

Inhibition of CD39 Enzymatic Function at the Surface of Tumor Cells Alleviates Their Immunosuppressive Activity

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Abstract

The ectonucleotidases CD39 and CD73 hydrolyze extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to generate adenosine, which binds to adenosine receptors and inhibits T-cell and natural killer (NK)-cell responses, thereby suppressing the immune system. The generation of adenosine via the CD39/CD73 pathway is recognized as a major mechanism of regulatory T cell (Treg) immunosuppressive function. The number of CD39⁺ Tregs is increased in some human cancers, and the importance of CD39⁺ Tregs in promoting tumor growth and metastasis has been demonstrated using several *in vivo* models. Here, we addressed whether CD39 is expressed by tumor cells and whether CD39⁺ tumor cells mediate immunosuppression via the adenosine pathway. Immunohistochemical staining of normal and tumor tissues revealed that CD39 expression is significantly higher in several types of human cancer than in normal

tissues. In cancer specimens, CD39 is expressed by infiltrating lymphocytes, the tumor stroma, and tumor cells. Furthermore, the expression of CD39 at the cell surface of tumor cells was directly demonstrated via flow cytometry of human cancer cell lines. CD39 in cancer cells displays ATPase activity and, together with CD73, generates adenosine. CD39⁺CD73⁺ cancer cells inhibited the proliferation of CD4 and CD8 T cells and the generation of cytotoxic effector CD8 T cells (CTL) in a CD39- and adenosine-dependent manner. Treatment with a CD39 inhibitor or blocking antibody alleviated the tumor-induced inhibition of CD4 and CD8 T-cell proliferation and increased CTL- and NK cell-mediated cytotoxicity. In conclusion, interfering with the CD39-adenosine pathway may represent a novel immunotherapeutic strategy for inhibiting tumor cell-mediated immunosuppression. *Cancer Immunol Res*; 3(3); 254–65. ©2014 AACR.

Introduction

Extracellular release of purine nucleotides, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), plays a

fundamental role in regulating inflammation and tissue homeostasis through the activation of purinergic P2 receptors (1). The conversion of ATP/ADP to adenosine results from the coaction of cell-surface ectonucleotidases CD39 and CD73. The P1 adenosine receptor family encompasses the A1, A2A (the main adenosine receptor expressed by T cells), A2B, and A3 G-protein-coupled receptors. The adenosine-A2A receptor axis is a critical and nonredundant immunosuppressive mechanism that dampens inflammation and protects normal tissues from inflammatory damage and autoimmunity (2). However, this immunosuppressive pathway is aberrantly activated in tumor tissues, notably in response to hypoxia, and provides protection for cancer cells against the immune system (3). Chronic activation of this pathway and accumulation of extracellular adenosine in tumor tissues produce an immunosuppressive and proangiogenic niche that is favorable to tumor growth (4–8). The principal role of this pathway in mediating cancer is evidenced by the complete rejection of large immunogenic tumors in A2AR-deficient mice (6, 9) as well as the tumor-resistant phenotype of CD39 or CD73 knockout mice (10–13).

The catabolic activity of CD39 and CD73 represents the primary source of adenosine. CD39, or ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD1), hydrolyses extracellular ATP and ADP into adenosine monophosphate (AMP; refs. 14, 15). AMP is then processed into the anti-inflammatory adenosine, essentially by the ecto-5'-nucleotidase CD73. Upon binding to A2A receptors on T cells, adenosine induces the accumulation of

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intracellular cyclic AMP, thereby preventing TCR-induced CD25 upregulation and inhibiting effector T-lymphocyte proliferation and inflammatory cytokine secretion (16). Adenosine also blocks the cytotoxic activity and cytokine production of activated natural killer (NK) cells (17). Therefore, as pointed out by Sitkovsky and colleagues (3), modulation of this immunosuppressive pathway is an attractive strategy for cancer therapy. Although they act in synergy, CD39, CD73, adenosine, and adenosine receptors are not equal and each represents a unique target with singular profile. For instance, blocking CD39 will prevent the generation of adenosine as well as the hydrolysis of extracellular ATP (a potent immunoactivator released by dying cells; refs. 18 and 19). Targeting CD73 will not prevent the decrease of extracellular ATP levels but may decrease breast cancer cell migration and intravasation in an adenosine-independent manner (20). Alternatively, adenosine deaminase drugs may be used to degrade peritumoral adenosine, whereas selective antagonists of adenosine receptors may allow more fine-tuned regulation. Therefore, a thorough investigation of the expression and the role of each molecule is required to better design future therapeutic strategies.

The role of the adenosine and A2A receptors in cancer and their potential as targets for cancer therapy has been initiated by Ohta and Sitkovsky (2) and reviewed recently (3). The expression of CD73 in tumor tissues has been thoroughly described and CD73 is expressed by various cells, including tumor cells, endothelial cells, and stromal cells (21, 22). Less is known regarding the expression of CD39 in tumors. The most frequently reported source of CD39 in tumor tissues is regulatory T cells (Treg; refs. 23, 24). The number of CD39⁺ Tregs is increased in human cancers, and these cells participate in immunosuppression by generating adenosine (25–29). CD39 promotes melanoma and colon cancer growth and metastasis in mice (12, 13, 30), and CD39 disruption or blockade facilitates NK cell-mediated tumor eradication *in vivo* (13). CD39 is also expressed by other cell types in the tumor environment, such as stromal cells (31) and endothelial cells (32), and may stimulate tumor progression. Furthermore, results from a recent study suggested that, similar to CD73, CD39 might be expressed by some tumor cells (33). Hausler and colleagues (33) reported that two ovarian cancer cell lines express CD39 and produce adenosine, which suppresses T- and NK cell-mediated antitumor responses. Here, we sought to confirm and broaden these findings to other cancer types by assessing CD39 expression in 500 normal and tumor histologic specimens. We evaluated whether CD39⁺ tumor cells mediate immunosuppression via the adenosine pathway and whether treatment with CD39-blocking antibody, currently in preclinical development, or CD39 inhibitors reverse this immunosuppressive effect.

Materials and Methods

Cell culture

All media and reagents were purchased from Invitrogen. All cells were kept in a 5% CO₂ 37°C incubator. K-562, P815, Raji, BJAB, BL-41, B104, EHEB, RAMOS, OAW-42, MCF7, ASPC-1, HCT116, and T-47D cells were cultured in RPMI medium supplemented with 10% FBS, 2 mmol/L glutamine, 10 mmol/L HEPES, and 40 µg/mL gentamycin. SK-MEL-5, SK-MEL-28, MDA-MB-436, and A-375 cells were cultured in DMEM medium supplemented with 10% FBS, 2 mmol/L glutamine, 10 mmol/L

HEPES, and 40 µg/mL gentamycin. MDA-MB-231 cells were cultured in DMEM medium supplemented with 15% FBS, 2 mmol/L glutamine, 10 mmol/L HEPES, and 40 µg/mL gentamycin. MDA-MB-468 cells were cultured in DMEM medium supplemented with 10% FBS, 2 mmol/L glutamine, and 40 µg/mL gentamycin. MEC2 and CFPAC-1 cells were cultured in Iscove MDM supplemented with 10% FBS. HPAC cells were cultured in DMEM-F12 medium supplemented with 10% FBS and 40 µg/mL gentamycin. SK-OV-3 cells were cultured in McCoy medium supplemented with 10% FBS, 2 mmol/L glutamine, 10 mmol/L HEPES, and 40 µg/mL gentamycin.

Cell line authentication

All cell lines were tested monthly and were *Mycoplasma* free. Cells were kept in culture for a period not exceeding 2 months. Cell morphology and growth characteristics were checked on a weekly basis and remained unchanged. HCT-116, SK-OV-3, MCF7, T-47D, MDA-MB-231, MDA-MD-436, MDA-MB-468, CFPAC-1, ASPC-1, HPAC, and A-375 were purchased from the American Type Culture Collection (ATCC). OAW-42 was purchased from SIGMA. MEC2 and EHEB were purchased from Leibniz-Institute DSMZ (Braunschweig, Germany). Their morphology in culture was consistent with the description of the ATCC/provider. No additional authentication was performed.

