical trial [5], patients with high-risk localized prostate cancer (defined as cT2b or T3a or PSA $\geq$ 15 ng/ml or Gleason grade $\geq$ 4+3) underwent study-specific biopsies that were snap frozen then received four cycles of docetaxel and mitoxantrone followed by radical retropubic prostatectomy. The overall design and initial clinical results from this study have been reported [6]. The gene expression profiles from specific cellular compartments obtained through laser capture microdissection of snap frozen biopsy and subsequent prostatectomy tissue were compared using cDNA microarrays. Using this approach, we previously discovered that GDF15 is significantly upregulated by docetaxel, and contributes to docetaxel resistance [5]. Our continued analyses of the gene expression profiles derived from these chemotherapy-treated samples identified a cohort of genes with functional roles involved in regulating inflammatory processes.

Inflammation is associated with the development of solid malignancies [7]. As reviewed recently by Seruga, *et al.* [8], tissue homeostasis is regulated by the active balance between pro- and anti- inflammatory mechanisms. The disruption of this delicate equilibrium is often associated with cancer development that is accompanied by immune activation and chronic inflammation. Cytokines are important signaling mediators of the inflammatory response. Although various types of immune cells contribute both pro- and anti- inflammatory factors, cancer cells can also express specific cytokines and chemokines that in turn support a pro-tumorigenic microenvironment [9].

The mechanisms of action for taxane-based therapy is to alter microtubule dynamics causing cell cycle arrest and apoptosis [1]. Docetaxel binds to microtubules with high affinity, which leads to the activation of JNK signaling and Bcl2 phosphorylation such as at serine 70 (Ser70) [10,11]. Subsequently, the phosphorylation of Bcl2 inactivates its anti-apoptotic function, which results to the activation of caspase 3 and cell death [12,13].

Chemokine, CCL2, is responsible for the recruitment of immune cells such as macrophages to sites of inflammation [14], and has been shown to have a direct effect on endothelial cells in promoting angiogenesis [15,16]. More recently, the association between CCL2 and prostate cancer has been established. *In vitro* under serum-free conditions, CCL2 was shown to stimulate prostate cancer cell proliferation and resistance to apoptosis [17]. These effects were positively associated with the activations of ERK/MAP kinase and PI3K/AKT signaling cascades. Further, CCL2 was shown to induce expression of the cellular anti-apoptotic protein survivin [18,19]. *In vivo*, CCL2 siRNA in PC-3 cells has shown to reduce tumor growth in a mouse model [20], and the treatment of mice with an anti-CCL2 monoclonal antibody significantly suppressed the progression of prostate cancer bone metastasis in intracardiac and intratibial models [21]. Further, tumor expression of the CCL2 receptor CCR2 was positively associated with prostate cancer progression in patients [21].

In the current study, we extended our previous efforts to identify mechanisms contributing to the resistance of prostate carcinoma to chemotherapy based on *in vivo* comparative gene expression analyses. We found that inflammatory cytokines including CCL2 are significantly upregulated by chemotherapy in cancer cells. We report that the upregulation of CCL2

represents an inducible mechanism that contributes to chemotherapy resistance through the modulation of cell signaling pathways involving MAP kinase and AKT that influence apoptotic responses.

Materials and Methods

Prostate tumor transcript profiling by cDNA Microarray analysis

Briefly, prostate cancer specimens acquired before and after chemotherapy were used for laser-capture microdissection using methods we have previously reported in detail [5]. The pre-chemotherapy samples were acquired from needle biopsies, and the post-chemotherapy samples were from radical prostatectomy specimens. Total RNA was extracted from the microdissected tumor epithelium, amplified using a linear amplification method, and used for cDNA synthesis [5]. The Cy3 or Cy5 labeled cDNA (after treatment *vs.* before treatment from the same individual) were hybridized in a head-to-head fashion simultaneously to custom-made microarray comprised of ~6,800 clones derived from the Prostate Expression DataBase (PEDB) [22].

Cell culture and chemicals

Prostate cancer cell lines LNCaP (ATCC) and LAPC-4 (originally derived by Rob Reiter and Charles Sawyers at UCLA, and a gift from Dr. John Isaacs of Johns Hopkins School of Medicine) were cultured in regular RPMI containing 10% FBS and 1% PS. For serum-starved condition, cells were first plated in regular medium. Twenty-four hours later, cells were washed with PBS, and basal RPMI without FBS and PS was used to replace the regular medium. Docetaxel (DTX) was purchased from Sigma (St. Louis, MO), dissolved in DMSO. The frozen stock aliquots were stored at -80°, further diluted with medium right before adding to the cells. The NF-κB inhibitor (parathenolide) was purchased from Sigma, the JNK inhibitor (SP600125) and PI3K/AKT inhibitor (Ly294002) were purchased from Biomol International (Plymouth, PA). The recombinant human CCL2 protein was from R&D systems (Minneapolis, MN).

qRT-PCR and Western Blot

The qRT-PCR and western analysis were conducted based on previously published procedures [23,24] using the delta-delta Ct method. With the exception of CCL2, all antibodies used for western blot were purchased from Cell Signaling (Danvers, MA). The CCL2 antibodies were purchased from R&D Systems (Minneapolis, MN).

CCL2 over expression and siRNA

The CCL2 expression vector was purchased from Open Biosystems (Huntsville, AL). Subsequently, the CCL2 coding region was subcloned into pcDNA 3.1, PCR-amplified with EcoRV restriction site on both ends of 5′ and 3′ primers. The PCR products were inserted downstream of the elongation factor 1a promoter with EcoRV digestion of a lentiviral expression vector (gift from Dr. Linzhao Cheng at Johns Hopkins School of Medicine). The lentiviral delivery and establishment of LNCaP with stable CCL2 expression was done as previously described [25]. The CCL2 siRNA was purchased from Dharmacon, Inc. (Lafayette, CO), and siRNA transfection using DharmaFect 3 was done following the manufacturer's protocol.

