Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

Biology of Human Tumors

Clinical Cancer Research

CD73 Expression Is an Independent Prognostic Factor in Prostate Cancer

Bruno G. Leclerc[1,2], Roxanne Charlebois[1,2], Guillaume Chouinard[1], Bertrand Allard[1,2], Sandra Pommey[1], Fred Saad[1], and John Stagg[1,2]

Abstract

Purpose: CD73 is an adenosine-generating ecto-enzyme that suppresses antitumor immunity in mouse models of cancer, including prostate cancer. Although high levels of CD73 are associated with poor prognosis in various types of cancer, the clinical impact of CD73 in prostate cancer remains unclear.

Experimental Design: We evaluated the prognostic value of CD73 protein expression and $CD8^+$ cell density in 285 cases of prostate cancer on tissue microarray (TMA). Normal adjacent and tumor tissues were evaluated in duplicates.

Results: Univariate and multivariate analyses revealed that high levels of CD73 in normal adjacent prostate epithelium were significantly associated with shorter biochemical recurrence (BCR)-free survival. Notably, CD73 expression in normal epithelium conferred a negative prognostic value to prostate-infiltrating $CD8^+$ cells. Surprisingly, high levels of CD73 in the tumor stroma were associated with longer BCR-free survival in univariate analysis. *In vitro* studies revealed that adenosine signaling inhibited NF-κB activity in human prostate cancer cells via A2B adenosine receptors. Consistent with these results, CD73 expression in the prostate tumor stroma negatively correlated with p65 expression in the nuclei of prostate tumor cells.

Conclusions: Our study revealed that CD73 is an independent prognostic factor in prostate cancer. Our data support a model in which CD73 expression in the prostate epithelium suppresses immunosurveillance by $CD8^+$ T cells, whereas CD73 expression in the tumor stroma reduces NF-κB signaling in tumor cells via A2B adenosine receptor signaling. CD73 expression, including in normal adjacent prostate epithelium, can thus effectively discriminate between aggressive and indolent forms of prostate cancer. *Clin Cancer Res; 22(1); 158–66. ©2015 AACR.*

Introduction

Prostate cancer is the most common non-skin cancer among men in western countries. Despite good detection methods, it is still difficult to predict the natural progression of the disease (1–3). Deciding who needs to be screened and who needs to receive treatment remains an important challenge. In addition, although localized forms of prostate cancer can be successfully treated, a significant proportion of patients having undergone interventions are at risk of relapse. Thus, considerable efforts have been made to discover new biomarkers that can accurately predict disease relapse and lead to better targeted treatments.

Recent studies suggest that biomarkers associated with antitumor immunity could complement the clinicopathologic parameters traditionally used in the prediction of cancer progression (4–7). The presence of tumor-infiltrating $CD8^+$ T cells, for instance, has been associated with a favorable prognosis in various types of cancer (6). In prostate cancer, Vesalainen and colleagues (8) and Sorrentino and colleagues (9) reported that high levels of tumor-infiltrating $CD8^+$ T cells were associated with improved disease-free survival. In contrast, Kärjä and colleagues (10) showed that high lymphocytic densities were associated with shorter recurrence-free survival. Consistent with this, Ness and colleagues (11) recently reported that high levels of tumor-infiltrating $CD8^+$ T cells were significantly associated with shorter biochemical recurrence (BCR)-free survival in a cohort of 535 patients with prostate cancer.

One factor that can influence the prognostic value of immune infiltrates is the immunosuppressive status of the tumor microenvironment (TME). T-cell function in prostate tumors can be severely attenuated, as evidenced by their inability to mediate cytotoxic function and secrete cytokines, and by the expression of exhaustion markers (12–13). One of the most recently described immunosuppressive pathways involved in tumor progression is the CD73–adenosinergic pathway (14–28). The CD73–adenosinergic pathway is driven by tissue hypoxia and soluble factors frequently found in the TME, including type I IFNs, TNFα, IL1β, TGFβ, and Wnt activators. Tumor-infiltrating immune cells are inhibited in hypoxic and extracellular adenosine-rich tissues as a result of adenosine 3′,5′-monophosphate (cAMP)-mediated signaling triggered by high affinity A2A and low-affinity A2B adenosine receptors. Notably, recent studies published by Sitkovsky and colleagues (14) demonstrated the potential of respiratory hyperoxia in reversing immunosuppressive caused by the hypoxia–adenosinergic pathway.

Several studies, including from our group, have highlighted the importance of the CD73–adenosine axis in tumor immune escape

[1]Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Institut du Cancer de Montréal, Montréal, Quebec, Canada. [2]Faculté de Pharmacie, Université de Montréal, Pavillon Jean-Coutu, Montréal, Quebec, Canada.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: John Stagg, Centre de recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), 900 Rue Saint Denis, 10ième étage, Montréal H2X0A9, QC, Canada. Phone: 514-890-8000, ext. 25170; Fax: 613-9656-1411; E-mail: john.stagg@umontreal.ca

doi: 10.1158/1078-0432.CCR-15-1181

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

Translational Relevance

One of the most recently described immunosuppressive pathways involved in tumor progression is the CD73–adenosine pathway. We here report the prognostic impact of CD73 expression in prostate cancer. Our study revealed that high levels of CD73 in normal adjacent prostate epithelium are associated with early biochemical recurrence and increased risk of prostate cancer bone metastasis. Our study also demonstrated that extracellular adenosine, the metabolite produced by CD73, suppresses NF-κB activity in human prostate cancer cells via activation of the A2B adenosine receptor. Finally, we provide evidence that CD73 expression confers a negative prognostic value to intraepithelial $CD8^+$ cells.

(15–28). Using a transgenic mouse model of prostate cancer (i.e., TRAMP mice), we recently demonstrated that CD73 deficiency is associated with a significant reduction in prostate tumor growth and increased infiltration of $CD8^+$ T cells (29). Our data thus suggested that CD73 may be associated with prostate cancer progression and decreased antitumor immunity.

The objective of this study was thus to assess the prognostic value of CD73 expression in human prostate cancer. Using a TMA composed of 285 cases of prostate cancer, we analyzed CD73 protein expression and CD8 cell density in stromal and epithelial areas of tumor tissue and matched normal adjacent tissues. Our results revealed that CD73 analysis in the stromal and epithelial compartments could significantly stratify patient outcome, that CD73 expression confers a negative prognostic value to intraepithelial $CD8^+$ cells, and that coanalysis of CD73 and CD8 expression, including in normal prostate tissue, is highly prognostic.