BJAB and B104 cells were obtained from CelluloNet Centre de Ressources Biologiques UMS3444/US8 (Lyon, France); Raji, RAMOS, and BL-41 from the International Agency for Cancer Research (Lyon, France). SK-MEL-5 and SK-MEL-28 cells were obtained from Dr. Nicolas Dumaz (INSERM U976, Paris, France) and are regularly sequenced for BRAF V600E mutation. K-562 was purchased from the ATCC and was validated as a highly sensitive *in vitro* target for the NK assay. P815 is a murine mastocytoma cell line able to bind Fc portion of the murine Ig and used for the anti-CD3 mAb-redirection killing assay. Cells were obtained from U976 cell bank and were checked for their ability to bind murine Ig.

Peripheral blood mononuclear cells (PBMC) were collected from healthy donors (Etablissement Français du Sang). PBMCs were isolated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology). PBMCs were cultured in RPMI medium supplemented with 10% AB human serum, 2 mmol/L glutamine, 10 mmol/L HEPES, and 40 µg/mL gentamycin. CD4⁺CD25⁻ or CD8⁺ T cells were negatively isolated using magnetic beads (CD8 isolation kit, CD4 isolation kit II, and CD25 microbeads; Miltenyi Biotec; >90% purity). CD56⁺ cells were positively isolated using magnetic beads (CD56 MicroBeads human; Miltenyi Biotec; >90% purity).

Flow cytometry

Data were acquired on a FACSCanto cytometer and results were analyzed using the FlowJo software.

CFSE labeling

Incubation was carried out with 1–2 × 10⁷ cells/mL with 0.75 µmol/L 5,6 CFSE (Molecular Probes) for 12 minutes at 37°C.

T-cell activation

CFSE-labeled PBMCs or CD4 or CD8 T cells (4 × 10⁴) were cultured in flat-bottom plates in the presence of immobilized anti-CD3 antibody (10 µg/mL, clone UCHT1). When indicated, 2

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or 4×10^4 irradiated SK-MEL-5 cells (100 Gy) were added to the culture. ARL (100 $\mu\text{mol/L}$), SCH58261 (100 nmol/L), OREG-103/BY40, or control isotype antibody D6212 (10 $\mu\text{g/mL}$) were added at days 1 and 3. Proliferation of CD4 and CD8 T cells was analyzed by flow cytometry at day 4 or 5.

Expression of CD39 by human cancer cell lines

Human cell lines were stained with PE Cyanine 7–coupled anti-CD39 antibody (clone A1; eBioscience) for 30 minutes at 4°C . Cells were washed and resuspended in 300 μL of PBS containing 2% BSA (10 g/L) and 0.2% NaN_3 . Cells were analyzed by flow cytometry.

Expression of Foxp3 by human CD4 T cells

Human CD4 T cells were stained with PE-coupled anti-human Foxp3 antibody (clone 236/E7; eBioscience) according to the manufacturer's instructions. Cells were analyzed by flow cytometry.

Expression of CD107a by human CD8 T cells

Five-day cultured CD8 T cells were activated with PMA (10 ng/mL) and ionomycin (1 $\mu\text{g/mL}$) and incubated with PE Cyanine 7–coupled anti-CD107a antibody (clone H4A3; eBioscience). After 1 hour, BD Golgi Stop was added and cells were analyzed 4 hours later by flow cytometry.

CTL cytotoxic activity assay

PBMCs (5×10^6) were cultured with 10^6 irradiated (80 Gy) SK-MEL-5 cells seeded in 6-well plates for 6 days in the presence of 20 UI/mL of IL2 alone or associated with the CD39 inhibitor POM-1 at 100 $\mu\text{mol/L}$. Half medium volume is changed after 3 days with fresh IL2 and POM-1. CD8^+ T cells were then purified and their cytotoxic activities tested by a retargeted cytotoxic assay using anti-CD3 mAb and mouse P815 target cells as described previously (34). Anti-CD3 stimulation bypasses MHC restriction and the original antigen specificity of the CTL allowing measurement of the total level of CTL activity. To this aim, P815 cells were labeled with ^{51}Cr at 100 $\mu\text{Ci}/10^6$ cells (PerkinElmer) for 90 minutes at 37°C and extensively washed before cocultured at different ratio with purified CD8^+ T cells preliminary stained with either control or anti-CD3 antibodies. Cells were incubated in 96-well V-bottom plates for 4 hours and supernatants, after plate centrifugation, were harvested and transferred into Lumaplate-96 (PerkinElmer) to measure ^{51}Cr release. Control wells contained only P815 cells to measure spontaneous ^{51}Cr release or P815 cells plus 0.1% Triton X-100 to measure maximal ^{51}Cr . The percentage of specific lysis was calculated as follows: $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. Each condition was performed in triplicate.

NK cytotoxic activity assay

Isolated CD56^+ cells from PBMCs were plated at 4×10^4 cells per well in round-bottomed 96-well plates. When indicated, 4×10^4 SK-MEL-5 cells, treated or not with POM-1 at 100 $\mu\text{mol/L}$ for 16 hours, were added to the culture for 2 hours. K562 cells (target cells) were incubated for 1 hour at 37°C with chromium (^{51}Cr) at 100 $\mu\text{Ci}/10^6$ cells (sodium chromate; PerkinElmer). After washes, 0.8×10^4 K562 cells per well were added to CD56^+ cells cocultured or not with SK-MEL-5 for 4 hours. A total of 100 μL was collected and counted in a gamma counter. Control wells contained only K562 cells to measure spontaneous ^{51}Cr release or

K562 cells plus 0.1% Triton X-100 to measure maximal ^{51}Cr release. The percentage of specific lysis was calculated as follows: $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. Each condition was performed in triplicate.

Immunohistochemistry

Tissue Microarray (MC5002; US Biomax) was used to analyze expression of CD39. It contains 500 cores with 18 types of cancer (20–25 cases/type) and normal controls (5 cases/type). The tissues were formalin-fixed, paraffin-embedded. Immunohistochemical (IHC) staining was performed using CD39 antibody at 1.52 $\mu\text{g/mL}$ (clone 22A9; Abcam). Manual scoring of intensity, location, and cell types of staining was completed by a pathologist. The intensity (strength, 0–3) of CD39 staining was scored as negative (0–0.5), moderate (1), or strong (2–3).

ATPase activity measured by ATP hydrolysis

Cells were cultured in complete medium alone or treated with antibodies (5 $\mu\text{g/mL}$), ARL (100 or 250 $\mu\text{mol/L}$), or POM-1 (100 $\mu\text{mol/L}$) for 16 hours as indicated. Cells were then washed and cultured in complete medium or treated with antibodies (5 $\mu\text{g/mL}$), ARL (100 or 250 $\mu\text{mol/L}$), or POM-1 (100 $\mu\text{mol/L}$) for 30 minutes with 10 $\mu\text{mol/L}$ ATP. The concentration of "unhydrolyzed" extracellular ATP was determined using the ATPlite luminescence ATP Detection Assay System (PerkinElmer) according to the instructions of the manufacturer.

