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14. ABSTRACT

Metastasis is responsible for most prostate cancer (PCa) related deaths; therefore, therapies designed to prevent the spread of cancer cells and prognostic tests that better predict the outcome of patients are greatly needed. The reason(s) why prostate carcinomas (in some patients) preferentially metastasize to lymph nodes, bone, and brain is not fully understood. While our laboratory and others have shown a propensity of prostatic cancer cells to express CXCR4 and CCR9, we have recently found that PCa cell lines and prostate tissue differentially express CXCR5. The ligand for this receptor is CXCL13, which is expressed by stromal cells and an important chemokine that B cells use to navigate lymphatic endothelium. Here we show that, CCR9-CCL25 interactions mediate cell-signaling cascades involved in PCa progression. We report that CXCL13 stimulates CXCR5-dependent PI3K, ERK, FAK, Src and modest NF-kB activation for adhesion, invasion and survival. These studies on PCa cell involvement with lymphatic, vascular, and inflammatory host components will provide important and new information regarding the cellular and molecular mechanisms, following CXCL13-CXCR5 interaction, that modulate PCa bone-specific metastasis. Importantly, these studies will lead to new (CXCL13 or CXCR5) directed therapies and diagnostics to inhibit and monitor, respectively, PCa progress.

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None listed.

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INTRODUCTION:

The molecular mechanisms leading to tumor cell invasion and migration after chemokine and chemokine receptor interaction have only recently been explored. It has been shown that CXCL12 (SDF-1α) affects the growth and the spread of cancer cells through interactions with CXCR4 (1). CXCR4 is expressed by pancreatic cancer cells (as well as cell lines) and endothelial cells around tumor lesions (2). Others have shown that CXCR4 may influence migration in the peritoneum, a major route for cancer cell spread to lymph nodes (1, 3). Indeed, neutralizing CXCR4 Abs significantly impair breast cancer cell metastasis to regional lymph nodes and lung (1) and progression of non-Hodgkin's lymphoma in mice (4).

Studies have also suggested that PCa cells and other neoplasms may use the CXCL12-CXCR4 axis to spread to bone (5). Neutralizing the interaction between CXCL12 and CXCR4 with Ab significantly impaired PCa cells spread to the bone. However, these interactions alone do not explain the pattern of PCa metastasis, in its entirety, or the potential of neoplastic PCa cells to migrate and invade other tissues. We have recently shown that PCa cells, but not normal prostatic epithelial (PrEC) cells, express functional CCR9 (6). CCL25 differentially modulated the expression of MMP-1, -13, -10, -11, and -2, but not MMP-3, -7, -8, -9, -12, or -14 in PCa cells. Neutralization of CCL25-CCR9 interaction impaired the migration and invasion of the LNCaP and PC3 cell lines. We have also confirmed CXCR4 expression by PCa cells and that CXCL12-induces MMP-1, -13, -2, -9, -3, -10, -11, and -14 expression by PC3 cells, but not PrEC cells (7).

The ability of PCa cells to metastasize is not limited to mechanisms of motility and invasion. After migration, neoplastic cells must first adhere to and then penetrate the basement membrane, invade the interstitial stroma by active proteolysis, survive, and proliferate in a "new" environment to complete the metastatic process. Hence, this proposal will address an important question of *"How does CXCL13-CXCR5 interaction mediate prostate tumor cell migration, adhesion and invasion?"* This is a fundamental question to understand how we would better prevent, diagnosis, and treat PCa.

It has become evident that tight signal integration between growth factors/chemokines and their receptors, cell-cell adhesion molecules, cell-matrix receptors, and intracellular signaling proteins, are required to coordinate these properties. A number of kinases have been shown to play a role in leukocyte (as well as cancer cell) adhesion, motility and invasion. Chemokine-induced integrin clustering and affinity upregulation as well as chemotaxis by lymphocytes via F-actin polymerization and lamelli-podia formation depends on a signaling network involving Rac, Cdc42 and Rap (8-11). Rac and/or Rap activity have been linked to the enhancement of integrin-dependent adhesion (12, 13). In context, different signal transduction proteins may regulate multiple invasion events, including adhesion, de-adhesion, motility, and invasion using pathways such as the Src-ERK, FAK/PYK2-ERK, PI3K-AKT, and/or DOCK2-ELMO for Rac and/or Rap activation cascade(s).

