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PRINCIPAL INVESTIGATOR: Michael R. Shurin, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

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Michael R. Shurin, M.D., Ph.D.

Email: shurinmr@msx.upmc.edu

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14. ABSTRACT
Specific active immunotherapy is based on the principle that malignant cells contain immunogenic sites against which an antitumor immune response can be induced. Dendritic cells (DC) that acquire antigens from tumor cells are able to induce and regulate specific antitumor immunity. However, it is still unclear why the circulating DC do not induce efficient antitumor immunity in cancer patients. While successful immunotherapy requires a functional immune system, a defect in the immune response may contribute to tumor growth. Our major hypothesis was that loss of expression of chemokine CXCL14 by prostate cancer (PCa) cells plays an important role in PCa escape from immune recognition. To gain new insights into the functional interaction between DC and neoplastic cells, we proposed to analyze the effects of PCa on the chemotaxis of human DC *in vitro* and *in vivo*. Our Specific Aims were: 1. Characterize chemotactic activity of CXCL14 towards human DC *in vitro.* 2. Examine whether restoration of CXCL14 expression in PCa increases chemoattraction of DC both *in vitro* and *in vivo.* 3. Characterize epigenetic mechanisms of regulation of CXCL14 expression in PCa. The results of our studies allow us to conclude that epigenetic regulation of chemokine expression in PCa is a principal new mechanisms of tumor escape.

15. SUBJECT TERMS
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INTRODUCTION

Significant experimental evidence has recently accumulated that chemokines play an important role in regulating both metastatic properties of malignant cells and initiation of specific antitumor immune responses (Vandercappellen et al., 2008). However, characterization of biological significance of chemokines expressing by prostate carcinoma has not been yet elucidate. Surprisingly, dendritic cells (DC), which play a vital role in the induction and maintenance of specific antitumor immune responses, do not efficiently infiltrate and home the prostate cancer tissue, a crucial step for initiation of antitumor immunity (Shurin et al, 2005). We hypothesized that prostate cancer cells lose expression of a novel dendritic cell attracting chemokine CXCL14, which is normally expressed by virtually all non-malignant tissues, including the non-malignant prostate gland. The main goal of this proposal was to determine the molecular mechanisms of the regulation of CXCL14 expression by prostate cancer cells and test whether recovery of CXCL14 expression in tumor cells might be accomplished by attraction of dendritic cells and initiation of the effective antitumor immune responses. Our second goal was to verify whether expression of CXCL14 in prostate adenocarcinoma is regulated by epigenetic mechanisms. Since, novel therapies that can correct the dendritic cell activity without compromising normal host cell-mediated immunity are desirable, identification of mechanisms regulating dendritic cell trafficking and homing in prostate cancer will be critical for the development of the next generation of comprehensive vaccine systems.

BODY

Recent research has identified chemokines responsible for neutrophil and monocyte trafficking into inflamed tissues and lymphocyte homing to lymphoid organs (Thelen and Stein, 2008). Less was known about the trafficking of DC, particularly the recruitment of DC to the tumor site (Alvarez et al., 2008). A few chemokines, including MIP-3α, MCP-1 and RANTES, have been shown to be expressed in different tumors. However they are not critical determinants of the recruitment of tumor-associated DC. Our data demonstrated that expression of a new DC chemokine CXCL14 was lost in prostate cancer, in association with reduced infiltration of tumors by DC (Shurin et al., 2005). We speculated that low levels of prostate cancer infiltration by DC may be due to a low or lost expression of CXCL14 in tumor cells, which, in turn, results in low recognition of tumor cells by antigen-presenting DC and in failure to initiate antitumor immune responses. Task 1 for the first year of support focused on *in vitro* studies aiming to characterize CXCL14-induced DC chemotaxis. Thus our OBJECTIVE 1 was to characterize the chemotactic activity of CXCL14 towards human DC *in vitro.* Specifically, we proposed to determine: (i) expression of CXCL14 chemokine in different human prostate tissues, (ii) chemotactic potential of CXCL14 for human DC, and (iii) attraction of immature versus mature DC towards CXCL14 chemokine.

<u>(1) Expression of CXCL14 chemokine in different human prostate tissues</u>

Immunohistochemical analysis of tumor-infiltrating DC in prostate cancer using CD83 marker for human DC revealed a significant reduction of DC numbers in the tumor tissues when compared to the non-malignant tissues (N=10). BPH specimens, used as an additional control, also demonstrated high levels of infiltrating DC. These data indirectly support our working hypothesis that DC migration into the prostate cancer tissues might be deficient if compared with DC migration to the non-malignant tissues. Next, we tested whether decreased infiltration of PCa by DC may be associated with decreased expression of certain DC chemokines, we measured expression of CXCL14 protein in different prostate cancer tissues by immunohistochemistry. Our results revealed that normal prostate (N=7) and BPH tissues (N=7) were strongly positive

for CXCL14, whereas prostate cancer tissues (N=10) were negative for CXCL14 staining (Fig.1).

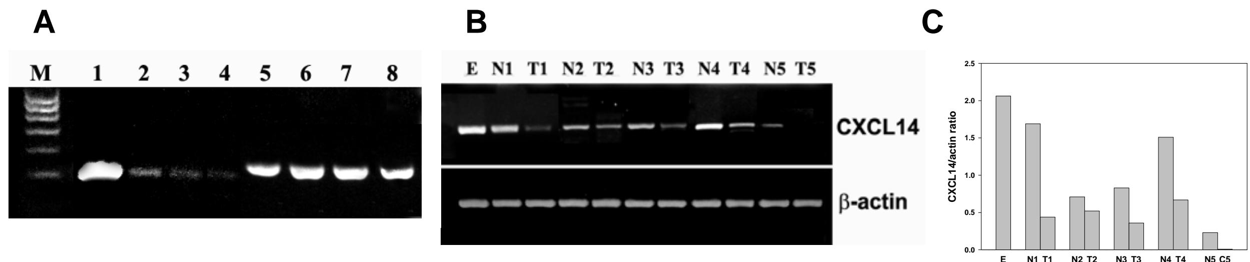
Analysis of CXCL14 mRNA expression in prostate cancer cell lines confirmed the down-regulation of CXCL14 expression in malignant cells, whereas normal prostate epithelial cells expressed high levels of CXCL14 mRNA (Fig. 2A). PCa cells obtained from primary human tumor specimens by needle microdissection technique demonstrated lower or no CXCL14 mRNA expression, whereas, adjunct normal prostate cells expressed higher levels of CXCL14 mRNA (Fig. 2B). Figure 2C demonstrates the densitometric analysis of these data shown as pair of prostate adenocarcinoma and adjunct normal prostate tissue. All evaluated specimens showed significantly reduced levels of CXCL14 expression in PCa tissues when compared to the normal adjunct areas. Thus, these data indirectly support the hypothesis that reduced infiltration of PCa by DC may be associated with an absence of expression of DC chemokines such as CXCL14.

