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CD73 Is Associated with Poor Prognosis in High-Grade Serous Ovarian Cancer

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Abstract

The cell surface nucleotidase CD73 is an immunosuppressive enzyme involved in tumor progression and metastasis. Although preclinical studies suggest that CD73 can be targeted for cancer treatment, the clinical impact of CD73 in ovarian cancer remains unclear. In this study, we investigated the prognostic value of CD73 in high-grade serous (HGS) ovarian cancer using gene and protein expression analyses. Our results demonstrate that high levels of CD73 are significantly associated with shorter disease-free survival and overall survival in patients with HGS ovarian cancer. Furthermore, high levels of CD73 expression in ovarian tumor cells abolished the good prognosis associated with intraepithelial CD8$^+$ cells. Notably, CD73 gene expression was highest in the C1/stromal molecular subtype of HGS ovarian cancer and positively correlated with an epithelial-to-mesenchymal transition gene signature. Moreover, *in vitro* studies revealed that CD73 and extracellular adenosine enhance ovarian tumor cell growth as well as expression of antiapoptotic BCL-2 family members. Finally, *in vivo* coinjection of ID8 mouse ovarian tumor cells with mouse embryonic fibroblasts showed that CD73 expression in fibroblasts promotes tumor immune escape and thereby tumor growth. In conclusion, our study highlights a role for CD73 as a prognostic marker of patient survival and also as a candidate therapeutic target in HGS ovarian cancers. *Cancer Res; 75(21); 4494–503. ©2015 AACR.*

Introduction

High-grade serous (HGS) ovarian cancer is the most common and lethal histotype of epithelial ovarian cancers, with a 5-year survival rate of 30% to 40% (1). Despite good initial responses to chemotherapy, the majority of patients with HGS ovarian cancer relapse and develop drug-resistant disease, emphasizing the need for new treatments.

Tumor-infiltrating intraepithelial lymphocytes (TIL), in particular CD8$^+$ T cells, correlate with improved clinical outcomes in epithelial ovarian cancers (2, 3). Interestingly, correlation between TILs and prognosis is particularly linked to the serous histotype of ovarian cancer (4). Within HGS ovarian cancer, studies from the Australian Ovarian Cancer Study (AOCS) and The Cancer Genome Atlas (TCGA) have identified four molecular subtypes: the mesenchymal/stromal (C1) subtype, characterized by a reactive stroma and poor prognosis; the immunoreactive (C2) subtype, characterized by intraepithelial TILs and favorable prognosis; the differentiated (C4) subtype, characterized by a low stromal response; and the proliferative (C5) subtype, characterized by negligible TILs and poor prognosis.

Overcoming immunosuppressive mechanisms that restrict antitumor T-cell function is a potential treatment strategy for ovarian cancer, as recently shown in clinical trials (5). Nevertheless, increasingly evident is the need to target multiple immunosuppressive mechanisms to overcome the high level of redundancy that exists in tumor immunity (6, 7). We and others have demonstrated that the CD73–adenosinergic pathway contributes to tumor immune escape in animal mouse models of cancer, including ovarian cancer (8–11). CD73 is a GPI-anchored nucleotidase that catabolizes the production of extracellular adenosine. Inspired by landmark studies from Sitkovsky and colleagues, we and others have underscored the therapeutic potential of blocking CD73 and adenosine receptors for cancer therapy (12–15). In the ID8 mouse model of ovarian cancer, Zhang and colleagues reported that CD73 gene silencing, or targeted blockade of CD73 with a monoclonal antibody (mAb) or a small molecule inhibitor, enhances antitumor T-cell responses and improves animal survival (11). Others have shown that *in vitro* treatment of alloantigen-primed human T cells with an anti-CD73 mAb enhances T-cell cytotoxicity against human ovarian cancer cells (16).

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CD73 overexpression has been associated with worse clinical outcomes in different types of cancer (15, 17–19). In triple negative breast cancer, high levels of CD73 gene expression are associated with poor prognosis and resistance to immunogenic-cell death induced by anthracyclines (15). The clinical importance of CD73 in ovarian cancer remains, however, unclear. High levels of CD73 have been reported to be associated with better prognosis in patients with ovarian cancer (20); however, this study aggregated various histotypes and relatively few cases of poor-outcome HGS were included. Because immunosurveillance of ovarian cancer is linked to the serous histotype, we hypothesized that the prognostic value of CD73 may be different in HGS ovarian cancer. We thus investigated the clinical importance of CD73 expression specifically in patients with HGS ovarian cancer. We also considered the impact of CD73 expression in ovarian cancer cells and cancer-associated fibroblasts (CAF).

Materials and Methods

Gene expression analysis

The Australian Ovarian Cancer Study (AOCS; GSE9899) cohort (21) was stratified into two groups based on CD73 or CD39 gene expression level. Samples with expression level higher than a threshold (median $+ 0.5 \times$ median absolute deviation) were classified as "High" and samples lower than a threshold (median $- 0.5 \times$ mad) were classified as "Low." Statistical significance of the difference in survival was computed using the Cox proportional hazard model and the log-rank test P value reported on Kaplan–Meier survival curves. The association between CD73 (NT5E) or PD-L1 (CD274) gene expression and the four molecular subtypes defined by gene expression profiling were assessed as previously described (21). The association between CD73 gene expression and EMT gene signature were assessed using the mean expression of the EMT genes: BMP1, CDH2, COL1A2, FN1, FOXC1, GNG11, ITGA5, ITGAV, MMP2, MMP3, MMP9, MSN, SNAI1, SNAI2, TCF4, TGFB1, TWIST1, VCAN, VIM, ZEB1, ZEB2, as previously described (22).

For the meta-analysis, gene expression and survival data from 13 independent ovarian cancer gene expression datasets were standardized as described in ref. 23. We identified 1,581 patients with high-grade, late-stage, serous ovarian carcinoma. The patient data were merged into a single pooled dataset using quantile normalization. Expression of CD73 in this pooled dataset was stratified into high, mid, and low tertiles. We performed a Cox proportional hazards model on the CD73 tertiles and performed statistical testing using the likelihood ratio test. Survival analysis was performed using R version 3.1.2 and package survcomp (24). To investigate the survival stratification of CD73 in combination with CD39, we grouped patients based on median-dichotomized expression values of CD73 and CD39. Patients with both genes expressed in the respective higher quantiles were assigned to the "CD39$^+$/CD73$^+$" group, and patients who did not highly express CD73 or CD39 were assigned to the "other" group.

