Emerging data highlight the significance of chemokine (C-X-C motif) ligand 12/chemokine (C-X-C motif) receptor 4 (CXCL12/CXCR4) signaling axis in the chemoresistance of several malignancies, including prostate cancer (PCa); however, underlying mechanisms remain largely elusive. Here, we demonstrate that CXCL12 treatment rescues the PCa cells from docetaxel (DTX)-induced toxicity by overriding its effect on cell cycle (G2/M phase arrest). We further demonstrate that the chemoprotective effect of CXCL12 is abolished upon pharmacological inhibition or RNA interference-mediated silencing of CXCR4. Moreover, microtubule stabilization caused by DTX is suppressed in CXCL12-stimulated PCa cells as revealed by immunofluorescence and immunoblot analyses. The effect of CXCL12 on microtubule stabilization is abrogated when PCa cells are pre-treated with a CXCR4 antagonist. In additional studies, we show that the chemoprotective action of CXCL12/CXCR4 signaling is mediated by p21-activated kinase 4 (PAK4)-dependent activation of Lim domain kinase 1 (LIMK1), and inhibition of either PAK4 or LIMK1 leads to re-sensitization of PCa cells to DTX-induced tubulin polymerization and cellular toxicity even in the presence of CXCL12. Altogether, our findings uncover a novel mechanism underlying CXCL12/CXCR4 signaling-induced PCa chemoresistance and suggest that targeting of this signaling axis or its downstream effector pathway could lead to therapeutic enhancement of DTX.
data show that low CXCR4-expressing LNCaP cells are (data not shown). Next, we treated PCa cells with DTX
CXCL12 (range between 0.2 to 1.0 pg/ml/10
we observed that PCa cells produced very low level of
benign prostate epithelial cells (Figure 1A). Moreover,
expression was detected in normal/
benign prostate epithelial (RWPE1 and 2) cell lines. An
aberrant expression of CXCR4 was observed in all PCa
cell lines, while no expression was detected in normal/

RESULTS

Activation of CXCL12/CXCR4 signaling relieves docetaxel-induced G₂/M phase cell cycle arrest

We first examined the expression of CXCR4 and its sole ligand, CXCL12, in a panel of PCa and normal/
benign prostate epithelial (RWPE1 and 2) cell lines. An aberrant expression of CXCR4 was observed in all PCa cell lines, while no expression was detected in normal/
benign prostate epithelial cells (Figure 1A). Moreover,
expression level of CXCR4 in PCa cell lines does not precisely correlate with docetaxel sensitivity, which could be due to the presence of additional resistance
mechanisms. Nonetheless, we observe that the silencing
(using specific siRNAs; Figure 1C) or inhibition (by
AMD3100) of CXCR4 leads to abrogation of CXCL12-
duced chemo-protection of CXCR4 in C4-2 and PC3 cells (Figure 1D and 1E). In next set of experiments,
examined whether activation of CXCL12/CXCR4
signaling had any effect on DTX-induced G₂/M phase
cell cycle arrest, which is an established mechanism
of chemotoxic action of docetaxel [20]. C4-2 and PC3 cells were treated with DTX alone or in presence of CXCL12
and/or AMD3100, and analyzed by flow cytometry for
their cell cycle distribution. Consistent with previous
reports [20], the data show an arrest of cells in G₂/M phase of cell cycle upon DTX treatment. We observe that
61.6% and 62.1% of DTX-treated C4-2 and PC3 cells,
respectively, were present in G₂/M phase as compared to
11.52% and 16.0% of control (vehicle treated) C4-2 and PC3 cells, respectively (Figure 2). Interestingly, our data
demonstrate that CXCL12 treatment rescued the PCa
cells from DTX-induced G₂/M mitotic arrest, and this
effect was abolished upon pretreatment of PCa cells with
AMD3100 (Figure 2). Altogether, our results suggest that
CXCL12/CXCR4 relieves DTX-induced G₂/M phase cell
cycle arrest in PCa cells and, thus may protect them from the
cytotoxic effect of DTX.