The current dogma for chemokine receptor signaling involves Gαi protein, PI3K, and phospholipase C activation that leads to Ca^{2+} flux required for Src and FAK activation. There is no doubt that PI3K(s) can be a key signal after CXCL13-CXCR5 in PCa cells. In this regard, certain isoforms of p110 act to catalyze the formation of $PI(3,4,5)P_3$ and subsequent production of $PI(4,5)P_2$ by PTEN or $PI(3,4)P_2$ by SHIP, which leads to the activation of AKT(s) and ERK(s) to regulate cell functions including: proliferation, survival, membrane trafficking and cytoskeletal structures (14). Four related p110 catalytic subunits of class I PI3Ks have been identified in mammalian cells, which have been dived into 2 subclasses – class IA (α, β, and δ) and IIB (γ) (15). Inhibition of PI3K(s) by wortmannin prevents the upregulation of CXCL12-mediated integrin firm adhesion (16); however the precise PI3K p110 subunits responsible for CXCL13-mediated integrin firm adhesion as well as motility and invasion remain unknown.

Src activity is involved in cell invasion (and possibly motility) through its central role in the scaffolding complex of signaling molecules at the focal adhesion signaling organelle (17). Activation by auto-phosphorylation of FAK (focal adhesion kinase) induces another pathway for motility and/or invasion (17-19). PI3K(s), Src, and FAK/PYK2 can also activate ERK(s) for migration, adhesion and invasion (18, 20-23). It has been suggested that integrin avidity downregulation on leukocytes appears to be mediated by ERK (16). AKT binds to the phospholipids produced by PI3K and recruits this kinase to the plasma membrane, where it is activated by phosphorylation. Translocation of activated AKT promotes cell motility in a Rho-dependent fashion (24), which has also been shown to be involved in fibrosarcoma cell invasion via MMP-9 expression (25). Indeed, over expression of wild type or constitutively active AKT in a human pancreatic cancer cell line resulted in enhanced invasiveness as well (26).

The non-universal role of PI3K in PCa cell motility, adhesion, and motility has important implications for the development of new targets against metastatic cancers. While B cell migration seems to be mediated in a predominately PI3K-independent and DOCK2-dependent manner, other lymphocytes do not appear to require DOCK2 (27). While PI3K and (Src and FAK) events have been shown to play a role in PCa cell motility, adhesion, invasion, and survival (28, 29), the role of DOCK2 in PCa progression has not been addressed and the precise role of PI3K in CXCL13-CXCR5 mediated events remain uncertain. DOCK2 deficient lymphocytes

show no detectable chemokine-induced Rac activation and exhibit poor chemotaxis (30). However, DOCK2$^{-/-}$ mice form defined T and B cell zones suggesting a DOCK2-independent pathway for lymphocyte migration and integrin activation (31, 32). ELMO-1, -2, and -3 have been shown to interact with DOCK2 in leukocytes to mediate DOCK2 recruitment and actin polymerization. *Hence, either PI3K-dependent and/or PI3K-independent and DOCK2-dependent pathways may be involved in CXCL13-triggered motility, integrin activation, and invasion.*

BODY:

In this award, we previously mentioned that we would complete specific aim one during first 16 months of this grant cycle and specific aim two would be carried out in months 17-36. However, I moved my laboratory from Morehouse School of Medicine, Atlanta GA, where this proposal was initially awarded to the University of Louisville. It took > 8 months for my new laboratory and office to be completed, i.e., November 1, 2006. There were also delays in setting up the laboratory and getting IACUC approval from the University of Louisville; IACUC, IBC, etc. protocols were approved by the CDMRP in April 2007. Therefore, we decided to conduct some of the aim two experiments from November 1, 2006 to May 9, 2007. (***Aim Two: determine the cell-signaling cascades involved in CXCR5-dependent PC3 cell adhesion to human bone marrow, lymphatic, and/or vascular endothelial cells***).