Patients and tissue microarray

For CD73 protein analysis, formalin-fixed paraffin-embedded (FFPE) oophorectomy tissue blocks were collected and banked following appropriate consent from patients undergoing surgery within the Division of Gynecologic Oncology at the Centre Hospitalier de l'Université de Montréal (CHUM) from 1992 to 2012. A pathologist scored tumor grade and stage and a gyneco-

logic oncologist scored tumor residual disease according to criteria from the International Federation of Gynecologists and Obstetricians (FIGO). Clinical data on progression-free interval were defined according to computed tomographic (CT) imaging, alone or combined with blood CA125 levels (25). Overall survival was defined as the time from diagnosis to death from ovarian cancer. Patients known to be still alive at time of analysis were censored at time of their last follow-up. Patient disease-free survival (DFS) was calculated from the time of surgery until the first progression. Eligibility criteria for inclusion in the study were as follows: no preoperative chemotherapeutic treatment for ovarian cancer, high-grade tumors, serous histopathology subtype, and completed informed consent. Patients who died from nonovarian cancer-related causes were censored at time of last follow-up. Ethics approval was obtained by the local institutional Ethics Board. Areas of tumor were selected based on review of a hematoxylin–eosin-stained slide. FFPE tumor blocks were then biopsied twice using a 0.6-mm-diameter tissue arrayer (TMArrayer; Pathology Device Inc.) and resultant cores were arrayed into a grid in a recipient paraffin block. The tissue array was composed of 208 HGS ovarian cancer samples in duplicate. This tissue microarray (TMA) was then sectioned, stained with hematoxylin–eosin, and received another pathology review to confirm tumor content.

Protein expression analysis

Sections of the TMA were deparraffinized and rehydrated prior to immunostaining. CD73 expression and epithelial regions were revealed using a mouse antihuman CD73 primary antibody (1:500, Clone 1D7; Abcam), DAPI (50 ng/mL) staining, and a mix of mouse anti-cytokeratin-7 (1:100, Clone Ab-2/OV-TL1 230; NeoMarkers) and mouse anti-cytokeratin-18 (1:100, Clone DC-10; Santa Cruz) primary antibodies. CD8$^+$ cells, nuclei, and epithelial regions were revealed using a mouse antihuman CD8 primary antibody (1:40, Clone 4D11; Novus Biologicals), DAPI staining and a mix of mouse anti-cytokeratin-7 (1:100, Clone Ab-2; NeoMarkers) and mouse anti-cytokeratin-18 (1:100, Clone DC-10; Santa Cruz) primary antibodies. Cross-contamination between CD73 or CD8 and cytokeratin staining was avoided by proceeding sequentially with a first primary–secondary labeling, and then blocking of any remaining mouse antigens with the Mouse On Mouse reagent (Vector, MKB-2213). The slides where coverslip mounted using ProlongGold (Life Technologies) and allowed to dry overnight at room temperature. Slides were imaged with a 20×/0.75NA objective using an Olympus VS110 Slide Scanner running FW-AS software. The scanner performs stitching of a collection of individual images to build super images (.vsi file format) of the whole slide. For immunohistochemistry, slides were treated with antigen retrieval solution (Dako 10×; #S2367), microwaved 11 minutes at 900 W and 15 minutes 400 W, cooled, and incubated in a Ventana Benchmark XT autostainer with primary anti-CD73 mAb (Abcam; clone 1D7; 1/600) diluted in antibody dilution buffer (Ventana #ADB250) for 1 hour at 37°C. UltraView Universal DAB Detection Kit (Ventana, #760-500) was used for detection and slides were imaged with a 20×/0.75NA objective using an Olympus VS110 Slide Scanner running FW-AS software.

Image analysis

Super images were imported into Visiomorph software (Visiopharm) where individual cores are separated and automatically

Turcotte et al.

labeled with a patient identification number using the Array Imager module. Threshold values were calculated by separating the image histogram of intensities at the interface between the background and the features, providing epithelia and stroma segmentation. Threshold values were stored in binary images subsequently processed and converted to regions of interest thus allowing quantification of CD73 expression or the count of CD8$^+$ objects in the epithelial regions. All cores are batch-processed ensuring unbiased classification and measurement. CD73 mean expression intensity (MFI) was calculated as the integrated intensity of the CD73 channel divided by the number of pixels present in the epithelial or stromal compartment. For the stromal compartment, highest 20% CD73 expression was used as an arbitrary cutoff. Epithelial CD8$^+$ cell density for each core was evaluated as the count of CD8$^+$ cells present in the epithelium over the corresponding epithelial area (number of CD8$^+$ cells/μm^2 of epithelia). Correlation between duplicates was highly significant for both CD73 and CD8 expression (Pearson $\rho > 0.7$; $P < 0.001$).

Animals and cell lines

Ovarian cancer cell lines were obtained from the laboratory of Anne-Marie Mes-Masson and were cultured in OSE medium (Wisent) with the addition of 10% bovine serum (FBS; Wisent). SKOV3 cells were purchased from ATCC. Cell lines were not authenticated. Mouse embryonic fibroblasts (MEF) were harvested from wild-type and CD73-deficient C57BL/6, immortalized using a lentiviral vector encoding a p53 gene-silencing element (GSE22) provided by Dr. Francis Rodier (Université de Montréal), and cultured in DMEM with 10% FBS. Wild-type C57BL/6 mice were purchased from Charles River Laboratory (Montreal, Canada). CD73-deficient C57BL/6 mice were obtained from Dr. Linda F. Thompson (Oklahoma Medical Research Foundation, Oklahoma City, OK) and were bred and housed at CHUM. The mouse ovarian cancer cell line ID8 was cultured in complete DMEM medium with 10% FBS and gene modified to express ovalbumin (OVA) using a retroviral vector, with an internal ribosome entry site and the green fluorescent protein (GFP). CD73 gene silencing of SKOV3 cells was performed using lentiviral vectors expressing a short-hairpin (sh)RNA-encoding plasmid targeting human CD73 (TRCN0000048755; Thermo Fisher Scientific) or GFP as control (target sequence: 5′-GCAAGCT-GACCCTGAAGTTCAT-3′) followed by one week of selection in 1 μg/mL puromycin. Stable silencing of CD73 was assessed by flow cytometry.

Cell proliferation assay

For three-dimensional (3D) assays, spheroids were formed using a modified protocol of the hanging drop method (26). Briefly, SKOV3 cells were trypsinized, counted, and 4,000 cells cultured in inverted drops (30 μL) for 5 days in OSE medium (Wisent) with 10% FBS. Spheroids (5 per group, in triplicates) were harvested, pooled, and trypsinized for 15 minutes at 37°C. After inactivation of trypsin with complete medium, cells were centrifuged and subjected to Cell Titer 96 Aqueous Cell Proliferation Assay (Promega) following the manufacture's protocol. For 2D assays, cells were plated at 2,000 cells per well (in quadruplicates) in complete media and subjected to Cell Titer 96 Aqueous Cell Proliferation Assay after 3 days. For CD73 reexpression, lentiviral vectors encoding human CD73 cDNA were used.