CXCL12/CXCR4 signaling counteracts
docetaxel-induced microtubule stabilization

DTX is a microtubule-stabilizing agent, which
causes mitotic arrest following binding to polymerized
tubulins and subsequent blockage of their depolymerization
[6, 21]. Therefore, we investigated if CXCL12/CXCR4
signaling had an effect on DTX-induced stabilization of
microtubulins. For this, we performed immunofluorescence
staining using an antibody against detyrosinated (glu-)
tubulin, a specific marker of polymerized tubulin [22]
on PCa cells either untreated or treated with DTX alone
or in the presence of CXCL12. Our data demonstrate
that PCa cells treated with DTX exhibit extensive
formation of microtubulins, an effect that is almost
completely abrogated in CXCL12-treated cells (Figure
3A). Furthermore, our data show that the pre-treatment
of PCa cells with a CXCR4 antagonist, AMD3100,
neutralizes the effect of CXCL12 and thus restores the
stabilization of microtubulins (Figure 3A). To further
confirm these findings, we examined the expression of
tubulin polymerization markers [glu- and ace- tubulin]
by immunoblot assay. Our data show an increased expression of both glu- and ace- tubulin in
DTX-treated cells, which is suppressed in cells co-treated with CXCL12. Similarly, the pre-treatment of PCa cells with AMD3100 overrides the suppressive effect of CXCL12 (Figure 3B). Taken together, these findings demonstrate that the activation of CXCL12/CXCR4 signaling rescues the PCa cells from DTX-induced G2/M phase cells cycle arrest by counteracting its effect on microtubule stabilization.

**Inhibition of LIMK1 abrogates the effects of CXCL12 on docetaxel sensitivity and microtubule dynamics**

We next explored the molecular mechanisms by which activation of CXCL12/CXCR4 signaling counteracts DTX-induced microtubule stabilization. Our specific focus was LIMK1, which is an important downstream effector of CXCL12/CXCR4 signaling [23] and is known to regulate the stability of microtubules through direct binding [24]. Our data show that the phosphorylation of LIMK1 is increased in a time-dependent manner following CXCL12 stimulation in PCa cells (Figure 4A). Moreover, we observe that CXCL12-induced LIMK1 phosphorylation is abrogated following pretreatment with AMD3100, thus, suggesting that this effect is mediated through CXCR4 activation (Figure 4B). In next set of experiments, we treated PCa cells with LIMKi3, a LIMK1 inhibitor; prior to the treatment with CXCL12 and/or DTX and determined its effect on overall cell survival and stability of microtubules.
Our data demonstrate that inhibition of LIMK1 neutralizes the rescue effect of CXCL12/CXCR4 signaling on DTX-induced cytotoxicity in both PCa cell lines (Figure 4C). The data also show that the inhibition of LIMK1 leads to abrogation of the counteracting effect of CXCL12/CXCR4 signaling on the DTX-induced microtubule stabilization (Figure 4D). Moreover, our data from immunoprecipitation assay reveal a direct interaction of LIMK1 with tubulin, which is decreased upon CXCL12 stimulation (Figure 4E). Together, these findings suggest that CXCL12/CXCR4 signaling impedes DTX-induced microtubule stabilization by promoting the phosphorylation-mediated dissociation of LIMK1 from microtubules.

**Figure 2: Activation of CXCL12/CXCR4 signaling relieves docetaxel-induced G₂/M cell cycle arrest.** Synchronized C4-2 and PC3 cells were treated with PBS (vehicle control), docetaxel (DTX; 20 nM) alone or in combination with AMD3100 (5 µg/mL) and/or CXCL12 (100 ng/mL). After 24 h of treatment cells were fixed, stained with propidium iodide and analyzed using flow cytometry. Data show a G₂/M phase-arrest in DTX-treated cells. CXCL12 abrogated DTX-induced G₂/M arrest, which was reversed in the cells pre-treated with AMD3100. Nocodazole (1 µM) was used as positive control.