Normal prostate | BPH | Prostate carcinoma

<u>Figure 1</u>. Loss of CXCL14 expression in prostate cancer specimens. Five-μm sections were pretreated with EDTA buffer pH 8.0 in a microwave oven, washed in PBS/Tween20, and stained with anti-CXCL14 antibodies. Biotinylated secondary antibodies were added for 30 min and positive reaction was developed using ABC technique.

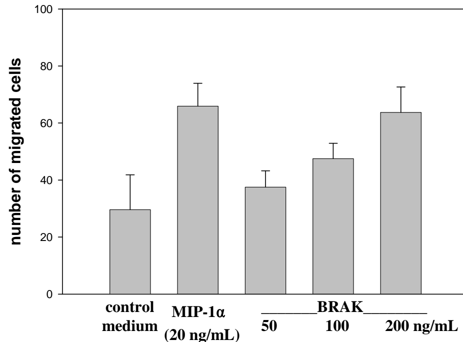
<u>(2) Chemotactic potential of CXCL14 for human DC</u>

Recently, we demonstrated that CXCL14 and CXCL14-positive HNSCC (Head and Neck Squamouse Cell Carcinoma) cell lines were potent inducers of DC chemoattraction *in vitro*, whereas CXCL14-negative HNSCC cell lines did not chemoattract DC in a Transwell assay.



<u>Figure 2</u>. (A). PCa cells display down-regulated expression of CXCL14. Total RNA was extracted from PCa cells. Lanes 2 and 6, LNCaP; Lanes 3 and 7, DU145; Lanes 4 and 8, PC3. Normal prostate epithelial cells served as a positive control (Lanes 1and 5). RT-PCR was carried out to evaluate the expression of CXCL14 (Lanes 1-4) and β-actin (Lanes 5-8) mRNA (313 bp and 323 bp).
(B). Expression of CXCL14 mRNA is down-regulated in prostatic adenocarcinoma in comparison to the benign prostatic glands. Total RNA was extracted from paired malignant glands of adenocarcinoma (T1-T5) and histologically benign prostatic glands (N1-N5). The expression of CXCL14 mRNA was assessed by RT-PCR.
(C). Densitometric analysis of expression of CXCL14 mRNA. Data are presented as pair of prostate adenocarcinoma (T1-T5) and adjunct normal prostate tissue (N1-N5). E, normal prostate epithelial cells served as control. All evaluated specimens showed significantly reduced level of CXCL14 expression in PCa tissue when compared to the normal adjunct areas.

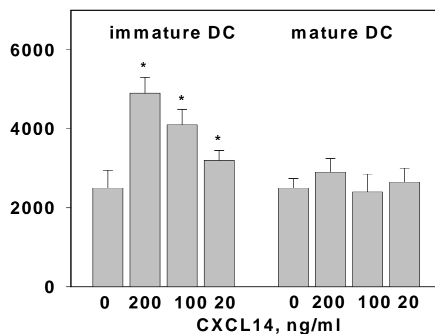
Here, to expand these observations, DC migration was evaluated by a different chemotaxis assay using microwell Boyden chambers: BW200S (Neuroprobe) and polycarbonate filters (5 µm pore size; Osmonics Inc). We determined that CXCL14 is a potent DC chemoattractant *in vitro* (Fig. 3). As shown in Figure 3, CXCL14 induced migration of monocytes-derived DC across polycarbonate filters in a dose-dependent manner. For example, the number of migrated DC stimulated by 50 ng/ml of CXCL14 was 37.5 ± 5.4, while CXCL14 at concentration of 200 ng/ml increased the DC migration to 63.7 ± 8.9 cells. The spontaneous migration of DC to a control medium was 26.1 ± 8.2 ($p<0.05$, Fig. 3).



<u>Figure 3</u>. CXCL14 chemokine dose-dependently chemoattracts human DC *in vitro*. DC were generated from monocytes, and their chemotaxis was assessed in Boyden microchambers. MIP-1α served as a positive control. Results are expressed as the mean number of migrated cells in five not-overlapping high-power microscope fields from each well. Three experiments have shown similar results. *, $p<0.05$.

<u>(3) Attraction of immature versus mature DC towards CSCL14 chemokine</u>

Next question was whether CXCL14 chemoattracts both immature and mature DC. CD14-derived DC were generated from PBMC isolated from buffy coats by Ficoll gradient centrifugation. The PBMC were further plated at 10^7 cells/well in 2 ml of AIM V medium (GIBCO) in 6-well plates. After 1-h incubation at 37ºC in a humidified 5% CO_2 atmosphere, non-adherent cells were removed and adherent monocytes were gently washed with warm AIM V medium. Adherent monocytes were cultured with recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 1000 U/ml, PeproTech) and IL-4 (1000U/ml, PeproTech) in complete RPMI medium for 7 days. Maturation of DC was stimulated by additional supplementation with 20 ng/ml of tumor necrosis factor-α (TNF-α, PeproTech) on day 6. Figure 4 demonstrates that only immature, but not mature DC, are chemoattracted by CXCL14. These data are in agreement with the general concept that immature DC are attracted to non-lymphoid tissues where a number of potent DC chemokines, including CXCL14, may be ubiquitously expressed.