AMP and adenosine measurement by mass spectrometry

Cells (10^5) were cultured in complete medium in the presence or not of CD39 inhibitors for 16 hours. Cells were then washed in cold PBS and resuspended in PBS supplemented with 50 $\mu\text{mol/L}$ ATP in the presence or not of CD39 inhibitors for 30 minutes at 4°C . After centrifugation, AMP and adenosine levels within cell supernatants were analyzed by mass spectrometry. Briefly, an internal standard working solution was prepared by mixing Guanosine ($m/z = 284.09$) and GMP ($m/z = 364.06$) with matrix solution (5 mg/mL α -cyano-4-hydroxycinnamic dissolved in 70% acetonitrile/0.1% TFA). Equal volumes of analyte and internal standard solutions were mixed and two microliters spotted in quadruplicate onto the MALDI-TOF target plate. For each spot, a 4800 Plus MALDI-TOF/TOF Proteomics Analyzer (ABSciex) was used to automatically acquire 40 mass spectra (50 shots/spectrum) in positive reflector ion mode in the m/z 250 to 370 range. To insure quantitative measurement, the laser power was automatically adjusted to avoid saturation of signal intensities. Only the spectra for which the maximum peak height was within a specific interval were kept. The spectra were averaged and the analyte/internal standard peak area ratios were calculated as response factors by averaging four measurements. A calibration curve obtained with pure adenosine (0–10 $\mu\text{mol/L}$) and AMP (10–50 $\mu\text{mol/L}$) was used to calculate the concentration in each sample.

Statistical analysis

In Figs. 4 and 5, results were compared using a two-tailed paired *t* test. A *P* value of <0.05 was considered as statistically significant. Analyses of statistical significance were performed using Prism Software (GraphPad Software).

Results

CD39 is overexpressed in human cancers

To elucidate the importance of CD39 in cancer-associated immunosuppression, we first assessed the expression of CD39 via IHC in 500 human tumor and normal histologic samples from 18 of the most common types of cancer. Staining was performed on paraffin-embedded tissues using an anti-human CD39 antibody suitable for IHC (clone 22A9). The antibody validation is presented in Supplementary Fig. S1. A previous study demonstrated that CD39 is expressed by endothelial cells and some immune cells (35). Indeed, we found that the vascular endothelia always stained positive for CD39 in both normal and tumor tissues, serving as an internal control (Supplementary Fig. S2). Furthermore, as expected, some lymphocytes in lymph node tissues stained positive for CD39 (Supplementary Fig. S2). Interestingly, CD39 expression was higher in many tumor tissues than in the normal specimens. The scoring of the intensity of CD39 expression in normal and tumor tissues is depicted in Fig. 1, and representative images are provided in Fig. 2. CD39 expression was significantly upregulated in kidney, lung, ovarian, pancreatic,

thyroid, and testicular tumor tissues compared with normal tissues. CD39 expression was also higher in endometrial tumors, melanoma, and prostate tumors than in normal tissues, but these differences did not reach statistical significance. CD39 was expressed in some normal lymph node cells, as expected on the basis of previous reports (35, 36), but its expression was significantly higher in lymphoma.

The distribution of CD39⁺ cells also differed between normal and cancer specimens, as summarized in Fig. 1 and displayed in Fig. 2. In normal tissues, CD39 expression was either negative (staining score of 0–0.5) or positive but was mostly restricted to the vascular endothelia or lymphocytes, as described previously (35, 36). In tumor tissues, CD39 was also expressed by endothelial cells and lymphocytes and additionally expressed by tumor-associated stromal cells: e.g., in ovarian, pancreatic, and testicular tumors. Interestingly, CD39 is expressed by tumor cells in kidney, lung, testicular, and thyroid tumors, as well as in lymphoma and melanoma, as illustrated by the membranous staining of the cancer cells (Fig. 2). We confirmed these IHC results using another anti-human CD39 antibody (HPA014067) from the Human Protein Atlas (37). Similar to the results described above, this

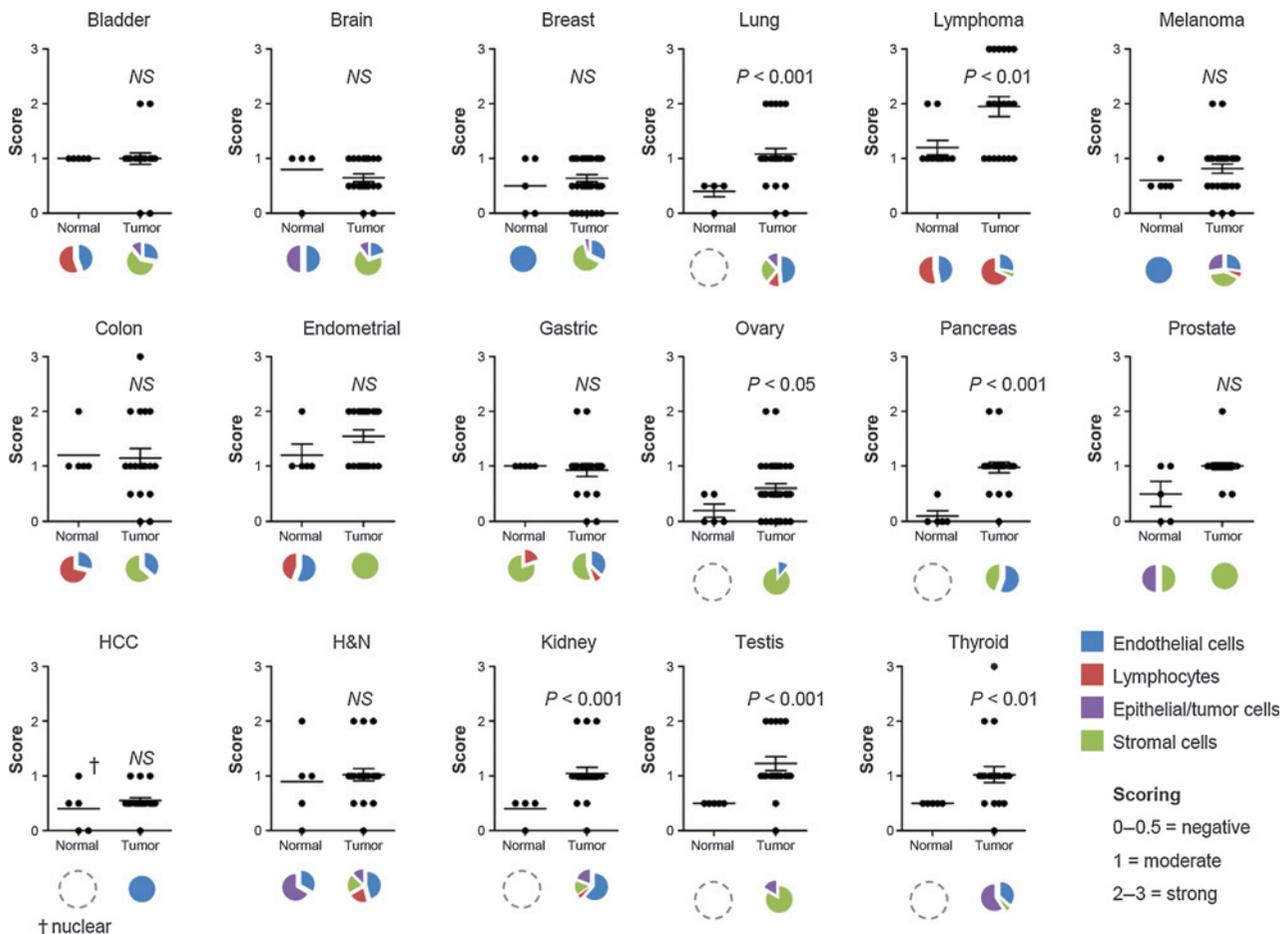
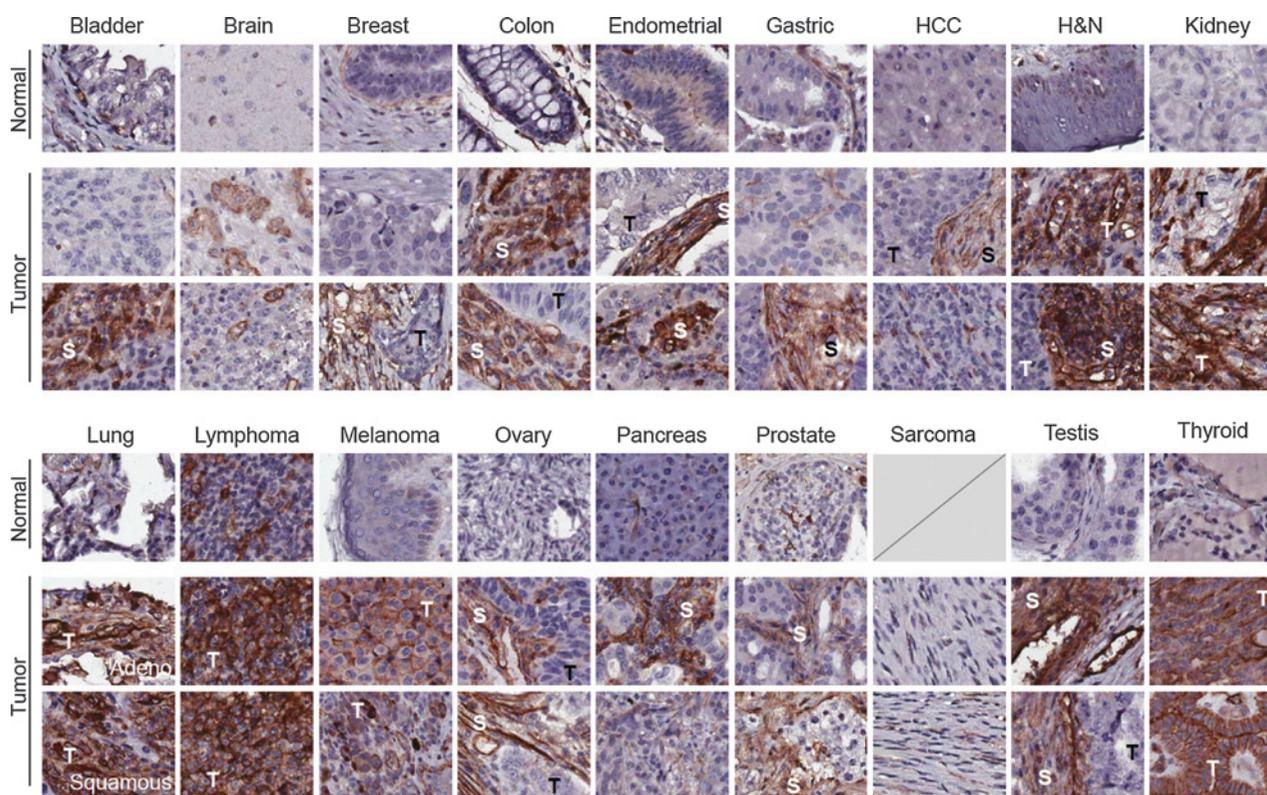