**Activation of LIMK1 by CXCL12/CXCR4 signaling is mediated through PAK4**

Having observed a role of LIMK1 in CXCL12/CXCR4 signaling-induced DTX resistance, we next sought to identify the protein kinase involved in its phosphorylation. For this, we focused on PAK4, which is known to cause LIMK1 phosphorylation [25]. The data show that CXCL12 treatment induces the phosphorylation of PAK4 in a time-dependent manner, which is abrogated upon pre-treatment of PCa cells with a CXCR4 antagonist (AMD3100) (Figure 5A and B). Next, we investigated if PAK4 mediates the
phosphorylation of LIMK1 in response to CXCL12 treatment. For this, PAK4 was silenced using specific siRNAs prior to CXCL12 stimulation and its effect on LIMK1 phosphorylation was examined. We observed substantial silencing of PAK4 after 24 h of transfection in both the PCa cell lines treated with siPAK4 and this effect was sustained at least until 72 h of transfection (data not shown). Furthermore, we observed that the effect of CXCL12 on LIMK1 phosphorylation was abolished in PAK4-silenced PCa cells (Figure 5C). Our data also reveal that the effect of CXCL12/CXCR4 signaling on DTX-induced microtubule stabilization is nullified upon silencing of PAK4 in PCa cells (Figure 5D). Together, these data suggest that CXCL12-induced LIMK1 phosphorylation and DTX-resistance is mediated through PAK4 in PCa cells through its effect on microtubule stability.

DISCUSSION

The present study provided mechanistic support for the chemoprotective action of CXCL12/CXCR4 signaling against DTX toxicity in PCa cells. CXCL12/CXCR4 signaling-induced DTX-resistance was caused by overriding the effect of DTX on cell cycle (G2/M phase arrest) due to its counteracting effect on DTX-induced microtubule stabilization. Additionally, we observed that PAK4-mediated LIMK1 activation was important in the rescue effect of CXCL12/CXCR4 signaling on DTX toxicity.

Overexpression of CXCR4 in PCa and its association with poor patients’ survival has been well reported [13]. It has also been demonstrated that CXCL12/CXCR4 signaling plays an important role in the invasion and metastasis of PCa cells [16, 18], which ultimately
promotes DTX-resistance in PCa [7]. Moreover, a recent study performed by Domanska et al. provides direct support to our findings, in which they have also shown the chemoprotective role of CXCL12/CXCR4 signaling in PCa cells [19]. Furthermore, Hatano et al. have reported that the DTX-mediated activation of the CXCR4, ERK1/2, and c-Myc signaling loop provides survival advantage to the PCa cells in the presence of DTX [26]. Furthermore, a role of CXCR4 signaling in the ligand-independent activation of AR has also been demonstrated, which could indirectly promote DTX-resistance by promoting cell survival [27]. Our findings further add to the list of this supporting literature by providing mechanistic support for a chemoprotective role of CXCL12/CXCR4 signaling in PCa. Our data also provide support to the notion that CXCL12/CXCR4 signaling serves as the common molecular link for the metastatic and therapy-resistant nature of the PCa cells.

We observed that the treatment with CXCL12 effectively relieved DTX-induced G2/M phase cell cycle arrest, an effect that was mediated through CXCR4. DTX induced G2/M phase arrest is mediated by inhibiting the microtubule depolymerization [20]. Microtubules are polymeric cytoskeletal structures made up of...
α-β-tubulin heterodimers and play an important role in the chromosomal segregation during mitosis [21, 28]. In relation to this, our data revealed that the CXCL12-mediated activation of CXCR4 counteracted DTX-induced microtubule stabilization in PCa cells. This finding is supported by an earlier study, in which a role of CXCL12/CXCR4 signaling has been demonstrated in the regulation of microtubule dynamics of immune cells [29]. Furthermore, indirect support comes from another prior report demonstrating the induction of mitotic catastrophe in ovarian cancer cells upon inhibition of CXCR4 activation [30].

Microtubules are highly dynamic structures and their intracellular dynamicity is tightly regulated by various microtubule-associated proteins (MAPs). MAPs physically interact with microtubules and promote their stabilization and/or destabilization [31]. In our study, we observed that the phosphorylation of LIMK1 was increased in PCa cells upon CXCL12 stimulation. Furthermore, data from our immunoprecipitation study