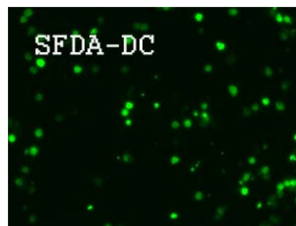
<u>Figure 4.</u> Immature (GM-CSF + IL-4, Day 6), but not mature (GM-CSF + IL-4 + TNF-α, Day 8) DC migrate towards CXCL14 chemokine in vivo. DC were generated from hematopoietic precursors in cultures and co-incubated with CXCL14 in different concentrations in Transwell chambers for 4 h. DC transmigration was evaluated by FACScan. The representative results are shown (Mean ± SEM) (N=4). *, $p<0.05$, (one-way ANOVA and Student *t*-test).

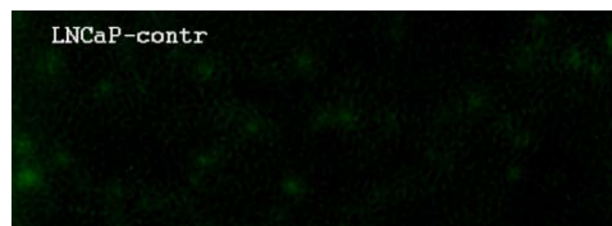
<u>(4) Functional analysis of different DC subtypes in chemotaxis assay</u>

We finalized Task 1 by comparing chemotaxis of DC generated in vitro from different precursor cells. All studies carried out during Year 1 (Task 1) utilized DC generated from CD14+ monocyte precursors cultured with GM-CSF and IL-4. Here, we prepared DC from CD34+ hematopoietic precursors generated with a cocktail of cytokines and growth factors as we described earlier (Shurin et al. 2003). DC attraction by CXCL14 was assessed in a Transwell system as we described earlier (Shurin et al. 2005). The results of these studies revealed that CD34-derived DC are attracted by CXCL14 in vitro and no differences between CD14-derived and CD34-derived DC in terms of their migration towards CXCL14 were noticed. Thus, both monocyte-derived DC and hematopoietic cell-derived DC express CXCL14 receptors and might be attracted by this chemokine in vitro.

(5) Analysis of DC migration towards CXCL14-positive and negative prostate cancer cells growing in vivo

The goal of these studies was to compare and contrast the ability of CXCL14-expressing and CXCL14-negative prostate cancer cells to chemoattract human DC in vivo. Based on the results of Task 1, LNCaP cell line was chosen and CXCL14-negative cell line, which did not attract DC in vitro in standard conditions. Using immuno-compromized SCID mice, we first demonstrated that CXCL14 cells growing in vivo did not chemoattract human DC that we generated ex vivo from CD14-precursors, labeled with green fluorescent dye PKH and injected intravenously in tumor-bearing mice (5-10 x 10^6cells/0.5 ml HBCC) 2 weeks after tumor cell inoculation or when tumor reached 30-50 mm^2 size. 24, 48 and 72 hours later, tumor mass was harvested, frozen, and cutted for the confocal microscopy evaluation. The results of two independent experiments revealed no significant migration of exogenous DC to the tumor site (Fig.5). However, if LNCaP cells were forced to secrete CXCL14 when growing in SCID mice prior to administration of fluorescent-labeled human DC, homing of DC at the tumor site was visible and significant in comparison to CXCL14-negative tumors. These results suggest that CXCL14 chemokine might serve as DC chemoattractant in vivo. Another conclusion was that loss of CXCL14 expression by prostate cancer cells might serve as a mechanism of immune escape.





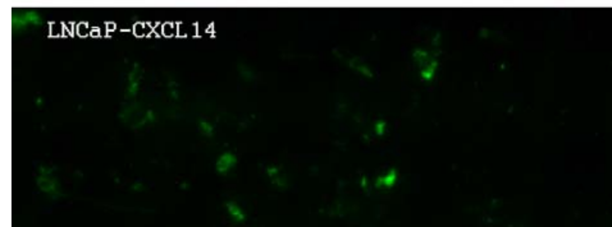


Figure 5. Chemoattraction of human DC to CXCL14-expressing PCa in vivo. DC were labeled with SFDA (left panel) and injected in SCID mice bearing CXCL14-negative LNCaP (upper right panel) or CXCL14+ LNCaP (lower right panel) cells.

(6) Analysis of human DC in human prostate carcinomas in SCID mice by immunohistochemistry

Conformation of chemoattractive role of prostate cancer-derived CXCL14 for human DC in vivo was obtained by visualizing DC in the tumor section by immunohistochemistry (IHC). DC staining was done as described earlier using CD11c and CD83 antibodies (Perez et al., 2005). As expected, expression of CXCL14 in tumor cells in vivo was associated with increased

homing of DC at the tumor bed (Fig.6). Interestingly, our preliminary data suggest that the areas of accumulation of tumor-associated DC (TADC) corresponded to the areas of CXCL14 expression inside of the tumor mass. This attractive observation requires further confirmation and verification and this analysis is in progress in the lab in collaboration with experienced pathologist. Thus, altogether, the results of Task 2a studies support our main hypothesis that CXCL14 is an important DC attracting chemokine whose expression is lost in malignant prostate tissues. Replacement of CXCL14 expression in prostate cancer cells resulted in attraction and accumulation of TADC. The next question was whether this replacement might have any therapeutic benefit.