Figure 1.

IHC analysis of CD39 expression in human normal and tumor tissues. CD39 expression in a panel of 500 normal and cancerous clinical samples was assessed via IHC using multiple tissue microarray analysis (MC5002; US Biomax). IHC staining was performed on formalin-fixed, paraffin-embedded tissues using an anti-CD39 antibody suitable for IHC (clone 22A9; Abcam). The staining intensity was scored as follows: 0–0.5: negative; 1: moderate; and 2–3: strong. The distribution of CD39 expression is also indicated: blue, vascular endothelial cells; red, lymphocytes; purple, epithelial or tumor cells; and green, stromal cells. HCC, hepatocellular carcinoma; H&N, head and neck; NS, not statistically significant.

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**Figure 2.**

Representative IHC staining of CD39 expression in human normal and tumor tissues. For each cancer type, representative images of immunostaining of one normal and two tumor samples are shown. Brown staining indicates the expression of the CD39 protein. "S" and "T" indicate the stromal and tumor regions, respectively. HCC, hepatocellular carcinoma; H&N, head and neck.

antibody stained normal lymph node cells and endothelial cells, again serving as an internal control (Supplementary Fig. S3). The expression pattern was remarkably similar to that using the 22A9 antibody (Supplementary Fig. S4). In normal tissues, CD39 expression is primarily restricted to vascular endothelial cells and lymphocytes, whereas CD39 expression is increased in several cancers, including kidney, lung, pancreatic, thyroid, and testicular tumors, as well as melanoma and lymphoma. A moderate increase in CD39 expression was detected in ovarian and prostate tumors. The membranous expression of CD39 on tumor cells was, again, detected in kidney, lung, testicular, thyroid, and prostate tumors, as well as lymphoma and melanoma. Altogether, these data demonstrate that CD39 expression is upregulated in a wide variety of human cancers and provide strong evidence that tumor cells express CD39.

Direct evidence of CD39 expression at the cell surface of human cancer cell lines

To directly demonstrate CD39 expression at the cell surface of cancer cells, we analyzed CD39 expression in several human cancer cell lines via flow cytometry using an appropriate anti-CD39 monoclonal antibody (clone A1). CD39 was strongly expressed on the cell surface of three of five B lymphoma cell lines (Fig. 3A), two of three melanoma cell lines (Fig. 3B), two B-cell chronic lymphocytic leukemia cell lines (Fig. 3D), and one of two ovarian cancer cell lines (Fig. 3E). In contrast with a previous report (33), CD39 was not expressed by SK-OV-3 cells, and this result was confirmed by an independent group using an inde-

pendent source of SK-OV-3 cells. CD39 was not expressed in the HCT116 colon carcinoma cell line (Fig. 3C), the five examined breast cancer cell lines (Fig. 3F) as well as the three pancreatic cancer cell lines (Fig. 3G). Altogether, these results, along with the IHC data, demonstrate that CD39 is expressed by tumor cells of various cancers, whereas CD39 expression is not detected in the corresponding normal cells.

CD39 in cancer cells displays ATPase activity and, together with CD73, generates adenosine

Next, we assessed the ability of CD39⁺ tumor cells to hydrolyze extracellular ATP, by measuring extracellular ATP degradation in cell culture supernatants. As illustrated in Fig. 4A, CD39⁺ lymphoma cells (i.e., B104, BL-41, and RAMOS cells) catalyzed the hydrolysis of ATP, as evidenced by decreased ATP levels in the culture supernatants. This result is in sharp contrast with that using CD39⁻ lymphoma cells (BJAB), which displayed negligible ATPase activity. Similarly, SK-MEL5 and SK-MEL-28 melanoma cells and OAW-42 ovarian cancer cells express CD39 and hydrolyze ATP. Similar results were found by measuring the release of free phosphate resulting from the CD39-induced degradation of ATP in the cell culture supernatants (Supplementary Fig. S5). Consistent with the lack of CD39 expression at the cell membrane (Fig. 3F), human breast cancer cell lines displayed negligible ATPase activity (data not shown). To further assess whether CD39 is directly involved in the ATPase activity of CD39⁺ cancer cells, we exposed CD39⁺ tumor cells to ARL 67156 (ARL), a chemical inhibitor of CD39, and found