For coculture experiments, ID8 cells were stained with CFSE dye (Life Technologies), mixed at a ratio of 1:1: with wild-type or CD73-deficient MEF (total 10^5 cells), and incubated for 48 hours. Cocultures were then exposed 1 hour to BrdUrd (Sigma) and analyzed by flow cytometry with an anti-BrdUrd antibody (Invitrogen). Samples were analyzed on LSRII Fortessa based on CFSE to discriminate ID8 cells and BrdUrd to assess the percentage of cells in proliferation. Data were analyzed with FlowJo software.

Flow cytometry analysis of CD73 and CD39 expression

Cells were incubated with PE-conjugated mouse antihuman CD73 mAb (BD Biosciences, 550257), PE-conjugated antihuman CD39 (BD Biosciences, 555464), or PE-conjugated IgG1k isotype control (BD Biosciences, 555749). Samples were analyzed on a Fortessa flow cytometer (BD) and data analyzed with FlowJo software.

CD73 activity

Malachite Green Phosphate Detection Kit (R&D Systems; #DY996) was used to measure CD73 enzymatic activity. Cells were plated in complete media in a flat-bottom 96-well plate (10^4 cells per well) 20 hours before the assay and washed twice with phosphate-free buffer (2 mmol/L MgCl$_2$, 125 mmol/L NaCl, 1 mmol/L KCl, 10 mmol/L glucose, 10 mmol/L HEPES pH 7.2). AMP (40 μmol/L in phosphate-free buffer; Sigma) was then added and cells were incubated for 90 minutes at 37°C. Where indicated, the CD73 inhibitor APCP (50 μmol/L; Sigma) was added. Inorganic phosphate levels were measured following the manufacturer's instructions.

Animal studies

For *in vivo* studies, 5×10^6 ID8 cells and 5×10^6 MEF were resuspended in 200 μL of PBS and coinjected intraperitoneally into WT C57BL/6 mice or Rag2$^{-/-}$γc$^{-/-}$ mice. MEF were reinjected biweekly for 8 weeks. Weights of mice were measured three times per week. In one of two experiments, mice were euthanized at day 60 for peritoneum nodules count. For survival studies, mice were excluded when weight gain exceeded 40% of initial weight. For tumor-infiltrating lymphocyte analysis, mice were injected subcutaneously with a mix of 5×10^6 ID8-OVA cells and 5×10^6 MEF in 400 μL of an equal volume of PBS and cold Matrigel (BD Biosciences). After 24 days, tumors were collected and exposed to a solution of collagenase type IV (Sigma) and DNAse type I (Sigma). Single cell suspensions were then analyzed with a panel of antibodies consisting of anti-CD8α (BD Biosciences), anti-TCRβ (Abcam), and H-2K^b-SIINFEKL tetramer (from the CHUM). Vitality dye 7-AAD (BD Biosciences) was added 20 minutes prior to analysis. Samples were analyzed on LSRII Fortessa and data were analyzed with FlowJo software.

Quantitative PCR

RNA were extracted from cell pellets using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions and quantified on a Nanodrop spectrometer (Thermo Scientific). One micrograms of RNA were reverse transcribed with qScript cDNA Supermix (Quanta Biosciences). Real-time PCR were performed using Taqman Master Mix and Gene Expression Assays primers and probes (human BCL2: Hs00608023_m1, BCLXL: Hs00236329_m1, MCL1: Hs01050896_m1, CD73: Hs00159686_m1, 18S: Hs03003631_g1 ADORA1: Hs00379752_m1, ADORA2A: Hs00169123_m1, ADORA2B: Hs00386497_m1,

ADORA3: Hs00252933_m1, and GAPDH: Hs02758991_g1; Applied Biosystems) on the StepOnePlus instrument and analyzed with StepOne software V.2.3 (Applied Biosystems). Every reaction was performed in triplicates and the relative expression of each gene was normalized to 18S or GAPDH and relative to the shGFP control of the cell line.

Results

CD73 gene expression is associated with poor prognosis and a C1/reactive stromal gene signature

Tumor CD73 gene expression levels were correlated with DFS in HGS ovarian cancer patients from the Australian Ovarian Cancer Study (21). As shown in Fig. 1A, patients with high levels of CD73 had a significantly worse prognosis than patients with low levels of CD73 ($P = 0.00055$). CD73 gene expression also positively correlated with CD39 gene expression (Supplementary Fig. S1A), an ectonucleotidase that hydrolyzes extracellular ATP to AMP. Consistent with this, high levels of CD39 gene expression showed a trend toward association with poor prognosis (Fig. 1B; $P = 0.0507$). In a meta-analysis of 13 independent datasets representing 1,581 patients with late-stage HGS ovarian cancer (Supplementary Fig. S1B), high levels of CD73 significantly associated with worse overall survival (Fig. 1C; $P = 0.008$). Patients with both CD73 and CD39 genes expressed in the respective higher quantiles also had a worse prognosis (Supplementary Fig. S1C). We next assessed whether CD73 gene expression was associated with a specific molecular subtype of HGS ovarian cancer, as defined by Tothill and colleagues (21). As shown in Fig. 1D, CD73 levels were highest in the C1 molecular subtype, characterized by a