Materials and Methods

Patients and TMA

For construction of the tissue microarray (TMA), prostate cancer specimens were obtained from radical prostatectomy of 285 patients treated at the Centre Hospitalier de l'Université de Montréal (CHUM) between 1993 and 2006. An informed consent form was signed by every patient. Cores (0.6 mm) of benign prostate tissues adjacent to the tumor and malignant tissues were spotted in duplicates. A pathologist reviewed each core and misclassified cores were reclassified. After pathologist's review, 15 specimens were excluded from the analysis. A total of 285 patients were evaluable. Patients' clinicopathologic characteristics are detailed in Table 1.

Table 1. Patient characteristics and clinicopathologic parameters

Age at diagnosis	62 years
Overall follow-up	108 months
PSA pre-op	8.6 ng/mL
Time to biochemical recurrence	35 months
Bone metastasis	
No	260
Yes	25
Death	
All	35
From prostate cancer	13
Biochemical recurrence	
No progression	172
Progression	113
Progression at 5 years	88
Gleason score (surgery)	
≤3+3	139
3+4	94
4+3	19
≥4+4	29
Castration resistant	
No	262
Yes	23
Lymph node invasion	
No	195
Yes	10
Not available	80
Extraprostatic extensions	
No	206
Yes	79
Seminal vesicle involvement	
No	254
Yes	31

Immunohistochemistry

An automated immunohistochemistry (IHC) protocol was used for the staining of $CD8^+$ T cells. Briefly, TMA slides were deparaffinized and rehydrated with xylene and alcohols. Then, endogenous peroxidases were blocked using diluted hydrogen peroxide and an antigen retrieval step was performed in boiling target retrieval solution pH 9 (Dako). Staining with anti-CD8 (Novus Biological, 4B11, 1/40) primary antibody was performed with a BenchMark XT automated stainer (Ventana Medical System Inc.). Revelation was done with the UltraView Universal DAB Detection Kit (Ventana Medical System Inc.) and slides were stained with haematoxylin, dehydrated, and mounted. TMA slides were imaged using the automated slides scanner VS-110 (Olympus).

Immunofluorescence

Multicolor immunofluorescence (IF) was performed to simultaneously stain CD73 and cytokeratins. The same steps as described in the IHC protocol were used from deparaffinization to antigen retrieval. Then, tissue sections were blocked with a solution containing 10% of horse serum and 1% of BSA for 30 minutes at room temperature. Anti-CD73 (Abcam, clone 1D7, 1/400) primary antibody was incubated over night at 4°C. Anti-p65 (Santa Cruz Biotechnology; clone sc-8008, 1/125) primary antibody was incubated at 37°C for 60 minutes on another slide. For CD73, staining with an anti-mouse secondary antibody coupled to Alexa Fluor 594 was performed (1/250; Life Technologies). For p65, staining with an anti-mouse secondary antibody coupled to Cy5 was performed (#A10524, 1/250; Life Technologies). To block mouse antibody binding sites, slides were blocked for 60 minutes with Mouse-On-Mouse blocking reagent (1 drop in 250 μL PBS, MKB-2213; Vector Laboratories). For epithelial staining, anti-PSA (Santa Cruz Biotechnology, clone A67-B/E3, 1/100), anti-CK18 (Santa Cruz Biotechnology, clone DC-10, 1/100), and anti-CK19 (NeoMarkers MS-190-P0, clone A53-B/A2.26, 1/100) antibodies were mixed and applied to the slides followed by secondary antimouse antibody coupled to Alexa Fluor 546 (Invitrogen). Slides were incubated with 0.15% sudan black in 70% ethanol. Slides were mounted with #1.5 coverslips using Prolong Gold, which stained nuclei using DAPI. The stained TMA slides were then imaged with the scanner VS-110 (Olympus) for each color channel.

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

Staining quantification

For IHC, slides were visualized with the OlyVIA software (Olympus) and the numbers of $CD8^+$ cells were manually counted in both stromal and epithelial areas in each core. Cells included within or touching epithelial acini was considered as part of the epithelial compartment. The percentage of the core occupied by the stroma and the epithelium compartment was estimated for each core. $CD8^+$ T-cell density was then determined for each compartment (epithelium and stroma) by dividing cell count by the percentage of the core occupied by the compartment. Mean $CD8^+$ cell density of duplicates was calculated and used for analysis.

For IF, expression levels were determined by the mean fluorescence intensity (MFI) in stromal and epithelial areas in each core for CD73, and in the nuclear (DAPI+) compartment in the stroma and epithelial areas for p65. Scanned images were imported into VisioMorph software (Visiopharm) and each TMA core was separated and automatically labeled with their respective patient number and corresponding tissue (Benign/tumor). The cytokeratin staining was used to create an epithelial/stromal mask allowing for the distinction between stromal and epithelial areas in each core. The total fluorescence intensity of the CD73 staining under each mask was measured. The MFI of CD73 staining in epithelial versus stromal compartments was obtained by dividing the total CD73 fluorescence intensity measurement by the area of its respective compartment. The mean MFI values of duplicate cores for each case were calculated. The intraclass correlation (ICC) between the duplicate cores was 0.80 for CD73 and 0.68 for p65 ($P < 0.001$ Spearman).

NF-κB activity assay

PC-3 human prostate tumor cells were transfected with the 3κB-conA-Firefly plasmid as previously described (30) and Luciferase activity measured at indicated time-points using the manufacturer's instructions (Promega) with a multiplate luminometer (BMG Labtechnologies, Inc.). Where indicated, cells were treated with 5′-*N*-ethylcarboxamidoadenosine (NECA; Tocris), PSB1115 (Tocris), or BAY-60-6583 (Tocris).

Statistical analysis

GraphPad Prism software was used for mean comparison. To compare CD73 expression in tumor and begnin adjacent compartments, a Kruskal–Wallis test with Dunn multiple comparison test was used. To compare CD8 density, a one-way ANOVA followed by a Bonferroni multiple comparison test was used. SPSS software (SPSS Inc.) was used for correlation and Kaplan–Meier analyses. CD73 MFI and $CD8^+$ cells densities were correlated to patients' clinicopathologic characteristics using a two-tailed spearman correlation test. Biochemical recurrence-free survival was determined using the Kaplan–Meier estimator and statistical significance was calculated by a log-rank test. The median values of CD73 expression or $CD8^+$ cell density were used as cutoffs to generate the different group of patients compared for survival. Patients with values lower than the median were considered as having low CD73 expression or a low density of $CD8^+$ cells and vice versa.

