CD73-deficient mice are resistant to carcinogenesis.

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Abbreviations: ASGM1, asialoGM1; DC, dendritic cells; IFN-γ, interferon-gamma; mAb, monoclonal antibody;
Abstract:

CD73 is a cell surface 5’-nucleotidase that converts AMP to adenosine, an immune suppressive molecule. CD73 may promote immune escape in cancer by contributing to the degradation of extracellular ATP released by dying cancer cells in hypoxic tumors or following chemotherapy. However, whether CD73 exerts a critical oncogenic function during tumorigenesis is unknown. In this study, we used genetically deficient mice to investigate its contribution to autochthonous tumor formation. CD73 deficiency suppressed the development of 3-methylcholanthrene (MCA)-induced fibrosarcomas through by mechanism relying upon IFN-γ, natural killer (NK) cells and CD8+ T cells. Similarly, CD73 deficiency also suppressed prostate tumorigenesis in TRAMP transgenic mice. Importantly, treatment with an anti-CD73 monoclonal antibody effectively suppressed growth of established MCA-induced tumors or TRAMP-C1 prostate tumors and inhibited the development of TRAMP-C1 lung metastases. The therapeutic activity of anti-CD73 mAb against primary tumors was dependent on CD8+ T cells, whereas its anti-metastatic activity was dependent on host CD73 expression independent of T cells or NK cells. Taken together, our findings indicate that CD73 is a critical factor in tumorigenesis and that anti-CD73 antibodies may offer a novel generalized strategy to blunt immune escape and treat cancer.
Introduction

The tumor microenvironment is an important regulator of immune functions that impact cancer progression and metastasis. While increased infiltration of tumors with lymphocytes correlates with prolonged survival in a variety of epithelial cancers (1), several immunosuppressive pathways inhibit anti-tumor immune responses. One immunosuppressive component of the tumor microenvironment is elevated levels of extracellular adenosine (2-3). Landmark studies by Sitkovsky and colleagues have demonstrated that accumulation of extracellular adenosine in tumors suppresses anti-tumor immune responses, essentially via the activation of A2A adenosine receptors (4-6). Activation of A2A adenosine receptors on T cells inhibits T cell-mediated cytotoxicity, cytokine production and T cell proliferation (6-7), and has been associated with T cell anergy (8). A2B adenosine receptors have also been shown to promote tumor immune escape. Activation of A2B adenosine receptors enhances tolerogenic factors produced by dendritic cells (DCs) and promotes tumor growth and metastasis in vivo (9-10).

We and others have recently demonstrated that one of the mechanisms contributing to the immunosuppressive accumulation of extracellular adenosine in tumors is the expression of CD73 by tumor cells (11-12). These studies revealed that CD73 expression on breast cancer and ovarian cancer cells significantly suppress adaptive anti-tumor immunity. CD73 expression on cancer exosomes can further suppress T cell functions (13). In addition to tumor-derived CD73, host CD73 also negatively regulates tumor immunity. Three independent groups, including ours, recently demonstrated the resistance of CD73-deficient mice to the development of CD73
negative transplanted tumors (14-16). The pro-tumorigenic effect of host CD73 is associated with its expression on hematopoietic and non-hematopoietic cells. In particular, CD73 expression on Foxp3+ Tregs is important for their suppression of anti-tumor immunity (15). Notably, host CD73 has been shown to protect against acute graft-versus-host disease and to inhibit graft-versus-leukaemia effect (17). Taken together, these studies suggest that CD73 may be a valid therapeutic target in order to enhance anti-tumor immunity. In proof-of-concept studies we and others demonstrated that pharmacological blockade of CD73 with a selective inhibitor or anti-CD73 monoclonal antibody (mAb) can significantly reduce growth and metastasis of transplanted tumors (11-12). However, experimental tumors transplanted into healthy mice behave differently compared with tumors that arise spontaneously. Spontaneous de novo tumors develop over many weeks, which allows for the establishment of a unique tumor microenvironment similar to that seen in cancer patients. We here investigated the role of CD73 on de novo tumorigenesis.
Materials and methods

Mice

Inbred C57BL/6 wild-type (WT) and CD73-deficient (CD73−/−) mice (kindly provided by Dr. Linda H. Thompson, Oklahoma Medical Research Foundation, Oklahoma) were bred and maintained at the Peter MacCallum Cancer Centre as previously described (15). C57BL/6 CD73-deficient TRAMP transgenic mice (CD73−/− TRAMP) were generated by backcrossing C57BL/6 TRAMP with CD73−/− mice until CD73−/− TRAMP mice were generated. Female CD73−/− TRAMP mice were then bred with male CD73−/− mice and offspring screened for the TRAMP transgene as described below. All mice were routinely screened for viruses, parasites and other microbes and tested negative over the entire course of the experiment. All experiments were performed in accordance with guidelines set out by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee.