Figure 1.
CD73 gene expression is associated with poor prognosis in HGS ovarian cancer. A, we assessed the prognostic value of CD73 (*NT5E*) expression in the AOCS dataset. Significance of differences in survival between patient groups was estimated by log-rank test. B, prognostic value of CD39 (*ENTPD1*) expression in the AOCS dataset. C, prognostic value of CD73 in a meta-analysis of 13 independent datasets composed of 1581 HGS ovarian cancer cases. Expression of CD73 was stratified into high, mid, and low tertiles. Cox proportional hazards model on the CD73 tertiles and likelihood ratio test were performed (HR1 is mid vs. low; HR2 is high vs. low). D, association between CD73 gene expression and HGS molecular subtypes ($P < 0.0001$ by Kruskal–Wallis test). E, association between PD-L1 (*CD274*) gene expression and HGS molecular subtypes ($P < 0.0001$ by Kruskal–Wallis test). F, correlation between CD73 gene expression and EMT gene signature (Pearson coefficient = 0.73; $P < 0.0001$). The line of best fit is shown in red.
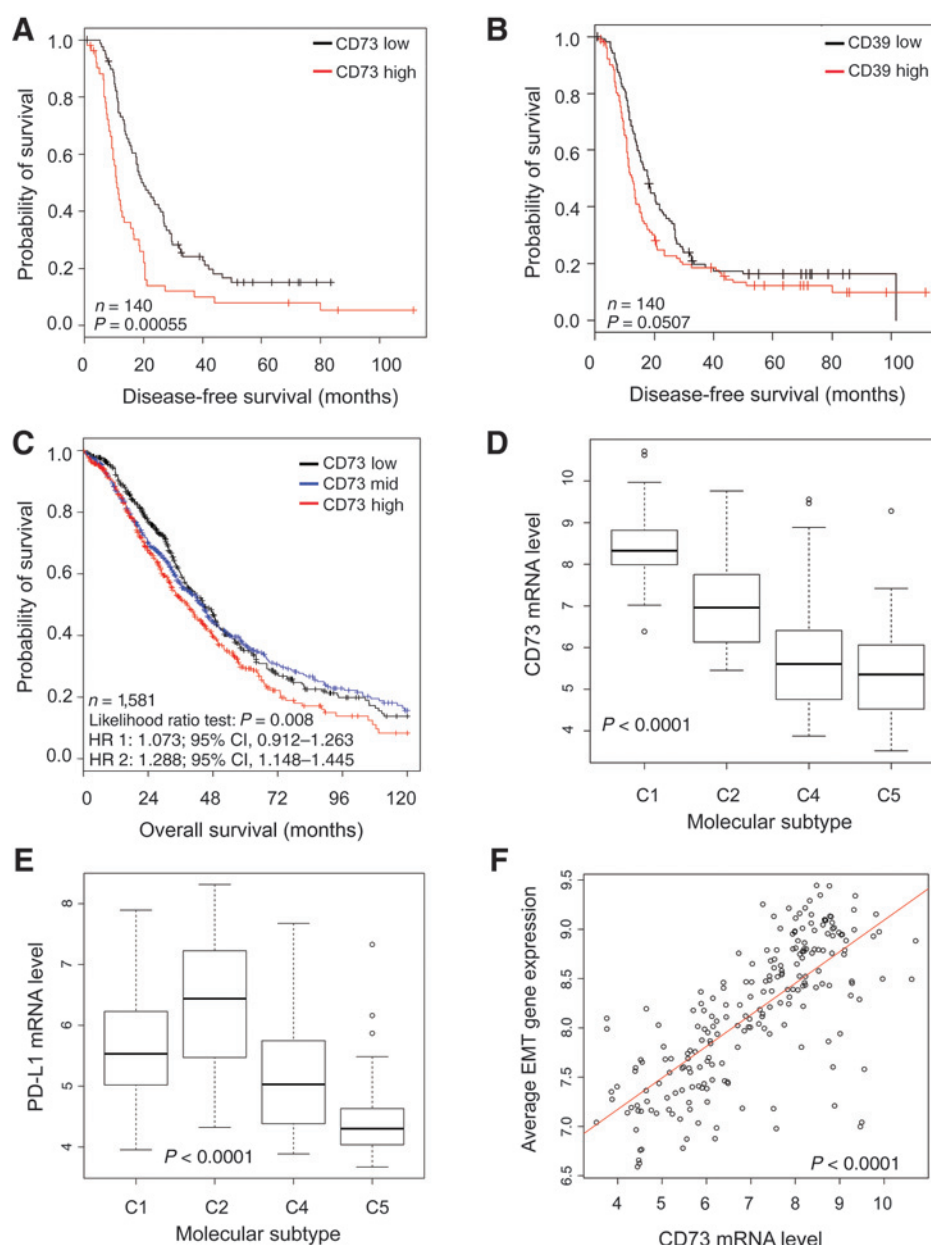
Turcotte et al.

Table 1. Clinicopathological characteristics of the TMA sample set ($n = 208$)

	Median (range) or *n* (%)
Age, y	61 (34–89)
FIGO stage	
I	13 (6)
II	23 (11)
III	147 (71)
IV	25 (12)
Grade	
2	29 (14)
3	179 (86)
Histological type	
Serous carcinoma	208 (100)
Debulking	
Optimal	96 (46)
Non-optimal	91 (44)
Unknown	21 (10)
Chemotherapy	
Platinum-based	191 (92)
Taxane alone	2 (1)
Unknown	15 (7)
Follow-up time (months)	36 (1–156)
Disease progression time (months)	14 (0–76)

reactive stromal gene signature (21). In comparison, PD-L1 expression was highest in the C2 molecular subtype, characterized by an immune gene signature (Fig. 1D). CD73 gene expression also strongly correlated with an EMT gene signature (Fig. 1E) and was independent of copy-number variations (Supplementary Fig. S1).

CD73 expression in ovarian tumors is associated with poor prognosis

To validate our gene expression analysis, we next assessed the prognostic value of CD73 protein expression in an independent cohort (Table 1). A total of 208 HGS cases were analyzed by quantitative immunofluorescence on TMA. To specifically assess the impact of CD73 expression on ovarian tumor cells, we performed co-immunofluorescence of CD73 and epithelial cytokeratin-7/18 (Supplementary Fig. S2A) and determined the intensity of CD73 expression per epithelial surface unit (Fig. 2A). Standard immunohistochemistry was also performed on selected cases for validation (Supplementary Fig. S2B and S2C). As shown in Fig. 2B and C, high levels of CD73 in tumor cells (above median) were associated with a significant shorter DFS (mean $\pm$ SD: 31.7 $\pm$ 4.7 months vs. 47.1 $\pm$ 5.5 months; $P = 0.004$ by log rank) and decreased overall survival (62.7 $\pm$ 6.1 months vs. 78.6 $\pm$ 6.4 months; $P = 0.048$ by log rank). CD73 expression in tumor cells was also associated with worse DFS in multivariate Cox regression analysis (Table 2). Consistent with our immunofluorescence analysis, CD73 was expressed on a majority of cell lines derived from serous human ovarian tumors (Fig. 2D; Supplementary Fig. S3).