Results

CD73 expression is associated with biochemical recurrence and bone metastasis

We analyzed CD73 protein expression by quantitative IF on 285 independent cases of prostate cancer. Expression levels in tumor tissues and normal adjacent tissues were evaluated in duplicates. Coanalysis of CD73 and cytokeratins-18/19/PSA allowed for specific assessment in epithelial and stromal compartments (Supplementary Fig. S1). Paired analysis of tumor and normal tissues revealed that CD73 was modestly, albeit significantly, higher in the tumor stroma compared with normal stroma (Supplementary Fig. S2). When the prognostic value of CD73 was evaluated, we found that high levels of CD73 (above median) in normal adjacent epithelium were significantly associated with shorter BCR-free survival (Fig. 1A) and shorter bone metastasis-free survival (Fig. 1B). In multivariate analysis (Table 2), CD73 expression in normal prostate epithelium was also an independent negative prognostic factor of BCR-free survival (HR, 2.75; $P = 0.001$). In contrast, high levels of CD73 in the TME (Fig. 1C and D), in particular in the tumor stroma, were associated with longer BCR-free survival in univariate analysis (not significant in multivariate analysis). Similar results were obtained using a receiver-operating characteristics (ROC)-based cutoff (Supplementary Fig. S3).

Prostate-infiltrating $CD8^+$ cells are associated with worse prognosis

We next investigated the prognostic value of $CD8^+$ cell density. Paired analysis of tumor and normal tissues revealed that $CD8^+$ cell densities were reduced in tumor epithelium compared with normal adjacent epithelium. In contrast, $CD8^+$ cell densities were increased in tumor stroma compared with normal stroma (Supplementary Fig. S2). When the prognostic value of $CD8^+$ cell density was evaluated, we found that high levels of $CD8^+$ cells (above median) were significantly associated with worse prognosis (Fig. 2; Supplementary Fig. S4). Total $CD8^+$ cell density (in normal adjacent and tumor tissues) was also significantly associated with shorter BCR-free survival (Fig. 3A).

Prognostic value of a combined score of CD73 and CD8 expression

In normal adjacent prostate epithelium, CD73 expression and $CD8^+$ cell density were positively correlated (Spearman ρ, 0.181; $P = 0.005$). When we evaluated the prognostic value of a combined score (Fig. 3B; Supplementary Fig. S5), we observed that patients with high levels of both CD73 and CD8 (CD73/CD8 high) had the shortest BCR-free survival (mean, 100 ± 11.9 months), whereas patients with low levels of both CD73 and CD8 (CD73/CD8 low) had the longest BCR-free survival (147 ± 11 months). Patients with high levels of both CD73 and CD8 also had shorter bone metastasis-free survival (Supplementary Fig. S5).

CD73 expression confers a negative prognostic value to $CD8^+$ cells

Considering the immunosuppressive effects of CD73-derived adenosine, we evaluated whether CD73 expression influenced the prognostic value of intraepithelial $CD8^+$ cells. We observed that high densities of $CD8^+$ cells were associated with poor prognosis only in cases where CD73 was expressed at high levels in normal adjacent prostate epithelium (Fig. 3C and D). Consistent with these results, CD73 expression in normal adjacent epithelium, but not $CD8^+$ cell density, was an independent prognostic factor in multivariate analysis (Table 2). Our data thus suggest that CD73 expression in the prostate epithelium regulates the prognostic impact of infiltrating $CD8^+$ cells.

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

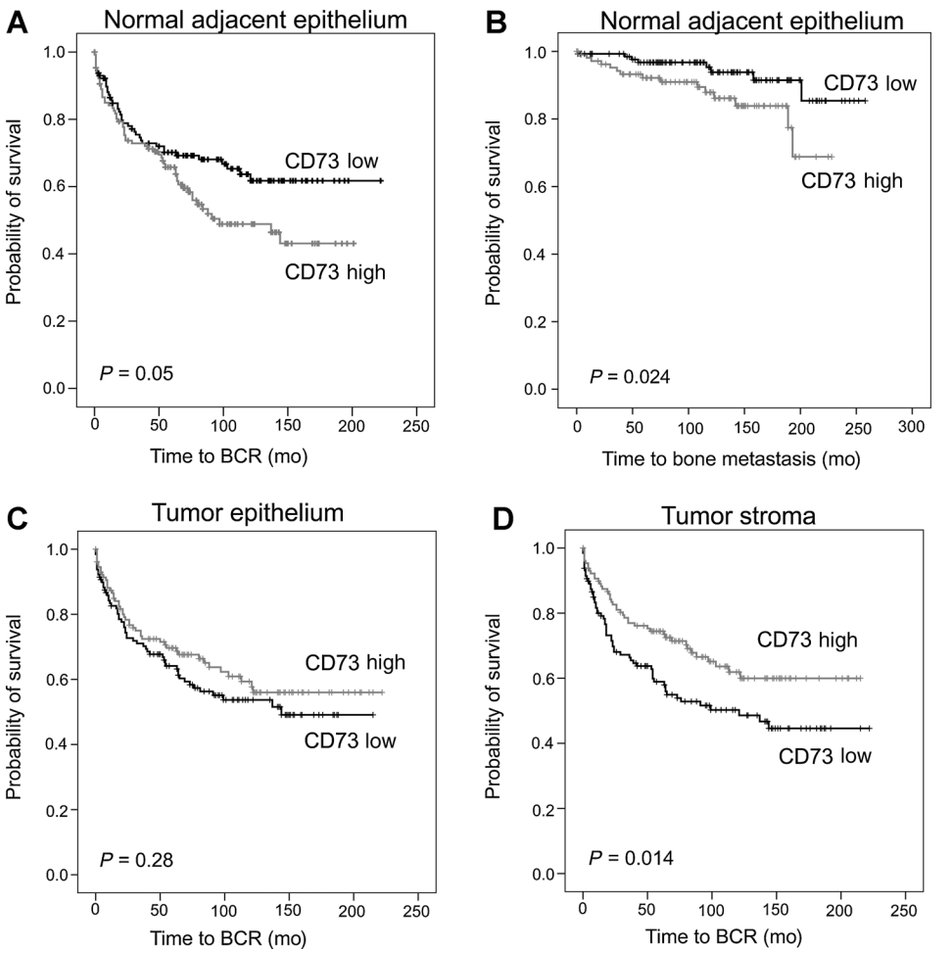

Figure 1.
Prognostic impact of CD73 in prostate cancer. CD73 protein expression in normal adjacent prostate tissue (A, B) and tumor tissue (C, D) was evaluated by IF in 285 cases of prostate cancer on TMA. Correlation between CD73 expression levels (above or below median) and BCR-free survival (A, C, D) or bone metastasis–free survival (B) was evaluated by log-rank test (*P* values are shown).