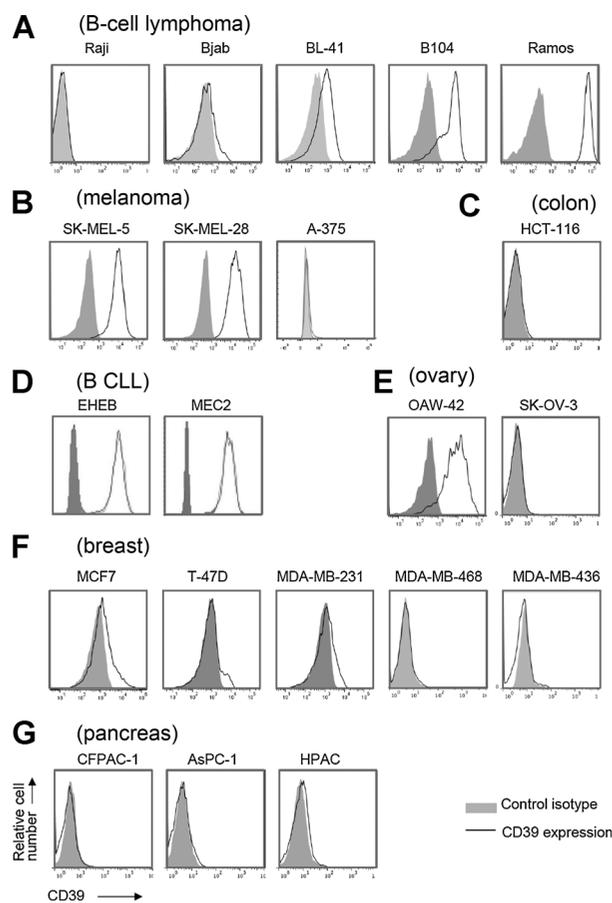


Figure 3. Expression of CD39 by human cancer cell lines. A, B-lymphoma cells (Raji, BJAB, BL-41, B104, and RAMOS); B, melanoma cells (SK-MEL-5 and SK-MEL-28 and A375); C, colon cancer cells (HCT116); D, B-cell chronic lymphocytic leukemia cells (B CLL; EHEB and MEC-2); E, ovarian cancer cells (OAW-42 and SK-OV-3); F, breast cancer cells (MCF7, T-47D, MDA-MB-231, MDA-MB-468, and MDA-MB-436); G, pancreatic cancer cells (CFPAC, AsPC-1, and HPAC) were stained using an anti-CD39 antibody (clone A1; eBioscience), and CD39 expression was analyzed via flow cytometry. The results are representative of at least three independent experiments.

decreased ATPase activity in CD39⁺ tumor cells in a dose-dependent manner (Fig. 4B). Similar results were obtained using POM-1, another CD39 inhibitor (Fig. 4C).

CD39 acts in concert with CD73 to ultimately generate adenosine. As CD73 is expressed in tumor tissues by various cell types, including tumor cells (21), we speculated that CD39⁺CD73⁺ cancer cells might be able to mediate the entire process from ATP to adenosine. To address this hypothesis, we quantified the levels of key metabolites of this pathway via mass spectrometry. We found that CD39⁺CD73⁺ SK-MEL-5 cells (Fig. 4D) generated both AMP and adenosine (Fig. 4E) in the presence of extracellular ATP, whereas no significant levels of AMP or adenosine were detected in the supernatant of CD39⁻CD73⁻ BJAB cells (Fig. 4D and E). Similar purine metabolism, including the production of adenosine, was detected using the CD39⁺CD73⁺ RAMOS lymphoma cell line (data not shown). Altogether, these results demonstrate that the CD39 that is expressed by some tumor cells is functional and, in concert with CD73, induces the generation of adenosine.

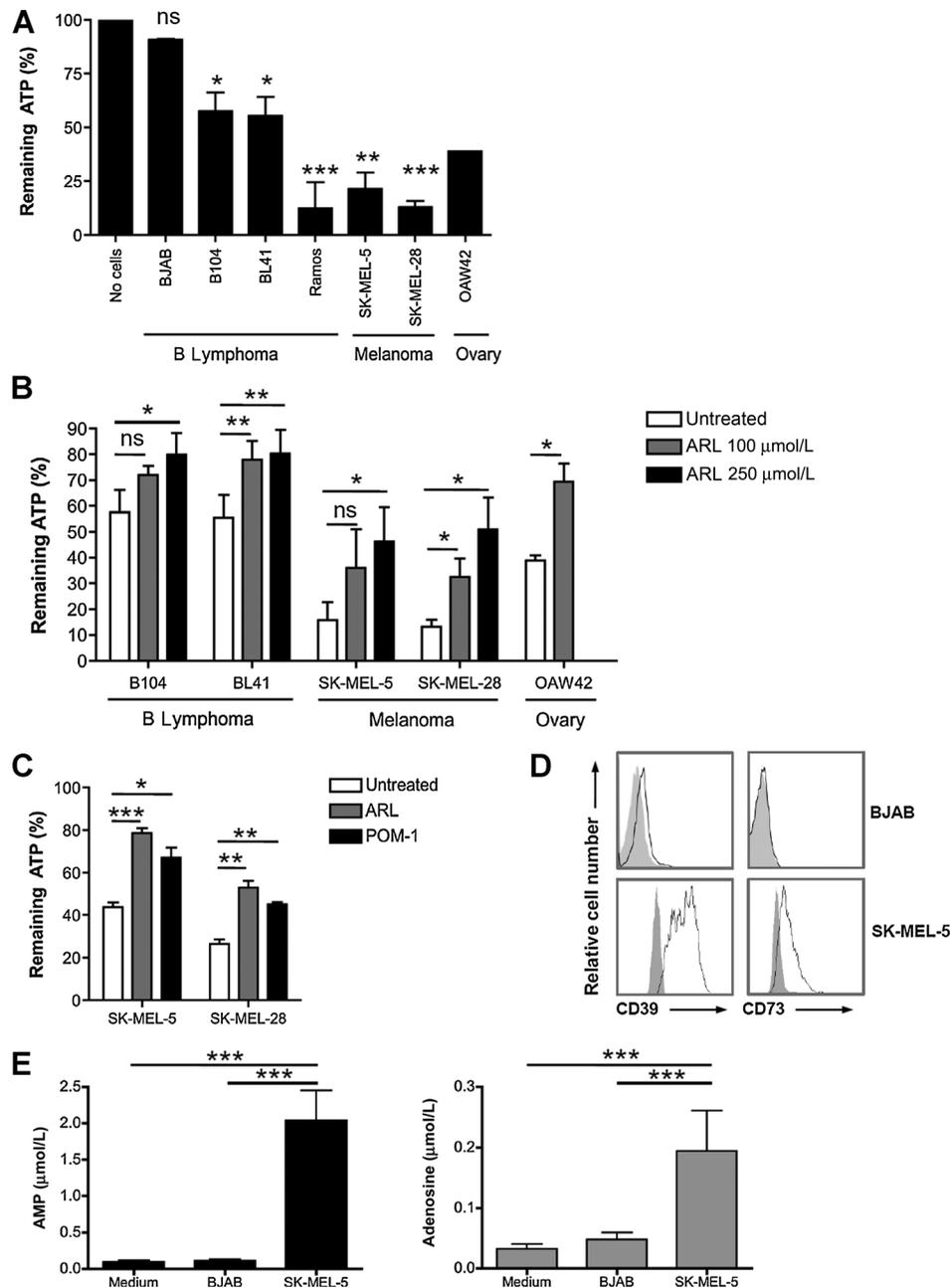
CD39⁺CD73⁺ cancer cells suppress the proliferation of CD4 and CD8 T cells and the generation of effector CTLs in a CD39- and adenosine-dependent manner

As CD39⁺CD73⁺ cancer cells catalyze the hydrolysis of ATP and the generation of adenosine, we speculated that these cells would mediate immunosuppression in a CD39- and adenosine-dependent manner. To test this hypothesis, we cocultured CD3-activated T cells with irradiated (i.e., proliferation-inhibited) CD39⁺CD73⁺ SK-MEL-5 melanoma cells. SK-MEL-5 cells suppressed CD4 and CD8 T-cell proliferation in a dose-dependent manner (Fig. 5A and B). Importantly, the suppression mediated by SK-MEL-5 melanoma cells varied between individual PBMC donors (see Figs. 5A and 6A). Next, to evaluate the role of CD39 and adenosine in melanoma cell-induced suppression of T-cell proliferation, we cocultured CD3-activated CD4 and CD8 T cells with irradiated SK-MEL-5 cells in the presence of various concentrations of CD39 inhibitor ARL, the drug candidate monoclonal antibody OREG-103/BY40 that blocks CD39 enzymatic activity (38), or the adenosine receptor antagonist SCH58261. ARL or mAb OREG-103/BY40 inhibited CD39 enzymatic activity as demonstrated by decreased SK-MEL-5-dependent production of AMP (Fig. 5C) and restored CD4 and CD8 T-cell proliferation (Fig. 5D), whereas treatment with an isotype control antibody displayed no effect. Similar results were obtained using the adenosine receptor antagonist SCH58261 (Fig. 5D). Taken together, these results suggest that the suppression of T-cell proliferation by CD39⁺CD73⁺ melanoma cells is largely mediated by the generation of adenosine and requires both CD39 activity and adenosine signaling. Importantly, we found that the CD39-dependent inhibition of CD4 and CD8 T-lymphocyte proliferation by SK-MEL-5 melanoma cells (Fig. 6A) was not due to the induction of Foxp3-expressing CD4⁺ T cells (i.e., Tregs; Fig. 6B). The inhibition of CD8 T-cell proliferation was accompanied by decreased expression of CD107a (also referred to as lysosomal-associated membrane protein-1, LAMP-1), a reliable marker of CTL function (39), suggesting that CD39⁺CD73⁺ melanoma cells inhibited the generation of effector CTLs (Fig. 6B).