**Figure 5: PAK4 is involved in CXCL12/CXCR4-induced LIMK1 phosphorylation.** (A) C4-2 and PC3 cells were grown in 6-well plate and treated with CXCL12 (100 ng/mL) for various time intervals (0-60 min). Thereafter, total protein was isolated, resolved, and subjected to immunoblot analysis to determine the expression of p-PAK4 and total-PAK4. (B) Cells were treated with AMD3100 (5 µg/mL) 1 h prior to the CXCL12 (100 ng/mL) treatment for 30 min and, effect on the expression of p-PAK4 and total-PAK4 examined by immunoblot assay. (C) C4-2 and PC3 cells were transfected with PAK4 targeting or non-targeting siRNAs (NT-siRNA). After 24 h of transfection, cells were treated with CXCL12 for 60 min. Post treatment, phosphorylation status of LIMK1 was determined by immunoblot assay. (D) PCa Cells were transfected with PAK4 specific- or NT -siRNAs prior to the DTX (20 nM) and CXCL12 (100 ng/mL) treatment. After 24 h of treatment total protein was isolated and expression of glu-tubulin was examined by immunoblot assay. β-actin was used as an internal control.
Figure 6: Schematic diagram for the role of CXCL12/CXCR4 and its downstream signaling in DTX resistance. CXCR4 is activated upon binding to its sole ligand, CXCL12, which then leads to PAK4 phosphorylation. Activated PAK4 phosphorylates LIMK1 causing its dissociation from microtubules (MTs) and thus enhancing dynamicity of MTs. In this state, MTs are less likely to bind to DTX and consequently less susceptible to DTX-mediated stabilization. This results in suppression of anti-mitotic effect of DTX and promotes tumor cell survival.

revealed a physical interaction between LIMK1 and tubulin, which is decreased upon CXCL12 stimulation of PCa cells. We further observed that the inhibition of LIMK1 abolished the protective effects of CXCL12/CXCR4 signaling on DTX-induced cytotoxicity as well as on microtubule stabilization. A similar finding is reported in a recent study, where inhibition of LIMK1 in HeLa cells promoted stabilization of microtubules and subsequently led to the cell death [24]. Together, these data suggest that LIMK1 acts as a MAP in its unphosphorylated state to promote the stability of microtubules.

CXCL12/CXCR4 signaling has been shown to activate a number of downstream signaling molecules, which are involved in the regulation of growth, aggressive phenotypes and therapy resistance.
in cancer cells [14–16]. Here, we observed increased phosphorylation of PAK4, a member of the p21-activated kinases (PAKs) family, in CXCL12-treated PCa cells. In concordance with our data, previously Haddad et al. 2001 have also reported the increased activity of PAK4 in CXCL12 stimulated T lymphocytes [32]. To date, the exact molecular mechanism(s) of CXCL12/CXCR4-mediated PAK4 activation is not clear. However, the roles of several proteins kinases such as Rac/Cdc42, PI3K/Akt, PKC, which are the downstream effectors of CXCL12/CXCR4 signaling, have been identified in the activation of PAK4 [33]. In a recent study, Park et al. identified an association of PAK4 activation with increased tumorigenic potential and therapy resistance of PCa cells [34]. Furthermore, our data provide strong evidence that silencing of PAK4 abrogates the effects of CXCL12 on LIMK1 phosphorylation and microtubule dynamics, and thus promotes re-sensitization of the PCa cells to DTX toxicity. PAK4 interaction with LIMK1 leading to its enhanced phosphorylation has also been reported previously [25] along with demonstration of its pathological significance in PCa [35]. In the same line, our findings now shed new light on the importance of this PAK4-LIMK1 axis and establish it as an essential mediator in the chemoprotective action of the CXCL12/CXCR4 signaling pathway.

In summary, our research findings have demonstrated an important role of the CXCL12/CXCR4 signaling axis in DTX-resistance of PCa cells through a novel mechanism (Figure 6). This new information provides additional support towards the candidacy of this signaling node as a useful target for PCa therapy.

**MATERIALS AND METHODS**

**Cell lines, antibodies and siRNAs**

All the cell lines used in this study were obtained, cultured and validated as described earlier [36]. Anti-CXCR4 (rabbit polyclonal), anti-detyrosinated-tubulin (glu-tubulin) and anti-acetylated-tubulin (ace-tubulin) antibodies (mouse monoclonal) were from Abcam (Cambridge, MA). Anti-phospho-LIMK1, anti-LIMK1, anti-PAK4 (rabbit polyclonal) and anti-phospho PAK4 (mouse monoclonal) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-α-tubulin (mouse monoclonal; for immunoprecipitation and rabbit polyclonal; for immunoblot), normal mouse IgG and all horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin antibody (mouse monoclonal) was from Sigma-Aldrich (St. Louis, MO). Non-target (ON-TARGET plus Non-targeting pool) and CXCR4 and PAK4 specific (ON-TARGET plus SMART pool) siRNAs and transfection reagent (DharmaFECT) were from Dharmacon (Lafayette, CO).