CD73 expression negatively correlates with NF-κB activity

Consistent with previous studies (31), we observed a strong positive correlation between CD73 gene expression and several TGFβ genes in human prostate tumors (Supplementary Fig. S6). We next investigated the potential mechanism by which CD73 in the TME negative regulates prostate cancer progression (Fig. 1C and D). We hypothesized that CD73-derived adenosine could be associated with decreased NF-κB activity in prostate tumor cells. NF-κB has been shown to promote prostate tumor cell survival, proliferation, and invasiveness, and to be associated with poor prognosis in patients with prostate cancer (32). Interestingly, extracellular adenosine can alter NF-κB signaling via activation of adenosine receptors (33–35). To test our hypothesis, we first assessed whether CD73 expression levels negatively correlated with NF-κB activity using nuclear p65 expression. As shown in Fig. 4A, CD73 expression levels in the prostate tumor stroma negatively correlated with nuclear p65 expression in prostate tumor cells. We next measured NF-κB activity in PC-3 human prostate tumor cells treated with the pan-adenosine receptor agonist NECA. As shown in Fig. 4B, NECA significantly inhibited endogenous NF-κB activity in human prostate tumor cells. Real-time PCR analysis revealed high levels of A2B adenosine receptor expression in PC-3 tumor cells (Fig. 4C). To assess the role of A2B adenosine receptor in NF-κB regulation, PC-3 cells were treated with a selective A2B receptor agonist (BAY-60-6583) or an A2B receptor antagonist (PSB-1115). As shown in Fig. 4B, treatment with the selective A2B receptor agonist significantly suppressed NF-κB activity, whereas treatment with the selective A2B receptor

Table 2. Multivariate Cox regression analysis of CD73 (A) or CD8 and CD73 (B) for BCR-free survival

Factor	HR (95% CI)	*P*
A		
pT stage	0.897 (0.294–2.737)	0.849
Gleason score	1.253 (0.995–1.578)	0.056
PSA pre-op (>10 ng/mL)	1.615 (0.956–2.728)	0.073
Lymph node involvement	1.869 (0.778–4.489)	0.162
Extraprostatic extension	3.364 (1.006–11.243)	0.049
Positive surgical margin	1.869 (1.054–3.311)	0.032
CD73 normal adjacent epith	2.753 (1.483–5.109)	0.001
CD73 normal adjacent stroma	0.584 (0.316–1.077)	0.085
CD73 tumor epith	1.018 (0.534–1.941)	0.957
CD73 tumor stroma	1.002 (0.526–1.910)	0.995
B		
pT stage	1.052 (0.346–3.199)	0.929
Gleason score	1.210 (0.970–1.509)	0.091
PSA pre-op (>10 ng/mL)	1.588 (0.943–2.674)	0.082
Lymph node involvement	1.661 (0.680–4.055)	0.266
Extraprostatic extension	2.708 (0.824–8.899)	0.101
Positive surgical margin	1.734 (1.003–2.999)	0.049
CD73 normal adjacent epith	1.654 (1.000–2.735)	0.050
CD8 normal adjacent epith	1.356 (0.830–2.218)	0.224

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

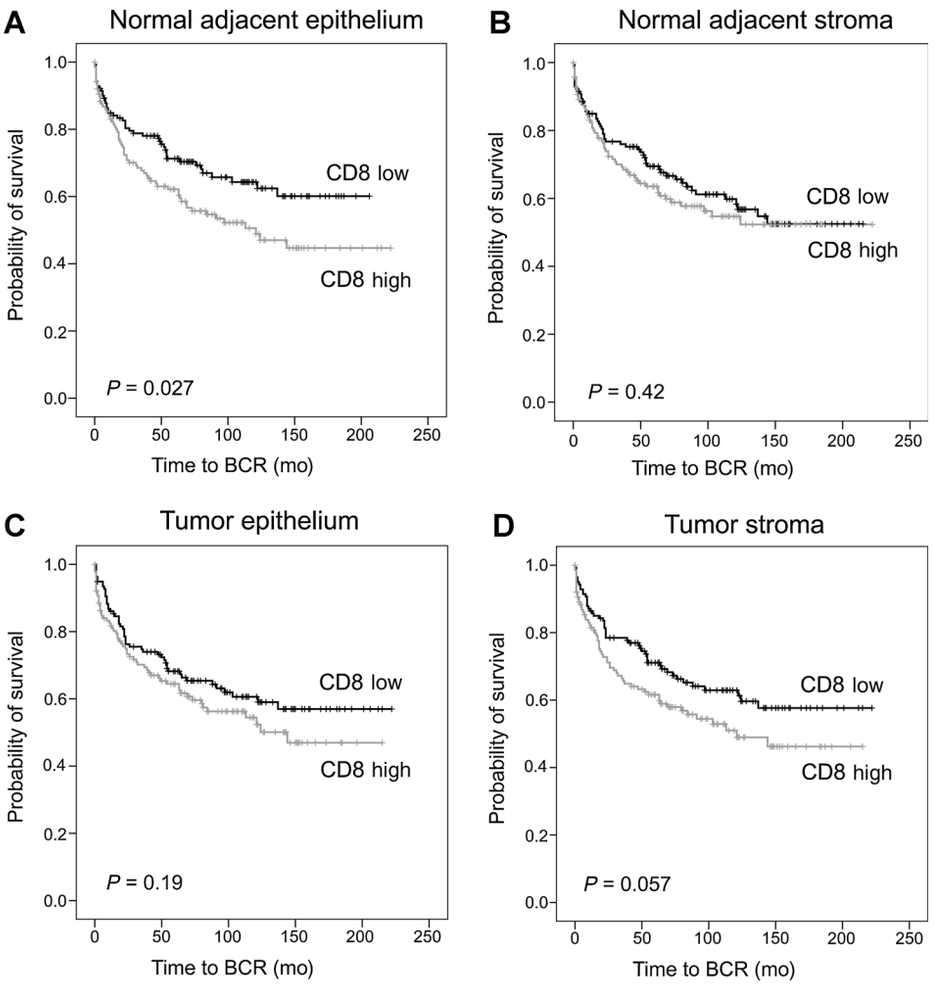

Figure 2.
Prognostic impact of $CD8^+$ cell density in prostate cancer. $CD8^+$ cell density in normal prostate adjacent tissue (A, B) and tumor tissue (C, D) was evaluated by IHC in 285 cases of prostate cancer on TMA. Hematoxylin counterstaining allowed for specific assessment in epithelial (A, C) and stromal (B, D) compartments. Correlation between $CD8^+$ cell density (above or below median) and BCR-free survival was evaluated by log-rank test (*P* values are shown).

antagonist rescued the suppressive effect of NECA on NF-κB activity. Take together, our data demonstrated that extracellular adenosine inhibits NF-κB activity in human prostate cancer cells via A2B adenosine receptor.