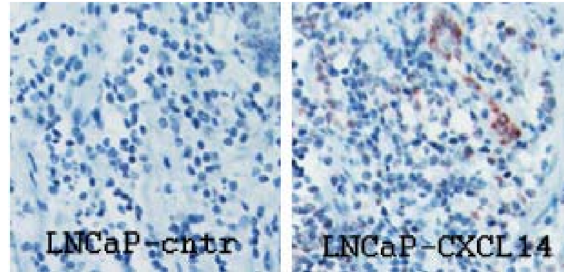
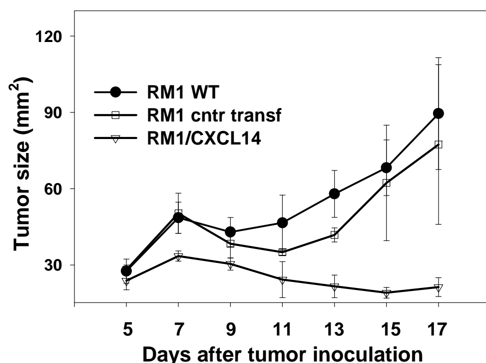


Figure 6. IHC analysis of human DC in CXCL14- and CXCL14+ LNCaP tumors growing in SCID mice.

(7) Analyze the therapeutic potential of CXCL14 expression in prostate cancer

We next tested if transfection of murine prostate cancer cell lines with the CXCL14 gene alter tumor growth in immunocompetent syngeneic mice. Initially two murine cell lines were used for these studies – RM1 and TRAMP-C2. Both cell lines were transduced with the CXCL14 gene or control empty vector and cells were cultured for selection as we described in the original application for other murine cell lines. CXCL14 positive and negative RM1 cell lines were obtained as expected. However, very slow growing TRAMP-C2 cells were not selected after three attempts due to the very low expression of CXCL14 protein, as determined by Western blot. This might be explained by their low growth rate or some additional unknown features. The results obtained with RM1 cells revealed that enforced expression of the chemokine in tumor cells was associated with marked and statistically significant inhibition of tumor growth in vivo. An important control included the analysis of treated and intact tumor cell line growth in vitro to rule out the possibility of direct inhibition of tumor longevity by CXCL14. No differences in tumor cell proliferation in vitro, assessed by MTT assay, were determined. Thus, these data suggest that CXCL14 has a potential antitumor activity in vivo.



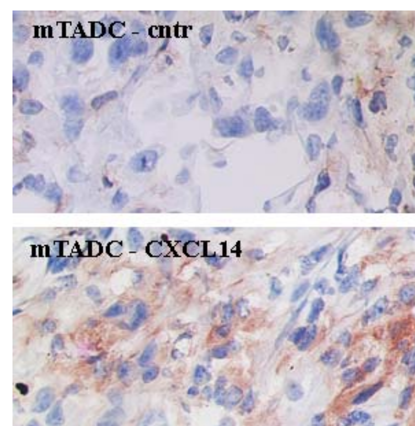


Figure 7. Induced expression of CXCL14 chemokine in prostate cancer is associated with significant inhibition or rejection of tumor growth in vivo. Immunocompetent mice received wild type, control-transfected or CXCL14-transfected RM1 tumors and tumor growth was assessed every two days (left panel). Immunohistochemical analysis of tumor tissues revealed the presence of tumor-associated DC in CXCL14-expressing tumor mass.

Next important question was whether CXCL14-mediated antitumor effect was linked to the induction of antitumor immunity. To test this, we first harvested all tumor tissues and analyzed them for the presence of tumor infiltrating leukocytes, since the appearance of these cells at the tumor site was shown to be associated with antitumor immune responses (Dunn et al, 2007). Immunohistochemical evaluation of TILs (CD4+ and CD8+ T cells) and tumor-associated dendritic cells (TADC) revealed higher levels of DC and T cells, especially CD8+ lymphocytes, in CCL14-expressing tissues when compared to intact or control-transduced tumors. These data suggest that CXCL14 may attract DC to the tumor site, where DC can engulf tumor antigen, process it, migrate to the regional lymph nodes, present tumor antigen to antigen-specific T cells and, thus, induce antitumor immune responses. Together with additional data showing the formation of tumor-specific T cells in lymph nodes of mice inoculated with CXCL14-positive tumor cells, our results support our hypothesis about important role of DC chemokine CXCL14 in immunosurveillance.

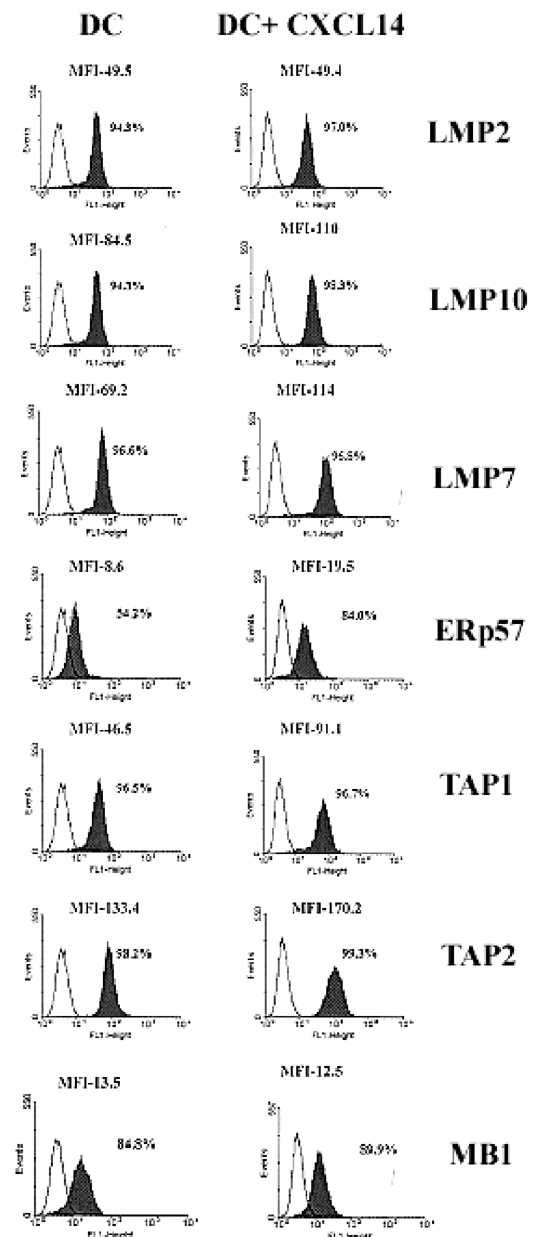


Figure 8. Up-regulation of expression of MHC class I antigen-processing machinery proteins in human DC by CXCL14. DC were treated with CXCL14 chemokine for 48 h and expression of APM components in DC was assessed by FACScan.