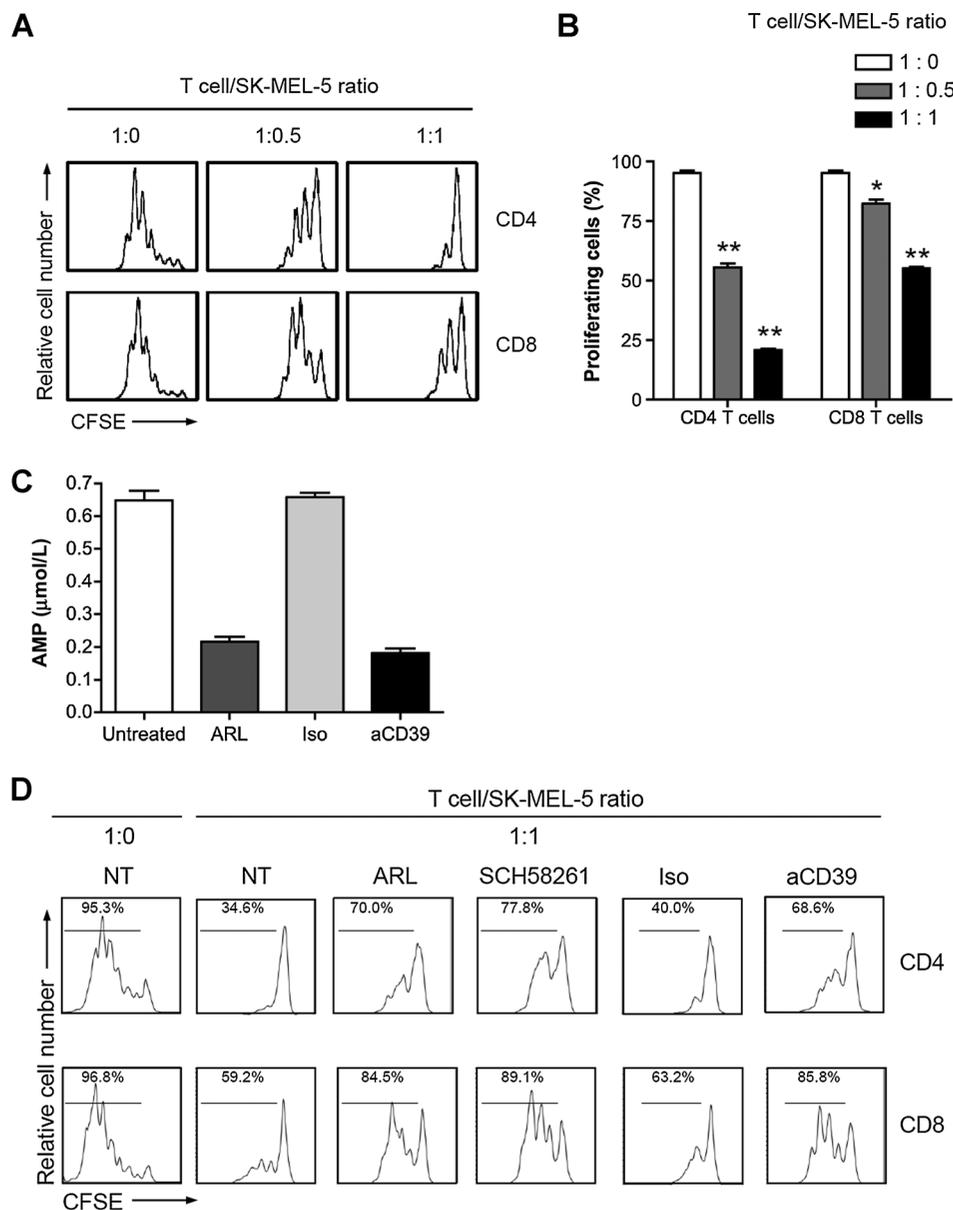
Blockade of CD39 increases CTL- and NK cell-mediated killing

Next, we evaluated whether treatment with the CD39-blocking agent POM-1 restores CTL activity during a 6-day coculture of freshly isolated PBMCs with irradiated CD39⁺CD73⁺ SK-MEL-5 melanoma cells. The level of cytotoxic activity was measured according to the standard anti-CD3 mAb-redirected killing of P815 murine mastocytoma cells labeled with ⁵¹Cr. As expected on the basis of the results shown in Fig. 5, we found that the number of CD8 T cells generated in the presence of tumor cells was low but was significantly increased (by ~2-fold) in the POM-1-treated cocultures (data not shown). Importantly, we found that the total CTL activity of an equivalent number of CD8 T cells was completely inhibited by SK-MEL-5 tumor cells but was increased via pharmacologic inhibition of CD39 (Fig. 7A). Similar results were obtained when freshly separated peripheral blood CD56⁺ NK cells were cocultured with SK-MEL-5 melanoma cells, either pretreated or not with the CD39 inhibitor POM-1, before coculture with the standard K562 NK target cells. The lytic activity of CD56⁺ NK cells was completely suppressed by preincubation in CD39⁺CD73⁺ SK-MEL-5 melanoma cells, and addition of the pharmacologic inhibitor of CD39 restored efficient K562 cell-mediated lysis based on ⁵¹Cr-release cytotoxicity assays (Fig. 7B). In conclusion, we demonstrated in this study that CD39 expressed

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**Figure 4.**

A, the ATPase activity of human cancer cells. A total of 5×10^4 lymphoma cells (BJAB, BL-41, B104, or RAMOS), melanoma cells (SK-MEL-5 or SK-MEL-28), and ovarian cancer cells (OAW-42) were cultured for 30 minutes in the presence of 10 μ mol/L ATP. The concentration of nonhydrolyzed extracellular ATP was determined using the ATPlite assay (PerkinElmer). All of the cell lines are CD39⁺, except for BJAB, which is CD39⁻. The results are expressed as the mean of three independent experiments performed in triplicate, except for OAW-42, in which the results are expressed as the mean of a triplicate experiment. B and C, the ATPase activity of CD39⁺ cells is dependent on CD39. B, a total of 5×10^4 CD39⁺ cancer cells (B104, BL-41, SK-MEL-5, SK-MEL-28, or OAW-42) were cultured alone (white bars) or in the presence of 100 μ mol/L (gray bars) or 250 μ mol/L (black bars) of the CD39 inhibitor ARL. During the final 30 minutes of culturing, 10 μ mol/L ATP was added, and the concentration of nonhydrolyzed extracellular ATP was determined using ATPlite. C, a total of 5×10^4 CD39⁺ cancer cells (SK-MEL-5 or SK-MEL-28) was either not treated (white bars) or treated with 100 μ mol/L ARL (gray bars) or 100 μ mol/L of another CD39 inhibitor, POM-1 (black bars), for 16 hours. During the final 30 minutes of culturing, 10 μ mol/L ATP was added, and the concentration of nonhydrolyzed extracellular ATP was determined using ATPlite assay. D, the expression level of CD39 and CD73 in SK-MEL-5 and BJAB cells. Cells were stained using an anti-CD39 (clone A1; eBioscience) or anti-CD73 antibody (clone AD2; BD), and the levels of CD39 and CD73 expression were analyzed via flow cytometry. E, AMP and adenosine production by SK-MEL-5 cells in the presence of extracellular ATP. SK-MEL-5 cells were incubated in 50 μ mol/L ATP. AMP and adenosine generation were quantified in the supernatant via mass spectrometry. The results are expressed as the means \pm SEM of at least two independent experiments performed in triplicate for B, C, and E and at least three independent experiments for D. For A, B, C, and E, the data were compared using a two-tailed paired *t* test (ns, not statistically significant; *, *P* < 0.05; **, *P* < 0.01; and ***, *P* < 0.001).