Genetic screening of mice

DNA was extracted from the tails of 10 – 21 day old litters of TRAMP mice and CD73−/− TRAMP mice using the Puregene DNA Purification Kit (Flowgen Bioscience Limited, Wilford, Nottingham, U.K.), as per their instructions. Presence of the TRAMP transgene was detected using PCR using an annealing temperature of 55 °C. The sequences of the primers used for TRAMP detection were (5’ → 3’): 994, CCGTGTCGACCGGAAGCTTCCACAAGTGCATTTA and 995, CTCCTTTCAA GACCTAGAAGTAAGTA, which produced a 600 bp PCR fragment from TRAMP tg DNA. Primer sequences to ensure integrity of the PCR reaction were (5’ → 3’): 992, GATGTGCTCCAGGCTAAAGTT and 993, AGAAACGGAATGTTGTGG AGT,
which generated a PCR product of 500 bp. Presence of the CD73 mutant allele was detected using PCR using an annealing temperature of 55 °C. The sequences of the primers used for wild-type CD73 detection were (5’ → 3’): 2062, TCTTTAGGGACAAATTTAGTC and 2063, AGAAAGGTGGTGGAGGTCTCT, which produced a 188 bp PCR fragment from DNA. Primer sequences for the mutant CD73 allele (neo) were (5’ → 3’): 2060, CTTGGGTGGAGAGGCTATTC and 2061, AGGTGAGATGACAGGAGATC, which generated a PCR product of 280 bp.

**Cell lines and antibodies**

TRAMP-C1 mouse tumor cell lines were maintained as previously described (18). MCA.WT100-5 and MCA.WT100-7 cells were derived from WT mice that had been inoculated with 100 μg of MCA. CD73 expression was assessed using phycoerythrin (PE)-conjugated anti-mouse CD73 mAb (clone TY/23; BD Bioscience). PE-conjugated anti-mouse CD45.1 (A20), fluorescein (FITC)-conjugated anti-mouse Gr1 (RB6-8C5), Pacific Blue-conjugated anti-mouse CD4 (L3T4), PE-conjugated anti-mouse CD25, allophycocyanin (APC)-conjugated anti-mouse CD8 (53-6.7) were purchased from BD Bioscience, FITC-conjugated anti-mouse CD11b (M1/70), FITC-conjugated anti-mouse Foxp3 and (APC)-conjugated anti-mouse CD45.2 (104) were purchased from eBioscience. Purified anti-CD73 mAb (clone TY/23; provided by Dr. Linda H. Thompson, Oklahoma Medical Research Foundation, Oklahoma), anti-CD4 mAb (GK1.5), anti-CD8β mAb (53-5.8) and control Ig (clone MAC4) were produced in house.
MCA-induced fibrosarcoma

Groups of 15-20 male WT or CD73<sup>−/−</sup> mice were inoculated s.c. in the hind flank with 25, 100, or 400 μg of 3-methylcholanthrene (MCA; Sigma-Aldrich, St. Louis, MO) in 0.1 ml of corn oil as described (Swann et al. 2008). Some WT mice received control Ig or weekly depletion/neutralization of CD8β<sup>+</sup> T cells (53.5.8), NK cells (anti-asialo-GM1) or IFN-γ (H22, kindly provided by Dr. Schreiber, St Louis, MO, USA) from the time of MCA inoculation to day 56 (100 μg i.p.). Development of fibrosarcomas was monitored weekly over the course of 300 days. Measurements were made with a caliper square as the product of two perpendicular diameters (mm<sup>2</sup>) and individual mice are represented. For percent survival tumors > 100 mm<sup>2</sup> in size were recorded as lethal.