CD73 expression in tumor cells hinders the prognostic impact of tumor-infiltrating CD8$^+$ cells

The presence of tumor-infiltrating CD8$^+$ T cells has been associated with improved survival in HGS ovarian cancer (3).
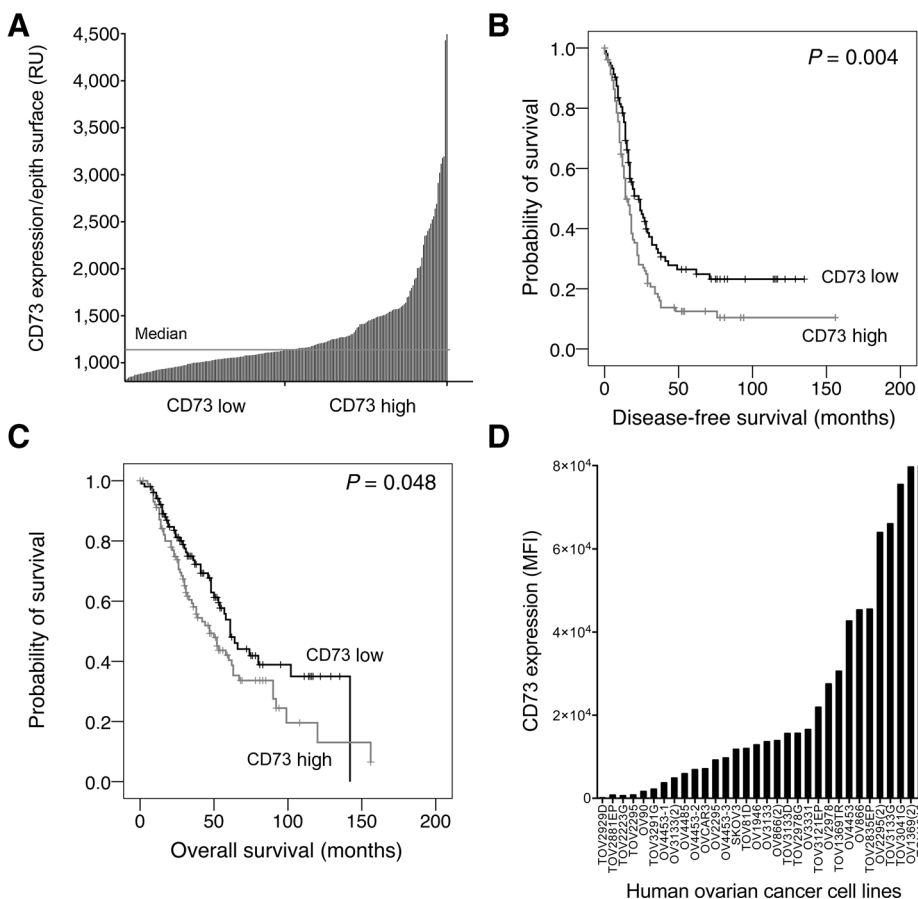
Figure 2.
CD73 protein expression in ovarian tumor cells is associated with poor prognosis. A, CD73 protein expression in cytokeratin 7/18$^+$ tumor cells was quantified by immunofluorescence in 208 cases of HGS ovarian cancer on TMA. Expression per epithelial surface unit was calculated as the integrated intensity of CD73 in the cytokeratin compartment divided by the number of pixels present in the cytokeratin compartment (bars, individual cases). B and C, association of CD73 expression in tumor cells with disease-free survival (B) and overall survival (C). Significance of differences in survival was estimated by log-rank test. D, flow cytometry analysis of CD73 expression on 32 human serous ovarian cancer cell lines.

Table 2. Disease-free survival Cox regression analysis

	Univariate		Multivariate	
	HR (95% CI)	P	HR (95% CI)	P
CD73	1.581 (1.152–2.171)	0.005	1.479 (1.052–2.078)	0.024
Age	1.005 (0.993–1.018)	0.393	1.010 (0.994–1,027)	0.216
FIGO stage	2.070 (1.602–2.674)	0.000	1.796 (1.330–2.425)	0.000
Residual disease	2.658 (1.886–3.746)	0.000	1.861 (1.276–2.715)	0.001

We hypothesized that high levels of CD73 might hinder the prognostic value of intratumoral CD8^{+} T cells (27). Using the same TMA as described above, we analyzed tumor-infiltrating CD8^{+} cell density (Supplementary Fig. S2D) and evaluated its prognostic value, alone and in combination with CD73 expression. As shown in Fig. 3A and B, patients with a high density of intratumoral CD8^{+} cells (i.e., above median) showed a trend toward longer DFS (47.8 ± 6.1 months vs. 32.8 ± 4.3 months) and a significant increase in overall survival (83.6 ± 6.7 months vs. 56.3 ± 5.0 months). In support of our hypothesis, coanalysis of CD73 expression in tumor cells and CD8^{+} cell density significantly improved the prognostic value of either biomarker (Fig. 3C and D); patients with high CD73 expression in tumor cells and low CD8^{+} cell density had the shortest DFS (20.0 ± 3.0 months) and overall survival (45.5 ± 5.8 months), whereas patients with low CD73 expression in tumor cells and high CD8^{+} cell density had the longest DFS (60.7 ± 9.6 months) and overall survival (94.8 ± 10 months).

We next evaluated whether the prognostic value of CD73 was different in tumors infiltrated with CD8^{+} cells compared with tumors with low CD8^{+} cell density. We hypothesized that CD73 expression, due to its immunosuppressive effects, would be most prognostic in tumors with high CD8^{+} cell density. The prognostic value of CD73 expression was indeed superior in tumors with high CD8^{+} cell density compared with tumors with low CD8^{+} cell density (log-rank P = 0.008 and 0.07, respectively; Fig. 3C and D; Supplementary Fig. S4A and S4B). Conversely, the association between intraepithelial CD8^{+} cells and good prognosis was restricted to tumors with low levels of CD73 expression (log-rank P = 0.025 and 0.199, respectively; Fig. 3C and D; Supplementary Fig. S4C and S4D). Our data thus strongly suggest that CD73 expression in HGS ovarian tumors contributes to suppress the function of tumor-infiltrating CD8^{+} T cells.