Based on the above information, we speculated that CXCL14 might also potentiate antigen-presenting ability of DC. To obtain more important translational data, this hypothesis was tested using human DC generated from CD14+ precursor cells. In these studies, we evaluated ability of CXCL14 to alter expression of antigen-processing machinery (APM) components in DC, which correlate with antigen-presenting function of DC. APM components in DC were assessed by flow cytometry as we described earlier (Tourkova et al., 2005). Immature DC were treated with medium alone (control) or CXCL14 for 24 hours, harvested, washed and stained for different components of APM pathways, including proteasome, chaperon, and TAP proteins. The results were expressed as the percentage of cells expressing a protein and mean fluorescence intensity (MFI) reflecting the level of expression (Figure 8). Our data demonstrated that CXCL14 was able to slightly up-regulate the percentage of positive cells with the exception of Erp57, where the percentage of positive DC was doubled. Furthermore CXCL14 significantly increase the MFI values for many APM components, suggesting its unique role in up-regulating antigen presenting capacity of DC.

In summary, these and other results of Task 2 demonstrated that restoration of CXCL14 expression in prostate cancer cells was associated with increased attraction of DC both in vitro

and in vivo, which, in turn, was accomplished by induction of antitumor immune responses in vivo. The next key question, which will be investigated in Task 3, is the mechanism responsible for low or no expression of CXCL14 chemokine in prostate cancer cells.

(8) Blockage of hypermethylation restores expression of CXCL14 in LNCaP cells *in vitro* and *in vivo* and attracts human DC to the tumor site *in vivo*

The main question of Task 3 and the chief question of the entire proposal was associate with the mechanism of regulation of CXCL14 chemokine expression in prostate adenocarcinoma. Based on our preliminary data, we hypothesized that CXCL14 expression in PCa might be regulated by epigenetic mechanism, i.e., by the hypermethylation of the gene promoter. Thus, we have initiated studies focusing on the mechanisms involved in the regulation of CXCL14 expression in PCa cells. We first tested whether the expression of CXCL14 in PCa cells might be regulated by the epigenetic mechanisms, i.e. hypermethylation. Figures 9A and 4B show that the treatment of PCa cells with 10 and 25 μM of a demethylating agent 5-aza-dC *in vitro* resulted in restoration of CXCL14 mRNA (Fig. 9A) and protein (Fig. 9B) expression. Although the levels of restored expression of CXCL14 varied in different cell lines, these data suggest a role for epigenetic regulation of chemokine expression in PCa cells.

The results of studies utilizing the chimeric tumor model revealed that administration of 5-aza-dC (100 μg/day i.p. for 7 days) in LNCaP-bearing SCID mice restored expression of functional CXCL14 in CXCL14-negative tumor cells *in vivo* (Fig. 10B) and resulted in the attraction of i.v. injected human DC to the tumor site (Fig. 10A and B). As shown in Fig.10A and B, the numbers of SFDA/SE-labeled DC and CD1a+ human DC in tumors obtained from 5-aza-dC -treated mice were significantly higher than the numbers of DC detected in control tumors,

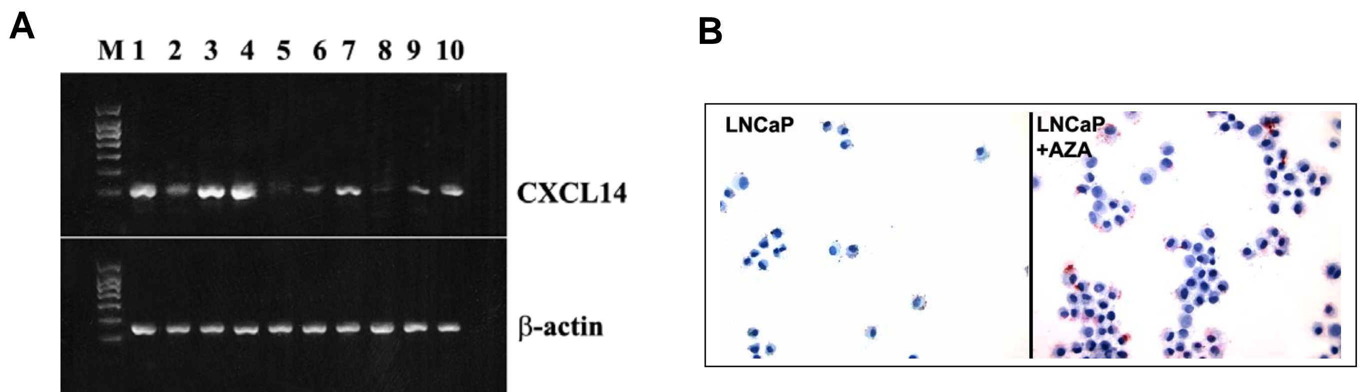
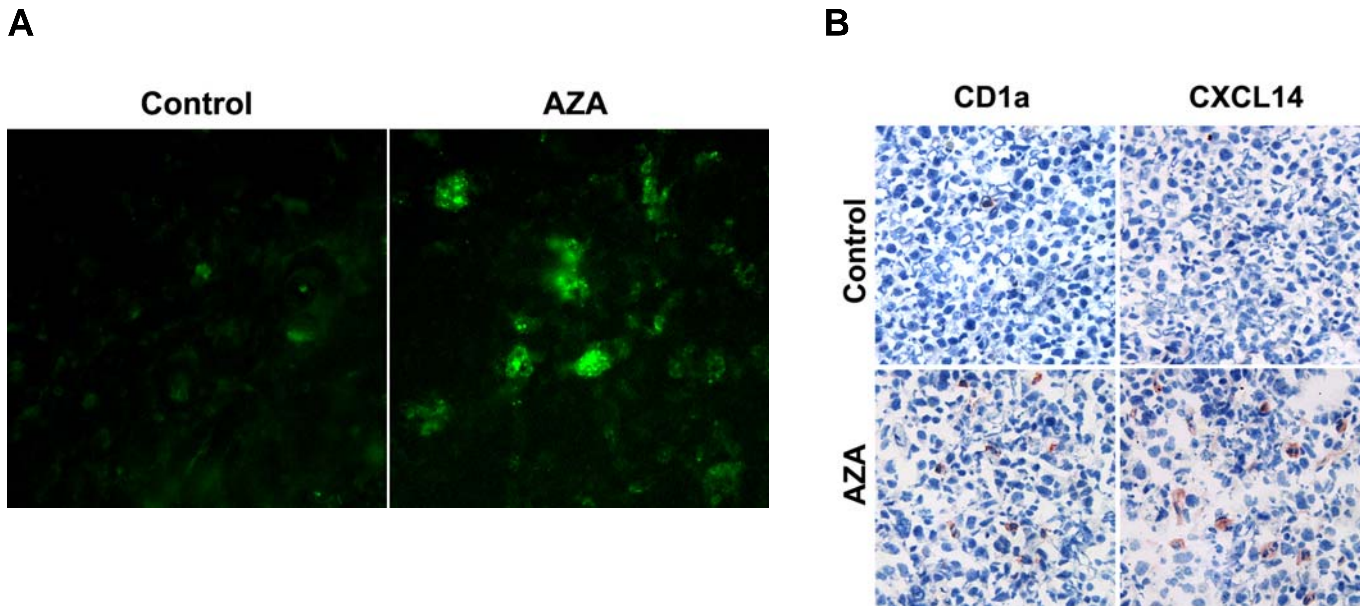