Proliferation assay

The proliferation assays were performed as previously described [26]. Briefly, equal numbers of cells (~50,000 cells per well) were seeded into 12 – well plates. A portion of the attached and viable cells were counted with a hemocytometer, and set as day 0 control. The remaining

cells were treated and counted at later time points. The viability and growth under specific conditions were calculated by comparing to that of day 0 control.

Statistical Analysis

Differences in transcript abundance between pre- and post-treatment samples analyzed using the cDNA array were determined by random variance t-test as we have previously described [5]. The results of cell proliferation and survival were analyzed with Student's t-test, with P values < 0.05 being considered significant.

Result

Inflammatory cytokines are upregulated in prostate cancer cells by chemotherapy

To identify the molecular alterations associated with chemotherapy, we compared the gene expression patterns of 31 patients with high-risk localized prostate cancer before and after a neoadjuvant chemotherapy trial using docetaxel and mitoxantrone. We used laser capture microdissection to specifically isolate tumor cells with the same Gleason grade from both pre- and post- treatment tissues (5). Previously, we have shown that surgical manipulations of radical prostatectomy tissues can impact gene expressions on the cellular stress pathways, and established a cohort of 441 genes whose expression can be influenced by surgical tissue ischemia. In the current study, we excluded these 441 genes from our microarray analysis (5). We found that several genes encoding mediators of inflammation including CCL2, CXCL10, IL-1β, IL-6 and IL-8 were significantly up-regulated in the post-chemotherapy specimens (fig 1a). To confirm that these alterations reflected tumor cell responses to chemotherapy, we treated LNCaP and LAPC4 prostate cancer cell lines with increasing doses of either docetaxel or mitoxantrone and measured mRNA levels for these genes by qRT-PCR. Docetaxel-treated cells had dose-dependent increases in CCL2, CXCL10, IL-6 and IL-8 mRNAs in LNCaP cell line (fig1b) and LAPC-4 cell line (data not shown). Mitoxantrone did not induce similar alterations (data not shown).

CCL2 is up-regulated by docetaxel via JNK and NF-kB pathway

To investigate the role of CCL2 in modulating prostate cancer resistance to docetaxel, we first confirmed that CCL2 was upregulated following chemotherapy using qRT-PCR to measure CCL2 transcripts. We found a significant difference in CCL2 transcripts between pre- and post-treatment patient matched cancer samples ($p<0.002$, paired t-test, $N=31$) (fig 2a). We also confirmed that both intracellular and extracellular CCL2 protein levels were increased in the prostate cancer cell line treated with docetaxel (fig 2b). In macrophages, CCL2 expression is co-regulated by JNK and NF-kB pathways [27]. Since JNK pathways are activated by chemotherapy-induced stress [28], and NF-kB is overactive in cancer [29,30], we hypothesized that these two pathways were also responsible for the up-regulation of CCL2 in prostate cancer cells. We used SP600125 (1 μM) to inhibit JNK signaling. After LNCaP cells were co-treated with SP600125 and docetaxel, the docetaxel-induced CCL2 gene up-regulation was abolished (fig 2c). Lactone compounds such as parathenolide have been reported to inhibit NF-kB signaling. 10 μM of parathenolide inhibited the basal CCL2 expression in LNCaP cells (fig 2d), more importantly, when parathenolide was co-administered to LNCaP with docetaxel, it dose-dependently inhibited the docetaxel-induced CCL2 expression. Besides LNCaP, similar results were observed using LAPC4 and breast cancer cell line MCF-7 (data not shown). Neither JNK nor NF-kB inhibitors induced significant amount of cytotoxicity with the dose and time employed in the current experiments, we therefore conclude that JNK and NF-kB pathways are possible signaling mechanisms that are involved in the CCL2 upregulation by docetaxel.

Inhibition of CCL2 enhances docetaxel cytotoxicity

We next hypothesized that inhibition of docetaxel-induced CCL2 would enhance the docetaxel cytotoxicity toward prostate cancer cells. To test this hypothesis, we used RNAi to transiently knock-down CCL2 expression in the LNCaP cell line. qRT-PCR and western blot confirmed that docetaxel-induced CCL2 expression was significantly inhibited in cells treated with siRNA for 48 hours followed by 3 nM of docetaxel for another 48 to 120 hours (fig 3a). Similar experiments were performed to measure docetaxel-induced cytotoxicity with CCL2 suppression by RNAi. LNCaP cells were transfected with siRNA against CCL2 (siCCL2) or scrambled control (siControl), and allowed to recover for 24 hours. Viable cells were counted with a hemocytometer (considered as day 0 control), and cultured with serum-free media containing 0, 1 and 3 nM of docetaxel for 5 days. The growth and viability of cells were calculated by counting viable cell numbers at day 5, and compared to those of day 0 control (fig 3b). In the absence of growth factors and without docetaxel exposure, cells transfected with CCL2 siRNA had significantly less viability relative to cells expressing a scrambled siRNA control (* in fig 3b). In cells received siControl, treatment with 3 nM docetaxel significantly reduced the cell viability compared to the solvent control. Importantly, treatment with 3 nM docetaxel plus CCL2 siRNA resulted in a significant reduction of cell viability compared to 3 nM of docetaxel alone (# in fig 3b) or CCL2 siRNA alone (** in fig 3b). Similar results were also seen in LAPC4 cells, in which 3 nM of docetaxel significantly reduced the cell viability in siCCL2 cells than siControl cells (fig 3c). These data suggest that the induction of CCL2 by docetaxel can confer docetaxel resistance in prostate cancer cells, and inhibition of CCL2 can enhance the cytotoxicity of docetaxel.