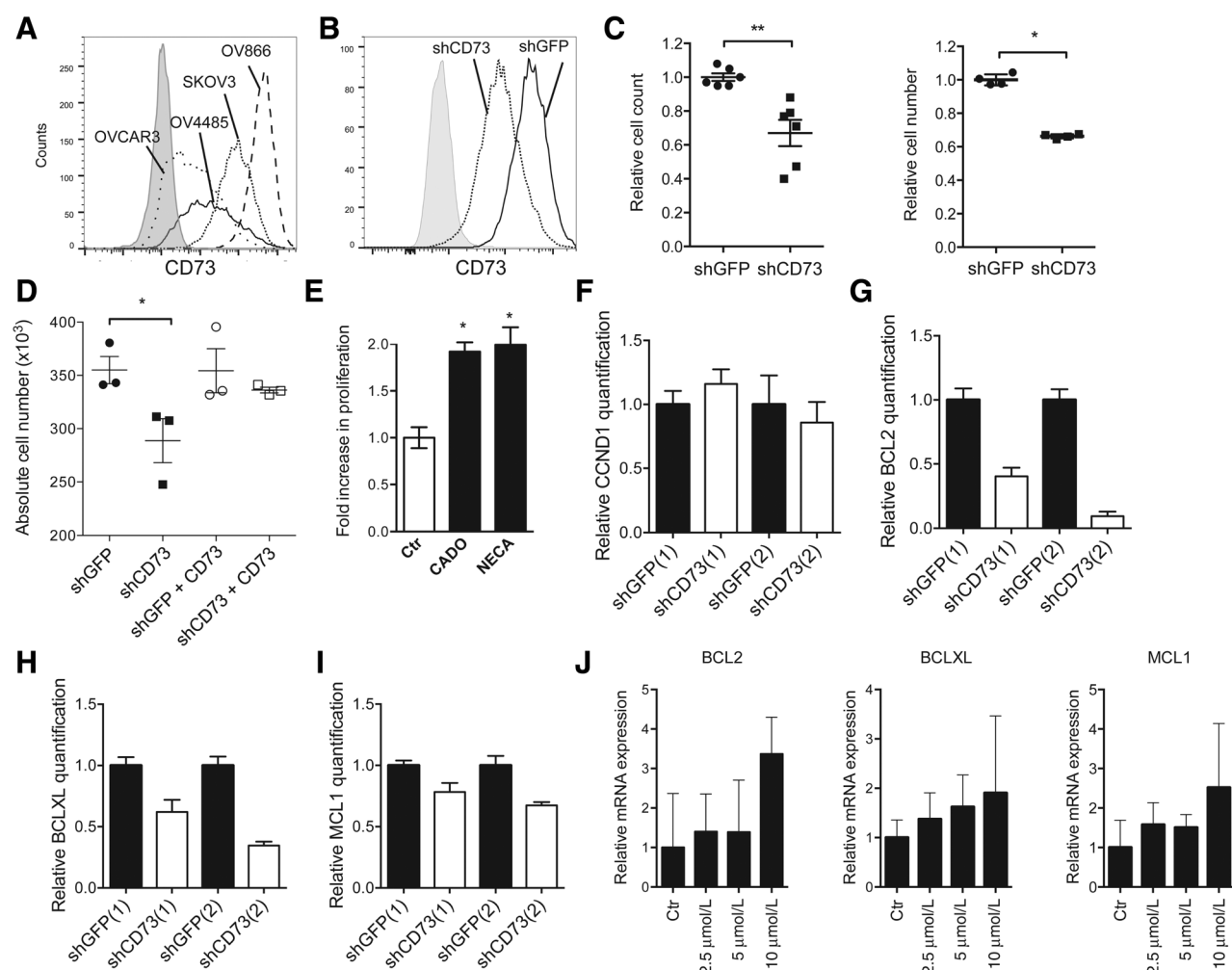### CD73 promotes ovarian tumor cell growth

Because ovarian tumor cells can express high levels of CD73 (Fig. 4A), we next investigated the impact of CD73 expression on tumor cell growth using shRNA gene silencing (Fig. 4B). As shown in Fig. 4C, CD73 gene silencing (Fig. 4B) significantly suppressed the proliferation of SKOV3 cells. Similar results were obtained

Figure 3.
CD73 expression in ovarian tumor cells enhances the prognostic value of intraepithelial CD8^{+} cells. A and B, intraepithelial CD8^{+} cell density was measured in the HGS TMA and its prognostic impact assessed. C and D, association of CD73 expression in tumor cells and intraepithelial CD8^{+} cell density with disease-free survival (C) and overall survival (D). Significance of differences in survival between patient groups was estimated by log-rank test. Medians were used as cutoffs.
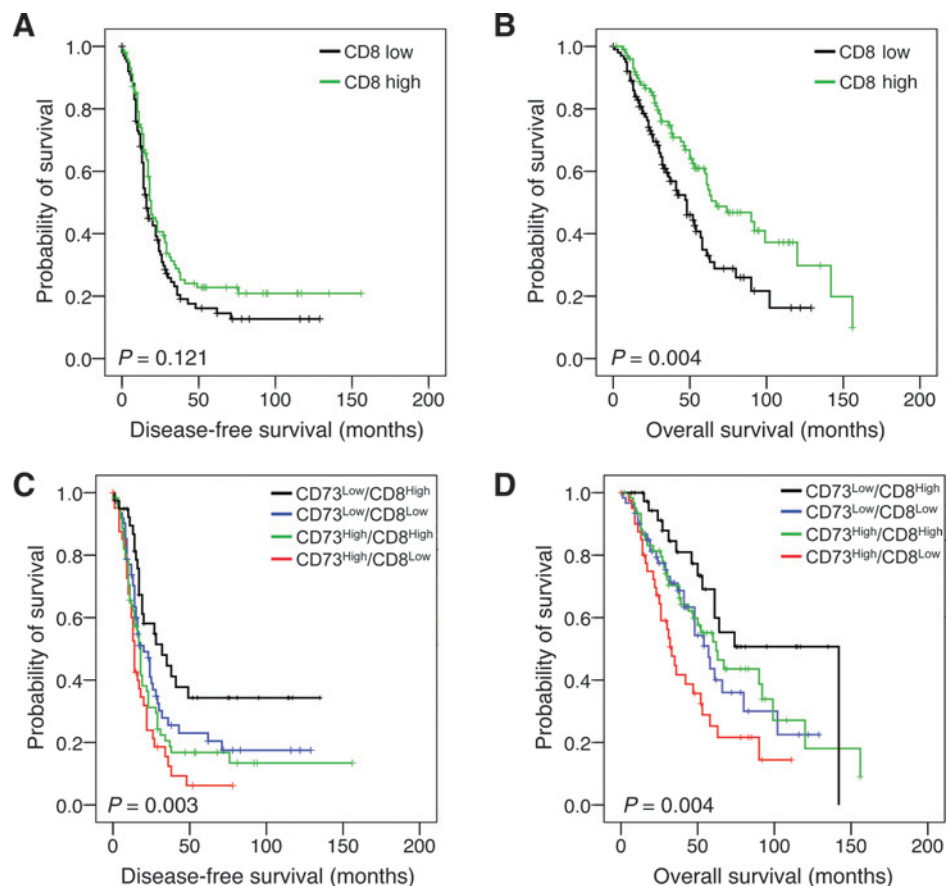
Figure 4.
CD73 promotes ovarian tumor cell proliferation. A, CD73 expression levels on human ovarian cancer cell lines were measured by flow cytometry. B, CD73 gene silencing in SKOV3 cells was performed using a shRNA-encoding plasmid targeting CD73 (or GFP as control). C, SKOV3-shCD73 and SKOV3-shGFP cells were maintained in 3D cultures (left) or standard 2D cultures (right) and proliferation measured after 5 or 3 days, respectively (3D data represent a pool of two independent experiments; 2D data represent individual replicates of one representative experiment; *, $P < 0.05$; **, $P < 0.01$ by Mann–Whitney; means ± SEs are shown). D, CD73 reexpression in SKOV3-shCD73 restored cell proliferation in 5 days assays (*, $P < 0.05$; means ± SEs are shown). E, treatment of SKOV3 cells with CADO (20 µmol/L) or NECA (100 µmol/L) increased cell proliferation in 3 days assays (*, $P < 0.05$; means ± SEs are shown). F–J, real-time PCR was performed on SKOV3-shCD73 and SKOV3-shGFP cells (F–I) or SKOV3 cells treated with CADO for 3 days (J). Expression of each gene was normalized to 18S and relative mean expression to SKOV3-shGFP cells or control is shown (error bars, 95% CI).