**Figure 5.**

Suppressive effect of CD39⁺CD73⁺ melanoma cells on T-cell proliferation, and reversion of this effect via treatment with the CD39-blocking antibody, CD39 inhibitor, or adenosine receptor inhibitor. A and B, CD39⁺CD73⁺ melanoma cells inhibited CD4 and CD8 T-cell proliferation in a dose-dependent manner. A total of 4×10^4 CFSE-labeled PBMCs were incubated in the presence of a coated anti-CD3 antibody. When indicated, irradiated SK-MEL-5 cells were added at a 1:0.5 or 1:1 ratio (T cells:SK-MEL-5 cells). The CFSE profiles (A) and the percentage of proliferating cells (at least one division; B) were analyzed via flow cytometry after 4 days of culturing. For B, the results are expressed as the means \pm SEM of at least three experiments and were compared using a two-tailed paired *t* test (*, $P < 0.05$; **, $P < 0.01$). C, AMP production by SK-MEL-5 cells is inhibited by treatment with the CD39-blocking antibody OREG-103/BY40 or ARL. A total of 10^5 SK-MEL-5 cells were either not treated (white bars) or treated with ARL (dark gray bar, 100 µmol/L), the CD39-blocking antibody OREG-103/BY40 (aCD39, dark bar, 5 µg/mL), or a control isotype antibody (Iso, light gray bar, 5 µg/mL) for 16 hours. SK-MEL-5 cells were then incubated in 50 µmol/L ATP with or without ARL or antibodies at the same concentration. AMP generation was quantified in the supernatants via mass spectrometry. The results are expressed as the means \pm SEM of at least two independent experiments performed in triplicate. D, treatment with the CD39-blocking antibody OREG-103/BY40, ARL (CD39 inhibitor), or SCH58261 (A_{2A} adenosine receptor inhibitor) reversed the melanoma cell-mediated suppression of CD4 and CD8 T-cell proliferation. A total of 4×10^4 CFSE-labeled PBMCs were cultured in the presence of 4×10^4 irradiated (100 Gy) SK-MEL-5 cells. As indicated, ARL (100 µmol/L), SCH58261 (100 nmol/L), the anti-CD39 antibody OREG-103/BY40 (aCD39, 10 µg/mL), or a control isotype antibody (iso, 10 µg/mL) was added on the first and third days of culturing. After 5 days, the proliferation of CFSE-labeled CD4 and CD8 T cells was measured via flow cytometry. The experiments were performed in duplicate, and the results are representative of two independent experiments.

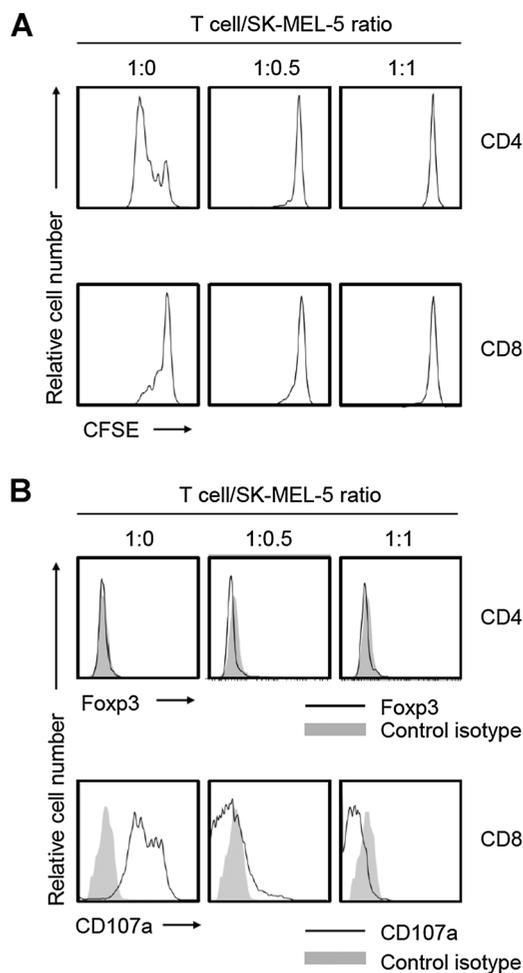


Figure 6. Modulation of T-cell phenotype and function by $CD39^+CD73^+$ tumor cells. A total of 4×10^4 CD4 or CD8 T cells were incubated in the presence of a coated anti-CD3 antibody. When indicated, irradiated SK-MEL-5 melanoma cells were added at a 1:0.5 or 1:1 ratio (T cells:SK-MEL-5 cells). A, The proliferation of CFSE-labeled CD4 and CD8 T cells, and B, the expression of Foxp3 by CD4 T cells and CD107a by CD8 T cells, were analyzed via flow cytometry after 5 days of culturing. The results are representative of at least two independent experiments.

by tumor cells inhibits *ex vivo* CTL activity and NK activity mediated by freshly isolated $CD56^+$ lymphocytes.

Discussion

Many tumors are infiltrated by immune cells, including cytotoxic T cells and NK cells, but no effective antitumor response occurs. Indeed, an emerging hallmark of cancer cells is their capacity to evade immune-mediated destruction (40) via the upregulation of multiple negative regulators of the immune response, termed immune checkpoints, such as CTLA4 and PD-1/PD-L1. Antibody-mediated blockade of these immunoregulatory pathways has provided outstanding clinical results with durable responses and survival benefits for some patients with advanced cancer, placing immunotherapy among the most promising anticancer treatments (41). Recently, the CD39/CD73-adenosine pathway has emerged as an important regu-

lator of immune effector function, through its modulation of the levels of extracellular ATP and adenosine within the tumor microenvironment.

We and others have reported that CD39 is expressed by a subpopulation of Tregs, and its expression is increased in pathologic settings, such as infectious disease (38) and cancer (25–29). CD39 is the rate-limiting component of an enzymatic cascade that catalyzes hydrolysis of ATP and participates in the production of extracellular adenosine. ATP is released by cells undergoing various types of cell death, including apoptosis, and efficient ATP release is required for chemotherapy-induced antitumor immune responses (42). Released ATP binds to P2X7 receptors on dendritic cells, thereby activating the inflammasome and the