***Methods*:**

Signaling cascades mediated by CXCL13-CXCR5: PC3 and LNCaP cell lines signaling cascades given 0 or 100 ng/ml of CXCL13 were quantified by FACE assay (fast active cell-based ELISA assay). Fast activated cell basted ELISA (FACE™) is a new method to monitor protein activation by phosphorylation and enable modification-specific analysis directly within the cell. PCa cells were cultured in 96 wells plate and stimulated with CXCL13 (100 ng/ml) for 5 and 10 minutes and un-stimulated cells were used as controls. Medium was removed and cells were fixed with 100 μl of 4% formaldehyde in phosphate buffer saline (PBS) for 20 minutes at room temperature. After fixation, cells were washed three times (5 minutes each) with 200 μl of wash buffer. After washing cells were incubated at room temperature for 20 minutes with 100 μl of quenching buffer. Next, cells were washed two times for five minutes each with 200 μl of wash buffer. After washing, cells were incubated for one house at room temperature with 100 μl of antibody blocking buffer. Next, antibody blocking buffer was removed and cells were washed with 200 μl of wash buffer and incubated at 4 °C over night with 40μl of primary antibody (anti-PI3K, -ERK, -FAK, -SRC, or -NF-kB) and antibody dilution buffer was added in negative control wells. After completion of overnight incubation with primary antibody cells were washed three times with wash buffer and incubated at room temperature for one hours with 100 μl of secondary antibody. Next, secondary antibody was removed and cells were washed three times with 200 μl of wash buffer followed by two washing with PBS and 50 μl of chemiluminescent working solution was added in each well and red using luminometer within 10 minutes. After reading chemiluminescence, cells were washed twice and taped on paper towels to remove excess of liquid and air dried at room temperature for 5 minutes. Next, 100 μl crystal violet solution was added in each well and incubated for 30 minutes at room temperature. Crystal violet was removed and 1% SDS was added in each well after three washing, and incubated on shaker for one hour at room temperature. Absorbance was measured at 595 nm by spectrophotometer. The measured OD_{595} indicate relative number of cells each well and used for normalizing kinases expression.

CXCL13-mediated αvβ3 and CXCR5 clustering. PC3 cells received no additions (NA), 1 nM of pokeweed mitogen A (PMA), 100 ng/ml of CXCL13, 100 ng/ml of pertusis toxin (PTX), and/or 5 nM of wortmanin for 0, 5, or 10 min. After staining with PE-conjugated anti-CXCR5 or FITC-conjugated anti-αvβ3 antibodies, cell were fixed using formaldehyde and cytospin for fluorescence microscopy.

***Results*:**

Signaling cascade mediated by CCL25-CCR9: Changes in the phosphorylated/active and total PI3K (total p85, phospho-Tyr p85), ERK1/2 (total or phospho-Thr202 and -Tyr204 of ERK1 as well as Thr185 and Tyr187 of human ERK2), FAK (total or phospho-Tyr 397), Src (total or phospho-Tyr418 kinases and NF-kB (total NFkB, phospho-NFkB S536 and phospho-NFkB S536) transcriptional factor were quantified following PCa cell line treatment with or without CXCL13. PCa cells (LNCaP and PC3) showed a significant increase and temporal increase in FAK, ERK, PI3K p85, Src and NFkB activity after CXCL13 treatment (Figure 1). Interestingly, the highest expression of active Src was found in PC3, compared to LNCaP cells, suggesting a role for PYK2 in the CXCL13-CXCR5 axis.

CXCL13-mediated αvβ3 and CXCR5 clustering: CXCL13 treatment of PC3 cells induced both CXCR5 and αvβ3 aggregation. Interestingly, this clustering effect is both PI3K- and Gi protein-independent, since wortmanin and pertussis toxin, respectively, did not inhibit this aggregation. Thus, alternative pathways for chemokine receptor activation and integrin clustering for adhesion and invasion are mediated by the CXCL13-CXCR5 axis.

Key Research Accomplishments:

- CXCR5 stimulation mediates PI3K p85, ERK, FAK, Src, and NF-kB activation.
- CXCL13-CXCR5 interaction promotes αvβ3 clustering in a PI3K- and Gi protein- independent manner.

Reportable Outcomes:

Abstract presented at the 2006 American Urological Association Meeting, Atlanta, GA

Conclusions:

CXCL13-CXCR5 interactions activate PI3K, ERK, FAK, SRC, and NF-kB pathways to support PCa progression.

CXCL13-treated PCa cells cause integrin clustering to allow for firm cell adhesion at the source of the CXCR5 agonist.