with OV4485 cells (Supplementary Fig. S5A). Consistent with these results, CD73 rescued expression (Supplementary Fig. S5B) in SKOV3 cells, or treatment with exogenous CADO, a stable adenosine analogue, significantly enhanced tumor cell proliferation (Fig. 4D and E). Although CD73 gene silencing had minimal impact on cyclin D1 gene expression (Fig. 4F), it significantly downregulated antiapoptotic BCL-2 and BCL-XL expression and slightly albeit not-significantly downregulated MCL-1 expression (Fig. 4G–I). Consistent with these results, exogenous CADO enhanced BCL2, BCLXL, and MCL-1 gene expression (Fig. 4J). Taken together, our data demonstrated that CD73 expression and extracellular adenosine promote ovarian tumor cell growth. Further studies are required to decipher the role of CD39 and adenosine receptors, which can be expressed on ovarian tumor cells (Supplementary Fig. S6).

CD73-expressing CAFs promote tumor growth in mice

Our immunofluorescence analysis revealed that CD73 could be expressed on tumor cells (Fig. 2A) and CAFs (Supplementary Fig. S7A). When we evaluated the prognostic impact of CD73 expression on CAFs, we observed that high levels were associated with worse prognosis (Fig. 5A; mean ± SD: 37.85 ± 6.6 months vs. 28.71 ± 6.6 months; $P = 0.05$ by log rank). To test whether CD73 expression on CAFs was involved in suppressing antitumor immunity, we utilized the ID8 mouse model of ovarian cancer, which expresses CD73 at low levels and produces low levels of adenosine in vitro (Supplementary Fig. S7B), and primary MEF derived from wild-type and CD73-deficient mice as a model of CAFs. Flow cytometry confirmed CD73 expression on wild-type fibroblasts (Supplementary Fig. S7C). Although CD73 expression on MEF had no effect on ID8 cell proliferation

Figure 5.
CD73-expressing CAFs promote ovarian cancer. A, CD73 protein expression in CAFs (cytokeratin-7/18^{neg}) was quantified by immunofluorescence and its prognostic value evaluated. Significance of differences in survival between patient groups was estimated by log-rank test (high, top 20%). B, ID8 cells (5×10^6) with our without MEF (5×10^6) were resuspended in 200 μL of PBS, coinjected intraperitoneally into wild-type C57Bl/6 mice ($n = 10$ per group), and overall survival estimated by log-rank test (**, $P < 0.01$). C, same as B, except that mice were euthanized at day 60 for peritoneum nodules count (means ± SEs are shown; *, $P < 0.05$ by Mann–Whitney). D, same as C, except that immunodeficient Rag2$^{-/-}$γc$^{-/-}$ mice were injected. E, wild-type C57Bl/6 mice were injected subcutaneously with a mix of 5×10^6 ID8-OVA cells and 5×10^6 MEF, tumors were collected after 24 days, and single cell suspensions analyzed by flow cytometry. Percentages of OVA-specific tumor-infiltrating CD8$^+$ specific are shown (means ± SEs are shown; **, $P < 0.01$ by Mann–Whitney).
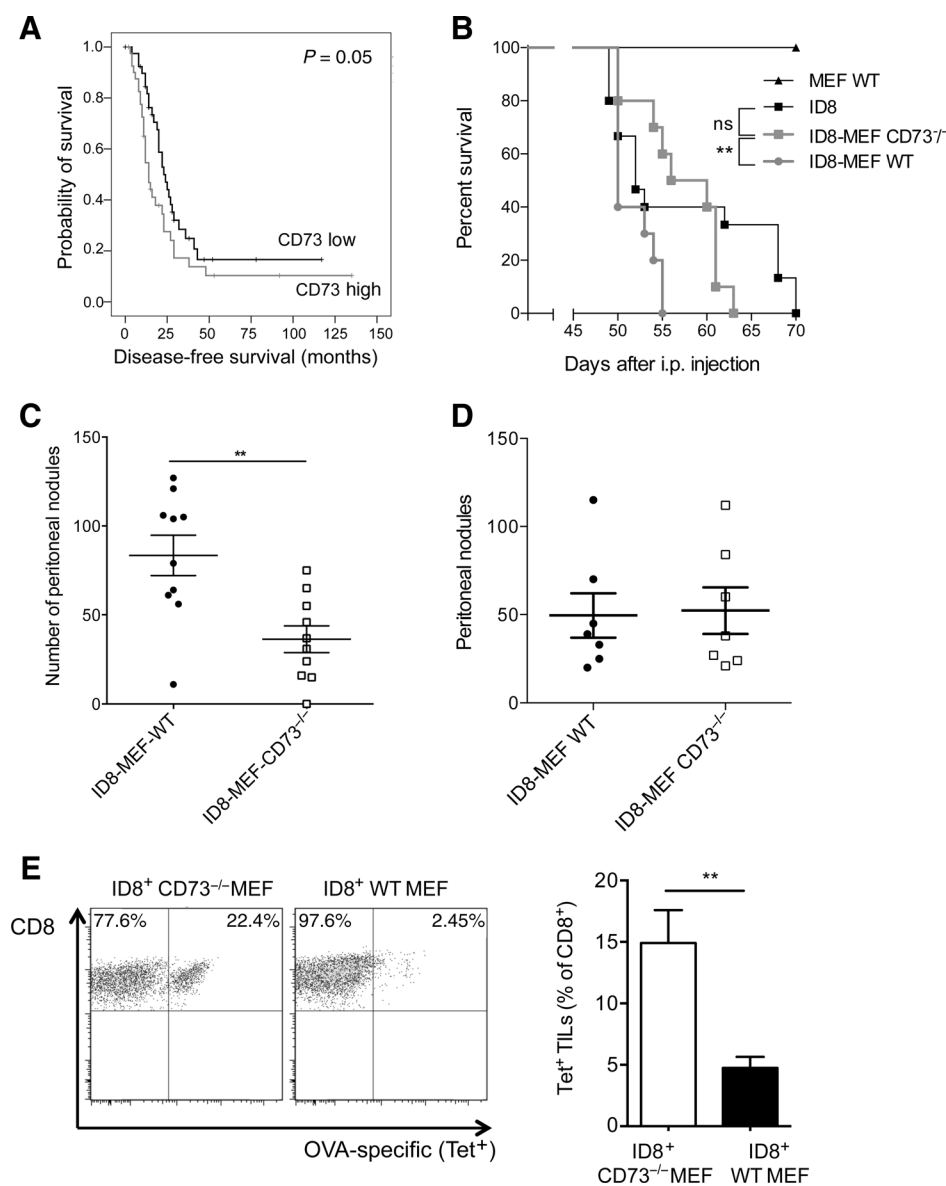
in vitro (Supplementary Fig. S7D), CD73 expression on MEF significantly enhanced ID8 tumorigenesis in immunocompetent mice (Fig. 5B and C; Supplementary Fig. S7E). As shown in Fig. 5D, this protumorigenic effect of CD73-expressing MEF was lost in immunodeficient Rag2$^{-/-}$ γc$^{-/-}$ mice, suggesting an immune-dependent mechanism.