Discussion

Given the heterogeneity of prostate cancer and concerns regarding overtreatment, significant effort is currently being deployed to identify biomarkers capable of distinguishing aggressive and indolent forms of the disease. The objective is to develop improved classification methods that will help its clinical management.

Biomarkers associated with immune infiltrates (e.g., CD8 and CD3) and immunosuppressive molecules (e.g., PD-L1) have recently emerged as highly promising in various types of cancer (4–7). Immune biomarkers also have the potential to identify cancer patients most likely to respond to immunotherapy (7). In several types of cancer, the presence of $CD8^+$ T cells is associated with better clinical outcomes. In prostate cancer, however, the prognostic value of tumor-infiltrating lymphocytes has long been unclear. Earlier studies reported that prostate tumor-infiltrating lymphocytes were either not associated with prognosis or associated with better outcomes (8–10). Recent studies in large cohorts have however challenged this notion. In their analysis of 535 cases on TMA, Ness and colleagues (11) demonstrated that high densities of $CD8^+$ T cells (in tumor epithelium only or in total tumor tissues) were independent negative prognostic factors of biochemical recurrence (HR, 1.45 and 1.57, respectively).

Our results support the findings of Ness and colleagues (11). Indeed, we also demonstrated that high levels tumor-infiltrating $CD8^+$ T cells were associated with shorter BCR-free survival. In addition, we demonstrated that high levels of $CD8^+$ T cells in normal adjacent prostatic epithelium were also associated with poor prognosis. As suggested by Ness and colleagues (11), one of the potential explanations is that $CD8^+$ T cells might display immunosuppressive functions in the prostate. Several studies have indeed described the presence of immunosuppressive $CD8^+$ T cells in tumors, including in prostate tumors (36–38). The mechanisms of immunosuppression by prostate tumor-infiltrating $CD8^+$ T cells include the production of extracellular adenosine by ecto-nucleotidases (39), CTLA-4 expression and production of IL35 (38). Interestingly, the prostate microenvironment has been implicated in the conversion of effector $CD8^+$ T cells into immunosuppressive $CD8^+$ T cells (40). Although the underlying mechanism of such conversion remains undefined, we

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

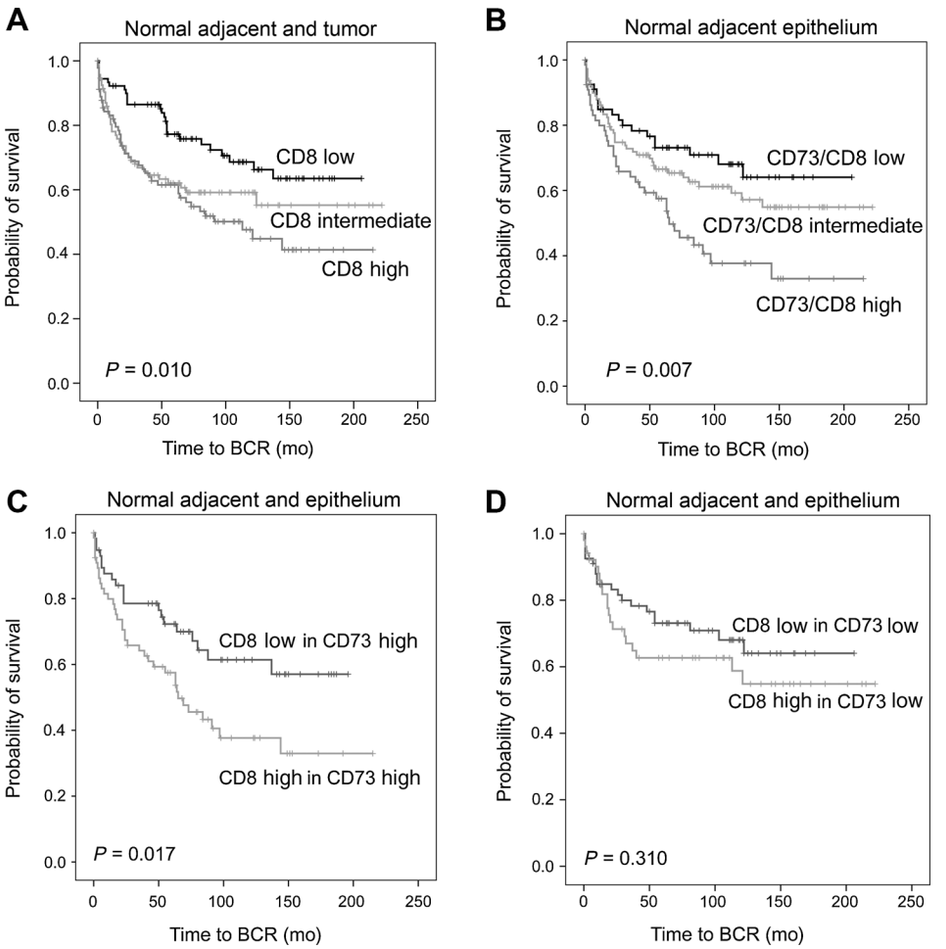

Figure 3.
Prognostic impact of total $CD8^+$ cell density and a combined CD73/CD8 score. A, correlation between total $CD8^+$ cell density (in tertiles) and BCR-free survival was evaluated by log-rank test (*P* value shown). B, correlation between a combined CD73/CD8 score (based on median expression) and BCR-free survival was evaluated by log-rank test (*P* value shown). C and D, correlation between $CD8^+$ cell density (above or below median) and BCR-free survival was evaluated in CD73-high (above median) and CD73-low (below median) cases by the log-rank test (*P* values are shown).

believe CD73 expression and extracellular adenosine may be involved. This is supported by our observation that high levels of CD73 in the prostatic epithelium confers a negative prognosis to $CD8^+$ cells, and by the fact that extracellular adenosine can convert $CD4^+$ T cells into immunosuppressive cells (41–42). Clearly, further work is needed to decipher the potential role of the CD73–adenosinergic pathway in the generation and function of $CD8^+$ T suppressor cells.