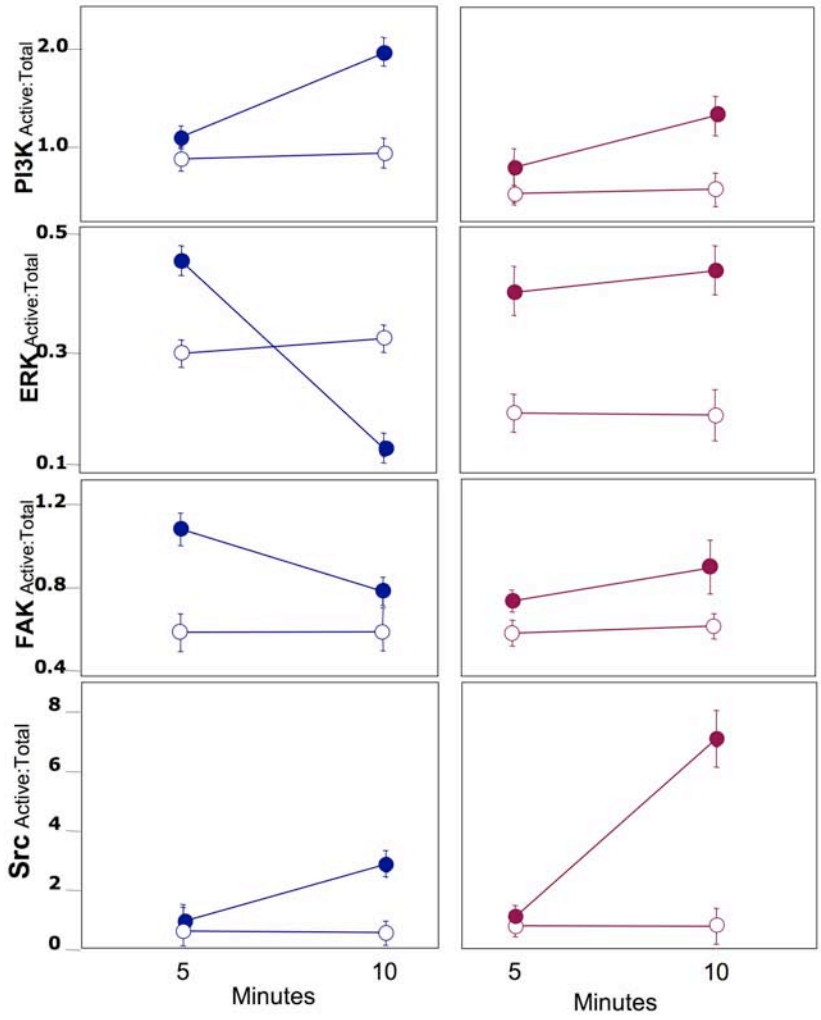
References:

1. Muller A, Homey B, Soto H*, et al.* Involvement of chemokine receptors in breast cancer metastasis.[comment]. Nature 2001;410(6824):50-6.
2. Koshiba T, Hosotani R, Miyamoto Y*, et al.* Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. Clinical Cancer Research 2000;6(9):3530-5.
3. Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR. Epithelial cancer cell migration: a role for chemokine receptors? Cancer Research 2001;61(13):4961-5.
4. Bertolini F, Dell'Agnola C, Mancuso P*, et al.* CXCR4 neutralization, a novel therapeutic approach for non-Hodgkin's lymphoma. Cancer Research 2002;62(11):3106-12.
5. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, McCauley LK. Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Research 2002;62(6):1832-7.
6. Singh S, Singh UP, Stiles JK, Grizzle WE, Lillard JW. Expression and Functional Role of CCR9 in Prostate Cancer Cell Migration and Invasion. Clin Cancer Res 2004;10(24):8743-50.
7. Singh S, Singh UP, Grizzle WE, Lillard JW. CXCL12-CXCR4 interactions modulates prostate cancer cell migration, metalloproteinase expression and invasion. Laboratory Investigation 2004;84(12):1666-76.
8. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature 2002;420(6916):629-35.
9. Laudanna C, Kim JY, Constantin G, Butcher E. Rapid leukocyte integrin activation by chemokines. Immunological Reviews 2002;186:37-46.
10. Dustin ML, Bivona TG, Philips MR. Membranes as messengers in T cell adhesion signaling. *Nature Immunology* 2004; *5*:*363*.
11. Giagulli C, Scarpini E, Ottoboni L*, et al.* RhoA and zeta PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing. Immunity 2004;*20*:25.
12. Gu Y, Filippi MD, Cancelas JA*, et al.* Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. *Science* 2003;*302*:*445-9*.
13. Shimonaka M, K. Katagiri, T. Nakayama*, et al.* Rap1 translates chemokine signals to integrin activation, cell polarization, and motility across vascular endothelium under flow. *Journal of Cell Biology* 2003;*161*:417.
14. Cantrell DA. Phosphoinositide 3-kinase signalling pathways. Journal of Cell Science 2001;114(Pt 8):1439-45.
15. Vanhaesebroeck B, Leevers SJ, Ahmadi K*, et al.* Synthesis and function of 3-phosphorylated inositol lipids. Annual Review of Biochemistry 2001;70:535-602.
16. Weber KS, G. Ostermann, A. Zernecke, A. Schroder, L. B. Klickstein, Weber. C. Dual role of H-Ras in regulation of lymphocyte function antigen-1 activity by stromal cell-derived factor-1alpha: implications for leukocyte transmigration. *Molecular Biology of the Cell* 2001;*12*:*3074*.
17. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM. Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. Journal of Cell Biology 1996;135(6 Pt 1):1633-42.
18. Braga V. The crossroads between cell-cell adhesion and motility. Nature Cell Biology 2000;2(10):E182-4.
19. Ilic D, Furuta Y, Kanazawa S*, et al.* Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 1995;377(6549):539-44.
20. Fashena SJ, Thomas SM. Signalling by adhesion receptors. Nature Cell Biology 2000;2(12):E225-9.
21. Xue L, Murray JH, Tolkovsky AM. The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons. J Biol Chem 2000;275(12):8817-24.
22. Liotta LA, Kohn EC. Angiogenesis: role of calcium-mediated signal transduction. Cancer Res 1995;55(9):1856-62.
23. Masiero L, Lapidos KA, Ambudkar I, Kohn EC. Regulation of the RhoA pathway in human endothelial cell spreading on type IV collagen: role of calcium influx. Journal of Cell Science 1999;112(Pt 19):3205-13.

24. Servant G, Weiner OD, Herzmark P, Balla T, Sedat JW, Bourne HR. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science 2000;287:1037-40.
25. Kim D, Kim S, Koh H*, et al.* Akt/PKB promotes cancer cell invasion via increased motility and metalloproteinase production. FASEB Journal 2001;15(11):1953-62.
26. Tanno S, Mitsuuchi Y, Altomare DA, Xiao GH, Testa JR. AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. Cancer Res 2001;61(2):589-93.
27. Nombela-Arrieta C, Lacalle RA, Montoya MC*, et al.* Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. Immunity 2004;21(3):429-41.
28. Bonaccorsi L, Marchiani S, Muratori M, Carloni V, Forti G, Baldi E. Singaling mechanisms that mediates invasion in prostate cancer cells. Annals of the New York Academy of Sciences 2004;1028:4693.
29. Shulby SA, Dolloff NG, Stearns ME, Meucci O, A. F. CX3CR1-fractalkine expression regulates cellular mechanisms involved in adhesion, migration, and survival of human prostate cancer cells. Cancer Res 2004;*64*:*4693*.
30. Fukui Y, Hashimoto O, Sanui T*, et al.* Hematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature 2001;412:826.
31. Nishihara H, Maeda M, Oda A*, et al.* DOCK2 associates with CrkL and regulates Rac1 in human leukemia cell lines. Blood 2002;100:3968.
32. Sanui T, Inayoshi A, Noda M*, et al.* DOCK2 regulates Rac activation and cytoskeletal reorganization through interaction with ELMO1. Blood 2003;*102*:*2948*.