CD73 expression by CAFs promotes tumor immune escape in mice

To validate that CD73 expression on MEF promoted ID8 tumor immune escape, we generated ovalbumin-expressing ID8 cells to monitor ovalbumin-specific CD8$^+$ T-cell responses (Supplementary Fig. S8), and coinjected ID8-OVA tumor cells with wild-type or CD73-deficient MEF. As shown in Fig. 5E, CD73-expressing MEF significantly inhibited tumor-specific CD8$^+$ T cells responses against ID8-OVA tumors. Our data thus demonstrated that CD73 expression by MEF significantly suppressed CD8$^+$ T-cell–

mediated immunosurveillance of ID8 ovarian cancer in mice, thereby promoting tumor growth.

Discussion

HGS ovarian cancer is the most common and lethal histotype of all ovarian cancer with a 5-year survival rate of only 30% (28). There has been little improvement in overall survival for several decades, emphasizing the need for new treatments (29).

Several studies have shown that TILs, in particular CD8$^+$ T cells, are associated with improved clinical outcomes in HGS (2, 3, 30). This suggests that HGS ovarian cancer may be amendable to immune-based therapy. Nevertheless, the microenvironment of ovarian tumors is highly immunosuppressive. Accordingly, T regulatory cells (Treg), B7-H4 expressing macrophages, and PD-L1 expression on tumor cells have all been associated with

Turcotte et al.

poor survival in patients with ovarian cancer (31–33). Another immunosuppressive pathway potentially involved in ovarian cancer is the production of extracellular adenosine by the ecto-nucleotidase CD73 (34).

CD73 is a GPI-anchored enzyme that generates extracellular adenosine, a potent immunosuppressive metabolite in the tumor microenvironment (reviewed in ref. 35). CD73 has been shown to suppress antitumor T cells in the ovarian cancer setting (27, 36). Monoclonal antibody treatment targeting CD73 has been shown to delay ovarian tumor growth in mice and to rescue human T-cell functions when cocultured with CD73-expressing human ovarian cancer cells (11, 16).

Our current study revealed that CD73 is significantly associated with a poor prognosis in HGS ovarian cancer. Both gene expression and protein expression analyses confirmed the prognostic importance of CD73 in HGS ovarian cancer. Interestingly, we observed that CD73 gene expression, in contrast to PD-L1, was highest in the C1 molecular subtype, characterized by an activated stroma and poor outcome. The C1 subtype contains markers of activated myofibroblasts, vascular endothelial cells, pericytes, and enrichment of pathways defining extracellular matrix production and angiogenesis (21). A important feature of the C1 subtype is that despite its poor prognosis, it displays a prominent immune gene expression signature (21), suggesting the presence of immunosuppressive mechanisms keeping antitumor immunity at check. The fact that C1 tumors express high levels of CD73 may explain why C1 tumors have poor prognosis despite having TILs. In support of an immunosuppressive role for CD73 in HGS ovarian tumors, we found that the prognostic value of tumor-infiltrating CD8^{+} cells was restricted to tumors with low levels of CD73. Although we recognize that core samples used in TMAs may not account for tumor heterogeneity, our data suggest that when CD73 is expressed at high levels in tumors, tumor-infiltrating CD8^{+} cells fail to control tumor progression. This is further supported by the fact that CD73 expression by tumor cells (11) and associated fibroblasts (Fig. 5) inhibit CD8^{+} T-cell–mediated immunosurveillance in preclinical models of ovarian cancer.

Stromal expression of CD73 has been previously described in the mammary gland (37), but its effect on tumorigenesis was never investigated. Using the ID8 mouse model of ovarian cancer and primary MEF derived from wild-type or CD73-deficient mice as a model of CAFs, we demonstrated that CD73 expression in MEF significantly enhanced ID8 tumorigenesis *in vivo* and promoted immune escape from CD8^{+} T cells. Our study is thus consistent with recent reports that in the tumor microenvironment, CAFs are important regulators of tumor immunity (38).

Another finding of our study is the observation that CD73 gene expression is strongly correlated with an EMT gene signature, consistent with a recent study in gallbladder cancer cells (18). CD73 has been shown to be upregulated on human mammary epithelial cells stably expressing the EMT-inducers Twist, Snail, or TGFβ1 (39). As EMT is increasingly being recognized as an important process in ovarian cancer, especially in promoting the invasion of ovarian tumor cells into the mesothelial cell lining of the peritoneal cavity (40), further work is required to decipher the role of CD73 in EMT. Interestingly, tumors that express a mesothelial clearance-EMT gene signature generally fall into the C1 molecular subtype of ovarian cancer (40). This is consistent with our observation that CD73 expression is associated with

both C1 and EMT gene signatures. Inhibiting EMT is a potentially promising therapeutic approach, as it may maintain tumor cells in a lower-grade state, thereby increasing efficacy of standard treatments. However, the development of EMT inhibitors remains a challenge, as current targets consist of transcription factors.

In conclusion, our study validates CD73 as a potential target in HGS ovarian cancer. We found that when CD73 is expressed at high levels in HGS ovarian tumors, patients' survival is decreased and, importantly, the presence of tumor-infiltrating CD8^{+} cells no longer correlates with better prognosis. Taken together with previous studies (11), our study suggests that CD73 blockade might be a relevant strategy to enhance the antitumor function of CD8^{+} T cells in HGS ovarian cancer. Our study thus sheds new light on the protumorigenic effects of CD73 in ovarian cancer.

Disclosure of Potential Conflicts of Interest

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

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CD73 Is Associated with Poor Prognosis in High-Grade Serous Ovarian Cancer

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