Our multivariate analysis highlighted the negative prognostic impact of CD73 expression in normal adjacent prostate epithelium, exceeding the prognostic value of pathologic staging, Gleason score or preoperative PSA levels. Consistent with our previous studies in TRAMP transgenic mice (29), CD73 expression in normal prostatic epithelium negatively influenced the clinical impact of prostate-infiltrating $CD8^+$ T cells.

Because CD73 can be induced by TGFβ (31), we investigated the potential association between CD73 and TGFβ genes in human prostate tumors. Our gene expression analysis revealed a strong positive correlation between CD73 and several TGFβ genes, suggesting coregulation. TGFβ is classically defined as a tumor suppressor in the early stages of carcinogenesis and a tumor promoter in established cancers. In prostate cancer, TGFβ forms a growth barrier in response to PTEN deletion (43). Paradoxically, it also promotes the expression of prometastatic genes and the development of bone metastases (44). TGFβ is also important in the normal prostate, where cellular interactions between luminal epithelial cells and stromal cells determine the amount of active TGFβ, in order to maintain tissue homeostasis (45). It is our assertion that CD73 expression in normal prostatic epithelium may also be regulated by TGFβ. Because androgen ablation is associated with an increased production of TGFβ in the prostate, CD73 may also be induced by androgen deprivation therapy (ADT). This possibility merits further investigation, especially considering that ADT is known to increase prostatic T-cell infiltration without affecting clinical outcomes (46–47).

In contrast to the poor prognosis associated with CD73 expression in normal adjacent prostatic epithelium, our data revealed that high levels of CD73 in prostate tumors resulting for expression by stromal cells were associated with better prognosis. Our correlative clinical data and *in vitro* studies support the notion that high levels of CD73 in the prostate TME contribute to reducing NF-κB activation in prostate cancer cells. The NF-κB complex is a key transcription factor involved in the regulation of proinflammatory genes, aberrantly activated in prostate cancer (30). Gannon and colleagues demonstrated that nuclear p65 expression, a surrogate of NF-κB activity, could predict early BCR in prostate cancer patients (32). Our current study demonstrated that extracellular adenosine produced by CD73 suppresses NF-κB activity in human prostate cancer cells via activation of the A2B adenosine receptor.

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

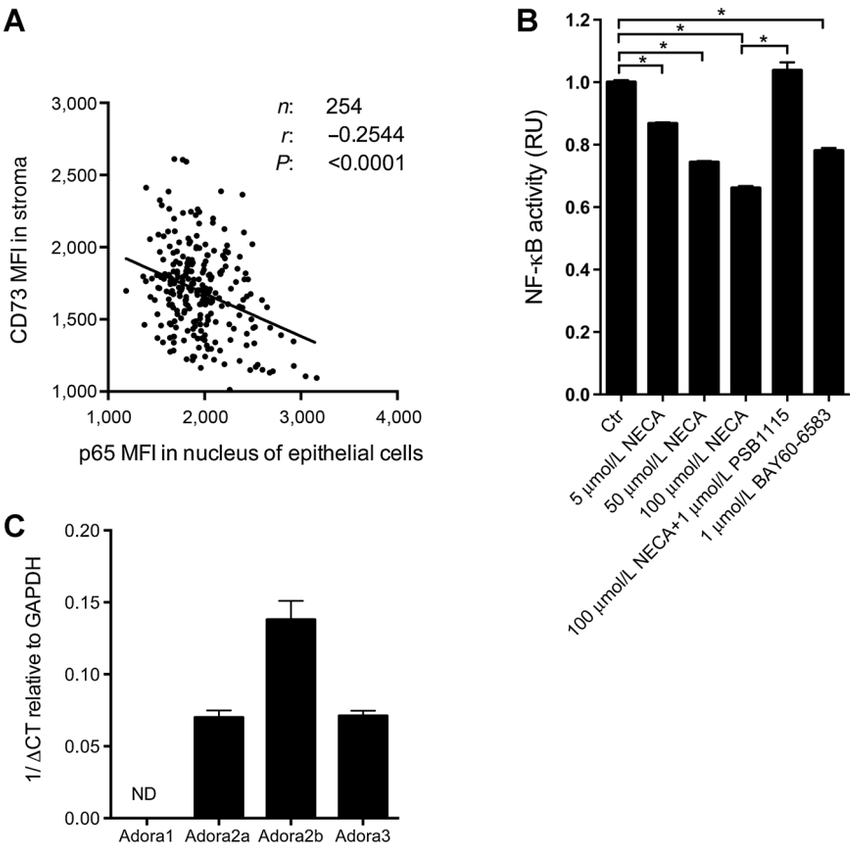

Figure 4.
Adenosinergic signaling via A2B adenosine receptor inhibits NF-κB activity in human prostate cancer cells. A, correlation between CD73 expression levels in prostate tumor stroma and nuclear p65 expression in prostate tumor cells on TMA ($n = 254$) was evaluated (Spearman ρ and P value are shown). B, PC-3 prostate tumor cells were transfected to express the 3κB-conA-Firefly NF-κB reporter plasmid. Cells were then treated with NECA, BAY-60-6583, or PSB-1115, and luciferase activity was measured 32 hours later (mean relative units ± SE of triplicates are shown; *, $P < 0.001$ by the Student t test). C, real-time PCR analysis of *ADORA1*, *ADORA2A*, *ADOAR2B*, and *ADORA3* mRNA expression in PC-3 human prostate tumor cells (relative to *GAPDH*).

Another mechanism by which CD73 and extracellular adenosine may contribute to improve clinical outcomes in prostate cancer is through tumor cell apoptosis. As described above, CD73 expression is strongly correlated with TGFβ in prostate tumors. An important function of TGFβ is to maintain genomic stability via activation of DNA damage responses (DDR; ref. 48). Interestingly, a recent study revealed that p53 activation can induce the expression of A2B adenosine receptor and stimulation of A2B receptor can amplify p53-induced apoptosis (49). Thus, in addition to its inhibitory effect on NF-κB signaling, extracellular adenosine may eliminate prostate tumor cells via p53 activation.

Because of its short-range paracrine effects, we hypothesize that extracellular adenosine generated by CD73 in the normal prostatic epithelium suppresses the effector function of infiltrating $CD8^+$ T cells without affecting distant tumor cells. In contrast, adenosine produced by CD73 in the tumor stroma may suppress both immune cells and tumor cells, thus favoring better clinical outcome (Supplementary Fig. S7). Whether the prognostic impact of CD73 expression would be different in metastatic castration-resistant prostate tumors or in the context of immunotherapy remains unknown.