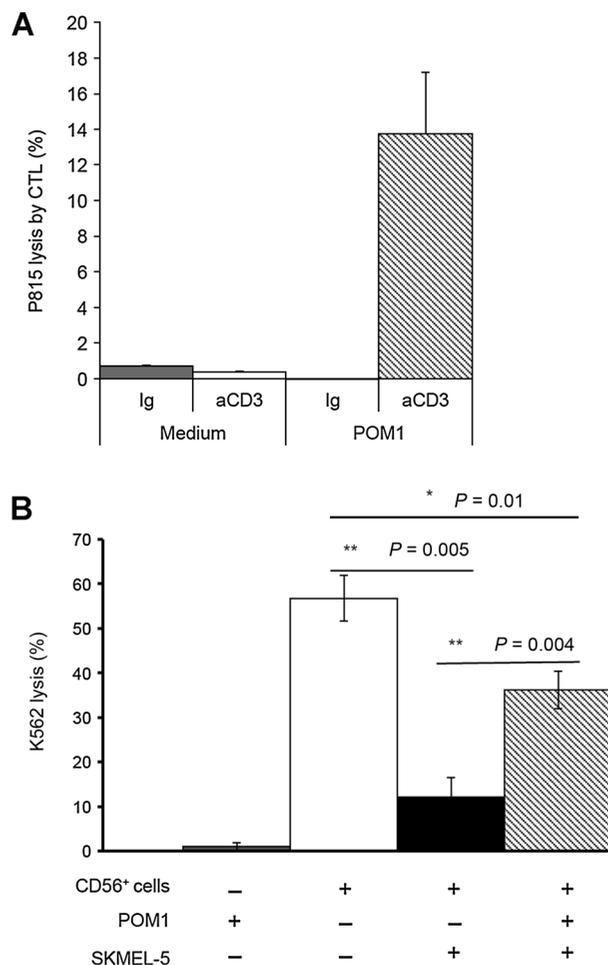


Figure 7. Modulation of the cytotoxicity of CTLs and $CD56^+$ cells by $CD39^+CD73^+$ melanoma cells. A, PBMCs were cocultured with SK-MEL-5 cells (MLR) and IL2 and were pretreated or not with POM-1. The $CD8^+$ cells were then sorted, stained using an antibody (control or anti-CD3 mAb), and cocultured in target P815 cells at a 10:1 ratio (effector cells:target cells). Retargeted CTL activity was measured via the ^{51}Cr -release assay. The results are representative of two experiments performed in triplicate. B, $CD56^+$ cells were cocultured with SK-MEL-5 cells pretreated or not with POM-1 and were then cocultured in target K562 cells at a 5:1 ratio ($CD56^+$ cells:K562 cells). Cytotoxicity was measured via the ^{51}Cr -release assay. The results are expressed as the means \pm SD and are representative of at least two independent experiments performed in triplicate. Comparisons were performed using a two-tailed paired *t* test (*, $P < 0.05$; **, $P < 0.01$).

secretion of IL1 β required for the generation of tumor-specific and IFN γ -producing CD8 T cells (43). In cancer cells that display impaired release of ATP or that are located in a CD39-rich environment, chemotherapeutic agents fail to elicit an immunogenic response unless CD39 inhibitors are used (18). ATP also serves as a "find-me signal" for the recruitment of monocytes to apoptotic sites via P2Y2 receptors (44). Therefore, extracellular ATP plays an important role in the immune antitumor response. In addition to its capacity to decrease immune-activating ATP, CD39 also promotes, together with CD73, the generation of immunosuppressive factor adenosine. Via its binding to adenosine receptors that are expressed by immune effector cells, adenosine induces reduced cytotoxicity of NK cells, decreased proliferation and cytotoxicity of CD8 T cells, increased differentiation of CD4 T cells into FOXP3⁺ Tregs, and M2-polarization of macrophages (4). The importance of adenosine in antitumor immune responses is illustrated by the finding that A2A-deficient mice reject large established tumors (9). In summary, CD39 regulates the outcome of antitumor responses and immunogenic cell death by modulating the balance between the extracellular ATP and adenosine levels.

Using a large cohort of human normal and cancer tissues, we report that CD39 is absent from or weakly expressed in normal samples, with the exception of endothelial cells, whereas CD39 expression is upregulated in several human cancers, including kidney, lung, ovarian, pancreatic, thyroid, testicular, endometrial, and prostate tumors, as well as lymphoma and melanoma. Infiltration of CD39⁺ immune cells, likely including Tregs or Th17 cells, was clearly detected in some specimens, as reported by other authors (25, 26, 45), although we did not further characterize these cells in the present study. Interestingly, in some cancers, such as kidney, lung, testicular, and thyroid cancer, and lymphoma and melanoma, CD39 was strongly expressed by the tumor cells. A similar expression pattern in human tumors and in tumor cells was evidenced using another IHC dataset accessible from the Human Protein Atlas. Furthermore, the expression of CD39 by tumor cells was directly validated via flow cytometry using several human cancer cell lines. In line with previous reports (21, 22), we found that some cancer cell lines also express CD73, suggesting that CD39⁺CD73⁺ cells may exert potent immunosuppressive functions via adenosine. CD39 and CD73 expression by tumor cells was also reported recently in ovarian cancer (33), further strengthening our conclusions. Moreover, it has been reported that cancer exosomes released by various human cancer cell lines express CD39 and CD73 on their surface and mediate immunosuppression via the generation of adenosine (46), thereby supporting the finding that some cancer cells express both CD39 and CD73.

Another interesting finding from this microarray study is that CD39 is also strongly expressed in the tumor-associated stroma in numerous cancers. This phenomenon suggests that, in some cases, tumor-associated stromal cells may potentially hydrolyze ATP and, in concert with CD73, generate adenosine, thereby producing an "immunosuppressive environment" that favors tumor growth. This expression of CD39 by tumor-associated stromal cells has been detected previously in ovarian (33) and endometrial tumors (31) and requires further investigation.

We showed that CD39⁺ tumor cells hydrolyze ATP (i.e., that the CD39 enzyme is functional) and generate adenosine in the presence of CD73. Using a proprietary anti-CD39 antibody (OREG-103/BY40) currently under preclinical development, we showed that CD39 inhibition alleviates the CD39⁺ tumor cell-

mediated suppression of CD4 and CD8 T-cell responses and tumor cell-associated AMP production. In line with the inhibitory effect of tumor-expressed CD39 on immune effector cells, we also demonstrated that pharmacologic inhibition of CD39 increases the cytotoxic activity of CTL and NK cells, leading to tumor cell killing. Altogether, this study contributes new insight into the potential for CD39 as an anticancer target. Previous studies using CD39 knockout animals have suggested the potential benefit of inhibiting CD39 to treat cancer. Melanoma and colon cancer growth and metastasis are markedly decreased in CD39 knockout mice and in WT animals treated with the CD39 inhibitor POM-1 (12, 13). Interestingly, the authors reported that disruption of CD39 function resulted in decreased immunosuppressive activity of Tregs, increased cytotoxicity by NK cells (13) and defective angiogenesis (12), which may be another anticancer property of CD39 antagonism. The promising value of this pathway has been further emphasized by elucidating the role of CD73 in breast cancer. High expression of CD73 is a poor prognosis factor in human triple-negative breast cancer and promotes resistance to anthracycline (47). Anti-CD73 antibody therapy inhibits the growth and dissemination of breast cancer in mice (10, 48) and increased effectiveness of chemotherapeutic agents (47). Importantly, anti-CD73 therapy has been shown to strongly synergize with other immunotherapy treatments such as CTLA-4- or PD-1-blocking antibodies (49). Similarly, CD73 or adenosine receptor blockade has been found to decrease the growth of B16F10 melanoma cells, and both treatments synergize with treatment with an anti-CTLA-4 antibody (50). These results suggest that disrupting the CD39/CD73-adenosine pathway may potentiate the therapeutic strategies that target immune checkpoints. Altogether, these data suggest that blocking CD39 may represent a promising therapeutic strategy for cancer.

Disclosure of Potential Conflicts of Interest

N. Bonnefoy and A. Bensussan have ownership interest (including patents) in OREGA Biotech and are consultant/advisory board members for the same. G. Alberici has ownership interest (including patents) in OREGA Biotech. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Bastid

Study supervision: J. Bastid, N. Bonnefoy, A. Bensussan, J.-F. Eliaou

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Cancer Immunology Research

Inhibition of CD39 Enzymatic Function at the Surface of Tumor Cells Alleviates Their Immunosuppressive Activity

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