CCL2 gain-of-function reduces docetaxel efficacy

To further investigate the role of CCL2 in prostate cancer response to docetaxel, we established a line of LNCaP cells that stably overexpress CCL2. Under serum-free conditions, the growth patterns of LNCaP-CCL2 cells (CCL2) and LNCaP-empty vector control cells (Ev) were very similar (fig 4a). In contrast, when these two cell lines were treated with low-dose of docetaxel (3 nM) for 5 days, the CCL2 cells showed higher viability than the Ev cells (* in fig 4a). Next, we determined if exogenous CCL2 protein can influence prostate cancer cell responses to docetaxel. Under serum-free condition, both 10 and 100 ng/ml of CCL2 significantly enhanced the growth of LAPC4 cells over the course of 5 days (* in fig 4b). Low-dose of docetaxel (3 nM) was growth inhibitory and induced a significantly higher sub-G1 dead cell populations in flow cytometry analysis (fig 4c); however, recombinant CCL2 countered the docetaxel-induced inhibition and greatly enhanced the cell viability and growth (# in fig 4b, * in fig 4c). Similar observations were also made using LNCaP cell line (data not shown). The CCL2-induced resistance was less robust at high-concentrations (10 nM) of docetaxel. It is likely that the level of damage caused at this concentration was sufficient to overcome the growth and/or survival advantage provided by CCL2. *In vivo*, the pharmacokinetics of docetaxel suggests that tumor cells are only exposed to low nanomolar concentrations of the drug between rounds of therapy, therefore, the effect of CCL2 in the presence of low concentrations of docetaxel maybe more relevant to the mechanism of resistance.

CCL2 modifies proteins of the apoptotic pathways

To gain additional molecular insights into CCL2's role in mediating docetaxel chemotherapy response and resistance, we treated LNCaP cells with docetaxel, recombinant CCL2, or the combination of these agents for 48 hours under serum free conditions. Western blot analysis revealed that docetaxel was able to activate the JNK signaling cascade as evidenced by the increase of phosphorylation of JNK1 and c-Jun at Thr183 and Ser63, respectively (fig 5a). CCL2 can also activate the JNK pathway, but it strongly induced the phosphorylation of Erk and AKT, indicating effects through both MAP kinase and PI3K/AKT. When CCL2 was

combined with docetaxel, the Erk and AKT phosphorylation was maintained, but the JNK1 and c-Jun phosphorylation was reduced compared to single agent treatment (fig 5a), which suggests that there are antagonistic effects between docetaxel and CCL2 regarding the JNK signaling pathway. Downstream of JNK, docetaxel alone induced Bcl2 phosphorylation at Ser70. CCL2 had no effect on Bcl2 as single agent, but in combination, CCL2 attenuated the docetaxel-induced Bcl2 phosphorylation (fig 5b). The protein Bad is pro-apoptotic. The survival signals from the MAP kinase and AKT can phosphorylate Bad at different residues and inactivate its pro-apoptotic function [31]. In our current experiment, docetaxel slightly induced Bad phosphorylation at serine 112, a target of MAP kinase. Addition of exogenous CCL2 protein increased the phosphorylation of Bad (fig 5b). These data suggest that CCL2 can exert its potential chemoresistance function by activating AKT and MAP kinase pathways and countering docetaxel-induced JNK activation.

Targeting PI3K/AKT enhances docetaxel efficacy

To further identify the mechanism for the antagonistic effect of CCL2 on docetaxel, we hypothesized that the activation of AKT by CCL2 were responsible for the inhibition of docetaxel-induced Bcl2 phosphorylation and caspase 3 activation. Treatment of LNCaP cells with docetaxel induced Bcl2 phosphorylation and caspase 3 activation, but recombinant CCL2 reduced these two events (Fig 6a). Importantly, the PI3K/AKT inhibitor (Ly294002) restored the Bcl2 phosphorylation and caspase 3 activation, while Bad phosphorylation at Ser112 (a target of MAP kinase) was unchanged (fig 6a). These data is consistent with a pathway, in which the therapy-induced CCL2 activates PI3K/AKT signaling to promote survival and protect cells from chemotherapy-induced toxicity. Therefore, we hypothesized that targeting PI3K/AKT pathway can enhance the effect of chemotherapy by in part inhibiting the CCL2 pathway. We treated LNCaP cells with low dose of docetaxel, Ly294002 or the combination of these agents. Individually each treatment resulted in 20%-30% growth inhibition. Combining docetaxel with PI3K/AKT inhibition reduced cell growth by ~75% (fig 6b). This suggests the combination between low-doses chemotherapy and a PI3K/AKT targeted approach can achieve an additive antitumor effect.

Discussion

In the treatment of advanced, castration resistant prostate cancer, docetaxel-based chemotherapy is empirically deployed. Responses were seen in about 50% of the patients and emergence of treatment resistance is universal. When used preoperatively in the treatment of high risk disease, docetaxel chemotherapy can reduce serum PSA, but has not resulted in pathologic complete responses [4,32,33]. These results illustrate the need to identify docetaxel-resistance mechanisms in prostate cancer. In this study, we sought to identify mechanisms by which prostate cancer cells resist the toxic effects of docetaxel. A comparison of transcript profiles from prostate cancer cells obtained before and after chemotherapy exposure *in vivo* identified the robust up-regulation of several inflammatory cytokines and chemokines including CCL2. The cytotoxicity of taxane-based chemotherapy has previously been shown to occur in part through activation of stress-induced JNK signaling pathways [34]. The primary molecular function of docetaxel is to bind and disrupt microtubules depolymerization [1]. As a part of the cell stress response mechanism, the disruption on microtubule dynamics is sensed by microtubule interacting proteins [35,36], which in turns activate JNK/SAPK signal transduction pathways that are capable of modifying Bcl2 and inducing apoptosis [12,13,37]. We found that that docetaxel was able to up-regulate tumor cell CCL2 expression *via* the JNK pathway. We determined that docetaxel-induced CCL2 can act in an autocrine fashion to stimulate the Erk/MAP kinase and PI3K/AKT signaling to promote tumor cell survival, which was associated with the increased phosphorylation of Bad, decreased phosphorylation of Bcl2, and reduced activation of caspase-3. The effects of CCL2 on Bcl2, caspase-3 and viability were