Figure 9. Epigenetic regulation of CXCL14 expression in PCa cells. (A). The methylation inhibitor 5-aza-dC dose-dependently restores expression of CXCL14 mRNA in PCa cells. Total RNA was extracted from PCa cells before and after treatment with 5-aza-dC for 6 days. RT-PCR was carried out to evaluate the expression of CXCL14 and β-actin mRNA (313 bp and 323 bp). CXCL14 mRNA was restored in all three tested PCa cell lines by the treatment with 25 μM 5-aza-dC *in vitro*. Lane M: markers; Lane 1: normal prostate epithelial cells served as a positive control; Lanes 2, 5, and 8: untreated LNCaP, DU145, and PC3 cells, respectively; Lanes 3, 6, and 9: LNCaP, DU145, and PC3 cells treated with 10 μM of 5-aza-dC, respectively; Lanes 4, 7, and 10: LNCaP, DU145, and PC3 cells treated with 25μM of 5-aza-dC, respectively. **(B). Treatment of PCa cells with demethylating agent 5-aza-dC *in vitro* resulted in restoration of CXCL14 protein expression.** Non-treated (left panel) LNCaP cells and LNCaP cells treated with a demethylating agent 5-aza-2'-deoxycytidine *in vitro* were stained with anti-CXCL14 antibodies. Positive staining was determined in LNCaP cells treated with 5-aza-dC and is shown as red color.

where only single cells were seen per power view field ($p<0.05$).

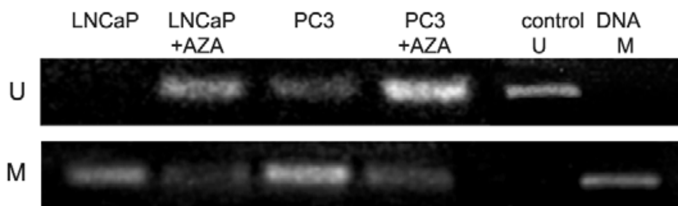
Thus, treatment of PCa cells with 5-aza-dC *in vitro* and administration of 5-aza-dC in LNCaP-bearing mice results in re-appearance of CXCL14-expression in tumor cells *in vitro* and *in vivo*. Furthermore, infiltration of LNCaP by DC was significantly higher in mice pretreated with 5-aza-dC.



<u>Figure 10.</u> Administration of 5-aza-dC in LNCaP-bearing mice results in re-appearance of CXCL14-expression in tumor cells *in vivo*. (A). Treatment of LNCaP-bearing SCID mice with demethylating agent 5-aza-dC (100µg/day×7days) is associated with attraction of fluorescent labeled human DC following their i.v. administration. **(B).** Treatment of LNCaP-bearing SCID mice with demethylating agent 5-aza-dC (100µg/day×7days) results in restoration of CXCL14 expression in the tumor mass (right panels) and chemoattraction of i.v. injected human CD1a+ DC (left panels), as was determined by immunohistochemical analysis of tumor tissues with corresponding specific antibodies. The results from a representative experiment are shown (N=2).

<u>(9) The CXCL14 gene promoter methylation in prostate cancer cells</u>

Next, we focused on the primary mechanisms of down-regulation of CXCL14 expression in PCa cells and tested whether promoter hypermethylation was involved in the loss of CXCL14 expression in tumor cells. DNA methyaltion patterns in the CpG islands of the CXCL14 gene were determined by MS-PCR. PCR primers were designed to amplify CXCL14 promoter region with CpG islands. Specifically, forward primers were constructed to differentiate methylated and unmethylated promoter sequences after bisulfite modification. Reversed primers were identical and designed to anneal outside the CpG islands. Both tested cancer cell lines (LNCaP and PC3) revealed methylation of CXCL14 promoters by MS-PCR, but the extend of methylation was different. LNCaP cells showed complete methylation, while PC3 cells showed partial methylation of promoters (Fig. 10). Treatment of both cell lines with 25 µM 5-aza-dC resulted in demethylation of CXCL14 promoters. Universal unmethylated DNA was fully unmethylated, resulting in a single band in the MS-PCR corresponding to unmethylated allele, whereas universal methylated DNA was fully methylated, respectively (Fig. 10). These data directly demonstrated hypermethylation of CpG islands in the CXCL14 gene promoter region and its reversibility.