TRAMP transgenic mice

CD73<sup>+/+</sup> TRAMP and CD73<sup>−/−</sup> TRAMP mice were monitored twice weekly and when the abdomen became distended, the age of the mouse recorded and a post-mortem performed. Mean prostate weight ± standard error of the mean at various ages (13, 20 and 25 weeks) were calculated and probability of significance determined using a Mann-Whitney Rank Sum U-test.

Immunofluorescence

Prostates were harvest from 20 week old CD73<sup>+/+</sup> TRAMP and CD73<sup>−/−</sup> TRAMP mice, frozen in OCT media, sectioned and stored at -80C. For immunofluorescence staining, sections were thawed at room temperature prior to fixation in ice cold acetone for 10 minutes. Sections were then washed in PBS and then incubated with anti-CD4 (RM4-5: BD Pharmingen), anti-CD8 (53-6.7: BD Pharmingen) or anti-CD73 (TY/23) diluted
1 in 200 in Dako antibody diluent. After 90 minutes, sections were washed in PBS and then probed with Alexa Flour 488 conjugated goat anti-rat (Invitrogen-A11006) diluted 1 in 1000. Sections were incubated for 75 minutes, washed in PBS and then mounted with Prolong Gold plus DAPI (Invitrogen). CD4/CD8 cells were quantified by recording the average number of cells within a 10x objective. Images were taken from 4-6 fields of view per section and 3 sections per prostate using a BX-51 Olympus microscope.

**Subcutaneous tumors**

TRAMP-C1 tumor cells were injected subcutaneously into syngeneic C57BL/6 wild-type (WT) or C57BL/6 CD73<sup>-/-</sup> mice at the indicated doses. Where indicated, mice were treated with control Ig (cIg) or anti-CD73 mAb (TY2/3).

**Metastatic tumor models**

To examine metastatic tumor growth, WT or CD73<sup>-/-</sup> mice were inoculated intravenously (i.v.) with increasing doses of TRAMP-C1 prostate carcinoma cells. Mice were monitored, harvested and lung metastases quantified as previously described (15). Some mice were treated with control Ig (Mac-4) or anti-IFN-γ, anti-CD8β (53.5.8) and anti-CD4 (GK1.5) or anti-asialoGM1 (Wako Chemicals) (as indicated in the legends) to neutralize IFN-γ or deplete cell subsets.

**Tumor infiltrating lymphocytes**

MCA-induced fibrosarcomas were excised, minced with scissors and incubated 1 h at 37° for in PBS containing collagenase type 4 (Worthington Biochemical) and DNase I (Roche). Tumor cell suspensions were passed through a 70-μm cell strainer, washed
twice in PBS and resuspended in PBS containing 2% serum for flow cytometry analysis. Anti-CD16/32 mAb (clone 2.4G2) was used to block Fc receptors. Flow cytometry was performed on a LSR II (BD Bioscience) and analysed using the software program FCS Express.

Statistics

Statistical analyses were performed using Graph Pad Prism software. Significant differences in tumor growth and metastases were determined by a Mann-Whitney test. Statistical differences in % mice survival were determined by a Log Rank Mantel-Cox test. Values of $p < 0.05$ were considered significant.
Results and Discussion:

CD73-deficient mice are resistant to MCA-induced tumor initiation.

We assessed the importance of CD73 in the MCA induction model of fibrosarcoma, where host immunity plays a critical factor in suppressing tumor initiation and progression. As shown in Fig. 1A, host CD73 deficiency caused a significant reduction in the induction of fibrosarcomas by MCA. Tumor incidence in CD73-deficient mice was significantly reduced at all doses of MCA tested (Supp. Fig. S1A-B). We also assessed the effect of CD73 deficiency on the growth rates of tumors that developed in CD73-deficient and WT mice. We observed that tumor growth rates were significantly suppressed in CD73-deficient compared to WT mice (Fig. 1B). We next determined the effect of CD73 deficiency on immunosurveillance of MCA-induced tumors. For this purpose, we compared tumor initiation in WT (Fig. 1C) and CD73-deficient mice (Fig. 1D) depleted of NK cells and/or CD8+ T cells, or treated with a neutralizing anti-IFN-γ mAb from the time of MCA inoculation. Our results revealed that depletion of NK cells and IFN-γ abolished the protective effect of CD73 deficiency on MCA-induced tumor initiation, while depletion of CD8+ T cells did not reach statistical difference (Fig. 1D).