in direct conflict with effects induced by low-doses of docetaxel. In essence, the therapy-induced growth inhibition can be countered by the therapy-induced stress adaptation. In our studies, the CCL2-induced proliferation and phosphorylation were masked by serum in the media. The cytoprotective role of CCL2 in a more complex *in vivo* system within the context of chemotherapy has not been fully elucidated. Further, the potential cytoprotective functions of additional inflammatory cytokines induced *in vitro* and *in vivo* have not been evaluated, though our findings indicate that such studies merit evaluation.

The interaction between CCL2 and its receptor CCR2 during prostate cancer bone metastasis has been studied. Prostate cancer bone metastasis is positively associated with tumor CCR2 expression [21]. The mechanistic rationale behind this association is that bone marrow cells have high level of CCL2 expression; therefore, high expression of tumor CCR2 can promote metastatic migration and growth in the bone environment. It is unknown whether docetaxel chemotherapy could further upregulate CCL2 expression from bone marrow cells as a mechanism contributing further to therapy resistance. A targeted approach using monoclonal antibodies against CCL2 has demonstrated antitumor activity in preclinical models of prostate cancer [21]. Our study further emphasized the potential benefit of targeting CCL2 in the context of chemotherapy. Our results suggest that targeting docetaxel-induced CCL2 expression using combinations of docetaxel and a CCL2 specific antibody or by other means may mitigate therapy-induced resistance and enhance docetaxel activity and thereby enhance the utility of this drug in the treatment of prostate cancer.

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References

1. Mackler NJ, Pienta KJ. Drug insight: Use of docetaxel in prostate and urothelial cancers. Nat Clin Pract Urol 2005;2(2):92–100. quiz 101 p following 112. [PubMed: 16474654]
2. Pienta KJ, Smith DC. Advances in prostate cancer chemotherapy: a new era begins. CA Cancer J Clin 2005;55(5):300–318. quiz 323-305. [PubMed: 16166075]
3. Beer TM, El-Geneidi M, Eilers KM. Docetaxel (taxotere) in the treatment of prostate cancer. Expert Rev Anticancer Ther 2003;3(3):261–268. [PubMed: 12820771]
4. Garzotto M, Myrthue A, Higano CS, Beer TM. Neoadjuvant mitoxantrone and docetaxel for high-risk localized prostate cancer. Urol Oncol 2006;24(3):254–259. [PubMed: 16678060]
5. Huang CY, Beer TM, Higano CS, True LD, Vessella R, Lange PH, Garzotto M, Nelson PS. Molecular alterations in prostate carcinomas that associate with in vivo exposure to chemotherapy: identification of a cytoprotective mechanism involving growth differentiation factor 15. Clin Cancer Res 2007;13(19):5825–5833. [PubMed: 17908975]
6. Beer TM, Garzotto M, Lowe BA, Ellis WJ, Montalto MA, Lange PH, Higano CS. Phase I study of weekly mitoxantrone and docetaxel before prostatectomy in patients with high-risk localized prostate cancer. Clin Cancer Res 2004;10(4):1306–1311. [PubMed: 14977829]
7. De Marzo AM, Platz EA, Sutcliffe S, Xu J, Gronberg H, Drake CG, Nakai Y, Isaacs WB, Nelson WG. Inflammation in prostate carcinogenesis. Nat Rev Cancer 2007;7(4):256–269. [PubMed: 17384581]

8. Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. Nat Rev Cancer 2008;8(11):887–899. [PubMed: 18846100]
9. Balkwill F. Cancer and the chemokine network. Nat Rev Cancer 2004;4(7):540–550. [PubMed: 15229479]
10. Basu A, Haldar S. Microtubule-damaging drugs triggered bcl2 phosphorylation-requirement of phosphorylation on both serine-70 and serine-87 residues of bcl2 protein. Int J Oncol 1998;13(4): 659–664. [PubMed: 9735392]
11. Haldar S, Basu A, Croce CM. Serine-70 is one of the critical sites for drug-induced Bcl2 phosphorylation in cancer cells. Cancer Res 1998;58(8):1609–1615. [PubMed: 9563469]
12. Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 1998;273(9):4928–4936. [PubMed: 9478937]
13. Yamamoto K, Ichijo H, Korsmeyer SJ. BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol Cell Biol 1999;19(12):8469–8478. [PubMed: 10567572]
14. Loberg RD, Ying C, Craig M, Yan L, Snyder LA, Pienta KJ. CCL2 as an important mediator of prostate cancer growth in vivo through the regulation of macrophage infiltration. Neoplasia 2007;9 (7):556–562. [PubMed: 17710158]
15. Barcelos LS, Talvani A, Teixeira AS, Cassali GD, Andrade SP, Teixeira MM. Production and in vivo effects of chemokines CXCL1-3/KC and CCL2/JE in a model of inflammatory angiogenesis in mice. Inflamm Res 2004;53(10):576–584. [PubMed: 15597153]
16. Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV. CCL2 regulates angiogenesis via activation of Ets-1 transcription factor. J Immunol 2006;177(4):2651–2661. [PubMed: 16888027]
17. Loberg RD, Day LL, Harwood J, Ying C, St John LN, Giles R, Neeley CK, Pienta KJ. CCL2 is a potent regulator of prostate cancer cell migration and proliferation. Neoplasia 2006;8(7):578–586. [PubMed: 16867220]
18. Roca H, Varsos Z, Pienta KJ. CCL2 protects prostate cancer PC3 cells from autophagic death via phosphatidylinositol 3-kinase/AKT-dependent survivin up-regulation. J Biol Chem 2008;283(36): 25057–25073. [PubMed: 18611860]
19. Roca H, Varsos ZS, Mizutani K, Pienta KJ. CCL2, survivin and autophagy: new links with implications in human cancer. Autophagy 2008;4(7):969–971. [PubMed: 18758234]
20. Lu Y, Chen Q, Corey E, Xie W, Fan J, Mizokami A, Zhang J. Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. Clin Exp Metastasis 2009;26(2):161–169. [PubMed: 19002595]
21. Loberg RD, Ying C, Craig M, Day LL, Sargent E, Neeley C, Wojno K, Snyder LA, Yan L, Pienta KJ. Targeting CCL2 with systemic delivery of neutralizing antibodies induces prostate cancer tumor regression in vivo. Cancer Res 2007;67(19):9417–9424. [PubMed: 17909051]
22. Nelson PS, Pritchard C, Abbott D, Clegg N. The human (PEDB) and mouse (mPEDB) Prostate Expression Databases. Nucleic Acids Res 2002;30(1):218–220. [PubMed: 11752298]
23. Myrthue A, Rademacher BL, Pittsenbarger J, Kutyba-Brooks B, Gantner M, Qian DZ, Beer TM. The Iroquois Homeobox Gene 5 Is Regulated by 1,25-Dihydroxyvitamin D3 in Human Prostate Cancer and Regulates Apoptosis and the Cell Cycle in LNCaP Prostate Cancer Cells. Clin Cancer Res 2008;14(11):3562–3570. [PubMed: 18519790]
24. Qian DZ, Kato Y, Shabbeer S, Wei Y, Verheul HM, Salumbides B, Sanni T, Atadja P, Pili R. Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589. Clin Cancer Res 2006;12(2):634–642. [PubMed: 16428510]
25. Qian DZ, Wei YF, Wang X, Kato Y, Cheng L, Pili R. Antitumor activity of the histone deacetylase inhibitor MS-275 in prostate cancer models. Prostate 2007;67(11):1182–1193. [PubMed: 17520666]
26. Qian DZ, Wang X, Kachhap SK, Kato Y, Wei Y, Zhang L, Atadja P, Pili R. The histone deacetylase inhibitor NVP-LAQ824 inhibits angiogenesis and has a greater antitumor effect in combination with the vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK787/ZK222584. Cancer Res 2004;64(18):6626–6634. [PubMed: 15374977]

27. Wolter S, Doerrie A, Weber A, Schneider H, Hoffmann E, von der Ohe J, Bakiri L, Wagner EF, Resch K, Kracht M. c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene. Mol Cell Biol 2008;28(13):4407–4423. [PubMed: 18443042]

28. Wang Q, Wieder R. All-trans retinoic acid potentiates Taxotere-induced cell death mediated by Jun N-terminal kinase in breast cancer cells. Oncogene 2004;23(2):426–433. [PubMed: 14724571]

29. Richmond A. Nf-kappa B, chemokine gene transcription and tumour growth. Nat Rev Immunol 2002;2(9):664–674. [PubMed: 12209135]

30. Suh J, Rabson AB. NF-kappaB activation in human prostate cancer: important mediator or epiphenomenon? J Cell Biochem 2004;91(1):100–117. [PubMed: 14689584]

31. She QB, Solit DB, Ye Q, O'Reilly KE, Lobo J, Rosen N. The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells. Cancer Cell 2005;8 (4):287–297. [PubMed: 16226704]

32. Febbo PG, Richie JP, George DJ, Loda M, Manola J, Shankar S, Barnes AS, Tempany C, Catalona W, Kantoff PW, et al. Neoadjuvant docetaxel before radical prostatectomy in patients with high-risk localized prostate cancer. Clin Cancer Res 2005;11(14):5233–5240. [PubMed: 16033841]

33. Magi-Galluzzi C, Zhou M, Reuther AM, Dreicer R, Klein EA. Neoadjuvant docetaxel treatment for locally advanced prostate cancer: a clinicopathologic study. Cancer 2007;110(6):1248–1254. [PubMed: 17674353]

34. Mhaidat NM, Zhang XD, Jiang CC, Hersey P. Docetaxel-induced apoptosis of human melanoma is mediated by activation of c-Jun NH2-terminal kinase and inhibited by the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 pathway. Clin Cancer Res 2007;13(4):1308–1314. [PubMed: 17317842]

35. Li Y, Bachant J, Alcasabas AA, Wang Y, Qin J, Elledge SJ. The mitotic spindle is required for loading of the DASH complex onto the kinetochore. Genes Dev 2002;16(2):183–197. [PubMed: 11799062]

36. Li Y, Elledge SJ. The DASH complex component Ask1 is a cell cycle-regulated Cdk substrate in Saccharomyces cerevisiae. Cell Cycle 2003;2(2):143–148. [PubMed: 12695666]

37. Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H, Wimalasena J. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem 1999;274 (12):8208–8216. [PubMed: 10075725]