<u>Figure 10.</u> Methylation-Specific PCR of CpG islands of the CXCL14 gene promoter. Genomic DNA isolated from LNCaP and PC3 cells was subjected to bisulfite modification, followed by MS-PCR with methylation-specific primers (**M**) and non-methylation specific primers (**U**). Lanes LNCaP and PC3: modified DNA isolated from cultured LNCaP and PC3 cell lines; Lanes LNCaP + AZA and PC3 + AZA: modified DNA isolated from LNCaP and PC3 cells treated with 25 μM of 5-aza-dC. CpGenome universal unmethylated (control DNA/U) and methylated (control DNA/M) DNA sets were used as control for MS-PCR.

(10) <u>Direct proof of the CXCL14 gene promoter methylation in prostate cancer cells</u>

To obtain direct evidence of promoter hypermetylation in the CXCL14 gene in human prostate cancer cells, we finally use sequence analysis of control, treated and modified samples prepared from different PCa cell lines. The methylation status in the promoter region of the CXCL14 gene was determined in genomic DNAs isolated from LNCaP, PC3, DU145, and normal prostate epithelium cells by chemical treatment with sodium bisulfite and subsequent methylation-specific PCR (MSP) analysis. First, DNAs were denatured to create single-stranded DNA and then modified with sodium bisulfite followed by PCR amplification using primers that are specific for the modified DNA, but do not contain any CpG sites in their sequencing. The resulting PCR product was sequenced directly (Figure 11). MSP analyses were conducted using primers for the –423 to –154 region in CXCL14 promoter. 5'CpG hypermethylation has been shown to be the mechanism underlying the aberrant expression of CXCL14 gene in PC3 cells (from –423 to –154 regions). Moderate methylation of CXCL14 gene was observed in LNCaP cells. While the CXCL14 genes in DU145 and normal prostate epithelium cells were found to be unmethylated.

Thus these studies concluded Task 3 and provide direct evidence to proof our chief hypothesis. Altogether, the results from this proposal demonstrate for the first time that expression of chemokines in prostate cancer can be regulated by hypermethylation. This mechanism of chemokine regulation has important biological significance, which was demonstrated by the role of CXCL14 in attraction of key immune cells – dendritic cells. Therefore, we showed that reversal of CXCL14 expression in prostate cancer was associated with attraction of dendritic cells to the tumor site and induction of effective antitumor immunity and, in turn, inhibition of tumor growth. Our data thus open new opportunity to alter prostate cancer microenvironment by regulating gene hypermethylation in a way that is beneficial for tumor cell recognition by the immune cells and elimination through powerful immunological mechanisms. In addition to describing novel basic mechanisms of prostate cancer progression, our results offer new approach for treatment of patients with prostate cancer, which, of course, require further evaluation in clinical trials.

File: 3130_061108_D01_Shurin_2_R1.ab1 Run Ended: Jun 11, 2008, 17:53:46 Signal G:89 A:1988 T:1392 C:2503 Comment:
Sample: Shurin_2_R1 Lane: 7 Base spacing -13.40 308 bases in 15145 scans Page 1 of 2

File: 3130_061108_H01_Shurin_4_R1.ab1 Run Ended: Jun 11, 2008, 17:53:46 Signal G:85 A:305 T:203 C:387 Comment:
Sample: Shurin_4_R1 Lane: 15 Base spacing 13.51 239 bases in 14931 scans Page 1 of 2

File: 3130_082608_H08_Shurin_11R.ab1 Run Ended: Aug 27, 2008, 3:32:50 Signal G:24 A:590 T:404 C:749 Comment:
Sample: Shurin_11R Lane: 16 Base spacing -13.40 234 bases in 16031 scans Page 1 of 2

File: 3130_090408_H05_Shurin_4_R.ab1 Run Ended: Sep 5, 2008, 2:18:14 Signal G:64 A:452 T:298 C:541 Comment:
Sample: Shurin_4_R Lane: 15 Base spacing -13.40 241 bases in 15538 scans Page 1 of 2

<u>Figure 11</u>. Sequence analysis of the CXCL14 gene promoter hypermethylation in human prostate cancer cell lines. All samples were prepared as described in the text and the results are shown in the following order from the top to the bottom: Du145, PC3, normal prostate epithelium, LNCaP cells.

KEY RESEARCH ACCOMPLISHMENTS

- Prostate carcinoma mass are low infiltrated by dendritic cells, key immunological cells responsible for initiation of antitumor immunity
- Prostate cancer cell lines can be characterized by low expression of CXCL14 chemokine protein determined by Immunohistochemical methods
- Prostate cancer cell lines can be characterized by low expression of CXCL14 chemokine mRNA assessed by RT-PCR. Together with our data showing low expression of CXCL14, these results support the hypothesis that loss expression of certain chemokines within prostate cancer bed may be associated with attraction of immune cells and, thus, deficient antitumor immunity
- Prostate cancer tissue obtained from cancer patients express low levels of CXCL14 chemokine, which confirms the conclusions shown above
- CXCL14 chemokine is a potent chemoattractant for human DC as was shown by two different methods: Transwell insert migration and Boyan microchember migration
- Only immature human DC express receptors for CXCL14 chemokine since mature DC were not chemoattractive to CXCL14 protein in vitro.
- Prostate carcinoma cells that loss expression of CXCL14 chemokine do not attract DC in vivo in the chimeric tumor model
- Induced expression of CXCL14 in prostate cancer cell lines recovered their ability to chemoattract DC in vivo in the chimeric mouse model
- Chemoattraction of exogenous fluorescent-labeled DC to CXCL14-positive tumor mass growing in mice was determined by confocal microscopy and confirmed by immunohistochemistry
- Growth of CXCL14-positive prostate cancer cells in vivo in immunocompetent animals was significantly slower that growth of CXCL14-negative tumor cells
- CXCL14-positive prostate carcinomas growing in vivo were infiltrated by DC and T lymphocytes
- Inhibited growth of CXCL14-positive tumor cells was associated with the induction of local and systemic antitumor immunity in mice
- CXCL14 chemokine is not only a potent chemoattractant for DC, but it also a potent inducer of expression of antigen processing machinery components in DC
- Inhibition of hypermethylation in prostate cancer cells resulted in recovery of CXCL14 chemokine expression on both mRNA and protein levels
- Blockage of hypermethylation in human prostate cancer cells was associated with increased infiltration of the tumor mass by dendritic cells in vivo
- Methylation of the CXCL14 gene promoter in prostate cancer caused inhibition of expression of CXCL14 chemokine.