**Treatments and transfection**

PCa cells were grown in 96- and/or 6- well plates and allowed to attain 60–70% confluence. Thereafter, cells were treated with DTX (LC labs, Woburn, MA) in the presence or absence of CXCL12 (100 ng/mL) (R&D Systems, Minneapolis, MN) as indicated in the pertinent figure legends. To dissect the role of CXCR4 and LIM kinase 1, cells were pre-incubated for 1 h with AMD3100 (CXCR4 antagonist; 5 µg/mL) (Sigma-Aldrich) and LIMKi3 (LIM kinase 1 inhibitor; 25 µM) (EMD Millipore, Billerica, MA), respectively. For the knockdown of CXCR4 and PAK4, cells were cultured in 6-well plates and transiently transfected with 50 nM of non-target or target-specific siRNAs using DharmaFECT as per the manufacturer’s protocol.

**Cell growth assay**

Cells were seeded in 96 well plate (3×10³ cells/well) a day prior to treatment. Cell growth was then examined after 24 and 48 h of treatment using WST-1 assay kit (Roche Applied Science, Indianapolis, IN) and percent growth was calculated as described previously [37].

**Immunoblot analysis**

Total cells protein was extracted using NP-40 lysis buffer supplemented with protease and phosphatase inhibitors and western blotting was performed as described earlier [38]. Immunodetection was carried out using specific primary antibodies (1:1000). Thereafter, blots were incubated with respective HRP-labeled secondary antibodies (1:2500), washed and processed with ECL plus® Western Blotting detection kit (Thermo Scientific, Logan, UT) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

**Cell cycle analysis**

Cells were synchronized by two courses of incubation (for 48 h) in serum-deprived culture media with intermittent culturing (for 24 h) in serum-containing media and then various treatments were performed. After 24 h of treatment (as indicated in figure legend), cells were washed, trypsinized and fixed with 70% ethanol overnight at 4°C. Cells were then washed with cold PBS, stained using PI/RNase kit (BD Bio Sciences, San Jose, CA) and analyzed by flow-cytometry on a BD-FACS Canto™ II (BD Bio Sciences). The percentage of cell population in various phases of cell cycle was calculated using Mod Fit LT software (Verity Software House, Topsham, ME).
**Immunofluorescence assay**

PCa cells (2 × 10^3) were grown on glass-bottom FluoroDish until sub-confluence and treated with AMD3100, CXCL12 and DTX as described above. After treatment cells were fixed in ice-cold methanol, washed, blocked and incubated with glu-tubulin antibody (1:50) for 90 min at room temperature followed by washing. Cells were then incubated with FITC-conjugated goat anti-mouse secondary antibody (1:500) for 60 min. Thereafter, cells were washed, mounted with antifade Vectashield mounting medium (Vector Labs) and observed under Nikon A1rsi Confocal Microscope System (Nikon Instruments Inc, Melville, NY).

**Immunoprecipitation assay**

Total protein from the control and CXCL12-treated PCa cells was collected and estimated using DC Protein Assay Kit (Bio-Rad, Hercules, CA). Subsequently, protein lysates were incubated overnight at 4°C with anti-α-tubulin (mouse mAb) or normal mouse IgG antibodies (100:1) followed by incubation with Protein A agarose beads (Thermo Scientific, Rockford, IL) for next 2–3 h. Resulting antigen-antibody complex was centrifuged at low speed (2500 g), washed and eluted. Thereafter, whole cell lysate (input) and immunoprecipitated proteins were subjected to electrophoresis and immunoprobed for α-tubulin and LIMK1.

**Statistical analysis**

All the experiments were performed at least three times. The data obtained were expressed as ‘mean ± standard deviation’ and subjected to unpaired two tailed Student’s t-test. A value of p < 0.05 was considered as significant.

**ACKNOWLEDGEMENTS**

We would like to acknowledge the funding support from Department of Defense [PC110545 (to APS)], NIH/NCI [CA137513, CA167137, CA175772, CA185490 (to APS) and CA169829, CA186233 (to SS)] and USAMCI.

**Conflict of interest**

No potential conflict of interest to disclose.

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