CD73 inhibits adaptive immunity in established MCA tumors and can be targeted for treatment.

We next investigated the role of CD73 on the immunosurveillance of established MCA-induced tumors. In these experiments, mice injected with MCA were depleted of NK cells and/or CD8+ T cells after tumor initiation (i.e. from palpable tumor formation). As shown in Fig. 2A, depletion of CD8+ T cells, but not NK cells, after...
tumor initiation completely abolished the protective effect of CD73 deficiency on tumor growth rates. We next assessed whether targeted blockade of CD73 could effectively delay growth rate of established MCA-induced tumors. Groups of mice were inoculated with MCA and treated with bi-weekly injections of a control Ig or anti-CD73 mAb starting at tumor onset. As shown in Fig. 2B, treatment with anti-CD73 mAb significantly suppressed the growth of established MCA-induced tumors. Individual growth curves of treated mice are shown as supplementary data (Supp. Fig. S1C-D).

**Tumor and host CD73 are targeted by anti-CD73 mAb treatment**

We next investigated whether CD73 was expressed on MCA-induced tumor cells. Tumor cell lines were established from WT mice inoculated with MCA and analysed by flow cytometry for CD73 expression. CD73 was heterogeneously expressed amongst different MCA fibrosarcoma cell lines. The majority of MCA tumor cell lines were negative or expressed low levels of CD73 (Supp. Fig. S2). We also investigated whether CD73 was expressed on various tumor-infiltrating immune cells. Consistent with previous studies (14-16), CD73 was expressed on tumor-infiltrating CD8+ T cells, CD4+ T cells and Foxp3+ T regulatory cells, but absent on tumor infiltrating CD11b+ or CD11c+ cells (Supp. Fig. S3). We next sought to determine the role of tumor and host CD73 on anti-CD73 mAb therapy of MCA-induced tumors. For this purpose, we compared treatment activity of anti-CD73 mAb against CD73+ and CD73null MCA tumors in WT and CD73−/− mice. Treatment of CD73+ tumors was effective in both WT and CD73−/− mice (Fig. 2C), while treatment of CD73null tumors was only effective in WT mice (Fig. 2D). Taken together, our results suggest that treatment of MCA tumors is mediated by targeting both tumor and host CD73.
**CD73 promotes prostate cancer in TRAMP transgenic mice**

We next assessed the role of CD73 in the development of de novo prostate tumors in TRAMP transgenic mice. Previous studies suggested that CD73 is associated with malignant transformation of prostate epithelial cells (19). TRAMP transgenic mice develop mild to severe prostate hyperplasia by 12 weeks of age and by 24 weeks of age, approximately 100% of male mice have poorly differentiated and invasive adenocarcinomas (18). We generated CD73^{-/-} TRAMP transgenic mice and compared prostate weights with CD73^{+/+} TRAMP mice. As shown in Fig. 3A, CD73 deficiency in TRAMP mice was associated with a significant reduction in prostate weights as early as 13 weeks of age. Significant reduction in prostate weights was also observed at 20 and 25 weeks of age. Immunohistochemistry (IHC) revealed that TRAMP prostate tumors expressed CD73 (Supp. Fig. S4). We next assessed the effect of CD73 deficiency on TRAMP tumor infiltrating lymphocytes. As shown in Fig. 3B, CD73 deficiency was associated with an increased ratio of tumor infiltrating CD8{+} to CD4{+} T cells (Fig. 3C).