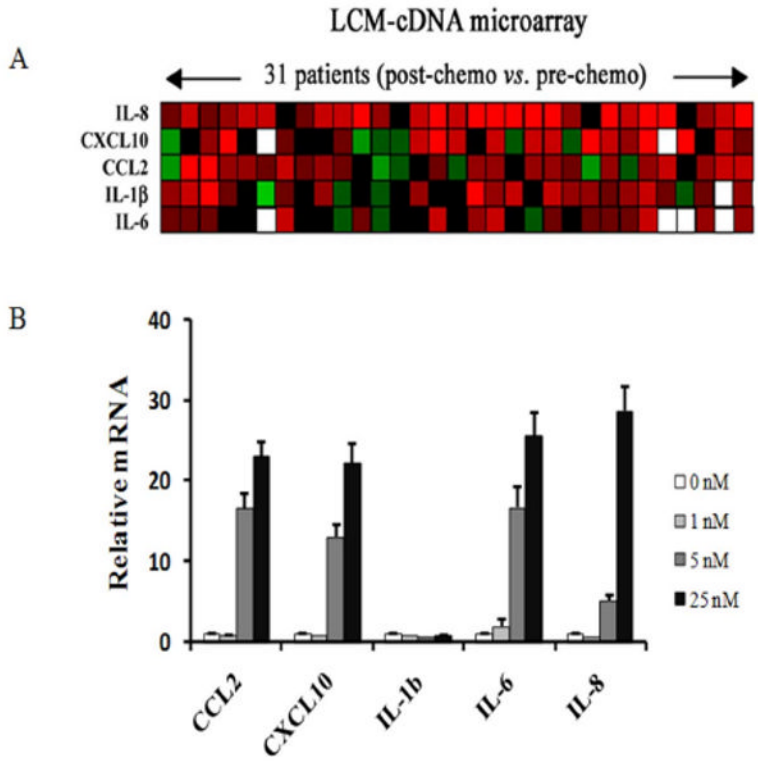

Figure 1. The modulations of inflammatory genes in vivo and in vitro

A. The heatmap of 5 most significantly upregulated inflammatory cytokine gene expression in prostate cancer epithelium from 31 patients underwent chemotherapy. Tumor epithelium was laser-capture microdissected from post-treatment radical prostatectomy samples and pre-treatment needle biopsy samples. cDNA were synthesized as previously described. The expression level was expressed as post-treatment *vs.* pre-treatment. Rows represent genes and columns represent individual patients. Red: upregulation, Green: down-regulation, Black: no change, White: no data.

B. Docetaxel induced the expression of inflammatory genes. LNCaP cells were treated with 0, 1, 5, and 25 nM of docetaxel for 48 hours. RNA was extracted, and gene expressions were measured by qRT-PCR, analyzed using the delta-delta Ct method and normalized to samples treated with 0 nM docetaxel.

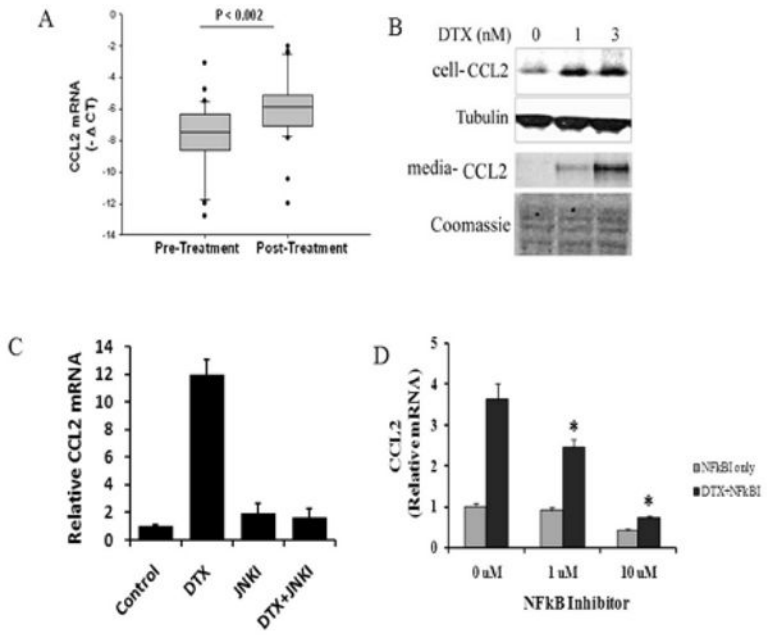

Figure 2. JNK and NF-kappaB pathways mediated the docetaxel-induced CCL2 gene expression

A. The CCL2 upregulation in post-treatment cancer epithelium was confirmed by qRT-PCR. The cDNA of 31 patients from post-treatment radical prostatectomy samples and pre-treatment needle biopsy samples were used. $P < 0.002$, paired-t-test.

B. Docetaxel induced CCL2 protein expressions in LNCaP cells. Cells were treated with 1 and 3 nM of docetaxel (DTX) for 72 hours. Whole cell lysates and proteins in LNCaP extracellular media were extracted and probed with an anti-CCL2 antibody by western blot. The intracellular and extracellular CCL2 loadings were controlled by tubulin and coomassie stain, respectively.

C. LNCaP cells were treated with solvent, 5 nM of docetaxel, 1 μM of JNK inhibitor (SP600125), or combination of both for 48 hours. Gene expression was measured by qRT-PCR, normalized to solvent control, and expressed as relative mRNA.

D. LNCaP cells were treated with solvent, 5 nM of docetaxel, 1 uM or 10 uM of NF-kB inhibitor (parthenolide), or combinations for 48 hours. Gene expressions of CCL2 were measured, normalized to solvent control, and expressed as relative mRNA. * $P < 0.05$, ANOVA.