High levels of CD73 in tumors have generally been associated with poor clinical outcomes (16), including in melanoma, colorectal cancer, triple negative breast cancer (TNBC), and high-grade serous (HGS) ovarian cancer (50). Our current study in prostate cancer sheds new light and highlights the complexity of the adenosinergic pathway in cancer. The prognostic impact of CD73 and extracellular adenosine levels probably depends on several factors, such as tumor immunogenicity, activation of specific oncogenic pathways and loss-of-function of tumor suppressor mechanisms. Given the role of extracellular adenosine in regulating p53 activity, we hypothesize that targeted inhibition of the CD73–adenosinergic pathway will be most effective in cancers with p53 loss-of-function. In support of this, tumoral CD73 expression has been associated with poor prognosis essentially in cancers harboring frequent p53 loss-of-function, such as TNBC (22) and HGS ovarian cancer (50).

In conclusion, our study demonstrated that CD73 protein expression in normal adjacent prostate epithelium is an independent negative prognostic factor of BCR-free survival in prostate cancer. Our study also provides evidence that prostate-infiltrating $CD8^+$ T cells are associated with worse prognosis in prostate cancer, in support of recent studies (11). We propose that CD73 expression in the prostate epithelium suppresses immunosurveillance by $CD8^+$ T cells and converts them into tumor-promoting cells. Future studies are required to better define the impact of CD73 expression in various cell types and adenosine signaling on prostate cancer outcomes.

Disclosure of Potential Conflicts of Interest

J. Stagg reports receiving a commercial research grant from, has ownership interest in, and is a consultant/advisory board member for Surface Oncology. No potential conflicts of interest were disclosed by the other authors.

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

Authors' Contributions

Conception and design: F. Saad, J. Stagg
Development of methodology: B.G. Leclerc, R. Charlebois, G. Chouinard, S. Pommey
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.G. Leclerc, R. Charlebois, F. Saad
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.G. Leclerc, R. Charlebois, G. Chouinard, B. Allard, F. Saad
Writing, review, and/or revision of the manuscript: B.G. Leclerc, B. Allard, F. Saad, J. Stagg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.G. Leclerc, R. Charlebois, F. Saad
Study supervision: J. Stagg

Grant Support

This work was supported by operating grants to J. Stagg from Prostate Cancer Canada and The Terry Fox Research Institute. B.G. Leclerc was supported by the Canadian Institutes of Health Research Doctoral Award program. J. Stagg acknowledges support from the Institut du Cancer de Montréal "Rapatriement de Cerveaux" program and support from the Fonds de Recherche Québec–Santé.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 20, 2015; revised July 22, 2015; accepted July 26, 2015; published OnlineFirst August 7, 2015.

References

1. Saad F, Miller K. Current and emerging immunotherapies for castration-resistant prostate cancer. Urology 2015;85:976–86.
2. Basch E, Loblaw DA, Oliver TK, Carducci M, Chen RC, Frame JN, et al. Systemic therapy in men with metastatic castration-resistant prostate cancer: American Society of Clinical Oncology and Cancer Care Ontario clinical practice guideline. J Clin Oncol 2014;32:3436–48.
3. Gartrell BA, Saad F. Managing bone metastases and reducing skeletal related events in prostate cancer. Nat Rev Clin Oncol 2014;11:335–45.
4. Jiang L, Zhao Z, Jiang S, Lin Y, Yang H, Xie Z, et al. Immunological markers predict the prognosis of patients with squamous non-small cell lung cancer. Immunol Res 2015;62:316–24.
5. Giraldo N, Becht E, Pages F, Skliris GP, Verkarre V, Vano Y, et al. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. Clin Cancer Res 2015;21:3031–40.
6. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 2013;39:11–26.
7. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, et al. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol 2015;16:375–84.
8. Vesalainen S, Lipponen P, Talja M, Syrjänen K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. Eur J Cancer 1994;30A:1797–803.
9. Sorrentino C, Musiani P, Pompa P, Cipollone G, Di Carlo E. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. Clin Cancer Res 2011;17:1571–81.
10. Kärjä V, Aaltomaa S, Lipponen P, Isotalo T, Talja M, Mokka R. Tumour-infiltrating lymphocytes: a prognostic factor of PSA-free survival in patients with local prostate carcinoma treated by radical prostatectomy. Anticancer Res 2005;25:4435–8.
11. Ness N, Andersen S, Valkov A, Nordby Y, Donnem T, Al-Saad S, et al. Infiltration of $CD8^+$ lymphocytes is an independent prognostic factor of biochemical failure-free survival in prostate cancer. Prostate 2014;74:1452–61.
12. Bishop JL, Sio A, Angeles A, Roberts ME, Azad AA, Chi KN, et al. PD-L1 is highly expressed in Enzalutamide resistant prostate cancer. Oncotarget 2015;6:234–42.
13. Schweizer MT, Drake CG. Immunotherapy for prostate cancer: recent developments and future challenges. Cancer Metastasis Rev 2014;33:641–55.
14. Hatfield SM, Kjaergaard J, Lukashev D, Schreiber TH, Belikoff B, Abbott R, et al. Immunological mechanisms of the antitumor effects of supplemental oxygenation. Sci Transl Med 2015;7:277.
15. Young A, Mittal D, Stagg J, Smyth MJ. Targeting cancer-derived adenosine: new therapeutic approaches. Cancer Discov 2014;4:879–88.
16. Allard B, Turcotte M, Stagg J. Targeting CD73 and downstream adenosine receptor signaling in triple-negative breast cancer. Expert Opin Ther Targets 2014;18:863–8.
17. Young A, Mittal D, Stagg J, Smyth MJ. Antimetastatic effects of blocking PD-1 and the adenosine A2A receptor. Cancer Res 2014;74:3652–8.
18. Allard B, Turcotte M, Spring K, Pommey S, Royal I, Stagg J. Anti-CD73 therapy impairs tumor angiogenesis. Int J Cancer 2014;134:1466–73.
19. Beavis PA, Milenkovski N, Stagg J, Smyth MJ, Darcy PK. A2A blockade enhances anti- metastatic immune responses. Oncoimmunology 2013;2:26705.
20. Allard B, Pommey S, Smyth MJ, Stagg J. Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Clin Cancer Res 2013;19:5626–35.
21. Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, et al. Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. Proc Natl Acad Sci U S A 2013;110:14711–6.
22. Loi S, Pommey S, Haibe-Kains B, Beavis PA, Darcy PK, Smyth MJ, et al. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. Proc Natl Acad Sci U S A 2013;110:11091–6.
23. Stagg J, Allard B. Immunotherapeutic approaches in triple-negative breast cancer: latest research and clinical prospects. Ther Adv Med Oncol 2013;5:169–81.
24. Beavis PA, Stagg J, Darcy PK, Smyth MJ. CD73: a potent suppressor of antitumor immune responses. Trends Immunol 2012;33:231–7.
25. Stagg J. The double-edge sword effect of anti-CD73 cancer therapy. Oncoimmunology 2012;1:217–218.
26. Stagg J, Thompson LF, Dwyer KM. Ectonucleotidases in cancer and inflammation. J Biomed Biotechnol 2012;2012:951423.
27. Allard B, Turcotte M, Stagg J. CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth. J Biomed Biotechnol 2012;2012:485156.
28. Stagg J, Divisekera U, Duret H, Sparwasser T, Teng MW, Darcy PK, Smyth MJ. CD73- deficient mice have increased antitumor immunity and are resistant to experimental metastasis. Cancer Res 2011;71:2892–900.
29. Stagg J, Beavis PA, Divisekera U, Liu MC, Möller A, Darcy PK, et al. CD73-deficient mice are resistant to carcinogenesis. Cancer Res 2012;72:2190–6.
30. Péant B, Diallo JS, Dufour F, Le Page C, Delvoye N, Saad F, et al. Over-expression of IkappaB-kinase-epsilon (IKKepsilon/IKKi) induces secretion of inflammatory cytokines in prostate cancer cell lines. Prostate 2009;69:706–18.
31. Regateiro FS, Howie D, Nolan KF, Agorogiannis EI, Greaves DR, Cobbold SP, et al. Generation of anti-inflammatory adenosine by leukocytes is regulated by TGF-β. Eur J Immunol 2011;41:2955–65.
32. Gannon PO, Lessard L, Stevens LM, Forest V, Bégin LR, Minner S, et al. Large-scale independent validation of the nuclear factor-kappa B p65 prognostic biomarker in prostate cancer. Eur J Cancer 2013;49:2441–8.
33. Majumdar S, Aggarwal BB. Adenosine suppresses activation of nuclear factor-kappaB selectively induced by tumor necrosis factor in different cell types. Oncogene 2003;22:1206–18.
34. Jijon HB, Walker J, Hoentjen F, Diaz H, Ewaschuk J, Jobin C, et al. Adenosine is a negative regulator of NF-kappaB and MAPK signaling in human intestinal epithelial cells. Cell Immunol 2005;237:86–95.
35. Csóka B, Németh ZH, Rosenberger P, Eltzschig HK, Spolarics Z, Pacher P, et al. A2B adenosine receptors protect against sepsis-induced mortality by dampening excessive inflammation. J Immunol 2010;185:542–50.