**Anti-CD73 mAb therapy inhibits TRAMP-C1 tumor growth and metastasis**

We next investigated the role of host CD73 in the progression and metastasis of prostate cancer by inoculating WT and CD73^{-/-} mice with TRAMP-C1 tumors cells. The growth of primary TRAMP-C1 tumors was significantly, albeit modestly, delayed in CD73^{-/-} mice (Fig. 4A) in a CD8{+} T cell-dependent manner (Supp. Fig. S5). Remarkably, CD73^{-/-} mice were greatly resistant to experimental TRAMP-C1 lung metastases (Fig. 4B). Based on these observations, we next assessed the therapeutic activity of anti-CD73 mAb against TRAMP-C1 prostate tumors. As
shown in Fig. 4C, anti-CD73 mAb therapy significantly delayed the growth of primary TRAMP-C1 prostate tumors in a CD8+ T cell-dependent manner. We next assessed the anti-metastatic activity of anti-CD73 mAb therapy. As shown in Fig. 4D, anti-CD73 mAb therapy significantly suppressed lung metastasis of TRAMP-C1 prostate cancer cells. Notably, this anti-metastatic activity was dependent on host CD73 expression, independently of T cells or NK cells. (Fig. 4D).

In conclusion, we demonstrated that CD73 promotes carcinogen-induced tumor initiation and tumor growth by suppressing immunosurveillance via IFN-γ, NK cells and CD8+ T cells. We also demonstrated that CD73 promotes de novo prostate tumorigenesis. Furthermore, we provided the first evidence that anti-CD73 mAb therapy can suppress prostate tumor growth and metastasis. Our current findings might have significant impact for prostate cancer treatment. The recent successes of cancer immunotherapies such as Sipuleucel-T (Provenge, Dendreon Corporation, WA, USA) and ipilimumab (Yervoy, Bristol Meyers Squibb, NY, USA) are paving the way for new immune-based treatments. Promising approaches include antagonists of immune checkpoint inhibitors, such as anti-PD-1 mAb, and immune-activating anti-CD137 mAb (20). Our study suggests that immunotherapy of prostate cancer may be significantly improved by combining these approaches with targeted blockade of CD73.
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References


Figure Legends

Figure 1. Host CD73 promotes the formation of 3-methylcholanthrene (MCA)-induced fibrosarcomas. A. Groups of 20 male WT and CD73−/− mice were injected s.c. with 400 μg MCA and monitored for tumor development over 300 days. Kaplan-Meier survival curves of mice in each group are shown. Statistical difference was determined by Log Mantel Cox test. B. Individual growth rates of tumors (mm²/day) from panel A were scatter plotted with the mean ± SEM shown for each group. Statistical difference was determined by Mann-Whitney test. C-D. Groups of 15 male WT (C) and CD73−/− (D) mice were injected s.c. with 100 μg MCA and received either 250 μg control Ig (cIg; clone MAC4) or 250 μg anti-IFN-γ mAb (clone H22), 100 μg anti-CD8β mAb (clone 53.5.8) and/or 100 μg anti-asialo GM1 i.p. on day -1, 0 and weekly for 6 weeks. Mice were monitored for tumor development over 300 days. Kaplan-Meier survival curves of mice in each group are shown. Statistical difference (compared with cIg) was determined by Log Mantel Cox test.

Figure 2. Host CD73 inhibits adaptive anti-tumor immunity against MCA tumors and can be targeted for treatment. A. Groups of 8-9 male WT and CD73−/− mice were injected s.c. with 400 μg MCA and received either 100 μg control Ig (cIg) or 100 μg anti-CD8β mAb (clone 53.5.8) or 100 μg anti-asialo GM1 i.p. weekly from tumor initiation (range: day 70-168). Mice were monitored for tumor development over 300 days. Individual growth rates of tumors (mm²/day) were scatter plotted with the mean ± SEM shown for each group. Statistical difference was determined by Mann-Whitney test. B. Groups of 15 male WT mice were injected s.c. with 400 μg MCA and treated with 100 μg control Ig (cIg) or 100 μg anti-CD73 mAb (clone
TY/23) injected i.p twice weekly from palpable tumor formation (day 77-105) for 6 weeks. Statistical difference was determined by Mann-Whitney test. **C-D.** WT and CD73−/− mice were injected subcutaneously with (C) 5 x 10^5 MCA.WT100-5 tumor cells or (D) 5 x 10^5 MCA.WT100-7 tumor cells and tumor size measured over time. Mice were treated on days 8, 10, 12, and 14 with either 100 µg control Ig (cIg) or 100 µg anti-CD73 mAb (clone TY/23) injected i.p. (means of 5 mice per group ± SEM are shown).