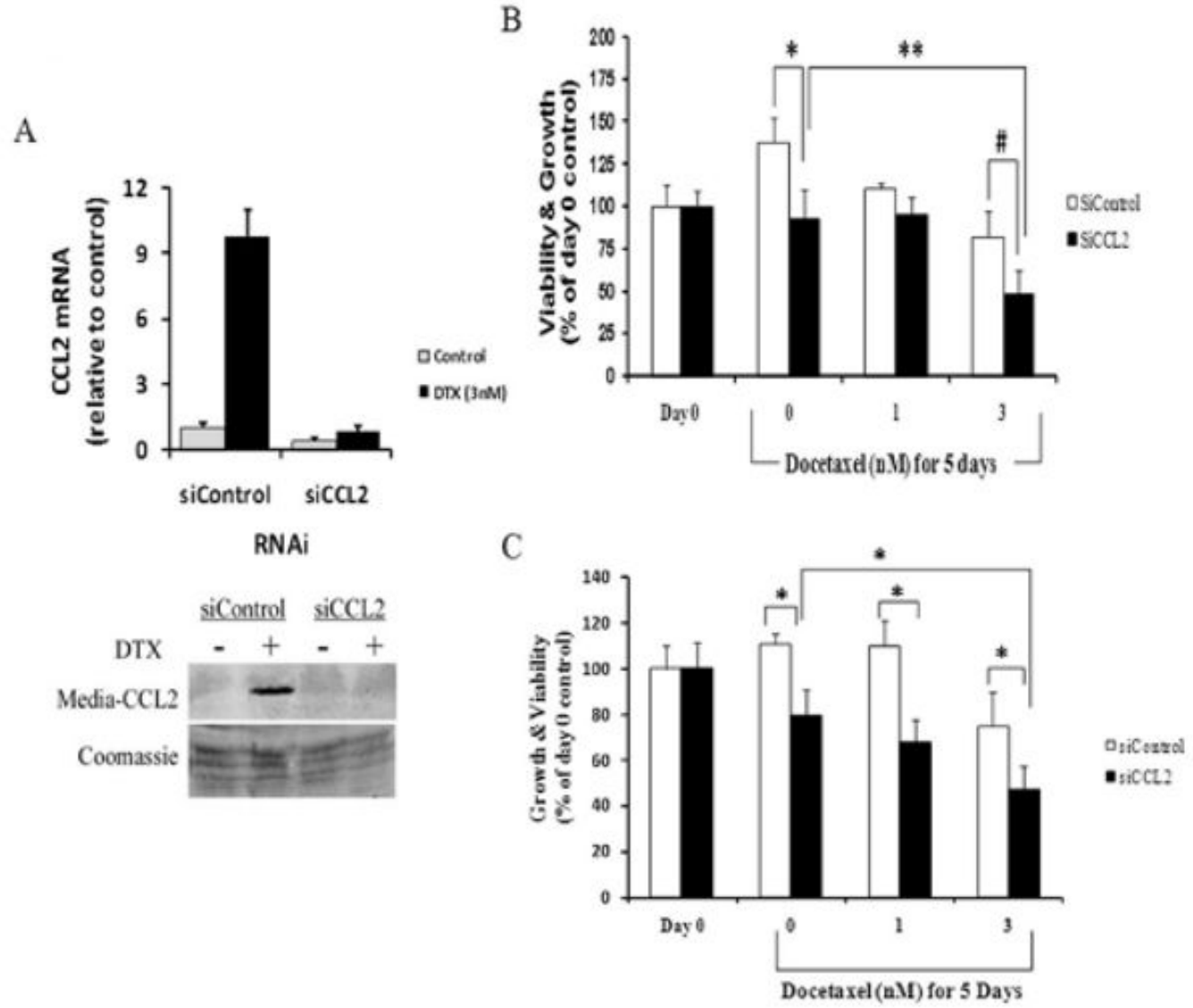

Figure 3. CCL2 loss-of-function by siRNA enhanced prostate cancer cells sensitivity toward low-dose of docetaxel

A. LNCaP cells were transfected with scramble control (siControl) or siRNA oligo targeting CCL2 (siCCL2) for 48 hours. Then cells were treated with 3 nM docetaxel. The expressions of CCL2 were measured by qRT-PCR in cells and western blot in cell culture media.

B. & C. Prostate cancer cells LNCaP (B) and LAPC-4 (C) were transfected with a CCL2 specific siRNA oligonucleotide. 24 hours later viable cells were counted, cultured in serum free media and treated with low-doses of 1 and 3 nM of docetaxel for 5 days. The viable cells of siRNA control and siCCL2 were counted and normalized to that of respective day 0 control. *, #, **, $P < 0.05$, t-test.

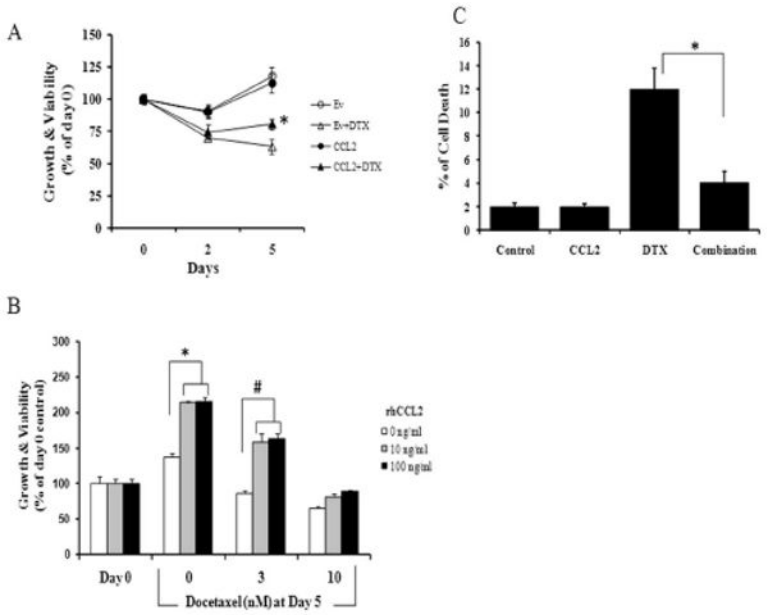

Figure 4. CCL2 gain-of-function decreased prostate cancer cell sensitivity to low-dose of docetaxel

A. LNCaP cells were transfected with either a CCL2 over-expression vector or an empty vector control. Then cells were treated with 3 nM of docetaxel for 5 days. The viable cell numbers of empty vector cells or CCL2 cells were counted at day 0, 2 5 of docetaxel treatment, and normalized to that of respective day 0 controls. * *vs.* Ev+DTX, $P < 0.05$, t-test.