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

36. Kiniwa Y, Miyahara Y, Wang HY, Peng W, Peng G, Wheeler TM, et al. CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer. Clin Cancer Res 2007;13:6947–58.
37. Olson BM, Jankowska-Gan E, Becker JT, Vignali DA, Burlingham WJ, McNeel DG. Human prostate tumor antigen-specific CD8+ regulatory T cells are inhibited by CTLA-4 or IL-35 blockade. J Immunol 2012;189: 5590–601.
38. Churlaud G, Pitoiset F, Jebbawi F, Lorenzon R, Bellier B, Rosenzwajg M, et al. Human and Mouse CD8(+)CD25(+)FOXP3(+) Regulatory T Cells at Steady State and during Interleukin-2 Therapy. Front Immunol 2015; 6:171.
39. Parodi A, Battaglia F, Kalli F, Ferrera F, Conteduca G, Tardito S, et al. CD39 is highly involved in mediating the suppression activity of tumor-infiltrating CD8+ T regulatory lymphocytes. Cancer Immunol Immunother 2013; 62:851–62.
40. Shafer-Weaver KA, Anderson MJ, Stagliano K, Malyguine A, Greenberg NM, Hurwitz AA. Cutting Edge: Tumor-specific CD8+ T cells infiltrating prostatic tumors are induced to become suppressor cells. J Immunol 2009; 183:4848–52.
41. Zarek PE, Huang CT, Lutz ER, Kowalski J, Horton MR, Linden J, et al. A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. Blood 2008;111:251–9.
42. Ohta A, Kini R, Ohta A, Subramanian M, Madasu M, Sitkovsky M. The development and immunosuppressive functions of CD4(+) CD25(+) FoxP3(+) regulatory T cells are under influence of the adenosine-A2A adenosine receptor pathway. Front Immunol 2012;3:190.
43. Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, Zhang J , et al. SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression. Nature 2011;470:269–73.
44. Fournier PG, Juárez P, Jiang G, Clines GA, Niewolna M, Kim HS , et al. The TGF-β signaling regulator PMEPA1 suppresses prostate cancer metastases to bone. Cancer Cell 2015;27:809–21.
45. Salm SN, Koikawa Y, Ogilvie V, Tsujimura A, Coetzee S, Moscatelli D , et al. Generation of active TGF-beta by prostatic cell cocultures using novel basal and luminal prostatic epithelial cell lines. J Cell Physiol 2000;184:70–9.
46. Sorrentino C, Musiani P, Pompa P, Cipollone G, Di Carlo E. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. Clin Cancer Res 2011;17:1571–81.
47. Gannon PO, Poisson AO, Delvoye N, Lapointe R, Mes-Masson AM, Saad F. Characterization of the intra-prostatic immune cell infiltration in androgen-deprived prostate cancer patients. J Immunol Methods 2009; 348:9–17.
48. Barcellos-Hoff MH, Cucinotta FA. New tricks for an old fox: impact of TGFβ on the DNA damage response and genomic stability. Sci Signal 2014;7:re5.
49. Long JS, Crighton D, O'Prey J, Mackay G, Zheng L, Palmer TM , et al. Extracellular adenosine sensing-a metabolic cell death priming mechanism downstream of p53. Mol Cell 2013;50:394–406.
50. Turcotte M, Spring K, Pommey S, Chouinard G, Cousineau I, George J, et al. CD73 is associated with poor prognosis in high-grade serous ovarian cancer. Cancer Res 2015 Sep 11. [Epub ahead of print].

Published OnlineFirst August 7, 2015; DOI: 10.1158/1078-0432.CCR-15-1181

Clinical Cancer Research

CD73 Expression Is an Independent Prognostic Factor in Prostate Cancer

Bruno G. Leclerc, Roxanne Charlebois, Guillaume Chouinard, et al.

Clin Cancer Res 2016;22:158-166. Published OnlineFirst August 7, 2015.