Figure 3. **CD73 promotes de novo TRAMP prostate cancer.**

**A.** Prostate weights of CD73+/+TRAMP and CD73−/−TRAMP mice were compared at 25 weeks, 20 weeks and 13 weeks of age. Individual prostate weights were scatter plotted with the mean ± SEM shown for each group. Statistical difference was determined by Mann-Whitney test. **B.** Immunofluorescence for CD4 and CD8 expression (in green) was performed in 20-week old TRAMP prostate tumors. Representative microphotographs of each genotype are shown (10X magnification). **C.** Ratios of tumor-infiltrating CD8 to CD4 cell s per field were scatter plotted with the mean ± SEM shown for each genotype (n=9 tumors per group). Statistical difference was determined by Mann-Whitney test.

Figure 4. **Host CD73 promotes TRAMP-C1 tumor growth and metastasis and anti-CD73 mAb therapy inhibits TRAMP-C1 growth and metastasis.** **A.** WT and CD73−/− male mice were injected subcutaneously with 5 x 10^5 TRAMP-C1 tumor cells and tumor growth monitored over time (means of 5 mice per group ± SEM are shown; *: p < 0.05 by Mann-Whitney). **B.** WT and CD73−/− male mice were injected intravenously with dose-escalating TRAMP-C1 tumor cells and macroscopic lung...
metastases were counted after 14 days (symbols represent individual mouse; means ± SEM are shown; statistical differences were determined by Mann-Whitney test). C. WT male mice were injected s.c. with 5 x 10^5 TRAMP-C1 tumor cells and received either 100 μg control Ig (cIg) or 100 μg anti-CD8β mAb (clone 53.5.8), 100 μg anti-CD4 mAb (clone GK1.5) or 100 μg anti-asialo GM1 i.p. on days 5, 6, 13, 20 and treated with 250 μg control Ig (cIg; left panel) or anti-CD73 mAb (clone TY/23; right panel) injected i.p on days 6, 8, 10, 12, 14, 16, 18 (means of 5 mice per group ± SEM are shown; *: P < 0.05 by Mann-Whitney). D. WT and CD73^-/- male mice were injected intravenously with 2 x 10^5 TRAMP-C1 tumor cells and received either 100 μg control Ig (cIg) or 100 μg anti-CD8β mAb (clone 53.5.8), 100 μg anti-CD4 mAb (clone GK1.5) or 100 μg anti-asialo GM1 i.p. on days -1 and 0 and treated with 250 μg control Ig or anti-CD73 mAb (clone TY/23) injected i.p on days -1,0,4, and 7. Macroscopic lung metastases were counted after 14 days (symbols represent individual mouse; means ± SEM are shown; statistical differences were determined by Mann-Whitney test).
Figure 1

A

Percent survival

Days after MCA inoculation

WT

CD73\(^{-/-}\)

*** P < 0.0001

B

Tumor growth rate mm\(^2\)/day

WT

CD73\(^{-/-}\)

*** P < 0.0001

C

Percent survival

Days after MCA inoculation

WT + clg

WT + αIFN\(_γ\)

WT + αCD8

WT + αasGM1

WT + αCD8/asGM1

D

Percent survival

Days after MCA inoculation

CD73\(^{-/-}\) + clg

CD73\(^{-/-}\) + αIFN\(_γ\)

CD73\(^{-/-}\) + αCD8

CD73\(^{-/-}\) + αasGM1

CD73\(^{-/-}\) + αCD8/asGM1

* P < 0.05

** P < 0.001
Figure 2

A

Tumor growth rate mm²/day

WT × clg
CD73⁻/⁺ × clg
WT × αCD6
CD73⁻/⁺ × αCD6
CD73⁻/⁺ × αsGM1

P < 0.05
P < 0.001

B

Tumor growth rate mm²/day

clg
anti-CD73

** P < 0.001

C

MCA.WT100-5

% max

CD73

WT + clg
WT + anti-CD73
CD73⁻/⁺ + clg
CD73⁻/⁺ + anti-CD73

D

MCA.WT100-7

% max

CD73

WT + clg
WT + anti-CD73
CD73⁻/⁺ + clg
CD73⁻/⁺ + anti-CD73

Mean tumor size (mm²)

Days after MCA.WT100-5 inoculation

Days after MCA.WT100-7 inoculation
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