REPORTABLE OUTCOMES

PUBLICATIONS:

1. Shurin M.R., Shurin G.V., Lokshin A., Yurkovetsky Z.R., Gutkin D.W., Chatta G.S., Zhong H., Han B., Ferris R.L. Intratumoral cytokines/chemokines/growth factors and tumor infiltrating dendritic cells: Friends or enemies? *Cancer Metastasis Reviews*, 25(3): 333-356, 2006.

2. Zhong H., Shurin M.R., Han B. Optimizing dendritic cell-based immunotherapy for cancer. *Expert Review of Vaccine*, 6(3): 333-345, 2007.
3. Aalamian-Matheis M., Chatta G.S., Shurin M.R., Huland E., Huland H., Shurin G.V. Inhibition of dendritic cell generation and function by serum from prostate cancer patients: Correlations with serum free PSA. *Adv. Exp. Med. Biol.*, 601: 173-182, 2007.
4. Song E.Y., Shurin M.R., Tourkova I.L., Chatta G.S., Gutkin D.W., Shurin G.V. Epigenetic Regulation of Dendritic Cell Chemokine CXCL14 Expression in Human Prostate Carcinoma. Resubmitted.

PRESENTATIONS:

- Shurin M.R. “Epigenetic mechanisms of immune escape”. Department of Pathology Seminar Series. Pittsburgh, PA. December 2005.
- Shurin M.R. “Tumor cells and dendritic cells: How to break the survival of the fittest”, Immunology lecture series, Pittsburgh, PA. April 2006.
- Shurin M.R., Song E.Y., Tourkova I.L., Perez L., Dutkin D.W., Shurin G.V. Epigenetic down-regulation of CXCL14 expression in tumor tissues is associated with low attraction of dendritic cells both in vitro and in vivo. Keystone Symposia on Chemokines and Chemokine Receptors, p.79. Snowbird, Utah, 2006.
- Shurin M.R. “Mechanisms and therapeutic reversal of dendritic cell dysfunction in cancer”. Molecular Targets in Cancer Therapy: Fourth Biennial Meeting “Mechanism and Therapeutic Reversal of Immune Suppression in Cancer”, Clearwater Beach, FL. January 2007.
- Shurin M.R. “Dendritic cells in the tumor microenvironment: from experiments to therapy”. The 4th Intl Conference on Tumor Microenvironment: Progression, Therapy and Prevention. Florence, Italy. March 2007.
- Shurin M.R. “Dendritic cell-based cancer vaccines”. Chest Hospital, Shanghai, China, May 2007.
- Shurin M.R. “Regulation of the dendritic cell system in cancer”. Inst. for Immunology, 2nd Medical Military Academy, Shanghai, China. May 2007.
- Shurin M.R. “How do dendritic cells mediate immune escape in cancer?” 2nd Intl. Immune-Mediated Diseases Congress, Moscow, Russia. September 2007.
- Shurin M.R., Shurin G.V., Gutkin D.W. Regulation of CXCL14 Expression and Dendritic Cell Attraction in Prostate Cancer. DoD IMPaCT meeting, Atlanta, GA, September 2007.
- “Role of dendritic cells in immune-mediated diseases”. New Advances in Diagnosis & Treatment of Immune-Mediated Diseases Conference, Pittsburgh, PA. October 2007.
- “Dendritic Cells in the Tumor Microenvironment”, Invited lecture, Hebrew University, Jerusalem, June 2008.
- “Tumor microenvironment and cancer immunotherapy”, 4th Charter Member meeting of the Intl Cancer Microenvironment Society. Safed, Israel. June 2008.
- Gutkin D., Shurin M.R. Epigenetic mechanisms of regulation of chemokine expression in prostate cancer. US CAP meeting, Boston, 2009.

CONCLUSIONS

During three years of support, we developed a marked progress toward the main goal of our proposal – understanding the mechanisms of chemokine regulation in prostate cancer. Specifically, we revealed that prostate cancer cell lines and tissues obtained from cancer

patients express low or no CXCL14 chemokine protein and mRNA, which might results in low infiltration of the tumor mass by dendritic cells. Importantly, if dendritic cells are not attracted to the prostate cancer tissues, no antitumor immune responses may be generated due to the absence of tumor antigen recognition, processing and presentation. We also revealed the role of CXCL14 chemokine in regulating DC attraction and homing in prostate cancer tissue in vivo. We demonstrated that neither murine nor human DC migrate towards CXCL14-negative tumors in vivo, but could be attracted by induced expression of CXCL14 in tumor cells. Migration of DC to the tumor site was associated with increased tumor mass infiltration by T cells and inhibition of tumor growth in syngeneic murine tumor models. Finally we showed that blocking hypermetylation in prostate cancer cells resulted in reversal of lost expression of CXCL14 chemokine and attraction of dendritic cells to the tumor site both in vitro and vivo. Using methylation specific PCR and nucleotide sequencing analyses, we proved that prostate cancer cells could loss expression of CXCL14 chemokine due to hypermethylation of the CXCL14 gene promoter. This is the first evidence of epigenetic regulation of chemokine expression in prostate cancer.

List of personnel

Michael R. Shurin, MD, PhD. Role: PI

Irina L. Tourkova, PhD. Role: Research Associate

Galina V. Shurin, PhD. Role: Co-Investigator

Lori Perez. Role: Research technician

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Appendices