B. LAPC4 cells were cultured at serum-free condition, and viable cell numbers were counted as day 0 controls. Then cells were treated with 3 or 10 nM of docetaxel, in combinations with 0, 10 or 100 ng/ml of recombinant human CCL2. After 5 days of treatment, viable cells in each CCL2 group were counted and normalized to that of respective day 0 control. *, #, $P<0.05$, t-test.

C. LAPC4 cells treated with control, 3nM docetaxel (DTX), 10 ng/ml CCL2 or combination in (B) were stained with PI and subjected to flow cytometry. The % of dead cells in sub-G1 phase was plotted. Mean and standard deviation. *, $P < 0.05$, t-test.

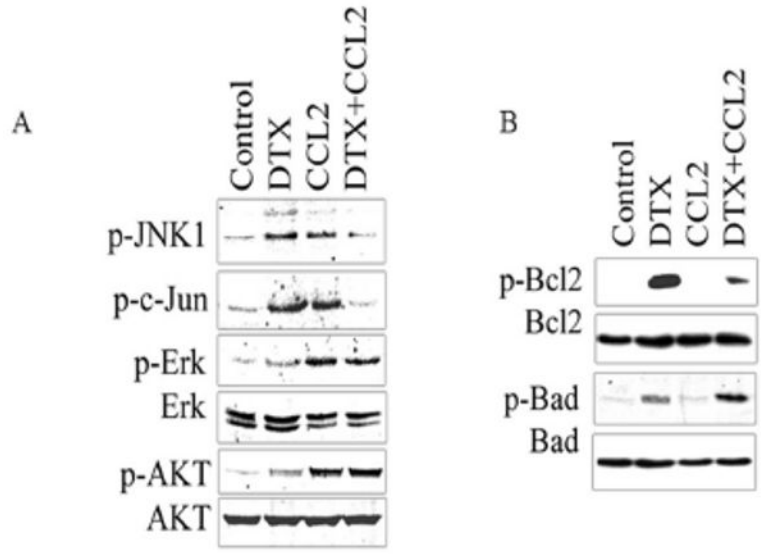

Figure 5. CCL2 modified signal transduction

A. CCL2 increased Erk and AKT signaling, and decreased docetaxel induced JNK signaling. LNCaP cells were treated with docetaxel, CCL2 or combination for 48 hours. Whole cell lysates were prepared, resolved on 12% SDS-PAGE gel, and probed with antibodies specific for JNK1, c-jun, Erk and AKT phosphorylation. Total Erk and AKT were used as loading controls.

B. CCL2 activated Bcl2 and inactivated Bad. Cells and proteins were prepared as in A, and probed with antibodies specific for Bcl2 phosphorylation at serine 70, Bad phosphorylation at serine 112. The total Bcl2 and Bad were used as loading controls.

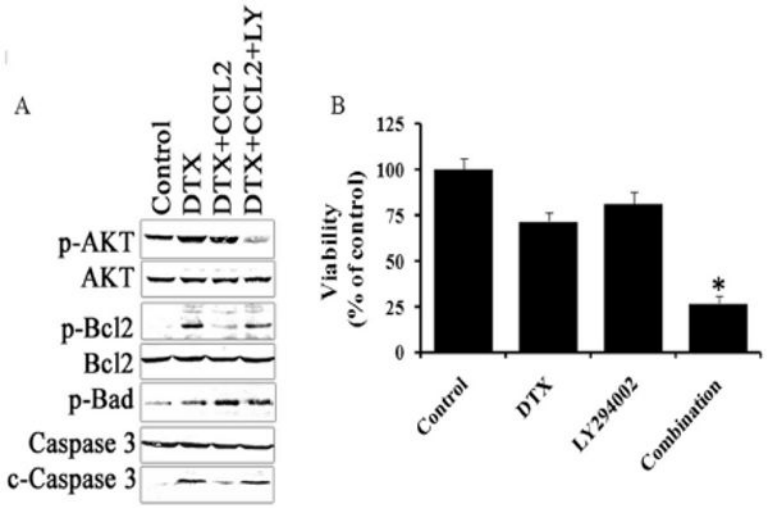

Figure 6. PI3K/AKT is utilized by CCL2 to confer survival advantage during chemotherapy

A. Inhibition of PI3K/AKT restored the docetaxel-induced Bcl2 phosphorylation and caspase 3 activation. Cells were treated with docetaxel or docetaxel with CCL2 as in figure 5A, plus PI3K/AKT inhibitor Ly294002 (5 μM) was added into the media of docetaxel and CCL2 co-treated cells.

B. PI3K/AKT inhibitor enhanced the growth inhibitory effect of low-dose docetaxel. LNCaP cells were treated with solvent control, low-dose of docetaxel (1 nM), Ly294002 (5 μM), or combination for 72 hours. Viable cells were counted and expressed as % of solvent control. * $P < 0.05$ vs. docetaxel, Ly294002, t-test.