Appendices:

Supporting Data:



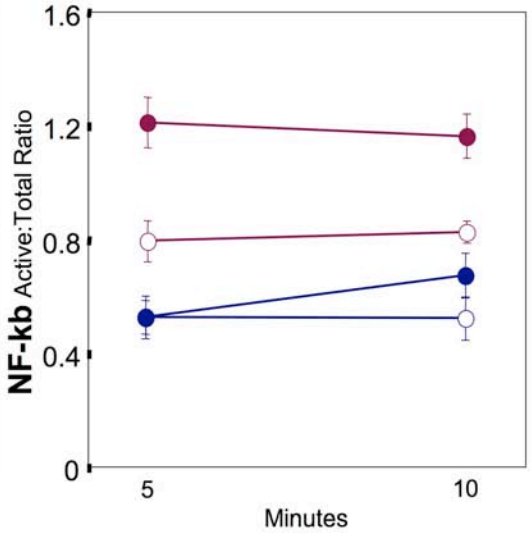


Figure 1. CXCL13-CXCR5 Cell Signaling Cascades. LNCaP (●,❍) or PC3 (●,❍) cells received no additions (❍,❍) or 100 ng/ml of CXCL13 (●,●) for 5 or 10 minutes. FACE™ assays (Active Motif, Carlsbad, CA) were used to detect both active and inactive (total) PI3K, ERK, FAK, Src kinase and NFkb proteins, 5 or 10 minutes after stimulation. Ratios of active (phosphorylated) to total proteins are presented ± SEM from 3 separate experiments performed in triplicate.

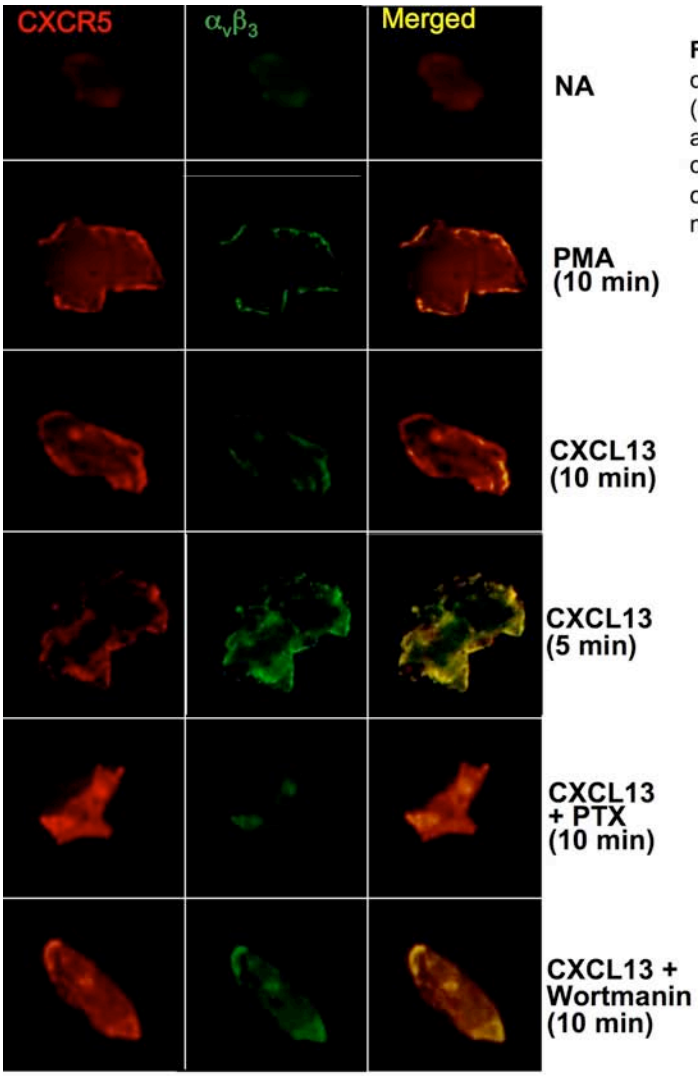


Figure 2. CXCL13-mediated $\alpha_v\beta_3$ and CXCR5 clustering. PC3 cells received no additions (NA), 1 nM of pokeweed mitogen A (PMA), 100 ng/ml of CXCL13, 100 ng/ml of pertusis toxin (PTX), and/or 5 nM of wortmanin for 0, 5, or 10 min. After staining with PE-conjugated anti-CXCR5 or FITC-conjugated anti-$\alpha_v\beta_3$ antibodies, cell were fixed using formaldehyde and cytospin for fluorescence microscopy.